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Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA to monitor changes in mouse gut bacterial communities during *Salmonella enterica* serovar Enteritidis latent infection

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ABSTRACT. Changes in intestinal microbial flora during a 4-week period of *Salmonella enterica* serovar Enteritidis colonization in resistant mice (latent carrier animals) were evaluated using a culture independent method involving denaturing gradient gel electrophoresis. The contents of the ileocecal portion of the intestines produced 26 bands. Fifty-seven percent of the bands were expressed in more than 80% of the samples. Forty percent of the bands present in the negative control were common to all samples, and 60% differed from those obtained 12 h and 1, 5, 10, and 28 days post-inoculation (PI). A dendrogram distinguished the negative control as the external group, and 2 clusters were formed with 76% similarity, separating the 12-h PI and 3-day PI time points from the others. These groupings were also revealed through multivariate analysis in a principal component analysis and the

Venn diagram. The production of interferon γ 12 h and 3 days PI may explain this brief imbalance in microbiota that was quickly reversed in the subsequent days. These findings demonstrate that *S. enterica* serovar Enteritidis can colonize the gut and persist in balance with the microbiota of resistant hosts.

Key words: *Salmonella* infection; Asymptomatic carrier; Latent hosts; Intestinal microbial community; DGGE; Pathogen-host interaction

INTRODUCTION

The intestinal microbiota is molded by symbiotic interactions between the host organism and autochthonous microorganisms. These interactions are influenced by many conditions inherent to the gut, such as nutrient availability, pH, and host immunity (Suzuki et al., 2004). During an intestinal infection, the pathogen-host interaction may result in varying degrees of inflammation related to the levels of cytokines produced (Kaiser et al., 2000). Depending on the inflammation level, the intestinal microbial community may be imbalanced, which favors pathogen growth (Santos et al., 2009).

One of the mechanisms used by pathogenic *Salmonella* to invade a host organism involves the induction of an immune defense that shifts the balance of protective microbiota in favor of pathogen entry (Stecher et al., 2007). In latent carriers, however, *Salmonella* invades the intestinal mucosa without triggering an intensive host immune response, intercalating periods of intracellular and extracellular dissemination. Evidence of imbalance in the gut microbiota is lacking in the setting of bacterial invasion of latent hosts, but confirmation of such a mechanism is important because *Salmonella* eventually becomes part of the microbial flora in latently infected animals, which continue to shed the disease-causing agents intermittently without showing symptoms of infection (Wallis, 2006; Pasmans et al., 2008).

The objective of this study was to evaluate the dynamics of the intestinal bacterial community during the colonization of *Salmonella* in a latent host. The study was carried out during a 4-week period of *S. enterica* serovar Enteritidis (SE) infection in resistant mice, which are latent carrier animals. Because the majority of intestinal microorganisms cannot be cultured using conventional techniques (Suau et al., 1999; Vaughan et al., 2000), we used molecular polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis with 16S ribosomal DNA (rDNA) amplicons.

MATERIAL AND METHODS

Experimental infection

All animal procedures were approved by the Local Animal Ethics Committee and performed according to the legal requirements of the scientific community. A group of 27 C57BL/6-Bcgr mice aged 7 weeks and weighing 16-20 g were inoculated intragastrically with 5 x 10^8 CFU SE phage type 1 (SE PT1). Another group of 9 mice was inoculated intragastrically with an equal volume of water as negative controls (NC). At 12 h and 1, 2, 3, 4, 5, 10, 15, and 28 days post-inoculation (PI), 3 mice from the infected group and 1 from the NC

group were killed. The ileum, cecum, and content of the ileocecal portion of the intestine were collected for detection of SE PT1 using a real-time quantitative PCR assay on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Foster City, CA, USA), following a procedure published by Maciel et al. (2011).

To test the intermittent shedding of *Salmonella*, which characteristically occurs in latent carriers, we performed bacteriological culture of feces at each PI time point following a procedure published by the American Public Health Association (1992). Clinical signs, such as depression and diarrhea, were evaluated throughout the experimental period.

