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Meet the editor

Professor Xiaojun Liu obtained his PhD in animal reproduction from China Agricultural University, China, in 1998. He then worked at Roslin Institute and the University of Edinburgh, UK, where he conducted researches on QTL mapping, molecular genetics, and functional genomics of poultry and model animals. He is currently a full professor in the Animal Genetics, Breeding and Reproduction Department and head of International Joint Research Laboratory for Poultry Breeding of Henan, Henan Agricultural University, China. His main research interest has been identifying and characterizing the genomic variations, epigenetic factors, and transcriptional differences influencing lipid metabolism, growth, and reproduction in poultry, which directed him toward understanding the genetic determinants of complex traits important for poultry breeding.

Contents

Preface

The development of new-generation sequencing technologies and computer-based bioinformatic analysis methods, genetics, and genomics in poultry have been the most rapidly advancing subjects, which largely promote the progress of extensive areas in poultry science, such as high-throughput single nucleotide polymorphisms (SNP) detection, quantitative trait loci (QTL) mapping, genome-wide association studies (GWAS), disease resistance control, genomic manipulation, etc. As a result, the regulative molecular mechanisms of the economically interesting traits are being discovered. The purpose of this book is to provide readers with a comprehensive overview of the current progress in the application of genetics and genomics in poultry science. A total of ten chapters are divided into three sections. The contents cover genetic variation detection, selection methods for breeding, transgenesis and genome editing, genetic basis of disease resistance, control of gene expression and regulation, reproduction and meat quality, etc. The book should prove useful to researchers and students working in related fields.

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Section 1

Genetic Variation, Breeding and Transgenesis

Chapter 1

Detection and Utility of Genetic Variation in Chinese Local Chicken Breeds

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Additional information is available at the end of the chapter

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Abstract

China has a wide variety of indigenous chicken breeds. Most of these local chicken varieties have valuable genetic features. These resources could provide valuable breeding material for the poultry industry in China and even for the rest of the world. Assessment of genetic differences of these important chicken genetic resources is an important prerequisite to establish efficient conservation and utilization. Up to now, several types of genetic variations have been identified across genomes, and the area of genetic variation in the chicken genome seems to be a rapidly growing research topic in China. These research data can also provide additional evidence for our understanding of chicken genome variation, developing molecular markers, and elucidating the association between genetic variations and phenotypes in the future. This chapter reviews the research progress of molecular genetic variation in Chinese native chicken breeds in recent years.

Keywords: SNP, INDEL, CNV, Chinese native chicken breeds

1. Introduction

In China, with its long history of animal husbandry and diversified geographical conditions, there is a wealth of chicken genetic resources with more than 107 different indigenous chicken breeds. However, many Chinese native breeds are characterized by slow growth, late maturity, and low production performance. At present, the majority of these chickens are maintained in small populations. Due to underutilization and a lack of protective measures, many favorable alleles have been lost. Most of these breeds have unique meat and/or egg qualities, disease resistant, and other useful characteristics. For example, in recent years, blue-shelled layers and

black-bone chickens have gained popularity, and their eggs generate greater profit as the consumption demand diversifies. Xichuan black-bone chicken (XC), Yunyang black-bone chicken (YY), and Silkie fowl (SY) are well-known black-bone chicken breeds, and XC and Lushi chicken (LS) are popular blue-eggshell chicken breeds in China [1]. Such indigenous breeds may contain genes and alleles pertinent to the adaptation to particular environments and local breeding goals and needs to maintain genetic resources permitting adaptation to unforeseen breeding requirements in the future and a source of research materials [2]. Therefore, a study on the genetic diversity of Chinese chicken breeds has important significance for protecting and using local breeds and resources.

As a result, identifying genetic determinants of economically important traits is one of the main focuses of chicken genetic studies, which requires a comprehensive knowledge of DNA sequence variations as well as the development of numerous informative genetic markers. The near-complete chicken genome has made it possible to systematically study genetic variations. Genetic variation takes many forms and ranges from large microscopically visible chromosome anomalies to single-nucleotide changes. Up to now, several types of genetic variations have been identified across genomes. Genetic variation can be divided into different forms according to the size and type of genomic variation underpinning genetic change. Small-scale sequence variation (<1 Kbp) includes base-pair substitution and insertion and deletion. Large-scale structural variation (>1 Kbp) can be either copy number variation (loss or gain) or chromosomal rearrangement (translocation, inversion, or segmental acquired uniparental disomy) [3]—namely, single-nucleotide polymorphism (SNP), insertion and deletion (INDEL), and copy number variations (CNV).

SNP is a variation in a single nucleotide that occurs at a specific position in the genome, where each variation is present to some appreciable degree within a population (e.g., >1%). For example, at a specific base position in the genome, the C nucleotide may appear in most individuals, but in a minority of individuals, the position is occupied by a T. This means that there is an SNP at this specific position, and the two possible nucleotide variations—C or T—are said to be alleles for this position [4].

INDEL is a molecular biology term for an insertion or deletion of bases in the genome of an organism. It is classified among small genetic variations, measuring from 1 to 10,000 base pairs in length [5]. A microindel is defined as an INDEL that results in a net change of 1–50 nucleotides [6]. In domestic animals, INDELs are also found to be responsible for a number of traits and diseases, such as double-muscle trait [7] in cattle and immotile short-tail sperm defect in pig [8]. In chicken, INDELs of 9–15 bp in PMEL17 gene are causative mutations for plumage color (dominant white, dun, and smoky) [9], and an INDEL mutation in the growth hormone receptor (GHR) gene causes sex-linked dwarfism [10]. With the rapid advance of sequencing technology, considerable progress has been made in INDEL discovery in chicken genome. Three chicken breeds were partially sequenced by capillary sequencing and 2.8 million SNPs were identified by aligning the resultant reads to the reference genome, and about 10% of these variations are actually INDELs [11]. The segregating short indels in unique sequence of the chicken genome are on average 5% as common as SNPs [12]. Recently, genome-wide INDELs in 12 Chinese diverse chickens were detected by next-generation

sequencing (NGS) and their potential influence on gene functions were examined [13]. The transcriptomic SNPs and INDELs in Chinese Gushi chickens were detected by Ribo-Zero RNA-Seq technology [14].

CNV is a type of structural variation: specifically, it is a type of duplication or deletion event that affects a considerable number of base pairs [15]. This variation accounts for roughly 12% of human genomic sequence and each variation may range from about 1 kb (1000 bp) to several megabases in size [16]. Compared with the most frequent polymorphisms of SNPs, CNVs have potentially larger effects by disrupting genes and altering gene dosage, disturbing coding sequences and perturbing long-range gene regulation [17].

Over the past decade, there were quite a few studies that have been done on CNV distribution, function, and role in disease of DNA segments in the human genome. Recently, it has been reported that there is a genome-wide presence of CNVs not only in human beings but also in domestic animals. Previous studies have discovered that CNV was responsible for phenotypic changes in chicken. Examples of phenotypes associated with a CNV in the chicken include late feathering on chromosome Z (GGAZ) [18], pea comb on GGA1 [19], dark brown plumage color on GGA1 [20], and dermal hyperpigmentation on GGA20 [21]. A total of 7.6 million SNPs and 8839 CNVs were identified in the mapped regions; hundreds of shared and divergent structural CNVs were also identified in the genomes of two breeds—Silkie and the Taiwanese native chicken —by Illumina sequencing [22].

Using different technological platforms, substantial progress has been made in identifying DNA sequence variations in chickens. Array comparative genomic hybridization (aCGH) [23], SNP array [24, 25], and next-generation sequencing [26] technologies are efficient and reliable methods for analyzing changes in DNA sequence variations.

2. Association between polymorphisms of candidate gene and economic traits in Chinese chicken breed population

2.1. Association between polymorphisms of candidate gene and economic traits in an F_2 population of Gushi chicken cross Anak chicken

In 2004–2005, Hennan Agricultural University bred an F_2 resource population from Gushi (G) chicken (24 hens and 2 roosters) and Anak (A) broilers (12 hens and 4 roosters). The F_2 population consisted of four cross families (A-roosters mated with G-hens) and three reciprocal families (G-roosters mated with A-hens). To build the F_2 population, nine F_1 females were selected from each of seven families (six unrelated rooster families and one half sib). The 63 F_1 females were mated with 7 F_1 males from 7 families. It included 42 grandparents, 70 F_1 parents, and 860 F_2 chickens. Growth traits including body weight were individually measured every 2 weeks from birth to slaughter, and body size indices including shank girth, chest depth, chest width, breastbone length, breast angle, body slanting length, and pelvis breadth were measured every 4 weeks. At the age of 84 days, 13 carcass traits were measured, such as carcass weight, semi-evisceration weight, evisceration weight, fat bandwidth, skin fat

thickness, abdominal fat weight, breast muscle weight, leg muscle weight, and so on. The meat quality traits, muscle fiber traits, and serum indices were also measured. The measuring methods have been previously described [27].

In the beginning, we mapped quantitative trait loci (QTL) associated with growth traits in this F_2 population by 19 microsatellite markers on chromosomes 8–11, and 13; for 32 growth traits, the QTL significant at the genome-wide level that affected body weight at all ages were identified on chromosome 8. The QTL related to BW at early ages were identified on chromosomes 10 and 11, only one QTL-affected body weight was located on chromosome 13 [28]. And mapped QTL associated with growth traits, carcass traits, and meat quality traits on chromosomes 1–5, 7–11, 13 [in Chinese, not shown].

Then, association study between polymorphisms of 20 candidate genes including PR domain 16 (PRDM16), visfatin, Krüppel-like factor 15 (KLF15), patatin-like phospholipase domain containing 3 (PNPLA3), the paired box 7 (Pax7), pro-melanin concentrating hormone (PMCH), thyroid peroxidase (TPO),Adiponectin Receptor 2 Gene (ADIPOR2), lncRNA-pouBW1 and lncRNA-pouMU1 (new gene found), ankyrin repeat and SOCS box-containing 15 (ASB15) gene, and so on, along with economic traits were done in the F_2 population. Meanwhile, association between SNP of eight microRNAs and production traits were studied in the F_2

Gene name	Gene symbol	Trait(s)	References
PR domain containing 16	PRDM16	Weight gain	[28]
Patatin-like phospholipase domain-containing protein 3	PNPLA3	Carcass	[31]
Pro-melanin-concentrating hormone	PMCH	Meat tenderness	[33]
Thrombopoietin	TPO	Growth and carcass	[34]
Adiponectin Receptor 2	ADIPOR2	Weight	[35]
lncRNA-pouBW1	lncRNA-pouBW1	Weight	[36]
Ankyrin repeat and SOCS box 15	ASB15	Growth and carcass	[37]
Cyclin-dependent kinase inhibitor 2A	CDKN2A	Barring	[39]
Endothelin 3	EDN3	Silky/Silkie	[40]
Sonic hedgehog	SHH	Polydactyly	[41]
Flavin-containing Monooxygenase 3	FMO3	Fishy taint	[42]
miR-1657	miR-1657	Growth and carcass	[49]
Dopamine D2 receptor	DRD2	Egg number	[52]
Vasoactive intestinal peptide receptor-1	VIPR-1	Broodiness	[53]
Growth hormone secretagogue receptor	GHSR	Growth and development	[53]
Growth hormone	GH	Growth and development	[53, 58]
Pituitary-specific transcription factor-1	PIT-1	Growth	[54]
Insulin-like growth factor I receptor	IGF1R	Growth and carcass	[55]

Table 1. SNPs of candidate genes with reported associations for chick traits.

population [27, 29–38]. The SNPs of candidate genes in the F2 population [39–45] are summarized in **Table 1**.

Among these, authors found that allele D (9-bp deletion) of the visfatin gene had a negative effect on skeletal growth, while a 31-bp deletion had a negative effect on chicken growth and carcass traits and positive effect on meat quality traits [29, 32]. The INDELs of candidate genes in chickens [46–48] are summarized in **Table 2**.

2.2. Association between polymorphisms of candidate gene and economic traits in Chinese chicken breeds

In recent years, the SNP mutation of candidate gene in Chinese local chickens was widely studied in China. For example, Xinghua chicken, Ningdu Yellow chicken, Qingyuan partridge chicken, Taihe Silkie Fowls, Kangle yellow chicken, Langshan chicken, Sichuan black-bone chicken, Erlang mountain chicken, Caoke chicken, and Tibetan chicken were used in the experiment to study the relationship between SNP_S of prolactin receptor (PRLR), vasoactive intestinal peptide-receptor 1 (VIPR-1), growth hormone secretagogue receptor (GHSR), insulin-like growth factor 1 receptor (IGF1R), prolactin (PRL), pituitary-specific transcription factor (PIT1), growth hormone (GH) gene, and many other genes and chicken reproductive traits, growth traits, and fat traits by some researchers from research institutions and agricultural universities/colleges [49–57]. These studies have laid a good genetic foundation for the development and utilization of Chinese native chicken population. Due to the limitation of this chapter, we are not going into details here.

Gene name	Gene symbol	Trait(s)	References
Premelanosome protein 17	PMEL17	Dominant white	[9]
Growth hormone receptor	GHR	Dwarfism, sex-linked	[11]
Prolactin receptor	PRLR	Early/late feathering	[17]
SRY (sex determining region Y)-box 5	SOX5	Pea-comb phenotype	[18]
SRY (sex determining region Y)-box 10	SOX10	Dark brown	[19]
Visfatin	Vis	Body weight	[29]
Krüppel-like factor 15	KLF15	Growth and carcass	[30]
Paired box 7	PAX7	Growth	[32]
miR-16	miR-16	Body weight	[43]
Tyrosinase	TYR	Recessive white	[44]
Solute carrier family 45, member 2	SLC45A2	Silver Z-linked	[45]
Melanocortin 1 receptor	MC1R	Extended black	[46]
Homeodomain protein 2	MNR2	Rose comb	[47]
Solute carrier organic anion transporter family member 1B3	SLCO1B3	Blue eggshell	[48]

Table 2. INDELs of candidate genes with reported associations for chick traits.

3. Genome-wide association study of production traits in Chinese local chicken

Chicken genomics is likely to have major applications and benefits in comparative genomics, evolutionary biology and systematics, models of development and human disease, and agriculture. Genomic study is required to study genome-wide patterns of DNA variation for dissecting the genetic basis of phenotypic traits. In order to identify genes and chromosome regions associated with body weight, a genome-wide association study using the chicken 60 k SNP panel in a chicken F_2 resource population derived from the crossbreeding between Silkie Fowl and White Plymouth Rock was performed. Results showed that a chicken chromosome 4 (GGA4) region approximately 8.6 Mb in length (71.6–80.2 Mb) had a large number of significant SNP effects for late growth during weeks 7–12. The LIM domain-binding factor 2 (LDB2) gene in this region had the strongest association with body weight for weeks 7–12 and with an average daily gain for weeks 6–12. GGA1 and GGA18 had three SNP effects on body weight with genome-wide significance [58].

A total of 12 different chicken breeds including 7 Chinese indigenous chicken (Beijing You (BY), Dongxiang (DX),Luxigame (LX), Shouguang (SG), Silkie (SK), Tibetan (TB), and Wenchang (WC)) and four commercial breeds (Cornish (CS), Rhode Island Red (RIR), White Leghorn (WL), and White Plymouth Rock (WR)) were selected and the next-generation sequencing methods were applied at an average effective depth of 8.6. Over 1.3 million nonredundant short INDELs (1–49 bp) were obtained. Both the detected number and affected bases were larger for deletions than insertions. Many of them are associated with economically important traits [13].

A total of 78 domestic chickens (36 Tibetan fowls from the Qinghai-Tibet Plateau and 42 domestic fowls from Szechwan Basin) from 17 populations were sequenced to an average of 18-fold coverage for each bird. By combining these data with publicly available genomes of five wild red jungle fowls and eight Xishuangbanna game fowls, a comprehensive comparative genomics analysis of 91 chickens from 17 populations were conducted. Approximately 6.44 million (M) SNPs were identified for each population [59].

In our group, we performed genome re-sequencing identification of genetic mutations in five XC chickens with 229.73 G bp of clean data, and average genome coverage depth of all samples were over 28-fold. The reads were mapped onto the chicken reference genome to 98.73% genome coverage for the five chickens with percentages of Q30 showing >92%. The number of SNPs detected in each chicken varied from 4,998,304 to 5,127,695 in five birds, with an average of 5,062,529 (2,918,565 heterozygous and 247,054 homozygous),1,593,603 INDELs (693,235 insertions and 900,368 deletions), and 11,437 SVs (7156 insertions and 2418 deletions) were identified in the XC chicken genome. SNPs, Small INDEL and SVs were located in 9732, 2710, and 397 genes, respectively (not public).

All the earlier-mentioned data are vital for population genetics and further studies on chickens, and they serve as a valuable resource for investigating diversifying selection and candidate genes for selective breeding in chickens.

4. Linkage and association study of appearance traits in Chinese local chicken breeds

China Agricultural University's (CAU) chicken resource population was derived using an F_2 design from reciprocal crosses between Silkie and White Plymouth Rock chickens. The Silkie is considerably different from other breeds with its feathers and black skin. The feathering is soft and downy, covering practically the whole body with the exception of the beak. Some Silkies have a crested head and are bearded and muffed The Silkie has a bluish-black beak, black eyes, fifth toe, small wattles, and very small walnut or cushion combs. The Silkie are known to go broody and lay few eggs. Using this F_2 population, that crest phenotype of Silkie is located on the E22C19W28 linkage group, and that it shows complete association to the HOXC-cluster on this chromosome by linkage analysis and genome-wide association [60]. Other several different appearance traits have been identified and located such as pigmentation [61], rose comb [62], silky [63], Polydactyly [64], muffs, and beards [65] in Chinese Silkie chicken and other breeds.

Dongxiang chicken is from Dongxiang town, Jiangxi province of China. It is characterized by blue eggshell, single comb, and black feather. Lushi chicken is another local breed laying blue-shelled egg from Lushi town, Henan province of China. For a study on blue eggshell, Chinese indigenous blue-shelled chicken breeds and an American blue-shelled breed, Araucana, were selected to use for blue eggshell study—results indicated that the blue eggshell is caused by an Avian endogenous retrovirus elements insertion that promotes the expression of SLCO1B3 gene in the uterus (shell gland) of the oviduct in chicken, and that the insertion site in the blue-shelled chickens from Araucana is different from that in Chinese breeds [66].

In our group, using F_2 resource population of Gushi chicken and Anak broiler, we established the shank color extreme phenotype mixing pool—yellow shank DNA pooling and willow shank DNA pooling and conducted 200× deep sequencing at the 10 Mb interval with Chr. Z 67.1–72.3 Mb as the core region, on the two pools by targeted next-generation sequencing at target region. By SHOREmap and differences observed in mutation sites analysis, we mapped the inhibitor of dermal melanin (Id) gene at the interval for 71.58–72.18 Mb of chromosome Z in chicken, which reduced the interval of inhibitor of dermal melanin gene and laid the foundation for mutation of willow shank in chicken. According to the results of linkage analysis, expression of tissue, and biological information, we conclude that the CDKN2A/B gene was the candidate gene of inhibitor of dermal melanin gene in chicken (not public).

5. Identification and functional characterization of copy number variations in Chinese diverse chicken breeds

A detailed analysis of the copy number variants in locally raised 11 Chinese chicken breeds identified using CGH was presented. The 11 chicken breeds (one male and one female in each breed) used in this study were the Silkie (WJ), Tibet (ZJ),Chahua (CH), Bearded (HX), Jinhu (JH), Anak (AK),Beijing fatty (BY), Langshan (LS), Qingyuan partridge (QY), Shek-Ki (SQ),

and Wenchang (WC) varieties. A total of 833 copy number variants contained within 308 copy-number variant regions were identified. Principal component analysis and agglomerative hierarchical clustering revealed the close relation between the four locally raised chicken breeds, Shek-Ki, Langshan, Qingyuan partridge, and Wenchang [67].

In 2014, we reported a genome-wide analysis of CNVs in five chicken breeds including XC, SK, LS, GS chicken, and one French commercial breed, Houdan chicken (HD) by aCGH. A total of 281 CNVRs across the WUGSC2.1/galGal3 genome sequence was identified, while 216 (76.87%) CNVRs were reported for the first time in our study. A total of 231 genes within the identified CNVRs were retrieved from galGal4 database. Additionally, 83 CNVRs partially or completely overlapped with 143 QTLs, which involved in many important traits including growth traits, carcass traits, meat quality traits, reproductive traits, and disease-related traits. In EDN3 locus, we concluded that there were heterozygote Fmfm and homozygote Fmfm of black skin genotype in XC chicken. Then our results confirmed that this EDN3 locus may be a molecular marker to selection of skin color in poultry production [68].

Two copy number polymorphisms (CNPs) related to different traits in the genome level were identified in chickens by AccuCopy® and CNVplex® analyses. Notably, five white recessive rock (CN = 1, CN = 3) variant individuals and two Xinghua (CN = 3) variant individuals contained a CNP13 (chromosome 5: 10, 500,294–10,675,531), which overlapped with SOX6. The results of Q-PCR and knockdown of the SOX6 suggest that the number of CNVs in the CNP13 is positively associated with the expression level of SOX6 [69].

6. Conclusion

To date, many complete and partial genome-wide scans for genetic variation in Chinese local chicken have been published. Appearance trait is one of important traits due to the old Chinese diet culture, especially in chickens. In this report, analysis of linkage and association has been shown to be effective at identifying appearance traits including black skin, crest, shank color, pigmentation, rose comb, silkie, toe numbers, shank feather, muffs, and breads in Silkie chicken, Xichuan chicken, Lushi chicken, and other Chinese chicken breeds, and molecular marker of these traits have been developed and applied in the breeding programs. On the other hand, many Chinese native breeds are characterized by slow growth, late maturity, and low production performance. In terms of genetic variation and effect for these traits, genome-wide association studies (GWAS) have been deemed successful for identifying statistically associated genetic variants of large effects on complex traits. Past studies have found enrichment of trait-associated SNPs in functionally annotated regions. However, no systematic examination of connections between genomic regions and predictive ability of complex phenotypes has been carried out. Overall, although lot of efforts has been taken and a variety of assays were developed, very few of them are successfully applied in breeding and selection. The reasons are in addition to low heritabilities, the polygenic nature, and the strong environmental influences on these traits. For further research, fine mapping of QTL regions should be extended in order to narrow QTL intervals to reduce the number of positional candidate genes with regard to quantitative trait. A combination of fine mapping and candidate gene

approaches for promising chromosomal regions is a straightforward strategy. At present, whole-genome prediction methods allow predicting complex traits, irrespective of knowledge of their molecular basis. This suggests that whole-genome prediction methods are able to capture signals from the most useful genomic regions. Thus, use of all markers of genome wide seems the way to go, if interest is on prediction of complex traits.

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Conflict of interest

The author has no conflicts of interest to report.

Notes

It should be noted that there has been a large number of studies on genetic variation in Chinese native chicken population, especially on candidate gene for production traits. However, due to limited space, here, we can only introduce some of the main research content.

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Chapter 2

Selection Methods in Poultry Breeding: From Genetics to Genomics

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Additional information is available at the end of the chapter

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Abstract

Scientific and technological advancements have led to great expansion of poultry sector in last few decades. The development of genetically superior stocks capable of higher production, even under adverse climatic conditions, has transformed poultry from rural farming to full-fledged industry within 30–35 years. Increase in production volume and productivity per bird may largely be attributed to the combined crossbred and purebred selection (CCPS). The superior purebred lines were evaluated for their nicking ability by specialized cross-breeding program, and the best nicking male and female lines were used for developing four-way commercial crosses. With advancement in molecular techniques, the DNA marker technology emerged as a finer tool for assessing the genetic variability. Genome-wide scan using microsatellites led to identification of quantitative trait loci (QTL) for their use in marker-assisted selection (MAS). Subsequently, the single nucleotide polymorphisms (SNPs) were discovered as third generation of genetic markers. Recent "next-generation sequencing" technique led to the development of high-density SNP arrays as powerful tool for genetic analysis. Predicting genomic estimate of breeding value (GEBV) of individual using SNPs across the whole genome paved way to conceptualization of "genomic selection" which emerged as the most advanced technology to revolutionize the animal production.

Keywords: quantitative genetics, purelines, microsatellites, SNP, next generation sequencing

1. Introduction

Identifying the superior animals based on performance or phenotype, for breeding, has been practiced since ages. Of course, this practice was followed without the knowledge of underlying

principles of genetics. G.J. Mendel in the year 1866 demonstrated that "factors" (now called genes) are responsible for the inheritance of characters from parent to offspring and "law of heredity" proposed by him, gave the scientific bases for inheritance. Mendelian traits are determined by a single gene and were described as qualitative traits, which follow discontinuous distribution in population and may be subjected to standard genetic analysis. In contrast, the other class of traits exhibits gradual variation following a continuous pattern in population, e.g., body size, milk yield, wool yield, etc. Such traits were described as quantitative traits. Later, Fischer [1] explained the inheritance and variation in quantitative traits as simultaneous segregation of many Mendelian factors (now called genes). The quantitative traits follow multigenic inheritance and each has small allelic and additive effects with substantial environmental influence on phenotype. Mostly, the performance traits are quantitative in nature and controlled by many genes. Therefore, for these quantitative traits, identifying or selecting the superior individuals, different procedures based on theories of quantitative genetics principles were developed. The statistical models and selection theory used in animal breeding are based on the infinitesimal genetic model of quantitative genetics [2]. For quantitative traits the genotype is not able to be observed, it can only be measured through phenotypic value. As a result, specific knowledge of the genetic architecture is not essential for these phenotype-based methods to be effective. The infinitesimal model assumes the trait is affected by a large (infinite) number of unlinked genes with very small and additive effects. But, infinitesimal model has limitations as most of the assumptions of this model are known to be false with regard to the poultry genome [3]. The number of loci in the poultry genome is finite. Directional dominance and linkage may affect the normality of distribution. Economic traits in poultry have considerable genetic nonadditivity. Low-frequency genes with large negative effects have been observed for some fitness traits [4] and many examples of major genes affecting economic traits exist [5, 6].

Expected Breeding Value (EBV) estimated from phenotype has been effective in implementing the selection program and achieving the genetic improvement over the generations. But, a lot of limitations are also being faced *viz.* the ability to accurately and timely recording the phenotypes on candidates and/or their close relatives; the cost of recording the data; and onset of most of the production traits late in life hampers genetic progress per unit time. Heritability estimate of the trait is decisive in deciding the method of selection to be practiced. In long-term selection, the additive genetic variation keeps reducing over the generations resulting in reduced estimation of heritability, and genetic gains in each generation. This necessitates the need of evolving finer tool to assess the genetic variation more accurately, which would help in accurate assessment of the breeding value of the individual. Recent molecular tools may be effectively used for assessing the variations at the genomic level and estimating the breeding values of an individual.

2. Selection methods

The selection and breeding program in poultry has been changing as per the knowledge gained and the needs. In 1940s, the individual poultry flocks were evaluated and after retaining the

selected birds, the surplus culled birds were sold as a terminal product. The concept of two-, three-, or four-way crosses in poultry was adapted from maize improvement program in 1980s, which transformed the poultry breeding for production of the high-yielding modern layer and broiler strains. The purebreds were also replaced by commercial hybrids as terminal cross as well the specialized egg and meat type birds replaced dual type birds. The negative correlations in production and reproduction traits necessitated the need of development specialized male and female lines both in layer stocks and broiler stocks. These specialized lines were developed in meat type stocks [7] and egg type stocks [8]. These specialized male and female lines usually have very different foundation genetic sources [9]. Cornish Game breed was most favored for developing a male line of meat type chicken, whereas developing female lines Plymouth Rock (barred, Columbian, or white) breeds were the most chosen ones for producing commercial broiler across the world. Similarly, for developing brown egg layer male lines, predominantly Rhode Island Red and New Hampshire were used. Plymouth Rock lines were used as female lines. For developing, white shelled egg layers varieties of White Leghorn were used as male and female lines. The modern commercial lines across the world are now a composite of the founding breeds having minor contributions from other suitable breeds [9].

The present poultry breeding, therefore, involves both pure-line selection (PLS) and crossbreeding program. The selection in poultry is therefore combined crossbred and purebred selection (CCPS). Purebred performance and crossbred performance (r_{pc}) are treated as genetically correlated traits assuming the infinitesimal model [10]. Depending on genetic parameters like heritability estimates and correlations, the method such as phenotypic selection primarily followed for improving body weights, whereas for egg production, the index selection (Osborne index) was employed in PLS. The number of traits is now included in selection program, the modern programs, therefore, rely on breeding value estimation with animal model best linear unbiased predictor (BLUP).

i. Pure-line breeding for development of specialized lines:

Specialized sire and dam lines were developed through unique selection program based on different set of traits for sire and dam lines. The dam lines are selected for their reproductive performances, e.g., egg production, egg size, egg weight, shell quality, age at sexual maturity, and hatchability besides juvenile growth. The sire lines are primarily selected for improving the rate of growth, body confirmation, feed conversion ratio, and carcass quality and fertility. Therefore, with the involvement of these specialized lines in the development of commercial broiler stocks thrive toward lowering the cost of production. Crossing of these genetically diverse lines results in gene recombination producing a heterotic effect in progeny for different economic traits. Therefore, intense selection within pure-lines and crossing those genetically diverse lines is the most characteristic feature in broiler breeding program. While practicing the artificial selection, care is taken to minimize the inbreeding, and its related consequences in the population. A control population with the same increment in inbreeding as the selected population may be maintained for comparison and evaluation of the selected population.

a. *Layers*: for layer, the objectives primarily is "To obtain maximum number of saleable eggs per hen housed at low feed cost per egg or per kg egg mass and the eggs should have optimal internal and external qualities. Stock should have low mortality and high adaptability to different environments." Layer breeders apply selection to improve over 30 traits important for commercial egg production. Breeders today select for (or at least monitor) the age at sexual maturity, the rate of lay, livability, egg weight, body weight, feed conversion, shell color, shell strength, albumen height, egg inclusions (blood and meat spots), and temperament. The selection strategies to improve egg production include part-time egg production records, persistency of lay, clutch length, FCR/Residual feed consumption (RFC), skeletal problems (majorly osteomalacia and osteoporosis) [11].

b. *Broilers*: for broilers, selection strategies concentrate on rapid growth and carcass traits. The most practiced strategy for broiler PLS is "selection at commercial weight," which employs selection at a weight that matches the market weight and the age at selection becomes progressively earlier as growth potential increases. The other two strategies are the selection at a commercial age and multi-stage selection. Different breeding and selection technologies at different period of time were employed for the genetic improvement of poultry (**Table 1**). Breast muscle weight, meat quality, and FCR are major traits; in addition to these, thrust is also being given on skeletal abnormalities, metabolic disorders and welfare. The selection basing on breast area measured through length and width of the breast using a pachymeter along with body weight resulted in a genetic gain of 277% per generation while keeping feed conversion and fertility in the actual levels. The nondestructive means like needle catheters, ultrasonic apparatus etc. were found more accurate for measuring the thickness of the breast muscle. The other non-invasive methods like computed tomography scan (CT scan), magnetic resonance imaging (MRI), and echography are more accurate for measuring the muscle thickness and dimensions of internal organs etc., but these methods are far more expensive [13, 14]. Therefore, among the various non-invasive means, ultrasound offers a viable and advanced solution for breast muscle analysis [15]. In developing or maintaining a strain of broilers, geneticists must consider a balance of characteristics related to growth versus reproduction (**Table 2**).

Utilization of these specialized sire and dam lines in commercial layer and broiler enterprises minimizes the production cost and the gene recombination in these crosses produced a heterotic effect in progeny for different economic traits. While practicing the artificial selection, care is taken to minimize the inbreeding, and its related consequences in the population. A control population with the same increment in inbreeding as the selected population may be maintained for comparison and evaluation of the selected population.

(ii) Combined crossbred and purebred selection

Development of synthetic lines using specialized selection program and their utilization through cross-breeding has been the vital tools for the progress made in poultry production.

Exhaustive literature suggests that including the information recorded on pure as well as crossbreds in selection criterion helps in the improving response to selection in crossbreds

Technique/methodology	Decade
Mass selection	1900
Trap nesting	1930
Hybridization	1940
Artificial insemination	1960
Osborne index in layers	1960
Family feed conversion testing	1970
Selection index	1980
Individual feed conversion testing	1980
BLUP breeding value estimation	1990
DNA markers	2000

c.f: [12].

Table 1. Journey of selection methodology through time.

Growth-related traits	Reproduction
1. Growth rate	1. Egg number
2. Weight-for-age	2. Egg size
3. Feed efficiency	3. Hatchability of fertile eggs
4. Meat (breast) and carcass yield and body conformation	4. Fertility
5. Livability	5. Libido
6. Skeletal integrity	6. Mature weight and age
7. Feathering-cover, rate, and color	7. Aggressiveness (±)
8. Adaptation to heat distress	8. Adaptation to heat distress

Table 2. Characteristics most often considered in selecting pure-line breeders [16].

[17–20]. Using the information of both pure as well as crossbreds, the estimated breeding value (EBV) for r_{pc} can be determined, which may also be used as basis for selection [18, 19]; such a selection strategy is known as combined crossbred purebred selection (CCPS). The instance where the selection is based only on the information obtained on pure-line is called as PLS. Genetic correlation between purebred and r_{pc} and heritability of crossbred (h^2_C) are important parameters for optimizing and evaluating crossbreeding systems [21], especially when applying a combined crossbred and purebred selection method to achieve genetic progress in crossbreds [19]. When estimating breeding values for the purebred selection candidates, the information on their crossbred half-sibs can be included in the EBV, which results in a higher accuracy of selection [10]. Bell [22] suggested that r_{pc} is the most reliable indicator of the relative merit of information obtained on purebred versus that received from crossbred when selecting for r_{pc} . When the breeding goal is r_{pc} and the genetic correlation between

purebred and r_{pc} is low, the information coming from crossbred half sibs will dominate the EBVs of selection candidates [10]. Low or negative estimates of r_{pc} indicate the existence of non-additive genetic effect suggesting that reciprocal recurrent selection (RRS) will be more effective. Superiority of CCPS over PLS increases and over crossbred selection (CS) decreases with decreasing r_{pc}.

Wei and van der Werf [19] compared the CCPS was compared with PLS and CS methods. The CCPS was found better than PLS or CS when a fixed number of purebred progeny is tested. However, at very high values of r_{pc} (>0.8) CCPS was worse than PLS. The lesser the estimate of r_{pc} the higher the superiority of CCPS over PLS and decreases over CS. Response of CCPS and CS increase with increasing estimate of h^2_C is (relative to an estimate of purebred heritability). At decreasing values of r_{pc} the difference between actual and optimal response increases but at large r_{pc} values it is small (e.g., for $r_{pc} > 0.7$, the difference between responses is <3%). Furthermore, the expected response has been found to be smaller than the actual response at large values of r_{pc} and $h^2_C > h^2_P$. Finally, for positive values for r_{pc}, the actual response to CCPS is larger than the optimal response to PLS.

The modern commercial poultry strains sustaining the present day production have been developed by crossing the selected parent lines. Crossbreeding exploits both additive and non-additive gene action thereby tends to increase heterozygosity. The resulting crossbreds, therefore, are expected to have uniformity and are least influenced by environmental factors compared to their parent lines. The stocks that complement one another effectively, crossbreeding is the most economical method for combining them.

For the successful crossbreeding program, estimation of crossbreeding parameters and identifying the superior cross combination of lines is essential. A number of experimental design e.g., diallel cross analysis, three-way cross analysis, analysis of double-cross hybrids, line x tester analysis, north Carolina designs, recurrent selection, and RRS have been designed to estimate crossbreeding parameters. Of these diallel or partial diallel cross have been most extensively used for estimation of general and specific combining abilities, which have helped in maximizing the genetic gains through identification of best lines and cross combinations. Systems such as RRS [23] are being widely used for evaluations of purebred and crossbreds. Statistical tools continue to evolve and their improvements have been a hallmark of the continued success of genetics applied to animal breeding [24]. Presently, the most efficient selection method employs the BLUP as a statistical tool. The data from different sources *viz.* individual's phenotype data and family information in a pedigree matrix, may be combined and analyzed.

