Gastric Microbiota Alteration in Klebsiella pneumoniae-Caused Liver Abscesses Mice

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Abstract

Gastric microbiota provides a biological barrier against the invasion of foreign pathogens from the oral cavity, playing a vital role in maintaining gastrointestinal health. Klebsiella spp. of oral origin causes various infections not only in gastrointestinal tract but also in other organs, with Klebsiella pneumoniae serotype K1 resulting in a liver abscess (KLA) through oral inoculation in mice. However, the relationship between gastric microbiota and the extra-gastrointestinal KLA infection is not clear. In our study, a 454 pyrosequencing analysis of the bacterial 16S rRNA gene shows that the composition of gastric mucosal microbiota in mice with or without KLA infection varies greatly after oral inoculation with K. pneumoniae serotype K1 isolate. Interestingly, only several bacteria taxa show a significant change in gastric mucosal microbiota of KLA mice, including the decreased abundance of Bacteroides, Alisptipes and increased abundance of Streptococcus. It is worth noting that the abundance of Klebsiella exhibits an obvious increase in KLA mice, which might be closely related to KLA infection. At the same time, the endogenous antibiotics, defensins, involved in the regulation of the bacterial microbiota also show an increase in stomach and intestine. All these findings indicate that liver abscess caused by K. pneumoniae oral inoculation has a close relationship with gastric microbiota, which might provide important information for future clinical treatment.

Key words: Klebsiella pneumoniae serotype K1, defensins, gastric mucosal microbiota, High-throughput pyrosequencing, liver abscess

Introduction

Klebsiella pneumoniae as one of the most important pathogens leads to many infections including nosocomial and community-acquired infections. K1 or K2 serotype K. pneumoniae-caused pyogenic liver abscess (KLA) is a highly invasive community-acquired infection and often develops serious complications (Siu et al. 2012). Previous studies show that liver abscess infection can be caused by many pathogens and that K. pneumoniae causes less than 10% liver infection (Lee et al. 1991; Zibari et al. 2000). Recently the rate of KLA infection has increased greatly even up to 70% in some regions (Kuo et al. 2013). And drug-resistant K. pneumoniae isolated from KLA infection has been reported to be increased in Beijing (Li et al. 2014).

It is well known that oral-gastrointestinal tract is one of the main pathways for the foreign pathogen, which...
causes human infection. Klebsiella spp. of oral origin causes various diseases not only in the gastrointestinal tract but also in other organs. Atarashi et al. (2017) hold that oral Klebsiella spp. colonizing the intestines causes inflammatory bowel disease (IBD). It has also been identified that oral inoculation of K. pneumoniae K1 or K2 serotype isolates causes liver abscess infections in mice (Tu et al. 2009; Chen et al. 2014).

Gastric microbiota provides a biological barrier against the invasion of foreign pathogens entering the oral pathway, playing a vital role in maintaining gastrointestinal health. (Bik et al. 2006). For example, Helicobacter pylori can successfully colonize stomach tissues via the oral pathway what results in infection. It has been identified that H. pylori infections can change the overall composition of stomach microbiota (Lofgren et al. 2011). Probiotics have been used for clinical treatment of gastritis caused by H. pylori (Sheu et al. 2002; Lesbros-Pantoflickova et al. 2007). But the relationship between the microbiota in gastric mucosal and extra-gastrointestinal liver abscess infection caused by K. pneumoniae through the oral pathway is not clear.

### Experimental

#### Materials and Methods

**Mouse model of KLA infection.** In our previous study, K. pneumoniae K1 serotype clinical isolate Kp1002 was identified to cause KLA infection in C57BL/6 mice by oral inoculation (Chen et al. 2014). In the present study, six-week-old male C57BL/6 mice were orally inoculated with 10^6 CFU of Kp1002 using a 21-gauge feeding needle as previously described, with the phosphate buffered saline (PBS) given to the healthy control. All the mice were killed 48 h after oral inoculation. The serum, stomachs and cecum samples were retrieved for the following analysis. Mice with Kp1002 inoculation were divided into two groups: mice with KLA infection (KLA group) and mice without KLA infection (NKLA group) according to the criterion in our previous study (Chen et al. 2014). Five mice were randomly selected from each group and their samples were prepared for the following analysis. Mice in this study were raised in the Specific Pathogen Free (SPF) animal house. All the animal experiments in this study were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhejiang Medical University (2013–012) and the Animal Welfare and Ethical Committee of Hebei University (2018026).

