ENVIRONMENT, HEALTH, AND BEHAVIOR

Microflora Ecology of the Chicken Intestine Using 16S Ribosomal DNA Primers

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ABSTRACT The microflora in the gastrointestinal tract of broiler chickens influences digestion, health, and well-being. Analysis of chicken gut microflora has been mainly by culture-based methods. Studies using these techniques have been useful for identification and analysis of specific groups of bacteria, however, the use of enrichment medium precludes even relative quantitation of bacterial species. Recent advances in ribosomal DNA-based molecular techniques make it possible to identify different bacterial populations in environmental samples without cultivation. In this study, the intestinal microflora was examined using 16S ribosomal DNA (rDNA) targeted probes from bacterial DNA isolated from intestinal and cecal contents of chickens at 4, 14, and 25 d of age. The ribosomal gene sequence was amplified using PCR with universal primers to determine total bacterial DNA and specific primers directed at 6 bacterial species: *Lactobacillus, Bifidobacterium, Salmonella, Campylobacter, Escherichia coli,* and *Clostridium*. The use of universal primers extends these methods to allow determination of relative proportions of different bacterial species.

The results indicated that in young chicks the major species present in the small intestines and ceca was *Lactobacilli,* with a *Bifidobacteria* population becoming more dominant in the ceca at older age. *Clostridium* was detected in some segments of the small intestine in young chicks. In older chickens, *Salmonella, Campylobacter,* and *E. coli* species were found in the ceca. This study has demonstrated the use of molecular techniques for determining relative proportions of bacterial species and monitoring pathogens in the chick gastrointestinal tract.

(*Key words*: bacteria, cecum, chicken, intestine, microflora)

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INTRODUCTION

The gastrointestinal tract is the major digestive and absorbing organ and it plays a crucial role in chicken growth. A diverse microbiota is found throughout the tract and is most extensive in the cecum (Barnes, 1972; Barnes et al., 1972; Mead and Adams, 1975; Mead, 1997). This microflora has a role in nutrition, detoxification of certain compounds, growth performance, and protection against pathogenic bacteria. The gut microflora influences health and well-being of host animals (Nurmi and Rantala, 1973; Mead, 1997; Vispo and Karasov, 1997; van der Wielen et al., 2002).

In poultry, the absence of normal microflora in the cecum has been considered a major factor in the susceptibility of chicks to bacterial infection (Barrow, 1992). Although the alimentary tract of the newly hatched chick is usually sterile, organisms rapidly gain access from the mother and the surrounding environment. Previous studies have documented the changes in microflora during the posthatch period. Large numbers of anaerobic bacteria capable of decomposing uric acid comprise the cecal flora of chicks 3 to 6 h after hatching (Mead and Adams, 1975). During the first 2 to 4 d posthatch, streptococci and enterobacteria colonize the small intestine and cecum. After the first week, lactobacillus predominate in the small intestine, and the cecum is colonized mainly by anaerobes (*Escherichia coli* and Bacteroides) with lower numbers of facultative aerobes (Lev and Briggs, 1956; Mead and Adams, 1975).

A typical microflora of adult birds in the small intestine is established within 2 wk, however, it was found that the adult cecal flora, which was mainly obligate anaerobes, took up to 30 d to develop. At that age, bifidobacteria and bacteroides predominate (Barnes et al., 1972).

Until recently, intestinal microflora were analyzed by culture-based methods (Barrow, 1992), and microbial ecologists relied largely on techniques requiring the growing of organisms on selective media. Several limitations are associated with culture-based approaches, par-

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Abbreviation Key: rDNA = ribosomal DNA; rRNA = ribosomal RNA.