(iii) Evaluation of crosses under specific climatic conditions (G × E interactions)

As the phenotype depends on genotype and environment, the environmental effects also need due emphasis while selecting the stocks, and planning the breeding strategy. The ultimate aim of the breeding scheme is to evolve a commercial cross that performs optimally under specific climatic conditions. Therefore, the cross needs to be evaluated under specific climatic condition before releasing it for commercial exploitation.

For a better understanding of G × E, it is important to differentiate microenvironment from macro-environment and also the intrapopulation genotypes from interpopulation genotypes. Diets, ambient temperature, and climatic differences between seasons and regions constitute macro-environments. However, within population, random environmental differences are categorized as micro-environments. Therefore, for deciding breeding strategies particularly between wider ranges of environments, the phenomenon of G × E interactions is important to be considered. The success of particular cross in a particular environment depends on its ability to adapt and perform in particular environment or climatic zone.

The available evidences for G×E interactions in performance analysis of modern broilers and various suboptimal conditions emphasize the need for breeding programs aimed at improving the performance under particular environment. The potential importance of G × E interactions to both the poultry breeder and the producer appears to have been recognized as early as 1936 by Munro. Most of the experiments reported have compared layer and broiler chicken, commercial hybrids, purebreds vs. crossbreds have kept under different rearing and housing systems, climatic conditions etc.

3. Molecular approaches

After discovery of double helical model of DNA, the molecular genetics approaches started making a humble beginning. The advent of the era of molecular genetics in 1970s provided new opportunities to enhance breeding programs through the use of DNA markers associated with traits of interest. Number of type I markers *viz*. RFLPs, ESTs, and SNP and type II markers such as RAPDs, micro- and minisatellites, AFLP, etc. were identified. Because of being highly polymorphic and abundant in the genome, the type II markers are more preferred ones, however, the use of SNPs, the third generation marker is also becoming popular in various genetic applications including.

i. *QTL identification and genome wide scans*: the genetic control of quantitative traits is expected to be distributed throughout the genome and the numerous regions of the genome, which control the quantitative trait of interest, were described as quantitative trait loci (QTL). These QTLs were identified using specialized experimental crosses, which were specifically developed for the purpose. The identification of QTL and the development of DNA tests were the important steps in the practical application of QTL through marker-assisted selection (MAS) i.e., selection on a combination of information derived from genetic markers associated with QTL and the traditional phenotypic information. Most of these QTL searches were done using 200–350 MS markers and crosses between very diverse breeds, such as heavy meat-type birds and lighter egg-laying varieties or specialized inbred lines [25]. The implementation of MAS in breeding programs was, however, limited for various reasons [26], *viz.* (a) Majority of work on QTL is restricted to experimental crosses having wider linkage disequilibrium rather than original populations undergoing genetic improvement program. (b) The effects identified by QTL analysis are able to explain a limited amount of genetic variation affecting a trait. (c) Due to

the several genetic and non-genetic variations as well as interactions, replication of many associations determined through QTL analysis are difficult to be replicated. And (d) the high-cost of routine genotyping yielding few markers also limits the application of QTL analysis in large breeding operations. Existence of negative correlations between traits of commercial interest also hurdles MAS.

ii. *Candidate gene approach*: the "candidate genes" are the gene with direct and large effect on the trait of interest. Basing on prior information certain gene (the candidate) may be hypothesized to be responsible for a known major genetic effect. The sequence variations in that gene are identified and then finally various alleles are associated with variation in a trait(s). The genes which are directly associated with production traits like growth hormone (cGH), growth hormone receptor (cGHR), insulin-like growth factor-1 (IGF-1), IGF-1R,TGF betas, myostatin, etc. have been the candidate genes analyzed and molecular markers like SNPs, indel/dels were identified [27–35]. Three physiological candidate genes (i.e., genes for cGH) and gonadotropin-releasing hormone receptor (GnRHR) and neuropeptide Y (NPY) were analyzed to find out their association with egg production, number of double yolk egg, and age at first egg [36, 37]. SNP and deletions were detected in these genes [36–38], and Polymerase chain reaction-restriction length Fragments (PCR-RFLP) was done to determine genotype frequency.

iii. *High-density SNP genotyping for whole-genome selection*: development of "next-generation" sequencing technologies and high-throughput genotyping platforms has led to the creation of high-density SNP array as a state-of-the-art tool for genetics and genomics analyses of domestic animals. The most promising applications of these arrays in agriculture could be genomic selection for the improvement of economically important traits [39]. Genomic selection is an advanced form of marker-assisted selection (MAS), which concentrates on all markers across the whole genome [40, 41]. It precisely predicts the breeding values of animals by utilizing the information related to the distribution of abundant SNPs across the genome (genomic estimated breeding value, GEBV), with an assumption that abundant SNPs are scattered throughout the genome and there exist LD relationships between SNPs and QTL. The large number of SNPs essentially required for the design and construction of arrays can be obtained through different methods and resources e.g., predicted SNPs generated from genome sequencing and HapMap studies, completing reduced representation library (RRL) sequencing [42, 43] downloading SNP information from dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/projects/SNP/) etc. The candidate SNPs for array design should be validated and have high minor allele frequency (MAF) in the testing populations. The two biggest and most competitive SNP chip genotyping platforms are Illumina's BeadArray based on single-base extension or allele-specific primer extension (http://www.illumina.com) and Affymetrix's GeneChip based on molecular inversion probe hybridization (www.affymetrix.com). Currently, majority commercially released SNP arrays for domestic animals (dog, cattle, horse, pig, and sheep) are constructed using the BeadArray platform with Illumina's iSelect Infinium technology [39]. Aviagen started developing its first SNP panel for chicken; the chip density increased from 6 K [44], to 12 K [45], 42 K [46], and ultimately to 600 K SNPs [47]. Chicken 60 K SNP array (Illumina Inc., San Diego, CA) [48] was developed using financial assistance from two breeding companies (Cobb-Vantress, and Hendrix

Genetics, the Netherlands), which became proprietary. Heavy restrictions were imposed on its availability to non-academic samples [25]. A second chicken SNP chip (42 K SNP, Illumina Inc.) was subsequently developed completely with private funds [EW Group (Visbeck, Germany), consisting of Aviagen (Huntsville, AL), Hy-Line International (West Des Moines, IA), and Lohmann Tierzucht (Cuxhaven, Germany)]; this was also not available publically [25].

Capitalizing on historical linkage disequilibrium (LD) detected from a genome wide association studies (GWAS), the major QTLs were identified, which utilized to implement MAS. However, the other limitations such as difficulties in detecting and validating QTL and finally the larger proportions of genetic variance for the main quantitative traits of economic importance still remain unexplained, hurdling its application. Several alternate statistical methods have been used in GWAS for determining the association of SNPs with QTL. Single SNP models which employ fitting of each SNP separately as a fixed effect has been most extensively used. The BLUP animal model that accounts for the family structure of the data by fitting a polygenic effect with pedigree-based relationships, found most suitable [49, 50]. Hayes et al. [51] used the mixed linear model methodology to estimate the proportion of genetic variance associated with each genomic region of 50 SNP. The Bayesian methods that have been developed for genomic selection have also been used for GWAS. Several criteria have been used to identify important SNP or genomic regions using these methods current models for genomic selection and GWAS primarily fit additive models but Bayesian variable selection models that fit dominance [52] and even epistatic effects [53] are available or possible. Genomic selection models do not solve the problems of low-accuracy for traits with low heritability and a limited number of records [54]. This is especially advantageous in breeding programs for layers where there is no information available on males before they have records on offspring performances [55]. Application of single step genomic prediction in general leads to increased accuracy of predicted breeding values for both genotyped and non-genotyped individuals in the broiler [56]. An alternative derivation of the single step prediction model based on Bayesian principles were presented by [57]. The main challenge to genomic selection was the high cost of large-scale genotyping due to in large breeding populations and despite the cost of genotyping per SNP is reduced; the overall price per selected candidate that is to be genotyped was relative stable since the density was increasing. The main snag with genomic selection is that, it phenomenally incurs a huge cost for application on the large-scale basis as in involves higher selection candidates. Even though on individual basis SNP cost is reducing, this may not witness the same in overall cost, which is attributed to its relatively high density. Genotyping the animals with a sparse panel comprised of equally spaced markers [58]—the low-density strategies for genomic selection offered a viable solution to the problem.

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Chapter 3

Transgenesis and Genome Editing in Poultry

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Additional information is available at the end of the chapter

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Abstract

The transgenic approach and precise editing of specific loci in the genome have diverse practical uses in animal biotechnology. Recent advances in genome-editing technology, including clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) have helped to generate highly valuable and quality-improved poultry. The production of transgenic and genome-edited birds mainly depends on primordial germ cells (PGCs), which are the progenitor cells of gametes, due to the unique system that is quite different from the mammalian system. This chapter introduces the basic physiology of avian PGCs and the latest PGC-mediated methodologies in transgenesis and genome editing of birds. Based on these techniques, future applications of precisely genome-modulated poultry are discussed to provide opportunities and benefits for humans.

Keywords: avian primordial germ cells, CRISPR/Cas9, genome editing, poultry, transgenesis

1. Introduction

The ability to genetically modify and precisely edit the genomes of animals has revolutionized various fields in which the genotypes, phenotypes, and traits of animals can be easily modified. Traditional animal breeding has been dependent on selective or artificial breeding for improvements in productivity, food quality, and other economical traits of the offspring [1]. However, transgenic and precise genome-editing tools facilitate improvements in genetic traits of animals when combined with conventional breeding systems. Recent technological progress of programmable nucleases, particularly the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, has enabled much higher frequencies of homologous recombination events and targeted mutagenesis through

a highly efficient generation of double-stranded DNA breaks (DSBs) in specific regions and genetic modifications at targeted loci in the genome [2, 3]. This system has been adopted for programmable genome editing in various organisms, including humans [4]. More recently, programmable one-base pair conversion into another without DNA cleavage has been reported [5, 6]. This novel base editing system overcomes the low efficiency of correcting a point mutation using Cas9-mediated genome editing by delivering a homology-directed repair (HDR) donor template.

Germ-line modification is essential for the application of transgenic and genome-editing technologies in animals and to transmit modified and improved genetic traits from generation to generation. Germ-line modification methods differ between mammals and birds. The first transgenic mouse was generated by microinjecting the target DNA into the pro-nucleus of a fertilized embryo [7]. Livestock, including rabbits, sheep, and pigs, have been genetically modified using this technique [8]. This classical strategy is still widely used in animal transgenesis despite several disadvantages, such as the low efficiency of producing founder animals and the random integration of foreign DNA. Another popular method is the use of embryonic stem cells (ESCs) to modify the germ line, especially in mice (**Figure 1a**). Following genetic modification of ESCs in vitro, the cells are injected into the recipient blastocyst. Then, germ-line chimeras composed of germ cells originating from both endogenous and exogenous sources are produced to generate transgenic offspring derived from genetically modified ESCs [9, 10].

Unlike mammals, a unique system is used for transgenesis and genetic modification in avian species (**Figure 1b**) due to their oviparity and the physiological properties of the ovum [11]. As the avian zygote is surrounded by a large amount of yolk and a small germinal disc, introducing foreign DNA or microinjecting avian ESCs into the zygote is quite difficult [12–14]. Alternatively, the first transgenic chicken was produced via retroviral injection into the subgerminal cavity of

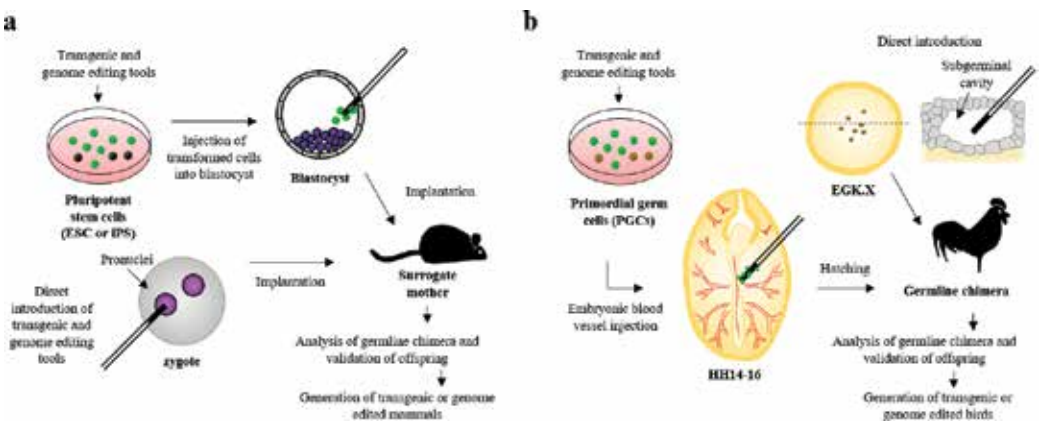

Figure 1. The different transgenic and genome-editing system between mammalian and avian species. (a) In mammals, transgenic and genome-edited systems are based on direct introduction of genome-editing tool into the zygote or microinjection of genome-edited ESCs into the recipient blastocyst. (b) In aves, those systems can be applied via injection of genome-edited primordial germ cells (PGCs) into the blood vessel of recipient. This figure is adopted from [135].

Eyal-Giladi and Kochav (EGK) [15] stage X embryos [16]. Then, various strategies, including viral infection of stage X embryos [17–19], microinjection of transgenes into fertilized eggs [14, 19], and ESCs [20], have been applied to produce genetically modified transgenic birds. However, because of the low efficiency of germ-line transmission, these strategies have not been appropriate for the production of genome-modified birds until recently. As an alternative cell source comparable to ESCs in mammals, primordial germ cells (PGCs) in avian species have overcome this limitation [21]. Here we present an overview of PGC physiology, recent advances in transgenesis and genome editing, and potential strategies for programmable genetic modulation in poultry.

2. Physiological overview of avian PGCs

2.1. Early development of avian PGCs

Since the first examination of the origin of PGCs in chicken germinal epithelium [22], chickens have been used as a valuable germ cell model (**Figure 2**). In initial studies on the origin of avian PGCs, only the central region of the blastoderm was considered to give rise to PGCs [23, 24], until the discovery and tracing of the chicken VASA homolog (CVH) in 2000 [25]. CVH is used as a PGC marker during the early developmental stages in chickens. CVH mRNA and protein expression can be consistently detected during early embryogenesis, from functional oocyte to fertilized embryo. The CVH protein was observed in granulofibrillar structures surrounding the mitochondrial cloud and the spectrin protein-enriched structure of oocytes, suggesting a CVH-containing structure in the germplasm of chickens. During early cleavage, CVH was found in cleavage furrows and restricted to about 6–8 cells at the 300-cell stage. According to

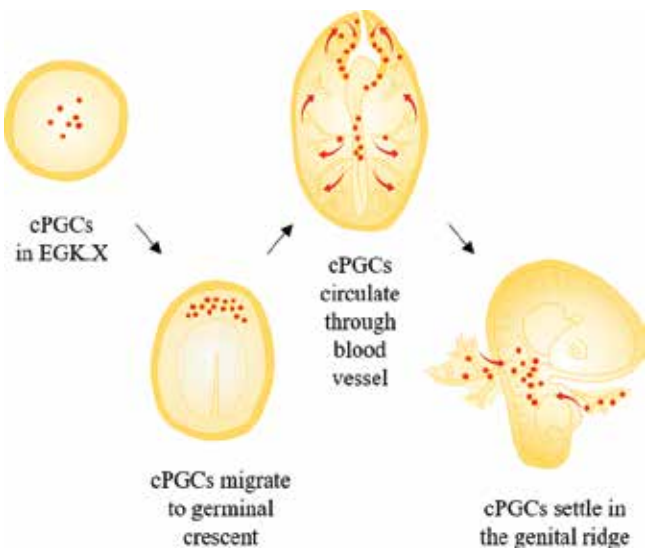

Figure 2. Schematic representation of the development and migration of PGCs in chicken. Chicken PGCs (cPGCs) are dispersed at stage X and move to the germinal crescent at HH stage 4. They then undergo circulation via extra-embryonic blood vessels until settlement in embryonic gonads at HH stage 17. This figure is adopted from [135].

these observations, the specification of germ cells in chickens seems to follow a pre-determined model from maternally inherited material. More recently, another germ cell marker detected in various species, deleted in azoospermia-like (DAZL), was identified in pre-PGCs of chicken embryos during intrauterine development prior to oviposition [26]. Using DAZL as an early germplasm marker, the germ granule was determined to be asymmetrically localized in oocytes, with a shift to a diffused form during early cleavage when the zygotic genome is activated. Moreover, knockdown of DAZL expression in chicken PGCs affects germ-cell integrity, such as proliferation, gene expression, and apoptosis. These findings further demonstrate that the origin of PGCs in birds is mediated by maternally inherited determinants, which is required to examine specific functions of germplasm components and to clarify the mechanisms.

In vertebrates, germ cells arise in a specific region of the embryo and then migrate to the genital ridges during early development [27]. Avian PGCs are clustered and derived from the epiblast layer [28, 29]. Then, the PGCs migrate toward the germinal crescent region at Hamburger and Hamilton (HH) stage 4 [30–32]. The PGCs are located in this extraembryonic region from HH stages 4–10 during formation of the primitive streak [15, 31, 33]. Using chicken fibroblast cell-line DF-1 and PGC transplantation into the embryos, PGCs are shown to passively reach the anterior region but, later, are actively incorporated into the germinal crescent compared to DF-1 [32]. However, the detailed mechanism of active migration, which may be guided by attractive and repulsive cues, remains poorly understood. Several studies have shown that migrating and in vitro-cultured PGCs generate pseudopodia, suggesting germ-cell migration in birds occurs via amoeboid movement [34–36]. Subsequently, PGCs enter the embryonic blood vessels through the anterior vitelline vein during HH stages 10–12 [37, 38], contrary to mammalian PGC migration from the hindgut endoderm to the mesentery [27, 39]. The PGCs enter blood vessels and are most abundant at HH stage 12 [35, 40]. PGCs circulating in the embryonic bloodstream start to settle in the genital ridge and invade the thickened coelomic epithelium during HH stages 15–18 [41, 42]. Research suggests that coelomic epithelium releases a chemical cue to attract PGCs to the gonads [43]. Later, the main factors guiding chicken PGCs to the genital ridges are chemokine stromal cell-derived factor 1 (SDF1) and its receptor, C-X-C motif chemokine receptor 4 (CXCR4) expressed by migrating PGCs [44]. One study revealed that the anterior vitelline vein plays a key role directly accumulating migrating PGCs, which reach the genital ridges during circulation [37]. Thus, compared with mammalian PGCs, the unique migratory pathway of avian PGCs through the bloodstream enables the generation of germ-line chimeras or genetically modified birds via an exogenous injection of PGCs into the blood vessel of a recipient embryo.

2.2. Germ-line chimera production via PGCs

Due to difficulties in the application of the mammalian system for highly efficient production of transgenic birds, many researchers have focused on improving the efficiency of germ-line transmission. In 1976, the colonization of germinal crescent-derived donor turkey PGCs was examined in recipient chicken gonads following intravascular injection, and a germ-line chimera chicken was produced from functional gametes derived from turkey PGCs [45]. In addition, PGCs from the germinal crescent have been successfully transplanted into recipient embryos to produce germ-line chimeras in quail [46]. Germ-line chimeras and donor-derived progeny

in birds have been generated by transplanted blood PGCs from the HH stage 14–16 embryos [47, 48] and gonadal PGCs (gPGCs) of HH stage 26–28 embryos [49, 50] in chickens and quail. Germ-line chimeras using cryopreserved PGCs or interspecific germ-line chimera enables the preservation of avian genetic resources and restoration of endangered bird species [51, 52]. Furthermore, endogenous PGCs are depleted in recipient embryos to improve the efficiency of germ-line chimera production. Various approaches, such as exposure to gamma rays [53], administration of busulfan to embryos [54], and removal of blood from recipient embryos at HH stages 14–15 [55], have been used to eliminate endogenous germ cells in birds. One report showed that the germ-line chimera efficiency of a busulfan-treated founder was approximately 99% [56]. These efforts have promoted the development of transgenic and genome-edited birds.

Many attempts have been made to develop an alternative system for producing germ-line chimera using other germ-line competent cells, such as blastodermal cells [57], embryonic germ cells [58], germ-line stem cells, and spermatogonial stem cells [59]; however, the efficiency of using these cells is lower than that of the PGC-mediated method. In addition to efficient germ-line chimera production, a PGC culture system, which has been optimized and proven to maintain germ-line competency after expansion in vitro [36, 60–62] despite differences in efficiency, provides many advantages of the use of PGCs in terms of transgenesis and precise genome modulation in birds. Although there are challenges to overcome, including the relationship between the in vitro culture of PGCs and germ-line competency and the absence of germ-line competency-associated markers, the PGC-mediated germ-line transmission system is the most efficient method to establish transgenic and genome-edited birds, until now.

3. Transgenesis and programmable genome editing in poultry

3.1. Primordial germ-cell isolation and in vitro culture in birds

Avian PGCs are generally obtained from three different stages, such as the germinal crescent in HH stage 4–8 embryos, embryonic blood in HH stage 14–16 embryos, and gonads in HH 26–28 embryos. Before the PGC cell-surface antigens were identified, PGCs were isolated using a density gradient-dependent centrifugation method [63, 64]. However, this method was limited due to low yield rates, purity, and viability of isolated PGCs. After the discovery of PGC-specific surface markers such as chicken stage-specific embryonic antigen-1 (SSEA-1) and the quail germ-cell-specific marker, QCR1, magnetic-activated cell sorting or fluorescence-activated cell sorting systems were used to isolate highly pure avian PGCs [65–67]. Nevertheless, isolating PGCs from wild or endangered birds, in which PGC-specific markers have not yet been fully determined, is difficult using such cell-sorting systems. To overcome this problem, a Transwell-mediated size-dependent isolation method was recently developed in various avian PGCs from HH stage 14–16 embryonic blood based on the larger size of PGCs compared to whole blood cells at that stage [68].

Since in vitro culture of PGCs without loss of germ-line competency was successfully established in 2006 [60], many studies have focused on optimizing PGC culture systems and revealing the detailed signaling mechanisms related to the proliferation and maintenance of

germ-line competency in vitro. For example, basic fibroblast growth factor is essential for in vitro proliferation and survival through the MEK/ERK signaling pathway in chicken PGCs [36, 61]. Furthermore, in vitro self-renewal of chicken PGCs requires complex pathways composed of MEK1, AKT, and SMAD3 signaling to maintain germ-line competency [69], and Wnt/β-catenin signaling is also required for the proliferation of PGCs in vitro [70]. This system could be developed for various avian species and would be useful to apply to PGC-mediated avian transgenesis and genome editing.

3.2. PGC-mediated transgenesis in birds

Before the establishment of in vitro PGC culture systems, the major method for transgenesis in birds relied on injecting viruses into EGK stage X embryos. The first transgenic chicken was generated by microinjecting recombinant avian leukosis virus into the subgerminal cavity of EGK stage X embryos [16]. In addition, transgenic quail were produced using direct injection of a replication-defective retrovirus into the embryonic blastoderm [71]. Due to frequent silencing of the transgene, which is randomly integrated in the genome of the transgenic animal [17, 71–73], the lentivirus-mediated method has been recognized as the most efficient viral transduction system for avian transgenesis. This system successfully produces diverse transgenic chickens without silencing gene expression [74–77]. In the case of zebra finch, microinjecting lentivirus into blastodermal stage embryos generated the first transgenic finch expressing green fluorescent protein (GFP) [78]. The transgenic birds, using PGCs from the germinal crescent of HH stage 5 chicken embryos, was firstly produced [79]. Furthermore, gPGC-mediated transgenesis in quail was successfully established via the lentiviral system [80]. In that study, although the efficiency of the gPGC-mediated method was comparable to the blastoderm-mediated method in quail, the production of transgenic birds through viral infection into non-cultivated PGCs after purification has been demonstrated.

Much effort has also been made to develop non-viral transgenic systems without PGCs, such as sperm-mediated gene transfection [81, 82] and direct microinjection of transgenes into fertilized eggs [14]. However, these approaches appear to have low germ-line chimerism and transmission efficiency compared with that of the PGC-mediated method. Furthermore, because of long-term *in vitro* PGC culture systems, it is possible to develop more optimal approaches to produce genetically modified birds compared with other germ-line cells. Combined with a culture system, the highly efficient non-viral transposable systems, such as *piggyBac* and Tol2, have been developed for stable transgene integration into the genome of chicken PGCs [83, 84]. The transgenic efficiency in cultured PGCs using lipofection or electroporation is remarkably higher than that of the virus-mediated methods to produce transgenic chickens. Moreover, using site-specific gene cassette exchange in transgenic chicken genomes via PGCs with the flippase recombinase system was introduced [85]. Alternatively, transgenic birds have been produced by direct transfection into circulating PGCs at HH stages 14–16 [86–88], although transgenic efficiency is usually lower than that of the cultured PGC-mediated method. This approach could be applied to establish a transgenesis system in avian species, as PGCs are difficult to manipulate in vitro in birds.

3.3. Precise genome-editing tools

Programmable DNA nucleases, such as ZFN and TALEN, have made genome editing in the target region possible over the last decade. Briefly, ZFN is the first programmable genome-editing tool. ZFN is a fusion protein with zinc finger proteins that bind to specific DNA and nuclease domains, such as the FokI endonuclease [89]. The second generation of programmable genome-editing tools is TALEN, in which the TAL effector of a DNA-binding domain derived from *Xanthomonas* is fused with a DNA cleavage nuclease domain [90]. Generally, ZFN and TALEN specifically recognize target sequences, resulting in the generation of DSBs to enable efficient gene targeting in specific genomic loci compared with natural homologous recombination, although the DNA binding mechanism is different between the systems. Compared to ZFN, TALEN is a more flexible tool for editing genome sequences in the target site because the TAL effector contains one repeat domain that binds to one nucleotide each [91]. Furthermore, customized TALEN can be easily synthesized using an assembly kit for precise genetic modifications [92–94].

The CRISPR/Cas9 system is considered the most revolutionary tool and has been developed to carry out highly efficient and specific genome editing as a simple RNA-guided platform. This system is derived from prokaryotic DNA, which is involved in acquired resistance against exogenous plasmid DNA and phages. These bacteria possess clustered repeats called CRISPRs that bind to the viral RNA to disrupt it with the Cas9 protein to defend [95]. CRISPR/Cas9 also leads to DNA cleavage at a specifically recognized target site, resulting in the generation of DSBs, similar to ZFN and TALEN. However, unlike ZFN and TALEN, which require paired units to induce DSBs at the target region, the CRISPR/Cas9 system, a type-II CRISPR system, includes the Cas protein, CRISPR RNA (crRNA), trans-activating CRISPR RNA (tracrRNA), and a protospacer adjacent motif (PAM) sequence for targeted genome editing [2] (**Figure 3a**). The PAM sequence is in the upstream region of the crRNA-binding site and guides the Cas9 protein to the target site. The target DNA sequence is specifically recognized by the CRISPR/Cas9 complex through base pairing with a guide RNA and subsequently induces DSBs at the targeted genomic loci. Then, these DSBs activate the cell's DNA repair system, which includes random indels at the site of DNA cleavage via non-homologous end joining or replacement of a homologous DNA template in the DNA surrounding the cleavage site via HDR. Compared to ZFN and TALEN, which rely on DNA-binding specificity and were developed through expensive and time-consuming processes, the CRISPR/Cas9 system is convenient for targeted genome editing because it is extremely easy to synthesize crRNA and tracrRNA and it is easy to construct thousands of customized CRISPR/Cas9 systems depending on the targeted genes. Furthermore, the CRISPR/Cas9 system is simple and practically easy to use with robust cutting activity, leading to a fast and cost-effective system for modifying the genomes of various organisms [96, 97]. Among the ZFN, TALEN, and CRISPR/Cas9 systems, CRISPR/Cas9 is now the most powerful method to precisely edit in a targeted manner and has been applied in diverse organisms, including animals, plants, and humans [4, 98, 99].

More recently, a programmable base editing system leading to precise and efficient nucleotide conversion was developed and applied to various species to minimize DNA damage

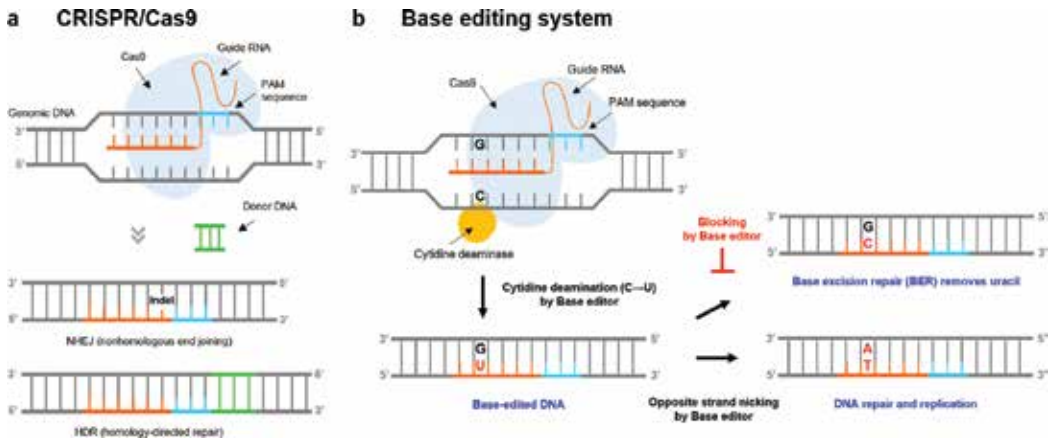

Figure 3. Principles of clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) and development of the base editing system. (a) CRISPR/Cas9 system enhances the DNA mutation through the creation of a double-stranded DNA breaks (DSBs) at a specific locus in the genome and generates highly efficient genetic modification in a targeted manner. (b) A single base editing system is generated by fusion of cytidine deaminase to the catalytically inactivated Cas9 (dead Cas9) and provides a valuable tool for precise genome editing with regard to highly targeted single-base changes. This figure is modified from [136].

and acquire point-mutation corrections without HDR donors during genome editing. The strategies for modifying a single base pair by CRISPR/Cas9 are difficult to apply for various purposes, although efficiency is 60% in cultured cells [100]. However, the base editing system is composed of a group of cytidine deaminases, including the apolipoprotein B mRNA editing enzyme (APOBEC) 1–4 or activation-induced deaminase, resulting in deamination of cytidine to uridine [101] (**Figure 3b**). The deaminases fuse with CRISPR/Cas9 substitute C in a target site with T (or G to A) without breaking the DNA [5, 102, 103]. A base editing system is advantageous because indel formation rates are <0.1% [5]. Until now, the improved base editing systems have been advanced up to four generations. The first-generation base editor (BE1) involves the rat APOBEC1 fused with the N-terminus of catalytically dead Cas9 by a 16-residue peptide of the XTEN linker [5]. BE1 converts C to U with an activity window of approximately five nucleotides. However, base excision repair removing U from DNA decreases intracellular efficiency. To increase the low-editing efficiency of BE1, BE2, which fuses uracil glycosylase inhibitor (UGI) to the C-terminus of BE1, was created. In human cells, BE2 increases the editing efficiency threefold compared with BE1. Moreover, BE3, which involves Cas9 nickase (A840H)-UGI-generated nicks in an unmodified DNA strand, results in 37% efficiency, which is from three to sixfold to that of BE2. Subsequently, BE4 was developed to increase efficiency to 50% compared with BE3 and decrease undesired products [104]. Moreover, the Mu protein Gam, which binds DSBs and protects their ends from degradation during base editing, was fused to the N-terminus of BE4 resulting in a reduction of indel frequency [104]. In addition, A·T to G·C conversion was recently developed to broaden the application of the base editing system [105]. This simple system for base conversion has been applied to precisely modify the human and mice genomes [106]. In addition, base editing has been successfully applied in various

plants [5, 6, 99, 107–113]. In the near future, the ability to modify single-base changes in the base editing system will be widely used for precise genome editing and specialized purposes by substituting amino acids.

3.4. The recent progress of genome-edited poultry

In birds, combining an in vitro culture system for PGCs and an efficient genome-editing system can produce programmable genome-edited poultry, especially chickens. Although total germ-line transmission efficiency from targeted PGCs is approximately 0.1% due to natural homologous recombination that occurs with very low frequency, the immunoglobulin gene knockout chicken was first produced via the PGC-mediated method in 2013 [114]. However, applying TALEN technology to in vitro-cultured PGCs improved germline transmission efficiency of mutant progeny to 8% of the donor-derived knockout chicks in the ovalbumin locus in 2014 [115]. This case is the first programmed DNA nuclease-mediated knockout chicken, and the TALEN-mediated gene knockout appeared to be much more efficient than the conventional homologous recombination-mediated system. Later, the CRISPR/Cas9 system was used to efficiently generate ovomucoid gene-targeted chickens by transferring transiently drug-selected PGCs into recipient embryos using gamma-ray irradiation to deplete endogenous PGCs [116]. Here, the G0 founders had 93% mutant sperm and produced 53% ovomucoid gene mutant offspring, indicating a highly efficient CRISPR/Cas9 system in birds. Furthermore, through HDR insertion of an additional loxP site into the loxP variable region segment previously inserted into the joining gene segment of the chicken immunoglobulin heavy chain (IgH) locus and Cre recombination, a 28-kb genomic DNA sequence at the IgH locus was deleted in CRISPR/Cas9-mediated genome-modified chickens [114, 117]. More recently, CVH gene-targeted chickens via the TALEN-mediated HDR system were produced using 2-week-recovered PGCs with GFP transgene knockin at the CVH locus with 8.1% efficiency [118]. They generated 6% CVH-targeted progeny from one G0 male founder showing 10% of genomic equivalents in its semen. Germ-line transmission efficiency varies among genome-edited PGC lines compared with TALEN- and CRISPR-mediated genome modification. Because of possible loss of germline competency during long-term in vitro culture and genetic modification, it is crucial to optimize the conditions for establishing stable PGC lines during genome editing. On the other hand, a recent method, called sperm transfection-assisted gene-editing based on direct delivery of the CRISPR/Cas9 complex, is a potential alternative for avian transgenesis and genome editing without culturing PGCs, despite the low efficiency of genome editing and germ-line transmission [119].

3.5. Further applications of genome editing in poultry

After completion of the chicken genome sequencing project in 2004 and the subsequently available genomic sequences of the zebra finch and turkey, infinite possibilities and multiple opportunities are available to access invaluable genetic information from birds [120]. The bird 10 K genome sequencing project was initiated in 2015 based on recent next-generation sequencing technology. The progress of efficient genome-editing technologies in birds synergizes the

value of avian genetic information by avian genome manipulation for the development of beneficial poultry breeds (**Figure 4**).

We expect to establish an efficient bioreactor system to produce valuable proteins through genome-editing technology in chickens, which has the well-known advantage that egg white protein is easy to purify and eggs are produced daily by chickens [11, 121]. Bioreactors producing target proteins under the strong ovalbumin promoter have interested researchers for a long time [77, 122]. Thus, HDR-mediated target gene insertion into the ovalbumin locus could be an ideal bioreactor system to cost-effectively produce more than 1 g of target protein from an egg. In addition, genome editing has been used to remove or enhance targeted nutrients in meat and eggs of chickens. Knocking out allergen-related genes, such as ovalbumin and ovomucoid, has been achieved and could be used to generate allergen-free chicken meat and eggs [115, 116]. Additionally, muscle-related genes, such as myostatin, could be used to generate double-muscled and muscle hypertrophied chickens via genome editing, as in other livestock [123–125]. The conventional genetically modified organism (GMO) containing a foreign gene has been a concern due to a safety issue from unknown allergic reactions or the use of antibiotic resistance genes. Genome-edited poultry can be produced with a controllable genome-editing system, which is similar to natural mutations rather than foreign gene insertion as in conventional GMO. Moreover, an advanced base editing system may be more suitable for slight modifications of nucleotides without HDR in some cases. After scientists convince the public that genome-edited animals are similar to naturally selected animals, genome-edited poultry will be profitable for consumers.

