



Title	Diversity changes of microbial communities into hospital surface environments
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1 **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

34 **Abstract**

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

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

56

57

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

60

61

62

## 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

79 that were sampled had a strong effect on contamination [11]. These findings suggest  
80 that numerous currently uncontrolled factors contribute to HAIs in Japanese hospitals  
81 [10-11].

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

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

94

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



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

118

#### 119 *Sampling locations and swab collection*

120 A total of 144 samples were collected from hospital environments. As mentioned above,  
121 sampling locations belonging to two ward types [Surgical (Su) and Internal (In)]  
122 included patient room (multiple) (4BT), patient overbed table (multiple) (4OT), patient  
123 room sink (multiple) (4S), patient room bed table (single) (SBT), patient overbed table  
124 (single) (SOT), patient room sink (single) (SS), nurse desk (ND), and nurse wagon  
125 (NW). Samples were collected from these environmental surfaces on weekday mornings.  
126 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

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

147

#### 148 *Amplicon sequence analysis*

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

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

166

#### 167 *Ethical considerations*

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

173

#### 174 *Statistical analysis*

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

178

179

## 180 **Results**

### 181 *Validity of sampling and analysis*

182 As described in the protocol outlined in Fig. 2, amplicon sequencing using Illumina  
183 MiSeq was undertaken to compare microbiomes on surfaces in three hospitals (“A”,  
184 “B”, and “C”) with distinct bed numbers. A total of 144 samples were randomly  
185 collected from various hospital environmental surfaces, according to a general swabbing  
186 protocol (see Methods). Based on the ability to PCR-amplify *Mycobacterium* 16S rRNA,  
187 50 samples were selected for next-generation sequencing targeting the hypervariable  
188 (V3–V4) region of the bacterial 16S rRNA gene. Illumina sequencing yielded 8,948,588  
189 sequences in total, with a mean of 178,971.76 sequences per sample and a mean  
190 sequence length of 419 bp. Sequence cleanup was performed using QIIME, creating

191 115,573 OTUs with a mean of 2,311 OTUs per sample. These data with sampling  
192 information are deposited as supplementary data (Online Resource 1).

193

#### 194 *Hospital microbiome diversity and population structure*

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

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

211

212

## 213 **Discussion**

214 Since microbial contamination of the hospital environment likely contributes to the  
215 spread of HAIs, surface disinfection and ward cleaning are typically prioritized to  
216 maintain a clean clinical environment [19-23]. However, whether environmental  
217 contamination has a role in the transmission of HAIs is poorly understood. Moreover,  
218 there is little accurate epidemiological evidence based on metagenomic analysis to  
219 support the belief that hospital cleanliness can specifically reduce the incidence of HAIs  
220 [19-23].

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

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

232 Furthermore, cluster analysis of sampling locations showed a cluster consisting of  
233 samples from hospital "C" and samples from some locations of the other hospitals. The  
234 results indicated that microbial population structures in these locations were very  
235 similar and unusually skewed with a single family *Enterobacteriaceae* (unclassified  
236 genera), members of which inhabit the human gut and can be associated with  
237 antibiotic-resistant HAIs, spreading via the fecal-oral route [1-8, 21-25]. Interestingly,  
238 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

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

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

267         In conclusion, we demonstrated diversity changes of hospital environmental  
268 microbiomes with a skewed population, presumably by medical staff pushing NWs and  
269 sinks shared between patients or visitors. This factor may help to explain variability in  
270 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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308

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393  
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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*

