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Molecular Approaches for the Study of Genetic Diversity in Microflora of Poultry Gastrointestinal Tract

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1. Introduction

Livestock production currently comprises approximately 40 per cent of the gross value of the world's agricultural produce. At the end of the 20th century, approximately 74 per cent of poultry meat and 68 per cent of eggs were produced in the industrial sector. It is predicted that the consumption, per capita, of poultry products will increase to 17.2 kg by 2020. These needs will require extensive scientific and technological development in many areas.

Because chicken protein plays such a huge role in the supply of human nutritional needs, much scientific research has been carried out to improve the productivity of these animal foodstuffs. The productivity of poultry, like all living birds, is influenced by genetics, the environment and interactions between these two factors. Accordingly, besides coherent and targeted programs that seek to increase the genetic potential of broilers, extensive research on environmental conditions, such as nutrition, physiology, hygiene, control of diseases and management improvement, must be carried out to improve productivity.

One of the most important factors affecting broiler productivity is the gut microbial flora that can play a very significant positive and negative role in the final yield of broilers. Our limited knowledge of the role of bacteria in the digestive tract of birds is largely derived from the little information obtained on the composition of chicken intestine microflora. Significant studies on the microbial flora of the digestive tract of poultry began in 1970, and currently, the microflora is being increasingly studied by researchers worldwide.

Microorganisms, especially bacteria, have considerable effects on the immune, nutritional and physiological processes of the host. Birds suffering from infections of harmful bacteria will cause heavy losses in the poultry herds and also in human communities. Investigation of the microbial flora of the gastrointestinal tract has a significant role to play in ensuring the health and safety of poultry products submitted for human consumption. Further, one of the most reliable methods for the quality control of poultry products is a survey of microorganisms in poultry herds (Skanseng et al., 2006).

Recently, the detection, differentiation and identification of microorganisms have been accomplished using methods such as phenotypic measurement, biochemical assays,

immunological assays and molecular methods. However, methods that are based on phenotypic and biochemical assays to identify microorganisms are typically vague and inaccurate (Settanni and Corsetti, 2007). Although phenotypic identification remains standard and is a commonly used method for identifying most bacteria, it is very cumbersome, requires excessive time, exceptional technical ability and proper technical standards to obtain accurate results (Rossello-Mora and Amann, 2001).

Moreover, it is difficult to process a large number of samples. Typically, at least 10 tests are carried out to identify and differentiate all of the species within a sample. Rapid and accurate diagnosis of pathogens allows the correct course of action to be taken and also enables better understanding of the pathogen epidemiology. Microbiological culturing takes time (at least 24-48 hours for microbial growth), and the methods are not specific and do not have the required precision; hence, the current effectiveness of these methods is very limited (Miyashita et al., 2004). Indeed, because these methods cannot be used for non-culturable bacteria and can only be used for culturable bacteria, our knowledge of microbial flora in the digestive tract of poultry remains inaccurate and incomplete. Most bacteria, due to their unknown requirements for growth, cannot be grown *in vitro*.

Consequently, many researchers have sought to develop methods that would allow the rapid and accurate identification of useful and harmful microorganisms. Certainly, commercial identification kits, such as API, and automated identification systems, such as VITEK, allow the identification of many bacteria to a species level, thus enabling the user to identify the bacteria relatively quickly. However, many bacteria cannot be detected with these conventional methods. This may be due to the specific needs of the bacteria, for example, the Lactic Acid Bacteria (LAB) (Ampe et al., 1999). Further, because existing commercial systems on the market have been developed to primarily identify human pathogenic bacteria, they may not accurately detect and identify bacteria from other sources, such as animal bacteria and food product bacteria (Settanni and Corsetti, 2007). It is impossible to identify new bacterial species and strains using these commercial systems.

With the development of methods based on molecular techniques, the speed and accuracy of diagnosis of many poultry diseases has been increased, leading to a reduced time to diagnosis and a consequent reduction in treatment costs. Ultimately, these methods will reduce and prevent the incidence of disease in the herd.

