

Metagenomic survey and whole genome sequencing of antimicrobial resistant bacteria in the sewage of a Japanese hospital

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Abstract

Background The dissemination of antimicrobial-resistant bacteria (ARB) and the transfer of antimicrobial resistance genes (ARGs) are a threat to public health. Antibiotics are indispensable therapeutic agents essential for the treatment of infectious diseases; however, inappropriate use of antibiotics leads to the emergence of ARB. It is established that hospitals are closely involved in the spread of antimicrobial resistance (AMR), which impedes antibiotic treatment and subsequently increases mortality. In addition, excrement of patients or healthy carriers of ARB are discharged from the hospital sewage through the wastewater treatment plant (WWTP) into the rivers, causing an AMR burden on the environment.

Method Metagenomic analysis was performed on the hospital sewage samples, followed by whole genome sequencing of the extended spectrum β -lactamase (ESBL)-producing organisms (EPOs). A comparative genome analysis was also performed between EPO isolates from sewage and clinical isolates.

Results Metagenomic analysis showed that the hospital sewage tanks had bacterial flora corresponding to the human gut. During the study period, the hospital was relocated to a newly constructed building with new sewage tanks; however, the presence of ARB/ARGs in the new hospital sewage tanks became markedly equivalent to that of the old hospital within one month. The ESBL *bla*_{CTX-M} and carbapenemase *bla*_{IMP} genes were not much detected in the original hospital sewage samples by metagenome analysis, but selection on CHROMagar ESBL increased the sensitivity to detect those β -lactamase genes. Comparative genome analysis between sewage and clinical EPO isolates revealed partial similarity; however, most EPO isolates exhibited a notable difference (≥ 50) in single nucleotide variations based on core-genome phylogeny. This result suggests that only some of the sewage EPO isolates were originated from the clinical patient. Therapeutic agents in the hospital sewage were analyzed and the concentration of levofloxacin and clarithromycin was 0.0325 and 0.0135 $\mu\text{g}/\text{mL}$, respectively.

Conclusions Whole genome analysis between sewage and clinical isolates suggested that healthy or asymptomatic carriers may be involved in the contamination of hospital sewage. Moreover, the hospital sewage tank may serve as a hotspot for the horizontal transfer of ARGs under the selective pressure of antimicrobial agents. Therefore, ARB monitoring in hospital sewage is expected to detect the presence of carriers prior to nosocomial ARB outbreaks. In addition, hospital wastewater should be treated suitably to bring ARB below detectable levels to reduce the environmental AMR burden.

Introduction

The rapid dissemination of antimicrobial-resistant bacteria (ARB) has become one of the major public health concerns in the world. In case novel antibiotics against ARB are not developed, the annual number of deaths due to bacterial infections by ARB is predicted to increase to 10 million by 2050 [1]. In addition to nosocomial ARB infections, other environmental sources of ARB should also be investigated. The Global Action Plan on Antimicrobial Resistance drafted by World Health Organization [2] states that there

is a need to understand the impact of human activities on the environment, particularly the spread and transfer of antimicrobial resistance genes (ARG) and strains. The Nippon National Action Plan on Antimicrobial Resistance One Health Report 2018 in Japan addressed that 12.4 – 27.5% of tested invasive *Escherichia coli* isolates were resistant to 3rd generation cephalosporins and 0.1% were resistant to carbapenems. Additionally, 5.7 – 9.4% of tested invasive *Klebsiella pneumoniae* isolates were resistant to 3rd generation cephalosporins and 0.3 – 0.5% were resistant to carbapenems [3]. The isolation rates of these resistant strains are lower in Japan compared to other countries, but are certainly increasing. Therefore, antimicrobial resistance (AMR) should be well monitored in the β -lactam-resistant Enterobacteriaceae family of bacteria.

