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**Diversity changes of microbial communities into hospital surface environments**

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30

31 **Running title:** Diversity changes of hospital environmental microbiome

32

33

## **Abstract**

Previous works have demonstrated considerable variability in hospital cleanliness in Japan, suggesting that contamination is driven by factors that are currently poorly controlled. We undertook 16S rRNA sequence analysis to study population structures of hospital environmental microbiomes to see which factor(s) impacted contamination. One hundred forty-four samples were collected from surfaces of three hospitals with distinct sizes (“A”: >500 beds, “B”: 100–500 beds, “C”: <100 beds). Sample locations of two ward types (Surgical and Internal) included patient room (multiple) (4BT), patient overbed table (multiple) (4OT), patient room sink (multiple) (4S), patient room bed table (single) (SBT), patient overbed table (single) (SOT), patient room sink (single) (SS), nurse desk (ND), and nurse wagon (NW). Total DNA was extracted from each sample, and the 50 samples that yielded sufficient DNA were used for further 16S rRNA sequencing of hospital microbiome populations with cluster analysis. The number of assigned bacterial OTU populations was significantly decreased in hospital “C” compared to the other hospitals. Cluster analysis of sampling locations revealed that the population structure in almost all locations of hospital “C” and some locations in the

other hospitals was very similar and unusually skewed with a family, *Enterobacteriaceae*. Interestingly, locations included patient area (4OT, 4BT, SBT) and nurse area (ND), with a device (NW) bridging the two and a place (4S and SS) shared between patients or visitors. We demonstrated diversity changes of hospital environmental microbiomes with a skewed population, presumably by medical staff pushing NWs or sinks shared by patients or visitors.

**Keywords:** Hygiene, Nosocomial infection, Hospital cleanliness, 16S rRNA sequence analysis, Microbiome

## 63    **Introduction**

64    CDC guidelines on controlling hospital-acquired infections (HAIs) prioritize  
65    environmental cleanliness, while recommending precautions for contact between  
66    patients and medical staff. In particular, the guidelines promote cleanliness of  
67    high-contact surfaces such as machines placed around patients [1]. Bacterial  
68    contamination of environmental surfaces is the leading cause of HAIs. Although  
69    extensive measures to promote cleanliness are routinely undertaken, HAIs are a  
70    persistent problem [2-8]. Thus, many hospitals implement monitoring of the  
71    effectiveness of cleaning procedures, with visual assessment of surfaces, assessment of  
72    residual fluorescent dye after cleaning, determination of aerobic colony-forming units,  
73    or detection of ATP on surfaces [9].

74        Previous assessments of hospital cleanliness in Japan, using ATP bioluminescence  
75    and stamp agar methods, revealed bacterial contamination to be highly variable and  
76    independent of time or ward type [10]. No significant relationship was observed  
77    between the number of patients or medical personnel in the hospital and organic or  
78    microbiological contamination [10]. However, the physical properties of the surfaces

that were sampled had a strong effect on contamination [11]. These findings suggest that numerous currently uncontrolled factors contribute to HAIs in Japanese hospitals [10-11].

Amplicon sequencing, targeting the hypervariable region of the bacterial small-subunit ribosomal RNA gene (16S rRNA), is currently a widely used technique to study environmental microbiomes. Data can be used quantitatively and qualitatively to compare environments, as well as provide direct diagnostic information that can inform therapeutic decisions [12, 13]. Microbiome analysis also has been applied in a wide range of research fields, including medicine, as well as ecology of indoor and marine environments and urban public spaces such as subways [14-18]. Thus, microbiome analysis is likely to provide pertinent data in attempts to understand the factors controlling hospital cleanliness and HAIs.

In the present study, we undertook amplicon sequencing to visualize and compare microbial population structures in three different hospitals, which differed in bed numbers.