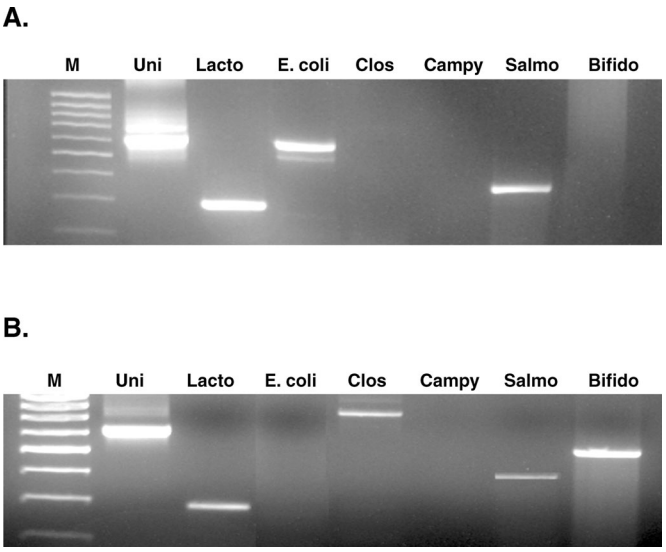

FIGURE 1. PCR products from 2 different animals (A. and B.) in a 2% agarose gel for each of the 6-primer sets that identify 6 bacterial groups (lanes 2 to 7) and the universal primers (lane 1). M = DNA size marker; Uni = universal primer (611 bp); Lacto = *Lactobacillus* (286 bp); E. coli = *Escherichia coli* (585 bp); Clos = *Clostridium* (722 bp); Campy = *Campylobacter* (857 bp); Salmo = *Salmonella* (396 bp); Bifido = *Bifidobacterium* (510 bp).

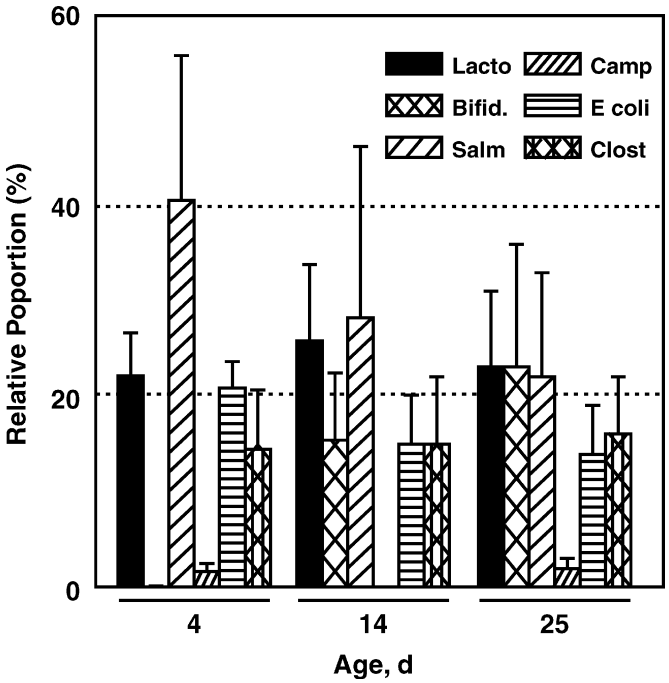

FIGURE 2. The proportion of PCR products of *Lactobacillus, Escherichia coli, Clostridium, Campylobacter, Salmonella*, and *Bifidobacterium*, relative to the universal PCR product calculated as percentage of the total examined bacteria in the chicken ceca with age. Lacto = *Lactobacillus*; Bifido = *Bifidobacterium*; Salm = *Salmonella*; Camp = *Campylobacter*; E. coli = *Escherichia coli*; Clost = Clostridium.

ticularly for surveying the intestinal ecosystem (Langendijk et al., 1995). In addition to being time and labor intensive, the use of selective media specific for different types of bacteria imposes an a priori bias. Various attempts have been made to determine the composition of the cecal microbiota in poultry, but the isolation methods used have not always been suitable for the oxygen-sensitive anaerobes, many of which are difficult to isolate and maintain (Mead, 1997).