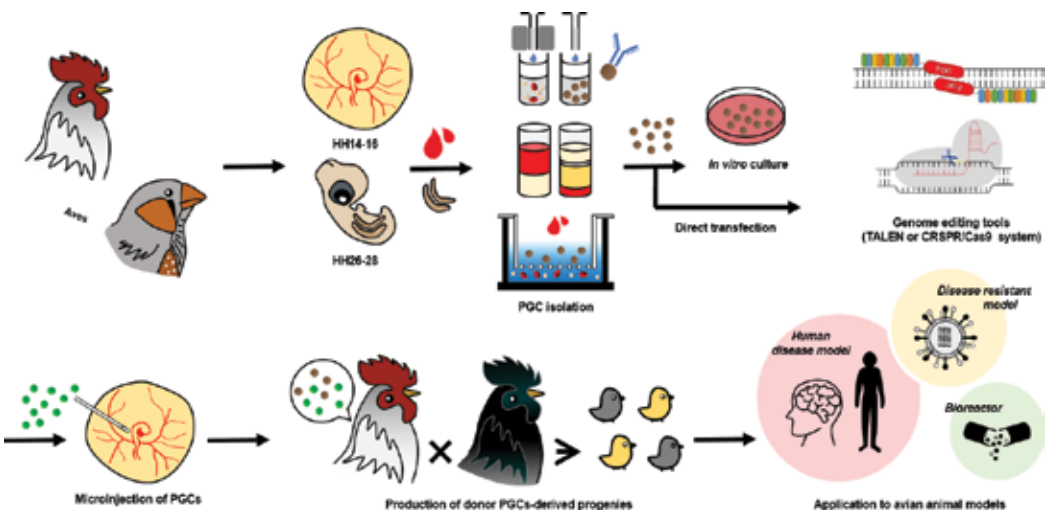

Figure 4. Strategies for the production of genome-edited poultries. PGCs in poultry can be obtained from embryonic blood and embryonic gonads. After the delivery of genome editing tools, genome-edited poultry can be established by microinjection of directly isolated or *in vitro* cultured PGCs into the blood vessels of recipient embryos. Avian genome editing systems can be applied to produce various avian models and poultry. This figure is adopted from [135].

In addition, it will be possible to control avian-specific diseases, such as avian influenza and Marek's disease, which cause serious problems in the poultry industry. Although understanding the mechanism of avian virus pathogenesis is essential for the application [126, 127] and limited in vitro results have been achieved [98, 128], avian genome-editing technology is expected to be used to develop avian disease-resistant birds by eliminating host factors or receptors of avian viruses. Lastly, because birds lay a large number of eggs and have a short ovulation cycle, they are considered the best model organism for studying human ovarian cancer [129]. With precise genome modulation of ovarian cancer-related genes in an avian model, especially chickens, it is possible to reveal the genetic mechanisms of ovarian cancer. In addition, avian genome-editing tools will gradually be applied to other birds, such as zebra finch, which is an exclusive non-human model organism for investigating the biological basis of speech learning and neurobehavioral research and disease [130–134]. Until now, direct injection of virus-mediated transgenesis into embryos has been used in zebra finch [132]. Genome-editing technology delivered by both in vivo and in vitro strategies will be widely applied to reveal the function and mechanism of neuron-related genes in zebra finch.

4. Conclusions

Poultry is important not only as a food resource but also as a valuable model animal for diverse disciplines, such as human disease, neurological research, and developmental biology. Until a few years ago, the difficulties in transgenesis and genome editing of birds limited their use as model animals. State-of-the-art technologies, such as CRISPR/Cas9 and the base editing system, have provided new insights into avian models when combined with PGC culture and other reliable germ-line systems. The novel genome-edited birds, including specific-gene knockout, human disease models, allergen-free, and disease-resistant poultry and egg-based bioreactors, are expected to be developed. Although the challenges in improving germ-line transmission strategies remain for many poultry species, programmable genome-editing tools will be useful in the development of genetically modulated poultry, together with efficient delivery and germ-line modification. Therefore, applying genome editing technology to birds will contribute to the poultry industry and ultimately provide benefits to humans.

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Conflict of interest

The authors declare that they have no competing interests.

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Section 2

Control of Gene Expression, Meat Quality and Reproduction

Chapter 4

Assessment of a Nutritional Rehabilitation Model in Two Modern Broilers and Their Jungle Fowl Ancestor

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Abstract

Inclusion of rye in poultry diets induces a nutritional deficit that leads to increased bacterial translocation, intestinal viscosity, and decreased bone mineralization. However, the effect of diet on developmental stage or genetic strain is unclear. Therefore, the objective of this chapter is to evaluate the effects of a well-established rye model diet during either the early or the late phase of development on performance, bone mineralization, and morphometric analysis. Furthermore, intestinal integrity evaluated by liver bacterial translocation, leakage of FITC-d, and gene expression of tight junctions across three diverse genetic backgrounds Modern 2015 (Cobb 500) broiler chicken, 1995 Cobb broiler chicken, and the Giant Jungle Fowl are also discussed.

Keywords: nutritional rehabilitation, chicken lines, compensatory growth, bone mineralization, morphometric analysis, intestinal integrity

1. Introduction

Multiple metrics of growth are utilized when determining a child's nutritional status [1]. Both height and weight relative to age are essential benchmarks when monitoring growth because these growth metrics exhibit similar trends across human development [1]. Stunting is defined as low height/length for a child's age and is often reflected in poor linear growth [1, 2], while wasting is low weight for length/height [1]. It should be noted that before the age of 2, a child's height is measured as length. The World Health Organization (WHO) defines stunting as two or more standard deviations (SD) below the standard height for children at that particular age, often referred to as Z score. The typical growth pattern for stunted

children is a sharp decline in height/length from birth until 24 months or until the child reaches 1.5–2 SD below the median and then plateaued till 59 months [1]. Globally, stunting, underweight or wasting are major contributors to morbidity and mortality in children [2]. A report from 2000 indicated that stunting has decreased by 14% globally since the 1980s; however, the reduction in stunting was unevenly distributed [3]. A projected trend for 2020 expects a decrease in the rate of stunting in Asia from 100 million to 68 million, while the number of stunted children in Africa is expected to increase from 60 million to 64 million as population increases [2, 3] .Globally, 26% of children under the age of 5 have a−Z score, two standard deviations below the average, indicating stunting [4]. Childhood stunting increases the risk of mortality from an infectious disease and reduces cognitive ability and lowers adult learning [4]. Undernutrition in infants 6 months of age or younger is often attributed to low birth weights and breastfeeding patterns [1]. The most vulnerable time for childhood stunting is from conception until 24 months [3]. During the first 2 years of life, children are more vulnerable to the long-term effects of stunting, like cognition, executive function, and school attainment [5]. Stunting that occurs after this time is less correlated with the long-term effects of stunting [5]. Therefore, the first 2 years of life is the time when interventions are more effective [1]. Therefore, it is evident that prenatal and early life interventions are required to ensure proper growth.

It has been estimated that 43% (250 million) of children under the age 5 subjected to poverty and stunting will not reach their development potential [5, 6]. There is a growing body of evidence that documents that healthy children tend to become healthy and wealthy adults [7, 8], and there is a positive correlation between higher birth weights and social, economic, and cognitive outcomes [7]. For example, anthropometric markers such as birth weight and child height are related to future schooling, employment, earnings, family formation, and health [9]. Low- to middle-income countries have a higher risk of children not reaching their developmental potential due to poverty and stunting [5]. Exposure to poverty early in life affects the individual's health and well-being as an adult which can lead to 19.8% lower income than those individuals not exposed to stunting [5]. Low socioeconomic status in early childhood has been associated with smaller hippocampal gray matter, which has been associated with low cognition, academic, and behavioral performance [10]. Early life stressors have long-term effects that reach into adulthood where there are low task-related activation of brain regions supporting language, cognition, memory, and emotional reactivity [11, 12]. Interventions to annihilate poverty have been shown to improve wage earning, intelligence, better health biomarkers, reduction violence, depression, and social inhibition [5]. The negative effects of early childhood stunting can also be attenuated when the child receives nurturing care [5]. Positive home environments had longer effects on cognition where children were more susceptible to their environment for up to 63 months [5]. Romanian children placed in foster care had a better cortisol response than those children who remained in an institution. The cortisol response may be the link between cognition and early childhood stressors [5]. Poverty often leads to exposure to multiple physical and psychological stressors, which can affect the child's physiological response as well as inhibit self-regulation and stress management [5]. For the first 2 years of life, macronutrient supplementation for the increase in intellectual development is required [5]. Nutrients promote healthy brain development [5].Therefore, it is evident that reducing the incidence of stunting

can have positive long-term effects on the health and the economy and may be able to break the cycle of poverty. The purpose of this chapter is to evaluate the etiology of stunting and present chickens as a viable model to study stunting in children.

2. Etiology of stunting

The top five predictors of childhood stunting in India were maternal stature and weight, maternal education, household incomes, and diversity in the diet [13]. Macro and micronutrient deficiencies also play a role in stunting. Semba et al. [14] found that stunted children in rural Malawi have a low circulating level of nine essential amino acids and lower levels of sphingomyelins. In addition, certain phosphatidylcholines were in lower concentrations in the serum suggesting that stunted children may also be deficient in choline [14]. Phosphatidylcholine and sphingomyelin play a major role in chondrogenesis, which can determine linear growth in bones [14]. This study suggests that the inefficiency of micronutrient and lipid supplementation in stunting may be due to the deficiency in essential amino acids [14]. There is also a direct relationship between systemic inflammation, growth hormone (GH) signaling, and linear growth [15]. Blocking tumor necrosis factors-alpha, via antibodies, reverses the GH signaling suggesting an interaction between GH and inflammation [16]. Systemic inflammation has been linked with higher levels of GH, and lower levels of IGF-1 and IGF binding protein-3 (IGFBP-3) systemically in the liver and lower linear growth [15]. The higher systemic levels of inflammation are likely caused by the recurrent infections in children subjected to poverty [15]. HsCRP (high-sensitivity C-reactive protein) has been utilized as a biomarker of mild inflammation during viral or bacterial infections [15]. Higher levels of serum hsCRP was correlated with higher systemic and hepatic GH and lower level of IGF-1 and IGFBP-3 [15]. Higher GH and lower IGFBP-3 were associated with short stature and states of undernutrition. This data suggests that both diet and environmental pathogen exposure can have direct effects on growth in children.

A longitudinal study was conducted on Malawian twins from age 0 to 3 in rural communities to evaluate the effect of genetics on child malnutrition [17]. Between sets of twin pairs, there was a high rate of discordance in the effect of severe and moderate malnutrition [17]. In addition, nutrition alone was not an effective treatment for stunting, as feeding interventions only improved growth by 30%, suggesting that stunting is a multifactorial disease [4]. Disease is another factor to consider when determining the etiology of stunting. Infections can affect nutrient absorption, which can lead to undernutrition and stunting. However, it should be noted that there is not a strong correlation between growth, diarrhea, and disease [4]. It has been theorized that unsanitary living environments lead to asymptomatic but chronic intestinal injury which results in immune stimulation and poor growth [4]. An effective intervention in reducing the incidence of stunting focuses on three core issues: water, sanitation and hygiene (WASH). Another factor that contributes to stunting is environmental enteropathy dysfunction (EED) [4, 18]. EED is a subclinical disease of the small intestine characterized by villous atrophy, crypt elongation, infiltration CD8+ T-cells in the lamina propria and increases intestinal permeability associated with intestinal inflammation [4, 18]. Also, there is

an inverse relationship between enteric inflammation and linear growth and vaccine efficacy [18]. EED is prevalent in low-income countries with poor sanitation and high environmental loads of enteropathogenic bacteria and is often associated with the pathogenesis of malnutrition [18]. The effects of EED are cyclic further proliferating growth faltering. The cycle starts with damage to the intestinal morphology causing a loss of barrier function, which triggers hyperstimulation of the immune system [4, 18]. This perpetuates the loss of barrier function and a reduction in absorptive function and secretion of digestive enzymes causing poor digestion and malabsorption [4, 18]. The etiology of EED is unclear but the continuous exposure to pathogenic bacteria and their enterotoxins causes villous atrophy that correlates with crypt hyperplasia [4]. This causes villous blunting which reduces absorption capacity and fewer secretions. Also, the high intestinal pathogenic load causes hyperstimulation of enteric T-cells which contribute to the crypt hyperplasia [4]. Therefore, both the host's immune system and the pathogenic bacteria are causing mucosal damage. The hyperstimulation of the cell-mediated immune response is thought to occur due to the high concentration of fecal microorganisms but may also be caused by severe nutritional deficiency, HIV, or mycotoxin exposure [4]. Certain pathogens and/or endotoxins can disrupt the intestinal barrier via tight junctions or by activating pro-inflammatory immune mediators. Chronic pathogen exposure causes chronic immune activation. Intravenous infusion of endotoxin administered to healthy humans increases gut permeability [19]. There is a correlation between intestinal permeability and stunted growth, where 55% of linear growth faltering occurred while Gambian infants had impaired intestinal permeability [20]. It is believed that there are three primary reasons for growth impairment during EED. First, the hyperstimulation of the immune response it metabolically expresses [20]. Second, proinflammatory cytokines can act to reduce growth-related hormones impeding growth [20]. Lastly, proinflammatory cytokines can attenuate bone remodeling causing more permanent stunting [20]. Regardless if the child had diarrhea, pathogenic bacteria was found in the stool of children with EED under the age of 60 months in both sub-Saharan Africa and South Asia [21]. Suggesting that there is subclinical inflammation caused by EED is a major contribution to stunting [21]. Malabsorption may also play a role if the severity of EED causes a high abundance of nutrient loss in the stool [21]. It is evident that regardless of etiology of EED, the disease is dependent on the environment. In areas where the incidence of EED is high, newborns do not have the intestinal histopathology associated with EED and when individuals removed environment, individuals were able to restore normal intestinal morphology and barrier function [22]. However, it should be noted that recovery of this disorder is relatively slow and individuals who presumably had the condition longer take longer to recover [4]. Bangladeshi children living in environmentally clean households had less severe EED and higher HAZ than children from contaminated households [23].

There are limited efficacious treatments for EED; antibiotics have been shown to have modest improvements in growth [18, 24]. Improving micronutrient status via supplementation did not affect linear growth [18]. Energy dense, micronutrient-fortified ready-to-use therapeutic food can accelerate short-term weight gain affecting metabolism by switching from fatty acid oxidation to amino acid oxidation, which increases fat deposition and weight gain [18]. There is a lack of information on the optimal time and duration of the nutritional intervention [18].

It is evident that stunting and malnutrition are multifactorial issues and that the microbiota plays a role in the mediating nutrition and pathogenesis of disease. It has been suggested

that in order to determine microbiota into equation, there needs to be a benchmark set to determine significant changes [18]. Microbiota for age Z is currently being defined as the degree of deviation of an unhealthy individual microbiota age from a reference cohort of a chronological age matching a child with normal growth. This data revealed that Bangladeshi and Malawian children had "immature" gut microbiota, which is similar to the bacterial profile of a younger child [25, 26]. They hypothesized that microbiota maturation is functionally linked to the growth rate of the host [25, 26]. Breast milk contains a lactose core and linked glucose, galactose, N-Acetyl galactosamine, fructose, and/or sialic acid residues [18]. These carbohydrate sources have prebiotic actions to promote colonization of bifidobacterial taxa. Bifidobacterium has multiple benefits to the host including improved vaccine response, enhanced gut barrier, and protection from enteric infection [18]. Gnotobiotic mice that were colonized with bacteria isolated from stunted infants in Malawi were supplemented with sialylated bovine milk oligosaccharide (BMO) or fructo-oligosaccharides and given a micro and macronutrients deficient diets [18]. They found that mice on the BMO diet had growth increase that was dependent on the microbiota [18]. Therefore, it is evident that microbiota play a key role in modulating growth and EED.

Animal agriculture has shown that low levels of antibiotics can reduce the number of pathogenic bacteria [4]. It is evident from animal trials that growth and intestinal morphology can be improved when the animals are placed in an environment with a low bacterial load and environmental immunogens. Research conducted in Bangladesh found that household with lower levels of parasites and less severe EED had better growth than those less hygienic environments [23]. This suggests that improved household sanitation and hygiene in areas with high parasitic loads can improve the severity of EED. However, in highly contaminated areas with little access to clean water, it was found that handwashing was not able to reduce the levels of subclinical mucosal damage and immune stimulation [27]. Infants' exploratory behaviors can lead to increased ingestion of pathogenic bacteria [28, 29]. Therefore, it is evident that still much is unknown about EED.

3. Compensatory gain

Compensatory growth, also known as catch-up growth or compensatory gain, is an accelerated growth of an organism following a period of slowed development, particularly because of nutrient deprivation. Growth may be measured with respect to weight, length, or height in humans [30, 31]. In some instances, body weights of animals under feed restriction will catch-up to control animals with ad libitum feed intake [32, 33]. In fact, high compensatory growth rates in feed restriction animals result in overcompensation due to excessive fat deposition and animals recover their normal weight without additional time [34, 35]. Nevertheless, when the nutrient restriction is severe, the growth period must be extended to reach the normal weight, but if the nutrient restriction is severe enough, permanent stunted growth may occur [36]. Compensatory growth has been reported in metazoans, plants, fungi, and even prokaryotes [35, 37–39]. Although the exact biological mechanisms for compensatory growth are poorly understood, it is clear that in some animals the endocrine system is involved [38]. During the first stages of starvation, there is a reduction of

basal metabolism [40]. The intestinal tract is the first organ to be reduced in both weight and activity [41, 42]. Then, as feeding is normalized, dietary protein and energy support intestinal growth, followed by muscular tissue and at the end adipose tissue [43]. Some of the factors that affect compensatory growth include composition of the restricted diet; severity of undernutrition; duration of the period of undernutrition; age; genotype; and gender among others [40, 44–46]. An epidemiological study determined that 56% of childhood mortality (aged 6–59 months) was attributed to malnutrition potentiating effects, and 83% of these was attributed to mild-to-moderate as opposed to severe malnutrition [36]. The present and other studies propose that malnutrition plays a major role in child mortality and suggest that strategies involving only the treatment of the severely malnourished are not enough to reduce the negative impacts of malnutrition [25, 47, 48]. Furthermore, malnutrition remains the major focus of nutritional intervention efforts, especially because dietary deprivation during early life can also have adverse effects on brain anatomy, physiology, biochemistry, and may even lead to permanent brain damage [49]. When diarrhea was followed by diarrhea-free periods, children exhibited compensatory gain and were put back on the growth trajectory [4].

4. Chickens as a model for research in humans

Using appropriate animal models is essential when studying human health [50]. Chicken has been an important experimental model in biology for more than 2000 years having led to many central discoveries [51–53] . However, with the latest advances in genetics and nutrition technologies, chicken has attained a superb model organism status [53, 54]. Hence, chickens are the system of choice for many vertebrate biologists, especially in the field of human sciences, who are interested in gene function [51, 55], as well as nutrition [53]. Typically, pigs are used as a model to study human nutrition because rodents have vastly different nutrient requirements and nutrient-nutrient interaction, and they are coprophagic and utilize different feeding strategies [56]. Roura et al. [56] reported that the mechanism of intestinal permeability and intestinal immunity system are well conserved across species; however, pigs often make an excellent model for humans due to their similarity in gastrointestinal anatomy. Although chickens have a shorter intestinal tract when compared to humans [57], there are many reasons as to why chicken are an appropriate model to study human nutrition. The first being the liver is the primary site of lipogenesis in both chicks and humans [58]. Also, both neonatal chicks and humans are able to efficiently utilize sucrose as an energy source. [59]. In both chickens and humans, iron is primarily absorbed in the duodenum [57]. Although the pig is the model organism for conducting human nutritional research, it appears that poultry have a more severe reaction to rye-based diets. It has been hypothesized that pigs are able to digest non-starch polysaccharides (NSP) better than poultry due to the high volume of the large intestine allowing for more fermentation and longer transit time of the digesta [60]. It also should be noted that chicks are able to double their starting body weight in 3 days, where it takes pigs 20 days and in children 5–6 months [50]. Therefore, from a practical standpoint, more trials can be completed in a shorter amount of time.

5. Chicken and rye

Starch and non-starch polysaccharides (NSPs) are primary carbohydrate sources in plants [61]. Cellulose arabinoxylans and β-glucans are the primary NSPs and require microbial digestion to be utilized by monogastric animals [61]. Arabinoxylans are the primary components of the thin lignified cell wall of the endosperm [62], and insoluble arabinose and endogenous enzymes do not efficiently degrade xylose residues. NSPs can be further classified by their water solubility, which is dependent on the chemical structure of these sugars [61]. The greater the solubility of the polysaccharide, the more viscous the digesta which reduces its nutrient availability. Rye is a cheap raw feed material to produce as it is tolerant to low temperature and drought, irregular soil pH, and requires less chemical treatments [61]. However, on a dry-matter basis, rye grain contains 9.7% soluble NSPs likely in the form of arabinoxylans. Hybrid rye variants have been bred to reduce the amount of anti-nutritional factors allowing rye to be added to poultry diets at a higher inclusion rate [61].

Soluble NSPs have beneficial effects on human health by lowering blood sugar levels, facilitating regular bowel movements, and reducing risk of heart disease and other metabolic syndromes [63]. Wild avian species consuming NSPs as whole grains prolong digestion time in the crop and allow for more microbial digestion, eliminating some of the anti-nutritional factors [61]. In modern agricultural animals, diets containing excessive amounts of NSPs negatively affect health and perpetuate a state of disease [61]. It is evident from the literature that broiler chickens consuming diets high in NSPs have increased ileal viscosity which leads to less interaction of endogenous enzymes and nutrients and reduces nutrient digestion [61]. Lower digestibility results in less energy available for growth, which reduces body weight (BW) and increases feed consumption that increases production costs [61]. It has also been reported that chickens consuming diets high in NSPs have increased feed intake in an attempt to maintain nutrient intake which increases the transit rate and increases intestinal viscosity even further [64]. Broilers consuming wheat-based diets had significantly higher gut viscosity, reducing AME and depressing growth and feed conversion efficiency [65]. Broilers and turkey poults consuming rye as the primary carbohydrate source had increased digesta viscosity, increased intestinal permeability, reduction in bone strength and mineralization, and changes in microbial composition [66–68]. The anti-nutritive effects of rye were attenuated when the diet was supplemented with bacillus-based direct-fed microbial [68]. Inclusion of 5% and 10% rye from d14 to d28 decreased performance and litter quality and increased gene expression of cellular growth and differentiation in cell survival processes [69]. Rye also upregulated complement and coagulation signaling pathway which is characteristically upregulated to eliminate infections [69]. Laying hens fed a diet containing rye, had a reduction in egg production, feed conversion efficiency, and eggshell cleanliness. The anti-nutritive effects of rye in laying hens could be improved when the diet was supplemented with a NSP degrading enzyme complex [70]. The higher digesta viscosity of soluble NSP diets also increases litter moisture, which can increase the incidence of footpad dermatitis [61].

There is a negative correlation between the digestibility of fat and dietary fiber inclusion [62]. The increased digesta viscosity associated with high NSP diets reduces fat digestibility by

interfering with emulsification and subsequent absorption [71]. The reduction in fat absorption of chicks consuming diets high in NSP also puts chicks at risk for fat-soluble vitamin deficiencies. It has been previously observed that hepatic vitamin E levels were significantly lower in rye-fed birds [72]. Higher viscosity also increases gastric passage rate, which can increase the possibility of pathogen proliferation. Higher potential pathogenic load within the lumen of the intestinal tract can increase risk of bacterial translocation stimulating the inflammatory response that increases intestinal leakage, and leads to higher amounts of bacterial translocation [61]. Rye-fed chicks also had a higher abundance of coliforms in the small intestine [68]. The higher abundance of coliforms in high NSP diets was also observed in an in vitro system [73]. Adding silage, rye, and chicken manure to a biogas reactor led to a high abundance of *Clostridia*, which plays a vital role in the digestion of polysaccharides and oligosaccharides [73]. There have been varied observations on the effect of soluble fibers on microbial population [61]. Diets containing 10% rye decreased population of commensal bacteria like *Lactobacillus* [69]. Certain populations of commensal bacteria can utilize resistant starches, NSPs, oligosaccharides, or proteins to produce short chain fatty acids (SCFA) which can be used as an energy source by the animal [61]. Particular types of SCFA are able to cross the lipid membrane of prokaryotes where they dissociate in the cytoplasmic, destroying the bacterial cells [61]. SCFA also reduce the luminal pH, which can limit pathogen proliferation [61]. Soluble fiber has also been reported to prevent the adherence of certain pathogenic bacteria to epithelial cells [74]. Mathlouthi et al. reported that wheat and barley consumption increases bacteria in the caeca—both commensal (*Lactobacillus* strains) and pathogenic (*E. coli*) [75]. Non-ruminant enzymes are unable to degrade arabinoxylans which enter the colon relatively intact where they stimulate growth of residing bacteria such as *Bacteroides, Bifidobacterium, Clostridium, Lactobacillus*, and *Eubacterium* [76]. Chickens consuming wheat/rye diets resulted in a higher abundance of mucosa associated bacteria especially enterobacteria and enterococci [77]. This indicates that the higher digesta viscosity associated with a rye-based diet results in an increase in the bacterial activity in the small intestine [77]. Also, NSPs containing diets that were not supplemented with enzymes had significantly more ileal volatile fatty acids, which indicates higher bacterial fermentation [78]. Furthermore, preliminary microbiome analysis from our laboratory found drastic differences in cecal microbiome profiles between chicks consuming rye and corn-based diets (**Figure 1**). Rye-fed chicks had a higher abundance of beneficial bacteria like *Lactobacillus and Bifidobacteria* but also a higher abundance of potentially pathogenic bacteria like *Clostridium* and *Proteus*, indicating dysbacteriosis. Corn-fed chicks had a higher abundance of SCFA-producing bacteria like *Faecalibacterium*, *Dorea*, *Oscillospira,* and *Blautia*, which maybe more representative of a "healthy" microbiota. Soluble NSPs have been reported to improve the development of the intestinal mucosa by increasing villus height and crypt depth in broilers consuming a diet containing 10% rye [69]. Insoluble fibers have also shown to improve intestinal morphology by increasing absorptive surface area [79]. Broilers fed a barley-based diet had changes in intestinal morphology compared to those birds fed corn-soy where there were shorter and thicker and atrophied villi and increased goblet cell size [80].

High-NSP diets supplemented with enzymes have shorter passage time by decreasing digesta viscosity [72]. Supplementation with starch degrading enzymes can ameliorate some of these negative side effects while again there was no observed effect of antibiotics supplementation on performance parameters [65]. The addition of a multicarbohydrase enzyme

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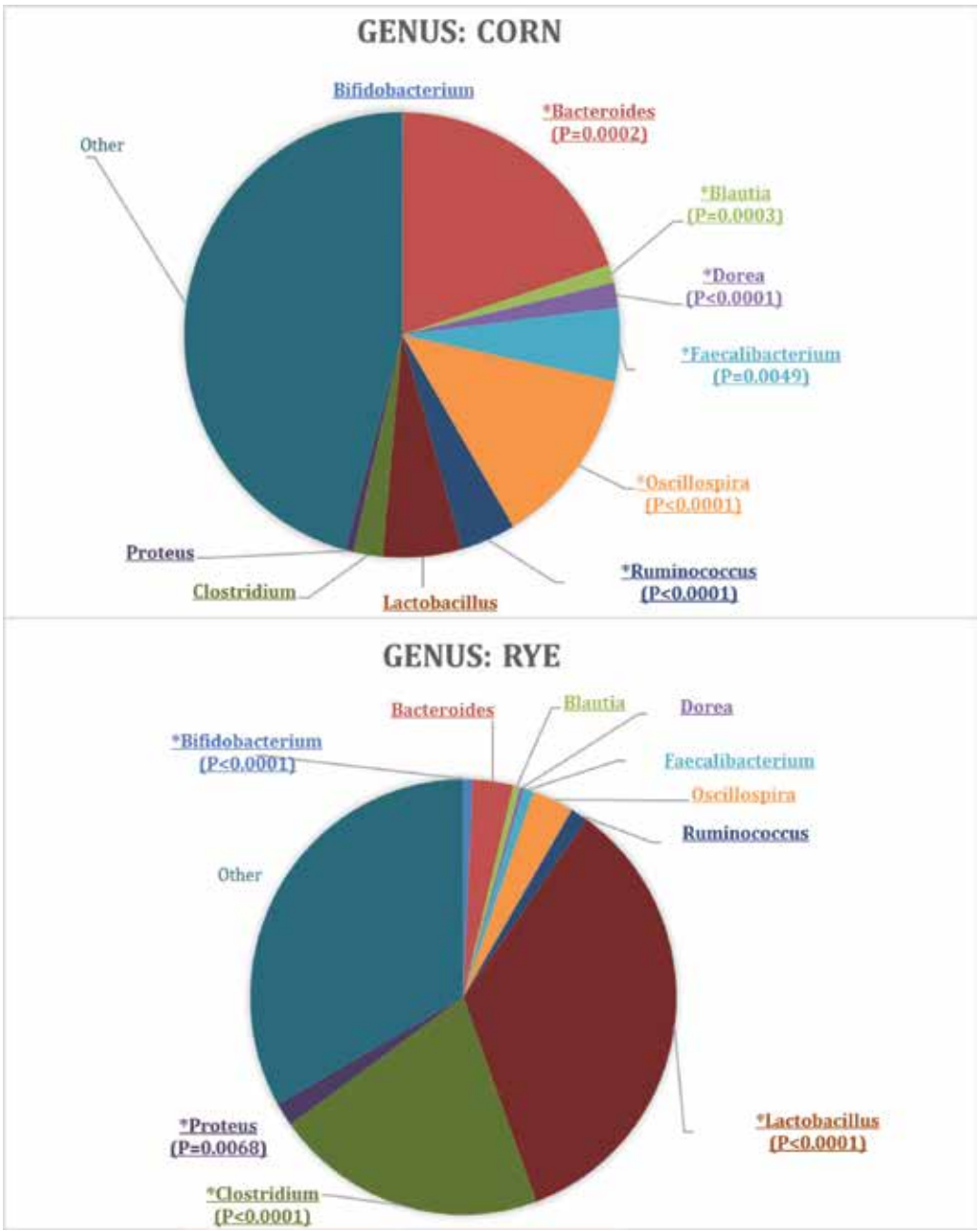

Figure 1. Cecal microbiome of 10-day-old broiler chicks consuming rye- or corn-based diets at the genus level. Data are expressed as relative abundance (%). *indicates significant differences in genera between chicks fed a corn- and rye-based diet.

complex containing both xyalanses and arabinofuranosidases improves digestibility of diets containing various amounts of different NSPs [62]. The higher apparent metabolizable energy of diets was attributed to the starch crude protein and crude fiber but the NSPs did not

increase energy availability [62]. The proposed mechanism of action of this enzyme complex is that the carbohydrases allow for improved endogenous enzyme and substrate interaction, allowing for improved digestibility [62]. Similar improvements in broiler performance were observed when the cereal grain was soaked in water where there was a decreased digest viscosity, increased growth parameters, increased villus height and reduced muscularis thickness and crypt depth proliferation, and increased volatile fatty acids (VFA) concentration in the ceca [81]. Pettersson et al. also found that steam-pelleting diets containing barley, wheat, and rye had increased digestibility in broiler chickens [82]. Young chicks are less tolerant to high-NSP diets because the higher digesta viscosity limits peristalsis which prevents the maintenance of digesta flow rate [64]. The inability of nutrients to move through the intestine rapidly prevents the absorption of nutrients to meet energy requirements for maintenance [64]. Antibiotics growth promoters are thought to improve performance via modulation

Figure 2. Visually comparing fecal viscosity and body weight of rye- and corn-fed jungle fowl and modern broilers. (A) Jungle fowl consuming a corn-based diet had feces with relatively normal consistency (red arrow) and a relatively clean vent. (B) Jungle fowl consuming a rye-based diet had feces pasted to the vent and diarrhea like consistency (red arrow). (C) Modern broilers consuming a rye-based diet had diarrhea pasted to the vent. (D) Illustrates the overall appearance and the size difference between modern broiler chicks fed a corn-based diet (red arrow) and a chick fed a rye-based diet (green arrow).

of the intestinal microbiome [83]. Previous research has suggested that antibiotics supplemented with rye-based diets attenuated some of these effects by eliminating the ability of the microbiota to ferment soluble NSP [84]. However, Choct and Annison did not observe any improvement in digestion and growth when antibiotics were added to the feed, which may be due to the antibiotic used in this study [78].

Figure 3. Litter quality and behavior observation of chickens consuming a rye- or corn-based diets (a) modern broiler maintained on a rye-based diet for 20 days of age had higher litter moisture and tended to huddle (red arrow). (B) Modern broiler maintained on a rye-based diet from 0 to 10 days of age and then the diet was switched to a corn-based diet from 10 to 20 days of age had a reduction in litter moisture. (C) Modern broiler maintained on a corn-based diet for 20 days of age and appear to have similar litter moisture to those chicks consuming rye in the first phase of the experiment. (D) Jungle fowl maintained on a rye-based diet exhibited diarrhea (seen in **Figure 1**); however, litter moisture did not appear to be affected by the diet.

The aim of future studies is to evaluate a nutritional rehabilitation in chickens to determine if it is an appropriate model to study interventions in childhood malnutrition. To the best of our knowledge, there is limited information on whether nutrition alone can facilitate intestinal recovery after the consumption of a rye-based diet in chickens. The model utilized rye and corn to evoke early- or late-phase malnutrition in three different genetic lines of chickens. To study early phase malnutrition, chicks were fed rye for the first 10 days and then switched to a corn-based diet. To study late phase malnutrition, chicks were fed a corn-based diet for the first 10 days and then switched to rye-based diet. The two control groups were maintained on a rye- or corn-based diet throughout the experiment. Preliminary results from our laboratory had comparable results to what has been reported in rye-fed chicks. **Figure 2** illustrates that rye fed Jungle fowl (B) and modern broilers (C) had feces pasted to their vents while the corn-fed birds had more normal fecal viscosity (A). As mentioned earlier, this is likely due to the higher digesta viscosity caused by diets containing high amounts of NSPs. **Figure 2**(**D**) also illustrates the drastic difference in body weight between modern broilers which were fed a corn-based diet (red arrow) and those fed a rye-based diet (green arrow). Litter quality was another qualitative observation made when chicks were fed high NSP diets. **Figure 3A** illustrates that modern broilers maintained on a rye base had higher litter moisture. Broilers in the early phase malnutrition group (**Figure 3B**) appeared to have comparable litter moisture with those broilers maintained on the control corn-fed diet (**Figure 3C**). This indicates that the modern broilers in the early phase malnutrition groups were able to reduce digesta viscosity which reduced litter moisture. Although the Jungle fowl had pasted feces (**Figure 2**), consumption of a rye-based

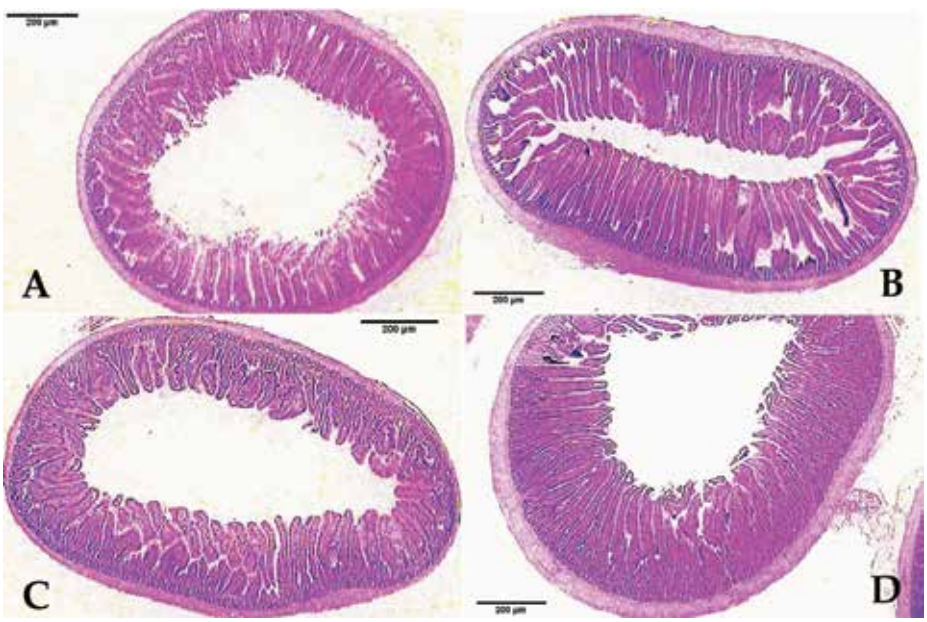

Figure 4. Histology of the ileum in modern broilers on a corn- or rye-based diet program. The histology of the ileum of chicks maintained on different combinations of rye- and corn-based diet. (A) Modern broiler maintained on a corn-based diet from hatch to 20 days of age. (B) Modern broiler maintained on a rye-based diet from 0 to 10 days of age and then the diet was switched to a corn-based diet from 10 to 20 days of age. (C) Modern broiler maintained on a rye-based diet from hatch to 20 days of age. (D) Modern broiler maintained on a corn-based diet from 0 to 10 days of age and then the diet was switched to a rye-based diet from 10 to 20 days of age.

diet appeared to have little effect on litter moisture (**Figure 3D**). There was observable difference in body weight, litter moisture, and pasted feces in the modern broilers; however, there are no obvious differences in histology of ileum consumption in the various diets (**Figure 4**).

