Next-generation sequencing: The future of molecular genetics in poultry production and food safety[1]

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ABSTRACT The era of molecular biology and automation of the Sanger chain-terminator sequencing method has led to discovery and advances in diagnostics and biotechnology. The Sanger methodology dominated research for over 2 decades, leading to significant accomplishments and technological improvements in DNA sequencing. Next-generation high-throughput sequencing (HT-NGS) technologies were developed subsequently to overcome the limitations of this first generation technology that include higher speed, less labor, and lowered cost. Various platforms developed include sequencing-by-synthesis 454 Life Sciences, Illumina (Solexa) sequencing, SOLiD sequencing (among others), and the Ion Torrent semiconductor sequencing technologies that use different detection principles. As technology advances, progress made toward third generation sequencing technologies are being reported, which include Nanopore Sequencing and real-time monitoring of PCR activity through fluorescent resonant energy transfer. The advantages of these technologies include scalability, simplicity, with increasing DNA polymerase performance and yields, being less error prone, and even more economically feasible with the eventual goal of obtaining real-time results. These technologies can be directly applied to improve poultry production and enhance food safety. For example, sequence-based (determination of the gut microbial community, genes for metabolic pathways, or presence of plasmids) and function-based (screening for function such as antibiotic resistance, or vitamin production) metagenomic analysis can be carried out. Gut microbialflora/communities of poultry can be sequenced to determine the changes that affect health and disease along with efficacy of methods to control pathogenic growth. Thus, the purpose of this review is to provide an overview of the principles of these current technologies and their potential application to improve poultry production and food safety as well as public health.

Key words: next-generation sequencing, food safety, poultry

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INTRODUCTION

The rapid technological advances in sequencing methodologies, their applications, and improved platforms highlight the importance of the past decade as the decade of genome research (Pareek et al., 2011). In the recent years, studies on the grounds of microbial experimental evolution have used sequencing as a powerful tool to investigate the biomedical and ecological role of microbes. The emergence and maintenance of drug resistance and the evolution of virulence among microbes has motivated researchers to explore the evolutionary processes in convenient experimental systems (Brockhurst et al., 2011). However, most of the studies are difficult to carry out as they examine a relatively small portion of the genome. The technological advances in the field of genomics, and especially high-throughput next-generation sequencing (**HT-NGS**), have opened new avenues of research with a wide range of applications including but not limited to chromatin immunoprecipitation coupled to DNA microarray (ChIP-chip) or sequencing (ChIP-seq), RNA sequencing (RNA-seq), whole-genome genotyping, de novo assembling and reassembling of the genome, genome-wide structural variation, mutation detection and carrier sequencing, detection of human diseases, DNA library preparation, paired ends and genomic captures, sequencing of mitochondrial genome, and personal genomics (Brockhurst et al., 2011; Pareek et al., 2011). The application of HT-NGS techniques as a powerful tool toward the understanding of microbial pathogen evolution in real-world environments, not only offers us more detailed

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information on the evolution of microbial pathogens and their mutational changes within and between hosts (Ellis and Cooper, 2010; Racey et al., 2010), but it can also offer a wide window on the evolution of pathogenic strains causing infections or epidemics in host populations (Schmid et al., 2000). Therefore, HT-NGS opens a brand new perspective to the approach and the integration of the laboratory studies of microbes with the study of patterns of evolution in microbial pathogens in different environments. Furthermore, insights into the development and control of microbial pathogens and acquisition of resistance can be understood on a whole-genome scale and in gut microbiota studies. An overview of the current sequencing technologies and available platforms with a focus on application to food safety and poultry science will be provided.

Historical Perspective of Sequencing

In 1975, Sanger and others introduced the concept of DNA sequencing called the chain-terminator method (Sanger and Coulson, 1975; Sanger et al., 1977). This first-generation technology is based on incorporation of fluorescently labeled deoxynucleoside triphosphate (**dNTP**) and primers into a PCR that set the stage for automated high-throughput DNA sequencing. With the information obtained from the last terminator base in the 4 individual base reaction tubes after size separation, the original sequence could be determined (Venter et al., 2001; Hert et al., 2008; Schloss, 2008). Around at the same time in 1977, Maxam and Gilbert developed a chemical degradation DNA sequencing method in which terminally labeled DNA fragments were chemically cleaved at specific bases and separated by gel electrophoresis (Maxam and Gilbert, 1977).

In the following years, another landmark was achieved using Polony sequencing, which was the first approach used to sequence the whole genome. Although the Sanger method remained the mainstay for the original sequencing of the human genome, a high number of limitations was associated with this technique, mainly associated with the low throughput of DNA sequences obtained and the high cost. For example, to sequence the first human genome took 10 yr of work and cost 3 billion US dollars (Schloss, 2008; Metzker, 2010).

