Diversity changes of microbial communities into hospital surface environments

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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

Introduction

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

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

Materials and methods

Hospitals

Because of their different sizes, three hospitals were selected, which have distinct numbers of beds (hospital “A”: >500 beds, hospital “B”: 100–500, hospital “C”: <100). Both hospitals “A” and “B” are general hospitals with 30 and 21 clinical departments, respectively, while hospital “C” specializes mainly in lung cancer treatment (including antibiotic treatment to prevent infectious diseases after surgery) with only 5 clinical departments. Because of distinct treatment policies, “Surgical” and “Internal” wards were selected for sampling locations. For each of the wards, mean hospitalization periods (days)/patient numbers are as follows: hospital “A”: 24 (Surgical 33.2, Internal 14.8)/49.5 (Surgical 50.1, Internal 48), hospital “B”: 15 (Surgical 16.3, Internal 13.7)/34.7 (Surgical 36.9, Internal 32.5), and hospital “C”: 12.3 (Surgical 10.8, Internal 13.7)/21.1 (Surgical 18.5, Internal 23.4). These hospitals are located in the Hokkaido-Tohoku area in Japan. Hospital features with relative ratios of patients with 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^2 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

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,

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.

Supporting Information

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

Declaration of interest

Consent for publication

Not applicable.

Availability of data and materials

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

Conflict interest

The authors declare that they have no conflict interests.

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Authors' contributions

The project was conceived by RY, TS, RW, SY, and HY. The samples were collected by RY and TS. All laboratory work was conducted by RY, TS, YK, SN, TO, and JM. Bioinformatics analysis was conducted by HY. The draft paper was critically edited by RY, TS, TO, JM, and HY. RY and HY wrote the paper.

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Figure legends

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

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

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

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

[1]ratio vs. Hospital A: patient numbers with lung cancer were obtained from data published as hospital index