95

## 96 **Materials and methods**

### 97 *Hospitals*

98 Because of their different sizes, three hospitals were selected, which have distinct  
99 numbers of beds (hospital “A”: >500 beds, hospital “B”: 100–500, hospital “C”: <100).  
100 Both hospitals “A” and “B” are general hospitals with 30 and 21 clinical departments,  
101 respectively, while hospital “C” specializes mainly in lung cancer treatment (including  
102 antibiotic treatment to prevent infectious diseases after surgery) with only 5 clinical  
103 departments. Because of distinct treatment policies, “Surgical” and “Internal” wards  
104 were selected for sampling locations. For each of the wards, mean hospitalization  
105 periods (days)/patient numbers are as follows: hospital “A”: 24 (Surgical 33.2, Internal  
106 14.8)/49.5 (Surgical 50.1, Internal 48), hospital “B”: 15 (Surgical 16.3, Internal  
107 13.7)/34.7 (Surgical 36.9, Internal 32.5), and hospital “C”: 12.3 (Surgical 10.8, Internal  
108 13.7)/21.1 (Surgical 18.5, Internal 23.4). These hospitals are located in the  
109 Hokkaido-Tohoku area in Japan. Hospital features with relative ratios of patients with  
110 lung cancer are summarized in Table 1. The numbers of patients with lung cancer were



obtained from data published as the hospital index, and the relative ratio of patients with lung cancer among all patients was then estimated. Cleaning was similar in all hospitals and followed CDC guidelines [1]. Cleaning was performed daily in several locations, as shown in Fig. 1, according to the following protocol. In brief, floors were cleaned with disinfectant (Fig. 1, asterisks). The overbed table or bed frame was wiped with detergent and then treated with disinfectant (Fig. 1, arrowheads). The handrail or window frame was also cleaned with detergent only (Fig. 1, arrows).

#### *Sampling locations and swab collection*

A total of 144 samples were collected from hospital environments. As mentioned above, sampling locations belonging to two ward types [Surgical (Su) and Internal (In)] included patient room (multiple) (4BT), patient overbed table (multiple) (4OT), patient room sink (multiple) (4S), patient room bed table (single) (SBT), patient overbed table (single) (SOT), patient room sink (single) (SS), nurse desk (ND), and nurse wagon (NW). Samples were collected from these environmental surfaces on weekday mornings. Sterile cotton swabs dipped in sterile saline were wiped over 400 cm<sup>2</sup> at each location.

127 To prevent contamination from hands, disposable sterilized plastic gloves were used for  
128 all sample collections.

129

#### 130 *DNA extraction*

131 Each cotton swab was intensely vortexed in sterile saline, and the resulting suspension  
132 was then centrifuged, yielding pellets that were used for total genomic DNA extraction.

133 Extraction was performed using the QIAamp DNA kit (Qiagen, Valencia, CA, USA),  
134 according to the manufacturer's instructions. DNA was eluted in 50 µl of the elution  
135 buffer supplied with the kit, and stored at -20 °C until use. In addition, all laboratory  
136 procedures were conducted in a safety cabinet with filtered airflow to prevent  
137 cross-contamination.

138

#### 139 *Quality checking of extracted DNA*

140 The quality of extracted DNA from each sample ( $n=144$ ) was confirmed by PCR  
141 amplification using specific primers that target the 16S rRNA gene of *Mycobacterium*  
142 sp., a species that inhabits most indoor and outdoor environments, including pond water

and soil [18]. Band densities of electrophoresed PCR samples on 2 % agarose gels were quantified using ImageJ software. DNA samples from which *Mycobacterium* sp. 16S rRNA could not be PCR amplified ( $n=94$ ) were omitted from the following amplicon sequence analysis because of insufficient DNA concentrations.

*Amplicon sequence analysis*

Analysis of samples that passed the quality check ( $n=50$ ) was performed according to the following general protocols. First, DNA amplicons were amplified by specific primer sets that target the V3–V4 region of SSU rRNA (supplied by Hokkaido System Science Co., Ltd.); PCR products were resolved on 2 % agarose gels and then purified. After indexing PCR adaptor sequences had been added to the amplicons, a DNA library for each sample was constructed, with a constant volume of amplicon solution (*i.e.*, concentrations were not adjusted), to permit comparisons between samples of read numbers produced by the Illumina 16S Metagenomic Sequencing Library Preparation kit (Illumina). Libraries were sequenced on the MiSeq Illumina sequencer platform, producing 300-bp paired-end reads. QIIME was used to process raw reads, with quality

scores >30, and perform OTU (Operational Taxonomic Units) clustering. OTU annotation was based on BLAST analysis with a baseline of >90 % similarity. Metagenomic analysis including quality filtering, OTU production, taxonomic classification, and phylogeny generation was all conducted by Hokkaido System Science Co., Ltd. Cluster analysis was performed by using Cluster 3.0 for Mac OS X (C Clustering Library 1.52). Phylogenetic trees generated from aligned population structures were constructed and then visualized in Java TreeViewX (version 0.5.0).