Among the molecular methods, the restriction fragment length polymorphism (RFLP) of total genomic DNA (generated by non-PCR techniques) method belongs to the first generation of molecular methods that were widely used for differentiating microorganisms (Rosselló-Mora and Amann, 2001). Southern blot gel electrophoresis (SBGE) was also among the first-generation methods that were widely used to identify the microflora. Today, second-generation molecular methods are used and include PCR-based methods, such as PCR-RFLP and randomly amplified polymorphic DNA (RAPD), for the detection and differentiation of bacterial isolates. Because the resulting profiles on ethidium bromide-stained gels do not contain specific bacterial sequence information, they must be compared with profiles of reference bacteria. If this method is used in isolation, it will not relate samples with reference species because it relies on the existence of specific PCR products and produce from a specific beginner primer pair (Rosselló-Mora and Amann, 2001). The

RAPD-PCR method has been successfully implemented to identify different microorganisms (Raclasky et al., 2006).

Targeted gene amplification has been increasingly studied by researchers as a valid method to identify bacterial species (Settanni et al., 2005). At present, many specific PCRs for identifying bacterial species have been confirmed and are used for identifying bacterial preparations. One of the most common PCR-based methods for identifying microorganisms is denaturing gradient gel electrophoresis (DGGE). This informative method can differentiate between polymorphic gene sequences. The data are obtained using an acrylamide gel with a denaturing gradient **(**Muyzer et al., 1993). Molecular or genetic methods for the identification of bacteria, in addition to or indeed instead of phenotypic assays, provide more sensitive and more specific detection. These methods decrease the errors that result from subjective interpretation of morphological and biological bacterial characteristics. Essentially, bacterial DNA remains almost unchanged throughout the life cycle and even while exposed to environmental stresses. Therefore, molecular methods for the identification of bacteria (and other microorganisms) that target genomic DNA are being rapidly developed and are of increasing significance. Recent advances in PCR methods that allow rapid and accurate diagnosis of a broad range of bacteria, such as molecular genetic methods, have become a key method for detecting microorganisms. It is many years since 16S ribosomal RNA (rRNA) sequencing was used as the main tool for determining phylogenetic relationships between bacteria. The molecular specificity of this method allowed the determination of phylogenetic relationships for both the detection and identification of bacteria used in clinical laboratories. Studies have now shown that sequencing determination is a valid method for identifying bacteria with slow, unusual or difficult growth requirements; moreover, such bacteria were inadequately identified using the bacterial culture methods (Zhu and Joerger, 2003). Recent developments in ribosomal DNA- and RNA-based molecular methods allow the identification of different bacterial populations from environmental samples without culturing. The PCR method for determining bacterial diversity in the population of samples is used. Available reports indicate that there is a good correlation between PCR-based methods and culture methods for those bacteria that can be grown in culture.

Accordingly, molecular methods have been used to detect bacteria in the digestive tract of broiler chickens reared in commercial conditions. Although molecular methods also have limitations, they can be referred to error possibility in separating, amplification and DNA simulations in some bacteria and special sequences; but nevertheless, this method provides the ability to investigate the diversity of microbial flora in a particular sample. Data obtained using molecular methods will allow us to easily compare different isolates of poultry digestive systems without requiring subjective comparisons of biochemical features. Gastrointestinal microbial ecology is the study of the prevalence and diversity of existing microorganisms, their activities and their relationships with each other and the host animal (competitive and coherence effects).

The increase in information on human gut microbial ecology is due to three main factors: 1) development of anaerobic culture techniques; 2) use of laboratory rodents to understand the relationship between intestinal bacteria and the host; and 3) use of animal models lacking in specific microbes or a microbial flora (Savage, 2001).

Although the ability to culture digestive system bacteria is relatively high (10-50% of total species, Vaughan et al., 2000), only a part of the total species are cultured.

The main reasons underlying this partial culturing include an absence of required bacterial growth substances, the choice of medium required, the stress of the culturing process, the need to remove oxygen to maintain anaerobic conditions and the difficulty of simulating interactions with other bacteria and the host tissues.

Solutions to address these problems in culture methods are required. During the last decade, there have been notable increases in the use of 16S ribosomal RNA methods to determine bacterial population diversity (Vaughan et al., 2000). Nucleic acid sequence comparisons of isolates from bacterial ecosystems can be used to determine the molecular characteristics and classification of the bacteria and also to predict their evolutionary relationships.

Molecular technology provides information on nucleic acid sequences. Using these sequences, microorganisms can be identified within a specific environment and further, their function, their performance and their importance or role in a specific environment can be evaluated.

The molecular ecology of microbial flora using molecular technology - mainly based on information related to nucleic acid sequences - microorganisms identified in a specific environment, tasks and their performance is evaluated and the importance or role in an environment where residents are being evaluated.