In contrast, the recent development of PCR techniques has allowed the rapid and specific detection of a wide range of bacteria and should become a key procedure for detecting microorganisms. For many years, sequencing of the 16S ribosomal RNA (rRNA) gene has served as an important tool for determining phylogenetic relationships between bacteria. The features of this molecular target that make it a useful phylogenetic tool also make it useful for bacterial detection and identification in the clinical laboratory. Several studies have shown that sequence identification is useful for slow-growing, unusual, and fastidious bacteria, as well as for bacteria that are poorly differentiated by conventional methods (Patel, 2001). Recent advances in ribosomal RNA- and DNA-based molecular techniques make it possible to identify different bacterial populations in environmental samples without cultivation (Harmsen et al., 2000). This PCR methodology has been used to determine variation in bacterial population in the human colon and feces (Langendijk et al., 1995; Wang et al., 1996; Franks et al., 1998; Harmsen et al., 2000), the bovine rumen (Nelson et al., 1998), and the chicken cecum (Gong et al., 2002; Zhu et al., 2002). Previous reports have indicated that there is good correlation between PCR-based techniques and culture methods for species growing in cultures without significant enrichment (Wang et al., 1996).

There is a great importance in examining and monitoring the intestinal microflora because many bacterial species with pathogenicity toward humans have been found in the gastrointestinal tract of chickens and can thus be introduced into the food chain (Reeves et al., 1989; Davies and Wray, 1996; Brandt et al., 1999; Moreno et al., 2001).

The objectives of this study were to use PCR-based methods for detecting and quantifying, in the different parts of the intestine, the 16S ribosomal DNA (rDNA) of 6 bacterial species, *Lactobacillus, Bifidobacterium, Salmonella, Campylobacter, E. coli,* and *Clostridium*, which have a major role in chicken performance and consumer health, and to monitor the relative changes in the microbial population in the cecum and small intestine with age.

MATERIALS AND METHODS

Collection of Microbial Samples and DNA Isolation

Fifty Cobb chickens were grown from hatching for 25 d on wood shavings and were fed a standard commercial diet.[2] At 4, 14, and 25 d of age, 8 chickens were randomly selected and killed by cervical dislocation. The intestine and cecum were removed and treated as described by

[2]Matmor Feedmill, D. N. Evtach, Israel.

TABLE 1. PCR primers used in the study

Bacterial group	Primers	Sequence (5′-3′)	Length (bp)	References
Universal	Unibac-f	CGTGCCAGCCGCGGTAATACG	611	
	Unibac-r	GGGTTGCGCTCGTTGCGGGACTTAACCCAACAT		
Lactobacillus	LAA-f	CATCCAGTGCAAACCTAAGAG	286	Wang et al., 1996
	LAA-r	GATCCGCTTGCCTTCGCA		
Escherichia coli	ECO-f	GACCTCGGTTTAGTTCACAGA	585	Candrian et al., 1991; Wang et al., 1996
	ECO-r	CACACGCTGACGCTGACCA		
Clostridium	Clos58-f	AAAGGAAGATTAATACCGCATAA	722	
	Clos780-r	ATCTTGCGACCGTACTCCCC		
Campylobacter	Camp-f	ATCTAATGGCTTAACCATTAAAC	857	Denis et al., 2001
	Camp-r	GGACGGTAACTAGTTTAGTATT		
Salmonella	Sal201-f	CGGGCCTCTTGCCATCAGGTG	396	
	Sal597-r	CACATCCGACTTGACAGACCG		
Bifidobacterium	Bif164-f	GGGTGGTAATGCCGGATG	510	Langendijk et al., 1995
	Bif662-r	CCACCGTTACACCGGGAA		

Zhu et al. (2002). The contents of each segment (duodenum, jejunum, ileum, and cecum) were inverted into a sterile 15-mL tube containing 9 mL of sterile PBS, and homogenized by vortexing with glass beads (4-mm diameter) for 3 min. Debris was removed by centrifugation at $700 \times g$ for 1 min, and the supernatant was collected and centrifuged at $12{,}000 \times g$ for 5 min. The pellet was washed twice with PBS and stored at –20°C until DNA extraction. For DNA purification, the pellet was resuspended in EDTA and treated with lysosyme[3] (final concentration of 10 mg/mL) for 45 min at 37°C. The bacterial genomic DNA was isolated with the Wizard Genomic DNA purification kit.[4] DNA concentration and purity was checked spectrophotometrically.

All procedures were approved by the Animal Care and Welfare Committee of our Institute.