The 454 Pyrosequencer developed by Life Science was introduced in 2000 as the first commercial HT-NGS platform, followed by the Genome Sequencer, GS 20, which was available in 2005. The success of this technique was based on the combination of single-molecule emulsion PCR with pyrosequencing (shotgun sequencing procedure; Schadt et al., 2010). The principle of pyrosequencing technique, also called sequencing by synthesis, permits the detection of pyrophosphate released when a nucleotide is incorporated in the chain resulting in detectable light in a real-time format. Improvements and development of this technology resulted in advances in the next-generation devices, leading to so-called second-generation HT-NGS platforms as marketed by Roche, Illumina-Solexa, Life Technologies, Helicos, and other companies.

The second generation NGS platforms methods rely on a parallel process in which each single DNA fragment is sequenced individually and separated in clonal amplicons for further analysis among the total sequences generated (Pareek et al., 2011). Yet, second-next generation sequencing methods still have drawbacks such as the long time associated with this technique in which the time-to-results are long (days), or the loss of synchronicity (dephasing) in a percentage of molecules (Whiteford et al., 2009) leading to an increase in noise and sequencing errors as the reads are extended (Metzker, 2010; Schatz et al., 2010). The amplification of DNA by PCR to obtain the template sequence also can introduce errors and increase the complexity and time to the sample preparation (Schadt et al., 2010). Some of the platforms of second generation or next-generation sequencing are briefly described below.

Ion Torrent Sequencing

The innovation of this molecular technique is the creation of a link between the chemical and digital information that allows sequencing in a faster, simpler way on a massive scale. Also called pH-mediated sequencing or silicon sequencing, the method follows the principle of a biochemical process, in which a nucleotide incorporated into a strand of DNA releases a hydrogen ion as a byproduct. As a consequence, the pH of the solution changes and can be detected directly by the ion sensor.

The Ion Torrent platform differs from other sequencing technologies in that no modified nucleotides or optics are used (DNA Electronics Ltd. developed by Ion Torrent Systems Inc. and released in February 2010). With this technique, labeling of nucleotides and optical measurements are avoided, because incorporation events are measured directly by electronics without scanning, cameras, and light. So signal processing and DNA assembly can then be carried out using computer software programs. The designed Ion Torrent Sequencing method allows the performance of a wide range of applications such as, multiplexing amplicons, transcriptome, small RNA, and ChIP-Seq paired-end sequencing and methylation. Furthermore, this method may be best suited for small-scale applications such as microbial genome sequencing, microbial transcriptome sequencing, targeted sequencing (polymorphism and mutation discovery), amplicon sequencing, or for quality testing of sequencing libraries and also determination of variations and changes in microbial populations. There are different instruments available in the market currently, including the Ion Torrent Personal Genome Machine (PGM) that fits on a benchtop without taking much space. This instrument and technology aims at making sequencing available for more researchers. Its low cost and high speed have advantages for use in

rapid screening of amplicons. However, if SNP or mutation analysis need to be determined, this may not be the best-suited platform.

454 Pyrosequencing

This is a 2-step sequencing method. First the DNA that is previously sheared is attached to oligonucleotides by using adaptors. Each obtained fragment is attached to a bead for further PCR amplification within droplets of an oil-water emulsion (Emulsion-PCR). This generates multiple copies of the same DNA sequence on each bead. Following that, the beads are captured for pyrophosphate sequencing. After each nucleotide incorporation, an inorganic pyrophosphate (**PPi**) is released that also leads to an enzymatic generation of protons. The enzymes adenosine triphosphate (**ATP**) sulfurylase, luciferase, and apyrase are used in a sequential format, and adenosine 5′ phosphosulfate (**APS**) and luciferin are used as substrates. When PPi is generated during chain elongation, the ATP sulfurylase incorporates PPi to APS and forms ATP. In the presence of luciferase, luciferin, and ATP, light is emitted and oxyluciferin is generated that allows for detection of the incorporated nucleotide. This technique provides an increased average read length of 400 bp (Medini et al., 2008).

Life Technologies: ABI SOLiD, SOLiD 4

The concept/approach of the amplification for this technology is very similar to 454 (bead-based/emulsion PCR), but the sequencing strategy is totally different. Before sequencing, the template DNA is prepared on beads and amplified by emulsion PCR. After beads are deposited onto glass slides, the sequence is determined by sequential hybridization and ligation of nucleotides provided with a specific base, which is then identified by a fluorophore. The fluorescent emission after the ligation of dye-labeled nucleotides permits its detection. The result is sequences of quantities and lengths comparable to Illumina sequencing. With this technique, the average of read lengths generated is at least 35 bp (Medini et al., 2008; Pareek et al., 2011).