The author of this chapter has performed many experiments on the molecular detection of poultry gastrointestinal tract microflora, has published many papers in notable journals and has also presented many papers in international conferences on this subject (Seidavi., 2008; 2009a; 2009b; 2009c; Seidavi & Chamani., 2010; Seidavi & Qotbi., 2009a; 2009b; Seidavi et al., 2007; 2008a; 2008b; 2008c; 2008d; 2008e; 2008f; 2008g; 2008h; 2008i; 2008j; 2008k; 2009a; 2009b; 2009c; 2009d; 2010a; 2010b; 2010c; 2011; Mirhosseini et al., 2008a; 2008b; 2009a; 2009b; 2009c; 2010). This chapter describes the molecular approaches that may be used for the study of genetic diversity in poultry gastrointestinal tract microflora.

2. Different methods used for the detection and identification of microbial flora

Several molecular genetics-based methods for the detection and identification of microbial flora in different samples have been developed, which have greater accuracy and are faster than classical methods.

2.1 Polymerase Chain Reaction (PCR)

Many of the molecular based polymerase chain reaction methods are used for the detection of microbial flora. Using specific primers, the PCR amplifies a specific sequence of DNA, thus confirming the presence of microorganisms. The principles and details of the polymerase chain reaction have been well described by various sources.

2.1.1 Multiplex PCR (mPCR)

One of the branches of applied molecular microbiology includes the monitoring and control of existing microorganisms in natural ecosystems (Settanni and Corsetti, 2007). Classical

technologies and mono-PCR are insufficient for studying several complex species simultaneously, including microbial flora, and the use of multiple PCR reactions is highly desirable. Today, the use of mPCR for the rapid identification of multiple isolates is a useful tool for studying the structure of the microbial population and the dynamics of microbial communities, such as changes in the microbial population during fermentation or in response to environmental changes. Additionally, mPCR is semi-quantitative because it can be used to estimate bacterial concentrations as the threshold for identification is very low (Settanni and Corsetti, 2007). Today, the mPCR method is used in different applied science fields to accelerate and direct the identification and differentiation of microorganisms with or without prior isolation and culture.

2.1.2 Technical aspects and molecular targets of mPCR

In order to ensure specificity of the system (a unique target sequence for each primer pair), it is necessary to use protocol designs with longer melting (T_m) steps than in typical PCR. Additionally, starter DNA sequences that bind the primers or each other should be avoided because denaturing these sequences will reduce their availability for amplification.

The magnesium concentration in the mPCR is an important factor that affects the efficiency of the reaction (McPherson and Moller, 2000). In general, the $MgCl_2$ concentration in mPCRs is higher than the concentration used in typical PCR reactions. Depending on the number of bacteria that must be recognised, mPCR generally requires a single amplification reaction (usually 4-5 bacteria) or a two-stage amplification reaction (usually 5-6 or more bacteria).

For the molecular diagnosis, identification and classification of bacteria (Grahn et al., 2003), mPCR generally targets the 16S rRNA genes. This gene is widely used to understand the phylogenetic relationships between bacteria (Rosselló-Mora and Amann, 2001). However, sometimes the 16S gene sequence is insufficient to identify related species (Torriani et al., 2001), and therefore, other genes should be considered in the mPCR design.

2.1.3 The role of mPCR in the study of microorganisms

The mPCR method is widely used for the identification and separation of microorganisms, and these are some of the reasons underlying its ability to provide a simple fingerprint of the bacterial population. For this method, specific DNA sequences for each bacterium are necessary to obtain an individual and a single band for each bacterium. The sizes of these specific bands should be different to allow straightforward band location. Using the sample DNA as the template, and provided no cross-amplification is observed, the system can be used for total DNA extracted from the sample. Thus, DNA fragments of amplified products of desired bacteria can be observed in the agarose gel. Ultimately, this method provides a fingerprint that is specific for the sample analysed (a series of bands related to the bacteria in the mPCR).

The validity of the mPCR measurement is based on the availability of desired bacteria DNA. The primer mix should include a cross-specific primer that produces a PCR product for all bacteria to be identified in the experiment (Zarlenga and Higgins, 2001).

