J Infect Chemother (2013) 19:673–676
DOI 10.1007/s10156-012-0541-1

ORIGINAL ARTICLE

Use of culture-independent analysis to reveal alteration of intestinal microflora by heat-killed *Lactobacillus pentosus* in a mouse model of endogenous sepsis

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Received: 25 September 2012 / Accepted: 16 December 2012 / Published online: 1 January 2013

Abstract In this study we evaluated alteration of intestinal microflora by terminal-restriction fragment length polymorphism (T-RFLP) analysis and quantitative PCR (qPCR) for specific microbes. The effects of orally administered heat-killed *Lactobacillus pentosus* strain b240 (HK-b240) in immunosuppressed mice with endogenous *Pseudomonas aeruginosa* sepsis was estimated. By T-RFLP analysis, 5 dominant operational taxonomic units (OTUs) including *Bacteroides* spp. (OTU460) and *Lactobacillus* spp. (OTU657) were consistently observed, irrespective of treatment, at all time points. A significantly higher population of segmented filamentous bacteria (SFB) was observed by qPCR after 3 weeks of HK-b240 administration; thereafter, the difference was not sustained during immunosuppression and progression of sepsis. Although not significant, *Lactobacillus* spp. accounted for a larger population in the HK-b240-treated group. In conclusion, this study demonstrated successful application of culture-independent assays for evaluating biological agents by detecting changes in microflora even if the protection was not sufficient to result in significant survival change.

Keywords Terminal-restriction fragment length polymorphism (T-RFLP) · Quantitative PCR (qPCR) · Lactobacillus · Filamentous bacteria · Intestinal microbiology

Introduction

Conventional methods for flora analysis that rely on cultivation of bacteria under anaerobic conditions are time-consuming and laborious, and more crucially, miss a large population of bacteria that are difficult to culture or cannot be cultured. Recent advances in techniques using 16S ribosomal RNA sequences, which enable us to estimate specific bacterial groups, have begun to yield new insights into the diversity, organization, and dynamics of gut microbiota in health and disease [1]. Terminal-restriction fragment length polymorphism (T-RFLP) analysis is a practical way to characterize microbiota, because the relative abundance of each operational taxonomic unit (OTU) is measured rapidly and reproducibly [2–4]. Quantitative PCR (qPCR) is another technique that is suitable for estimating populations of specific bacteria with greater sensitivity [4–7].

In this study we examined the effects of oral administration of heat-killed *Lactobacillus pentosus* (formerly *Lactobacillus plantarum*) strain b240 (HK-b240) on intestinal microflora and gut-derived *Pseudomonas aeruginosa* sepsis in immunosuppressed mice, in which *P. aeruginosa* bacterial translocation from the gut to blood was induced by disruption of intestinal microflora by administration of cyclophosphamide [8]. Alterations in intestinal microflora, including segmented filamentous bacteria (SFB), which were identified for their critical roles in maturation of gut and host immune systems[9, 10], were evaluated by culture-independent methods including T-RFLP and qPCR for specific organisms such as *Lactobacillus* spp. and SFB.

Electronic supplementary material The online version of this article (doi:10.1007/s10156-012-0541-1) contains supplementary material, which is available to authorized users.

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Materials and methods

Strains, culture, and preparation of bacteria

P. aeruginosa strain D4 [11] was grown on an NAC agar plate (Eiken Chemical, Tokyo, Japan) at 35 °C for 18 h, and suspended in sterile 0.45 % saline in a final inoculum of 1×10^7 CFU/ml. *L. pentosus* strain b240 (ONRIC b0240) was isolated from fermented tea leaves. It was cultured in MRS broth (Becton–Dickinson, Sparks, MD, USA) under aerobic conditions for 24 h at 33 °C, and then washed twice with sterile saline to remove any metabolic substances associated with it. It was then killed by autoclaving at 121 °C for 15 min. The heat-killed *L. pentosus* strain b240 (HK-b240) solution was freeze-dried and dissolved in 0.45 % sterilized NaCl solution before use.

Mice

Five-week-old specific-pathogen-free female ICR mice were purchased from Charles River Japan (Kanagawa, Japan). The experimental protocol was approved by the institutional animal care and use committee at Toho University School of Medicine (approval number 09-51-54).

Induction of gut-derived *P. aeruginosa* sepsis and treatment with HK-b240

Gut-derived *P. aeruginosa* sepsis was induced as described elsewhere with some modifications [8]. In brief, 10 mg/body weight of HK-b240 in 0.45 % sterile saline was administered via an esophagus tube once a day for 21 days (Day −21 to Day −1). A suspension of *P. aeruginosa* D4 was given to mice as drinking water for 3 days in a final inoculum of 6–7 $\times$ 10^6 CFU/ml (from Day 0 to Day +2). Then, 150–200 mg/kg cyclophosphamide (CY) was injected intraperitoneally on Day +3 and Day +7. A total of 0.1–0.2 ml heart blood was inoculated on an NAC agar plate to detect *P. aeruginosa* bacteremia.