Illumina SOLEXA

Solexa is now part of Illumina sequencing technology and is based on reversible dye-terminators. The DNA molecules are first attached to primers on a glass surface using fold-back PCR so that local clonal colonies are formed (bridge amplification). Following several cycles of solid-phase amplification, it is possible to create clusters of a 1,000 copies of single-stranded DNA molecules. Four types of reversible terminator bases are added, and nonincorporated nucleotides are washed away. After the incorporation of reversibly terminating nucleotides, a camera capture images of the fluorescence and the dye along with the terminal 3′ blocker is chemically removed from the DNA allowing the next cycle. The average read length is currently 40 bp (Medini et al., 2008; Pareek et al., 2011). Solexa's key technology, the Genome Analyzer, can sequence the equivalent of one-third of the entire human genome in a single run.

Illumina Sequencing Technology

With this method, sequencing templates are immobilized on a proprietary flow cell surface. This is designed to present the DNA in a way that facilitates access to enzymes while ensuring high stability of a surface bound template and low nonspecific binding of fluorescently labeled nucleotides. Solid-phase amplification creates up to 1,000 identical copies of each single template molecule in close proximity (diameter of 1 micron or less). Because this process does not involve photolithography, mechanical spotting, or positioning of beads into wells, densities on the order of 10 million single-molecule clusters per square centimeter are achieved.

This technology (sequencing by synthesis) uses 4 fluorescently labeled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel. And during each sequencing cycle, a single labeled dNTP is added to the nucleic acid chain. After each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Because all dNTP are present as single, separate molecules, natural competition minimizes incorporation bias. Furthermore, base calls are made directly from signal intensity measurements during each cycle, which greatly reduces raw error rates compared with other technologies. This method enables robust base calling across the genome, including repetitive sequence regions and within homopolymers.

These newer technologies are based on the fact that different strategies including combinations of template preparation, sequencing and imaging and methods for genome alignment and assembly were needed (Table 1). The ultimate research approaches are moving toward sequencing directly from a single DNA molecule, without PCR amplification to eliminate potential error in the results obtained (Schadt et al., 2010). To overcome some of these drawbacks, next-to-next generation or third generation sequencing (**TGS**) technologies were developed. Readers are referred to previously published reviews (Schadt et al., 2010; Brockhurst et al., 2011; Pareek et al., 2011) for more detailed information. The technologies are briefly described below.

Toward TGS

Utilizing the concept of sequencing-by-synthesis, newer TGS approaches based on single-molecule-sequencing technologies rely on sequencing without a prior amplification step. This is done to have advantages that include increased sequencing rate, higher throughput and read lengths, decreased complexity of sample

Table 1. Examples of differences between earlier molecular techniques and technology and more recent, updated sequencing techniques and technology (data from J. Santoyo, CIPF, Valencia, Spain, personal communication)

Previous technology	Updated technology
Clone DNA	Obtain DNA
Generate a ladder of labeled (colored) molecules that are different by 1 nucleotide	Attach it to a matrix
	Emulsion PCR (emPCR)
	(Polonator, 454, SOLiD)
Separate mixture on some matrix	Extend and amplify signal with some color scheme
Detect using autoradiography or detect fluorochrome by laser	Detect fluorochrome
Interpret peaks as string of DNA	Interpret series of spots as short strings of DNA
Strings are 500 to 1,000 letters long	Strings are 30 to 300 letters long
1 machine generates 57,000 nucleotides/run	Multiple images are interpreted as 0.4 to 1.2 GB/run (1,200,000,000 letters/d)
Assemble all strings into a genome	Map or align strings to one or many genomes

preparation, and reduction of the cost of the analysis (Schadt et al., 2010). Numerous companies that have adopted this developed technology are briefly described below.

Helioscope Single Molecule Sequencer

This technique was introduced by Braslavsky et al. (2003) as one of the first techniques for sequencing a single molecule of DNA. The Helioscope sequencer relies on a true single molecule sequencing concept, capable of sequencing more than 28 GB in a single run but with the disadvantage that the sequencing takes at least 8 d. Recently, Helicos has improved this technique, which allows one to obtain more accurate homopolymer and direct RNA sequencing (Ozsolak and Milos, 2011a,b).

Single Molecule Real-Time Sequencer

As mentioned above, the principle of this technique is the real-time sequencing of single molecules by a synthesis method. This occurs on a sequencing chip that contains thousands of zero-mode waveguides. A single DNA polymerase molecule performs the sequencing reaction leading to the attachment of the DNA fragment to the bottom of each zero-mode waveguide. When the fluorescent nucleotide is incorporated into the reaction, detection is observed that determines the DNA sequence with a CCD array in a real-time format [i.e., sequence is determined as the reaction proceeds (Levene et al., 2003; Eid et al., 2009)]. The single molecule real-time technology enables the generation of at least 100 GB per hour and in a single run is able to read longer than 1,000 GB.