In addition to clinically isolated ARB, comparable ARB/ARGs have been detected from various environments around the world, including the effluent from wastewater treatment plants (WWTP) [4, 5], lakes and rivers [6–8], and soil [9]. In particular, extended spectrum β -lactamase (ESBL)-producing *E. coli* or carbapenemase-producing organisms (CPOs) have been detected in rivers, lakes [10–12], and WWTP treated water [13–15] in several countries, posing a potential risk of infection, and Japan is no exception [16, 17]. Moreover, hospital wastewater is the most ARB-contaminated source in WWTP [5, 13, 18–22] and there is concern about the relationship between hospital wastewater and environmental pollution [23, 24]. In particular, hospital wastewater is contaminated by ARB and residual antibiotics present in the excrement of patients, visitors, and medical personnel, and is considered to be a hot spot for the growth and propagation of ARB [5].

In hospital wastewater, residual antimicrobial agents, limited nutrients, and the structure of the microbial community may generate selective pressure for the development of ARB [5, 25–28]. Genetic transfer of resistant genes from ARB to susceptible bacteria under this selective pressure may lead to the emergence of new populations of ARB [26, 29, 30]. ARB has been detected in subjects exposed to recreational water areas [31, 32], drinking water [33], seafood [34–37], and vegetables grown in irrigation water [3, 38]. Although it is not yet clear whether the hospital wastewater-related ARB disseminate into the water bodies, ARBs are observed to exhibit deleterious effects on human health. In the clinical setting, resistance to carbapenems in *Enterobacteriaceae*-related infections [39] is a serious concern, owing to limited treatment options [40, 41] and high mortality rates [42]. The presence of CPOs in hospital sewage [5, 13, 21, 22], urban sewage [43], and coastal water environments [31, 44] has also increased.

Therefore, active surveillance and management are necessary to avoid undesirable dissemination of ARB from hospital sewage to WWTP. Similar to the general sewage system, hospital sewage is also discharged into a public WWTP and released into rivers after primary purification, activated sludge process, and occasional chlorine disinfection. In recent years, technically and economically viable management strategies for ARB, ARG, and residual antibiotics discharged from hospitals have been studied in some countries [45–51]; however, there is a lack of similar reports from Japan. The aim of this study is to illustrate the contamination of hospital sewage with ARB containing resistant genes of clinical origin, and the effect of hospital relocation on the state of ARB/ARGs in the hospital sewage using comprehensive metagenomic sequencing. In addition, we compared the whole genome sequence of

extended spectrum β -lactamase (ESBL)-producing organisms (EPOs) from hospital sewage and clinical isolates.

Materials And Methods

Hospital setting

The study was conducted at the Ohashi Medical Center in Toho University, located at Jonan-area suburb of Tokyo, Japan. The Ohashi Medical Center (35.652573, 139.685833) was opened in 1973 with a single East building and expanded to Central, Administration, and West buildings to increase patient capacity. Eventually, the number of beds in this medical center was 430 (Fig. 1). The hospital included the following departments in the West building (BW); Cardiology, Respiratory, Internal medicine, and Neurology wards, Intensive Care Unit, examination (dialysis, MRI, endoscopy, and radiography) and operating rooms; Administration building (BA); outpatient clinics of Surgery and Pediatric departments, and administrative staff room; Central building (BC); laboratories, Cardiovascular surgery, Orthopedics, Gynecology, and emergency outpatient clinics and inpatient wards of Gastrointestinal medicine, Surgery and Orthopedics, and dining rooms; East building (BE); outpatient clinics of Internal medicine, Plastic surgery, Ophthalmology, Otorhinolaryngology, Dermatology, and Urology and inpatient wards of Cardiology, Neurosurgery, Ophthalmology, and private wards.