6. Conclusion

Almost half the children under the age of 5 are living in impoverished conditions, putting them at greater risk of becoming stunted. The short-term effects of childhood stunting increase risk of mortality from an infectious disease and has long-term effects like reduced cognitive ability and lower adult learning [4]. It is evident from case studies that the incidence of stunting can be reduced when people have access to primary health care and education, increased sanitation, improved wealth distribution, and access to food [85, 86]. However, treatment opportunities tend to be time sensitive and most effective in reducing long-term effects of stunting if implemented within the first 2 years of life. Multiple factors contribute to the etiology of stunting, making it difficult to find a treatment. There has been limited treatment success by improving diet alone, and high environmental load of enteropathogenic bacteria can affect nutritional state and growth [4]. The histopathology of EED includes villous atrophy, crypt elongation, increased intestinal permeability, and intestinal inflammation [4, 18] which can be observed in stunted patients. Also, there is an inverse relationship between enteric inflammation and linear growth and vaccine efficacy [18]. However, treating EED with antibiotics has had limited success in improving growth [18, 24]. There is a lack of information on the optimal time and duration of the nutritional intervention [18]. Therefore, to determine effective ways to treat stunting, a viable animal model is essential. Avian species are a common animal model for human research especially in the field of gene function, nutrition, immunology, and developmental biology. The physiological response of poultry to a rye-based diet is like what is observed in patients with EED. The inclusion of rye in poultry diets has been fraught with problems, principally related to the production of sticky droppings, malabsorption syndrome, poor growth performance, increased intestinal permeability, and intestinal bacterial overgrowth [66, 67, 87–89]. The similarities between poultry consuming a rye-based diet and patients with EED and stunted children suggest that chickens would make a viable stunting model to determine potential interventions and treatments.

Conflict of interest

There was no conflict of interest.

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Chapter 5

Flavor of Poultry Meat: A New Look at an Old Issue

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Additional information is available at the end of the chapter

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Abstract

In Japan, fast-growing broiler occupies over 90% of poultry meat production. Meanwhile, many of Japanese native breeds of chickens are on the verge of annihilation, because most of them have low meat yield and egg production. Recently, meat flavor produced from native chickens has been reevaluated in the Japanese market. Most high-quality chickens, "Jidori" in Japanese, were initially bred by crossing native Japanese breeds with highly selected lines with rapid growth rate or relatively high egg production. Japanese consumers recognize that the meat from Jidori chickens is more palatable than that from broiler chickens; however, the reason behind this rich flavor of Jidori meat has not been elucidated. We found that (1) the high arachidonic acid (ARA, C20:4n-6) content is a characteristic feature of Hinai-jidori meat, (2) chicken meat containing higher levels of ARA is more palatable than that containing low ARA content, and (3) single-nucleotide polymorphisms in the fatty acid desaturase 1 and 2 gene cluster are associated with ARA content in meat. Our findings predict the beginning of a new era that the flavor of commercial chicken meat can be designed according to a commercial breeding program.

Keywords: chicken, meat, flavor, arachidonic acid, genetics

1. Introduction

Globally, chicken meat is obtained from a few fast-growing broiler strains. Meanwhile, countries, especially in Asia, have been keeping native breeds of chickens. Since most native chicken breeds have low meat yield and egg production, many of these breeds have been in danger of disappearing in many countries. Meanwhile, in Japan, some consumers are willing to pay a high retail price for a palatable meat known as "Jidori" chicken. Jidori, that is translated as an indigenous chicken in a local, is far more delicious, firm in texture, and expensive than the affordable broiler chicken. Most Jidori chickens were bred by crossing native Japanese breeds

with highly selected lines with rapid growth. For example, the Hinai-jidori chicken, a popular brand in the Japanese market, is produced by crossing of Hinai-dori (a breed native to Akita prefecture, Japan) cocks and Rhode Island Red hens.

Most Japanese consumers recognize that the meat of Jidori chicken has a richer flavor than that of the broiler chickens. However, the underlying reason for this rich flavor has not been elucidated. Meat texture is an important factor, because most Japanese consumers believe that Jidori meat is characteristically tough. Other key factors are the presence of free amino acids (FAA) including glutamic acid (Glu) and purine compounds such as inosine 5′-monophosphate (IMP). As is well known, Glu and IMP salts are widely used as food additives for the purpose of enhancing the flavor of general foods. So, in the past three decades, studies have compared the content of FAAs such as Glu and IMP in broiler and Jidori meat in Japan.

For example, Karasawa et al. [1] reported that the Glu and total FAA content, in the gastrocnemius muscle from three types of Jidori chickens (Satsuma breed, Satsuma × broiler F_1 cross, and Kukin × broiler F_1 cross), is significantly higher than that in the muscle from broiler chickens, while subsequent studies have reported no significant differences between the FAA contents of Jidori and broiler chickens [2, 3]. Matsuishi et al. [4] reported that soup prepared from broiler meat has a significantly higher FAA and Glu content than that prepared from a Jidori (Nagoya breed) meat and the broiler soup is pleasanter than the Jidori soup. In other words, Matsuishi et al. [4] pointed out for the first time that the intensity of umami that are felt by Japanese consumers and the Glu content of Jidori meat are not necessarily proportional. Meanwhile, Fujimura et al. [2] reported that the IMP content in the meat from Hinai-jidori chicken was significantly higher than that of the meat from broilers, while other studies have not shown a significant difference in the IMP content between Jidori and broiler meat [1, 5]. Therefore, it has not been verified if the difference in the contents of FAA, Glu, and IMP is actually correlated with the flavor of Jidori chicken.

Reflecting on past studies, we concluded that to determine the flavor of chicken meat, it is necessary to define candidate substances related to characteristic differences between broiler and Jidori chicken meat.

2. Association of arachidonic acid content and flavor in chicken meat

To identify the candidate substances influencing chicken meat palatability, quantitative analyses were performed on general biochemical components, such as FAAs including Glu, IMP, and fatty acids in the thigh meat of Hinai-jidori and broiler chickens. Female chicks hatched on the same day were reared under identical environmental conditions for the same duration. The results showed that higher arachidonic acid (ARA, 20:4n-6) and docosahexaenoic acid (DHA, C22:6) content was a characteristic feature of Hinai-jidori chicken (**Table 1**) [6].

Actually, Fujimura et al. [3] compared fatty acid profiles of Hinai-jidori and broiler chicken meat; however, they did not detect the phenomenon. Causes of the discrepancy may be that

Item	Broiler, female (8 weeks of age)	Broiler, female (22 weeks of age)	Hinai-jidori, female (22 weeks of age)
ARA	1.42 ± 0.27[a]	1.26 ± 0.33[a]	1.92 ± 0.04[a]
DHA	0.20 ± 0.07[a]	0.24 ± 0.11[a]	0.38 ± 0.04[a]

[a]Within a row, means without a common superscript are significantly different ($P < 0.05$).

Table 1. ARA and DHA content (% of total analyzed fatty acids) in broiler and Hinai-jidori thigh meat (mean value ± SD).

[3] analyzed a limited number of samples per chicken strain and Hinai-jidori chickens in the 1990s and 2010s are different.

ARA is a polyunsaturated fatty acid (PUFA) which exists in animal fat. ARA serve as an intracellular second messenger in many cells, as well as a precursor for biologically active molecules, such as eicosanoids (molecules of oxygenated ARA metabolites that act as second messengers and/or local mediators) and anandamide (an endogenous cannabinoid) [7]. When we found that a high ARA content is a characteristic feature of Hinai-jidori chicken [6], we noticed that a report patented in the 1960s [8] documented that autoxidized ARA has a flavor like cooked chicken meat. In addition, Yamaguchi et al. [9, 10] reported the relationships between the levels of PUFA including ARA, and food palatability. For example, the addition of trace amounts of ARA to vegetable oil used for cooking enhance umami (L-glutamate taste), kokumi (continuity, mouthfulness, and thickness), aftertaste, and total taste intensity of such foods as fried potatoes, Chinese noodle soup, and Hamburg steak cooked with the oil. Therefore, we assumed that ARA is associated with chicken flavor.

To elucidate the relationship between the ARA content and the flavor of Hinai-jidori meat, Hinai-jidori chickens were administered diets containing 3 oils (palm oil (PO), corn oil (CO), and ARA-enriched oil (AO, SUNTGA40S, Nippon Suisan Co., Tokyo, Japan)) during rearing; meat was subsequently evaluated by biochemical and sensory analyses [11]. Each oil was mixed in a weight ratio of 7:3 with silicate (TIXOSIL38A, Rhodia Silica Korea Co., Seoul, South Korea) and three types of oil supplements were prepared. Five percent of the supplements (3.5% equivalent of each oil) was added to the finisher diet and Hinai-jidori chickens were fed the test diets for 2 weeks before slaughter. As a result, in thigh meat, the ARA content of the AO group was twice higher than those of the PO and CO groups. Contents of other fatty acid components excluding ARA were not significantly different among the three groups. Sensory evaluation of steamed minced meat revealed that umami, kokumi, aftertaste, and total taste intensity of the AO group were significantly higher than those of PO and CO groups (**Table 2**). These data suggest that the flavor of chicken meat might be improved by dietary ARA supplementation.

To elucidate the relationship between the ARA content and the flavor of broiler meat, we evaluated the effects of AO supplements on the fatty acid content and sensory perceptions of thigh meat [12]. We prepared four types of oil: CO; a 1:1 mixture of AO and PO (1/2 AO); a 1:3 mixture of AO and PO (1/4 AO); and a 1:7 mixture of AO and PO (1/8 AO). Each type of oil was then mixed with silicate in a weight ratio of 7:3 and added to the finisher diet at a

	Comparisons	
	PO vs. AO[1]	CO vs. AO[2]
Sourness	0.34*	0.03
Sweetness	−0.03	0.34*
Umami	0.59**	0.50*
Kokumi	0.66**	0.75**
Aftertaste	0.69**	0.50*
Total taste intensity	0.72**	0.56**

[1]An average of the AO score of each subject when PO score is 0.

[2]An average of the AO score of each subject when CO score is 0.

*Statistically significant at P = 0.05 level.

**Statistically significant at P = 0.01 level.

Table 2. Sensory evaluation of steamed minced meat of Hinai-jidori chickens fed experimental diets.

final proportion of 5% of fresh matter in the same way as described above. In this experiment, broiler chickens were fed the four diets for 1 week before slaughter. As a result, the ARA content in the thigh meat (y, mg/g) increased linearly with the increasing content of dietary AO. The ARA content in the thigh meat of the 1/2 AO and 1/4 AO groups was significantly higher than that in the thigh meat of the CO group. Contents of other fatty acid components excluding ARA were not significantly different among the four diet groups. Sensory evaluation showed that the flavor intensity, umami, kokumi, and aftertaste, of the 1/2 AO and 1/4 AO groups, were significantly improved compared with those of the CO group (**Table 3**). Additionally, there were significant positive correlations between the ARA content of the thigh meat and flavor intensity, total taste intensity, umami, and aftertaste. These data suggest that the flavor of broiler chickens can be improved by increased supplementation of dietary ARA as well shown in Hinai-jidori chickens.

In our previous reports [11, 12], we measured the Glu and IMP contents of the samples and calculated the "umami intensity" of each experimental group. The value was expressed as the content of monosodium glutamate (MSG; mg/100 mL), with respect to the synergistic effect between Glu and IMP, according to Yamaguchi [13]. In these reports [11, 12], the differences in the intensity of umami among the experimental groups were less than 1%. These data suggest that the differences, in the intensity of umami between the groups, cannot be attributed to the contents of Glu and IMP, because Yamaguchi [13] reported that the differential threshold of umami between samples was 21%. Thus, we conclude that the differences in chicken flavor, observed in our reports, were caused by ARA and not by water-soluble substances, such as Glu and IMP.

After our sequential reports [6, 11, 12] were published, we found a report showing that Korean native chickens (KNC) had a higher content of ARA than broiler chickens; however, the sensory acceptance was not significantly different between KNC and broilers [14]. It is clear that [14] reported only a tendency of ARA content in KNC, but the authors failed to find an association between the ARA content and meat flavor.

	Pairs[3]					
Item	CO vs. 1/2 AO[4]	CO vs. 1/4 AO[5]	CO vs. 1/8 AO[6]	1/8AO vs. 1/2 AO[7]	1/4AO vs. 1/2 AO[8]	1/8AO vs. 1/4 AO[9]
Flavor intensity	0.88*	0.63*	0.63	0.13	0.25	0.25
Umami[1]	0.88*	0.63*	0.50	0.25	0.13	0.00
Kokumi[2]	0.50*	0.75*	0.63	0.25	0.38	0.38
Aftertaste	0.75*	0.75*	0.50	0.13	0.25	0.50
Total taste intensity	1.00*	0.63	0.25	0.38	0.50*	0.00

[1]Umami = L-glutamate taste.

[2]Kokumi = continuity, mouthfulness, and thickness.

[3]CO = Corn oil; 1/2, 1/4, and 1/8 AO = 1/2, 1/4, and 1/8 arachidonic acid-enriched oil.

[4]An average of the 1/2 AO score of each subject when CO score is 0.

[5]An average of the 1/4 AO score of each subject when CO score is 0.

[6]An average of the 1/8 AO score of each subject when the CO score is 0.

[7]An average of the 1/2 AO score of each subject when 1/8 AO score is 0.

[8]An average of the 1/2 AO score of each subject when 1/4 AO score is 0.

[9]An average of the 1/4 AO score of each subject when 1/8 AO score is 0.

*Statistically significant at P = 0.05 level.

**Statistically significant at P = 0.01 level.

Table 3. Sensory evaluation of steamed minced meat of broiler chickens fed experimental diets.

3. Flavor: a new breeding target of chickens

As mentioned earlier, Glu and IMP are the typical active taste components of umami. Based on recent reports, comparing the quality of meat between broiler and Jidori chickens, it appears that the broiler has a higher Glu content than that of Jidori chicken. The comparison was made at the marketable age of each strain [4, 6, 15, 16]. Because the content of Glu decreases with the age in broiler chicken [6, 17, 18], the difference in the content of Glu between broiler and Jidori chicken might be a reflection of their marketing age. Further, Wattanachant et al. [19] reported that Thai indigenous chicken had higher Glu content than the broilers. Jayasena et al. [20] reported that there was no difference in Glu content between Korean native and broiler chickens. These data suggest that the Glu content varies among different genotypes of chicken. Furthermore, the FAA content in the chicken meat increased with postmortem aging and is responsible for improving the taste of the meat [21]. Watanabe et al. [22] reported that a reduction in dietary lysine increases the content of free Glu in the broiler meat and improves its taste. An elevation in dietary lysine also increases the content of free Glu in the broiler meat, thus improving its taste [23].

Jidori chicken exhibited a higher IMP content than that of the broiler when their meat was compared at the marketing age of each strain [6, 15]. Similar results were obtained when the meat of the broiler chicken was compared with three Chinese native breeds [24]. Because there was no significant difference in the IMP content between 22-weeks-old broiler and 22-weeks-old Hinai-jidori chicken [6], the difference in the IMP content between broiler and Jidori

chicken might reflect their marketing age. Furthermore, the IMP content can be increased by dietary supplementation of IMP [25], purine nucleotides, betaine, soybean isoflavones, and combinations thereof [26].

Terasaki et al. [27] reported that the IMP content of broiler breast meat reached maximal level at 8 h after slaughter, and then decreased gradually when the meat was stored at 4°C. Further, the flavor of the chicken was more pleasant at 8 h after slaughter than that of the chicken immediately after slaughter. Thus, postmortem aging may increase the content of IMP more effectively than that by the slaughter age and feed additives.

As mentioned previously, we experimentally proved that the ARA content in chicken meat is changeable by adding AO to the feed; however, AO is so expensive that it is not suitable for the daily use. Therefore, we examined the possibility of genetic regulation of arachidonic acid content in chicken meat.

As shown in **Figure 1** [28], ARA originates from both dietary sources and the elongation desaturation process of linoleic acid (LA, C18:2n-6) in animals. D6D exerts a catalysis on a change in γ-linolenic acid (GLA, C18:3n-6), which is elongated to dihomo-γ-linolenic acid (DGLA, C20:3n-6) by elongases.

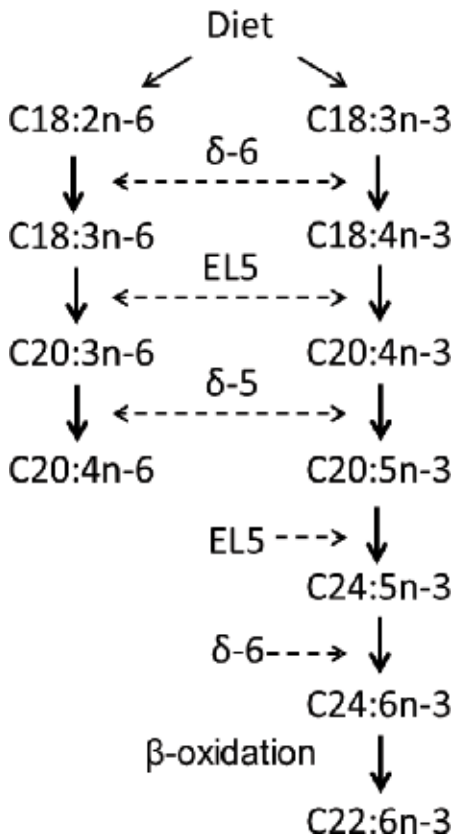

Figure 1. Metabolic pathway of n-6 and n-3 fatty acid synthesis.

Then, GLA is desaturated to ARA by D5D. As well as in the n-6 pathway, these enzymes are involved in the n-3 fatty acid pathway from α-linolenic acid (ALA, C18:3n-3) to DHA (**Figure 1**). D5D and D6D are known as encodes for fatty acid desaturase 1 and 2 genes (*FADS1* and *FADS2*), respectively. *FADS1/2* cluster is located at chicken chromosome 5 [29, 30]. Therefore, we supposed that *FADS1*, *FADS2*, and *FADS1/2* clusters are the keys that regulate ARA and DHA content in chicken meat.

We genotyped the polymorphisms of *FADS1* and *FADS2* and investigated their association with the fatty acid profile in the Hinai-jidori meat [31]. We found 71 and 46 SNPs in *FADS1* and *FADS2*, respectively. Of the SNPs, rs733003230 (A > G) and LC060926 (g.25 A > G) were chosen in *FADS1* and *FADS2*, respectively. Then, the Hinai-jidori female chickens, hatched on the same day and reared under identical environmental conditions over the same duration, were used for all analyses. In each SNP of *FADS1* and *FADS2*, the compositions of AA and DHA were significantly higher in the G, rather than in the A, allele, respectively (**Table 4**).

We also examined the association of the *FADS1* and *FADS2* haplotypes with the content of fatty acids. The AA and DHA content of the G-G-haplotype was significantly higher than that of the A-A-haplotype (**Table 5**).

Thus, we conclude that the SNPs, in the *FADS1* and *FADS2* gene cluster, increase the content of AA and DHA; this result may help to develop strategies for improving the flavor of Hinai-jidori chickens. Our reports predict the beginning of a new era that the flavor of commercial

Gene	***Fatty acid desaturase 1* (*FADS1*)**		***Fatty acid desaturase 2* (*FADS2*)**	
Locus	rs733003230 (A > G)		LC060926 (g.25 A > G)	
SNP allele	A	G	A	G
Allele frequency	0.453	0.547	0.813	0.188
ARA	1.01 ± 0.15	1.33 ± 0.07*	1.10 ± 0.07	1.55 ± 0.19*
DHA	0.25 ± 0.04	0.35 ± 0.02*	0.28 ± 0.02	0.40 ± 0.06*

*Statistically significant at P = 0.05 level.

**Statistically significant at P = 0.01 level.

Table 4. SNP effects of chicken fatty acid desaturase 1 and 2 genes (*FADS1* and *FADS2*) on ARA and DHA content (% of total analyzed fatty acids) in Hinai-jidori thigh meat (mean value ± SE).

Combined haplotype of *FADS1* and *FADS2*	**A-A**	**G-A**	**G-G**
Frequencies of plausible haplotype under linkage equilibrium	0.453	0.359	0.188
ARA	0.99 ± 0.12[a]	1.24 ± 0.15[a]	1.56 ± 0.24[a]
DHA	0.25 ± 0.04[a]	0.32 ± 0.04[a]	0.40 ± 0.07[a]

[a]Means within a row with different superscript letters are significantly different at P = 0.05 level.

Table 5. Haplotype effects of chicken fatty acid desaturase 1 and 2 genes *(FADS1* and *FADS2*) on ARA and DHA content (% of total analyzed fatty acids) in Hinai-jidori thigh meat (mean value ± SE).

chicken meat can be designed according to a plan. Thus, we are testing whether the flavor of Jidori meat can be improved by using molecular breeding and marker-assisted selection techniques.

4. A proposed mechanism to explain the effect of ARA on the enhancement of chicken flavor

The fact that the addition of AO to cooked foods improves the taste is widely recognized in Japan. To data, J-OIL MILLS, Inc. (Tokyo, Japan) sells AO-supplemented cooking and frying oils in the Japanese market. However, a mechanism to explain the effect of ARA regarding the enhancement of food taste has not been yet satisfactorily elucidated.

Alimentary fat is mainly in the form of triglycerides, which are not effective taste stimuli; however, on the tongue surface, free fatty acids (FFA) might be generated from triglycerides by lingual lipase and resultant FFA might be detected by FFA sensors [32].

To date, the CD36, GPR120, and GPR40 have been reported as putative FFA taste receptors [33, 34]. Because CD36 is expressed in some type II (sweet, bitter, and umami) receptor cells

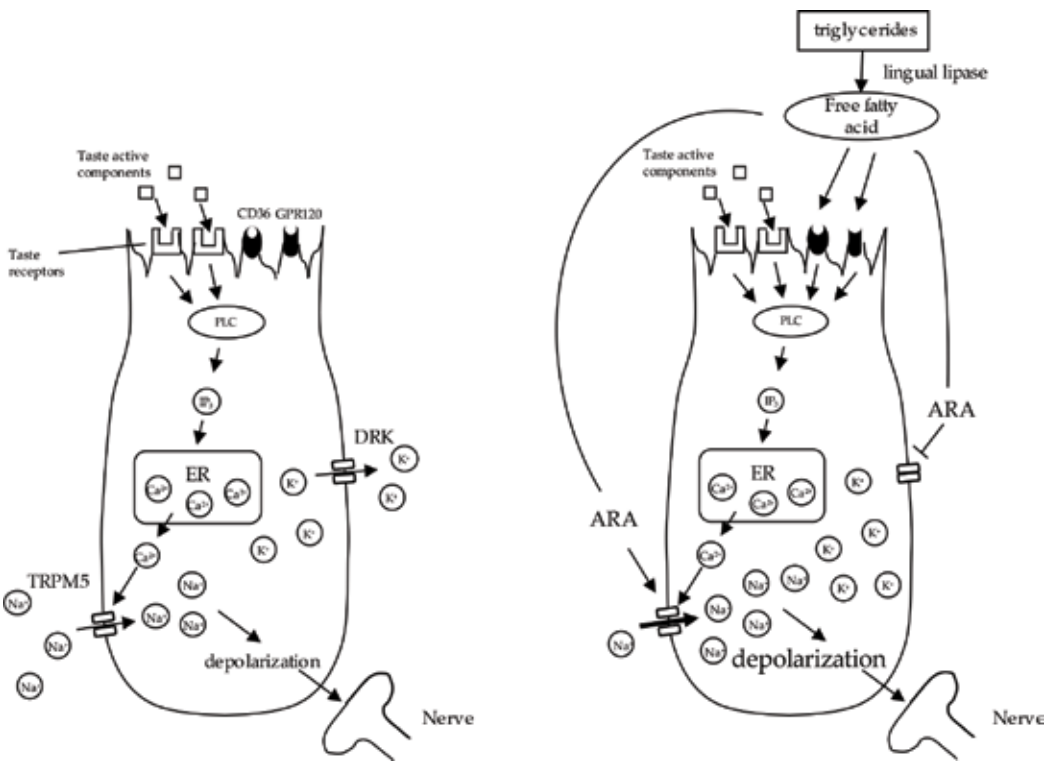

Figure 2. Proposed mechanism why ARA enhances taste perception in type II receptor cells [39]. DRK, delayed rectifying K+ channel; ER, endoplasmic reticulum; IP3, type 3 isoform of inositol 1,4,5-trisphosphate; PLC, phospholipase C.

in mouse taste buds [34] and GPR120 and GPR40 are mainly expressed in type II and type I (salty) receptor cells [33], FFA might affect taste perception of sweet, bitter, umami, and salty based on taste receptor distribution, although the presence of GPR40 has not been confirmed in the gustatory papillae of humans [35]. PUFAs, especially LA, ARA, DHA, and eicosapentaenoic acid (EPA), might inhibit the delayed rectifying K^+ (DRK) channel in taste receptor cells [36]. Resultantly, DRK channel inhibition might elicit a fast cell depolarization due to transient accumulation of positive charges in taste bud cells, since K^+ is the major intracellular monovalent cation in taste receptor cells. Oike et al. [37] reported that ARA activates the TRPM5 cation channel, which is an essential component of the sweet, bitter, and umami taste pathways of type II receptor cells. As a supporting evidence of TRPM5 function, it has been reported that TRPM5-null mice showed no licking response to a sweet tastant, a diminished preference ratio for sweet and umami tastants, and a reduced response to bitter taste [38]. Together, these data suggest that ARA may serve as a taste enhancer for type II receptor cells by modulating the TRPM5 channel and blocking the DRK channel (**Figure 2**). Details of the proposed mechanism were descried by Matsui and Takahashi [39].

Meanwhile, the Matsui-Takahashi model [39] lacks aspect of the effect of volatile odor generated from ARA, AO, and cooked chicken meat. Therefore, further studies are needed to characterize volatile odor components of ARA and to define whether the components affect sensory evaluation.

5. Conclusions

We have shown that the content of AA in chicken meat can be manipulated by dietary ARA supplements and by genetic selection, using *FADS1* and *FADS2* gene polymorphisms as selection markers; these approaches improve the flavor of chicken meat. We will be conducting studies on improving the flavor of Jidori meat and eggs, using molecular breeding and marker-assisted selection techniques, in the near future.

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Conflict of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Chapter 6

Research Progress in Genetic Control of Reproductive Performance in Chicken by High-Throughput Sequencing Technology

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Additional information is available at the end of the chapter

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Abstract

In chicken, egg production performance is a key trait to the production performance of chickens. Currently, low egg production performance is the major bottleneck, which restraints the development of indigenous chicken industry and blocks the cultivation of new chicken breeds. It has always been the focus of animal genetic breeding in detecting and studying the formation mechanism of complex traits. Due to the egg production is a complex trait determined by multiple genes, and regulated by heredity, environment, and the interaction between them, the mechanism regulating egg-laying performance is yet unknown due to its complexity. With the recent progresses of omics techniques, related researches on it have achieved considerable progress, making it possible to elucidate the molecular mechanism of egg-laying trait now. This article will provide an overall review about the recent research progress in genetic regulation of egg-laying performance in poultry through high-throughput sequencing technology.

Keywords: transcriptome sequencing, QTL, quantitative traits, egg-laying trait, genetic regulation, chicken

1. Introduction

To chicken, egg-laying trait is the vital part for playing the production performance in poultry industry. Although egg-laying trait is a quantitative trait controlled by multiple genes in chicken, it has almost reached their physiological extremity because of long-term selective breeding [1], for example, most breeds reach sexual maturity at an early stage; and the age of hens producing the first egg is advanced to 17 weeks; a modern egg-laying chicken (layer)

with a high laying rate can reach to one egg per day during the peak-laying period; the peak-laying period lasts a long time and produces approximately 300 eggs per year [2]. Besides, the egg quality is good enough, the quality of eggshell and uniformity of egg size are also eligible. To satisfy the increasing demands of world population to the protein obtained from animal, decrease the huge pressure caused by the increasing farming animals, and balance the economic growth with environment protection, the plan to cultivate a new breed, named as "longevity chicken," was firstly put forward in Europe. Basing on the guaranteed egg quality, the laying cycle of the commercial chicken is extended to 100 weeks, and the egg number could be reached to 500, which will lead to a decrease of breeding chicken production and a less need of land and feed; meanwhile it is an advantage to environmental protection [1]. Although the existing commercial layers show that genetic variation of the egg production-related performance is relatively little, the egg production and egg quality decline at the late stage of laying period; there are some individuals show high egg production with good egg quality at the late egg-laying stage. Therefore, it indicated that, to some extent, they possess the potential for breeding selection. Especially for the local chicken breed, the large genetic variation of egg production traits makes it owing wide space in selecting and improving. What is more important, with the rapid development of high-throughput sequencing technology platform, the elucidation of the molecular mechanism in regulating the egg-laying performance, and the application of new molecular breeding technology, the genetic progress of egg-laying trait selection could be further accelerated in poultry.

2. Application of high-throughput sequencing technology in quantitative traits

2.1. Quantitative trait locus (QTL) mapping remains to be the common way in the genetic analysis of quantitative traits

A locus affecting a quantitative trait is termed a quantitative trait locus (QTL) [3]. Dating back to 1918, based on the correlation between phenotype and genotype in cooperation with statistical method and genetics analysis, Ronald Fisher inferred the genetic basis of phenotype and proposed the basic thought of QTL mapping. However, until the presence of molecular markers in the 1980s, it provided the possibility for the intensive investigation the genetic basis of quantitative traits. Along with the exploitation and application of microsatellite, restriction fragment length polymorphism (RFLP) and other related genetic markers which are based on the polymorphism chain reaction (PCR), and the development of computer technology, QTL was developed to be the main method to identify the chromosome segments associated with the complex traits. In 2002, Tuiskula-Haavisto mapped the QTL related with laying performance in a F2 resource population produced by the cross between White Leghorn chicken and Rhode Island Red chicken and for the first time revealed the QTLs of some laying traits including age at first egg, egg weight, egg-laying number, as well as the eggshell quality [4]. Subsequently, related investigations were extensively researched. In China, basing on the genetic location resource of native chicken breeds, scholars performed the QTL mapping including the egg weight, egg quality traits, and so on, which promote our knowledge of the

formation and the molecular regulation mechanism of important economic traits in chicken [5, 6]. Until now, in the animal QTL databank, more than 890 QTLs associated with the laying performance have been verified basing on the diverse hybrid populations [7]. Among those, there are 248, 98, 53, 34, and 26 QTLs correlated with egg weight, egg number, age at first egg, laying rate, and body weight at first egg, respectively [8]. However, owing to the low density genetic markers in the past, majority of reports about the QTLs were previously mapped with a wide confidence interval [9]. In view of the constant study on chicken genome, the development of molecular genetic technology, and the exploitation and application of the high-density single nucleotide polymorphism (SNP) genetic marker, the accuracy of QTL mapping for egg production is significantly increased. Nonetheless, it is difficult to identify the potential reason that caused variations and exert a role in commercial breeding using the conventional QTL mapping method; it is mainly due to the relatively few recombinant individuals that are merely generated through the limited hybrid generations derived from their two original parents [10].

2.2. Genome-wide association study (GWAS) becomes an effective method for rapid identification the major genes controlling quantitative traits

In 1996, Risch group put forward the concept of genome-wide association studies (GWAS) while studying the complex diseases of human [11], which triggered the research upsurge of GWAS. After the completion of the whole genome-wide sequencing of cattle, sheep, pigs, and chickens, the GWASs on livestock and poultry have been gradually carried out. With the discovery of many high-density SNP molecular markers that cover the whole genome, the construction of high-throughput genotyping method, the development of computer technology, the GWAS method, which performing the regression analysis of phenotypes with the genotypes of each marker, and then the determination of significance of each marker have become the international mainstream and new strategy in identifying the complex (quantity) traits [12–14]. Chinese scholar Yang et al. took the lead in using GWAS method studying the variation of egg laying and egg quality in White Leghorn and brown shell egg dwarf layers, eight SNPs related with egg-laying performance and egg quality were discovered, and several egg-laying performance-related candidate genes were identified [15]. Furthermore, the egg production traits of brown shell egg-laying hens, white leghorn chicken, and Dongxiang green eggshell chicken were studied through GWAS; the genetic parameters of the corresponding traits and a number of candidate genes associated with egg-laying performance were obtained [10, 16–20]. Although GWAS has shown great advantages in genetic analysis of complex traits, most studies at the early stage only used the phenotypic value in single time point and could not reveal the genetic regulation mechanism of laying traits in the whole laying cycle. Therefore, it is necessary to further study the genetic structure of egg production traits from a more comprehensive perspective [21, 22]. In fact, in a cycle of egg laying, many egg-laying performance traits such as egg weight and laying rate can be detected repeatedly to obtain longitudinal data. A series of studies have proved that taking advantage of longitudinal data can identify sites, which are time-dependent and continuous. In addition, compared with the lateral analysis of a single time point, the multi-time points longitudinal joint analysis can improve the effectiveness of the statistical verification. Yi et al. divided the

laying period at the stages of 32–60 weeks into nine time points according to the egg weight, four variation sites were distributed on three chromosomes with independent contribution to egg weight at different periods, and five candidate genes were identified using the univariate, multivariate, and conditional GWAS [10]. Using the similar method, Yuan et al. identified nine variation sites that were significantly relevant with the egg number at the stage of 21–72 weeks [22]. Further analysis showed that GTF2A1 and CLSPN gene may be the candidate genes that influence the function of the ovary and uterus [22]. These findings help to understand the genetic basis of longitudinal traits, identify mutagenesis, and guide the selection breeding of egg-laying trait as molecular markers.

2.3. Whole genome resequencing providing new idea for mining and identification quantitative trait-related genes

The continual improvement of biological genome maps opened the prelude of the post-genome era, while because of the presentation of the next-generation sequencing technology, the genome sequencing has gradually become a routine tool to life science research; the combination of them makes the system research in genome level undergo a tremendous change. After long-term natural selection and high-intensity artificial selection of modern breeding techniques, the genetic polymorphism of animal strains will change enormously. The genome sequence variant regions between wild and domesticated breed were compared using the genome re-sequencing technology, and the gene and loci that are related to high-intensity selection traits can be identified through annotation and analysis to the genes and other functional elements in these regions. In 2010, Rubin group performed genome resequencing on red jungle fowl, the wild ancestors of domestic chicken, and other eight different types of domestic chicken using pools of genomic DNA sample; about 700,000 SNPs and more than 1300 deletions and numerous selective sweeps were observed [23]. One of the most popular selective sweeps occurred in thyrotropin receptor (TSHR) locus of all domestic chickens, which play a core role in metabolism regulation and the photoperiodic controlled reproductive regulation in vertebrate [23]. At the same time, the selective sweep in the broiler genome was found to overlap with the genes in controlling the growth, appetite, and metabolism; therefore a few important candidate genes were identified, and the cause mutations were directly separated [24]. This landmark work provides a new idea for mining and identification of complex quantitative trait-related genes.