Primer Design and PCR Amplification of Bacterial 16s rDNA

Primers for *Lactobacillus, Clostridium, Campylobacter, Salmonella,* and *Bifidobacterium* were designed using the16S rRNA region for each bacterial group. Potential primer targets for *Lactobacillus, E. coli, Clostridium, Campylobacter, Salmonella,* and *Bifidobacterium* were identified by comparing the complete 16S rRNA sequences of bacterial groups using the programs BLAST, Seqweb, and RDPII (http://www.ncbi.nlm.nih.gov/BLAST/, http://seqweb.huji.ac.il/gcg-bin/seqweb.cgi,http://rdp.cme.msu.edu/html/). The target sites for the primers were identified as sequences that are invariant, or nearly so, in all members of a particular bacteria group, but differ significantly from all the representatives of the other 5 groups. The GenBank program BLAST was used to ensure that the proposed primers were complementary with the target species but not with other bacterial groups.

The primers used in this study are shown in Table 1. Universal primers identifying all known bacteria were designed using the invariant region in the 16s rDNA of the bacteria. The universal primer set was used for determining the total microflora population. Primers targeting *Lactobacillus* species were designed according to Wang et al. (1996), and primers for *E. coli* were modified from Candrian et al. (1991) by deleting 4 to 6 bases at the 5′ end to fit our PCR conditions. The primer set for *Clostridium* was designed from 58bp and 780bp in the rDNA sequence (GenBank accession # AF316589). The primer set for *Campylobacter* species was according to Denis et al., (2001). Primers targeting *Salmonella* species were designed from the 201bp region and the 597bp region of the rDNA sequence (GenBank accession # AF332600), and the *Bifidobacterium* primer set was as described by Langendijk et al. (1995). For validation, each primer set was tested with known bacterial cultures.

For PCR amplification of the bacterial targets from intestinal contents, 5 μL of DNA extract was added to 45 μL of PCR mixture containing 27.5 μL of nuclease-free water, 5 μL of each primer, 1.5 μL of nucleotide (dNTP) mix, 5 μL of PCR buffer, and 1 μL of Taq polymerase. The PCR was conducted in a DNA Thermal Cycler.[5] The amplification conditions were: 1 cycle of 94°C for 3 min, 35 cycles of 94°C for 30 s, 60°C for 1 min and 68°C for 2 min, and finally 1 cycle of 68°C for 7 min. The PCR reaction was run with different numbers of cycles (25, 30, 35, 40, 45, or 50) for each primer set and 35 cycles was in the center of the exponential increase in PCR products. Products of PCR were visualized by agarose gel (2%) electrophoresis containing ethidium bromide.

Figure 1 shows the PCR products obtained from 16S rDNA for each of the 6 bacterial groups tested together with the universal primers in a representative sample from the chicken cecum. Background subtraction of gel images was performed and densitometric evaluation of the different bands was done with Gel-Pro Analyzer.[6]

The evaluation of the different PCR products was normalized to the density of the PCR product of the universal primers by densitometer scanning and was exhibited as arbitrary units (AU). To evaluate the relative proportion of each examined bacteria, all products were expressed relative to the content of the universal primer product and proportions of each bacterial group are presented where the total of the examined bacteria was set at 100%.

[3]Sigma Aldrich Co., St. Louis, MO.
[4]Promega Corporation, Madison, WI.
[5]MJ Research Inc., Waltham, MA.
[6]Media Cybernetics, L.P., Silver Spring, MD.

Results are presented as means ± SE and data were examined by the GLM procedure of SAS (SAS Institute, 1986) after arcsin transformation to test for significance, and returned to the original scale.

RESULTS

Microbial Distribution in Chicken Ceca at 4, 14, and 25 d

Analysis of chicks at 3 different ages (Figure 2), showed different relative proportions of the bacteria examined. In chicken ceca at 4 d, the relative proportion of *Lactobacilli* was about 25% of the total examined bacteria and *Bifidobacterium* was not detected. Relatively high proportions of *Salmonella* were detected (40%) and *Campylobacter* was present in minor amounts (2%). Almost one-third of the bacteria in the chicken ceca at this age consisted of *E. coli* and *Clostridium* species.