As part of a renovation plan, a new hospital building (BN) (35.652578, 139.683959) with 319 beds was constructed approximately 50 m away from the old hospital (35.652573, 139.685833) and was inaugurated on June 20, 2018. Since May 2018, we introduced a policy of restricted hospitalization of seriously ill patients, and transferred outpatients to neighboring hospitals to reduce the number of patients at the time of relocation. We stopped emergency services from June 6 to June 20 and outpatients from June 16 to June 19, and transferred the hospitalized patients to the BN on June 16. We started accepting outpatients and hospitalized patients on June 20. The BN attends to 1000 outpatients per day and has a staff count of 2000 employees.

In both the old and new hospitals, stool and urine were stored in the underground sewage tanks without mixing with other drainage and were pumped to the sewage system several times a day (Fig. 1). In the old hospital, each building had respective sewage tanks (STW, STA, STC, and STE as shown in Fig. 1); however, the new hospital had two connected storage tanks of 22.5 m³ collecting all the sewage (STN shown in Fig. 1). It was impossible to quantify daily inflow and outflow of sewage tanks as there is no system for regular measurement. The sewage discharged from the tanks was sent to the wastewater treatment plants (WWTP) and treated using filtering, and microbiological and biochemical mechanisms. After treatments, the effluent was discharged into the nearby river.

Sampling

Collection of water samples

In the period from May 8, 2018 to Jun 12, 2018, sewage water samples were collected from four separate sewage tanks of the old hospital (STW, STA, STC, and STE) once a week. In the period from Jun 6 to July 17, sewage samples were collected from the sewage tank of the new hospital (STN) once a week. At 9 a.m., 20 ml of sewage sample was collected in sterile bottles from the manhole of the sewage tank and processed for analysis within 2 h.

DNA-seq analysis

Metagenomic analysis for whole organisms in original hospital sewage: First, 5 ml of sewage was centrifuged at 5,000 x g for 5 min and the resultant cell pellet was vortexed with a remaining 500 µl of sewage water. Next, the cell suspension was mixed with 500 µl phenol/chloroform/isoamylalcohol (PCI) in a microcentrifuge tube with 2 ml of beads. Cell breaking was performed by GenoGrinder 2010 by shaking at 1,500 rpm for 5 min. The PCI mixture was centrifuged at 8,000 rpm for 5 min, followed by DNA purification using a Gel DNA Recovery Kit, Zymoclean-96 (ZYMO RESEARCH, [Irvine, CA, USA](#)). A metagenome DNA-seq library was prepared using the QIAseq FX DNA library prep kit (Qiagen: [Venlo, Netherlands](#)), followed by sequencing using NextSeq 500 (Illumina) with NextSeq 500 mid output kit v2.5 (300 cycle).

Metagenomic analysis for EPOs on CHROMagar-ESBL: Two microliter of sewage sample was diluted with 100 µl phosphate buffered saline (PBS), plated on CHROMagar-ESBL (bioMérieux, Marcy-l'Etoile, France) for selection of EPOs, and incubated at 36°C overnight. Cultured colonies were harvested and mixed with 1000 µl of PBS. Next, 100 µl of the bacterial suspension from CHROMagar-ESBL was transferred to a 2 ml ZR BashingBead™ Lysis tube, and mixed with 500 µl PCI and 500 µl *elution buffer* (10 mM Tris pH 8.0). The tube was shaken by GenoGrinder 2010 at 1,500 rpm for 5 min. A metagenome DNA-seq library was prepared using the QIAseq FX DNA library prep kit (Qiagen: [Venlo, Netherlands](#)), followed by sequencing using NextSeq 500.

EPO isolates in hospital sewage

To selectively grow EPO isolates from sewage samples, 50 µl of sewage from the glycerol stocks prepared on May 22 and July 17 were diluted with 1000 µl PBS, followed by plating on CHROMagar-ESBL, and incubation at 37°C overnight. Subsequently, 80 colonies were selected as potential EPOs and streaked on fresh CHROMagar-ESBL plates cultured at 37°C overnight to obtain pure cultures. ESBL producing bacteria were cultured in tryptic soy broth, and the cell suspension was transferred to 2 ml ZR BashingBead™ Lysis tubes, and mixed with 500 µl PCI. The tube was shaken by GenoGrinder 2010 at

1,500 rpm for 5 min. The PCI extract was centrifuged at 8,000 rpm for 5 min, followed by DNA purification using a Gel DNA Recovery Kit, Zymoclean-96 (ZYMO RESEARCH). A DNA-seq library was constructed by a QIAseq FX, followed by sequencing using NextSeq 500.