Single Molecule Real-Time (RNA Polymerase) Sequencer

Greenleaf and Block (2006) introduced a different single-molecule DNA sequencing approach based on the attachment of the RNA polymerase (**RNAP**) to a polysterene bead and the distal end of a DNA fragment to another bead. This assembly allows RNAP to interact with the DNA fragment, and the transcriptional motion of RNAP along the template continues. This technique detects changes in the length of the DNA that results in single-based resolution on a single DNA molecule and consequently obtains sequence information directly from a single DNA molecule.

Nanopore DNA Sequencer

Different from the described previously techniques, Nanopore DNA sequencing is free of nucleotide labeling and detection. In this technique, during the sequencing process an ion current passes through the pore that is blocked by the nucleotide. At the end of the process, different characteristics and parameters, such as diameter, length, and conformation of the molecule are determined (Astier et al., 2006). The current change depends on the size of the nucleotide and its associated properties.

Multiplex Polony Technology

This newly developed innovative technique recently developed by the privately funded personal genome project (http://www.personalgenomes.org), allows the determination of several hundred sequencing templates in parallel. This method simultaneously analyzes a high number of samples with a high order of several magnitudes with a great reduction of the reaction volume which results in a lower cost (Shendure et al., 2005).

Comparison of HT-NGS Platforms

Since the development of the first NGS technology labeled using the Sanger sequencing method, the fundamental mechanisms for DNA sequencing and data generation has changed radically. The improvement of these methods currently permits the acquisition of more sequence reads per instrument run and at significantly lower cost than their predecessors. However, the reduction of read length lowers the quality of reads; therefore, the revolution of the third generation technologies based on direct RNA and cDNA sequencing methods (single-molecule resolution) may offer several advantages in contrast to first and second HT-NGS

Table 2. Some examples and comparisons of characteristics among sequencing technology (data from Schadt et al., 2010; Pareek et al., 2011; Glenn, 2011)

Item	Ion torrent	454 Sequencing	Illumina-SOLEXA	SOLiD	References
Sequencing chemistry	Ion semiconductor sequencing	Pyrosequencing	Polymerase-based sequenced-by-synthesis	Ligation-based sequencing	Schadt et al., 2010
Amplification approach	Emulsion PCR	Emulsion PCR	Bridge amplification	Emulsion PCR	Schadt et al., 2010
Time per run	1.5 h	7 h	9 d	9 d	Schadt et al., 2010
Read length, bp	200	400	2 × 150	35 × 75	Schadt et al., 2010
Cost per run, US$	350	8,438	20,000	4,000	Pareek et al., 2011
Cost per Mb, US$	5.00	84.39	0.03	0.04	Pareek et al., 2011
Cost per instrument, US$	50,000	500,000	600,000	595,000	Pareek et al., 2011
Cost per run,[1] US$	500	8,439	8,950	17,447	Glenn, 2011; Pareek et al., 2011

[1]Total direct costs include the reagents and consumables, the labor, and the instrument amortization cost.

technologies: including i) a higher throughput, ii) faster turnaround time (hours versus days), iii) longer read lengths (1,000 bp and longer in commercial systems), iv) higher consensus accuracy to enable rare variant detection, v) small amounts of starting material, and vi) low cost. A comparison of the platforms is given in Table 2.

Applications and Advances of Sequencing Technologies for Animal Science Research

After the sequencing project of the human genome began in 2001, the animal genomic research phenomenon emerged (Lander et al., 2001; Venter et al., 2001). With the promise of HT-NGS to efficiently generate large amounts of sequence data at a reduced time and cost, multiple studies have focused on sequencing and assembly of the whole genome of food production animals, including bovine (Womack, 2006), pig (Mote and Rothschild, 2006), sheep (Cockett, 2006), equine/horse (Chowdhary and Raudsepp, 2006), and avian/chicken (Burt, 2006). These genome sequence improvements have provided a source of knowledge for understanding animal evolution and for the development of genetic tools within the domestic animal industries (Elsik et al., 2009). A clear example is the study on the cattle genome that has facilitated the identification of functions and regulatory systems that are beneficial for improved milk and meat production.

Technological advances and ongoing progress in the field of HT-NGS methods allow applications of these tools to a variety of different fields including examination of drug-resistance development in bacterial pathogens, genetic markers for specific phenotypes in livestock, the role of epigenetic DNA methylation in gene expression regulation, or polymorphisms in forensic samples of mitochondrial DNA (Joseph and Read, 2010). Accordingly, these current technologies can also be directly applied to improve animal production and enhance food safety. Furthermore, NGS has been paired with other methods such as QTL assay to understand functional relationships of genes. With QTL, groups of genes that work together to produce a specific phenotype have been discovered in several species of animals (Ramos et al., 2009). However, the complexity of genetic regulation of these genes and the identification of the specific genes that underlie genetic variation is a costly challenge in most cases, but NGS can facilitate an understanding of the regulation and functional relationships among genes in loci.