Subsequently, genome sequencing technology, DNA chip, and gene expression chip were extensively used to study the effect of high-intensity selection on the formation of agricultural animal characters basing on the evolution genomics aspect, including researches on chicken. Nätt et al. compared the differences of gene expression and promoter DNA methylation in the hypothalamus of red Jungle fowl and Leghorn chicken and found that those genes with different expression levels and DNA methylation levels are significantly overlapped with selective sweep regions, showing that some epigenetic mutations may be related to the laying performance [25]. Li et al. detected selection signatures from 385 white Leghorn hens, through locating the positive selection region to the genome, a group of genes related to laying, metabolic, and immune response, as well as some novel genes involved in important economic traits were identified [18]. Through analysis to the system scan results of selection progress of commercial layer and broiler obtained by genome resequencing, Saber et al. discovered

that genes including BCDO2 and TSHR were parallelly fixed in two strains [23]. Meanwhile, some candidate genes associated with the appearance and production performance were also identified, which suggests that the combination of genome resequencing with group genetic technology can effectively identify the genomic regions and functional genes related with different varieties of phenotype [23]. Recently, by comparing the data of two Korean native chicken breeds, Leghorn chicken, and 12 Chinese chicken breeds, Jeong et al. identified some candidate genes that are related to the local chicken phenotype in South Korea [26]. Roux et al. firstly combined the selective deletion location, SNP annotation of gene coding area, and cis-eQTL analysis method, to study the two strains of chicken obtained through the selection of fat deposition differentiation, and successfully identified the cause gene of abdominal fat deposition [27]. The team from China Agricultural University has made a series of important achievements in researching the gene location of chicken in recent years. Basing on the established F2 resource population, the candidate genome segments of the coronal and silk feather phenotypes were identified using linkage analysis and resequencing technology, and the causation gene and causation variation were determined by IBD localization and differential expression analysis of candidate genes [28, 29]. In addition, similar methods have been successfully applied to the precise localization of the cause gene in regulating the tassel and the over-deposition of melanin in chicken [30]. In conclusion, the comprehensive application of whole genome resequencing and evolutionary genetic analysis techniques, combined with QTL, GWAS, and RNA-seq analysis methods, is an effective way for mining and identifying the molecular mechanism of complex quantitative traits.

2.4. Transcriptome sequencing (RNA-seq) is a powerful tool to reveal the regulatory networks and molecular mechanisms of quantitative traits

Transcriptome sequencing (RNA-seq) is a new-generation sequencing technology used for transcriptional analysis. By using the RNA-seq technology, the transcripts of different breeds or individuals under the specific conditions or specific tissues can be obtained simultaneously. Through systematic study to the relationship between transcripts including known or novel mRNAs, miRNAs, lncRNAs, and circRNAs, and specific traits, respectively, the regulatory networks of quantitative trait can be revealed, and the causative genes can be discovered. Therefore, the RNA-seq technology is extensively applied for analyzing the molecular genetic mechanism of quantitative traits.

In recent years, transcriptome sequencing analysis technology has been extensively applied in studying the molecular regulation mechanisms related with growth, metabolism, disease resistance, and so on quantitative traits in agricultural animals [31–35]. In the field of chicken reproductive performance regulation-related research, Ayers et al. studied the mechanism of sex differentiation in chicken using the RNA-seq technology and found that most of the differentially expressed genes in different genders come from autosomal rather than sex-chromosome linkage, many of which are novel genes for sexual differentiation, such as CAPN5, GPR56, and FGFR3 [36]. Shen et al. studied the molecular regulation mechanism of broodiness in two breeds of chickens using RNA-seq technology and found that the mainly differentially expressed genes in pituitary tissue were steroidogenic and hormone-releasing-related genes during the physiological process transition from egg laying to broodiness, and

among those, SREBF2, NR5A1, and PGR transcription factors may serve as the central signal modifiers involved in the steroid biosynthesis process [37]. Wang et al. studied differentially expressed genes in different grades of Fork headbox L2 (FOXL2)-overexpressed granulosa cell transcriptome using RNA-seq technology and found that focal adhesion may be one of the key pathways to be activated during the differentiation of granulosa cells; FOXL2 may participate in the follicle selection by regulating the expression of cytokines and the concentration of cyclic adenosine monophosphate (cAMP) [38]. The lipid metabolism of the liver is closely related with egg production and other production performance in chicken [39]. Liu et al. systematically studied the hepatic lipid metabolism regulation network in the livers of pre-laying (20 weeks old) and peak-laying (30 weeks old) chickens, from the transcriptome, epigenetics, and other different levels using RNA-seq technology, and a number of genes related to chicken fat synthesis, lipoprotein assembly, and transport were identified, which laid the foundation for further study of the relationship between these genes and egg production performance [40–42]. The China Agricultural University established the promoter DNA methylation profiles and gene expression profiles of muscle tissues of AA broilers and Dehong jungle fowl by meDIP-chip and RNA-seq. It was found that promoter DNA methylation levels are highly consistent between Dehong jungle fowl and AA broiler, prompting that the promoter DNA methylation level is conservative during the domestication process [43].

3. Conclusion

With the completion of genome sequencing of livestock and poultry species, the individual genetic information including whole genome sequence map, transcriptome map, epigenomics map, and SNP map could be rapidly acquired. Meanwhile, with the emerging of some new technologies, such as DNA chips, RNA interference, and proteome analysis, researchers could comprehensively apply the theory and research means of genomics, molecular genetics, and bioinformatics subjects, combined with the methods including SNP scanning, whole genome association analysis, next-generation sequencing, computer-aid analysis, molecular clone, and gene expression regulation analysis to carry out the researches on the network regulation of genes. It greatly accelerated the speed of discovering and mining novel gene, making it possible in illuminating the molecular regulation mechanisms of complex economic traits such as egg production. This will greatly enrich the theory and knowledge of poultry breeding, accelerate the genetic progress of selection in chicken egg-laying trait, and provide important reference for analysis of the egg-laying trait in different poultry breeds and birds.

4. Future perspective

In the last few years, formerly with the adoption of microarrays and successively with the introduction and implementation of NGS platforms for RNA sequencing, transcriptome analysis has been completely revolutionized. Particularly, RNA-Seq can be used to simultaneously detect the whole gene expression levels and the diverse species of the RNA world. The availability of such a complete transcriptome profile has been a powerful tool for obtaining

the insights into the molecular mechanisms underlying the formation of complex traits. In recent years, the huge advances in the development of new high-throughput sequencing technologies, not only in transcriptomics but also in metabolomics, proteomics, epigenomics, and genomics, have increased the complexity of the analytical methods aimed at identifying the molecular basis of phenotypic traits, especially in complex traits.

Egg production as a complex trait is determined by multiple genes and regulated by heredity, environment, and the interaction between them; therefore the mechanism regulating egg-laying performance is yet unclear. Although the existing statistical methods have made it achieved considerable progress, current study needs to develop and improve multilevel data integrated analysis methods for multi-omic-derived data. Despite that multi-omic research is still challenging, it will accelerate the new discoveries and insights into elucidation of the molecular regulation mechanism of egg-laying trait in the future.

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Conflict of interest

The authors declare that they have no competing interests.

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Section 3

Disease and Disease Resistance

Chapter 7

Adenoviruses and Their Diversity in Poultry

Jowita Samanta Niczyporuk

Additional information is available at the end of the chapter

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Abstract

An investigation into the aetiology of fowl adenovirus strains and their distribution worldwide in populations of poultry flocks industry has been conducted. Pathogenic role of the viruses is not always clear. They can cause latent infection or several diseases and are the reason of economic losses in poultry flock industry. Ubiquity of adenovirus strains was commonly described, and stand-alone pathogenicity for a long time has been disputed. A globally emerging trend of adenoviruses and adenovirus-associated diseases has been increasing from year to year in all over the world. Mainly, type FAdV-4 is responsible for hydropericardium hepatitis syndrome (HP), type FAdV-1 for gizzard erosion and ulceration (GEU), and types FAdV-2, 8a, 8b, and 11 seem to be responsible for inclusion body hepatitis (IBH). Defining the spreading of the avian adenovirus strains in different types of fowl profile production, recognising their property and determining their types and molecular characterisation are very important from the epidemiological point of view and are considered as excellent basis for vaccine development and gene therapy implementation. This chapter provides a comprehensive review of FAdVs, including their epidemiology, pathogenesis, diagnostic, detection, and molecular characterisation. This comprehensive review is needed to better understand the latest progress in study of the viruses and prospects regarding disease control and implementation of gene therapy.

Keywords: CPA, FAdVs, HVRs region, LAMP, molecular characteristic, pathogenicity, RSCU

1. Introduction

Adenoviruses are a very diverse group of pathogens, causing diseases in domestic and wild birds. Fowl adenoviruses are common in healthy birds and can cause different diseases with pathogenicity from 10 up to 90%, depending on strain virulence, as demonstrated in poultry flocks in all over the world [44, 62]. Adenoviruses have been isolated from different types

of fowl profile production, and infections in fowl seem to be commonly widespread. Recent outbreaks of adenovirus infections (FAdV) in poultry flocks have been determined in many countries such as Europe, USA, Asia, and Australia, and are connected with economic loss in poultry production. Wild birds play some role in the transmission of several adenovirus types/species, which represent an important problem for poultry production [35]. Our knowledge about the role of wildlife reservoirs in the transmission of the adenoviruses is under exploration [35]. Worldwide distribution and outbreaks of adenovirus infection in 31 wild bird species have been reported by many authors, and types FAdV-1 and 4 infective for poultry are not infective for wild birds [45, 48]. To understand more, the evolution and transmission of FAdV viruses and detailed codon usage analysis was performed for FAdV strains representing five species FAdV-A-E and 12 types FAdV-(1–81-8b-11). High effective number of codons and indication of the presence of relative synonymous codon usage have been determined. The presence of mutations and their influence on codon usage was confirmed by the correlation between nucleotide compositions at the third codon position, HVRs1–4, and ENCs. This indicates some influence of natural selection and antigenic properties of examined FAdVs strains (Niczyporuk, Vol. 21 no3 (September 2018) of PJVS).

2. Morphology and biology/epidemiology

Adenoviruses, belonging to *Adenoviridae* family, are non-enveloped double-stranded DNA viruses [67]. The conserved domains are responsible for basement creation of the molecule and are responsible for trimmer formation [54]. The highly variable domains are mainly located outside the virion and are responsible for antigenic variation of the strains [7, 10]. Schematic concept of adenovirus genome is presented in **Figure 1**. The International Committee on Taxonomy of Viruses [32] separates the *Adenoviridae* into five genera: *Mastadenovirus*, *Aviadenovirus*, *Siadenovirus*, *Atadenovirus*, and *Ichtadenovirus*. Fowl adenoviruses are separated into five species designated as FAdV-A-E with 12 types of FAdV (1-8a-8b-11) [45, 48]. Adenoviruses are very divergent pathogens generally with low level of virulence; however,

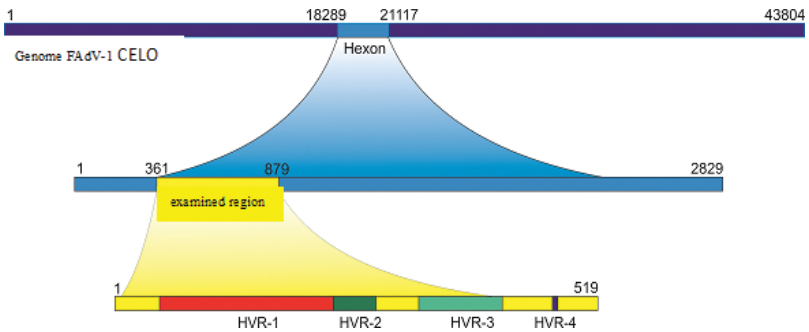

Figure 1. Schematic concept of adenovirus genome structure. Yellow colour indicates examined region of loop L1 hexon gene additionally divided into sections representing hypervariable regions HVR1–4. Niczyporuk, Vol. 21 no3 (September 2018) of PJVS.

under certain conditions, they can cause a variety of disorders in domestic and wild birds [33, 35]. Adenoviral infections can be found as asymptomatic or a complication factor in the course of different diseases [7].

Data connected with adenovirus structure, different protein functions, genome organisation, and replication are based mostly on human adenovirus research [58]. The adenovirus capsid is formed by three major proteins: hexon, penton base, and fibre. The hexon plays an important role in the genome organisation. Predicted amino acid sequences in hexon gene from human Ad2 and Ad5 and bovine adenovirus type 3 presented regions with high homology at the end of N and C regions, separated by central part with low homology. Both hexon proteins have a common structure. Hexon is the major capsid protein presented from 40 to 820 copies per virion. Every hexon has two characteristic parts: triangular peak with three towers and a basis with central part. To construct from conservative component: regions P1 and P2 are basements created of the particle and takes part in trimmer formation. Hypervariable regions (HVRs) of hexon protein creates loops L1, L2, and L4, which are located in external part of virion towers created; loop L1 is presented in (**Figure 1**). We can identify seven hypervariable regions HVRs1–7 [14]. Four of them are located in loop L1, two HVRs in loop L2, and one HVR in loop L4. Type-specific antigenic determinants are coded by HVRs from loops L1 and L2, existed on the hexon surface, and are strictly responsible for induction of immunological response. In loop L3, there are no such determinants [37]. This region can help in distinguishing the differences between strains of different types and point out their diversity. In a study conducted by Niczyporuk [52], the geographic analysis of adenovirus strains isolated from Poland, based on loop L1 region of hexon gene, has been described, and all the relative synonymous codon usages (RSCUs) in HVRs1–4 were designated (Vol. 21 no3 (September 2018) of PJVS). Alignment of consensus sequences clearly indicated the differences in hypervariable regions (HVRs) as described by Raue (2005). Four HVRs in loop L1 were identified as the regions of the highest sequence variability: HVR1 of about 191 bp long, HVR2 of about 50 bp long, HVR3 of about 90 bp, and HVR4 of about 18 bp long. The DNA sequences of HVRs are constant for every type, but there are major differences between FAdV types. Singh [64] indicated that timer stability in TAdV-3 fibre head monomer has the surface area in trimmer, and that fibre head from other adenovirus strains concerned with the stability of the trimmer are comparable. The melting temperatures of virulent and avirulent forms of the protein are 80°C at pH = 6, which indicates high protein stability. Stewart [68] indicated that adenovirus capsid amino acids are in symmetrical location in the inner and outer sides with the icosahedral symmetry. Each hexon has six nearest neighbours, and every hexon takes part in the construction of three hexon rows. Every triangular facet participates in forming three vertices, and every facet has three nearest neighbouring facets. Lenong [41] indicated that three structures of Ad type2 (Ad2) of species CE3-19 K/HLA-A2 complex showed the adaptation of a novel tertiary fold and uses a new binding surface on HLA-A2. Significant differences in genome size may have an influence on the genome organisation [10, 23]. The genome of avian adenoviruses is about 44–45 kb long, depending on the species/type. Adenovirus strains from the genus *Siadenovirus* with a genome of 26 kb length, genus *Mastadenovirus* with 31–36 kb, and the genus *Atadenovirus* with 27.7 kb [3, 38, 41] are analysed for comparisons. The shape of the trimeric hexon is not common and is divided into a hexagonal basis, which is reach in

β-structure, and every triangular top creates secondary structure [8]. They can be infectious for fish, reptiles, amphibians, birds, mammals, and were isolated from over 40 vertebrate species [7, 29]. Wild bird species [35] with adenovirus infection were documented by other authors and were found in falcons [65], common buzzards [22], *Milvus migrans* [24], tawny frogmouth [59], pigeons [67], and psittacines [40]. It is possible that under some conditions, adenoviruses can be more virulent in non-host-adapted species than in their typical host [24]. Some virulent FAdV strains can pass species barrier and can also infect new organisms [24].

3. Clinical features and pathogenesis

Adenovirus replication is based on two different mechanisms of the interaction between cell and the virus strains. Virus interaction with the cell starts from the entering into the pathway of the endocytosis to the host cells [49]. Domains, which are located at the end of the fibre, recognise the receptors, which are located on membrane, and binding process begins. We can suspect that the proteins from major histocompatibility complex (MHC) are also involved in this process. In the next step, the proteins, which are basis for penton, are involved in interactions with integrin cells that help in endocytosis (49, 70). During the next step, capsid disintegration takes place and genome that is connected with histone infiltrates into cell nucleus. This process is coordinated by microtubule of host cells (32, 39, 49). The replication is divided into early and late stages. Firstly, the transcript, which is created during the splicing, creates monocistronic mRNA, having the information about one protein. Next, the translation processes starts. Early genes are responsible for non-structural proteins, which are responsible for replication, regulation of cell metabolism, and changing the cell for the production of DNA viral protein and apoptosis prevention. During the beginning of replication, protein pre-terminal (pTP), polymerase DNA (pol), and protein DNA (DBP) binding are created. In the next step, heterodimer pol-pTP is created and is connected with 5'DNA, and replication gets started. The cell regulation transcripts like NFI/CTFI and NFIII/Oct-1 [43] are also involved. After the DNA replication starts, the transcription for late genes and formation of structural protein gets started. Virions stay in the nucleus and sometimes can create crystals and chromatin accumulation. The latency period for formation and maturation of the virus particles persists and the lysis of infected cells begins and virion particles are released [32, 39, 70]. Virus particles can appear after 14 h of post-infection—the first stage of particles releasing and increasing quick copy number of the virus particles with the highest point after 48–72 h. After 72 h, quantity of copy number of the virus particles decreases [45].

Adenoviruses can be transmitted horizontally [45, 48] and vertically [28]. Infections are most dangerous in younger birds with a predilection for birds less than 35 days of age. These viruses have the minor role in apoptosis, as type FAdV-1 has genes encoding anti-apoptotic peptides [28]. In embryonated eggs, virus can replicate causing death of the embryo or can be in latency period till hatching. Cell cultures, which are prepared from infected embryos, have the adenovirus infection. In chickens, hatched from these eggs, the infection can stay in latent phase and is indicated in the next chicken generation [18, 46, 69] which was indicated for types FAdV-4 [25] and FAdV-1 [28]. During the horizontal transmission with poultry litter,

water, or feeding staff in front of infected birds, adenovirus infections can have different courses of action. The infection can be latent, can be the implication factors for different diseases, or can be the reason of infection by themselves [48, 55]. In infected birds, the virus was found in faeces and in organs like trachea, kidneys, liver, spleen, and intestines. Adenovirus tropism depends on the age of the bird, its immunological status, and strain pathogenicity. Pathogenic activity in birds is not always clear. Even isolates from the same species show different pathogenicity depending on the strain, infective dose, and different environmental factors. In some cases, adenoviruses can be the opportunistic viruses [19], with a low level of virulence [61]. This mechanism is presented, e.g., FAdV-1 type for example pathogenic strains from that type are responsible for gizzard erosion and ulceration like strain FAdV-99ZH, but others like Japanese non-pathogenic strain FAdv-1-ote [19] did not induce indicated changes. Some strains cannot induce the disease by themselves; however, their pathogenic role is still important during the multi-aetiological syndromes. Adenoviruses can be isolated from sick and healthy birds. From one side the viruses can be the reason of the infections without any signs or can be the aetiological factor of syndromes like inclusion body hepatitis (IBH) [2, 17, 36, 55], hydropericardium hepatitis syndrome (HPS) [13, 34], haemorrhagic enteritis (HE) [56], marble spleen disease (MSD) [20], egg drop syndrome (EDS) [1], and gizzard erosion and ulceration (GEU) [54]. Period from first signs of infection is different and can be short 24–48 h. Antibodies produced after the infection with one virus type do not have any protection against the other adenovirus types [32]. However, serum with antibodies against type FAdV-9 is capable to neutralise the type FAdV-3. No cross-reactivity in opposite direction was noted [66]. For the adenovirus pathogenicity, genotype does not decide the type selection [27, 48] and pathogenic properties depend on the interaction between hexon proteins.

4. Detection

The presence of genetic material of adenovirus strains can be detected in samples of internal organs from sick or suspected for the diseased chickens. The sections of the liver, spleen, gizzard, kidneys, and intestines are collected and prepared as 10 w/v homogenates in a phosphate buffered saline (PBS). The homogenates for virus isolation are centrifuged and filtered through the 450-nm filters.

4.1. Virus neutralisation test

Serum samples from infected or suspected chickens are heat inactivated for 30 min at 56°C, and virus neutralisation test (VNT) is performed in 96-well plates containing CEF cells. After incubating for 5 days at 37°C in 5% CO_2 atmosphere, the cells are examined for cytopathic effects (CPE), virus titres are calculated, and titres above 3 $\log_2$ are considered positive.

4.2. Cell culture and virus isolation

Chicken embryo fibroblasts (CEFs) or chicken embryo kidney cells (CEKs) are prepared from 11-day-old or 19-day-old SPF chicken embryos (Lohmann-Tierzucht, Cuxhaven, Germany).

The cell suspensions are inoculated onto monolayer cultures, in which 0.2 mL suspension contains usually from 10^6 to 10^7 of live cells. Inoculated and control cultures are incubated at 37°C in a humid incubator containing 5% CO_2. Areas of cytopathic effects appear within 5–6 days; thereafter, the virus particles can be isolated.

4.3. Immunofluorescence assay

Usually, CEF cultures are infected with the third passage of the adenovirus strains. When CPE are observed after 5–6 dpi, CEFs are covered with 90% acetone cooled to −20°C. After 30 min, the acetone is removed and the plates are allowed to dry for the next 24 h. Then, the CEFs are washed three times with PBS buffer followed by the addition of 500 μL of blocking mix: 1× PBS, 5% bovine serum, and 0.3% Triton X-100. The plates are incubated for 1 h at 18–24°C, the blocking mix is removed, and 500 μL of mouse primary FAdV antibody, diluted 1:100 with PBS, are added. After incubation at 37°C for 18 h, the plates were washed three times with PBS (BioLab, Poland), a 1:200 PBS dilution of a secondary rabbit antibody against mouse IgG_1 conjugated with fluorescein isothiocyanate (FITC) was used and incubated at 18–24°C for 2 h in the dark. The fluid is removed and the plates are washed thrice with PBS buffer. The cells are observed under a fluorescence microscope. The presence of fluorescent cells of different sizes indicated a positive result of the IFA (**Figure 2**).

4.4. Molecular techniques

Molecular techniques such as restriction endonuclease analysis (REA) [50, 60], in situ hybridisation using DNA probes, polymerase chain reaction (PCR) [23, 52], real-time PCR [27], and real-time PCR Gunes, 2012 for the detection and quantitation of FAdV(A-E) species have been used. However, in the studies, primers were based on conserved nucleotide sequences within the 52 K gene with the efficiency of 98%, and regression squared values of $R^2 = 0.999$. Different real-time PCR and subsequent high-resolution melting curve analysis (HRM) of 191-bp region of the hexon gene and restriction enzyme analysis have been performed by

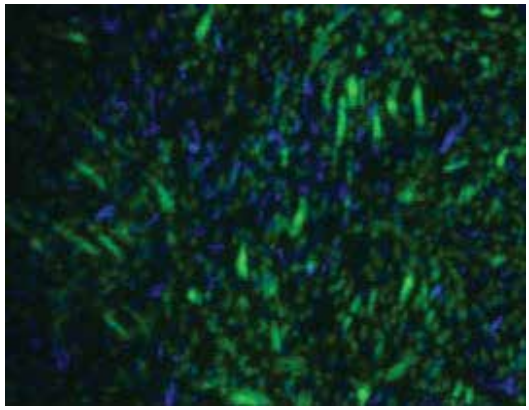

Figure 2. IF cytopathic effect of adenovirus strain type/species 1/A, IIIp. 96 h.p.i.

Steer [66] for differentiating all FAdVs species, and melting curve profiles were found to be mainly related to GC composition and distribution through the amplicons. High-resolution melting (HRM)-curve analysis [60, 67] was developed and applied as a specific, sensitive, and efficient detection method without cross-reactivity. For the latest years, the new effective and chipset techniques have been developed like loop-mediated isothermal amplification (LAMP) [51]) or cross priming amplification (CPA) by Niczyporuk [51].

5. Molecular study on HVRs1–4 and RSCU diversity

Data concerning the adenovirus genome, structure, function of selected proteins, and replication are based on human adenovirus strains [8]. In Poland, the presence of five species with eight types of adenovirus strains: FAdV-1/A, 2/D, 4/C, 5/B, 7/E, 8a/E, 8b/E, and 11/D were described recently [51]. The biggest group was formed by the strains classified as type FAdV-2/11/species D and the smallest group by the type FAdV-4/C [51]. Moreover, the relationship between type/species and the internal organs from which strains were isolated was found. It was demonstrated that types/species FAdV-2/11/D were most commonly isolated from the liver and intestines while type FAdV-4/C was represented less frequently [51]. Avian adenovirus genome size is 44–45 kb and depends on type/species representation. For comparison, mastadenoviruses having a genome size of 31–36 kb is chosen. Genome of adenoviruses belonging to different species has differences in quantity a pair of (GC) content. The value in avian adenovirus genome is between 53.8 and 66.9% and in mastadenoviruses it is 43.6 and 63.9% [8, 32]. Different sizes of the genome have a influence on gene organisation, depending on the type/species, and have 23–46 genes, which code several proteins [10]. Number of genes and their functions are different among strain-type/species; however, their basic genome organisation is common for the *Adenoviridae* family especially in the central part. Genome contains over a dozen of transcriptional units, which code 1–8 sequences with open reading frames (ORF). Owing to the alternative splicing, 50–70 different adenovirus proteins can be created. Till now, not all the genes and their functions are known [32]. Schematic concept of adenovirus genome and the location of hexon gene and hypervariable regions, HVRs1–4, are presented in **Figure 1**. Avian adenovirus genome has unique transcriptional units, so has the parts which are not well characterised yet. Adenoviruses have the ability to induce interferon that was demonstrated in experimentally infected animals [5] and cell cultures [4]. Adenovirus genome can also be used as a system for gene expression analysis [71]. Natural selection and mutation pressure have the influence on synonymous codon used during protein translation [11].

The relative synonymous codon usage (RSCU) is a simple measure of the heterogeneity in the usage pattern of synonymous codons. The value was calculated and performed by [11, 21, 26, 30, 31, 63]. RSCU value >1 means that the codon is more frequently used than expected and over-represented, while the RSCU value <1 means that the codon is less frequently used than expected [11]. The effect of mutational pressure on codon usage was confirmed by correlation between nucleotide compositions at the third codon position [64]. Sharp and Li [63] also defined the RSCU value as the ratio of observed frequency of a specific codon to the frequency expected, and the effect of mutational pressure was assessed by correlations of the third nucleotide position

in the codon. The presence of RSCU values for types FAdV-1/A and FAdV-7/E was published by Niczyporuk [53] and for types FAdV-2/11/D, 4/C, 5/B, 8a/E, 8b/E was described by Niczyporuk (Vol. 21 No3 (September 2018) of PJVS). However, all the types/species are different and all the molecular characteristics of the strains indicate diversity in each of the adenovirus type.

6. Adenovirus diversity

The degeneracy of the genetic code is established with in a group of 2–6 synonymous codons and some codons can be preferred than the others. This specific usage of codons is called codon usage bias, and between the various factors, natural selection, mutational pressure, RNA structure, and gene length are most important for codon usage bias [64]. Codon usage analysis is a well-established technique for understanding the process of evolution on molecular level [11]. In a study conducted by Niczyporuk, the nucleotide frequency in codons has been determined by the content of (GC) pairs and was different for different types/species (data was not published yet). The obtained data are comparable to the data reported by Raue [60]. It may be possible that genes of higher transcriptional importance could have higher (GC) content [16, 31]. Similar researches concerning different species have been done by Halder [30]. Every single deviation in codon usage is based on the codon preference by using the exact codon during the translation process. Specific codons are used more frequently than the other synonymous codons in genes with the high expression. Such codons are chosen as preferable or optimal one. This adaptation forces the expression, gene size, genome structure, and percentage of (GC) content or frequency of recombinations. In 2017, RSCU for FAdV-1/A and 7/E have been published by Niczyporuk [53], and preference of codons was defined.

The loop L1 with HVRs-1-4 is the main indicator of variability that was confirmed by authors of Refs. [14, 57, 60, 72]. Not every amino acid substitution has the influence on the structure and adenovirus protein function. Most substitutions are of similar size, charge, or hydrophobic properties. These are the amino acids of conserving function. This fact can explain that protein can have self-structure and function with different amino acid sequences coded by highly different nucleotide sequences. We can establish, that every codon can appear with equal frequency; however, Niczyporuk (data not published yet) indicated that from few possibilities, one codon can be preferable than others. These preferences can appear in genes with strong expression [6, 31]. The frequency of the presence of some codons in front of others (codon synonymous) is called relative synonymous codon usage (RSCU). It is indicated that the optimal codon can lead to the fast and exact or accurate translation, which is extremely important for the proteins synthesised in higher amounts [71], which has an impact in evolution. A deep analysis of examined consensus sequences based on the evaluation of mutation quantity, their localization, and possibility of the influence on protein tertiary structure (data not published yet) has been performed. Many researchers suggest, (Behura and Severson [6], Crawford-Miksza and Schnurr [14], Epstein et al. [16]) that the most important for the study are the mutations which are located on the first and second codon places. These are the mutations that influence amino acid coding, which have the impact on differences in protein structure and function. Changes in variability have in consequence an influence on virus pathogenicity. Principal component

analysis also supported that most codons showed biased effect on (G) and (C) at the third codon position and at the preferred codons that end with either (G) or (C). Halder [30] also indicated that positive significant correlation in gene expression parameters with a few amino acids such as Val (V), Arg (A), Ser (S), and Ile (I) might influence the gene expression.

Gu [26] in their comparative study described the RSCU of ovine 287(OAdV287) and human HAdV2/5 adenovirus strains. Gu [26] presented that OAdV287 had more conservative codon usage than HAdV 2/5. The preferred codons of HAdV2/5 mostly had (GC) ends. Das [15] indicated that one of the major determinants is the (GC) content in the third codon position C3S and G3S, and significant variations are observed in synonymous codon choice in structural and non-structural genes of HAdV. In a previous study, Niczyporuk [53] described RSCU in the loop L1 region of the hexon gene for types/species FAdV-1/A and FAdV-7/E. The most important mutations were those in the first and second codon positions, because these mutations are more likely to result in an amino acid change, affecting the structure and protein function. Nucleotide sequence analysis indicated that different codons can code the same amino acid, but only some of them are preferred. Codon analysis of the loop L1 region of the hexon gene indicated differences in codon preference patterns between adenovirus strains representing diverse types. It was found that (C) was the most frequent nucleotide for each type ranging from RSCU 29.3 to RSCU 34.4. The analysis indicated antigenic properties of examined viruses, the presence of relative synonymous codon usage, and the presence of mutations. The effect of mutational pressure of the codon can be tested in the future in order to understand its impact on FAdV pathogenicity.

7. Adenoviruses in poultry

Inclusion body hepatitis (IBH) mainly existed in broiler chickens from certain breeder flocks and is associated with many different serotypes. Concerning the type definition is misleading because of three classification systems: US, EU and Australia [8]. These systems indicated the type number and not the species designation. In the past years, viruses reported for IBH infection belongs mainly to species/type D/2, 11, 9, 3 or E/6, 7, 8a, 8b primarily affecting the liver and in haematopoietic system and presented intra-nuclear inclusion bodies in the hepatocytes and lesions in other organs [13]. Mortality may reach 10 till 30% occasionally [2]. The other syndrome caused by adenovirus infection is hydropericardium syndrome (HS) occurring in broilers, breeding, and laying flocks in pigeons and quails [13, 42]. It is connected to an infection with an type/species C/4. In HS cases, we observed an accumulation of clear, straw-coloured fluid in the pericardial sac with pulmonary oedema and enlarged kidneys. The vaccination against C/4 is commonly used. Gizzard erosions (GE) in broilers are caused by a type/species A/1 and E/8 with first cases described in Japan [34]. Gizzards are distended with haemorrhagic fluid and contain multiple black patchy erosions within the koilin layer.

Egg drop syndrome (EDS) or duck adenovirus 1 of genus atadenovirus of the *Adenoviridae family* is responsible for the syndrome in laying hens. Outbreaks of EDS in laying birds will cause significant loss of saleable eggs, and the disease is controlled by vaccination [1].

Other adenovirus disease in poultry is haemorrhagic enteritis (HE), which is an acute viral disease in turkeys characterised by depression, bloody droppings, and death. Marble spleen disease (MSD) is related to pheasants with 3–8 months of age [20]. The virus is serologically indistinguishable from HEV with diversity only at the genomic level, causing lung oedema, congestion, dyspnoea and death, and avian adenovirus splenomegaly (AAS) in broilers [8].

8. Discussion

Clear differences appeared between strains classified to different adenovirus types/species. All the data confirmed correctness in classification of the examined strains. The analysis of adenovirus genome was mostly based on properties of hexon gene, which is the biggest gene in adenovirus genome. Hexon gene has specific nature and structure with conservative and hypervariable regions HVR1–4, and is the object of most adenovirus studies based on taxonomy and characteristic antigenic properties [9, 16, 38, 50, 57, 60, 72]. It is very difficult or nearly impossible to conduct taxonomy studies based on conservative sequence, which is very similar and almost identical in all adenovirus types/species [23, 50, 57, 60]. That is why loop L1 HVRs1–4 region of hexon gene was used for analysis by many authors.

Mutations in genes, their quantity, and localisation can influence the protein structure. Mutations on the first and second places of codon are the most important, because these mutations can influence amino acid coding and subsequently change the structure and protein function. The differences are focused mainly in loops L1 and L2. Loop L1, together with HVR1–4 regions, is the longest loop in protein with complicated folding [14]. It also serves as the location of specific receptors [39, 58, 60]. Examinations on the regions that are responsible for the antibodies binding Pichla-Gollon [57] indicated that this bridge pile is responsible for it. Hypervariable regions indicate high differentiation between adenovirus species/types and between the adenovirus strains, which can infect different hosts [57]. Simultaneously comparison study on amino acid protein hexon sequences of adenovirus strains from different species conducted by Crawford-Miksza [14] indicated that amino acid sequences of HVR1 are specific for exact adenovirus host.

HVR1 region forms the structure called 'hairpin' [68, 71]. Analysis of the HVR1 structure suggests the presence of β-sheet structure, which is created by the 'hairpin'. This structure was found in all FAdV types. HVR3, similar to HVR1, also forms 'hairpin' structure, which forms above protein surface. HVR1 and HVR3 are positioned close to each other in shape of V. The HVR5 (sequence not in the loop 1) is positioned between these V-structure arms. Study by Pichla-Gollon [57] indicated that the side of hairpin structure of HVR1 is the main region for the neutralising antibody binding. All mutations, which are situated in HVR1–4 region, can led to avoidance of host mechanisms of immunity and face modifications of these regions, which can led to create vectors for clinical treatment.

9. Conclusions and future perspectives

The adenovirus area and their mechanisms of pathogenicity are still under the exploration and depend on virus type and species. To our knowledge, the pathogenicity is mediated by

interference with other antiviral immune responses, and fowl adenoviruses are widely considered as excellent platforms for vaccine development and gene therapy. The detailed outcome of the RSCU, (HVRs1–4) from adenovirus strains was examined for the presence of similarity and mutations on the first, second, and third codon positions as well as impact on the amino acid creation, tertiary structure, and spinal conformation. Analysis indicated specific sequence in loop L1 HVR1 region, which is strictly responsible for antibody binding. This review will help better understand the mechanisms of pathogenicity of adenovirus strains and provides a guide for disease control in birds. Further studies are needed to explain the possible predisposing factors, which may lead to the pathogenicity. Moreover, the detailed study on whole genome sequence and virulence of newly isolated strains should follow.

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Chapter 8

Analyses of the Polymorphisms in *E. coli* Strains Associated with Heat-Shock Proteins Hsp 55 Isolated from Bird Feathers

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Additional information is available at the end of the chapter

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Abstract

The bird feathers are often colonized by pathogenic microorganisms including mainly bacteria of the *E. coli* species. There is a grooving evidence that due to colonization of the pathogenic bacteria after slaughter material may lead to different zoonosis diseases that endanger human health. Poultry diseases are a very important issue both economically and epidemiologically in each country. Currently, in practice, EU postmortem monitoring programs are often used to eliminate breeding poultry infected with different pathogenic microorganisms, e.g., *E. coli* by introducing mandatory bird vaccination. The article describes the combination of genetic and genomic methods that were used in the analysis of species specificity of strains and their genomes, including specific pathogenic bacteria in bird feathers. The aim of the study was (i) to investigate DNA polymorphisms of the obtained bacterial strains isolated from avian feathers (ii) obtaining recombinant *Hsp55* protein and defining its role as a potential component of vaccines used in poultry diseases. For the detection and analysis of DNA polymorphisms, we have optimized a new innovative method based on the deficiencies of three molecular techniques, AFLP, PCR-MP, and PCR MP. This new method can be a useful tool used in the genotyping of bacterial *E. coli* serotypes present on avian feathers after the slaughter process. It also allows to effectively identify a number of early stages of infectious diseases from heterogeneous avian research material. Amplification of polymorphic regions was achieved by using a lower denaturation temperature of the primers and a reduction in the number of cycles in the classical PCR, which simplifies the procedure, preserving the quality and reproducibility of the obtained results. Research of recombinant *Hsp55* protein has allowed us to determine the optimal conditions for its production by the classical methods used in proteomic analysis.