#### *Ethical considerations*

Before samples were collected, the research design was explained to medical staff, and informed consent was obtained orally from medical staff. Privacy and confidentiality of personal information was protected according to the Helsinki Declaration [19]. Both the Ethics Committee of Hokkaido University and the ethics review committee of each hospital approved this study.

#### *Statistical analysis*

Comparisons of assigned numbers into OTU units among hospitals were assessed by single-factor ANOVA. A  $p$  value of  $<0.05$  was considered to be statistically significant. All calculations were executed in Excel for Mac (2001) with Statcel3C.

## Results

### *Validity of sampling and analysis*

As described in the protocol outlined in Fig. 2, amplicon sequencing using Illumina MiSeq was undertaken to compare microbiomes on surfaces in three hospitals (“A”, “B”, and “C”) with distinct bed numbers. A total of 144 samples were randomly collected from various hospital environmental surfaces, according to a general swabbing protocol (see Methods). Based on the ability to PCR-amplify *Mycobacterium* 16S rRNA, 50 samples were selected for next-generation sequencing targeting the hypervariable (V3–V4) region of the bacterial 16S rRNA gene. Illumina sequencing yielded 8,948,588 sequences in total, with a mean of 178,971.76 sequences per sample and a mean sequence length of 419 bp. Sequence cleanup was performed using QIIME, creating

115,573 OTUs with a mean of 2,311 OTUs per sample. These data with sampling information are deposited as supplementary data (Online Resource 1).

*Hospital microbiome diversity and population structure*

Microbial diversity was first compared among the three hospitals. Analyses of OTU numbers revealed considerable diversity in each hospital, with 543 species in 23 phyla in hospital “A”, 519 species in 20 phyla in hospital “B”, and 483 species in 17 phyla in hospital “C”. Hospital “C” was significantly less diverse than hospitals “A” and “B” ( $p=0.0063$ ) (Fig. 3). Population structure analysis among hospitals also showed that species in family *Enterobacteriaceae* accounted for 78.49 % of all OTUs identified only in the samples of hospital “C” (data not shown). Thus, in contrast to the population structure of samples from hospitals “A” and “B”, hospital “C” appeared to show a highly skewed population. Data were also analyzed by sampling location to understand the factors responsible for the unique population structure with less diversity found in samples from hospital “C”. Cluster analysis among sampling locations revealed that the population structure in almost all locations of hospital “C” and some locations in the

other hospitals formed a cluster, being very similar and unusually skewed with a single family, *Enterobacteriaceae* (Fig. 4, highlighted with an arrow). The locations included patient area (4OT, 4BT, SBT) and nurse area (ND), with a device (NW) bridging the locations and a place (4S and SS) shared between patients or visitors (Table 2).

## **Discussion**

Since microbial contamination of the hospital environment likely contributes to the spread of HAIs, surface disinfection and ward cleaning are typically prioritized to maintain a clean clinical environment [19-23]. However, whether environmental contamination has a role in the transmission of HAIs is poorly understood. Moreover, there is little accurate epidemiological evidence based on metagenomic analysis to support the belief that hospital cleanliness can specifically reduce the incidence of HAIs [19-23].

Comparison among assigned numbers into OTU units of hospitals revealed dramatic differences in diversity among hospitals, with hospital “C” being less diverse,

which specializes in cancer chemotherapy (see Table 1). Although several other factors (e.g., hospitalization periods, patient numbers, or cleaning protocol) than the use of chemotherapy may be responsible, chemotherapy may lead to altered microbial diversity. In fact, since cancer chemotherapy typically leads to physiological changes of the human gut, with considerable alteration of the intestinal microbiome [24, 25], it cannot be denied that microbial diversity may be the result of the influence of specific drugs on patients' microbiomes, presumably with environmental spread of fecal bacteria via toilets. Further studies, in particular gut microbiome analysis of patients, are needed to clarify this association.