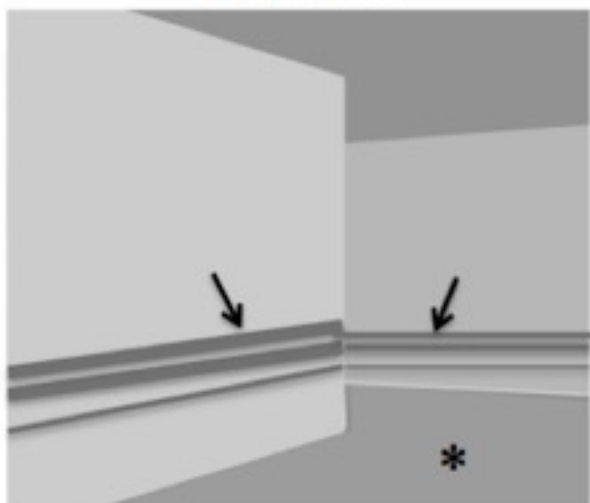
**Table 1 Hospital's size and summary**

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

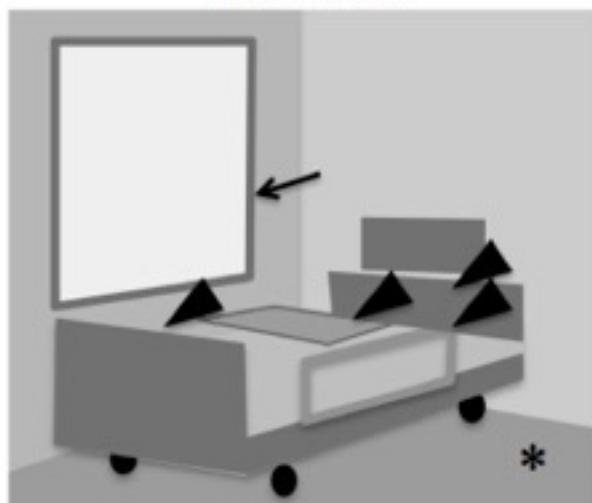