Figure 1

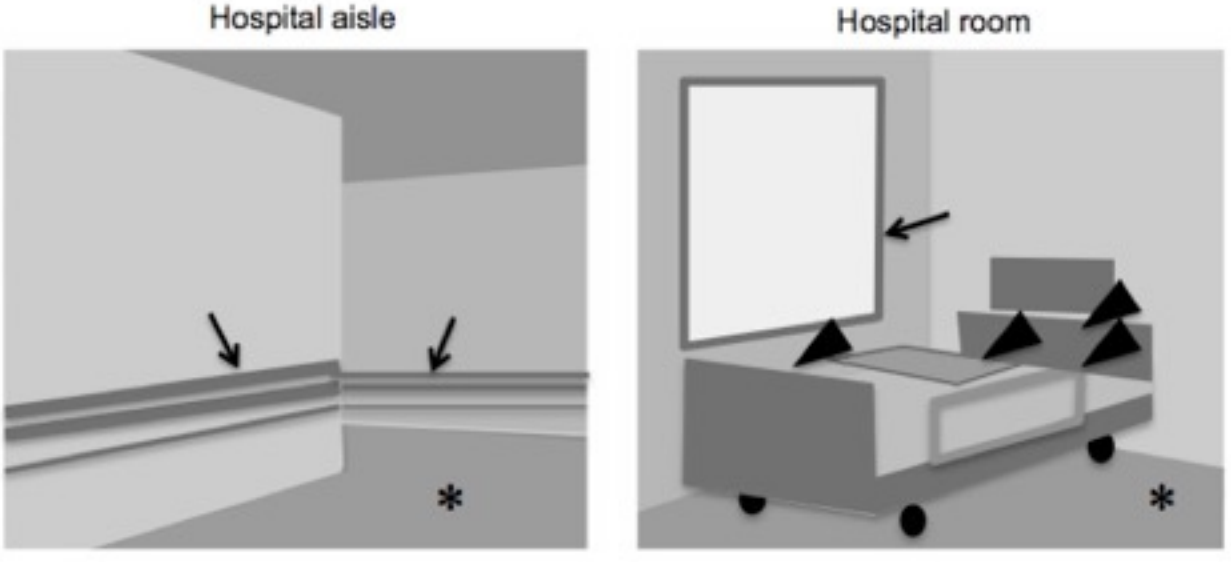

Figure 2

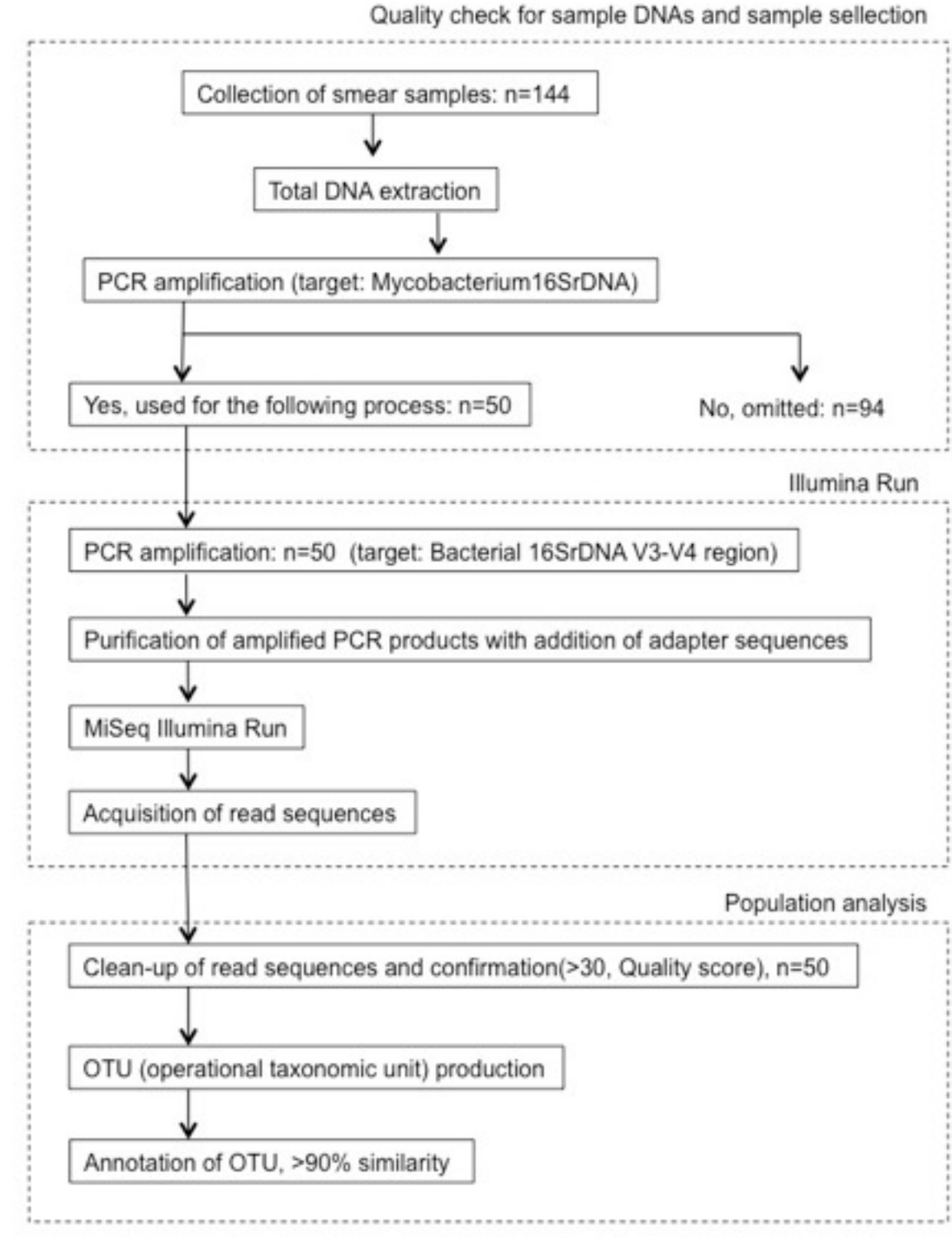
Quality check for sample DNAs and sample sellection
Collection of smear samples: n=144
Total DNA extraction
PCR amplification (target: Mycobacterium16SrDNA)
Yes, used for the following process: n=50
No, omitted: n=94
Illumina Run
PCR amplification: n=50 (target: Bacterial 16SrDNA V3-V4 region)
Purification of amplified PCR products with addition of adapter sequences
MiSeq Illumina Run
Acquisition of read sequences
Population analysis
Clean-up of read sequences and confirmation(>30, Quality score), n=50
OTU (operational taxonomic unit) production
Annotation of OTU, >90% similarity