2.2 Sequencing of SSU rDNA clone libraries with small subunits

rRNA libraries of the small Subunits are necessary to identify all bacteria in a specific environment. The sequenced SSU rRNA genes have become a standard method for the identification of isolates, such that the complete description of microbial populations is impossible without information on SSU rRNA sequences (Zoetendal et al., 2004). At present, over 79,000 16S rRNA sequences are available in the DNA database, more than any other gene (http://rdp.cme.msu.edu/html). rRNA sequences can be obtained from the SSU rRNA sequences directly or from their coding genes (SSU rDNA) by reverse transcription (RT) or conventional PCR. In practice, SSU rRNA sequences are determined from rDNA clone libraries rather than cDNA libraries. Following this determination, amplified sequences are determined and compared with the SSU rDNA sequences stored in the database (http://www.ncbi.nlm.nih.gov/BLAST/ and http://rdp.cme.msu. edu / html) and then phylogenetic analysis is conducted (Cole et al., 2003). SSU rRNA sequence clone libraries from human faeces (Suau et al., 1999), colon and ileum (Wang et al., 2003) and oral cavity (Paster et al., 2001) have shown that a considerable number of living bacteria had not been discovered in previous studies. Similarly, studies of the gastrointestinal tract of various animal species have been reported (Daly et al., 2001; Gong et al., 2002), and microflora identified (Zoetendal et al., 2004). It is also important to estimate the amount of actual variation in the SSU rDNA clone libraries. This estimate depends on how many operational taxonomy units (OTUs) are known. Unfortunately, the OTU has not yet been standardised and sequences within an OTU differ by up to 5%, which makes it difficult to compare between clone libraries (Martin, 2002).

In traditional methods, bacteria were categorised using phenotypic characteristics. Following the development of technologies based on nucleic acids, SSU rDNA sequences were developed as a standard tool for the phylogenetic classification of bacteria.

It should also be stated that SSU rDNA sequences that are stored in the DNA databases relate to a small part of the total bacterial isolates. Further, new microbiological methods, such as the analysis of the isolate cello-bio or butyrate production, have allowed the isolation of increasing numbers of previously unknown bacteria (Zeotendal et al., 2003). Thus, an accurate estimation of the ability to kill bacteria in the digestive system and the elimination of these ambiguities will be possible.

Although sequencing of amplified SSU rDNA clone fragments provides important information for the identification of non-cultivable bacteria, these data are not quantitative, and cloning procedures and PCR are not infallible. Several PCR methods have been proposed to minimise these errors. However, SSU rDNA clone libraries have made major contributions to the understanding of gastrointestinal microbial flora.

2.3 Fingerprinting methods SSU rDNA: DGGE, TGGE, TTGE, SSCP and T-RFLP

Because of the effort required and the cost of cloning and sequencing of SSU rDNA sequences of the microbial flora population, several fingerprinting methods have been developed that appear ideal for investigating changes in microbial populations, for example comparing different parts of the gastrointestinal tract of different animals.

Denaturing gradient gel electrophoresis (DGGE) was used to study the ecology of the diverse bacteria in marine ecosystems (Muyzer et al., 1993). In this study, a microbial

ecosystem was investigated using DGGE, temperature gradient gel electrophoresis (TGGE) and temporal temperature gradient gel electrophoresis (TTGE). Other methods include the analysis of microbial populations for single strand conformation polymorphisms (SSCPs) and terminal-restriction fragment length polymorphisms (T-RFLPs). DGGE, TGGE and TTGE are based on the specific melting behaviours of amplified sequences. The secondary structure of single-stranded DNA and SSCP-based T-FRLP is based on the specific targets of restriction enzymes. Interestingly, prior to their use in microbial ecology, these methods (except for T-FRLP) were used in clinical research, which demonstrates their efficacy. Today, with the development of statistical software packages, we can calculate the similarity indices and conduct cluster analyses of SSU rDNA profiles. Thus, these fingerprinting methods are very useful and allow the analysis and study of microbial populations over time (at all ages) and also allow study of the response of animals to diet. Several articles that review the details of fingerprinting methods have been published (Vaughan et al., 2000; Muyzer et al., 1993; Konstantinov et al., 2002).

DGGE, TGGE and TTGE analyses of SSU rDNA have been successful in determining the characteristics of human (Tannok et al., 2000; Seksik et al., 2003), cow (Kocherginskaya et al., 2001), dog (Simpson et al., 2002), rodent (Deplancke et al., 2000; McCracken et al., 2001) and chicken (Van der Wielen et al., 2002b; Zhu et al., 2002) intestinal bacteria populations. DGGE or TGGE methods are sufficiently sensitive to detect bacteria as a per centage of the total bacterial population (Zeotendal et al., 1998). The T-RFLP method has also proven useful for obtaining fingerprints of gastrointestinal microbial flora (Nagashima et al., 2003).