EPO clinical isolates

All 20 EPO clinical isolates obtained between May 8 and July 17, 2018 were subjected to whole-genome sequencing as described above, and comparative genomics. Among the 20 clinical isolates, 12 isolates were obtained from outpatients and 11 isolates were obtained from urological patients. The samples were from urine, sputum, and central venous catheter (75%, 10%, and 10%, respectively). Some specimens were obtained from the same patient through subsequent diagnosis. The ethical committee in Toho University Ohashi Medical Center waived the need for written consent regarding the research into bacterial isolates (Investigation No. pH). The personal data related to the clinical information were anonymized, and our procedure does not require a written consent from patients suffering from bacterial infections. Antibiotic susceptibility of samples cultured in the microbiology laboratory within the same period were determined using breakpoints standardized by the Clinical and Laboratory Standards Institute (CLSI). Screening (broth microdilution method) and confirmatory tests (the disk diffusion method) on the EPOs were conducted according to CLSI recommendations. (CLSI Performance Standards for antimicrobial disk susceptibility tests; Approved standard-13th edition CLSI document M02. Wayne, PA: Clinical and Laboratory Standards Institute; 2018).

Bioinformatics

To characterize microbial flora in the sewage samples, the sequencing reads were analyzed by MePIC2 [52] and Krona [53] and MEGAN v6 software [54]. To characterize isolates of bacterial species and identify antimicrobial resistance genes, sequencing reads were analyzed by multi locus sequence typing (MLST) [55] and ResFinder [56], respectively.

Principal Coordinate Analysis (PCoA)

The sequenced reads were assigned to a taxonomic hierarchy using MEGAN v6 software based on a megaBLAST nucleic acid homology search.

Core genome single nucleotide variation phylogenetic analysis

To compare the genotype of 37 *E. coli* strains isolated from patients and hospital sewage, the Illumina short reads, excluding low quality and adapter sequences, were aligned using BWA-MEM [57] against the complete chromosome sequences of *E. coli* STN0717-11, followed by extraction of single nucleotide variants (SNVs) using VarScan v2.3.4 [58]. The prophage and repeat regions were predicted by PHASTER [59] and MUMmer 3 [60], respectively, and the detected SNVs in these regions were excluded. Regions of recombination in the chromosome were predicted using Gubbins v. 2.3.4 [61], followed by masking SNVs in the recombination regions. A maximum likelihood phylogenetic tree was constructed from SNV sites in the core genome region using FastTree2. *De novo* assembly was performed using SKESA v.2.3.0 [62] with short reads of each strain, followed by analyzing sequence type, putative serotype, and AMR gene prediction using pubMLST (<https://pubmlst.org/escherichia/>), SeroTypeFinder [63], and Bacterial Antimicrobial Resistance Reference Gene Database (BioProject ID: PRJNA313047), respectively.

Measurement of concentrations of chemical compounds

Forty drug components in the sewage samples were analyzed using solid-phase extraction (SPE) and ultra-performance liquid chromatography - tandem mass spectrometry (LC-MS/MS) based on a previously described method [64] with minor modifications. Briefly, the sample was filtered by a polyethersulfone membrane (0.22 µm pore size, Merck) and 100 ml of the filtrate was spiked with 1 g/l ascorbic acid, 1 g/l EDTA, and a surrogate standard mixture, and then concentrated by SPE cartridge (Oasis HLB cartridges, 200 mg/6 cc, Waters, Japan). The analytes concentrated on the cartridge were extracted with 6 ml of methanol before being measured by LC-MS/MS and quantified by the alternative surrogate method [64].