At the age of 14 d (Figure 2), the relative proportion of *Lactobacilli* and *Bifidobacterium* increased and reached 40% of the total bacteria. In contrast, the relative proportion of *Salmonella* was reduced by approximately 10%. *Campylobacter* was present only in trace amounts and proportions of *E. coli* and *Clostridium* changed little. At 25 d of age, almost one-half of the bacteria in the chicken ceca were *Lactobacilli* and *Bifidobacterium* species. Furthermore, the relative proportion of *Salmonella* had decreased by approximately 50% compared with that at 4 d. Proportions of *Campylobacter* remained small, whereas proportions of *E. coli* and *Clostridium* remained approximately 30%.

Microbial Diversity Along the Digestive Tract

Analysis of the microbial luminal contents of the different small intestine sites examined indicated that among the 6 examined bacterial species, only *Lactobacillus* was consistently detected in all intestinal regions (Figure 3). The results indicated that at d 4, most of the bacterial species were not detectable in the small intestines. Proportions of *Lactobacilli* changed little along the small intestines at a young age (Figures 3A and 4A). However, at d 25, the posterior segments exhibited lower levels of *Lactobacilli* compared with the anterior segment (Figure 4B). In addition, at d 25 *E. coli* and *Clostridium* were detected in the duodenum and ileum (Figure 3B).

DISCUSSION

Molecular techniques were used in this study to follow the ontogeny of the microbial populations in the small intestines and ceca of broiler chicks and to monitor the presence of harmful species.

In this study, *Lactobacillus* was the major species present in the duodenum of young chicks. Some clostridia species were found in the jejunum and ileum, as has been described previously in older chicks using culture methods

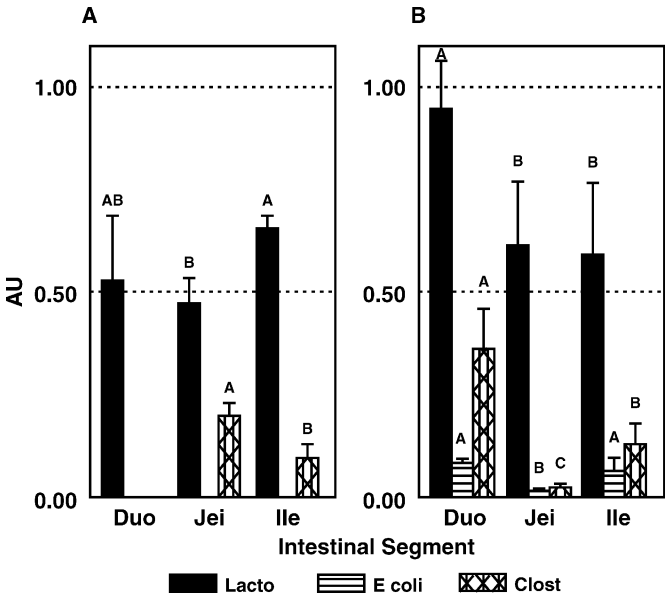

FIGURE 3. The proportions of bacterial populations in the content of the small intestines [duodenum (Duo), jejunum (Jej), and ileum (Ile)] at 4 d of age (A) and at 25 d of age (B). The evaluation of the different PCR products was normalized to the density of the PCR product of the universal primers by densitometer scanning and exhibited as arbitrary units (AU). Columns, for each bacteria, with different letters differ significantly among the intestinal regions ($P < 0.05$).

(Lev and Briggs, 1956; Barnes et al., 1972). The population in the cecum was more varied, with some *Salmonella* and *E. coli* species occurring, as has been previously observed using culture and molecular methods (Mead and Adams, 1975; Zhu et al., 2002). With age, the small intestine bacterial population remained predominantly lactobacilli, whereas in the cecum, *Bifidobacteria* began to develop and reached a stable proportion between 14 and 25 d. These results were again similar to previous reports that used culture methods (Barnes et al., 1972; Mead and Adams, 1975). In addition, it was demonstrated in this study that