**DNA extraction and high-throughput sequencing analysis.** After mice sacrificed, the stomachs of the mice were isolated, with the corpus tissues divided and used in DNA extraction. DNA was extracted from 25 mg corpus tissue using a PureLink™ Genomic DNA Mini Kit (Invitrogen, USA) according to the manufacturer’s instructions, with addition of lysozyme at the concentration of 20 mg/ml (Sigma-Aldrich, USA) at the beginning of cell lysis. DNA concentrations were tested by NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation). DNA integrity and size were checked by agarose gel electrophoresis. The DNA was stored at –80°C for the 16S rRNA gene sequencing analysis. 16S rRNA gene was amplified by PCR with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCGCGGCTGTGAC-3′) (Ling et al. 2015, Chen et al. 2018). The 16S rRNA gene amplification products were sequenced by the 454 Life Sciences genome sequencer FLX system (Roche, Switzerland). The metagenomic sequence data were deposited in the NCBI Sequence Read Archive under Accession number SRP111617. The sequencing data processing methods were described in our previous study (Ling et al. 2015, Chen et al. 2018).

**Biosample collection and analysis of defensins and cytokines.** After mice sacrificed, the stomachs and cecum of the mice were isolated. The contents of the stomachs or cecum were collected as previously described (Li et al. 2015). The contents were removed and washed off with an isopycnic 0.09% NaCl solution. The washout material was centrifuged at 10 000 rpm for 20 min at 4°C. Then, the supernatant was harvested for the analysis of the cytokines using an ELISA test kit (Mlbio, Shanghai, China). Serum samples were separated and stored at –80°C for the analysis of the cytokines using an ELISA test kit (Mlbio, Shanghai, China).

**Data processing and statistical analysis.** Mothur (http://www.mothur.org) was used for diversity and taxonomy-based analyses at a 97% similarity level as previously described (Ling et al. 2015, Chen et al. 2018). The Mann-Whitney U test was performed with the SPSS 16.0 software, and the ELISA analysis data statistical analyses were performed using One-way ANOVA analysis as implemented in the SPSS 16.0 software.

### Results

The overall structure of the gastric mucosal microbiota alteration after oral inoculation with K. pneumoniae K1 serotype isolate. Gastric mucus samples were obtained from KLA, NKLA, and Kp1002-untreated healthy control groups and the 16S rRNA gene was sequenced. Totally 153 397 high-quality reads were obtained after sequencing analysis, including 46 329 sequences from a healthy group, 53 863 sequences from NKLA group and 53 205 sequences from KLA.
Gastric microbiota alteration in KLA mice

The sequences had an average length of 477 bp (range from 400 bp to 521 bp). Operational taxonomic unit (OTU) was identified at the 97% similarity level. The coverage percentage was calculated by Good's method (Ling et al. 2015, Chen et al. 2018) and the coverage percentage values of healthy, NKLA and KLA groups were 98.27%, 98.54%, and 98.40%, respectively. These values showed sufficient sequencing depth in this study. The richness estimators (ACE and Chao1 value) and diversity indices (Shannon and Simpson indices) displayed no obvious difference between healthy and Kp1002-treated groups ($p > 0.05$) (Fig. S1). Rarefaction analysis estimates showed that species richness differed between the healthy and Kp1002-treated groups (Fig. 1A). The rank-abundance curves generated from OTU analysis also revealed differences between the healthy and Kp1002-treated groups (Fig. 1B). Beta-diversity indices analysis was performed by the unweighted UniFrac method and principal coordinate analysis (PCoA). Unweighted PCoA revealed obvious differences among the three groups, indicating variations in microbial communities (Fig. 1C). At the same time, PCoA of weighted UniFrac analysis also showed the different trends among the three groups (Fig. S2). A Venn diagram showed different OTU data in three groups and that the overlapping OTU data. Totally 3890 OTUs were obtained from three groups. In addition, 624 common OTUs were shared by the KLA and NKLA groups, which was higher than that shared by the healthy group and any of the other groups (Fig. 1D). These results suggested that the overall structure of gastric mucosal microbiota was obviously altered in Kp1002-treated mice in comparison with that in healthy mice, whereas it did not show a great difference between KLA group and NKLA group.