Figure 2

Quality check for sample DNAs and sample selection

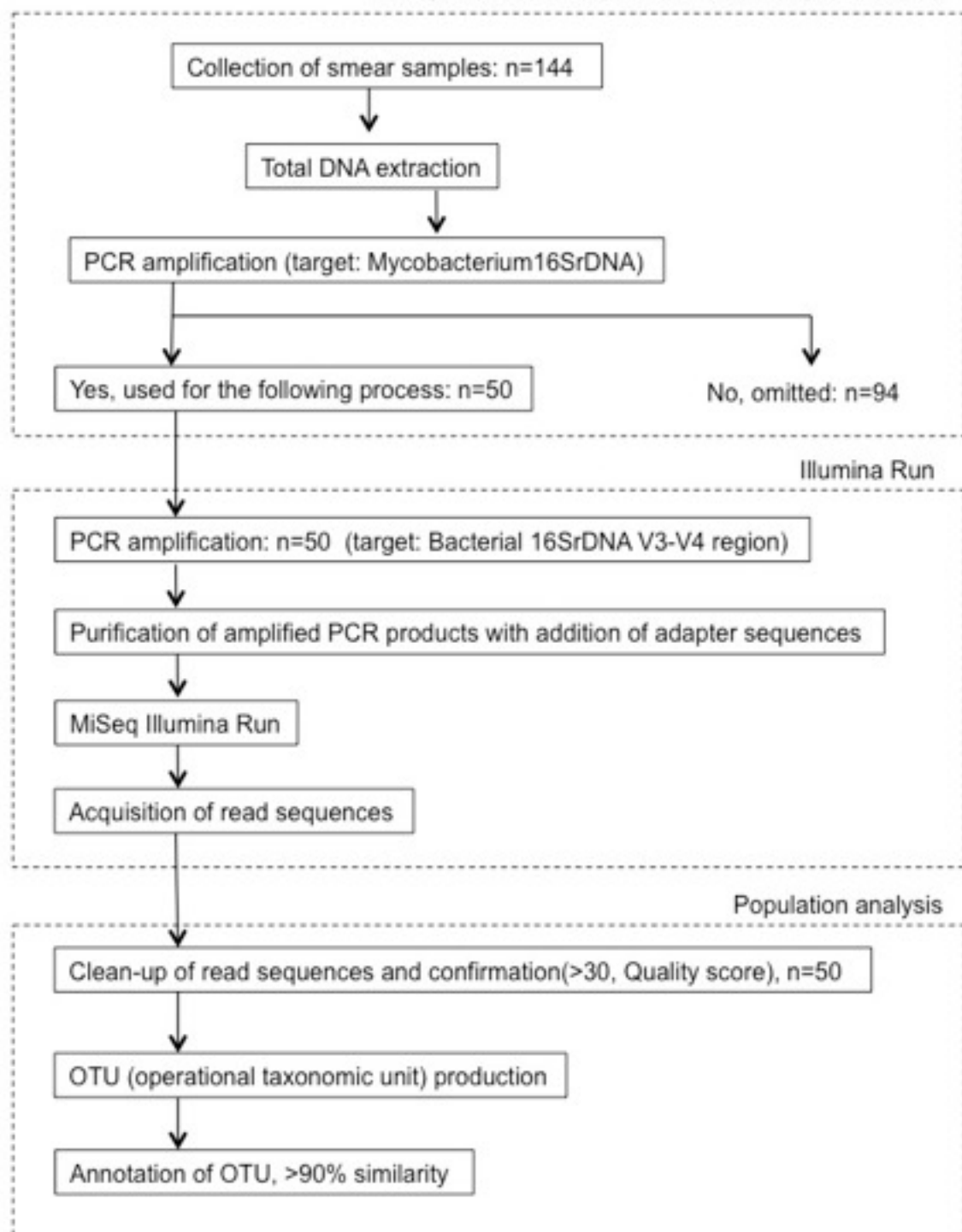
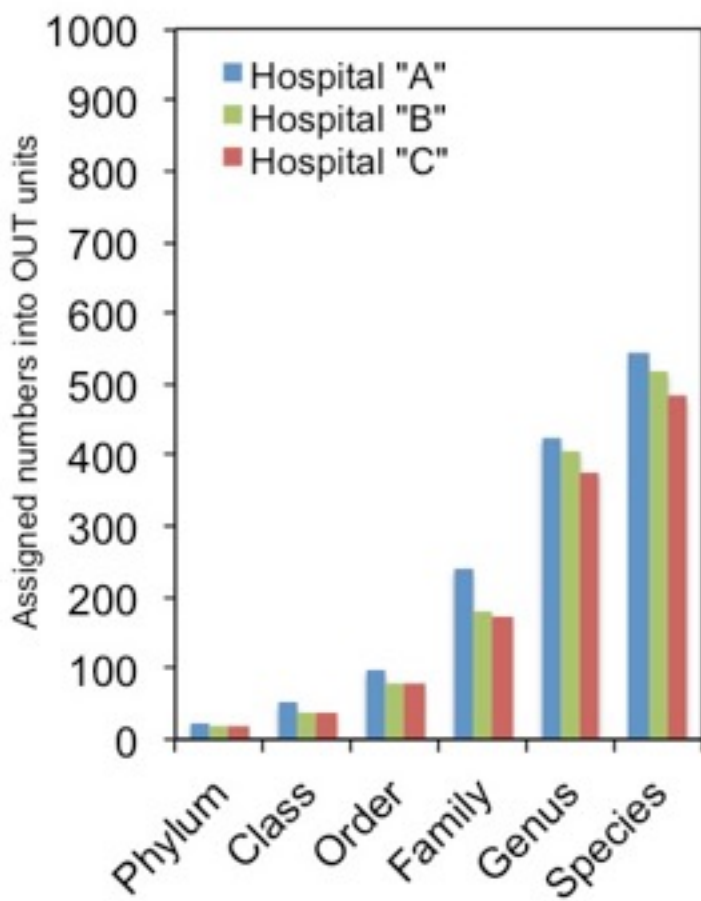