Furthermore, cluster analysis of sampling locations showed a cluster consisting of samples from hospital "C" and samples from some locations of the other hospitals. The results indicated that microbial population structures in these locations were very similar and unusually skewed with a single family *Enterobacteriaceae* (unclassified genera), members of which inhabit the human gut and can be associated with antibiotic-resistant HAIs, spreading via the fecal-oral route [1-8, 21-25]. Interestingly, the locations included patient area (4OT, 4BT, SBT) and nurse area (ND), with a device



239 (NW) bridging the locations and a place (4S or SS) shared between patients or visitors.

240 Undoubtedly, washing hands with disinfectant is critical to reduce the prevalence of

241 nosocomial infections [26]. Hence, CDC guidelines promote not only cleanliness of

242 high-contact surfaces such as machines placed around patients, but also careful

243 handwashing with disinfectants for nurses after treating each patient [1, 26]. However,

244 we found “sink” as well as “nurse wagon” in the cluster. Although the exact reason for

245 the unusually skewed cluster of *Enterobacteriaceae* remains to be clarified, it is likely

246 that unaware harmful behaviors of nurses while treating patient wastes or cleaning sinks

247 shared by patients or visitors result in the spread of bacteria originating from patients or

248 sinks to hospital wards. A recent study revealed that handwashing sinks were a possible

249 reservoir for KPC-2-producing *Klebsiella oxytoca* with a risk of hospital outbreak. Such

250 bacteria could persistently survive in the overflow trap of the sink and unknowingly be

251 transferred to individuals [26], even though cleaning according to CDC guidelines is

252 properly performed with disinfectant. Additionally, biofilms formed on the overflow

253 trap of the sink may facilitate bacterial survival [27]. Taken together, medical staff

254 pushing NW and sinks shared by patients or visitors likely not only changes the

microbiota in hospital environments, but also spreads bacteria present in patient wastes or the overflow trap of sinks to the hospital ward.

It may be unexpected that most of the OTUs identified from hospital “C” and some samples from the other hospitals should belong to Gram-negative *Enterobacteriaceae*, which have fragile cell walls. However, *Escherichia coli* can survive on stainless steel surfaces without any nutrients for at least 100 days [28], suggesting that Gram-negative bacteria can in fact remain viable for a long time on a wide range of artificial surfaces. Although the exact reason why NWs became contaminated with Gram-negative *Enterobacteriaceae* regardless of constant daily cleaning remains unknown, there could be unrecognized factors responsible for contamination on NW surface, which may be a significant factor determining the relationships between hospital contamination and HAIs as well as contaminated sinks.

In conclusion, we demonstrated diversity changes of hospital environmental microbiomes with a skewed population, presumably by medical staff pushing NWs and sinks shared between patients or visitors. This factor may help to explain variability in the relationship between hospital contamination and HAIs.

271

272

## 273 **Supporting Information**

274 **Online Resource 1.** Sample information (sampling location, ward type, hospital) and  
275 basic data obtained from 16S rRNA amplicon sequencing. Sample numbers,  $n=50$ .

276

277

## 278 **Declaration of interest**

## 279 **Consent for publication**

280 Not applicable.

281

282

## 283 **Availability of data and materials**

284 The datasets generated during and/or analyzed during the current study are available  
285 from the corresponding author upon reasonable request.

286

287

288    **Conflict interest**

289    The authors declare that they have no conflict interests.

290

291

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295

296

297    **Authors' contributions**

298    The project was conceived by RY, TS, RW, SY, and HY. The samples were collected by

299    RY and TS. All laboratory work was conducted by RY, TS, YK, SN, TO, and JM.

300    Bioinformatics analysis was conducted by HY. The draft paper was critically edited by

301    RY, TS, TO, JM, and HY. RY and HY wrote the paper.

302

303

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307   throughout this study.