Next-Generation Sequencing Tools: Applications for Food Safety and Poultry Production

The continued concern for the safety of animal-derived food, the sanitary status and welfare of farm animals and the maintenance of animal production systems have increased monitoring and traceability of foods with respect to food safety. Furthermore, the evolution of DNA-based techniques has permitted the development of better monitoring and early warning systems. In spite of this, foodborne diseases have not declined over the past decade (Harlizius et al., 2004). In the framework of preharvest food safety, HT-NGS tools will play an important role toward the improvement of the productivity of food animals, but also promise to improve health, product quality, efficiency, robustness, reproduction, and genetic disease resistance (Harlizius et al., 2004).

In the past, almost all the genome research efforts were focused and effectively adapted to traditional breeding program objectives (i.e., genetic improvement of economic traits through marker-assisted selection and gene-assisted selection programs). But recent research areas within poultry genomics are moving into 4 areas of interest as shown in Table 3. This new era of functional genomics (Lander and Weinberg, 2000) requires access to large resources (arrayed cDNA and BAC libraries, EST databases, and so on) and high-throughput technologies (sequencing, microarrays, and so on) with the final objective being the development of metagenomic tools in poultry production toward building stronger links among genomics, physiology, immunology, and developmental biology to identify genes controlling traits. Furthermore, the growing awareness of food safety issues focuses on reducing the use of chemicals, antibiotics, and other additives (Burt,

Table 3. Examples of research conducted utilizing next-generation sequencing for poultry research (data from Burt et al., 2002)

Areas of interest in poultry genomics	Objective
Isolation and mapping of genetic markers	Genome mapping Currently there is a linkage map of more than 2,000 loci covering most of the genome (Schmid et al., 2000). Genetic markers containing clones led to the integration of genetic and cytogenetic maps for 16 linkage groups.
QTL mapping	The goal is a map of genetic markers, in particular the use of microsatellite markers (Schmid et al., 2000).
Candidate gene identification	Finding a relationship between a candidate gene and the genetic trait. The definition of allelic variation at the causative gene provides the means for direct selection and for the trait of interest without family data (Schmid et al., 2000).
Gene discovery	Use of microarrays and other gene expression platforms to identify changes in gene expression in response to environmental stimuli (Lander and Weinberg, 2000).

2002). These new traits are difficult and costly to measure by conventional genetic selection methods and the development of HT-NGS techniques could offer a new insight into animal production.

For example, an application could include determining and resolving resistance of infectious pathogens related to broilers that reduce overall performance traits and BW or reduce the cost of vaccines and antibiotics by genetic selection methods. The identification of genes and virulence pathways, and the interaction with environmental changes could be helpful for the prediction of susceptibility to disease and drug response and, for instance, the early detection and molecular classification of diseases. This is desirable not only to the economy of the industry by saving costs because vaccines and antibiotics are becoming less effective, but is also helpful in the acquisition of genomic information that can result in therapeutic advances.

The Future of Molecular Genetics in Poultry Production

The release of the first draft of the chicken genome sequence in 2004 (International Chicken Genome Sequencing Consortium, 2004) has provided almost 7 million SNP (Rubin et al., 2010). The availability of these data and the continuous expansion of HT-NGS techniques are providing powerful tools to commercial breeding companies. Applications of HT-NGS at the breeder level include assessment of gut microbial community as an indicator of gut health and identification of genes and metabolic pathways. Specifically, gut microbial flora/communities of poultry can now be sequenced rapidly to determine any changes that affect health and disease and a detailed assessment of probiotics or prebiotic methods to control pathogenic growth. In addition, function-based metagenomic analysis can be used as a screening tool for antibiotic resistance patterns and vitamin production within microbial communities.

The application of HT-NGS sequencing is emerging and moving toward the development and the improvement of the poultry industry specifically and, at the same time, improving the food safety measures and the quality that consumers demand on these products. The application of metagenomic techniques in poultry production could lead to the development of novel alternatives to antibiotic growth promoters and comparative genome analysis [i.e., mechanisms of *Salmonella enterica* evolution and transcriptomics of *Salmonella enterica* plasmids (Johnson et al., 2012; Lin et al., 2012)]. Also, through the application of metagenomics, it is possible to uncover the contribution of microbial communities to gastrointestinal diseases in poultry (Gerardo Nava, Washington University, St. Louis, MO, personal communication). As an example, through the application of metagenomics it has been possible to obtain complete sequences of a new bacteriophage with antimicrobial properties (Avant, 2012). The applications and functionality of all these methods in the field of poultry production can enhance poultry health by circumventing foodborne pathogen colonization of the gut and can also improve food safety measures of the products (Table 4).

