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Application of Molecular Fingerprinting for Qualitative Assessment of Small-Intestinal Bacterial Diversity in Dogs

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The aims of this study were to evaluate the use of molecular fingerprinting for assessment of bacterial diversity in canine duodenal juice and to evaluate the variation in the small intestinal microflora at repeated sampling. Two groups of dogs were used. Duodenal juice was collected from eight dogs euthanized for an unrelated project (group 1). Duodenal juice was also collected endoscopically from six dogs at weekly intervals for a total of 3 weeks (group 2). The variable V6-V8 region of bacterial 16S ribosomal DNA was amplified, and PCR amplicons separated by denaturing gradient gel electrophoresis (DGGE). The reproducibility of DGGE profiles and variations in bacterial diversity between dogs were evaluated by comparing similarity indices (Dice's coefficient; 100% represents complete identity) of DGGE profiles from group 1 dogs. Weekly variations in the flora of the small intestine were evaluated by comparison of DGGE profiles from different time points within the same individuals in group 2. The mean (± standard deviation) similarity of DGGE profiles of duodenal juice between the dogs in group 1 was 38.3 ± 15.7% (range, 12.5 to 76.65%). There was a significantly higher variation in DGGE profiles between different dogs than between duplicates obtained from the same dog ($P < 0.0001$). DGGE profiles from samples collected at different time points varied within individuals, possibly due to variation over time or slight variation in sampling location. DGGE profiles indicate that dogs have a highly diverse microflora of the small intestine, with marked differences between individual dogs.

The domestic dog plays several important roles in modern human society. Dogs are commonly used as a model species for biomedical research as well as being commonly kept as pets. The normal canine microflora of the small intestine is not well characterized. Previous studies aiming to characterize the bacterial flora of the canine small intestine have focused on the enumeration and identification of bacterial species from direct cultivation of duodenal juice (2, 21, 27). This technique is considered the gold standard for the diagnosis of bacterial overgrowth of the small intestine in dogs. Bacterial culture, however, has limitations for assessing bacterial diversity in the gut. Samples of duodenal juice must be processed immediately in order to accurately represent the aerobic and anaerobic microbial flora of the small intestine. Thus, an on-site microbiology laboratory is required in order to obtain reliable bacterial counts when culturing duodenal juice.

It is increasingly recognized that the majority of microbial species present in biological samples escape identification by use of standard culture techniques alone (1, 3, 12, 13, 19, 25). Studies with a molecular biological approach, based on identification of 16S rRNA or 16S ribosomal DNA (DNA encoding the 16S rRNA), have identified a greater number of bacterial species than standard culture techniques, indicating that only a small proportion of bacterial species are cultivable with standard bacterial culture techniques (8, 19, 25). It has been estimated, for example, that 60 to 80% of bacterial organisms present in the gastrointestinal tract of humans and pigs have not yet been cultivated (12, 13). Based on these studies, it is very likely that a culture-dependent approach underestimates the bacterial diversity found in the fluid of the small intestine of dogs, and an approach based on molecular fingerprinting techniques may identify greater bacterial diversity of the small intestine in the domestic dog than previously reported.

Molecular fingerprinting techniques are commonly used to illustrate the genetic diversity in a complex microbial community (13, 24). Amplification of 16S rDNA with subsequent separation of amplicons by denaturing gradient gel electrophoresis (DGGE) has been used for assessment of bacterial diversity in environmental samples (15). DGGE is based on the principle of decreasing electrophoretic mobility of double-stranded DNA molecules when they are partially melted (16). Briefly, DNA is extracted from biological samples, and 16S rDNA is amplified with primers that target conserved regions (located up- and downstream of variable regions) within the gene encoding 16S rRNA. This approach allows amplification of unknown bacterial species. The mixture of PCR products, all approximately of the same length, are subsequently separated on a polyacrylamide gel containing a linear gradient of DNA denaturants (15). Sequence differences in the double-stranded DNA influence the melting behavior of the PCR amplicons, and, therefore, PCR amplicons with different sequences will stop migrating at different positions in the gel. This results in separation of amplicons, and the pattern of separated bands illustrates the bacterial diversity in the sample.

The aims of this study were to evaluate the utility of DGGE fingerprinting for the qualitative assessment of bacterial diversity of the microflora of the canine small intestine and to evaluate weekly variations in bacterial diversity.