308

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395

## 396 **Figure legends**

397 **Fig. 1.** Daily cleaning places in hospital aisles and patient rooms. Asterisks indicate  
398 floors cleaned with disinfectant. Arrowheads indicate overbed tables or bed frames  
399 wiped with detergent followed by treatment with disinfectant. Arrows indicate handrails  
400 or window frames cleaned with detergent only

401

402 **Fig. 2.** Flowchart illustrating experimental protocols for sampling, quality checks and  
403 sample selection, Illumina sequencing, and population analysis

404

405 **Fig. 3.** Differences in assigned numbers into OTU units (microbiome populations)  
406 among hospitals. Asterisks indicate statistically significant differences ( $p < 0.05$ ),  
407 estimated by single-factor ANOVA

408

409 **Fig. 4.** Cluster analysis of sampling locations shows population structures in almost all  
410 locations of hospital “C” and some locations in the other hospitals clustered together  
411 and were unusually skewed with *Enterobacteriaceae*

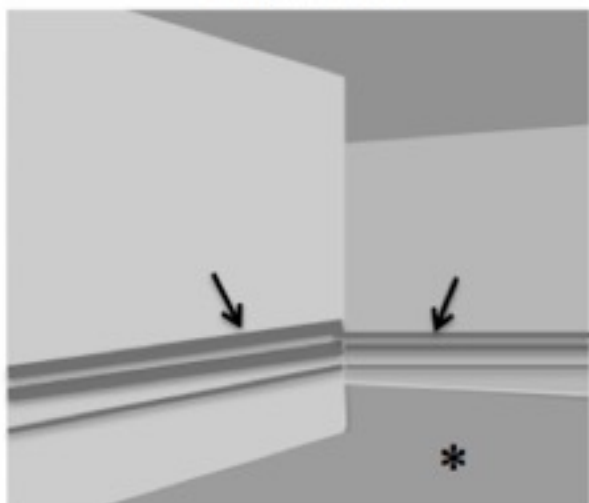
**Table 1 Hospital's size and summary**

| Hospital<br>(Speciality/Total bed number)  | Number of<br>clinical<br>department | Ward     | Bed number<br>per ward | Average<br>hospitalization<br>periods (Days) | Patient<br>numbers per<br>ward | Relative ratio of<br>patients with<br>lung cancer <sup>1</sup> |
|--------------------------------------------|-------------------------------------|----------|------------------------|----------------------------------------------|--------------------------------|----------------------------------------------------------------|
| A<br>(General hospital/500-1,000)          | 30                                  | Surgical | 60                     | 33.2                                         | 50.1                           | 1                                                              |
|                                            |                                     | Internal | 60                     | 14.8                                         | 48                             | 1                                                              |
| B<br>(General hospital/100-500)            | 20                                  | Surgical | 46                     | 16.3                                         | 36.9                           | 0.3                                                            |
|                                            |                                     | Internal | 40                     | 13.7                                         | 32.5                           | 0.5                                                            |
| C<br>(Lung cancer treatment/less than 100) | 5                                   | Surgical | 24                     | 10.8                                         | 18.5                           | 4.5                                                            |
|                                            |                                     | Internal | 25                     | 13.7                                         | 23.4                           | 2.4                                                            |

<sup>1</sup>ratio vs. Hospital A: patient numbers with lung cancer were obtained from data published as hospital index