In these studies, the role of environmental factors on the microbial population, such as disorders, physiological conditions, the part of the digestive system under investigation and the host animal species, has been investigated.

The stability of the bacterial ecosystem is directly related to its diversity. Reducing the diversity of the bacterial population decreases its stability. Fluorescent *in situ* hybridisation (FISH) and the bacterial population structure of the dominant TGGE in human faeces of healthy adults showed that the population composition remained relatively stable over time (Franks et al., 1998). Previous studies, based on culture techniques, have shown that in the case of human faecal microbial flora, the population changes usually occur in newborns and in the elderly (Hopkins et al., 2001), with similar changes expected for animals. More recent studies using molecular methods have confirmed these findings (Schwiertz et al., 2003). More molecular studies are required to confirm the relationship between microbes and gastrointestinal diseases in humans and animals.

For example, individual faecal microbial populations are affected by unstable Crohn's disease (Seksik et al., 2003). The important point here is to understand why bacterial population changes cause disease (or vice versa). Another study on the bacterial population in the ileum of piglets showed that there is an inverse relationship between bacterial diversity and sensitivity to pathogenic Clostridium bacteria (Deplank et al., 2002). The microbial flora of the ileum in neonates that were fed by their mothers showed a lower diversity and Clostridium density than neonates that were fed through intestine by the parents.

Comparison of faecal samples by TGGE fingerprinting in the adult human has demonstrated that the constituent dominant bacterial populations depend on the animal

host (Zeotendal et al., 1998). This finding was also noted in the stool samples of other individuals (Tannock et al., 2000) and in other animals, such as dogs, chickens and mice (Zhu et al., 2002; Toivanen et al., 2001; Vaahtovuo et al., 2003). These data show the dependence of the bacterial population on the host animal's digestive system. This dependence is a general phenomenon and is not restricted to a particular animal species. This effect could be due to the considerable influence of the animal host genotype on the bacterial population that for example, we can refer to Meta- neo-gene existing in digestive system in some vertebrate and invertebrate animals (Heksetin and Van Alen, 1996). This hypothesis has been investigated in humans by comparing the DGGE profiles of adult humans with relatives (people without kinship compared with identica twins, Zeotendal et al., 2001). The similarity between the DGGE profiles of mangos twins was significantly greater than between non-relatives, demonstrating the effect of genetic structure on the composition of microbial flora. Thus, the genetic makeup of the host animal, such as poultry, affects the microbes that interact with the host (the microbial flora) (Hooper et al., 2002).

These important findings indicate that specific effects on the host animal's intestinal microbial flora cannot be ignored. The ecology of the microbial flora of the digestive system, which varies in each part of the tract, is complex. Early studies that were based on fingerprinting demonstrated these differences in pigs (Simpson et al., 1999). The bacterial population in the colon mucosa is uniformly distributed; however, there are no significant differences with the faecal bacterial population (Zeotendal et al., 2001). There are a limited number of comparative and adaptive studies of different parts of the digestive tract of poultry and other animals. The observations discussed here show that faecal samples from other parts of the digestive system do not necessarily reflect the microbial flora population.

2.4 Diversity microarrays

DNA microarrays are a new, well-characterised method for the molecular identification of samples of environmental bacteria, and they include Biochips, gene chips or DNA chips. Typically, DNA microarrays consist of fragments of DNA that are covalently bound to several glass surfaces and that are available for hybridisation. DNA microarray technology has also been optimised for use in the studies of bacterial diversity in many ecosystems (Loy et al., 2002; El Fantrossi et al., 2003). The two main difficulties of DNA microarray analysis are hybridisation and quantitative signal determination. El Fantrossi et al. (2003) demonstrated that specific and nonspecific hybridisation can distinguish the differential thermal curve for each probe-goal pair. Initial efforts to develop DNA microarray studies of the gastrointestinal microbial ecosystem have been completed, and the technology appears promising (Wilson et al., 2002). Undoubtedly, further development of DNA microarray technology will occur, and the technology will be used in the study of the ecology of the gastrointestinal tract.

2.5 Non-SSU rRNA-based profiling

SSU rRNA-based profiles of several other methods, such as cellular fatty acid composition (Toivanen et al., 2001; Vaahtovuo et al., 2003) or C + G DNA content (Apajalahti et al., 2002), have been used successfully to investigate changes in bacterial populations of the

gastrointestinal tract. However, compared with SSU rRNA-based methods, these methods provide no phylogenetic information.