(Parts of this research were presented as an abstract at the

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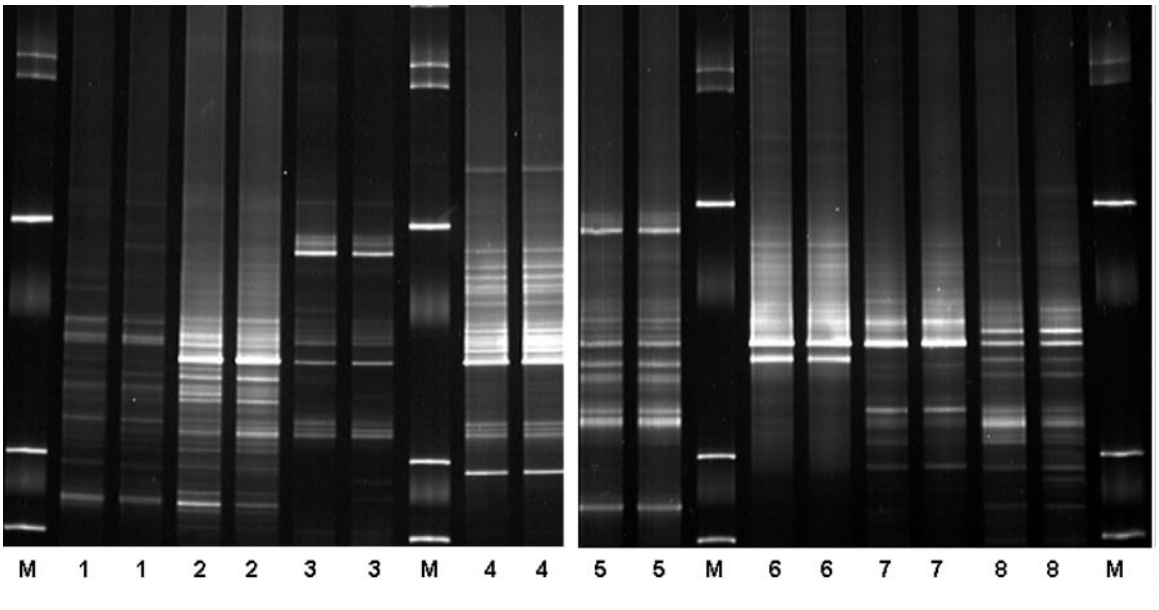

FIG. 1. DGGE profiles illustrating the bacterial diversity of the small intestine in eight dogs (lanes 1 to 8, in duplicate). Samples were collected in duplicate from approximately the same collection site in each dog and treated as independent samples to evaluate the reproducibility of DGGE profiles. Lanes M, markers.

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MATERIALS AND METHODS

Sample material. Duodenal juice samples were collected from two groups of research dogs, all with no clinical signs of gastrointestinal disease. The protocol for sample collection was approved by the University Laboratory Animal care committee at Texas A&M University (AUP 2002-103). Group 1 consisted of eight hound dogs (four male, four female) that were euthanized as part of an unrelated research project. The mean age was 3.9 years (range, 2.5 to 6.0 years). All dogs were housed in the same environment and fed the same regular canine maintenance diet. Food was withheld for 24 h before euthanasia.

Samples were collected in duplicate from each dog and treated as independent samples to evaluate the reproducibility of the DNA extraction, amplification of bacterial DNA by PCR, and the separation of amplicons by DGGE. Immediately after euthanasia the abdominal cavity was opened, the duodenum was isolated, and two samples, each of approximately 0.5 ml of duodenal juice, were collected from approximately the same collection site by needle aspiration with a sterile 16-gauge needle attached to a sterile 3-ml syringe.

Group 2 consisted of six healthy research beagles enrolled in an unrelated research project that required weekly anesthesia. The mean age of these dogs was 5.2 years (range, 2 to 6.8 years). These dogs were housed in the same environment and fed a regular canine maintenance once daily. Food was withheld for 24 h before induction of anesthesia on each study day. On the first day of the study, serum was obtained and serum cobalamin, serum folate, serum trypsin-like immunoreactivity, and serum unconjugated cholic acid concentrations were evaluated to screen for gastrointestinal disease.

On each study day, dogs were anesthetized, and approximately 0.5 ml of duodenal juice was aspirated with a sterile syringe attached to a sterile, 1-mm-wide, 150-cm tube introduced through the working channel of an endoscope (flexible endoscope, Olympus, Melville, N.Y.). The collection site was approximately 10 cm distal to the major duodenal papilla. Between each dog, the endoscope was cleaned (Megazyme, PEA Products Inc., Hunt Valley, Mass.) and disinfected (Cidex, Advanced Sterilization Products, Irvine, Calif.) according to the manufacturer's instructions. This sampling procedure was repeated once a week for a total of 3 weeks (samples 1, 2, and 3). In week 3, in addition to sample 3, a second sample of duodenal content was collected with a disposable sterile cytology brush (disposable cytology brushes, 1.7 mm by 160 cm, Horizons International Corp.) introduced through the working channel of the endoscope (sample 3A).

Storage of samples. All samples were immediately transferred to sterile cryotubes (Cryule 2 ml, Wheaton, Millville, N.J.), snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

Extraction of DNA. To each sample of duodenal juice, 500 μl of cell lysis solution (Puregene cell lysis solution, Gentra Systems, Minneapolis, Minn.), 200 μl of buffer-saturated phenol-chloroform-isoamyl alcohol (25:24:1, pH 7.2), and 300 μl of 0.1-mm zirconia beads (BioSpec Products Inc., Bartlesville, Okla.) were added. The tubes were positioned horizontally on a vortex adapter (Ambion Inc., Austin, Tex.) mounted on a standard vortexer, and the mixture was vortexed for 5 min at maximum speed. The tubes were centrifuged for 7 min at 12,000 × *g* at 4°C, and the supernatant transferred to a new, sterile, cryotube. Then 700 μl of phenol-chloroform-isoamyl alcohol was added, and the tube was vortexed for 30 s and centrifuged for 20 min at 12,000 × *g* at 4°C.