Results And Discussion

Hospital setting

The Ohashi Medical Center in Toho University is a general hospital opened in 1973. Prior to relocation, the hospital consisted of four buildings: West building (BW), Administration building (BA), Central building (BC), and East building (BE) and had a total of 430 beds, and accepted an average of 850 outpatients per day (Fig. 1). In the BW, there were intensive care wards (ICU, cardiovascular surgery, cardiovascular internal medicine, respiratory, and internal medicine), but no outpatient departments. In the BA, there were several outpatient departments, but the toilets were for only healthcare workers. In the BC, patients with digestive diseases, urinary diseases, and bone fracture were mainly accommodated, and the number of beds was the largest among the old hospital buildings. The BE included the highest number of outpatient departments with no intensive care wards.

The hospital relocated to the new building (BN) on June 20th, 2018. In the BN, all outpatient departments and wards were integrated into one building. The sewage from patients was not mixed with other

wastewater sources (laboratory and general wastewater) in the wastewater systems of the old and new hospital (Fig. 1).

The average number of hospitalized patients in May was 246, and the number of hospitalized patients in BC, BE, and BW building started to decrease in June. Consequently, 66 patients were transferred to BN on June 16 (Fig. 2). After the new hospital was opened on June 20, we started outpatient examinations and accepted a new hospitalized patient, and the number of inpatients gradually increased and exceeded 250 on July 2.

Metagenomic analysis of tank flora in each building

To elucidate the differential microbial flora in the hospital sewage tank (tank flora), metagenome DNA-seq analysis of sewage samples was conducted. The dominant bacteria in the sewage were classified according to the metagenomic data (Supplemental Data Set S1), wherein the most common genus was *Aeromonas*, followed by *Citrobacter* and *Bacteroidetes* (Fig. 3a). In addition, the proportion of genera varied depending on the building; the largest genus comprised of *Citrobacter* in STA, *Aeromonas* in STC, *Bacteroides* in STE, *Acinetobacter* and *Citrobacter* in STW, and variable genera in STN. Notably, less diversity was observed in STA (Fig. 3b) tanks because the toilet users in BA were limited to staff members, and the amount of excretion was also small.

In our hospital's sewage tank, bacteria originating from human gut flora were expected to be predominant as only stool and urine excreted from the toilet are stored. Composition of bacterial flora in hospital sewage has been reported to comprise of human gut flora *Bacteroides*, *Faecalibacterium*, *Bifidobacterium*, *Blautia*, *Roseburia*, and *Ruminococcus*, in addition to *Klebsiella*, *Aeromonas*, *Enterobacter*, *Prevotella*, and *Comamonas* [5]. In the gut flora of healthy Japanese adults, the anaerobes *Bifidobacterium*, *Blautia*, *Bacteroides*, *Faecalibacterium*, *Eubacterium*, *Ruminococcus*, and *Collinsella* are predominant [66]. However, hospital sewage is mainly influenced by the patients' gut flora, which is different from healthy individuals. Thus, flora in each tank at the old hospital (STA, STW, STC, and STE) exhibited different bacterial compositions depending on the characteristics of the patients present in each building (Fig. 1).

Since June 5th, only construction company and hospital staff visited the BN, and the STN was composed of a few genera such as *Citrobacter*, *Aeromonas*, *Escherichia*, and *Klebsiella*. Thereafter, *Arcobacter*, *Citrobacter*, *Aeromonas*, *Bacteroides*, and *Acinetobacter* comprised the main constituents, and the variety of genera increased with time. In the STN, the composition of bacteria was partially consistent with that in the STC, STE, and STW. It appears that all bacterial species detected in the old sewage tanks (STA, STC, STE and STW) were integrated into STN. For instance, *Aeromonas* originated from STC and *Citrobacter* and *Acinetobacter* originated from STW. In the STN, *Comamonas* and *Arcobacter* were significantly detected after the transfer of inpatients. *Comamonas* is a genus of aerobic proteobacteria, generally considered as environmental bacteria with less pathogenicity. It is reported that one of the