3. Different methods of investigation and quantification of microbial flora

3.1 Various methods of quantitative determination of SSU rDNA and SSU rRNA

Although PCR is the most sensitive method for the identification of sequences that have very low densities in the environment, many factors influence the amplification reaction, and fingerprinting methods alone cannot provide quantitative information to researchers (Von Wintzingerode et al., 1997). However, it is possible to determine the density of the SSU rDNA in the PCR reaction.

3.2 The RT-PCR method

Competitive PCR or the RT-PCR method for quantitative determination of the desired products can determine the amount of mRNA in human cells, which was the initial purpose of this method (Wang et al., 1989). In this method, a specific standard DNA fragment, at different concentrations, is added to a target (desired) product and amplified by PCR. The difference in the amount of the target and the standard is then quantified on an agarose gel. Using competitive PCR, the SSU rDNA of several bacterial species was determined in samples of cow rumen (Koike and Kobayashi, 2001, Reily et al., 2002).

3.3 Quantitative determination of amplified fragments in TGGE profiles and integration of quantitative PCR and constant-denaturant capillary electrophoresis (CDCE)

A similar method (to 3.2 above) for the quantitative measurement of individual components of the amplified fragments of TGGE profiles is used (Felske et al., 1998). The advantage of this method is that the amplified sample fragments and amplified standard fragments are similar, and differences between them can be identified by melting behaviour. Notably, when quantitative PCR and constant-denaturant capillary electrophoresis (CDCE) protocols were combined, similar results were obtained (Lim et al., 2001).

3.4 The most probable number PCR method

The Most Probable Number (MPN) PCR approach for determining the amount of SSU rDNA in environmental samples has been used successfully for human stool samples (Wang et al., 1996). The principles of this method are the same as the bacterial MPN count. Thus, DNA is diluted to a very low concentration and, using primers specific for a particular group or bacterial species, the DNA is then used as a PCR template.

This method is relatively quick and is suitable for determining the main groups of bacteria present, but it is not useful for the analysis of complex populations of species.

3.5 The real time PCR method

This method has received much recent attention and has been used to determine the characteristics of different samples of human and neonate pig gut and to successfully

determine rumen samples (Huijsdens et al., 2002; Malinen et al., 2003). Although the efficiency of real time PCR for complex bacterial populations requires further study, this method can be used to study very low numbers of bacteria (which is very difficult with other methods) and accordingly, its future is bright.

This method is based on the accurate and sensitive identification and quantitation of fluorescence, which shows an increased signal in proportion to the amount of PCR product. To identify more than a series of explicit microorganisms, recommended specific primers were used.

3.6 The dot blot electrophoresis method

The Electrophoresis Dot Blot (Blot spot or point) method is used to calculate the concentration of a population-specific 16S rRNA in a mixture. For this method, the total RNA is isolated and then filtered onto a Dot or Slot Blot (Blot fractured) and labelled with oligonucleotide probes. The relative rRNA concentration of hybridised material can be calculated by dividing the concentration of the general probe by the concentration of the specific probe (after normalisation of the rRNA signal with a control strain).

This method has been used to quantify rRNA from samples of human, horse and rumen (Sghir et al., 2000; Marteau et al., 2001; Daly and Shirazi - Beechey, 2003). Because PCR has no relation to other amplification methods, quantification determination is more accurate, and therefore, this method has a good reputation and is widely used. Recently, all of the data obtained from more than 700 probes was published, and the data are available in an online website (www.probebase.net), which facilitates the search for rRNA probes of microorganisms at the levels of family, genus and species.

Population studies have been conducted to compare the performance of quantitative PCR, Dot Blot or hybridisation; however, this type of population determination is relative (Rigottier-Gois et al., 2003a). The SSU rRNA and ribosomal density per cell are variable and depend on the species of bacteria, the growth stage and the activity level. Accordingly, Dot Blot electrophoresis can provide information on the number of bacteria, especially with regard to bacterial growth during culture. Similarly, genome size and the 16s rRNA gene copy number varies between different bacterial genomes, thus preventing accurate conclusions from these data.