The aqueous phase was transferred into a new sterile cryotube. To increase the DNA yield, 200 μl of buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.5]) was added to the remaining phenol and organic phase, the above-described extraction procedure was repeated, and both aqueous phases so obtained were combined. To remove RNA, 5.2 U of RNase (Puregene RNase, Gentra Systems) was added to the solution and incubated at 37°C for 30 min. The RNase was removed by phenol-chloroform-isoamyl alcohol extraction as described above. The aqueous phase containing DNA was mixed with 0.5 volume of 100% ethanol and applied to commercially available spin columns (GenElute bacterial genomic DNA kit, Sigma Chemicals, St. Louis, Mo.). Bound DNA was washed and eluted according to the manufacturer's instructions. Purified DNA was stored at −20°C until further use. A negative control containing H_2O instead of sample was purified parallel to each extraction batch to screen for contamination of extraction reagents.

PCR amplification of the V6-V8 region of 16S rDNA. Isolated DNA was subsequently used as a template to amplify the variable V6 to V8 region of 16S rDNA with universal bacterial primers F-968-GC (5′-GC-clamp+GAACGCGA AGAACCTTAC-3′) and R-1401 (5′-GGTGTGTACAAGACCC-3′) (26). The GC clamp (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACG GGG) incorporated into the forward primer prevents complete dissociation of the DNA double strand during the following DGGE analysis (16). The amount of DNA was quantified by measuring the absorbance at 260 nm. The reaction mixture (25 μl) consisted of reaction buffer (GeneAmp 10× PCR Gold buffer, Applied Biosystems, Foster City, Calif.) (final concentrations: 15 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$ [pH 8.0]), 1.25 U of *Taq* DNA polymerase (Amplitaq Gold LD, Applied Biosystems), 250 μM each of the deoxynucleoside triphosphates, 0.24 μM each primer, and 100 ng of DNA template. A negative PCR

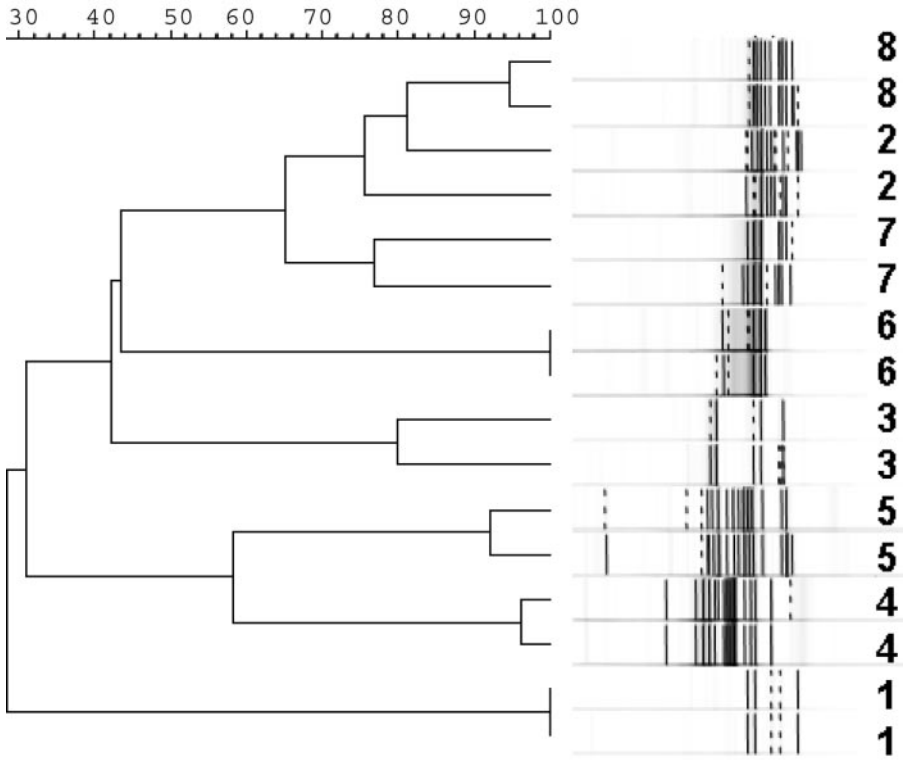

FIG. 2. Dendrogram (unweighted pair group method with arithmetic averages) generated from DGGE profiles obtained from canine duodenal juice from eight dogs (dogs 1 to 8 of group 1, in duplicate) representing similarities in banding patterns between duplicates obtained from each dog and between different dogs. The mean ± standard deviation similarity index between the eight dogs was 38.3 ± 15.7% (range, 12.5 to 76.65%). The mean ± standard deviation similarity index between the duplicates from each dog was 88.4 ± 12.3%. There was a significantly higher variation in DGGE profiles between dogs than between duplicates obtained from the same dog ($P < 0.0001$).

control, containing H_2O instead of the DNA template, was included to screen for contamination of PCR reagents.