Gut Health and Microbiota Development

Within the ecosystem of the gastrointestinal microflora there is a complex population of microbes that affect physiological and health aspects of the animal host (Fuller and Perdigón, 2003; Parker et al., 2007). Traditional methods of classical culturing of digestive microflora cannot be used because only 1% of all bacteria are culturable (Hugenholtz et al., 1998). An example of this can be seen in the work of Apajalahti et al. (2004) where they applied 16S rDNA gene sequencing analysis and observed that more than one-half of the 640 different species of bacteria could not be identified. A similar study carried out by Bjerrum et al. (2006) also observed that 85% of 557 cloned sequences from ileal or cecal microbiota of broilers from conventional or organic farms were not closely related to bacterial species previously identified by culturing methods. In concordance with previously reported results by Gong et al. (2002a,b), 25% of cloned sequences from cecum of 6-wk-old broilers possessed less than 95% similarity to database sequences.

Table 4. Examples of application of next-generation high-throughput sequencing (HT-NGS) in poultry production and food safety

Research field	HT-NGS assay	Objective	Results and applications	References
Alternatives to antibiotic growth promoters (AGP)	454 GS FLX sequencer	Identify intestinal bile salt hydrolase (BSH) inhibitors that promote feed digestion and BW gain	BSH as a specific substrate for *Lactobacillus salivarus* strain. Approach to replace AGP.	(Lin et al., 2012)
Comparative genome and plasmid analysis	CloVR-microbe pipeline CloVR-Comparative pipeline RNA-seq	Investigate *Salmonella* evolution Examined plasmid transcriptome of pAR060302 in *Salmonella* serovars	Identified sources of *Salmonella* evolution Analyze the cost of fitness of carrying these plasmids Understanding the behavior of this plasmid to circumvent *Salmonella* dissemination	(W. F. Fricke, Institute for Genome Science, University of Maryland, Baltimore, personal communication) (T. J. Johnson, K. Lang, and J. Danzeisen, University of Minnesota, Saint Paul, personal communication)
Assessment of gastrointestinal tract ecology in poultry	UPLC-MS profiling	Assessment of the gut microbiome Insight into gut physiological responses to dietary changes Parallel comparison of changes in the gut contents with microbiome and physiological responses	Diversity in the microbial populations increase with age/diet. Host gene can be regulated in concert depending on variations in diet.	(S. C. Ricke, University of Arkansas, Fayetteville; S. H. Park, University of Arkansas, Fayetteville; I. Hanning, University of Tennessee, Knoxville; A. Perrotta, Massachusetts Institute of Technology, Boston; B. J. Bench, Tyson Foods Inc., Springdale, AR; and E. Alm, Massachusetts Institute of Technology, Boston, personal communication)
Food safety	Dye-terminator chemistry on ABI 373 and 377 sequencing machines NGS molecular technology	Detection of genes implicated in the pathogenesis of *Campylobacter* spp. Traceback bacterial pathogens: linkage food sources and clinical isolates in *Salmonella Montevideo* serovars	Importance of variation of homopolymeric tracts in the survival strategy of *Campylobacter jejuni* High serovar diversity Phylogenetically differentiates a clonal lineage Development of novel multiple-locus variable number tandem repeat analysis targets	(Parkhill et al., 2000) (Allard et al., 2012)
	Paired-end Illumina reads to detect SNP	Analysis of genetic diversity in *Salmonella enterica*	Develop of NGS as a useful predictive tool at the outset of an outbreak	(McCann, 2011)

[1]UPLC-MS = ultra-performance liquid chromatography mass spectral.

Microbial interactions influence the intestinal environment and as a consequence affect the development and responses of the host immune system against pathogenic and nonpathogenic bacteria. To study these complex associations and their dynamic relations within the gastrointestinal ecosystems, several molecular techniques have been developed (Oviedo-Rondón, 2009; Kim and Mundt, 2011; Table 5). The application of the advantages of emerging HT-NGS methods and metagenomics in the study of the microbiota of chicken gut could offer a wide view on detecting and characterizing microbial populations, and the relationship between different microbial groups (S. C. Ricke, University of Arkansas, Fayetteville; S. H. Park, University of Arkansas, Fayetteville; I. Hanning, University of Tennessee, Knoxville; A. Perrotta, Massachusetts Institute of Technology, Boston; B. J. Bench, Tyson Foods Inc., Springdale, AR; and E. Alm, Massachusetts Institute of Technology, Boston, personal communication). Also, the implementation of more advanced statistical methods could be a useful tool for easily identifying deficiencies in the microbiota of chickens and molecular analysis of microflora responses in the host depending on diet variations (probiotics and prebiotics; Chambers and Gong, 2011; S. C. Ricke, University of Arkansas, Fayetteville; S. H. Park, University of Arkansas, Fayetteville; I. Hanning, University of Tennessee, Knoxville; A. Perrotta, Massachusetts Institute of Technology, Boston; B. J. Bench, Tyson Foods Inc., Springdale, AR; and E. Alm, Massachusetts Institute of Technology, Boston, personal communication). As an example, Feng et al. (2010) observed that during the development of necrotic enteritis in broilers, the inhibition of *Lactobacillus aviarius* was beneficial for *Clostridium perfringens* proliferation. The advanced research in metagenomics methods can provide a wide view toward the control of diseases in poultry as well as re-