3.7 Fluorescent hybridisation *in vivo*

To determine the amount of bacterial cells in environmental samples without the conventional culturing method, the FISH method may be used. In this method, oligonucleotide probes are used to target SSU rRNA. The combination of SSU rRNA probe hybridisation and epifluorescence, using light microscopy, confocal laser microscopy, or flow cytometry, allows the direct examination of a single bacterial population. This method can determine the relative prevalence of particular groups or genera of bacteria. FISH is being used more and more frequently to study the bacterial composition of the digestive system, and currently, probes for bacteria belonging to different genera, such as bifidobacterium, Streptococcus, Lactobacillus, Collineslla, Eubacteriuom, Fozobacterioum, Clostridium, Veillonella, Fibrobacter, and Rominokokus, have been described (Harmsen and Welling, 2002). To facilitate enumeration, automatic methods for FISH quantification and

computer programs that analyse the images have been generated (Jensen et al., 1999). Lastly, this is the best method for counting bacteria in the digestive tract. However, thus far, the FISH method has primarily been used for determining (Amann et al., 1990) the major bacterial groups in human faeces.

3.8 Cytometry

Recently, the efficacy of the flow cytometry method for the determination of faecal bacteria has been demonstrated (Rigottier-Gois et al., 2003a; 2003b; Zeotendal et al., 2002). Statistical analysis has shown that count results from two microscopic and cytometry methods are similar (Zeotendal et al., 2002). In the future, it is possible that both FISH and cytometry will be used to categorise bacteria without culturing. Although these bacteria are not alive, they can be used in molecular genetic studies. Limitations of the FISH method include the requirement for 16S rDNA sequence information from the database and the limited number of probes that can be used in each analysis. The FISH method depends on the permeability of the bacterial cells, the availability of target products and the construct number in each cell.

3.9 Densitometry

Densitometry measurements have many applications, especially for the measurement of SDS-PAGE protein gels (Zhang et al., 2007, Bromage and Kattari, 2007), but also for quantification of PCR products including mtDNA (Enzmann et al., 1999) and bacterial DNA (Amit-Romach et al., 2004) and many researchers have reported using this method.

4. Gene expression methods in microorganisms

Identifying the characteristics of the digestive system microbial flora is the first step in the study of this ecosystem because such data provide limited information on microorganism-microorganism and microorganism-host interactions. Given the complexity of microbial flora in the digestive system and the limited ability of many forms of bacterial culture, it is clear that determining the function of all of these microorganisms will be very difficult. However, some technical developments in bacterial analysis provide a good outlook for future research.

4.1 Gene expression analysis using RT-PCR in microorganisms

Functional gene expression is a suitable method for determining the activity of bacteria in an ecosystem. Using the RT-PCR method, Deplancke et al. (2000) identified mRNA expression of adenosine-5–phosphor soleplate in different parts of the digestive tract of rats. The RT-PCR method was also used to examine the effect of Helicobacter pylori infection on the expression of four genes in mouse and human gastric mucosa (Rokbi et al., 2001). The technology "gene expression *in vivo*" (IVET) is also a method for analysing gene expression in living organisms.

4.2 Gene expression evaluation in microorganisms using IVET

IVET allows the identification of promoters that are activated when bacteria are exposed to certain environmental conditions (Rainey and Preston, 2000). This method is typically used to study gene expression of pathogenic microorganisms. However, it was used recently to

identify gene promoters that were activated when exposed to lactobasirus in the mouse digestive system (Walter et al., 2003). Interestingly, the expression of three genes was associated with the establishment of Lactobacillus in the gastrointestinal tract.

4.3 Gene expression evaluation in microorganisms using microorganism DNA

Despite the use of complete genomic sequences, genomic comparisons and DNA microarrays for studying transcription in microorganisms, these methods remain in the early stages of development and are expensive. Thus, researchers are faced with obstacles and could instead use the genomic sequences of well-known cultured bacteria.

4.4 Gene expression evaluation using bacterial artificial chromosome and subtractive hybridisation

New methods for research into the function of genes, especially gene function in non-cultivated microorganisms, include the use of bacterial artificial chromosomes (BACs) and subtractive hybridisation. BAC-related cloning of large DNA fragments (more than one hundred pounds) is possible. Because environmental DNA libraries from soil (Liles et al., 2003) and sea (Beja et al., 2000) have been established, it is possible to use these libraries for the study of the diversity and metabolic potential of these complex ecosystems. Thus, it is possible to associate genes with SSU rRNA gene function, without culturing.

4.5 Gene expression evaluation in microorganisms using marked substitute materials

The use of substitute materials, marked with an isotope, provides another method for evaluating the performance of a specific microorganism in a complex population. Environmental samples are marked and grown with the substitute materials that contain environmental isotopes (stable or radioactive), and the microorganisms are then identified using extracted DNA or rRNA. (Boschker et al., 2001; Polz et al., 2003).