The samples were amplified in a thermocycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany) with a touchdown PCR protocol: initial denaturation step at 94°C for 3 min; nine touchdown cycles (denaturation at 94°C for 30 s, annealing for 30 s, and extension at 68°C for 1 min) with annealing temperature decreasing 1°C per cycle from 62 to 54°C; 25 cycles at 54°C annealing temperature (denaturation at 94°C for 30 s, annealing for 30 s, and extension at 68°C for 1 min), and a final elongation step at 72°C for 10 min. The purity and correct size of the resulting PCR amplicons (approximately 450 bp) were assessed on 1% agarose electrophoresis gels, stained with ethidium bromide (staining for 15 min and destaining in H_2O for 60 min), and visualized under UV light.

DGGE analysis of PCR amplicons. DGGE analysis was performed on a DGGE system (DCode, Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions. PCR amplicons were applied to 8% (wt/vol) polyacrylamide gels (16 by 16 cm; acrylamide-bisacrylamide, 37.5:1) in TAE buffer (40 mM Tris-acetate, 1 mM disodium EDTA, [pH 7.4]) with a linear denaturing gradient of 35 to 70% (100% denaturant was defined as 7 M urea and 40% [vol/vol] deionized formamide). To standardize DGGE gels, a commercially available DNA ladder (DNA ladder [log2], New England Biolabs Inc., Beverly, Mass.) was loaded as a marker. Electrophoresis was performed in TAE buffer at 60°C for 16 h at 70 V. Gels were stained with ethidium bromide for 12 min and subsequently destained in H_2O two times for 30 min. Gels were scanned (AlphaImager, Alpha Innotech Corporation, San Leandro, Calif.), and the banding patterns were analyzed with gel analysis software (Bionumerics 3.0, Applied Maths, Austin, Tex.). Similarity indices between the banding patterns were calculated with Dice's similarity coefficient ($D_{sc} = [2j/(a + b)] \times 100$, where a = number of DGGE bands in lane 1, b = number of DGGE bands in lane 2, and j = number of common DGGE bands, and $D_{sc} = 100\%$ demonstrates complete identity) (14). Dendrograms showing clustering according to the similarity of banding patterns of individual samples were constructed by the unweighted pair group method with arithmetic averages (6).

RESULTS

Group 1. The variable V6 to V8 region of 16S rDNA could be successfully amplified from all 16 samples. DGGE profiles illustrating the bacterial diversity in duodenal juice and the variation in bacterial diversity between duplicates and between dogs are illustrated in Fig. 1. Similarity indices of DGGE profiles between duplicates collected from the same collection site from each dog were 66.7, 76.9, 80.0, 92.3, 94.7, 96.3, 100.0, and 100.0% (mean ± standard deviation, 88.4 ± 12.3%). The mean ± standard deviation similarity index of DGGE profiles of duodenal juice between the eight dogs was 38.3 ± 15.7% (range, 12.5 to 76.6%). Student's t test revealed that there was a significantly higher variation in DGGE profiles between different dogs than between duplicates obtained from the same dog ($P < 0.0001$; Fig. 2).

Group 2. From 18 potential time points (three sampling periods in six dogs), 17 samples of duodenal content were obtained by aspiration. As no duodenal juice could be aspirated in one dog at time point 3 (sample 3), the corresponding sample of duodenal content collected at the same time point with the cytology brush (sample 3A) was excluded from further statistical analysis. In another dog, no 16S rDNA could be amplified from the DNA extracted at time point 1. DNA extracted from all other samples was successfully amplified. DGGE analysis revealed a sizable variation in similarity indi-

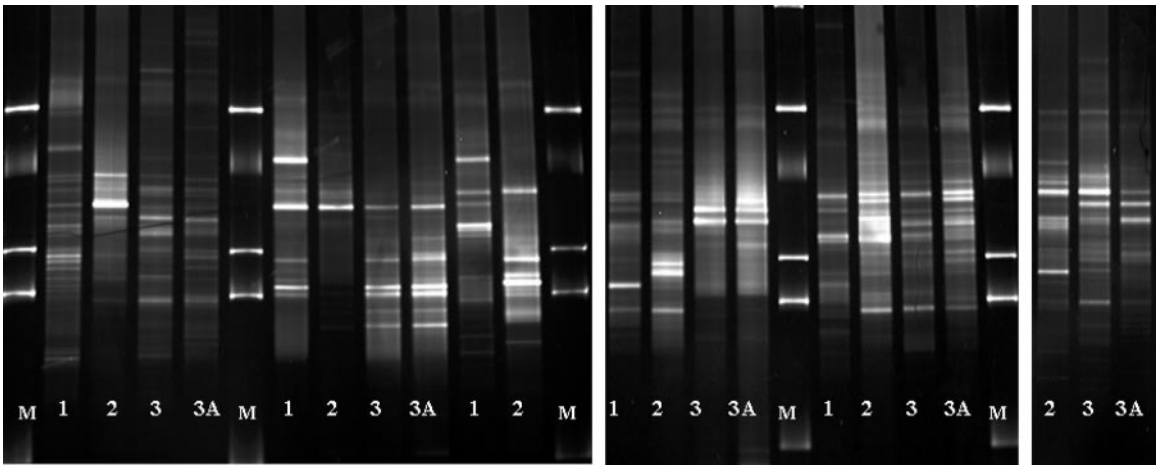

FIG. 3. DGGE profiles of duodenal juice collected endoscopically in six dogs (dogs A to F), illustrating a sizable variation in duodenal microflora between different time points within the same individual. Samples were collected by aspiration once weekly (samples 1, 2, and 3). In week 3 a second sample was collected with a cytology brush (sample 3A). Samples taken at the same time point from each dog (samples 3 and 3A) showed the highest similarity. No duodenal juice could be aspirated from dog C on week 3. No PCR amplicon could be generated from dog F on week 1. Lanes M, markers.