Intestinal bacterial diversity analysis

The microbial community of the contents of the ileocecal portion of the intestine was analyzed at various PI time points via PCR-DGGE. The DNA samples from each mouse were extracted at identical time points and purified using a Wizard® SV DNA Purification System (Promega Corporation, Madison, WI, USA) according to manufacturer recommendations. The DNA samples were then standardized to 100 ng and mixed in equal quantities for use as templates in PCR analyses. The mixed DNA samples were amplified using primers for the variable V3 region of the 16S rDNA. A region of approximately 200 bp was amplified with the primers F357 (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGTACGGGAGGCAGCAG-3') and R518 (5'-ATTACCGCGGCTGCTGG-3'). The underlined sequence in F357 indicates the GC clamp required for DGGE analysis (Muyzer et al., 1993). Each 50-µL PCR mixture contained 10 ng DNA sample, 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), 200 µM of each deoxyribonucleoside triphosphate (Invitrogen), 0.2 µM sense and antisense primers (Invitrogen), 3.0 mM $MgCl_2$ (Invitrogen), and 1.25 U Platinum® Taq DNA polymerase (Invitrogen). The amplification cycle consisted of an initial denaturation of 5 min at 94°C; followed by 35 cycles of 1 min at 94°C, 2 min at 60°C, and 1 min at 72°C; and a final extension of 30 min at 72°C. The DNA template of SE PT1 was used as a positive control in the PCR assay, whereas template DNA was omitted from the reaction mixture for the NC group. To visualize PCR products, we electrophoresed a 5-µL suspension on 1% agarose gels (Invitrogen) in 1X Tris-boric acid-ethylenediaminetetraacetic acid buffer and then stained the gels with SYBR® Safe (Invitrogen) and examined them under ultraviolet light. Bands were excised, and the DNA was purified from gel slices using a PCR Clean-Up System Kit (Promega).

A mutation detection system (MAXFILL, BioAgency, USA) was used for the DGGE analysis. A 20-µL sample of the PCR products of the rDNA sequences was analyzed on an 8% (w/v) polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) consisting of a 45-70% denaturing concentrate [100% denaturing agent corresponded to 7 M urea and 40% (v/v) deionized formamide]. The gels were run in 0.5X TAE buffer (20 mM Tris acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM disodium ethylenediaminetetraacetic acid; Merck, Darmstadt, Germany) at a constant voltage of 70 V (100 mA) for 16 h at 60°C. Bands were visualized via staining with silver nitrate (Merck), and images were acquired using the Image Master™ 2D Platinum software (GE Healthcare, Uppsala, Sweden) at a spatial resolution of 300 dpi. Each band was considered an operational taxonomical unit, and bands with identical migration positions were considered to be part of the same operational taxonomical unit. Reproducibility was assessed via electrophoresis of independent amplifications of the same DNA sample run on the same gel.

Interferon γ (IFNγ) detection in serum

Serum from the mice of each group described above was tested for the cytokine IFNγ with an enzyme-linked immunosorbent assay (Ready-Set-Go!®, ELISPOT Kits, eBioscience, Inc., San Diego, CA, USA) according to manufacturer instructions. All samples were assayed in duplicate, and optical density was measured on a VersaMax™ Microplate Reader (Molecular Devices Inc., Sunnyvale, CA, USA) at 450 nm. These data were analyzed with one-way analysis of variance followed by the Tukey multiple comparison test using the Prism GraphPad version 5.03 software. Differences were significant at $P \leq 0.05$.

Multivariate analysis

Three methods of multivariate analysis were applied to compare the results: cluster analysis, cluster analysis preceded by principal component analysis (PCA), and Venn diagrams. The cluster analysis was based on a binary matrix representing the presence/absence of bands in a sample. The similarity matrix was obtained according to the Dice similarity coefficient (S_D). S_D (values between 0 and 1) is an arithmetic determination of the degree to which banding patterns are alike (i.e., contain the same bands). S_D was calculated using the formula $S_D = 2n_{AB} / (n_A + n_B)$, where n_A is the number of DGGE bands in line A, n_B is the number of bands in line B, and n_{AB} is the number of bands common to lines A and B. Clusters were determined by sequentially comparing the patterns and constructing a relatedness dendrogram reflecting the relative similarities. The S_D calculation and cluster analysis were performed using the PAST software (Hammer et al., 2001). PCA was applied to reduce the dimension of the original variables using the StatSoft Statistica 8.0.550 software, and Venn diagrams were constructed manually, taking into account the intersections of the obtained DGGE bands.

RESULTS

All infected mice presented a latent *Salmonella* infection with absence of clinical symptoms and intermittent shedding of bacteria in feces (at 12 h and 1, 10, and 28 days PI) diagnosed via bacteriological culture. Analysis of the PCR-DGGE banding pattern of the microbes in the contents of the ileocecal portion of the intestines identified 26 distinct bands when all samples were combined; a mean of 17 distinct bands was observed across treatments (Table 1; Figure 1A). The percentage of samples containing a specific frequency of bands was calculated to characterize the distribution frequency of bands among various samples. The result revealed a similar bacterial community: most of the bands (57%) were expressed in ≥80% of the samples. Of all the bands present in the NC group, 40% were common to all samples and 60% differed from the 12-h PI and 1-, 5-, 10-, and 28-day PI time points when SE was present in the cecum (see Table 1).