4.6 Gene expression evaluation in microorganisms using micro autoradiography and FISH composition

Autoradiography and FISH composition are an additional successful method that generates phylogenetic information (Ouverney and Fuhrman, 1999). This method appears to be very promising. You should be able to remember some microorganism between different isotopes (Londry and Des Marais, 2003). However, it is clear that using and developing new methods such as those mentioned above will allow the examination of the normal microorganism interactions (microorganism-microorganism and microorganism-host).

5. Conclusion

Due to several difficulties, traditional and non-molecular based techniques do not provide the required quality for acceptance as a standard reference method for the identification and study of bacteria. Thus, researchers have disagreed over these methods, and the data have not been presented in any standardised or reliable format. These issues indicate the necessity for new and updated techniques. Microbial culture methods can theoretically detect a small number of live bacteria in the gastrointestinal tract of chickens. However, in many cases, it is necessary to use selective media and conduct enrichment. In practice, the

minimum required number of bacteria for identification by culture methods is much greater than with molecular methods (Bjerrum et al., 2006). Due to their specific requirements, in current media, many bacteria show slow growth or sometimes no growth. Therefore, their identification by culture methods is difficult and sometimes impossible. Many other studies of bacterial detection have reported on the superiority of the polymerase chain reaction over culture methods. One reason underlying this superiority is that PCR can detect target sequences and is not related to the growth of target cells, while culture-based methods are correlated with the growth of target bacteria.

In this regard, many researchers, including Kothary and Babu (2001), Bjerrum et al. (2006) and Apajalahti et al. (2004), have stated that in many cases, the number of bacteria in the digestive tract of poultry is very low, and it is therefore necessary to use highly sensitive methods for the detection of these bacteria. However, due to the use of specific media for the accurate identification and elimination of false negative bacteria during culturing, several enrichments of these samples can be carried out. Nevertheless, false positive results, due to the growth of non-target bacteria that have similar growth requirements to the desired bacteria, must always be considered. A typical solution for this issue is the use of a specific medium for the bacterium being studied. When a very low population for a specific bacterial species in a sample exists, the resulting inhibitory effects on the polymerase chain reaction must be overcome through the use of selective media (Jofre et al., 2005; Rossen et al., 1992; Al-Soud and Radstrom, 2000; Al-Soud and Radstrom, 2001; Lantz et al., 2000). Li et al. (2005) believed that negative results could occur in a polymerase chain reaction even with medium-associated bacterial enrichment. The growth requirements and the appropriate media for growth of many bacteria remain unknown.

With the development of specific polymerase chain reactions for the detection of bacteria in the digestive tract of chickens, separately or simultaneously, new horizons develop in the field of poultry science research. In recent years, many studies have been conducted related to nutrition and health of poultry instead of using traditional methods in such a process approach to research in animal science is very attractive. Accordingly, investigations of the effects of dietary components, processed diets, antibiotics, and proboscis and diet enzymes on the gastrointestinal microbial flora of poultry and related research using these methods will be initiated. Indeed, poultry veterinary medicine had recently been leaning toward using these new methods and fields of research in this area. In addition, portions of the digestive system of birds used in this study included the microbial flora of the state organs, such as the crop, and by-products, such as carcasses and eggs and even the surrounding environment, such as the bedding, water and rations. Bacterial presence can be studied using these methods. Furthermore, using such methods, the microbial flora of poultry strains can be compared, and the differences between indigenous and industrial (modified) strains may be investigated. Individual variation in the microbial flora of the digestive system will benefit from the introduction of flora. In the near future, researchers may even investigate breeding herds guided by the microbial flora of poultry, which is one of the traits that influences the health and economic performance of chickens.

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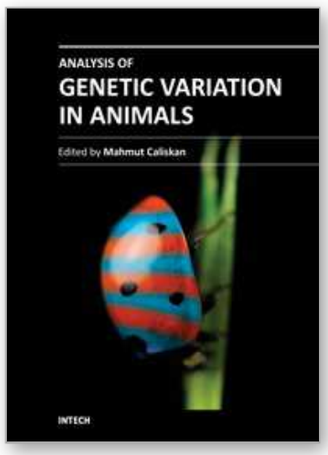

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Analysis of Genetic Variation in Animals includes chapters revealing the magnitude of genetic variation existing in animal populations. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs. In this concept molecular markers give valuable information. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in animals and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation in animals by presenting the thoughts of scientists who are engaged in the generation of new idea and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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