Figure 1

Hospital aisle



Hospital room

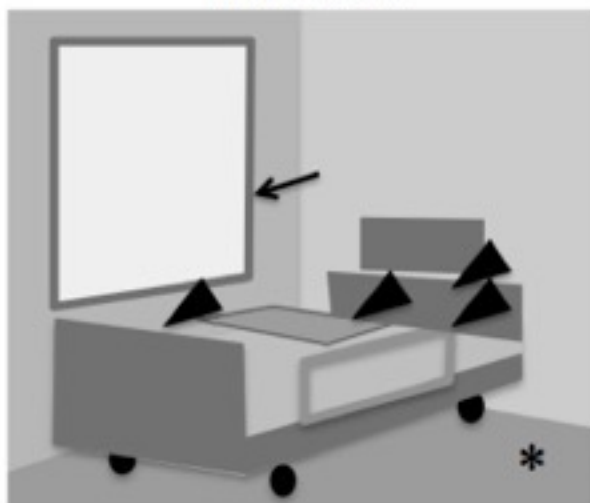


Figure 2

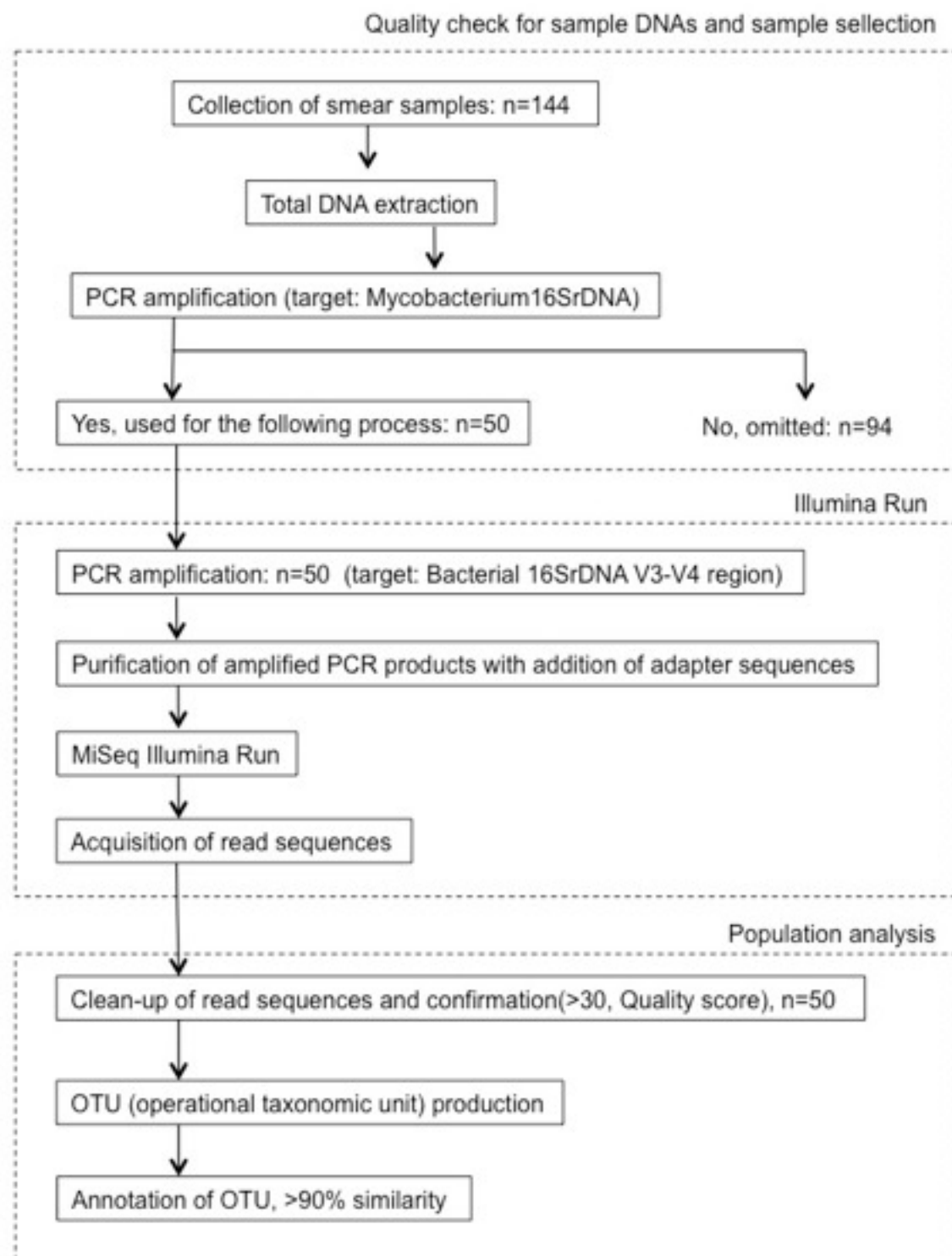