Figure 3

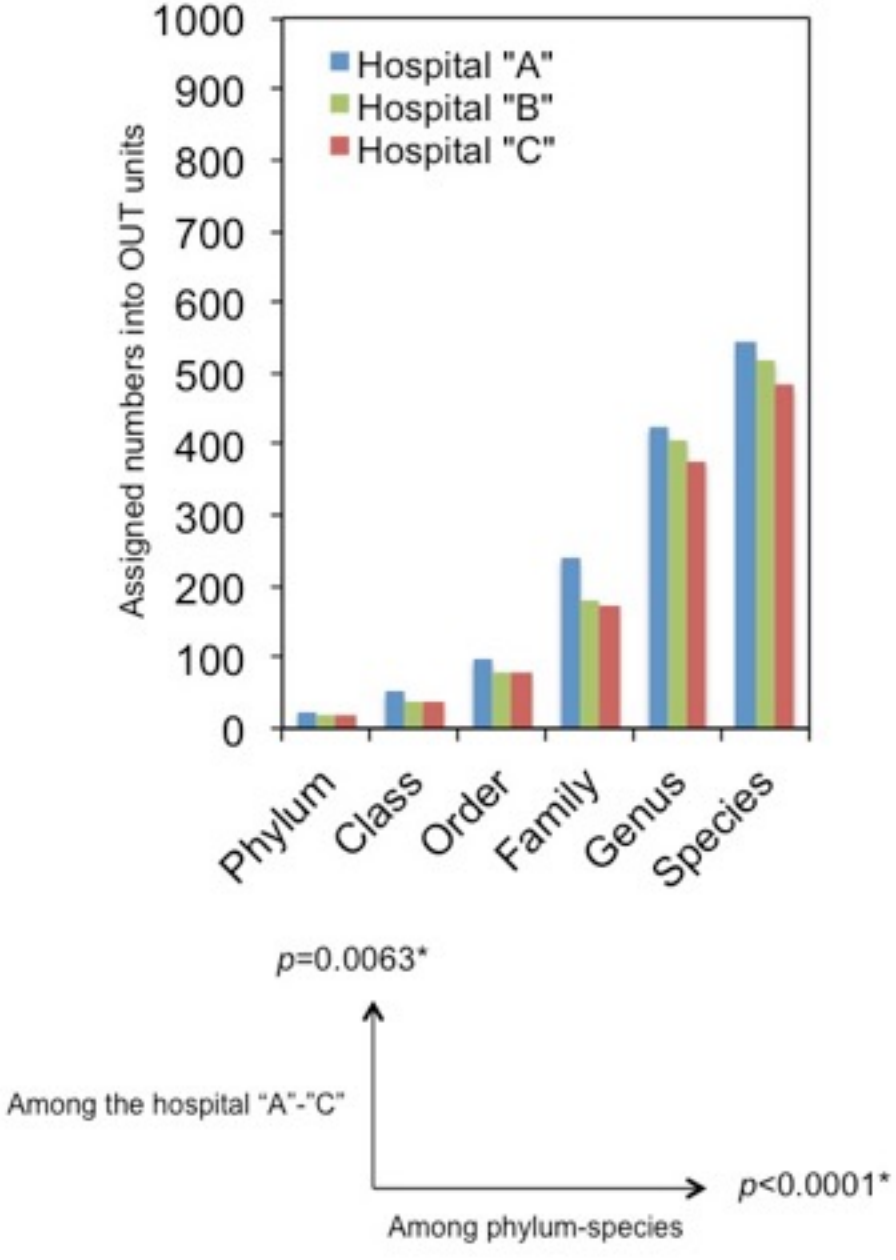
1000
900
800
700
600
500
400
300
200
100
0
Assigned numbers into OUT units
Hospital "A"
Hospital "B"
Hospital "C"
Phylum
Class
Order
Family
Genus
Species
p=0.0063*
Among the hospital "A"-"C"
p<0.0001*
Among phylum-species