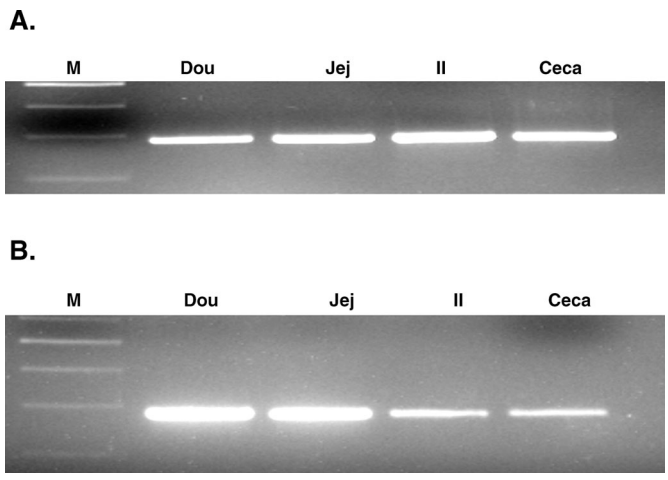

FIGURE 4. PCR products, in a 2% agarose gel, from representative chickens (A) 4 d old and (B) 25 d old, exhibiting *Lactobacillus* presence in different regions of the gastrointestinal tract. Lanes: M = size marker; Duo = duodenum; Jej = jejunum; Il = ileum; Caeca = cecum.

Salmonella and *Campylobacter* were present in some of the cecal samples.

In our study, we focused on 6 bacterial groups, some representing beneficial bacteria, (*Lactobacillus, Bifidobacteria*), some bacterial species potentially pathogenic to humans (*Salmonella, Campylobacter*), and some bacterial species possibly harmful to the chick (*E. coli, Clostridium*). *Lactobacillus* and *Bifidobacteria* are considered to be bacteria that stimulate growth and activity of other health-promoting bacteria and have been termed probiotic (Lucchini et al., 1998; Mikkelsen et al., 2003). *Salmonella* is a pathogen that causes gastroenteropathy in humans, has a broad distribution throughout the natural world, and a widespread occurrence in food animals, which may introduce this pathogen to the food chain (Davies and Wray, 1996; Reeves et al., 1989).

Thermotolerant *Campylobacter* is a common human enteric pathogen, which causes acute bacterial diarrhea worldwide, and which often originates from chick gut microflora (Moreno et al., 2001). Species of *Clostridium,* including *C. perfringens,* are widely distributed in the environment, inhabiting both human and animal gastrointestinal tracts (Brandt et al., 1999). *Escherichia coli* is an adaptive species that is both a commensal resident of the intestine and a versatile pathogen of humans and other animals, causing enteric infections and particular pathologies in different animal species (Dozois et al., 2003). Therefore, for animal and human health, there is great importance in developing a method that will enable accurate and rapid identification of the above bacteria. In the present report, we assumed that the universal primers are incorporated to all bacteria and thus could be used to quantitate the amounts of the different species. However, we restricted the analysis to detection of bacterial species without identification of specific subspecies including pathogens.

PCR-based techniques targeting the bacterial rDNA have been used to identify different bacterial species in fecal samples (Langendijk et al., 1995; Wang et al., 1996; Franks et al., 1998; Harmsen et al., 2000) However, the simultaneous use of universal primers directed at overall bacteria DNA has allowed us to extend this technique to estimate the relative proportions of different species present in the intestinal lumen. Some studies have used changes in the proportions of guanine plus cytosine to evaluate microbial populations (Apajalahti et al., 1998; Apajalahti et al., 2001). However, although this approach made it is possible to identify specific subgroups, it was not possible to determine species of bacteria in a mixed community and thus this method is less exact than the molecular identifications used here.

The use of molecular techniques has several advantages compared with the classical culture methods for enumerating bacteria, and does not introduce the bias of traditional methods. One major advantage is the rapidity and sensitivity of the determination compared with culture methods. Perhaps the most serious drawback of the culture-based methods is that only a small fraction of bacteria can be found; up to 99% of the bacteria in many environments fail to grow under artificial conditions (Amann et al., 1995; Hanson and Henson, 1996; Holben et al., 1998). This low recovery is due to the fact that growth requirements of most bacteria are still unknown and cannot be reproduced under laboratory conditions.

The results of this study demonstrate the application of PCR-based 16S rDNA techniques to determine changes in the microbiota in the chicken small intestines and ceca with age and to monitor the presence of potentially hazardous bacteria.

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