ces between the different time points within the same individuals (Fig. 3). The mean ± standard deviation similarity between the different time points (samples 1, 2, and 3) within individual dogs was 38.3 ± 13.3%. The mean similarity between samples taken in week 3 (sample 3) and their corresponding samples taken with the cytology brush (sample 3A) was 62.6 ± 13.4% (Fig. 4). There was a significantly higher variation between the different time points in individual dogs than between samples taken at the same time point 3 (sample 3 and sample 3A; $P = 0.003$; Fig. 5).

DISCUSSION

Traditionally, assessment of the microflora of the canine small intestine has been based on identification and enumeration of intestinal bacteria by bacterial culture (2, 21, 27). It is becoming increasingly apparent, however, that only a proportion of bacterial species present in the gut can be identified with traditional bacterial culture techniques (1, 3, 12, 13, 19, 25). The reasons for this inability to culture many bacterial species include nonviable or stressed microorganisms, obligate requirements for coexisting flora or host-derived products, bias due to selectivity of culture media, and our lack of knowledge regarding essential nutrients for some bacterial species (4).

Molecular approaches, based on assessment of human fecal samples, have revealed that the gastrointestinal microflora is more complex than previously documented (25). Based on sequence analysis of PCR products, new bacterial species not previously detected by cultivation have been identified in fecal samples of humans and the gastrointestinal tract in pigs (12, 13). Sequencing of single PCR clones, however, is a laborious and expensive procedure and not well suited to studying complex microflora or microbial dynamics due to environmental changes. Molecular fingerprinting techniques (e.g., DGGE) allow rapid assessment of the predominant bacterial species present in a sample. The DGGE profile typically represents up to 99% of the total bacterial community present in a biological sample (15). While molecular fingerprinting does not allow immediate discrimination between bacterial species, it does allow simultaneous analysis of multiple samples and, thus, direct comparison of microbial communities from different sam-

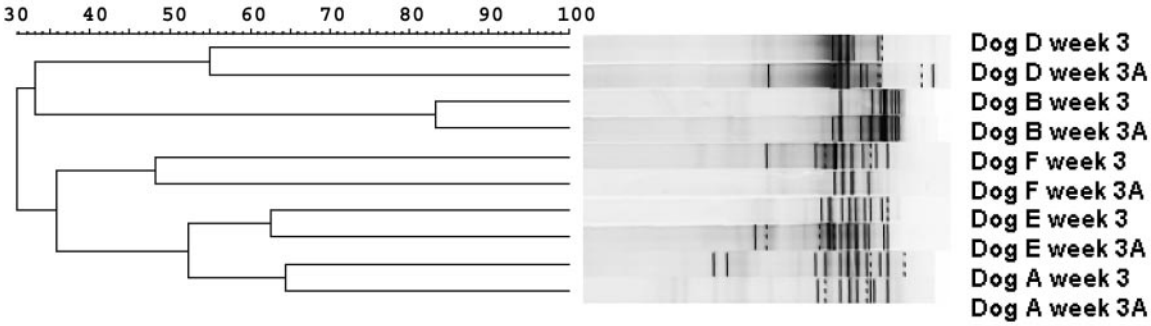

FIG. 4. Dendrogram (unweighted pair group method with arithmetic averages) generated from DGGE profiles representing similarities in banding patterns between the two samples obtained by different techniques in week 3. The mean similarity between aspirated samples (sample 3) and their corresponding samples taken with the cytology brush (sample 3A) was 62.6 ± 13.4%.