Figure 3

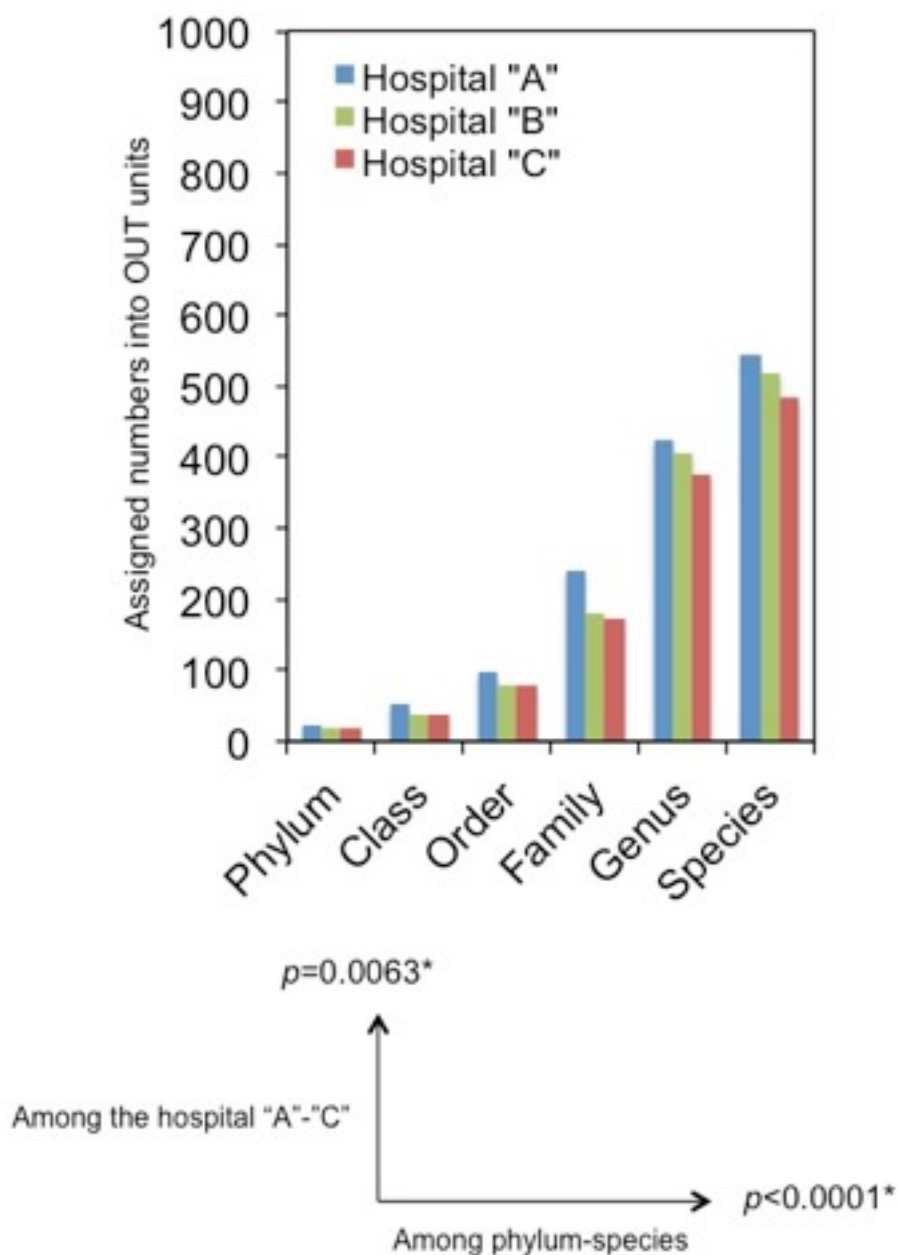
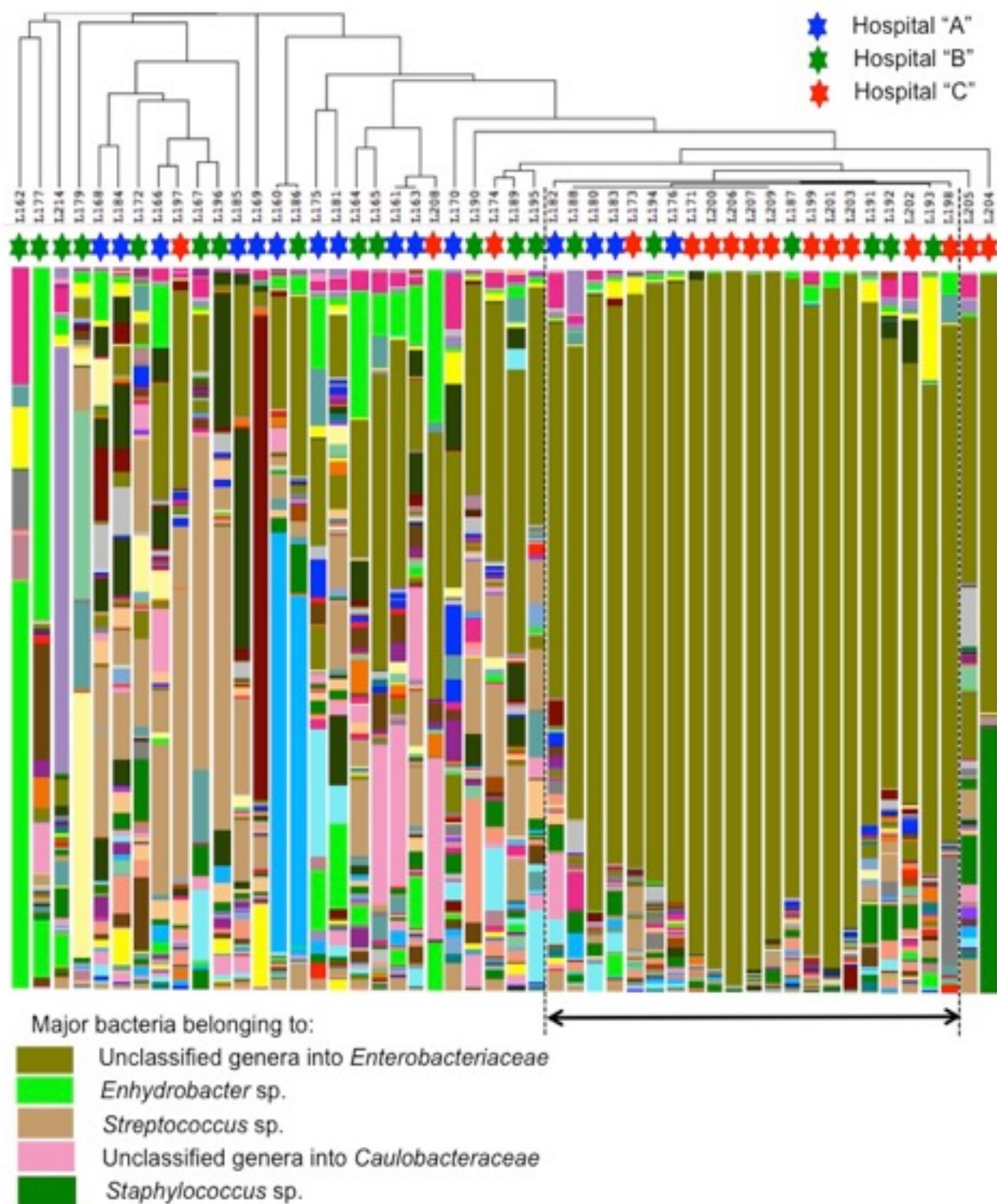