Table 5. Previous molecular techniques to studying microbial ecology (data from Zoetendal and Mackie, 2005)[1]

Approach	Applications	Limitations
Sequencing of rRNA genes		
16S rRNA gene sequencing	16S rRNA gene sequence collection	Bias in NA extraction; PCR and cloning laborious
RT-PCR	Specific gene expression	Bias in NA extraction and RT-PCR
Fingerprinting DGGE, TGGE, TTGE, T-RFLP, SSCP	Diversity profiles	Bias in NA extraction and PCR
Non-16S rRNA gene fingerprinting	Diversity profiles	16S rRNA approach required for identification
Quantification of 16S rRNA and its encoding genes		
Dot-blot hybridation	Relative abundance of 16S rRNA	Laborious at species level, requires 16S rRNA gene sequence data
qReal-time PCR	Relative abundance of 16S rRNA genes	Laborious and expensive
FISH	Enumeration of bacterial population	Laborious at species level, requires 16S rRNA gene sequence data
DNA microarray technology		
Diversity arrays	Diversity profiles	Laborious and expensive
DNA microarrays	Transcriptional fingerprint	Bias in NA extraction and NA labeling: expensive
Parallel sequencing technologies	Sequence of microbial genome	Cost, sequence to compare and identify correctly

[1]NA = nucleic acid; RT-PCR = real-time PCR; DGGE = denaturing gradient gel electrophoresis; TGGE = temperature gradient gel electrophoresis; TTGE = temporal temperature gradient gel electrophoresis; T-RFLP = terminal restriction fragment length polymorphism; SSCP = single strand conformation; FISH = fluorescence in situ hybridization.

search related to enteric foodborne pathogens (Frank and Pace, 2008).

Food Safety

Every year foodborne pathogens are responsible for 9.4 million cases of human illnesses in the United States that result in 60,000 hospitalizations and more than 1,300 deaths (Mead et al., 1999; Buzby and Roberts, 2009; Scallan et al., 2011). *Salmonella enterica* is recognized as one of the most relevant foodborne pathogens with poultry and poultry products being leading sources of this pathogen (Scallan et al., 2011). The investigation of these outbreaks related to the origin of the strains could be time-consuming in most of the cases. The available molecular tools, including the "gold standard" pulsed-field gel electrophoresis (**PFGE**) method, do not always distinguish the outbreak-related strains from other genetically similar strains unassociated with the same outbreak (Allard et al., 2012). Thus, NGS tools provide a powerful approach for epidemiological trace-back efforts. The tracking and tracing of living animals and animal-derived food within the food chain is part of the monitoring process, and pilot studies using microsatellite markers or SNP-based systems are in process (Harlizius et al., 2004). In addition to epidemiological tracing, NGS provides additional data to investigators that can include identification of specific markers for detection efforts and assessment of unique virulence factors that may be strain specific.

Some studies in these fields highlight the power of NGS tools to identify the genetic and evolutionary diversity of important serovars of *Salmonella enterica* along with the epidemiological sources of the strains related to the outbreaks (Holt et al., 2008; Chin et al., 2011). Also, by combining whole genome-based next-generation genomic technologies with other sophisticated software [e.g., SSKAE, SHARCGS, VCAKE, Newbler, Celera Assembler, Euler, Velvet, ABySS, AllPaths, and SOAPdenovo (Miller et al., 2010)], it is possible to achieve a highly specific and sensitive real-time monitoring of infectious diseases and early warnings of outbreaks or their detection (Janies et al., 2011; Lienau et al., 2011). As an example, the use of the Ion Torrent PGM sequencer led to the identification and genomic characterization of the enterohemorrhagic *Escherichia coli* O104:H4 strain responsible for the outbreak that occurred in May 2011 (Mellmann et al., 2011). Changes in the microbe gene expression triggered by different stressors and environmental factors as well as acid adaptation or stress response mechanism could also be detected using HT-NGS methods. The population genome data generated can be used for assessing antimicrobial susceptibility or detection of changes in resistance. This application could be helpful to confirm the occurrence of some mutations or polymorphisms in genes that could be associated with antimicrobial resistances (Joseph and Read, 2010) that may be difficult to determine using other methods. Other potential applications of the genomics and metagenomics techniques include the ability to provide an overview of the biodiversity within a sample including the detection of unknown or unexpected pathogens and unculturable bacteria. Knowledge of the complete genome sequence in combination with other genetic approaches (multiple-locus variable number tandem repeat analysis and PCR) could lead to a wealth of information for the discovery of new targets that could develop more effective strategies in the control, transmission, and spread of pathogenic microbes, acquisition of multidrug resistance, or a combination of these (Sukhnanand et al., 2005; Fitzgerald et al., 2007; McQuiston et al., 2008; Xi et al., 2008; Wise et al., 2009).