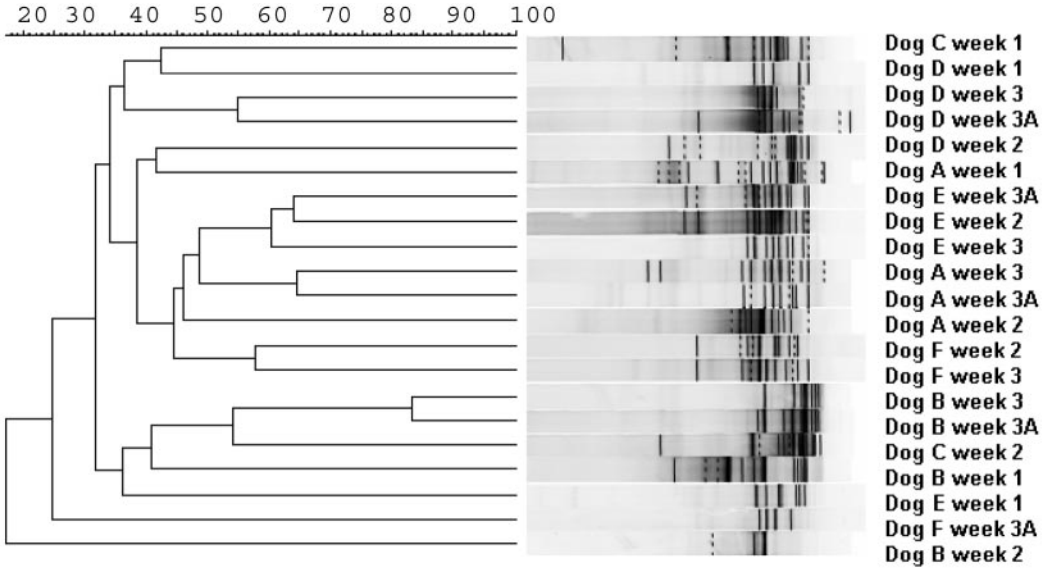

FIG. 5. Dendrogram (unweighted pair group method with arithmetic averages) generated from DGGE profiles representing similarities in banding patterns in samples from all time points collected endoscopically in six dogs (dogs A to F). The mean ± standard deviation similarity between the different time points (samples 1, 2, and 3) within individual dogs was 38.3 ± 13.3%. There was a significantly higher variation between the different time points in individual dogs than between samples taken at the same time point by two different techniques (sample 3 and sample 3A; $P = 0.003$).

ples (15). Molecular fingerprinting also allows the study of changes in individual microbial communities over time.

In this study we have demonstrated that DGGE profiles can serve as a rapid and reproducible tool for qualitative assessment of the bacterial diversity of the small intestine in dogs. Results were typically obtained within 48 h of sample collection. Duplicates of duodenal juice collected by laparotomy and needle aspiration from the same dog showed a mean similarity of 88%. This reproducibility is similar to previously reported DGGE profiles obtained from fecal samples, showing a reproducibility of 91% (23). The mean similarity of DGGE profiles from duplicates collected from the same dog was significantly higher than the mean similarity of DGGE profiles between dogs. The mean similarity of DGGE profiles between all eight dogs in group 1 was 38%, indicating that dogs have a highly diverse duodenal microflora with marked differences between individual dogs. This is consistent with previous reports suggesting, based on bacterial culture, that the microflora of the canine small intestine appears to be very variable between dogs (9). To our knowledge, molecular fingerprinting illustrating the bacterial diversity of the small intestine in dogs has not been reported previously in the literature. One study examined the influence of age, breed, and dietary fiber on bacterial diversity in fecal samples of dogs with DGGE profiles (22) and reported that individual dogs have stable and unique fecal flora.

While the DGGE profiles of duodenal juice collected by laparotomy and needle aspiration showed high reproducibility, the invasiveness of this sampling technique has obvious disadvantages. Studies of temporal variation in bacterial diversity of the small intestine and collection of clinical case material would be difficult with this method of collection. Therefore, a less invasive collection technique such as endoscopic collection of duodenal juice is preferable. Laparotomy with needle aspiration and endoscopic collection of duodenal juice showed significant correlation, based on qualitative and quantitative bacterial culture (10, 18).

We evaluated two different endoscopic collection methods, both utilizing the working channel of an endoscope: aspiration of duodenal juice through a sterile plastic tube and the collection of duodenal content with a cytology brush. We also evaluated weekly variations in the DGGE profiles. One problem that we encountered in evaluating the reproducibility of endoscopic sampling is the limited amount of duodenal juice that can be collected with the aspiration technique. At one time point, no duodenal juice could be collected at all in one of the dogs. While it was possible to collect approximately 0.5 ml of duodenal juice in all other dogs at all other time points, not enough duodenal juice was available to collect a duplicate sample at the same time point. Therefore, we were unable to evaluate the reproducibility of DGGE profiles from duodenal juice collected at the same time point by endoscopic aspiration.

In addition, in order to collect 0.5 ml of duodenal juice, a considerable amount of time (approximately 10 to 20 min) had to be invested per dog, making the collection of duodenal juice by endoscopic aspiration rather impractical for routine clinical application. Additionally, no PCR amplicon could be amplified from one sample obtained by endoscopic aspiration. This inability to amplify DNA is more likely due to carryover of inhibitory substances during DNA extraction rather than a causal effect of the collection technique. Exocrine pancreatic secretions contain considerable amounts of ribonucleases that might have not been sufficiently eliminated during DNA extraction, and these substances might have interfered in the subsequent PCR (11). Based on our experience during the development of the protocol for the DNA extraction from duodenal juice, it is crucial to obtain highly pure DNA.

The use of a cytology brush introduced through the working channel of an endoscope may be a superior method of sam-

pling the duodenal contents for several reasons: it would make collection of samples easier, faster, and, if taken in the same fashion, also more reproducible, as a more constant amount of sample would be collected. Also, in this study a PCR amplicon could be generated from DNA extracted from all cytology brushes. However, relatively few samples obtained by cytology brush were analyzed, and further samples collected with this method need to be evaluated.