Keywords: *E. coli* serotypes, bird feathers, DNA polymorphism, ALFP method, LM-PCR method, PCR MP method, Hsp

1. Introduction

Growing urbanization increases the risk of zoonosis diseases that endanger human health. These diseases are caused by microorganisms, parasites, and their toxic biological products. People working in the poultry industry or bird slaughterhouse are exposed by contact with bird feces and dust from feathers of domestic and farm birds. The lack of adequate hygiene and protective clothing can lead to direct skin or respiratory tract infections. Zoonoses actually are infectious transmitted by animals to humans [23, 63]. The people are more susceptible to zoonoses carried than birds, and they share more diseases with them. These infections as etiological factors can develop in humans in several ways: by the respiratory system, digestive tract, and skin laceration [23, 62]. Many microorganisms like bacteria of the *E. coli* strain need proper conditions and parameters to determine their host as receptor on the surface of the cells [23, 62]. These receptors can penetrate into cells, causing the development of infection. The mechanism determines the susceptibility of one animal species to a pathogen and resistance of another species. In the case of people who have been infected with an avian zoonosis, hospital treatment in isolation and prolonged administration of specific antibiotics is required [23, 62]. Avian zoonoses in humans may lead to a chronic disease or death. The first symptoms of a disease in a person appear after 2 weeks, resemble symptoms like food poisoning or influenza, and may also cause meningitis. These diseases are very dangerous for pregnant women or for those with reduced immunity and last for about 3 weeks. A small amount of bacteria introduced into the human body through contact with feathers or contained in feather dust can occur without visible symptoms. The above factors are responsible for the severity of the clinical course of infection, epidemic outbreaks in poultry, increasing antibiotic resistance, and economic losses. So far, the diagnosis of poultry infections has been based on clinical study, and bacteria carriage in bird feathers has not been tested [12].

The main goal of the work is developing the methods for DNA diagnostics toward *E. coli* from bird feathers that shall allow identifying their pathogenic serotypes so that it will be possible to control the environmental factors responsible for epidemic proper treatment in the future.

To better identify the microorganisms, we have decided to combine the three techniques that generate a very large number of polymorphic genomic DNA fragments into one optimization technique which can help significantly shorten the individual steps to obtain a reliable results of the analyzed material. These techniques belong to amplified length fragment polymorphism (AFLP), [1, 37, 39, 57], ligation-mediated PCR (LM-PCR) [17, 41], and PCR MP (polymerase chain reaction melting profiles) described in 2003 by [33] and modified by [24–28]. Currently, in a literature there are no data available concerning of analysis of avian-feathered microorganisms based on genetic polymorphism methods with using one of the available molecular biology techniques. These methods used separately can be helpful in identifying

the kinship between pathogenic strains when the information about the genome is unknown. Therefore, there is a need to clarify this issue.

AFLP as a unique fingerprint is generated for a particular genome and was first developed for plant studies [59]. Although they can generate large numbers of marker fragments for any organism is fraught with some errors. These include potential nonhomology and asymmetry in the probability by loss of fragments. Sometimes, they appear the problems in distinguishing heterozygote from homozygous bands [43, 44, 61]. LM-PCR technique which was discovered and used by [37, 41] for the following modification for footprinting of DMS-induced DNA damage is applied widely by [31]. LM-PCR is a procedure capable of detecting the presence of specially DNA sequences which are determined by a specific protein sequence of enzymes in the phosphodiester backbone of nucleic acid. Also, this technique has problems connected with the proofreading polymerase, which affects significantly the quality received bands [2]. The PCR MP method is based on genomic DNA digestion with only one restriction enzyme, namely, HindIII. This technique could also be used to check the stability of bacterial strains in industrial plants, microbial collections, and epidemiological studies [6, 34, 51]. At present "gold standards" in genomics of microorganisms are methods based on DNA–DNA hybridization, which are quite expensive and difficult to be carried out. But more widespread methods such as AFLP, LM-PCR, or PCR- MP despite their high universality power are less repetitive between laboratories, and their results are difficult to analyze [6, 13].

Based on the deficiencies of these techniques, we have optimized them in new one method in which uses multiple digestion sites to analyze DNA from environmental samples. This method can be a useful tool used to identify phenotypic characteristics of bacterial pathogen *E. coli* present in avian feathers after the slaughter process. This new compiled technique can be in the future a potential marker to distinguish epidemic from endemic strains and chance as a tool used routinely in microbiological diagnostics of bacterial genomes of unknown sequence from different sources as they described [23, 36, 18]. The high repeatability and shorter time of data analysis while preserving the quality of obtaining results facilitate the study of multiple gene loci at a reduced cost [38, 46].

This method is necessary to use DNA of high quality to obtain readable profiles and consists of only three short steps (**Figure 1**).

Briefly, in the first step, a total genomic DNA isolated from a bacterial strain is digested with one or two restriction enzymes giving 5′ or 3′ sticky ends at phosphodiester bond (**Figure 1**). In the second step, double-stranded synthetic short adapters with complementary sticky ends are ligated to the restriction fragments (**Figure 1**). These adapters including an enzyme-specific sequence are formed (leading ends) for complementary sequence of the restriction fragment (hanging ends). The ligated oligonucleotide is a template in PCR. In the third step, PCR amplification with one set of primers complementary to the end-specific target sequences of restriction fragments terminated with an adapter is amplified (**Figure 1**). Only part of the restriction fragments digested by EcoR1 and HindIII is denatured by becoming amplification templates. Next, the amplified DNA fragments are hybridized with the characteristic pairs of universal primers to 16S RNA sequence.

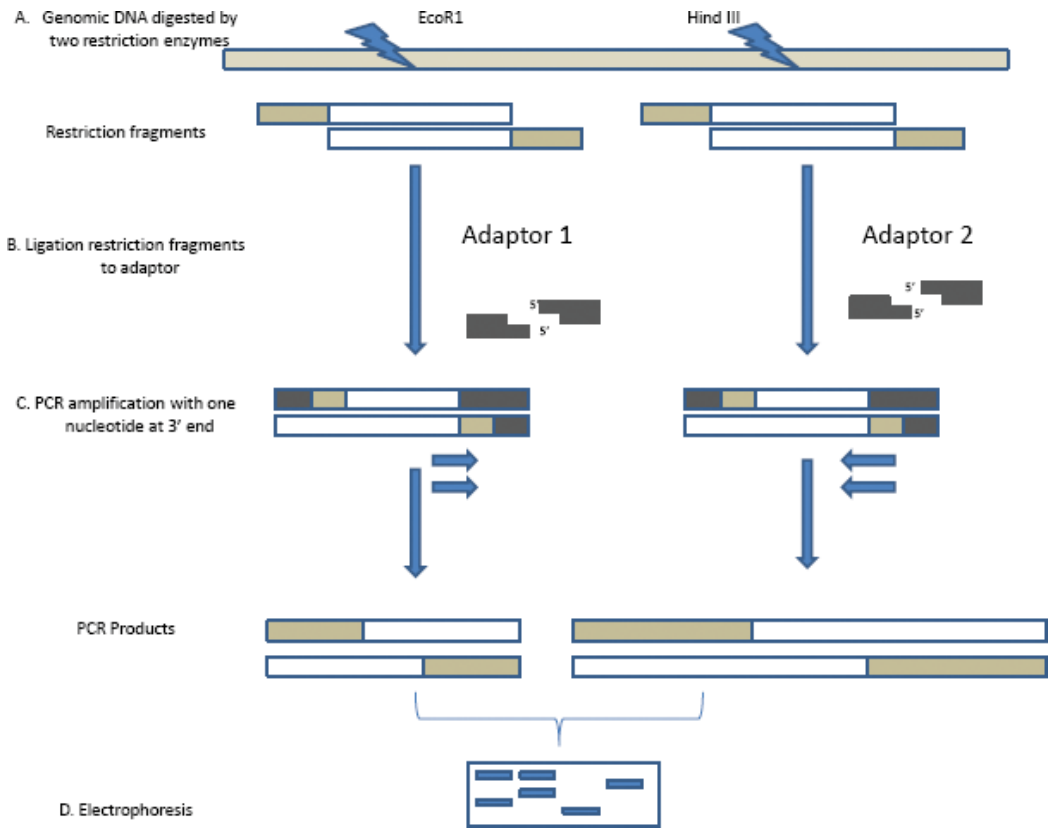

Figure 1. Multiple digestion sites to analyze DNA from environmental samples.

Using standard denaturation temperatures in the range of 90–95°C in PCR, all restriction fragments are terminated with suitable adapters. To obtain a limited amount of amplified fragments (selective amplification), a lower denaturation temperature of up to 90°C in 20 cycles of PCR is used. Due to the different thermal stability of the double-stranded restriction fragments in the analyzed biological material, the selection by lower denaturation temperature increases the distinction potential of analyzed profiles. Next, the products of PCR reaction of PCR, digested by two restriction enzymes of different lengths which are visualized on a polyacrylamide gel electrophoresis a stained with ethidium bromide as part of the analysis of the kinship of individuals analyzed strain. Differences in distances between specific paths in the gel are helpful in unique band pattern interpretation. Parallel with analyzed polymorphisms, we are looking for heat-shock proteins (Hsps) in bacterial cultures isolated from the bird feathers. The presence of these proteins is formed probably in a response to lower reaction temperature in analyzed bacterial cultures. The family of heat-shock proteins (Hsps) belongs to the seven groups of proteins with different molecular masses: 18, 30, 40, 60, 70, 90, and 110 kDa. These proteins are induced in response to cellular abiotic or biotic stress in prokaryotic and eukaryotic cells, mainly induced by elevated temperature. They participate in the stabilization of the cellular components [9].

The presence of Hsp60 and Hsp70 in bacteria strains can increase their pathogenic functions [16]. Amino acid sequence in Hsps proteins isolated from different microorganisms is very similar and shows high homologues 50% in its construction with eukaryotic proteins [52]. In *Escherichia coli*, Hsps possesses two chaperone systems containing 20% of all proteins at a temperature of 46°C [45], and they perform protective functions in the cell.

2. Materials and methods

2.1. Environmental samples and bacterial strains

In the first stage of the work, the waste and air samples contained 35 various kinds of feathers from poultry slaughterhouses in north-western Poland were used (**Table 1**).

Then, 50 g of each samples from the feathers was added to 450 ml of dilution fluid (0.85% NaCl +0.01% peptone), and the whole was shaken on a mechanical shaker for 2 hours. After this time from the feather samples, bacterial strains by using solid test dilutions were isolated. From this dilution 10^{-1}, further decimal dilutions were made up to (10^{-2} to 10^{-6}). Next, the types of bacterial strains were initially confirmed by:

1. Plate culture: 1 ml of slurry from each subsequent dilution was transferred to two parallel Petri dishes, poured about 15 ml of liquid, and cooled into TBX medium. After thorough mixing and solidification, the culture plates were incubated at 44°C for 18–24 h. The bacterial colony, during the chromogens accumulated in the cells, become blue or blue-green color. Characteristic colonies were isolated, and pure culture was conducted on TSA medium (incubation at 37°C for 24–48 h). Additionally, the bacterial cells were verified by microscope analysis.
2. Procedure with pre-selective multiplication on liquid MacConkey medium (MacConkey Broth). From subsequent decimal dilutions (10^{-2} to 10^{-6}), 1 ml to three parallel tubes containing Durham's tubes was plated. Incubation was carried out at 37°C for 24–48 h. From positive test tubes (yellow color change and gas presence), the reduction media was applied to solidified TBX media. Characteristic colonies based on their morphology were isolated, and pure cultures were grown on TSA and TBX medium (incubation at 37°C for 24–48 h) after 24 h of incubation at 44°C [6]. Additionally, the bacterial species were identified by biochemical testing in the HiBio-ID reader (Himedia Company, India). Preliminary identification of bacterial strains based on biochemical tests showed one type of bacteria, namely, *E. coli* (**Table 2**).

2.2. Isolation of genomic DNA

To confirm the accuracy belonging to the type of analyzed bacterial strains, the genomic DNA were isolated on the large scale by using the AccuPrep Genomic DNA Extraction Kit (Cat. No. K-3032R, Bioneer Company, South Korea) according to the manufacturer protocol (**Figure 2**).

Number of strain	Object
1–12	Chicken feathers
13–18	Turkey feathers
51–56	Chicken feathers
63–67	Turkey feathers
111–115	Duck feathers
116–121	Goose feathers

Table 1. Types of tested bird feathers.

Lp.	Biochemical feature	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	L-Glucose	—	+	+	+	+	+	+	+	+	+	—	—	—	+	+	+	+	+
2	D-Adonitol	—	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	—	—
3	D-Arabinose	—	+	+	+	+	—	+	+	+	+	—	+	+	—	+	+	+	+
4	L-Lactose	—	—	—	—	—	—	+	—	+	+	+	+	+	—	+	+	+	+
5	D-Sorbitol	—	—	—	—	—	—	—	+	—	—	+	+	+	+	+	+	+	+
6	D-Mannitol	—	+	—	—	—	+	—	—	—	—	+	—	+	—	+	+	+	+
7	D-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+	+	+
8	D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	+
9	D-Xylose	+	+	+	+	+	+	+	—	+	+	—	+	—	+	+	+	+	+
10	D-Melibiose	—	+	+	+	+	+	—	—	—	—	—	—	+	—	+	+	+	+
11	L-Raffinose	—	—	—	—	—	—	—	—	—	+	+	—	+	+	+	+	+	+
12	L-Trehalose	—	—	—	—	—	—	+	—	—	—	+	+	+	+	—	—	+	+
		51	54	55	56	63	66	67	111		112	113	114	115	116	117	118	119	121
1	L-Glucose	+	—	—	+	—	—	+	+		+	+	+	—	+	+	+	—	+
2	D-Adonitol	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
3	D-Arabinose	+	—	—	—	+	—	—	+		+	+	+	+	+	+	+	+	+
4	L-Lactose	+	+	+	+	+	+	—	—		+	+	+	—	+	—	+	+	+
5	D-Sorbitol	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
6	D-Mannitol	+	+	+	+	+	+	+	—		+	+	+	+	+	+	+	+	+
7	D-Rhamnose	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	—	+
8	D-Sorbitol	+	+	+	+	+	+	+	—		—	+	+	+	+	+	+	+	+
9	D-Xylose	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	—	+
10	D-Melibiose	+	+	+	+	—	+	+	—		+	+	+	+	+	+	+	+	+
11	L-Raffinose	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
12	L-Trehalose	+	—	—	—	+	+	+	+		+	+	+	+	+	+	+	+	—

Table 2. Example of biochemical characterization of strains isolated from the environmental samples.

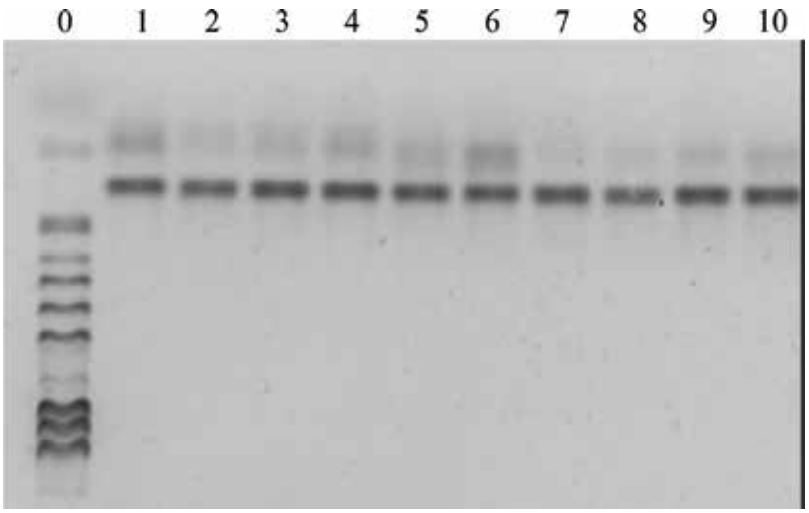

Figure 2. An example of a gel containing genomic DNA isolates. Lane 0, DNA 10 kb marker. Lanes from 1 to 10, DNA genomic samples. Genomic DNA concentration 150–200 ng/μl was determined by UV light absorbance at (A_{260}/A_{280} ratio). The obtained material was either used as a template in a multiplex PCR.

2.3. PCR amplification

To perform PCR amplified from isolated genomic DNA, AccuPower PreMix kits (Cat. No. K-2011, Bioneer Company, South Korea) was used according to the reaction condition recommended by the manufacturer with one pair of universal primers:

ECP79F-forward operating bases. 79–96; 5′-GAAGCTTGCTTCTTTGCT-3′ and ECR620R-(reverse targeting bases 602–620; 5′-GAGCCCGGGGATTTCACAT-3′).

In addition to the tested samples, negative control was performed without DNA, which confirmed the purity of the used reagents. To obtain the same specific product in all amplified DNA samples, optimal primer melting point temperature at 55°C was used. After PCR we received product size at 541 bp which was purified with using AccuPrep PCR Purification Kit (Cat. No. K3034, Bioneer Company, South Korea) as recommended by the manufacturer (**Figure 3**).

2.4. Electrophoresis of PCR products

After PCR, the samples were applied to a 1% agarose gel in 1× TBE buffer (Tris, boric acid, EDTA pH 8.0) containing ethidium bromide in an amount of 2 μl solution at a concentration of 10 mg/ml). The TBE solution was also used as an electrode buffer. In order to estimate molecular length of PCR products, a GeneRuler 1 kb DNA marker (Fermentas) was added to one of the gel pockets. Electrophoresis was performed for 40 min at 90 mV on a Bio-Rad PowerPac Basic. Upon completion of the electrophoresis, the gel was placed on a light-emitting diode (X = 302 nm) and photographed using a GelDoc-It Imaging System and UVP gel visualization system. The stage of electrophoresis informed of the length of the obtained products and confirmed the specificity of the primers used on the basis of the presence of a single gel band. Photos of gels were received and analyzed using Life Science Software Launch VisionWorks (**Figure 3**).

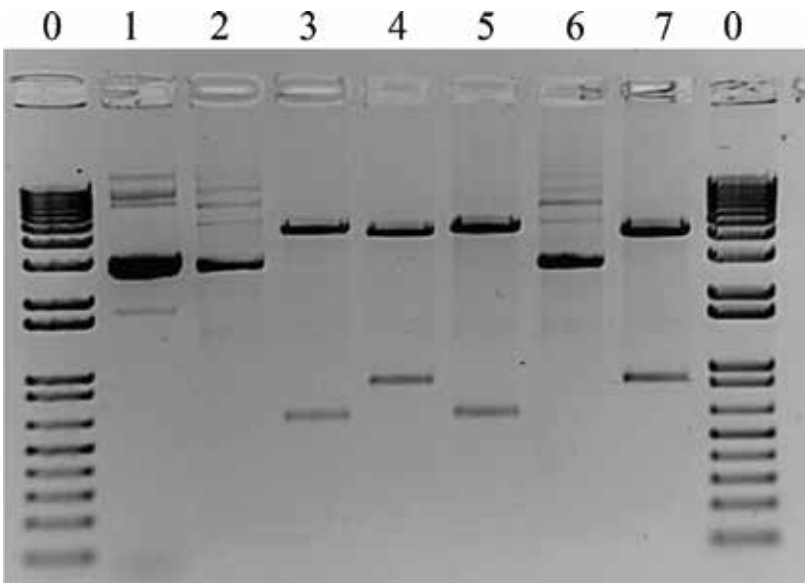

Figure 3. Example of polymorphism fragment in one of the selected strains digested with restriction enzymes EcoR1 and HindIII. Lane 0, DNA ladder 1 kb; lane 1, control DNA digested by both *E. coli* I HindIII enzymes; lanes 2 and 6, digested genomic DNA by both enzymes; lanes 3 and 5, digestion by EcoR1; and lanes 4 and 7, by HindIII.

2.5. Sequencing

Next, the PCR product was sequenced of the 16S rRNA with a universal set of primers; ECB75F (forward targeting bases 75–97; 5′-GGAAGAAGCTTGCTTCTTTGCTG-3′), ECR620R (described above) in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis Institute of Biochemistry and Biophysics, Polish Academy of Sciences. The received nucleotide sequences were compared with sequences located in the GenBank databases/EMBL (European Molecular Biology Laboratory)/DDBJ (DNA Data Bank of Japan) using the BLAST (Basic Local Alignment Search Tool) bioinformatic database. Obtained sequences confirm identification of all *E. coli* strains from ATCC group using biochemical methods including API tests. Among the analyzed sequences in the conserved domains, we have found 10, which had significant differences in the analyzed length of the DNA fragments.

2.6. Digestion by restriction enzymes

On the results of the sequenced DNA, 14 samples for further analysis of polymorphisms were selected. The 20 μg of each sample of genomic DNA samples was digested by combination of two restriction enzymes *EcoR1* and *HindIII* (10 U/μl each) (Fermentas, Lithuania) (**Figure 2**) to get sticky ends. The reaction was run for 2 h at 37°C in a buffer compatible with both enzymes (Tango Buffer: Fermentas, Lithuania) in volume 25 μl consistent with the manufacturer's protocols (**Figure 3**).

2.7. Ligation

After digestion of DNA with restriction enzymes, two pairs of synthetic oligonucleotides forming adaptor were included. First fixed part ligated oligonucleotides are POWIE 5′-CTCACT-CTCACCAACAACGTCGAC-3′ and HINLIG 5′-AGCTGTCGACGTTGG-3′ described by

Masny and Płócienniczak [33] and second fixed part POWIE 5′-CTCACTCTCACCAA-CAACGTCGAC-3′ and EcoR1LIG 5′-AATTGTCGACGTTGG-3′. Both adaptors (25 pmol each) were suspended in total volume 25 μl of ligation buffer (100 mM Tris–HCl, pH 8.2, 10 mM $MgCl_2$, 15 mM DTT, and 100 mM ATP; Bioneer South Korea). Next, the adaptors were ligated to the "sticky ends" created by both restriction enzymes. The reaction was incubated either for 5 min at 55°C and gradually cooled to room temperature. Then, 2 μl of ligase T4 2 U/μl (Cat No. E-3061, Bioneer, South Korea) was added, and reaction mixtures were incubated during 2–4 h at room temperature according to manufacturer's protocol. As a control, a sample of water was placed in place of the DNA fragment. Obtained construct in volume 5 μl was used for transformation of the competent cells of DH5alpha BL21 (Stratagene) as described by Sambrook and Maniatis [44]. Next, the transformation mixture was plated on LB medium with ampicillin (50μg/1 ml) and incubated at 37°C overnight. After incubation obtained cells including construct were frozen in Cryobank (Kucharczyk, Poland) and were stored for further molecular analysis at −80°C.

2.8. Colonial PCR

Cells including construct after defrosting were incubated at 37°C for 10 min and served as matrices to the colonial PCR. The initial primer concentration was 100 μM. The PCR mixture contained 60 pmol of primer POWAGCTT 5′-CTCACTCTCACCAACGTCGACAGCTT-3′ described by Masny and Płócienniczak [33]. To amplify the Hsp55 protein gene fragment, the PCR mixture in 25 μl volume composition was consistent with the manufacturer's protocols (*AccuPower® Pfu* PCR PreMix Cat No. K2024 Bioneer Company, South Korea). PCR program was as follows: (1) 90°C 4′, (2) 56°C 2′40″, (3) 72°C 3′40″, (4) goto1 1×, (5) 90°C 1′40″, (6) 52°C 2′40″, (7) 72°C 3′40″, (8) goto5 20×, (9) 72°C 7′40″, and (10) 4°C forever. The reactions were performed in Bio-Rad MJ Mini thermocycler. After completion of the PCR, the samples were transferred quickly into ice before loading on the 6% polyacrylamide gel.

2.9. Polyacrylamide gel electrophoresis

Next, the amplification PCR products (6 μl out of 50 μl DNA solution) were separated on 6% polyacrylamide gel electrophoresis in conditions 90 min under 120 V in the presence of TAE buffer (Cat. No. C-9004, Bioneer, South Korea) supplemented in 0.5 mg/L aqueous solution of ethidium bromide (Cat. No. C-9009 Bioneer, South Korea). All samples were runs the ladder Gen Ruller were runs the ladder GeneRuler 1000 bp (Cat. No. D-1040, Bioneer, South Korea) (**Figure 4**). The differences between digested templates were analyzed using ImageQuant software.

2.10. Production of recombinant *Escherichia coli* Hsp55 protein

Additionally, we also wanted to check whether the analyzed bacterium pellets, based on differences in DNA polymorphisms in 10 samples, are able to detect specific heat-shock proteins related to them. Based on genomic DNA obtained from bird feathers and digested with restriction enzymes as insert (as described in Section 2.6), we used a vector pET22b (5493 bp) (Novagen, Germany). A vector is based on the T7 promoter system, and its expression is controlled by the lactose operon. Additionally, to digestion the vector of the vector pET22b on sticky ends, the same types and portion of restriction enzymes EcoR1/HindIII were used on

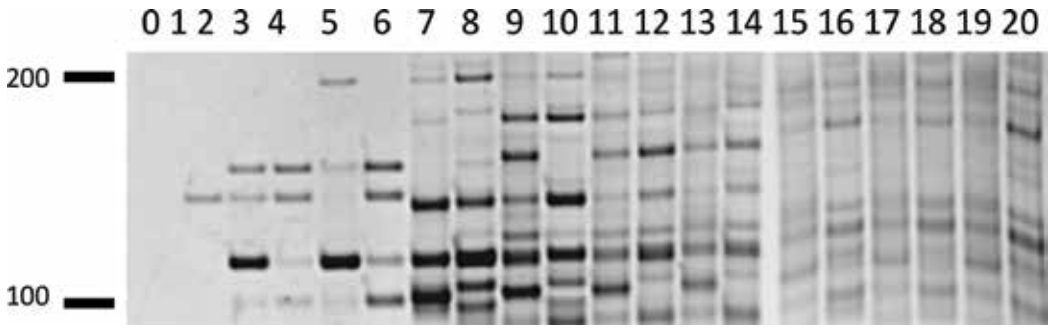

Figure 4. Analyses the differences between DNA templates isolated from *E. coli* strains with using the modified technique on polyacrylamide gels stained with ethidium bromide. Lane 0, molecular weight standard 2 kb (Bioneer). The remaining lanes 1–20 show digested profiles by both restriction enzymes: even numbers by EcoR1 and odd numbers by HindIII.

sticky ends (as described in Section 2.6). Next, the insert and vector were ligated with themselves by ligase 2 μl of ligase T4 2 U/μl in the same conditions (as described in Section 2.7). The obtained construct called hsp55pET2'b was transformed into competent cells of *E. coli* BL21, (Stratagene) (as described in Section 2.7). Next, the bacterial cells were used to express the hsp in autoinduction conditions according to Studier [50] (see Chapter 2.11).

2.11. Autoinduction

After overnight culture at 37°C, the bacteria were centrifuged for 20 min at 2500 × g, the supernatant was flushed, and the bacterial pellets were purified by affinity chromatography with a histidine oligopeptide (His-Tag) (see Chapter 2.12) under denaturing conditions (application of 4 M urea) on Sepharose Ni_2 gel. Next, the recombinant Hsp55 *E. coli* proteins obtained in the previous step were dialyzed in Tris-NaCl buffer. The dialysis buffer was changed five times. After dialysis, protein concentrations at (λ = 280) were determined.

2.12. Affinity chromatography

A chromatographic column Ni-NTA (Sigma-Aldrich, Germany) with a nickel agarose gel was used to purify the protein. The column has the properties of attaching molecules to the polyhistidine tail (His-Tag tail attachment). The protein was purified according to the Invitrogen protocol (Ni-NTA Purification System, Sigma-Aldrich, Germany). Quantitative evaluation of the protein was carried out on a regular basis using 100 μl Bradford Reagent (Bio-Rad). The concentration of proteins measured spectrophotometrically was ranged from 0.35 to 0.36 μg/ml.

2.13. Protein electrophoresis

For the separation of protein fractions obtained by affinity chromatography, SDS-PAGE was performed at 60 mA (**Figure 5a**). The gel composition was according to the manufacturer's protocols (Bio-Rad). The composition 20 μl of sample, 5 μl of dye, and additionally 7 μl of PageRuler™ Unstained Protein Ladder (Lab-JOT, New England, Great Britain) were applied

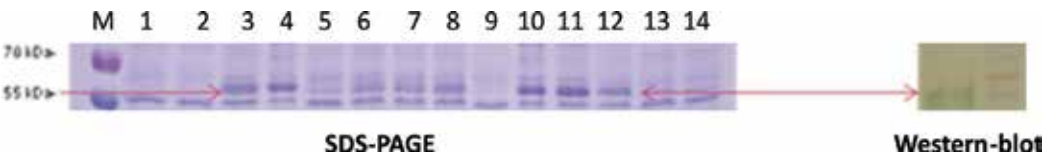

Figure 5. Analysis of the expressed proteins by SDS-PAGE (A) and Western blot (B). SDS-PAGE protein profiles of the *E. coli* strain stained with Coomassie Brilliant Blue. Lanes: M, molecular mass standard 6.5–200 kDa; 1–14 analyzed protein fractions. Red arrows show protein fractions of molecular weight 55 kDa.

to two gel lanes. The bands migrate at a height of 55 kDa relative to the protein ladder, and the absence of additional intermediate bands at this height of the template protein indicates a high degree of purification of the analyzed proteins. After electrophoresis the gel was stained with Coomassie Brilliant Blue (Sigma-Aldrich, Germany) according to the manufacturer's protocols. Next, the first gel was transferred on nitrocellulose membrane (Western blot) to confirm the acquisition of protein *Hsp*55 (**Figure 5b**), (see Chapter 2.14).

2.14. Western blot

Transfer from polyacrylamide gel to nitrocellulose was carried out by semidry method [53]. The gel after the electrophoresis was transferred to a soaked transfer buffer (30 mM Tris, 180 mM glycine, 25% methanol, pH 8.2) Whatman paper (Sigma-Aldrich, Germany) according to the scheme: upper transfer (−) 3 Whatman paper 4, 1 Whatman paper 1, gel, nitrocellulose membrane, 1 Whatman paper 1, and 3 Whatman 4 paper (+) down. The transfer was at a current of 1.8–2 mA/cm^2 of the membrane for 45 min. At the end of the transfer, nitrocellulose was blocked with a solution of PBS + 2% casein for 15 min. On the membrane goat anti-mouse primary antibody (IgG anti-His-Tag 1:2000, Sigma-Aldrich) were applied and were incubated overnight at 4°C. After incubation, the membrane was washed three times with PBS + 0.025% Tween 20, followed by a 1:2000 conjugate (Anti-Goat, Santa-Cruz, USA) conjugated with HRP and incubated for 1 h at room temperature. The next step was to rinse the membrane three times with PBS + 0.025% Tween 20 solution and to induce nitrocellulose (20 mg chloronaphthol dissolved in a minimum of 96% ethyl alcohol), added to 80 ml Tris–HCl buffer and then filtered through a paper filter. Protein samples after chromatography were conserved in 20% glycerol solution and frozen at −20°C. The resulting membrane, as well as the SDS-PAGE gel, was scanned and analyzed.

3. Results and discussions

The traditional polymorphism technique is a compilation of digestion by restriction enzymes and selective basic PCR, which can generate a wide spectrum of markers [24]. These markers are highly specific and needs typical sequence of the data base. Additionally, they require visualization platforms which can be intractable to analysis by specific banding patterns.

Our studies show that modification of these presented techniques can be an effective alternative to other PCR methods used to identify specific DNA sequence occurring in environmental origin from bacterial pathogens, e.g., bird features. Using these modified techniques, we observed that all 10 samples isolated from the same bird feathers treated by restriction enzymes presented different profiles. The reliability and repeatability of modified methods depend on optimizing the PCR conditions [24, 33] by experimental estimation: the amount of DNA of only 0.2 μg (0.2 pmol) of DNA per reaction is needed to obtain satisfactory signal intensity, the amount and concentration of reagents (what affects it reduced cost), the primer denaturation temperature 90°C, or the number of temperature cycles—only ×20, which significantly shortens the time of the experiment. We describe a method of finding differences in DNA sequence among bacterial species strains based on two enzymes EcoR1 and HindIII [24, 33, 61]. The method allows on analysis of DNA fragments, whose band pattern on the gel differs between strains of a bacterial species. The DNA sequences obtained from bird feathers are colonized by the serogroups of bacteria belonging to *E. coli* species (**Table 3**) [11, 23]. The most commonly isolated avian pathogenic *E. coli* strains were O6, O7, O26, O55, O127, and O157 which are the main causes of colicobacteriosis, inflammation of air sacs, polyserositis, sand bacteremia and epidermis [35]. Probably, the factor predisposing to the development of these infections is the PICV (circovirus) infection, which affects molting disorders, leading to immunosuppression and development of parenteral infections like lymphatic tissue in the fabrician bursa, thymus, spleen, and intestinal tract [3, 42, 23]. The most important virulence factors of *E. coli* colonizing bird feathers are fimbriae F1 and P, aerobactin inducer iron-sequestering system (AIS), and temperature-sensitive hemagglutinin [21]. They promote the development of generalized infections and inhibit phagocytosis in poultry lung alveoli in case of *E. coli* aspiration [49]. The obtained strains IAI 39 and 536 with serotypes O7:K1 and O6:K15:H31, respectively, are the etiological agents of pyelonephritis (ExPEC). Other strains EDL933 (O157:H7 serotype), CB9615 (O55:H7 serotype), APEC01 (01 serotype), and E2348/69 (O127:H6 serotype)

***E. coli* strain name**	**Serotype**	**Pathotype**	**Complete genome length G + C ratio [%]**
SS17	O157:H7	HUS	5.52 Mb/50.5%
SS52	O157*nonH7*(H16, H26, H39)	pEAF	5.488 Mb/50.5%
CB9615	O55:H7	Diarrhea	5.52 Mb/50.5%
E2348/69	O127:H6	Diarrhea	5.52 Mb/50.5%
APEC01	O157:H7	Diarrhea	5.498 Mb/50.5%
EDL933	O157: K1:H7	Diarrhea	5.498 Mb/50.5%
536	O6:K15:H31	Diarrhea	5.528 Mb/50.5%
39	O7:K1	Diarrhea	5.528 Mb/50.5%
EC4115	O157:H7	EHEC	5.57 Mb/50.5%
TW14359	O26:H11	HUS	5.528 Mb/50.5%

Table 3. Summary presentation obtained *E. coli* pathogenic strains including serotype.

are responsible for causing diarrhea, with symptoms of colicobacteriosis and secondarily of inflammation of air sacs, polyserositis sand bacteremia and epidermis (**Table 3**). Among the carriers of *E. coli* strains producing O157 (EC4115, EDL933, TW14359, SS17, SS52), we can distinguish the group determined as super-shedders—contamination O157 ≥ 10^4 CFU/g [7, 14, 23]. In addition, due to genomic similarity in the sequence length (5.49–5.53 Mb), G + C (50.4%–50.5%) proportion, and the average ORF (856–898 bp) length of *E. coli* strains, the reliable methods of genetic diagnostics that will allow to identify the pathogenic strains definitely are sought for (**Table 3**) [13, 23], which is consistent with the results obtained in other laboratories, which are described in the review paper [23].