**Major alterations in the composition of gastric mucosal microbiota in KLA mice.** The sequences obtained in this study belonged to twelve bacterial phyla, with eleven of them found in healthy mice, including the most abundant phyla Bacteroidetes and Firmicutes. At the genus level, 93 genera were obtained. The healthy group consists of 46 genera with four abundant genera (>1% of the total DNA sequences), including *Lactobacillus, Bacteroides, Alistipes,* and *Clostridium*.

![Fig. 1. Overall structure alteration in gastric microbiota.](image-url)

The species richness (A) and Rank abundance curve (B) were based on OTUs analysis of the three groups. (C) PCoA of weighted UniFrac analysis plot of gastric microbiota of three groups. (D) Venn diagram showed the OTUs overlaps among the three groups. (R, healthy group; S, NKLA group; T, KLA group).
Fig. 2. The differences in gastric microbiota composition between healthy and KLA groups by LEfSe analysis. Taxonomic cladogram (A) and linear discriminative analysis (LDA) scores (B) exhibited the different enriched bacteria taxa between healthy group (Red) and KLA group (Green). All of the taxa showed in the figure with LDA threshold value > 2.
Gastric microbiota alteration in KLA mice

There were 47 genera in the NKLA group with six abundant genera, and 59 genera in the KLA group with six abundant genera.

The linear discriminant analysis effect size (LEfSe) method was employed in this study to assess alterations in the composition of gastric mucosal microbiota in KLA mice. The gastric mucosal microbiota composition was significantly altered in KLA-infected mice when compared to that in healthy mice (Fig. 2). At the same time since the overall structure of gastric mucous microbiota showed differences between healthy mice and Kp1002-treated mice in Fig. 1, and for better understanding of the special changes in gastric microbiota from KLA infection mice, the significant differences in microbiota composition between NKLA and KLA mice were also explored. The specific changes of gastric mucosal microbiota in KLA mice were summarized in Fig. 3.

Fig. 3. Comparing the differences at the relative abundance of bacterial sequences at the different levels including phylum, order, family and genus level among three groups. (Mann-Whitney U test, *: p < 0.05).
The KLA and healthy mice showed significant differences in the abundance of phylum Bacteroidetes, a marked decrease in the KLA group compared with that in the healthy group \((p<0.05)\). In addition, the abundance of the non-predominant phylum Proteobacteria in the KLA group significantly increased \((p<0.05)\) (Fig. 3A). At the order level analyses, the great changes were observed in KLA infection mice. Compared with the healthy mice, the KLA infection mice showed an obvious decrease in the abundance of Bacteroidales and a significant increase in Enterobacteriales \((p<0.05)\) (Fig. 3B). At the family level, the data showed that the KLA infection mice exhibited a great decrease in the abundance of Rikenellaceae and a marked increase in the

![Fig. 4. Kp1002 oral inoculation changes gastric and intestinal defensins and serum inflammatory cytokines.](image)

Both β-defensin 1 concentration in the intestine (A) and stomach (E) showed a great increase. The concentration of β-defensin 2 (B), α-defensin 5 (C) and α-defensin 6 (D) in intestine showed a significant increase. The levels of serum cytokines IL-1β (F) and TNF-α (G) also increased. (One-way ANOVA analysis, *: \(p<0.05\)).
abundance of *Enterobacteriaceae*, *Pasteurellaceae*, and *Streptococcaceae* compared to the healthy mice (*p* < 0.05) (Fig. 3C). Further detailed analyses at the genus level showed that a great decrease in the abundance of *Bacteroides* and *Alistipes* and an obvious increase in *Klebsiella* and *Streptococcus* in the KLA group compared to the healthy group (*p* < 0.05) (Fig. 3D). The differences between the KLA and NKLA groups displayed trends in gastric mucosal microbiota composition at the phylum, order, family and genus level similar to that between the healthy and KLA groups (Fig. 3).