Figure 3

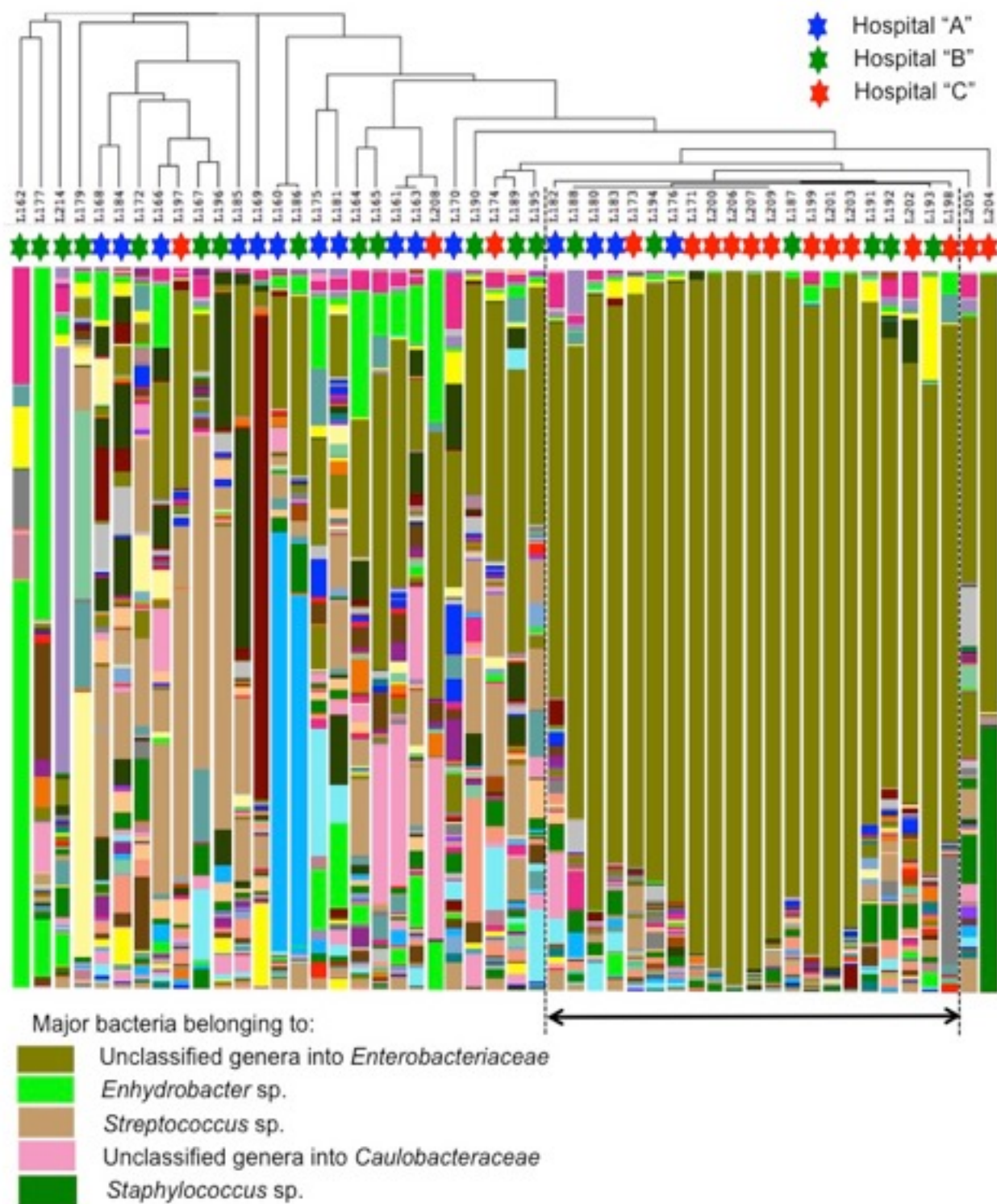


$p=0.0063^*$

Among the hospital "A"- "C"

Among phylum-species  $p<0.0001^*$

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.