Based on the analysis of the length of the restriction fragment, we can state that each analyzed lane is from 4 to 35 DNA fragments (bands) of approximately range from 150 to 2072 bp. Profiles found were different in relation to the presence or absence of at least one band, and all of them presented one band of approximately 1500 pb. The total number of the bands was 432, clearly separated and good visualized on gels only. Among primer combinations (see chapter primer selection 3.2), producing 14 polymorphic fragments, respectively, [57] reported that fragments amplified by modified method can detect from 60 to 100 fragments on a polyacrylamide gel. Genetic polymorphism, understood as the presence of more than one allele at a given locus in the population, can be identified by genetic markers [10, 57], using molecular methods based primarily on AFLP, LM-PCR, and PCR MP. In our modified methods, we can detect two times more fragments for revealing polymorphism. A high resolution of obtained gel stained in ethidium bromide revealed to be an efficient technique to visualize markers in modified method [33]. Samples belonging to the same biotype when tested by modified method presented small differences of the patterns. The differences between the two classes of represented isolates suggest probably a clonal relationship between the strains. It is well known that the presence of transposons, different types of mutation in sequences, may increase the variability of the profiles produced by the modified- three techniques. Modified technique may be useful for studying intraspecific variation and genetic relationships among different biotypes. According to the up-to-date reports, conventional PCRs or real-time PCRs are useful in detecting different pathogen strains [24]. DNA sequences that are characteristic of certain strains of a bacterial species can be very interesting from the biological and medical point of view. Bacterial genomes show considerable variation in GC content. This means that after genomic DNA digestion, fragments with different thermal stabilities are formed. The resulting sets of electrophoretic patterns are characteristic of the genome and restriction enzyme.

Nonetheless, they may serve as markers for species/strain identification or evolutionary studies. The classical genetic marker is characterized by the identified degree of variability of a feature (DNA nucleotide sequence) and may involve variation within encoding or noncoding sequences or single-nucleotide changes in DNA fragments. The sources of variation in DNA sequences are mutations, among others insertion, deletion, substitution, and duplication, and recombination during meiosis. Some of these changes at the genome level can lead to the variability observed at the phenotypic level, significant from an evolutionary point of view [57]. With respect to natural selection, markers may be neutral or not. It is assumed that neutral markers are not selectable, i.e., specific genotypes or alleles at a given locus or loci are not favored by selection. One of the most commonly used neutral markers are microsatellite sequences, which are used in population structure analysis. Microsatellite locus is a

DNA sequence consisting of short, one to six nucleotides, repetitive elements in the number of different copies for individual alleles. A variety of PCR-based methods for displaying DNA sequence polymorphism have been developed [24, 33]. Compilation of these methods allows detection of DNA polymorphisms distinguishing between strains of a species but does not exhibit high rates of reproducibility. Modified methods permit selective amplification of restriction fragments obtained from a total restriction enzyme digestion of a DNA and identification of DNA polymorphism within a species but require the use of sequencing gels and usually labeled primers because of the quantity of simultaneously amplified DNA fragments.

Our method allows simple identification of microsatellite loci by identifying flanking regions in individuals of the same species. Based on the experimental results, we can observe changes in modified patterns in 10 isolates of the environmental samples (avian feathers), which suggests that the technique is highly specific and presents high variability. The method can also be used for a further variety of epidemiological studies as a tool for the detection of cross-contamination, determination of the source of infection, and recognition of outbreaks and virulent strains [4, 15, 22, 23, 54]. One of the most common causes of bacterial infections in humans is *Escherichia coli*.

Pathogenesis and sources of *Escherichia coli* infection are described by [23, 54]. In poultry as a result of *E. coli* infection, there is a great economic loss, so monitoring programs have been introduced to limit them. Understanding the mechanism of pathogenesis of *Escherichia* species, including the penetration of rods into the intestinal epithelium, has contributed to many experimental studies. Elimination of cases of morbidity of *Escherichia* of morbidity of *Escherichia* has become a common poultry vaccination against avian influenza caused by [23, 54]. Initially used vaccines were based on full bacterial cells (live strains). This leads to numerous side effects, allergies, or toxic symptoms and to the conversion of the vaccine strain to the wild strain. With the development of the study, the attention of the investigators was reversed on bacterial cell fragments that contained a specific antigen [23]. Examples of such antigens may be proteins in the cell membrane of bacteria and intracellular proteins such as heat-shock proteins (HSP). Heat-shock proteins (Hsps) are stress proteins, which are characteristic of prokaryotic and eukaryotic cells. They are rapidly synthesized in response to shock induced by exposure on internal and external factors, e.g., heavy metal ions, high or low temperature, gamma and beta radiation, or various infectious agents. They play a role as stabilizer of cellular structures. In bacteria are documented Hsp60 and Hsp70 documented are Hsp60 and Hsp70 [16, 52]. So far has been identified six protein families with molecular masses between 18 and 30 kDa, 40, 60, 70, 90, and 110 in *Escherichia coli* have been identified, but so far there are no direct data about hsp55 protein. It is the heat-shock proteins that pay particular attention not only to the great conservatism of the construction but also to their high immunogenicity. The ability to use heat-shock proteins in the immune response and their potentially protective role in relation to Gram-negative infections has been the inspiration for the research. The method of production of recombinant Hsp60 protein based on literature data was used [52]. In addition, the results of optimization studies on the production of recombinant Hsps have been confirmed by [52]. The pET21b vector was used in the cloning process, and the resulting construct was cloned into competent cells of *E. coli* BL21 or DH5α.

These are universal competent cells in which effective expression of both membrane and intracellular proteins occurs. In addition, these cells are highly effective in autoinduction, as described by [50]. As a result of the optimization of the production conditions of the recombinant protein, the efficiency of the method used gave 0.35 μg. Available data from [47] present a recombinant protein production process using the Hsp60 protein derived from *Bacillus anthracis* (strain 34F2). For the amplification of the gene responsible for encoding the Hsp55 protein, suitable primers were used, and as a result, a 1635 bp product was obtained which was subjected to restriction analysis with EcoR1/HIII enzymes. The vector used in the cloning process was vector pET28a. The obtained construct was cloned into competent cells of *E. coli* DH5α. The expression process of the protein was based on the classical method using IPTG (synthetic lactose). The protein was purified with Ni-NTA in native conditions and then dialyzed in PBS with 10% glycerol added. The interaction between the *E. coli* pathogen and the host defense mechanism is stimulated probably by the immune system. The primary response to mobilizing the first line of defense (including macrophages) is to eliminate *E. coli* from the body, as demonstrated by [19]. Heat-shock protein 55 found in analyzed bird feathers may belong to the family of hsp present in prokaryotic cells such as *Helicobacter pylori* and *Mycobacterium leprae* [30, 47]. One of major heat-shock proteins in *H. pylori* is HSP60 [8, 58]. The HSP60 protein is expressed on the surface of bacterial cell using the adhesion process during exposition to elevated temperatures [8, 58]. HSP has several chaperones which can activate and regulate other proteins [60]. Some chaperones contain proteins HSP70 and HSP90 [60] which are helpful in supporting the establishment of protein complexes and prevention of unwanted protein aggregation [5, 56]. Finding the Hsp55 protein in various bacterial cultures from analyzed fractions may depend on many abiotic or biotic factors which may influence on the pathogenicity of bacteria. Among all analyzed samples, only eight samples had a clear band at a marker height of 55 kDa. The presence of Hsp55 protein observed only in lanes 3, 4, 6, 7, 8, 10, 11, and 12 of SDS-PAGE gel may indicate that this protein has a multidomain structure, as described by [32, 48]. Obtained results could suggest that the presence of these proteins can be an element of the bacterial cell adaptation to worse thermal condition factors of the environment. The overexpression of Hsp55 protein observed in selective fractions may indicate probably cross-reactions occurring in a homology between Hsp60 and another low-molecular-mass proteins or may be the result of degradation of proteins possessing higher molecular mass than 60 kDa in another microorganisms as described by [29, 55]. The strongest expression of Hsps occurred in the SDS-PAGE gel may suggest that the bacterial fraction was a target site for these proteins. Similarly, the strongest effect was observed in Western blot in selected bacterial cells with anti-Hsp55 antibodies. The positive reactions with Hsp55 protein may indicate a high degree of specificity in the *E. coli* proteins. Probably, in analyzed pathogenic bacterial cultures, stress factors which are crucial for expression of hsp are specific. Perhaps, the stress factors can change the localization of proteins in cells induced with specific gradient which indicates their differences in conformation and physical properties. This may lead to destabilization intracellular structures under stress conditions [20, 40]. The changes of temperature induced Hsps in bacteria, and it can also be a stress factor increasing the expression Hsps in *E. coli* bacteria. In these processes are genes activated responsible for encoding these proteins. Western blot shows that it is a suitable method of identifying this type of protein in analyzed bacterial strains.

To date, the effect of the Hsp55 protein on the immune cells has not yet been fully described. Much attention is devoted to research aimed at learning the response of immune system cells following infections with *E. coli*.

3.1. Selection of primers used in the modified method

One of the most important factors determining the success with modified ALFP and LM-PCR and PCR MP is the design of a gene-specific primer set. Three sets of specific primers must be used for analysis of bacterial strains to check the mapping of the modified method.

Primer set 1 is used for formation of adaptors and their complementary sequence described by [33], primer set 2 is used to make a hybridization probe, and primer set 3 is used for the PCR step. The three sets of primers must be in close proximity or preferably slightly overlapping to avoid repetitive DNA sequences in the bacterial genome and to avoid polymorphic sites. The initial criterion used to test the relationship between bacterial strains was the hybridization of total DNA supplemented with electrophoresis karyotypes and used profiles of restriction enzymes. The most distinctly different fragments were selected from the 6% polyacrylamide gel after silver staining with separated amplification products of individual bacterial strains depicted in the (**Figure 3**). Analysis of isolated strains started from the design of specific primers to obtain the same specific product in all genomic DNA amplified samples by PCR method. PCR primers have been designed to be longer than ligated oligonucleotide's normal primers [33]. Fragments that arise after genomic DNA digestion differ in the melting point and can be amplified directly in PCR after denaturation in 90°C. Using reduced denaturation temperatures, a smaller number of DNA fragments is obtained than at the standard temperature of 95°C, where all the fragments would be amplified. In procedure that uses Taq polymerase for primer extension, primer 1 must have a melting temperature considerably higher than 48°C to remain hybridized at this temperature. Primer 2 should have a melting temperature higher than primer 1 but similar to the linker primer in the PCR step, as they must work as a pair. Thus, for the standard protocol and linker, one needs to select primer 2 with a T_m of 55–59°C. The oligonucleotide used as primer 1 has AT-rich regions; longer primers may be needed to give the desired T_m. For many experiments involving comparisons between lanes, it is desirable to have roughly the same band intensity in each lane. The strain-specific primer used in the amplification step (primer 3) was designed to be downstream to primer 1, overlapping several bases with primer 1 to provide competition with any residual primer 1 that might be carried with the sample to the PCR step. The linker primer was the longer oligonucleotide (the 23–26 mer, T_m of 53.4°C) with a T_m very similar to that of primer 2. Only 20 cycles were performed, so as to ensure enzyme excess and minimize preferential amplification of short sequences. To completely extend all DNA fragments and uniformly add an extra nucleotide by the terminal transferase activity of Tag polymerase, an additional longer elongation step is part of standard procedure. This step can be important and should not be omitted; otherwise, double bands may appear due to some molecules having an extra 3′ base.

4. Conclusions

In conclusion, for the first time, we showed the developed modified AFLP, LM-PCR, and PCR MP methods as one powerful technique for the specific and selective detection of bacterial pathogen from environmental samples, for example, bird feathers. This new method is an

alternative for another research techniques based on PCR. It is more sensitive, much faster, and less labor intensive than the other existing methods. The advantages of this method can be applied to analyze specific fingerprints of obtained DNA (as PCR product) from different bacterial strains after specific restriction enzyme treatment and interaction between the recombinant Hsp55 protein and the humoral and cellular response of the breeding bird organisms. Compilation of these methods is a very useful and flexible tool in the analysis of microbiological strains. It allows analysis of many genomes coming from different sources in a short period of time in lower PCR temperature with small amount of DNA which is an alternative to differentiate between highly clonal strains. Typically, 60–100 restriction fragments are amplified in range size from 60 to 550 bp. The selection of the denaturation temperature is a very important factor for reliability of typing for each strain from previously purified bacterial colony.

The results of the Hsp55 *E. coli* protein evaluation study in birds from breeding farms indicate that it may be used in the future for the construction of component vaccines used in the pharmaceutical industry against various infections responsible for bird conditions. The results presented in this paper demonstrate the interaction between the recombinant *Hsp55* protein and the humoral and cellular response of the breeding bird organisms.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All the authors made substantial contributions to conception and design, analysis, and interpretation of data. In particular, PK designed this project, and PK, SMO, KC, AM, and AM1 also contributed to the laboratory analysis. PK is involved in drafting the manuscript. SMO and KC also participated in sampling and bacterial strain collection operations. PK, KC, SMO, AM, and AM1 reviewed the manuscript for intellectual content. All the authors have read and approved the final manuscript.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical statement

All applicable international, national, and institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Abbreviations

HUS	hemolytic-uremic syndrome
pEAF	adherence factor plasmid

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Chapter 9

Avian Cardiovascular Disease Characteristics, Causes and Genomics

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Additional information is available at the end of the chapter

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Abstract

Cardiovascular disease is common in avian species and increasing commercial economic losses and demand for healthcare in the household/smallholding veterinary sector has resulted in increased research into these disorders. This in turn has highlighted the importance of breeding, genetic testing and possibilities for future prognostic and diagnostic testing. Research into avian cardiovascular genetics has rapidly accelerated. Previously much work was undertaken in mammals with information extrapolated and transferred to birds. Birds have also been used to model cardiovascular disease and therefore knowledge has become enriched due to this endeavour. Increasingly, the avian genome is being analysed in its own right. This work is assisted by the growing number of avian genomes being published. In 2015, Nature published news on the 'Bird 10K' project, which aims to sequence 10,500 extant bird species. By 2018, the Avian Genomes Consortium had published the sequences of 45 species/34 orders. This review investigates a range of avian cardiovascular disorders in order to highlight their pathologies, epidemiology and genetics in addition to avian models of heart disease. With the availability of more reference genomes, increases in the number and magnitude of avian studies and more advanced technologies, the genetics behind avian cardiovascular disorders is being unravelled.

Keywords: avian, cardiovascular disease, genetics, pathology, epidemiology

1. Introduction to the cardiovascular system

Acute heart failure and chronic heart failure are of great concern in avian species. Heart failure represents the main cause of morbidity and mortality in broiler flocks after infectious

diseases [1]. The act of flying means that birds have a higher metabolic demand than mammals of a similar size. This means they have a higher cardiac output which is achieved by having larger hearts and pumping more blood per unit time. Additionally the cardiovascular system has become adapted in birds that dive, thus preventing inappropriate responses to submersion asphyxia [2]. Knowledge of the anatomical structure, histology and function of the avian heart are crucial in understanding how not only the healthy heart functions, but also in understanding the abnormal heart.

1.1. Structure and function of the avian heart

Similar to mammals, birds have a four-chambered heart surrounded by pericardium. The chambers effectively function as two separate pump systems to circulate blood around the body, with cardiac valves ensuring unidirectional flow of blood through the chambers and blood vessels [3]. The mammalian heart lies just into the left hand side of the thoracic cavity, whilst the avian heart lies slightly to the right of the midline [4, 5].

The heart itself it made up of multiple structural components. The cardiac muscle acts to contract rhythmically via coordination of the cardiomyocytes, potentiating movement of blood around the body [5]. The cardiac fibrous skeleton called the annulus fibrosus comprises of four connective tissue rings acting to separate the atria and the ventricles. The septum separates the heart into right and left halves, with the left side sending blood to the systemic circulation and the right side routing blood to the pulmonary circulation [6]. The septum itself contains a conduction system for the initiation and propagation of action potentials, allowing stimulation and consequent contraction of the myocardium [3, 5]. Finally, the heart is primarily supplied by two coronary arteries branching from the ascending aorta, whilst cardiac veins drain blood from the heart tissue into the right atrium, via the coronary sinus. The heart is also subdivided into atria cranially and ventricles caudally (see **Figure 1** for the histology of these structures). The atria receive the blood from the veins and then pump the blood through to the ventricles. Thinner walls are found in the atria as the blood is only pumped to the ventricles, which does not require as much muscle as that required to pump blood to the entire body [3]. Similarly, the right ventricle has a thinner wall than the left as blood is only pumped to the lungs, whereas the left ventricle pumps blood around the body [7].

1.2. Histology of the avian heart

The heart is made up of three distinct layers. The outermost layer is the epicardium, which is formed from a layer of mesothelial cells overlying adipose and connective tissue [8]. The epicardium acts as a protective layer, and contains nerves and blood vessels which supply the heart tissue [5]. The middle layer is the myocardium, which forms the greatest proportion of the heart tissue and is composed mainly from myocytes [9]. The innermost layer (endocardium) is composed of connective tissue, endothelium and smooth muscle cells and forms a protective lining over the valves and heart chambers [3].

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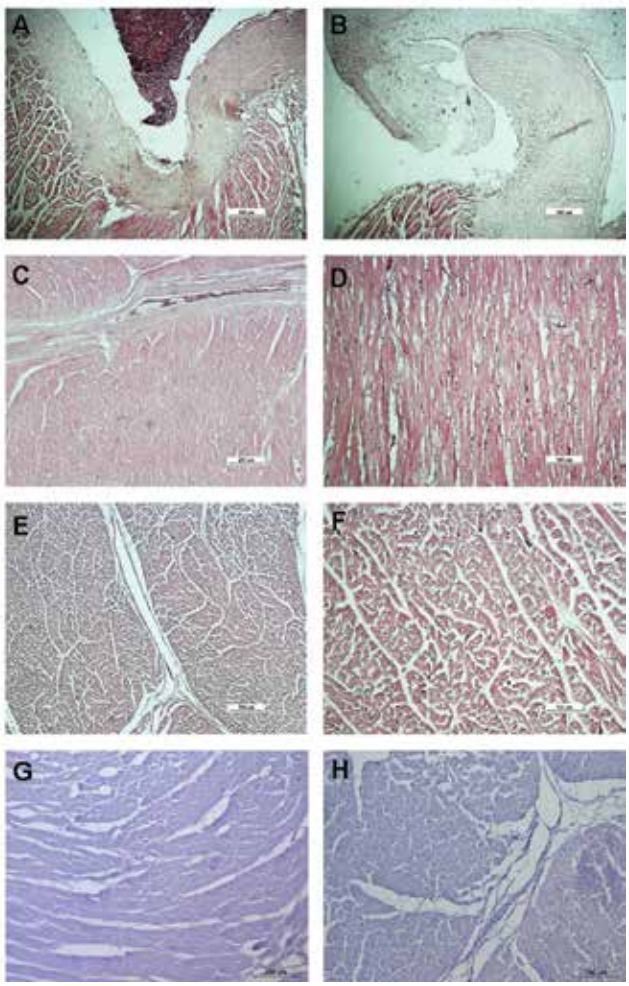

Figure 1. Chicken atrioventricular (AV) heart valve (A, B), ventricle longitudinal (C, D) and transverse (E, F) views, and the atrium (G, H). Scale bars represent 100 μm (A–C, E, G), 200 μm (H) and 250 μm (D, F). The heart histologically consists of three layers, which resemble their structure in blood vessels. The inside layer is endocardium, also covering valves. In the avian heart the right and left AV valves are in close proximity to the conduction system. The right AV valve is a single, spiral plane of myocardium, in remarkable contrast to the fibrous structure characteristic of the mammalian tricuspid valve. The vast inner part is myocardium, composed of cardiac muscle, specialised conductive tissue, valves, blood vessels and connective tissue. Cardiac muscle, the myocardium, consists of cross-striated muscle cells, cardiomyocytes, with one centrally placed nucleus. Nuclei are oval, rather pale and located centrally in the muscle cell which is 10–15 μm wide. Cardiac muscle does not contain cells equivalent to the satellite cells of skeletal muscle. Cardiac muscle cells often branch at acute angles and are connected to each other by specialisations of the cell membrane in the region of the intercalated discs. The most outer part is epicardium, consisting from mesothelial cells, submesothelial layer of connective tissue, adipose cells, nerves and vessels.

The cardiac muscle, blood vessels and endothelial lining are all derived from embryonic mesoderm, one of three primary germ layers of the early embryo [5]. The heart in turn is composed of several different cell types, all of which have a role in the function and maintenance

of the heart tissue [8]. Cardiac muscle is striated due to the arrangement of thick and thin myofilaments within the myocytes [10]. Thin filaments are composed of actin and are 6-8 nm in diameter, whilst thick filaments are composed of myosin II proteins and are around 15 nm in diameter [5, 10]. Myofilaments are organised within the sarcomere, which acts to form a single contractile unit [5]. The myocytes have central nuclei, unlike skeletal muscle fibres which have peripheral nuclei [11]. They also contain multiple mitochondria and glycogen granules, allowing for store and release of energy [5]. Cardiomyocytes are cylindrical and form a functionally continuous muscle fibre and may exhibit some branching. Unlike skeletal muscle, cardiac muscle fibres contain intercalated discs, which histologically appear as straight bands between opposing cells, but are not always visible under haematoxylin and eosin staining [5, 12]. Intercalated discs are regions containing gap junctions, which allow the transfer of ions between cardiomyocytes [13]. This enables cardiac muscle to act as a functional syncytium, by ensuring coordinated contraction of the muscle fibres [3, 12].

The endothelium is made up of simple, squamous epithelial cells known as endothelial cells, which encircle the lumen of blood vessels [14]. The cells respond to stimuli by interacting with the blood and connective tissue, and act to control functions such as permeability and blood flow [5]. Smooth muscle is derived from lateral plate mesoderm and is present in tissues throughout the body [5, 15]. Within the heart, smooth muscle is located in walls of the vasculature [16]. Smooth muscle tissue contains actin and myosin filaments similar to those seen in skeletal and cardiac muscle, however these fibres are not organised in sarcomeres and therefore do not demonstrate the same striated pattern. Smooth muscle cells are long with tapered ends and contain central nuclei. The cells are interconnected with gap junctions, enabling contraction of the muscle as a single sheet. This allows for controlled contraction or dilation of the blood vessel lumen [3, 5]. The blood vessels have the usual tunica intima (endothelial cells), media (smooth muscle cells) and externa (collagenous and elastic connective tissue) layers [5, 8].

One of the main differences between avian and mammalian hearts is that the right atrioventricular valve is muscular and does not contain chordae tendineae in birds. Also, the left atrioventricular valve is tricuspid in birds but bicuspid in mammals [4]. The physiology of how the heart works, however, is largely similar in mammals and birds. The avian vascular system is also similar to mammals apart from a shunt between the left and right jugular vein and an anastomosis between the femoral and ischiadic veins. In addition, when energy demand is high, blood can bypass the kidney [17]. It is also worth noting that avian erythrocytes are nucleated whereas mammalian erythrocytes are not [18].

2. Avian cardiovascular genetics

Cardiovascular disease has usually been reported to be much more common in pet birds compared to wild birds and it is thought that this is due to the longer life span of pet birds but may also be linked to exercise and diet. This review gives an overview of what has been studied and published about cardiovascular diseases in avian species and how this relates to clinical examination, management, cardiovascular genetics and cardiac development. In addition, the bird has proved to be a very informative model when undertaking cardiovascular research in

general. During development the heart is located outside of the thorax which makes it readily accessible. Therefore, avian species as research models are also discussed.

With over 10,500 bird species to choose from, prioritising the species is difficult. By 2018, 45 species/34 orders had been sequenced by the Avian Genomes Consortium under the 'Bird 10 K project [19, 20]. Although some birds such as the Red Jungle Fowl have been available since 2004, even this genome is under constant scrutiny and is updated to ensure accuracy. Since the genome was first published in 2004 there have been many improvements and complete genome builds with the latest version Gallus_gallus-5.0 being published in 2017 [21]. The arrival of next-generation sequencing presented more methods of identifying and developing what is already known, and what can be known about the genomes of each bird. In a recent publication the use of sequencing tools and bioinformatics is highlighted in relation to the ostrich genome [22]. The complexity of any genome, including the avian species is still being unravelled and the technologies used to undertake both the sequencing and bioinformatics are rapidly evolving and expanding areas. A number of innovative methods have also been used to edit the avian genome and these technologies are advancing greatly. They range from culturing and modifying primordial germ cells (PGCs) and direct in vivo transfection of PGCs, morpholino, gene targeting, TALEN, homology directed repair (HDR) and CRISP-Cas9 knockdown and knockout tools, direct injection and sperm transfection assisted gene editing (STAGE), in addition to combinations of these methods [23].

Naturally these techniques are vital in furthering our understanding of medical problems such as cardiac disorders, but they have been used for a wide range of observations. These include understanding genome evolution and ancestry, comprehending avian parasites, bacteria and viruses and is even helping towards understanding bird migration [24–27].

2.1. Difficulties in studying avian cardiovascular genetics

Despite the high levels of heart disease in many flocks of commercial birds, understanding avian cardiovascular disorders is not always easy. There is difficulty when examining a bird for cardiovascular defects. One reason for this is that birds do not have a palpable pulse so more thorough diagnostic techniques such as radiology, echocardiography, electrocardiography and post mortem investigation often need to be considered [28]. It is possible, however, to auscultate the heart but this would give little information that would help form a diagnosis [29]. Another issue is that signs of cardiovascular disease are often non-specific and could be caused by an overlying problem. Some general signs include lethargy, dyspnoea and exercise intolerance. It is also important to understand the bird's history, diet, environment and reproductive ability [28].

In many species, information relating the rate of cardiovascular disease has been investigated in both wild and captive populations and in humans. The number of papers reporting similar information is comparatively sparse in avian species. The incidence of congestive heart failure in a study of 269 Psittaciformes was as high as 9.7% [30]. Of the psittacines observed with cardiac problems, 58% had coronary heart failure considered as the cause of death, 10 with right ventricular or biventricular failure and the remaining 5 with left ventricular failure. The remaining 42% of birds affected by cardiac disease had lesions which were considered incidental or secondary [30]. Moreover, in a second study where 107 captive Psittaciformes (budgerigars (*Melopsittacus undulatus)* and Australian king parrots (*Alisterus scapularis*)) were compared,

36% had gross lesions of the heart and/or major blood vessels [31]. Interestingly 99% of the birds examined had some degree of pathological changes to blood vessels and/or the heart with 6% of the birds showed pericardial effusion, 15% showed serofibrinous coating on the pericardium and other 15% showed hypertrophy or dilatation of the ventricular myocardium [31]. It is interesting to note that these relatively long lived but captive birds have a high incidence of cardiovascular disease. The kakapo (*Strigops habroptilus*), a flightless member of the parrot family, can live for over 100 years and is kept under very close scientific and veterinary care due to the limited number alive. In addition, the kakapo is technically a wild population but benefits from extra support and care from humans due to its near extinction status, but without being fully captive. The cardiovascular system has not been specifically highlighted as a particular problem in the kakapo despite inbreeding and close monitoring. This is potentially because the data has not yet been published but it may also indicate that the data differs from other parrots, differing from the previously mentioned parrot studies. This could indicate that breed genetics play an important role when investigating cardiovascular disease in avian species.

Naturally the other difficulty with investigating cardiovascular disease in avian species is the infrequency with which most wild populations of birds are seen by veterinary surgeons/researchers. A number of studies into cardiovascular conditions have been carried out over the years but this number declines when looking at the genetics behind each disorder. Frequently the larger cardiovascular studies are carried out on commercial flocks. These flocks hold significant economic value worldwide but breeding genetics may also be part of the cause of the high incidence rates of cardiovascular conditions observed, therefore their findings may not be as applicable to wild populations and other avian species. In addition, although commercial birds hold significant value in the food industry, the value of each bird itself can be very low, therefore making individual veterinary care difficult to justify to companies/owners.

In addition to looking at captive and wild populations of birds in order to understand cardiovascular disease, chick embryos are commonly used in research because they are relatively inexpensive in comparison to larger mammalian models and have a four-chambered heart, similar to humans. Avian species also generally have a short life-span/rapid development phases so the whole gestation can be studied in a shorter period of time than in other species [32]. Chick heart models have been used often to study the outflow tract of the developing heart for example. This review aims to look at cardiovascular disorders seen in avian species and understand some of the models of heart disease. The review highlights some of the associations of cardiac conditions with environmental factors and links and associations to genetic causes.

2.2. Congenital heart conditions

There have been reported cases of congenital cardiac defects involving a wide range of avian species. Some defects appear to be genetically linked whilst others appear to result from environmental conditions *in ovo*. There is a delicate interplay that occurs whilst the embryonic heart develops, involving both environmental, *in ovo* conditions, and genetic factors. These all have an impact on how the cardiovascular system develops. Research into human congenital cardiac defects often relies upon data obtained from chick cardiac models, where chick embryos are subjected to a varying environmental temperatures, pressures, haemodynamics and gene manipulations [33]. An example was the work on vitamin A deficiency in the

developing chick showing that it is required for specification of cardiovascular tissues and regulation of a number of key genes including GATA-4 and heart asymmetry genes [34].

Congenital heart defects within avian species have also been associated with aluminium toxicity [35]. Ventricular septal defects and ventricular myocardial defects occurred when chick embryos were dosed with aluminium. Changes in the vitelline circulation were observed in the embryos injected with aluminium, and this altered circulation has been shown to have a significant impact on the physical development of macroscopic cardiac structures [35]. External deformities including changes to cardiac surface structure, cardiac shape and changes in ventricular wall thickness were found to occur in passerine bird species exposed to environments contaminated with polychlorinated biphenyls [36]. These deformities did not result from an *in ovo* contamination, but occurred in birds born and raised in heavily contaminated environments.

Another documented cause of heart failure reported in avian species is valvular dysplasia [37]. Valvular dysplasia describes a valve which is distorted, inflexible and often fused in some way to the cardiac wall. This inhibits or completely destroys normal function of the valve, affecting blood flow through the heart chambers. Turbulent blood flow around the distorted valve can lead to further wear and damage to the valvular tissue. A documented case of this occurred in a young captive African penguin (*Spheniscus demersus*) presenting with exercise intolerance and open mouth breathing. Upon necropsy it was found that the juvenile had congenital right atrioventricular valvular dysplasia, and as a result right atrial dilatation in addition to ventricular dilation and hypertrophy [37], although no genetic basis was suggested.

Hypoxia in the chick embryo has also been linked with cardiac disease. Restricting oxygen to the chick embryo has been shown to induce left ventricle dilatation and the breakdown of cardiomyocytes. Adult chickens that have been exposed to this hypoxia have also been shown to display cardiomyopathies, most significantly left ventricular dilatation in later life and an increase in myocardial collagen. These gross changes result in impaired contractility [38]. Most studies agree that altered haemodynamics during development can lead to heart defects. Studies have been undertaken that surgically alter blood flow in an embryonic model to resemble cardiac defects seen in newborn babies. Outflow tract banding is commonly used in chick embryos around the heart outflow tract to stimulate these cardiac defects. The heart outflow tract is important because it will eventually form the intraventricular septum and semilunar valves. Therefore, changes to the heart outflow tract should help understanding of congenital defects that involve the valves and septum [39]. One study investigated how collagen content varies with and without outflow tract banding. A subdivision atlas approach was used to visualise changes in levels of collagen. This approach allows more specific spatial recognition of proteins and could be expanded on in the future to go beyond the heart outflow tract [39]. Another study used outflow tract banding to assess how haemodynamic changes effect heart tissue. It was found that the tightness of the band affected the changes seen. As tightness increased, the prevalence of cardiac defects also increased [40]. Although other studies showed that altered blood flow during development can lead to heart disease, this was the first study to assess how the degree of band tightness has an effect on the clinical presentation. Techniques such as optical pacing have also been used to increase the heart rate, thus tiring the heart, resulting in cardiac regurgitant flow [41]. The result in developing quail hearts was a number of congenital heart disease outcomes including endothelial cushion defects, valve

and septal defects and hypoplastic ventricles [41]. Naturally, altered blood flow can be due to genetics or environmental conditions, for example teratogens.

Current knowledge about the role of cardiac neural crest cells during the development of the cardiovascular system has mostly come from using quail-chick chimeras. Ablation of cardiac neural crest cells in chick embryos caused several cardiovascular defects such as abnormal artery patterning, abnormal myocardial function and an abnormal cardiac outflow tract [42]. Observing the effects of abnormal blood flow on cells will help target treatments of developmental conditions.

2.3. Ventricular septal defects

A ventricular septal defect occurs when the wall in between the left and right ventricle does not fully develop, resulting in a left to right blood shunt. Often associated with this defect is ventricular hypertrophy which later develops in order to maintain cardiac output [43]. There have been a few notable reported exotic cases, in particular a captive houbara bustard (*Chlamydotis undulata*) and Humboldt penguin. The bustard in question died at 6 months old, presenting with retarded growth; upon necropsy it was found the heart was twice the anticipated size and a ventricular septal defect was located [43]. In this case it was believed to be a genetically linked defect, supporting the theory that avian species can develop defects both as a result of *in ovo* factors and also genetic factors [43]. In the incidence of the penguin, a ventricular septal defect was detected after the Humbolt similarly presented with retarded growth. There have been reports of ventricular septal defects in two other penguins, and research suggests this defect is also the most common congenital abnormality found in caged birds [44].

Alterations in cardiac blood flow can have dramatic implications to the developing embryonic cardiac tissue. In chick embryos, studies have indicated that ligation of the vitelline vein returning blood to the chick heart, resulted in 10–72% of tested embryos sustaining a ventricular septal defect [45–47]. Left arterial ligation restricts blood entering the ventricle, therefore decreasing cardiac load on the heart. This resulted in 25% of embryos sustaining a mild ventricular septal defect [48]. Outflow tract banding results in constriction of the tract allowing blood to leave the heart, therefore causing increased cardiac pressure. This alteration resulted in 100% ventricular septal defect occurring in the embryos [48]. These studies mimic physical defects and their resulting haemodynamic changes which can occur in developing chick embryos, and how these changes impact upon the final cardiac structure. The administration of retinoic acid to chick embryos has also been associated with the development of ventricular septal defects. When a solution of retinoic acid was applied to 41 different chick embryos all at the same stage of development, 11 of these embryos later developed a ventricular septal defect [49].

2.4. Right ventricular failure

Right sided heart failure is a problem particularly relevant within populations of broiler chickens. Fast growing broiler chickens cannot meet their oxygen demand as easily as a slower growing chicken, due to the mismatch between body mass and cardiac output [50]. When put in stressful environments the proportionally small broiler heart is often unable to cope, leading to cardiac failure, seen in cases of heat stress within broiler flocks [51]. 27% of

fast growing broiler chickens in one study were found to have arrhythmias, compared to 1% in slower growing breeds [51]. One cause of heart failure results from the upregulation of matrix metalloproteinases (MMPs) stimulated by cardiac stress, which cause collagen degradation and ultimately right ventricular dilation [50]. One 2017 study examined the impact cold temperatures had on broiler chicken hearts, and found that low temperatures activated MMP's leading to right ventricular dilation and heart failure [50]. Therefore, either extremes of temperature have been documented to induce cardiac failure in broiler species, a fact which has serious economic consequences.

The impact of diet on the risk of heart failure in broiler chickens has been much investigated, in order to find the optimum nutritional planes to maximise growth whilst reducing cardiac failure risk. It has been found that an increased salt intake resulted in right ventricular failure and mortality. The elevated salt levels resulted in an increased blood volume, leading directly to ventricular failure, and in the broiler chickens receiving the higher concentration salt solution the mortality rates reached 50% [52]. The same was not found in the slower growing white leghorn species. This research indicates that dietary control and components plays a pivotal role in maintaining the health of broiler chickens and reducing the risk of heart failure and that breed genetics may play a vital role in susceptibility. Broiler chickens are also shown to be more susceptible to blood volume expansion and consequently heart failure, potentially due to a reduced salt excretory capacity when compared with the white leghorn chickens [52]. Another study into dietary composition concluded that n-3 fatty acid supplements would act to increase the circulatory level of nitrous oxides, in turn stimulating vasodilation and therefore reducing mortality from cardiac failure [53].

The altitude that chickens are raised at also has a marked effect on their risk of developing right ventricular failure. Multiple studies have shown that high altitudes can result in a disorder called pulmonary arterial hypertension. This is due to hypoxia at high altitudes which results in compensatory hypertension, and as the chickens grow larger it becomes harder for cardiac output to meet the demand. Eventually the pulmonary arterial hypertension can develop into right ventricular dilation, and eventually cardiac failure. This is the most common cause of high altitude broiler flock death [53]. Interestingly low altitude may similarly affect the heart. In a study looking at right ventricular enlargement and ascites in broiler chickens it was thought that the condition resulted from the birds being raised at low altitudes, although no conclusion was drawn as to the true cause of the conditions. Right heart failure has been associated with consumption of Furazolidone, sodium chloride and P-dioxin, however in this study none of these compounds had been ingested. Therefore further studies regarding the impact on altitude on blood volume and heart failure in broiler chickens would be of economic and scientific interest [54]. To conclude, diet, environmental temperature and altitude, exposure to toxins and genetics all play a role in the prevalence of right ventricular failure in broiler chickens, and all factors must be considered the prevention and treatment of this condition.