S_D was determined and a dendrogram of the microbial profiles from the PCR-DGGE fingerprints was constructed (Figure 1B). The high percentage of common bands resulted in a high S_D (see Table 1) and clusters with a similarity index of >70% were formed (see Figure 1B). The dendrogram distinguished the NC group as an external group, and 2 clusters were formed with 76% similarity, separating the 12-h PI and 3-day PI time points from the other times (see Figure 1B). These groupings were also revealed by the PCA (Figure 1C). In a PCA score plot

(PC1 *vs* PC2), all samples were grouped together with positive scores in PC1. Along the PC2 axis, the 12-h PI and 3-day PI time points were grouped negatively, as well as the presence of IFNγ in serum, whereas the other time points were grouped positively (see Figure 1C).

Table 1. Distribution of *Salmonella* Enteritidis (SE) in intestine of resistant mice, titer of interferon gamma (IFNγ) in serum, and indices of diversity and similarity calculated from DGGE patterns of 16S rDNA from intestinal and ileo-cecal content.

	Time post-inoculation (PI)									
	12 h	1 day	2 days	3 days	4 days	5 days	10 days	15 days	28 days	NC
SE colonization[a]										
Intestine[b]	4×10^2	2×10^2	-	-	1×10^3	1.5×10^3	4×10^3	-	6×10^4	-
Ileo-cecal content	3×10^3	3×10^2	1.5×10^4	-	-	-	7.6×10^5	-	6×10^3	-
IFNγ in serum (pg/mL)	48.2	-	-	41.2	49.6	-	693	1334	-	-
Diversity index (total of distinct bands = 26)										
Species richness (*S*)	18	18	19	17	19	15	14	17	18	12
Similarity index										
Dice similarity (S_D)	0.77	0.84	0.86	0.75	0.80	0.82	0.78	0.86	0.75	0.74

NC = negative control. [a]Number of bacterial cells based on quantitative real-time assay, according to Maciel et al. (2011). [b]Presence of *Salmonella* in ileum at 4-, 5-, and 10-day PI and in cecum at 12-h, 1-, 5-, 10-, and 28-day PI time points.

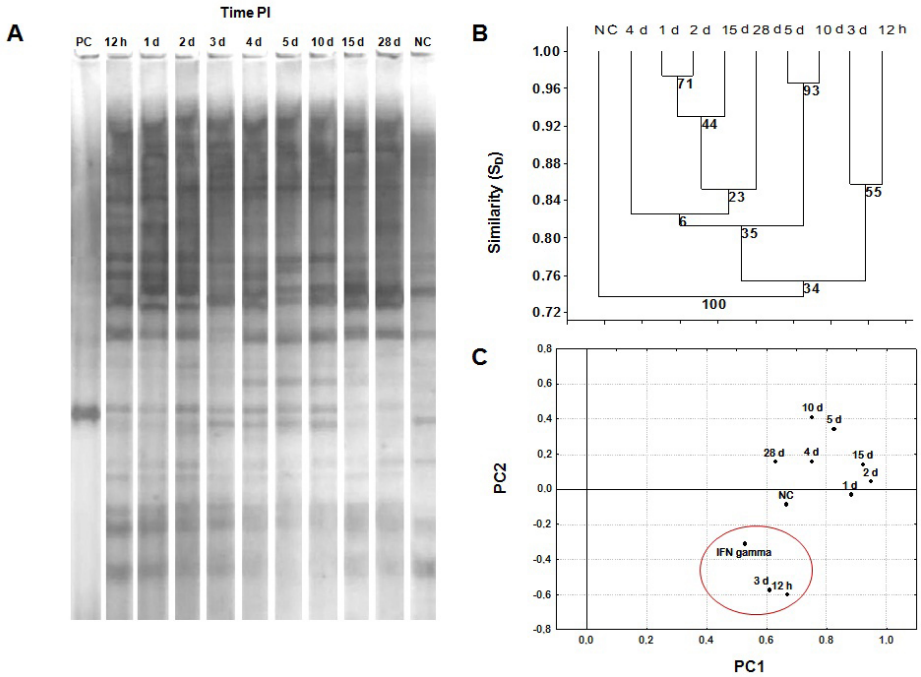

Figure 1. A. PCR-DGGE banding pattern of 16S rDNA from intestinal content of ileocecal portion after oral challenge with *Salmonella* Enteritidis (SE) in resistant mice. **B.** Dendrogram representing the analysis of Dice similarity (S_D) by paired groups of PCR-DGGE banding pattern between different times [hours (h) or days (d)] of SE post-inoculation (PI). **C.** Score plot of principal component analysis of PCR-DGGE profile and detection of IFNγ in serum. PC = positive control using SE phage type 1; NC = negative control using intestinal content of non-infected mice.