Figure 4

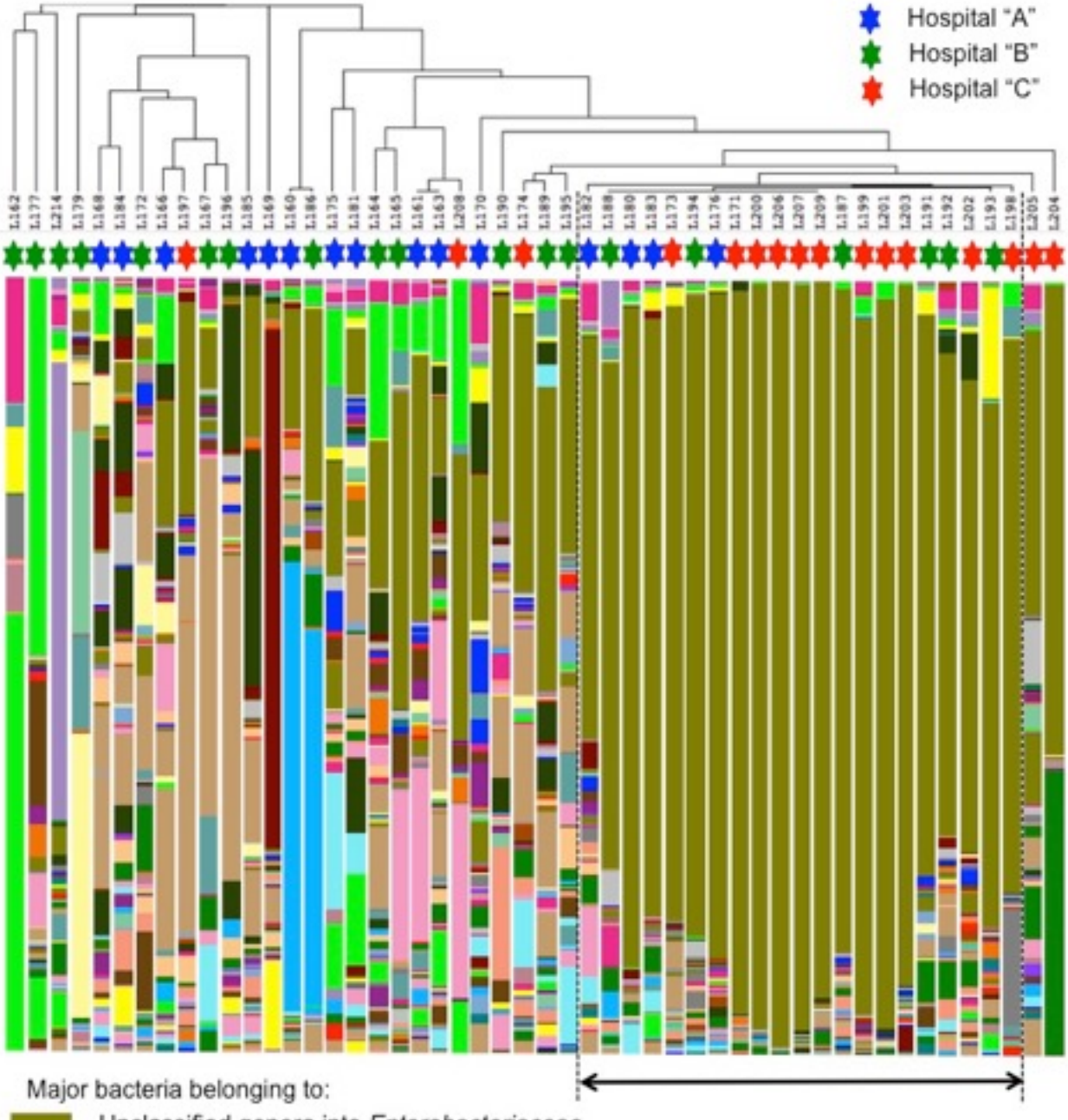
Hospital "A"
Hospital "B"
Hospital "C"
L162
L177
L214
L179
L168
L184
L172
L166
L197
L167
L196
L185
L169
L160
L186
L175
L181
L164
L165
L161
L163
L208
L170
L190
L174
L189
L195
L182
L188
L180
L183
L173
L194
L176
L171
L200
L206
L207
L209
L187
L199
L201
L203
L191
L192
L202
L193
L198
L205
L204
Major bacteria belonging to:
Unclassified genera into Enterobacteriaceae
Enhydrobacter sp.
Streptococcus sp.
Unclassified genera into Caulobacteraceae
Staphylococcus sp.

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

Hospital	Sample number	Sampling location[1]	Ward type[2]
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

[1]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). [2]Surgical, Su; Internal, In.