Terminal-restriction fragment length polymorphism (T-RFLP) analysis

DNA isolation, PCR amplification, restriction enzyme digestion, and T-RFLP analysis were performed according to the procedure of Nagashima et al. [3] using Ex Taq (Takara Bio, Otsu, Japan) and primers 5′-HEX-labeled 516f and 1510r (Supplemental table). The PCR product was then purified by use of the QIAquick-spin PCR purification kit (Qiagen, Chatsworth, CA, USA), and digested with *Bsl*I (New England BioLabs, Beverly, MA, USA). The fragments were subjected to electrophoresis on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using GeneScan Rox Standard 1200 (Applied Biosystems) as size standards. The results were analyzed on GeneMapper (Applied Biosystems).

Quantitative PCR (qPCR)

Quantitative PCR was performed on mouse fecal DNA by using SYBR Green ER qPCR SuperMix for ABI Prism (Invitrogen, Carlsbad, CA, USA). Amplification and detection were performed on an ABI Prism 7000 sequence detection system (Applied Biosystems). A standards curve was drawn on the basis of the C_T value of the stepwise-diluted purified reference PCR product of each partial 16S rDNA. Primers 27f and 1492r and boiled lysate of cultured *P. aeruginosa* D4 or *L. pentosus* ONRIC b0240 were used to generate reference PCR products for total bacteria, *P. aeruginosa*, and *Lactobacillus* spp. (Supplemental table). For SFB, which cannot be cultured in vitro, a reference PCR product was generated by primers SFB-F and SFB-R and DNA from a Day −21 fecal sample from a mouse treated with 0.45 % sterile-saline (Supplemental table). Nucleotide sequencing of the reference PCR product was performed by using the Big Dye Terminator v3.1 kit and an ABI 310 Genetic Analyzer (Applied Biosystems). The proportion of each bacterial group of interest was estimated by dividing the copy number of specific 16S rDNA by that of total bacterial 16S rDNA.

Statistical analysis

GraphPad Prism 5 (Graphpad Prism Software, La Jolla, CA, USA) was used for statistical analysis. A survival curve was estimated by use of the log-rank (Mantel–Cox) test. Group differences were compared by use of the two-tailed unpaired *t* test. A *P* value of <0.05 was considered significant.

Results

General trend of intestinal microflora in mice under immunosuppression

Mice intestinal microflora was analyzed by use of mouse fecal samples obtained on Day −21, Day 0, Day +4 and Day +7 (Fig. 1). The stool of mice before the experiment (Day −21) produced 5 dominant OTUs, namely, OTU657, OTU460, OTU166, OTU136, and OTU106. According to previous reports of 16S rDNA nucleotide sequences, OTU460 and OTU657 were *Bacteroides* spp. and *Lactobacillus* spp., respectively [1]. During the experimental period, the above 5 OTUs were consistently dominant in T-RFLP analysis, irrespective of treatment, at all time points.

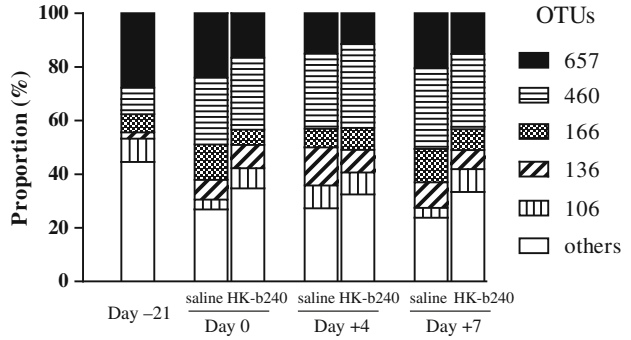

Fig. 1 Terminal-restriction fragment length polymorphism (T-RFLP) profiles of mice with endogenous gut-derived *Pseudomonas aeruginosa* sepsis. Each group of 5-week-old female ICR mice (6 fecal DNA samples per group) was given 10 mg/day heat-killed *Lactobacillus pentosus* strain b240 (HK-b240) or 0.45 % NaCl solution (saline) via an esophagus tube for 3 weeks (from Day −21 to Day −1). Thereafter, endogenous sepsis was induced by feeding drinking water containing 6–7 × 10^6 CFU/ml *P. aeruginosa* strain D4 for 3 days (from Day 0 to Day +2), and then 200 mg/kg cyclophosphamide was injected intraperitoneally twice (on Day +3 and Day +7). Fecal samples of mice on Day −21, Day 0, Day +4, and Day +7 were subjected to T-RFLP analysis. Average populations larger than 10 % in any specimen are shown in this figure. OTU 657 and OTU 460 are considered to be *Lactobacillus* spp. and *Bacteroides* spp., respectively