The Venn diagram shows that 13 bands were common to samples from 12 h and 1, 2, and 3 days PI (Figure 2A). Ten of these bands also appeared in the NC group, whereas the other 3 bands were present only in the 12-h PI and 1- and 2-day PI samples and absent in 3-day PI and NC samples (see Figure 2A). The samples from 12 h and 3 days PI showed 2 common bands that were absent in the other samples (see Figure 2A). Twelve bands were common to samples from 4, 5, 10, 15, and 28 days PI, and 8 were also present in the NC sample (Figure 2B). Three distinct bands were present only in samples taken 4 days PI and another 3 were present only in samples taken 28 days PI; 1 band was present only in samples taken 5 and 10 days PI (see Figure 2B).

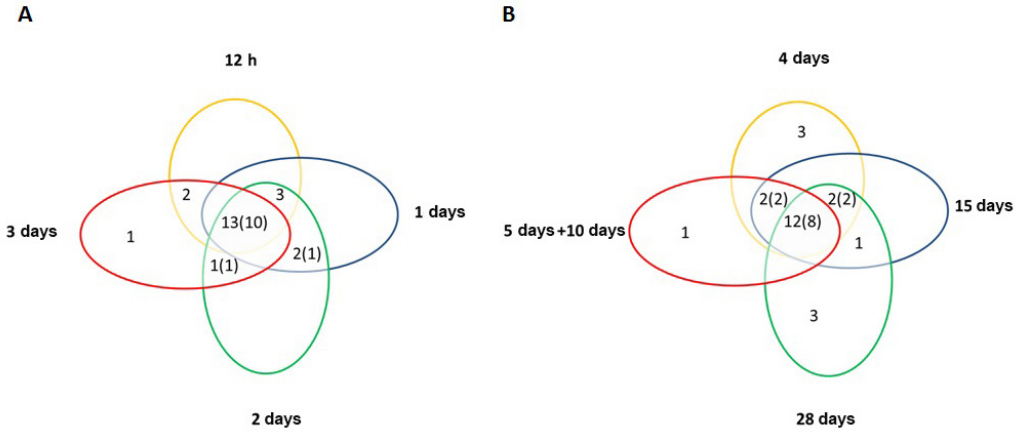

Figure 2. Venn diagram showing the richness of bands detected by DGGE profiles. Numbers in parentheses represent the number of bands common with the negative control samples. **A.** Comparison of bands in samples from 12 h and 1, 2, and 3 days post-inoculation (PI) with negative control. **B.** Comparison of bands in samples from 4, 5, 10, 15, and 28 days PI with negative control.

DISCUSSION

The molecular interaction between host immune systems and microbial pathogens may cause changes in the composition of the intestinal microbiota. A healthy intestine contains a balanced composition of bacterial symbionts, commensals, and pathobionts. During inflammatory processes, a reduction in symbionts or an increase in bacteria may be observed, reflected in the diversity and similarity indices (Round and Mazmanian, 2009). In the case of *Salmonella* infection, the production of IFNγ in the early phase of intestinal inflammation can alter luminal gut conditions, thereby causing an imbalance in the ecology of resident microbiota, which may favor the competitivity of pathogen growth (Stecher et al., 2007; Santos et al., 2009). In our experiment, the production of IFNγ during early periods (from 12 h until 3 days PI) may explain the brief imbalance in the microbiota, which was quickly reversed in subsequent days. The cluster and PCA analyses separated 12-h and 3-day PI time points from the other times (see Figure 1), showing a temporary change in the intestinal microbial community. Despite this perturbance, the high similarity indices (>75%) during all experimental observations indicated that the SE infection in resistant mice did not interfere significantly

with the ecology of resident microbiota and that, even within this balanced intestinal environment, SE could invade the intestine and persist for up to 28 days (see Table 1).

The majority of enteric pathogens cannot persistently colonize the gastrointestinal tract, possibly owing to the presence of an intact microbiota. Nevertheless, our findings demonstrate that *Salmonella* colonizes the gut and remains in balance with the microbiota of latent hosts. A much different situation occurs during salmonellosis gastroenteritis in susceptible hosts in which infectious bacteria disrupt the microbial ecology of the gastrointestinal tract and colonize the gut (Barman et al., 2008).

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