**K. pneumoniae** oral inoculation changes immune barrier. In our previous study, we found that cecal contents microbiota significant changes in KLA mice (Chen et al. 2018). Herein, we examined the immune barrier in the stomach and intestine. Fig. 4 shows alterations in defensins and cytokines in KLA mice. Compared with healthy mice, KLA infection mice showed significant increases in β-defensin 1, β-defensin 2, α-defensin 5, α-defensin 6 in the cecum, and β-defensin 1 in the stomach (*p* < 0.05). The levels of serum cytokines interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) also significantly increased in the KLA mice compared to that of healthy mice (*p* < 0.05). On the other hand, compared with healthy mice, NKLA mice also showed a great increase in β-defensin 1 and α-defensin 6 in the cecum, and β-defensin 1 in the stomach when compared to that of healthy mice (*p* < 0.05).

**Discussion**

Our previous study indicates that KLA infections are accompanied by changes in intestinal microbiota composition (Chen et al. 2018). The intestinal microbiota has been widely accepted to be an important biological barrier against foreign pathogens. The stomach has long been considered to be sterile due to the low pH of the gastric acid secreted by goblet cells. However, stomach actually consists of a microbial community, which provides a biological barrier against the invasion of foreign pathogens, especially pathogens of oral origin such as *H. pylori*, and maintains the gastrointestinal health (Bik et al. 2006; Andersson et al. 2008; Lofgren et al. 2011). To better understand the relationship between gastric microbiota and extra-gastrointestinal KLA infections, this study analyzes changes in gastric microbiota composition in KLA mice.

The present study observes great alterations in gastric mucosal microbiota composition in KLA mice. KLA mice show a significant reduction in Bacteroidetes in the gastric microbiota, with a great reduction in the abundance of Bacteroidetes in the gastric microbiota of *H. pylori*-infected mice (Lofgren et al. 2011). In human, positive *H. pylori* status is also associated with decreased abundance of Bacteroidetes (Maldonado-Contreras et al. 2011). Gastritis in male mice is accompanied by decreased *Bacteroides* species ASF519 colonization, indicating that inflammation-driven atrophy alters the gastric niche for the gastrointestinal commensal bacteria colonization (Lertpiriyapong et al. 2014). It seems that the decrease in the abundance of phylum Bacteroidetes or genus *Bacteroides* might be closely related to the gastrointestinal and extra-gastrointestinal infections caused by foreign pathogen through an oral pathway.

At the same time, the abundance of *Klebsiella* significantly increased in gastric mucosa of KLA infection mice. Sung et al. (2016) used 454-pyrosequencing to compare the microbiota composition between gastric fluid and gastric mucosa samples and found that using gastric mucosa samples was more effective in detecting meaningful bacteria, such as *H. pylori*, nitrosating or nitrate-reducing bacteria. In our study, the increase in the abundance of *Klebsiella* in gastric mucosa might be an effective bioindicator for KLA infection.

On the other hand, in the gastrointestinal tract, the constitutive expression of defensins imparts the regulation of the bacterial microbiota and immunomodulatory activity and is involved in the pathogenesis of various intestinal infection diseases (Wehkamp et al. 2005). α-defensin 5 has been reported to have microbicidal activity against various bacteria, including *Escherichia coli* and *Staphylococcus aureus* (Ouellette et al. 1994). The production of β-defensins 2, 3, and 4 is significantly higher in ulcerative colitis (Rahman et al. 2011). The present study also finds out that the defensin expression levels in the stomach or cecum are higher in KLA mice. Simultaneously, the elevated proinflammatory cytokines IL-1β and TNF-α could be induced by β-defensins (Ghosh et al. 2011). These findings show the changes in the gastrointestinal microbiota in response to *K. pneumoniae*, as well as an increase of the expression of defensins and cytokines.

It should not be ignored that Kp1002-treated mice without KLA infection also exhibited changes in the gastrointestinal microbiota and increased expression levels of defensins relative to that in healthy mice. We also find that after oral inoculation with the *K. pneumoniae* isolate, mouse infection rates continuously increase after 48 h (data not shown). These changes in gastrointestinal microbiota and defensins expression levels may be related to the infection process, thereby providing new insight into KLA treatment.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature


Supplementary materials are available on the journal's website.