Salmonella enterica

One of the approaches on the use of genetic tools is moving toward the control of the poultry carrier state (Calenge et al., 2011). *Salmonella* and *Campylobacter*, the 2 most prevalent foodborne pathogens, can both be carried in the intestinal tract of poultry. Therefore reducing carriage of these pathogens in chickens could reduce the transmission of foodborne pathogens to humans. One of the most promising research areas is the modulation and control of *Salmonella* in chickens (Chambers and Gong, 2011). Using HT-NGS could lead to the understanding of the mechanisms associated with *Salmonella enterica* colonization in the chicken gut (K. Venkitanarayanan, University of Connecticut, Storrs, personal communication) or comparison of microbiome and physiological changes in the gut content in response to dietary variations including probiotic and prebiotic treatments. These approaches may help to improve chicken health, performance, and as a consequence, chicken production.

Campylobacter spp.

Infections with *Campylobacter* are a primary concern for the public health. Consumption or handling of contaminated poultry meat is a main source of campylobacteriosis in humans (Jorgensen et al., 2002; Scallan et al., 2011). Given the low infectious dose of *Campylobacter jejuni* necessary to cause disease (about 500 cells) with the high levels of colonization in both intestinal contents (10^5 to 10^9 cfu per gram; Lee and Newell, 2006) and raw retail carcasses (10^2 to 10^5 cfu per carcass; Berndtson et al., 1992), poultry presents a high risk of the occurrence of *C. jejuni* infections to humans (Jacobs-Reitsma et al., 2008). In spite of the research efforts that have focused on tracing back the transmission, identification, and monitoring of this foodborne pathogen to develop control strategies and preventive measures at the farm level, the incidence of campylobacteriosis has not been reduced significantly since the initiation of select Government Programs including FoodNet (Humphrey et al., 2007).

The lack of a method for distinguishing *Campylobacter* strains makes it more difficult to trace back sources in outbreaks. Multiple typing methods have been developed including antibiotic resistance, phage typing, serotyping and several emerging genetically based methods (S. Pendleton, unpublished data). In fact, advanced research in the field of genome-based methods (multilocus sequence typing and microarrays) has allowed the identification of different clonal groupings of *C. jejuni* (Dingle et al., 2001). Also, the development of a real-time PCR Taqman allelic discrimination assays permitted the rapid detection of *Campylobacter jejuni* isolates and preliminary strain identification (Best et al., 2005). However, the high plasticity of *Campylobacter* relegates the use of some the genotyping technology in advance of more advantageous methods including whole-genome sequencing (Lefébure et al., 2010; S. Pendleton, unpublished data).

The development of whole genome sequencing of *C. jejuni* offers the opportunity of detection of genes and proteins involved in the pathogenesis of *Campylobacter* and also genes of unknown function (Parkhill et al., 2000; Stahl and Stintzi, 2011). Perhaps the understanding of the function of many of these proteins and genes could lead to the improvement of current practices and potential intervention strategies regarding the role of *Campylobacter* in the chicken gut, colonization, and immune response.

Conclusion

This review provides information on the different platforms and applications that could help to improve poultry production and food safety. Although the sequence-based characterization of genomes is still considered as a new approach for the poultry and food industries, these techniques will provide more knowledge to discover genes and the pathogenic pathways of bacteria that could enhance the understanding on the behavior of certain foodborne pathogens. In this framework, one of its future applications could be focused to reduce costs in production since the combination of these techniques could enhance the development of vaccines and therefore the reduction of antibiotic treatments in poultry production and also an exciting challenge to resolve food safety issues. Although some limitations of NGS remain such as the cost and the analysis of data, as these technologies become more widely used, these limitations will most likely be resolved. Finally the implementation of genomics will have to be cost effective, and the corresponding tools repeatable, tractable, and capable of dealing with the nature of poultry and the food safety requirements.

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