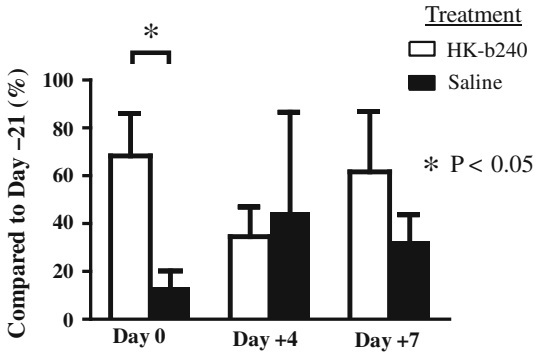

Fig. 2 Population of segmented filamentous bacteria (SFB) in mouse intestinal flora. The relative proportion compared with that before the experiment (Day −21) was assessed by quantitative PCR (qPCR) analysis specific for SFB (3 fecal DNA samples in each group). Values are means ± standard errors of the means. Treatments were as described in Fig. 1. *$P < 0.05$ between groups

Population of *Lactobacillus* spp. estimated by T-RFLP and qPCR analysis

We examined the relative population of *Lactobacillus* spp. (OTU657) found on Day 0, Day +4, and Day +7 compared with that found on Day −21 for each mouse. By T-RFLP analysis, the population of OTU657 was higher in the HK-b240 group at all 3 time points, although not statistically significant (Supplemental Fig. 1a). Consistently, the same trend was observed by qPCR analysis, when the population of *Lactobacillus* spp. was compared with that on Day −21 for each individual mouse (Supplemental Fig. 1b).

Altered population of SFB by qPCR analysis

Although the population of SFB was too small to yield a detectable signal in T-RFLP analysis, it could be estimated by qPCR: a reference PCR product of SFB 16S rDNA gene was successfully generated and sequenced, and was confirmed to be consistent with that of GenBank AP012209.

Interestingly, a significantly larger proportion of SFB was found on Day 0 in the HK-b240 group as compared with the level of Day −21 before administration of HK-b240 (Fig. 2). The difference in SFB signals between the untreated and HK-b240-treated groups disappeared at later time points.

Effects of HK-b240 on survival of immunosuppressed mice with gut-derived *P. aeruginosa* sepsis

In the mouse model of endogenous sepsis, mice began to die because of *P. aeruginosa* sepsis 3–5 days after the second injection of cyclophosphamide, as previously reported. Although *P. aeruginosa* was recovered from murine blood on the NAC agar plate, no detectable signal of *P. aeruginosa* was observed by either T-RFLP or qPCR at any timepoint. Survival in the HK-b240-treated group was slightly higher, but did not reach statistical significance (Supplemental Fig. 2).

Discussion

Recent advances in techniques using 16S rDNA sequences have shed new insight into the diversity, organization and dynamics of gut microbiota in health and disease [1, 12]. As shown in this study, culture-independent methods were useful to characterize microbiota in a model of gut-derived sepsis: T-RFLP was suitable for observing dynamic and global changes of microflora in hosts. In contrast, qPCR was necessary for more subtle and fine alterations such as those in SFB. Although expected, the proportion of *P. aeruginosa* was too subtle to yield detectable changes even by qPCR, which seems consistent with a past study [8].

A striking finding of this study was the significantly larger population of SFB in mice treated with HK-b240 on Day 0. Although the exact mechanisms underlying these changes in SFB population await future investigation, one possibility is that manipulations such as

P. aeruginosa challenge and cyclophosphamide treatment may attenuate the effects of HK-b240 on enhancement of SFB in mice. SFB are clostridia-related, spore-forming, non-culturable Gram-positive bacteria with unique morphology. The critical function of SFB in maturation of gut and host immune systems have begun to be elucidated, and SFB are reported to be tightly attached to the epithelial cells around Peyer's patches [9, 10]. It has also been reported that SFB colonization of intestinal tracts enhances luminal IgA production, although there are no IgA data available for mice with *P. aeruginosa* sepsis treated with HK-b240.

Although a significant improvement in survival by oral administration of HK-b240 was not demonstrated in our model, several investigators have reported beneficial actions of HK-b240 in different experimental models: Ishikawa et al. [13] reported a protective role of orally administered HK-b240 in a non-compromised mouse model of *Salmonella* intestinal infection. Tanaka et al. [14] showed that HK-b240 suppressed pneumonia induced by *Streptococcus pneumoniae* in healthy mice. In those two studies, immunocompetent mice were used to evaluate effects of HK-b240 whereas an immunosuppressed mouse model of *P. aeruginosa* sepsis was assessed for the first time in our study. Yamahira et al. [15] have reported that Peyer's patch cells stimulated with HK-b240 produce higher amounts of IgA, which may be associated with beneficial effects of HK-b240 in vivo. In this regard, cyclophosphamide treatment in our model may be one possible reason for the reduced effects of HK-b240, because this immunosuppressive drug is known to affect the numbers of a variety of cells, not only neutrophils but also other immune cells including lymphocytes and plasma cells.

In conclusion, in this study we used the culture-independent methods T-RFLP and qPCR to evaluate the intestinal microbial community in *P. aeruginosa*-gut derived sepsis. It is likely that oral administration of HK-b240 induces changes in intestinal microflora, although its efficacy and mechanism of action remained to be elucidated. Further study may be necessary to define the protective roles of HK-b240 in gut-associated infections in immunosuppressed individuals.

Acknowledgments The authors thank Dr Sanae Okada (Tokyo University of Agriculture) for his generous gift of *L. pentosus* strain b240.

Conflict of interest None.

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