Samples collected from the same dog at the same time point either by aspiration or cytology brush showed 62% similarity. While this similarity was significantly higher than the similarity between different time points in individual dogs, it was significantly lower than the similarity of duplicate duodenal juice collections with a syringe in the group 1 dogs. This could be partially explained by the differences in bacterial populations present in the intestinal lumen versus adherent to the intestinal mucosa. Quantitative and qualitative differences in microbial species between intestinal lumen and intestinal biopsy samples have been found based on bacterial culture (5). However, we speculate that collection with a cytology brush differs from an intestinal biopsy sample, since the collected sample consists mostly of intestinal fluid and some superficial mucosal cells. Therefore, this collection technique would represent a mixture of both microbial populations, and proper standardization of the sampling technique would allow comparison of the microbial community between healthy and diseased individuals.

The fecal microflora has been reported to be stable over time when analyzed by DGGE (22). In contrast, the results of studies with bacterial culture sampling at different time points suggest significant qualitative and quantitative fluctuations in the microflora of the small intestine (5, 7, 28). These studies are hampered by the fact that the repeated collection occurred either at long intervals (5, 28) or was performed in dogs with immunoglobulin A deficiency (28) or dogs that had undergone surgery that might have influenced the microflora of the small intestine (7). The authors of these studies also do not exclude the possibility that either culture techniques or ingested bacteria might have led to the observed variation.

In this study, samples of duodenal juice collected at weekly intervals in individual dogs showed sizable variations in their banding patterns. Since, for the reasons described above, no duplicate samples could be obtained at the same time point by aspiration, it is difficult to conclude if this variation was due to sampling technique or an inherent variation of the microflora of the small intestine over time. However, in dogs from group 2, approximately the same volume of duodenal juice was analyzed as in the dogs from group 1, where the duplicates showed 88% reproducibility. This would suggest that the variation in similarity between the different time points is less likely due to sampling method variation, instead reflecting genuine variation over time. The observation that "duplicate" samples collected at the same time points (samples 3 and 3A), albeit with two different collection techniques, showed significantly less variation than the weekly variation further suggests a genuine variation in the microflora of the small intestine over time. However, it cannot be definitively concluded from this study that the observed variation at different time points is solely due to changes in the small intestine microflora over time.

We attempted to collect samples from approximately the same sampling site on each study day with the distance markings located on the outside of the endoscope. However, it is possible that the bacterial composition differs between samples that are taken from locations that are only a short distance apart from each other. This variation of the microflora of the small intestine obtained during repeated sampling, regardless of the underlying cause, needs to be taken into consideration when collecting clinical samples.

With DGGE profiles, we have shown that dogs have a highly diverse bacterial microflora in the small intestine. However, important limitations of this PCR-based approach that might interfere with the interpretation of our results need to be acknowledged. Molecular detection techniques do not generally have the ability to determine whether an organism is dead or alive, and the DNA collected may at least in part be due to orally ingested microbial material (4). No information is available about the persistence of DNA in the canine intestine. Pancreatic juice contains considerable amounts of DNase, which may degrade DNA present in the lumen of the small intestine (11). Studies in mice have also shown that unfragmented aliquots of ingested DNA could be recovered from the small intestine for only up to 30 min after feeding; 18 h after feeding, no foreign DNA could be detected in the lumen of any gut compartment by Southern hybridization (17). Since food was withheld for 24 h in all dogs in this study, it appears unlikely that ingested DNA would have interfered with our results, assuming that all dogs had normal intestinal motility.

It is also assumed that DNA is equally extracted from all bacterial species. PCR may exhibit bias by targeting only predominant species that constitute more than 1% of the microflora (15). PCR, especially at higher cycle numbers, can introduce mutations, chimeras, and heteroduplexes (20). Some bacterial species are known to have multiple copies of the 16S rRNA gene, making a quantitative interpretation questionable. It has also been shown that DGGE has a limited resolving power for some PCR amplicons, as amplicons with closely related sequences may comigrate and denature at the same time. Thus, bands on DGGE may be comprised of several amplicons, underestimating bacterial diversity. Due to the exponential nature of PCR amplification, PCR is extraordinarily prone to iatrogenic contamination, and the use of negative PCR controls is crucial to monitor for contamination. Despite these limitations, DGGE profiles have been shown to be a powerful tool for assessment of bacterial diversity in environmental samples (16).

The molecular approach described in our study can facilitate the identification of bacterial species not previously cultured from the canine small intestine. Further elucidation of the complexity of the microflora of the small intestine will potentially allow us to understand the host-bacteria interactions leading to disease. The clinical significance of the diverse microflora of the small intestine in dogs and the alterations in bacterial diversity that may be present with gastrointestinal disease need to be explored further.