Figure 4



**Table 2 Hospital location and ward type of the samples being more similar to each other in a cluster**

| Hospital | Sample number | Sampling location <sup>1</sup> | Ward type <sup>2</sup> |
|----------|---------------|--------------------------------|------------------------|
| A        | L176          | SBT                            | Su                     |
| A        | L180          | 4OT                            | Su                     |
| A        | L182          | NW                             | Su                     |
| A        | L183          | ND                             | Su                     |
| B        | L187          | SBT                            | Su                     |
| B        | L188          | 4OT                            | Su                     |
| B        | L191          | NW                             | In                     |
| B        | L192          | 4BT                            | In                     |
| B        | L193          | 4S                             | In                     |
| B        | L194          | 4OT                            | In                     |
| C        | L171          | NW                             | Su                     |
| C        | L173          | SS                             | Su                     |
| C        | L198          | 4BT                            | Su                     |
| C        | L199          | SBT                            | Su                     |
| C        | L200          | ND                             | Su                     |
| C        | L201          | 4OT                            | Su                     |
| C        | L202          | SBT                            | Su                     |
| C        | L203          | 4S                             | Su                     |
| C        | L206          | SS                             | In                     |
| C        | L207          | 4OT                            | In                     |
| C        | L209          | 4BT                            | In                     |

<sup>1</sup>patient room (multiple), 4BT); patient overbed table (multiple), 4OT); patient room sink (multiple), 4S; patient room bed table (single), SBT; patient overbed table (single), SOT); patient room sink (single), SS); nurse desk, ND); nurse wagon (NW). <sup>2</sup>Surgical, Su; Internal, In.