2.5. Atherosclerosis

Atherosclerosis is a condition that has been reported to affect a variety of avian species. It is particularly recognised within parrot species, with a study carried out in 2013 finding the prevalence of advanced atherosclerotic cases to be 6.8% within a population of 7683 parrots

[55]. The authors noted that the incidence of advanced atherosclerotic lesions was similar to that in humans aged 45–75 years old and that advancing age was an important determinant in the birds, as with humans. Interestingly, in other smaller studies, the incidence rate varies from 1.9 to 92.4% but the authors highlighted that only five psittacine genera were studied from approximately 84. Long before these recent studies in parrots, the condition was known to naturally occur in birds such as chickens [56] and up to 90% of birds from captive exotic avian orders [57] and even 100% of White Carneau pigeons studied which decreased to virtually no cases in Slow Racer pigeons [58, 59]. The White Carneau Pigeon is also used as a clinical model to study the earliest stage of the disease within human populations [60]. A genetic predisposition in this species has been investigated and is found to be linked to a single gene defect which results in the build-up of unoxidised fatty acids. A polymorphism within pro-alpha-2(1) collagen was linked to atherosclerosis giving an autosomal recessive inheritance pattern [61]. Although dietary intake will also likely play a large role in whether the birds acquire lesions [62], when White Carneau and Slow Racer pigeons were compared on a similar diet, the Slow Racer pigeons were naturally resistant to atherosclerosis [63]. More recently is has been indicated that avian species have become less favourable as a model due to the varying artery size across avian species, instead pig and mouse models have grown in favour [64].

Atherosclerosis occurs when there is accumulation of fatty lipids and cholesterol within arteries. This initial deposition can lead to fibrosis and calcification, causing occlusion and narrowing of the blood vessel [65]. The disease itself is chronic and pro-inflammatory, affecting the lining of blood vessels, and can result in strokes, arterial disease and coronary artery disease. In a case involving a male white cockatoo, atherosclerosis was found to have caused an aneurysm of the right coronary artery. It was believed that the patients diet high in fats and low in vitamin A may have led to the initial atherosclerosis, which upon post mortem was found to be widespread in vessels across the patient's body [65]. A similar case was also reported involving a female macaw, presenting with dyspnoea, upon necropsy it was found she suffered with severe atherosclerosis of the aorta and brachiocephalic arteries [66]. Prevalence of the disease within the parrot population has been shown to increase with age and is more prevalent within females [55] [67]. A comparison of observed prevalence trends with cholesterol level revealed that a systemic cholesterol increase with age is associated with an increase in the prevalence of atherosclerosis. Differences in genera, fat nutritional requirements and lipid metabolism were also found to impact upon cholesterol levels and atherosclerotic risk [67]. Male parrots in one study were found to have only 69% of the atherosclerotic risk that females have [55]. This heightened female risk has been hypothesised to be attributed to reproductive dysfunctional diseases, although further and more specific studies are required to further investigate this [55].

Atherosclerotic lesions in avian species are comparable to human lesions and are classified in a similar method to that used in human medicine. Therefore avian models have been used in the past as clinical models to study the formation, development and treatment of human atherosclerosis disease [55]. A 2015 study into the impact that cholesterol and triglycerides have on the supra aortic trunk involved examining the effect that the diet of white leghorn chickens had upon the histological appearance of their aorta. Atherosclerotic lesions were classified similarly to the system used by the American heart association [68]. High fat and high cholesterol diets increased the prevalence of severe lesions within the supra-aortic trunk,

and decreased the age at which the lesions occurred, i.e. lesions developed quicker in chickens with a higher fat diet, with severe lesions seen in chickens as young as 6 months [68]. This clinical model has been applied to increase understanding of disease development within the human population, due to the comparable nature of the disease process in avian species and humans. The same clinical model involving chickens also helped to study the effectiveness of treatment of aortic lesions with atorvastatin therapy. It was believed that this regression of the severe lesions following treatment shown in the chicken population may also be replicated within the human population [68].

In human medicine aneurysms are a commonly reported condition, often resulting from atherosclerosis. However there have been very few reports of this condition in avian species. An aneurysm describes an outpouching, or thin or damaged section of a vessel wall. They can result from a congenital defect, be dietary, or result from bacterial infection damage. There is one documented congenital case of it occurring in a pigeon, in this incidence the pigeon died of unrelated circumstances; however, upon necropsy was found to have a right ventricular aneurysm. This would indicate that during development there was focalised area where the heart tissue failed to correctly develop. At the time of its reporting this was the first documented congenital avian aneurysm [69]. This is interesting to note given the high levels of similarity between humans and birds in the disease otherwise.

2.6. Hypoplastic left heart syndrome

Hypoplastic left heart syndrome (HLHS) has been investigated using chick embryonic models in several studies. It is often characterised by abnormal fetal development of the left hand side of the heart and can result in a smaller/under developed left ventricle, mitral or aortic valves not forming or being small, the ascending portion of the aorta being small/underdeveloped and atrial septal defects (hole in the heart) are common in affected offspring. Left atrial ligation is used to shunt the flow of blood from the developing left ventricle to the right ventricle. This produces heart models that are phenotypically the same as hypoplastic left heart syndrome [70]. The prognosis for hypoplastic left heart syndrome in humans is poor and several operations are required. The use of stem cells to differentiate into cardiomyocytes could remove the need for operations and postnatal care but needs further investigation.

Tissue hypoxia is necessary for normal development as it stimulates signalling pathways that lead to the development of the normal myocardial architecture [71]. If the developing tissue becomes too hypoxic, however, it can lead to defects. Many studies support that hypoxia leads to an increase in fibre due to increased expression of extracellular matrix proteins [72, 73]. There is speculation, however, about the order in which the defects develop. Most literature supports that it is altered haemodynamics that lead to hypoxia that leads to fibrosis but it is possible that there are other causes of the fibrosis [73].

2.7. Cardiomyopathies

The cardiomyopathies are a complex range of cardiovascular disease and occur naturally in many species. There have been many debates on the use of several different mammals as models for cardiomyopathy but many studies are carried out in avian species. Evidence indicates

that the avian heart carried many similarities to the human heart in terms of biochemistry, physiology, function and morphology [74]. Dilated cardiomyopathy in broiler chickens has been associated with avian leukosis virus, rapid growth and pulmonary hypertension [75]. Avian leukosis virus has been implicated to cause cardiomyopathy as it is linked to 11.1% rate of dilated cardiomyopathy in infected broiler chickens in comparison to ~1.4% in non-infected broilers [75]. Turkeys have also been noted to be affected by a similar virus and had a high incidence of DCM [76]. Cardiomyocyte hypertrophy has also been noted in Japanese native fowls exposed to the virus [77] and myocarditis has also been linked to it [78]. These links in birds are important as they help to shed light on the actions of the virus. It is likely that humans and other animals are similarly affected by viruses including human immunodeficiency virus (HIV-1) and simian immunodeficiency virus (SIV) [79, 80], however more studies are required in order to draw stronger conclusions. There are many theories as to how these viruses act but some studies have shown they can up and down-regulate genes and proteins via insertional mutagenesis, inflammatory changes, physical stimulation of matrix inclusion bodies, envelope protein-induced transformation and a number of other mechanisms [75, 77, 81, 82]. Environmental factors are also a consideration. A study into the effects of polychlorinated biphenyl (PCB) 77 showed that in the tree swallow (*Tachycineta bicolor*) *in ovo* exposure to the chemical results in higher levels of cardiomyopathy and ventricular wall abnormalities [83]. Age also had an effect on normal broiler roosters in comparison to mature roosters. As the roosters aged from 7 to 42 weeks, the incidence of increased right ventricle-to-total ventricle weight ratio was significantly larger in the older population, with over 36% of roosters at 42 weeks of age within ranges believed to result in right ventricular hypertrophy [84].

Dilated cardiomyopathy in turkeys has been linked to troponin T and phospholamban (PLN) variations [85]. This represents an important finding given that within the first 4 weeks of life 2–5% of captive turkeys have been reported to have cardiomyopathy, likewise the wild turkey is also affected by this cardiovascular disorder [85, 86]. Exon 8 skipping in affected individuals has been a suggested link in cardiac troponin T and it has also been indicated that wild turkeys have the similarly low molecular weight as exon 8 is spliced out [87]. This is also notable as humans carrying specific mutations in troponin T are also affected by cardiomyopathies [88]. Over 90 mutations have been described in the troponins in humans to date which result in heart disorders including hypertrophic, dilated and restrictive cardiomyopathy in addition to left ventricular non-compaction. It has also been highlighted that despite variable regions of troponin, there are some areas which are highly conserved between species including most mammals, turkeys and chickens [88]. The areas containing the most causative mutations in humans are the T1 terminal and the C-terminal and cardiac troponin T, therefore these regions make excellent candidates for future studies, not only in turkeys but in other birds too.

Avian species have also been used as models for human and non-human mammals. The Broad Breasted White turkey treated with furazolidine produced a dilated cardiomyopathy phenotype which shared many characteristic of human idiopathic dilated cardiomyopathy [74]. The white Leghorn has also been genetically altered to produce cardiomyopathy phenotypes. These have included morpholino induced knockdown of alpha myosin heavy chain causing an enlarged heart and septal defects [89]. Morpholino induced knockdown of embryonic myosin

heavy chain caused dilated cardiomyopathy, septal defects and electrical abnormalities in the developing chick heart [90]. Both proteins were also shown to be expressed from very early stages in development, when the heart is first developing *in ovo* and have also been shown to be expressed in human hearts at early developmental stages [89, 90]. It is worth noting that alpha and beta myosin mutations have also been linked to cardiomyopathies and atrial septal defects in humans [91–93], thus using the chicken models can give further insight into developmental, structural and physiological abnormalities. When looking back on the information about the structure, function and histology of the heart, it was notable that the myosins were an important feature of the avian heart, as they also are in mammalian hearts. To date no large scale study has investigated whether the disease causing mutations seen in humans and other mammals are also present in birds. In total the myosins, cardiac myosin binding protein C and troponin gene account for over 90% of the known causative mutations in human hypertrophic cardiomyopathy [94], for example. Tropomyosin I, which is associated with both actin and troponin T has also been shown to present with cardiomyopathy associated mutations in humans and more recently abnormal atrial septation, ventricular trabeculae and looping in genetic knockdown developing chickens [95]. Given the research already present in some of these genes in avian species, and their high sequence homology and expression patterns in avian cardiac tissue they present excellent candidate genes for further investigation. Given their similarities it is highly possible that further associations are present.

3. Conclusions and future directions

To conclude, a range of cardiovascular conditions have been reported that affect avian hearts. In many cases the ranges of clinical signs and treatments (where applicable) have been studied. Congenital defects, bacterial infections, dietary and environmental components have all be shown to have an impact on the presence of cardiac disease in a variety of avian species. Birds are difficult to study clinically and there are pronounced differences between wild and captive populations and differences between the species. There are also well known difficulties with using anaesthesia (often required to study or treat the birds), as the side effects of these drugs can include cardiac arrhythmias. Studying the incidence of heart disease and the genetic factors underlying the disorders has proved more problematic. Large flocks of birds produced for commercial use provide sufficient numbers of animals to study, which is usually good for genetic studies however few birds receive a post mortem in the commercial setting and most have a relatively short life. The question of inbreeding within these flocks also complicates the resulting incidence rates, and economic factors frequently affect the levels of care and veterinary interventions possible. Scientific funding is also difficult to establish given that the heart defects often present at advanced ages. Funding often relies on establishing a cost benefit, therefore would usually have to concentrate on commercial flocks rather than wild birds. The sequencing projects underway represent a big advancement towards the study of genetic disorders in avian species. They provide the much needed basic information about the avian genomes which will make it easier to study individual disorders. Appropriately powered studies using the most advanced genetic tools will highlight further causative and associated genes. Further information about the heart in general is also a key area to develop.

For example understanding the normal levels and locations of gene and protein expression during differing stages of development. Recently the transcription factor *Nkx2.5* was shown to be essential for blood vessel and cardiovascular development in the chicken, and its role has been well established in other animals, models and humans [96]. N-cadherin and retinoic A (vitamin A) are essential for appropriate cardiovascular development, without which appropriate looping, differentiation and symmetry are not achieved and chicken and quail embryos die very early in development [97]. In most animals cardiovascular disorders can affect both embryonic, young, adult and very advanced aged individuals. Vital loss of function of change of function of proteins at very specific time points can have an effect on cardiovascular health, in addition to proteins which are affected over longer time periods. Therefore understanding keys stages of development and the role that each gene plays is essential.

A large number of genetic techniques are being used in avian species in order to both understand the genome and in order to edit it. Although research into the heart is still in its infancy, there is much to be gained by continuing with the research. Many techniques are presently used in order to edit or manipulate the genome and these could be beneficial for not only animal health and welfare, but also for efficiency and economic gains. Gene editing techniques could support the food industry by enhancing meat production/egg laying traits, by inserting disease resistance and by identifying and possibly altering genetic disorder sequences. There are also possibilities for gene editing to insert characteristics into eggs (for example) to benefit human health. Naturally exploration of epigenetic modifications should also be an important factor moving forward. Studies in recent years have shown that both DNA and RNA epigenetics can have a large impact on an organism and more needs to be undertaken in the cardiovascular system in order to determine the impact in all species.

The importance of the bird as a part of the ecosystem, companion animals and as a food source mean that although they exist in great quantities, they have not been as well studied as other animals in the area of cardiovascular genetics. This is particularly true when addressing the incidence and genetic factors affecting cardiovascular disease. More research is required to quantify these diseases and to elucidate the true impact that these components have on heart development and disease.

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Conflict of interest

The authors declare no conflicts of interest.

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Chapter 10

Genetics of Disease Resistance in Chicken

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Additional information is available at the end of the chapter

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Abstract

Although poultry industry has gained momentum during the last few decades, there are still various impediments like improper infrastructure, unscientific management and above all various deadly infectious diseases which incur huge economic losses on poultry industry. These diseases include viral diseases like Avian Influenza, Marek's Disease, New Castle disease and bacterial diseases like Colibacillosis, Pasteurellosis and Salmonellosis, etc. Development of disease resistant poultry has been found successful practice over the use of drugs or vaccines for disease control. Studies involving genome wide associations to figure out certain candidate genes that are involved in disease resistance have also been carried out. Single nucleotide polymorphism studies to unveil the mechanisms underlying disease resistance in chicken show that SNPs and other candidate gene approaches play a vital role in providing disease resistance. Also, understanding the genes and biological pathways that confer genetic resistance to various infections will lead towards the development of more resistant commercial poultry flocks or improved vaccines against various diseases. This chapter shall focus on various factors involved in disease resistance in chicken that interact with the pathogen and provide resistance against the pathogen.

Keywords: chicken, genetics, pathogen, disease resistance

1. Introduction

Poultry is a principal component in the global agricultural economy by serving as one of the primary sources of proteins for humans. Worldwide egg and poultry meat production is close

to 73 million tons and 100 million tons respectively [1]. Despite such an increase in the growth of poultry industry, this industry is consistently threatened by various diseases, including those caused by viral, bacterial and parasitic infections. These diseases can lead to substantial economic losses in two ways, firstly there is a reduction in the production of poultry related products, and also the input costs like labour and feed get increased. The impact of these loses in poultry industry is more worse on the livelihood of poor people in the developing countries where up to 25% of monthly income may be lost due to poultry disease [2]. Chicken have developed different responses to counter these diseases. These responses include immunological and genetic responses of the poultry. The genetic interaction between the host and the pathogen is a key factor in deciding the disease resistance. The chicken karyotype includes 38 autosomes, many of which are relatively small and uniform in size, often termed microchromosomes. Current knowledge of chicken immunogenomics such as the quantitative trail locus (QTL) mapping of the combination of DNA variations, immune response by the host and the transcriptome can be used to identify disease resistant genes. Disease resistant genes are those encoding antibodies, microRNA and other materials that help the host resist the damage caused by pathogens. Recent advances in the field of molecular biology have led to the discovery of many disease resistant genes. In poultry, genes such MHC (major histocompatibility complex) genes, the Nramp1 (Natural resistance-associated macrophage protein 1) gene, IFN (Interferon) genes, Mx (Myxovirus-resistance) genes, anti-ALV (Avian leucosis virus) genes and the Zyxin gene have been linked to disease resistance [3]. In most of the multicellular organisms single-nucleotide polymorphisms, insertion/deletion polymorphisms, and copy number variations (CNVs) are the major sources of genetic and genomic structural variations [4]. These genetic variations may be exploited to study the diseases resistance levels in different organisms. Recent advances in the technology and cost effectiveness of genotyping, genomic selection approach has been followed extensively for animal breeding. The discovery of chicken genome and development of chicken transcriptome and proteome analysis has led to a better understanding of the mechanisms underlying the genetic susceptibly and resistance against different diseases. Genetic enhancement of the immune response can increase vaccine efficacy and disease resistance, thereby reducing drug residues in food. In order to reduce the drug residues in the food and introduce the genetic breeding programs for improving the disease resistance in the chicken we need to have a better understanding of the disease resistant genes. Also, breeding for disease resistance requires tools such as indicator traits or genetic markers that can be used for selection. Some diseases that have been found to cause serious economic loses to poultry industry in terms of morbidity and mortality are discussed below.

2. Salmonellosis

Among the different diseases occurring in poultry, those caused by the genus *Salmonella* is the most common, causing serious economic losses to the poultry industry in terms of mortality, reduced growth and loss of egg production. Two species are currently recognised in the genus *Salmonella*, *S. enterica* and *S. bongori*. These two species further comprise of about 2500 serovars. Chicken can get infected with S. enterica at any time during their life. However,

infections within the first hours and days of their life are epidemiologically the most important, as newly hatched chickens are highly sensitive to *Salmonella* [5]. Infection with most of the *Salmonella* serovars remains unnoticed as the birds do not show clinical signs, however infection with *S. gallinarum* and *S. pullorum* leads to clinical manifestations. Clinical signs that include lack of appetite, depression, respiratory distress, caseous core diarrhoea and early death are predominantly observed in young chicks. In laying hens symptoms include reduced egg production, fertility and hatchability [6]. The level of bacterial invasiveness depends upon the serovar that has caused the infection and host's immune status. Prophylactic measures, vaccination and use of antibiotics are insufficient to eradicate salmonellosis in poultry stocks, whatever the serotype involved. The major problems associated with the widespread use of antibiotics are the development of bacteria resistant to antibiotics, and the accumulation of antibiotic residues in food for human consumption [7]. In this context, selection of more resistant chickens can be considered as an alternative solution to decrease occurrence of the disease. In recent years, advances in molecular technology have created a new horizon for the genetic improvement of quantitative traits, particularly disease-resistant traits. The identification of direct or indirect molecular markers for these traits would facilitate the use of marker-assisted selection or gene introgression [8].

2.1. Genes involved in resistance to Salmonellosis

Many genes have been found to contribute towards resistance against bacterial infections. Some of the main genes involved are Major histocompatibility complex (MHC) genes, Caspase1 genes, NRAMP Family encoding genes, inducible nitric oxide synthase (iNOS) gene, genes encoding complement proteins and Toll-like Receptor 4 (TLR4) genes. Major histocompatibility complex studies have shown that different MHC-B haplotypes contribute differently towards the genetic resistance against salmonellosis. Also microsatellite analysis has shown that MHC-1 class has been linked to *Salmonella* colonisation [9]. Single-strand conformational polymorphisms (SSCP) and sequence polymorphisms have linked MHC I and MHC II to resistance against *salmonella* [10], and to antibody response kinetics [11]. The indigenous and commercial breeds of chickens in Vietnam also linked *Salmonella*-specific antibody responses to MHC-B haplotype [12]. Interleukins and chemokines also play a vital role in *Salmonella* infections. While comparing the interleukin mRNA expression in the heterophils of resistant and susceptible chickens, it was shown that the mRNA level of different interleukins like IL-6, IL-8 and IL-18 increased significantly in resistant chicken as compared to the susceptible chicken. The mRNA levels of transforming growth factor (TGFβ4) were found to decrease in heterophils of resistant chicken. Also the mRNA levels of interferon gamma (IFN γ) were found to be lower in susceptible chicken when compared to resistant chicken [13, 14]. Resistance to *Salmonella* has been linked to different genes like ILs, IFNγ, TLRs, iNOS and genes involved in apoptosis. Resistant chicken lines showed a higher expression of the interleukins like IL-2, IL-6, IL-8 and IFNγ in the small intestines as compared to susceptible chicken lines [15]. Interferon gamma gene expression was significantly lower in susceptible chicks as compared to resistant ones. Interferon γ expression level represents a valuable indication of immunodeficiency associated with persistence of *Salmonella* in the chicken digestive tract, and IFNγ thus represents a factor to consider in the development of prophylactic measures for the

reduction of *Salmonella* carrier state [16]. The natural resistance-associated macrophage protein 1 (NRAMP1) is a candidate gene associated with *Salmonella enteritidis* (SE) mediated immune response, and is related to the phagocytosis of SE. Studies have shown that the enhancement of host immunity mediated by the up-regulation of NRAMP1 mRNA in heterophil granulocytes and spleen might be more obvious and earlier in the SE infection resistant chicks as compared to susceptible chicks [17]. Different variations in the Nramp-1 gene have been associated with resistance to salmonellosis [18]. Natural resistance-associated macrophage protein 1 and 2 encoding genes (Nramp1 and Nramp2) are related to many diseases. Association analysis indicated that A24101991G is significantly associated with chicken salmonellosis resistance [19]. Certain genes like NRAMP1, TGFβ3, TGFβ4, and TRAIL have been found to be potent candidates for disease resistance against *salmonella* [20]. The candidate gene approach is a useful method to investigate genes that are involved in genetic resistance. Earlier studies showed that there is an involvement of 12 candidate genes in the pathogenesis of *Salmonella* in meat-type chicken [21]. These genes include NRAMP1, prosaposin (PSAP), inhibitor of apoptosis protein 1 (IAP1), inducible nitric oxide production (iNOS), Caspase-1 (CASP1), interferon-gamma (IFNγ), immunoglobulin light chain (IGL), interleukin-2 (IL2), transforming growth factors B2, B3 and B4 (TGFB2, B3 and B4) and ZOV3. *Salmonella enteritidis* infection was given to birds at 3 weeks post hatch. At day 7 post infection SE load was quantified in caecum, spleen and liver contents. In caecum nine out of 12 genes were found to be associated with bacterial load. These genes include CASP1, SLC11A1, IAP1, PSAP, iNOS, IL-2, TGFB2, TGFB4 and IGL. Five genes (SLC11A1, 1L2, CASP1, IGL and TGFB4) were found to be significantly associated with bacterial load in liver. Only one gene i.e. TGFB3 was found to show association with bacterial load in spleen. The above study confirmed polygenic nature of SE resistance. A quantitative trait locus (QTL) on chromosome 5 was identified that was involved in controlling bacterial load in spleen and was named as SAL1. This QTL was found to be involved in bacterial clearance by macrophages. Single nucleotide polymorphism studies have shown three SNPs in an exon of chTLR15. One of the SNPs was found to be associated with *Salmonella* infection. The 'T' allele in SNP C726T might be linked to resistance of *Salmonella* infection. The mRNA expression of TLR15 in heterophils of chickens infected with SE was lower than that of the control group at day 3 pi. However, TLR15 was up-regulated in the spleen of chickens infected by SE at day 3 pi [22]. The above discussed genes are potential candidates that can be used for selection programmes for increasing genetic resistance against *Salmonella* Enteritidis in chickens. A number of factors which include Nramp1, MHC, TLR4 and a novel genetic locus SAL1 determine the genetic resistance of chicken against SE. After analysing and comparing studies of Myeloid differentiation primary response gene 88 (MyD88), novel mutation G4810372T was found that was thought to have an effect on immune response of the individual. Further studies are needed to elucidate the molecular mechanisms that occur due to MyD88 gene polymorphisms. After correlating susceptibility towards *Salmonella* Pullorum and MyD88 polymorphisms, it was found that alleles in SNP1 locus and SNP1 and SNP3 genotypes show a significant effect against *Salmonella.* Also the advantaged haploid type (TTC) combined by SNP1, SNP3 and SNP4 loci played a very significant role in genetic resistance to *Salmonella* Pullorum infection. Myeloid differentiation primary response gene 88 polymorphisms or advantaged haploid type in a particular region had a positive effect against susceptibility to *Salmonella* Pullorum infection. From the above observations it can be concluded that MyD88 can be used as a candidate

gene which could provide a conceptual reference for marker assisted selected for poultry [23]. In a study that was based on the biological function and SE response of various genes, five candidate genes were selected that were found to have a role in *Salmonella enteritidis* infection. These genes include toll like receptor 4 (TLR4), macrophage migration inhibitory factor (MIF), T cell specific protein (CD28), tumour necrosis factor (TNF)- α factor (LITAF) and MD-2. In TLR4, CD28 and MD-2 single nucleotide polymorphisms were found. The SNPs were tested for associations between sire SNP and *Salmonella* enteritidis response. The association of sire SNP with cecum bacterial load and vaccine antibody response was found to be statistically significant. Association of MD2 SNP was statistically significant with bacterial load in spleen. The use of the above studied SNPs can be used in marker assisted selection and may result in improvement in diseases resistance in poultry [24].

3. Avian influenza virus

Avian/Bird flu, caused by avian influenza virus (AIV) belonging to Orthomyxoviridae family, is the most fearful viral disease of birds and has a potential to cause a detrimental effect on poultry flocks. This disease is of great economic, zoonotic importance and may also lead to pandemic threats. This virus can lead to disease that may range from subclinical symptoms to highly virulent pathogenicity in poultry birds. The frequent disease outbreaks caused by avian influenza virus (AIV) not only affect the poultry industry but also pose a threat to human safety. Based on the level of pathogenicity the disease has been categorised into two groups. The first group is highly pathogenicity avian virus (HPAI) which is highly contagious, and can affect multiple organs. This disease has a potential to spread across national boundaries and is a listed disease of World Organisation for Animal Health (OIE). The second group is low pathogenicity avian virus (LPAI) which is mild disease in poultry that causes mild clinical symptoms like depression and anorexia. Avian influenza virus (AIV) has caused a great economic loss across the globe [25]. The AIV mostly gets amplified in poultry at live poultry markets and finally disseminates to humans [26]. While replication of LPAI occurs in epithelial cells of respiratory and gastrointestinal tract, HPAI replicates in multiple tissues [27]. World Health Organisation has emphasised on the preventive measures to be taken in order to minimise the risk of pandemic influenza and also have highlighted the importance of elucidating the host factors that are related to infection [28]. Currently live or inactivated viral vaccines are used to reduce the incidence of AIV, but these measures are not promising as the efficacy of these vaccines is complicated by different factors which include age/ health status of bird and also the antigenic variant of the virus. So there is an urgent need to develop promising and long lasting strategies to combat these viral diseases. To complement current approaches against AIV, development of poultry flocks that are AIV resistant can be used as a proactive measure to control epidemics and pandemics of influenza in both avian and human populations.

3.1. Genes involved in avian influenza virus

Many studies have been carried out to figure out different disease resistant mechanisms and genes in AIV. Previous studies on Beijing-You chicken have revealed 39 SNPs associated with

different immunological traits against avian influenza virus. An important QTL was found on chromosome 16 that was related to total $I_g\gamma$ concentration. Also five candidate genes that were related to $I_g\gamma$ levels were found that might play a role in immune modulation of birds infected with AIV. Different candidate SNPs for marker assisted selection for disease resistance have been identified. The candidate genes play a vital role in regulating immunological response in chicken [29]. Approaches like RNA interference (RNAi) technology can be used to develop transgenic poultry that are resistant to AIV. Synthetic RNA duplexes (siRNA) can be used to trigger RNAi [30]. Also RNAi can be triggered by expression of RNA duplexes in hairpin structures (shRNA) [31] which by RNA endonucleases can be processed into siRNA. While working on cell lines, chicken embryos, synthetic RNA duplexes specific for conserved domains of the influenza virus genes have been found to inhibit replication of various influenza viruses. [32, 33]. Stable expression of influenza-specific shRNA via a lentiviral vector in a cell line renders the cells refractory to influenza virus infection [33]. After introduction of the above mentioned lentiviral vector into mouse lung, an inhibition in virus production was observed *in vivo*. From these studies we can conclude that there is a possibility to develop influenza resistant poultry flocks by transgenic expression of influenza-specific shRNA. Current proposals to develop influenza resistant chicken include using combination of transgenic and RNAi that can be used for AIV gene expression inhibition. Screening of siRNAs as candidate genes *in vitro* is the key step for transgenic breeding. A combination of bioinformatics and other online search tools to design siRNAs that target different mRNA sites of AIV H5N1 subtype. Five rational siRNAs were chosen, five U6 promoter driven shRNA expression plasmids that contained the siRNA genes were constructed that were used to develop stably transfected Madin-Darby Canine Kidney cells. Data obtained from Indirect Immunofluorescent Antibody (IFA), virus titration, PUI stained flow cytometry, Real time PCR and DAS ELISA revealed that all the five stably transfected cell lines when exposed to $CCID_{50}$ of AIV were resistant to viral replication. Finally transgenic chicken were developed from the plasmids (pSi604i and pSi 1597i). These findings provide baseline information for breeding transgenic chickens resistant to AIV in combination with RNAi [34].

4. Marek's disease

Marek's disease (MD) is a neoplastic disease in chickens, caused by the Marek's disease virus (MDV). Marek's disease virus (MDV) is an alpha herpes virus that targets avian species and establishes chronic infection. It is a highly contagious lymphotropic disease that remains an important source of economic losses to the world poultry industry since it was first reported by Joseph Marek [35]. Marek's disease signs include depression, wasting, loose watery stool, paralysis, lymphomas and severe immunosuppression. Although vaccination programs have been used to control onset of the disease, MDV still replicates in vaccinated chicks. These highly contagious cell free virions are continuously shed in the environment. This makes MDV environmentally persistent as well as a highly infectious [36]. Continuously more virulent MDV strains evolve that makes the current vaccination programs ineffective and urge for a need to develop strategies that will augment existing MDV control strategies [37].

4.1. Genes involved in resistance to Marek's disease

The genetics of the host response to the MDV have been studied for many years. Many loci have been known to be involved in disease resistance but only few genes have been identified to have an actual role. Major histocompatibility complex plays a vital role in resistance against MD [38]. Being a polygenic trait, many genes and gene loci have been reported to be involved in MD resistance. Major histocompatibility complex is one among gene/loci to be involved in genetic resistance against MD. Other genes that are non MHC in origin have also been linked to play a role in genetic resistance/susceptibility to MD. These genes include growth hormone gene, cytokines (IL 6 and IL 18) and the stem lymphocyte antigen 6 complex, LY6E gene. Loci rs14527240 and GGaluGA156129 have been reported to play a role in host resistance/susceptibility to MD. Also expression studies suggest a possible role of SMOC1 gene in MD susceptibility [39]. Nitric oxide which apart from being a promising antiviral agent, also plays a role in modulating immunological responses. While working on Marke's disease it was found that chickens resistant to MD have the ability to produce more nitric oxide than susceptible chicken lines. The above observation was made by measuring nitric oxide levels from the chicken fibroblasts that were taken from these chicken lines after treatment with LPS and recombinant Chicken IFN-γ. Further plasma nitric oxide levels were measured in chicken lines (N2a, P2a) inoculated with JM-16 strain of MDV. The levels of NO were found to be increased in N2a chickens in majority of the experiments carried out (four out of five). In comparison, in only one experiment the levels of NO were found to be elevated in P2a chickens that too at 10th day post infection. The level of the NO production was found to be associated with the range of virulence of the MDV strain. Inoculation with more virulent strains induced highest NO level which suggests the possible role of NO during the disease progression. Quantitative real time PCR studies show that IFNγ does not primarily induce iNOS gene expression during MDV infection. Nitric acid production and inducible nitric oxide gene expression are mediated during cytolytic phase of infection. These findings suggest that NO may play a role in increasing MDV virulence by suppressing immune system [40]. In order to breed chicken which are genetically resistant to the Marek's disease, we need to have an ample knowledge about markers that play a role in the resistance to MD. A study was carried to find out the MD resistant markers in chicken lines, copy number variation (CVN) were studied in inbred MD resistant and susceptible chicken lines. In four chicken lines 45 copy number variations were found, out of which 28 CVNs were involved in cellular proliferation and immunological responses. Also two CVNs that were found to be associated with resistance to MD were transmitted to the descendent recombinant congenic lines that differ in MD susceptibility. These observations may be useful for designing better and reliable strategies to improve genetic disease resistance in poultry.

5. Newcastle disease

The causative agent of Newcastle Disease is Newcastle Disease virus (NDV) which belongs to paramyxovirus and is a negative sense RNA consisting of about 15×10^3 nucleotides [41]. This is an enormous destructive and contagious disease that causes serious problems in poultry

industry across the globe. Among different poultry diseases NDV was reported to be the fourth most destructive disease that led to heavy loses to poultry industry [42]. Newcastle Disease was considered to be most widespread disease in animals along with rabies and bovine tuberculosis [43]. After infection with NDV the host comes up with non-specific symptoms which include ruffled feathers, depression, breathing problems, anorexia, hyperthermia and listlessness followed by death. Affected chicken show respiratory and neurological complications and also reduction in egg production. Chicken infected with NDV are able to raise an antibody and gene response. The antibody response varies in different chicken breeds, hence understanding the genetics of the immune response may help in improving diseases resistance in chicken [44].

5.1. Genes involved in resistance to Newcastle disease

A study was conducted to elucidate the host antibody response towards NDV. A novel QTL locus that was found to be associated with antibody response was found. From the proximal end of GGA1 this QTL region was located approximately 100 Mb away. This region was proposed to play an important role in immune response of the chicken. Two genes namely ROBO1 and ROBO2 were thought to be promising candidate genes that might have a role in modulating antibody response in chicken infected with NDV. For further confirmation of the role of these genes, studies that include silencing and over expression of ROBO 1& ROBO2 need to be carried out both *in vivo* and *in vitro* [44]. Host response towards NDV infection is poorly understood. In order to have a better understanding of the host pathogen interactions during NDV infection a transcriptional profiling study of chicken embryo cells that were infected with NDV strain D58 was carried out by quantitative real time PCR. Some of the genes under study were upregulated and some were down regulated. Genes such as IFN-α, IFN-γ, DDX-1 and MHC-1 were upregulated IL-6 gene was down regulated. The expression levels of the M and F genes of the virus were also measured. The genes that encode for pro inflammatory response, cellular responses and other genes that regulate interferons were found to be affected during the infection. These findings suggest the involvement of different signalling pathways that are involved in host response towards infection [41].

6. Conclusion

For effective control of different infectious diseases in chicken, the best and most reliable approach is the improvement of the genetics of disease resistance. Enhancement of immune responses may lead to improved efficacy of vaccines and disease resistance, hence reduction in drug residues in the food products. Introducing new technologies that will help us to unveil the underlying transcriptional and other molecular mechanisms for disease resistance in chicken is a promising tool to improve genetic resistance for diseases. Technologies that aid in identification of disease resistant genes include next generation sequencing, microarray analysis, RNA sequencing and high density SNP genotyping. The development and distribution of disease resistant poultry flocks represents a proactive strategy for controlling diseases in chicken and complements current approaches for disease control by drugs and vaccination.

Conflict of interest

The authors declare that they have no competing interests.

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Genetics and genomics in poultry have been the most rapidly advancing subjects since the completion of the chicken genome sequence in 2004 and have been extensively used to understand the genetic determinants of complex traits. This book intends to provide readers with a comprehensive overview of the current progress in the application of genetic and genomic science in the poultry field. The contents cover genetic variation detection, selection methods for breeding, transgenesis and genome editing, genetic basis of disease resistance, control of gene expression and regulation, reproduction and meat quality, etc. The book should prove useful to researchers and students working in related fields.

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