REFERENCES

1. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
2. **Batt, R. M., J. R. Needham, and M. W. Carter.** 1983. Bacterial overgrowth associated with a naturally occurring enteropathy in the German shepherd dog. Res. Vet. Sci. **35:**42–46.
3. **Brooks, S. P., M. McAllister, M. Sandoz, and M. L. Kalmokoff.** 2003. Cul-

ture-independent phylogenetic analysis of the faecal flora of the rat. Can. J. Microbiol. **49:**589–601.
4. **Collins, K., and L. O'Mahony.** 2002. The "unculturables," p. 25–33. *In* A. L. Hart, A. J. Stagg, H. Graffner, H. Glise, P. Falk, and M. A. Kamm (ed.), Gut ecology. Martin Dunitz Ltd., London, United Kingdom.
5. **Delles, E. K., M. D. Willard, R. B. Simpson, T. W. Fossum, M. Slater, D. Kolp, G. E. Lees, R. Helman, and G. Reinhart.** 1994. Comparison of species and numbers of bacteria in concurrently cultured samples of proximal small intestinal fluid and endoscopically obtained duodenal mucosa in dogs with intestinal bacterial overgrowth. Am. J. Vet. Res. **55:**957–964.
6. **Fromin, N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. Forestier, S. Teyssier-Cuvelle, F. Gillet, M. Aragno, and P. Rossi.** 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ. Microbiol. **4:**634–643.
7. **Harmoinen, J. A., J. M. Matto, M. L. Rinkinen, M. Wilsson-Rahmberg, and E. Westermarck.** 2001. Permanent jejunal fistula: promising method for obtaining small intestinal chyme without disturbing intestinal function. Comp. Med. **51:**252–256.
8. **Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace.** 1998. Novel division-level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. **180:**366–376.
9. **Johnston, K. L.** 1999. Small intestinal bacterial overgrowth. Vet. Clin. North Am. Small Anim. Pract. **29:**523–550.
10. **Johnston, K. L., A. Lamport, O. Ballevre, and R. M. Batt.** 1999. A comparison of endoscopic and surgical collection procedures for the analysis of the bacterial flora in duodenal fluid from cats. Vet. J. **157:**85–89.
11. **Kaneko, Y., H. Takeshita, K. Mogi, T. Nakajima, T. Yasuda, M. Itoi, H. Kuwano, and K. Kishi.** 2003. Molecular, biochemical and immunological analyses of canine pancreatic DNase I. J. Biochem. (Tokyo) **134:**711–718.
12. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. Wilkinson, and G. W. Welling.** 1995. Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61:**3069–3075.
13. **Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller.** 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. **68:**673–690.
14. **McCracken, V. J., J. M. Simpson, R. I. Mackie, and H. R. Gaskins.** 2001. Molecular ecological analysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. J. Nutr. **131:**1862–1870.
15. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
16. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek **73:**127–141.
17. **Palka-Santini, M., B. Schwarz-Herzke, M. Hosel, D. Renz, S. Auerochs, H. Brondke, and W. Doerfler.** 2003. The gastrointestinal tract as the portal of entry for foreign macromolecules: fate of DNA and proteins. Mol. Genet. Genomics **270:**201–215.
18. **Papasouliotis, K., A. H. Sparkes, G. Werrett, K. Egan, E. A. Gruffydd-Jones, and T. J. Gruffydd-Jones.** 1998. Assessment of the bacterial flora of the proximal part of the small intestine in healthy cats, and the effect of sample collection method. Am. J. Vet. Res. **59:**48–51.
19. **Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst.** 2001. Bacterial diversity in human subgingival plaque. J. Bacteriol. **183:**3770–3783.
20. **Qiu, X., L. Wu, H. Huang, P. E. McDonel, A. V. Palumbo, J. M. Tiedje, and J. Zhou.** 2001. Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Appl. Environ. Microbiol. **67:**880–887.
21. **Rutgers, H. C., R. M. Batt, F. J. Proud, S. H. Sorensen, C. M. Elwood, G. Petrie, L. A. Matthewman, H. A. F. Van Hijfte, A. Boswood, M. Entwistle, and R. H. Fensome.** 1996. Intestinal permeability and function in dogs with small intestinal bacterial overgrowth. J. Small Anim. Pract. **37:**428–434.
22. **Simpson, J. M., B. Martineau, W. E. Jones, J. M. Ballam, and R. I. Mackie.** 2002. Characterization of fecal bacterial populations in canines: effects of age, breed and dietary fiber. Microb. Ecol. **44:**186–197.
23. **Simpson, J. M., V. J. McCracken, H. R. Gaskins, and R. I. Mackie.** 2000. Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. Appl. Environ. Microbiol. **66:**4705–4714.
24. **Simpson, J. M., V. J. McCracken, B. A. White, H. R. Gaskins, and R. I. Mackie.** 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J. Microbiol. Methods **36:**167–179.
25. **Suau, A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D. Collins, and J. Dore.** 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. **65:**4799–4807.
26. **Temmerman, R., L. Masco, T. Vanhoutte, G. Huys, and J. Swings.** 2003. Development and validation of a nested-PCR-denaturing gradient gel electrophoresis method for taxonomic characterization of bifidobacterial communities. Appl. Environ. Microbiol. **69:**6380–6385.
27. **Willard, M. D., R. B. Simpson, E. K. Delles, N. D. Cohen, T. W. Fossum, D. Kolp, and G. Reinhart.** 1994. Effects of dietary supplementation of fructooligosaccharides on small intestinal bacterial overgrowth in dogs. Am. J. Vet. Res. **55:**654–659.
28. **Willard, M. D., R. B. Simpson, T. W. Fossum, N. D. Cohen, E. K. Delles, D. L. Kolp, D. P. Carey, and G. A. Reinhart.** 1994. Characterization of naturally developing small intestinal bacterial overgrowth in 16 German shepherd dogs. J. Am. Vet. Med. Assoc. **204:**1201–1206.