Comamonas species, *C. testosterone*, aerobically degrades testosterone when cultured in testosterone-containing media [67]. In the STN, the enrichment of *Comamonas* in sewage suggests the possibility of its role in decontamination of medical compounds. *Arcobacter* spp. are gram-negative rod-shaped bacteria of the family *Campylobacteraceae*. *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* have been implicated in severe acute gastrointestinal infections and bacteremia after exposure to shellfish and/or ingestion of contaminated water, or inadequately cooked meat/shellfish [International Commission on Microbial Specification for Foods (ICMSF, 2002)]. In the present study, there were no instances of infectious enteritis due to *Arcobacter* in the hospital; however, the causative bacteria of infectious enteritis are not easily identifiable, except in limited conditions. *Arcobacter* is detected in WWTP in several countries [68-71], and the presence of antimicrobial resistant *Arcobacter* has also been documented [70, 72]. *E. coli* and *Klebsiella* were not abundant, but were consistently detected, in all sewage tanks of buildings. It is unclear whether the difference in the bacterial composition of each tank reflects characteristics of each building (department, diseases, drugs administered). Unlike the sewage system in the old hospital (STA, STC, STE, and STW), the new hospital has a single sewage tank (STN). Thus, the bacterial composition of STN looks similar to that of STW, suggesting that the flora in STW was most influenced by STN.

Similarity and diversity of bacterial population amongst tanks was analyzed using principal co-ordinates analysis (PCoA) based on bacterial genus level (Fig. 4). A PCoA used a total of 25 sewage samples of each tank excluding STA, and showed that the STN and STW groups were closely plotted, while the STE and STC groups were largely separated. The BW and BN contain rooms where seriously ill patients are treated, such as intensive care unit (ICU), coronary care unit (CCU), and respiratory disease wards (Fig. 1). According to PCoA analysis, the distribution of flora in STN after the transfer of patients was similar to that of STW, indicated by the presence of *Aeromonas*, *Citrobacter*, and *Comamonas* (Fig. 4). The distribution of tank flora can be strongly influenced by the wards for patients who were prescribed strong doses of antibiotics and other therapeutic agents. Furthermore, tank flora can be instantly affected by excrement of hospital patient/staff/visitor because the bacterial composition of STN was comparable to that of STW within one month after the relocation.

In this study, the old hospital sewage tanks (STA, STC, STE, and STW) were separately installed in each building; thus, a marked difference was observed among tanks. In contrast, it was difficult to identify the relationship between the flora and specific departments in the new hospital sewage tank, STN, because of a single tank in the building. For the purpose of monitoring department-specific ARB, it may be beneficial to install department-specific sewage tanks.

Analysis of β -lactamase genes in flora of tanks

The metagenome next generation sequence (NGS) reads corresponding to β -lactamase genes were identified in original hospital sewage samples (Fig. 5a) and EPOs from each tank were selected on CHROMagar ESBL (Fig. 5b). In the original hospital sewage samples, various β -lactamase genes such as

*bla*_{GES}, *bla*_{OXA}, and *bla*_{CMY} were detected in flora of STC, STW, and STN, while almost no β -lactamase genes were detected in flora of STA and STE. This may be due to the limited sensitivity of metagenomic detection in this study. The *bla*_{IMP} gene was detected from STC and STW samples, and the *bla*_{CTX-M} gene was detected from STW and STN samples (Fig. 5a). CHROMagar ESBL selection facilitated the detection of EPOs as β -lactamase genes were detected in STA and STE using this technique. Moreover, *bla*_{IMP} and *bla*_{CTX-M} genes were present in flora of all sewage tanks (Fig. 5b).

Genome comparison between sewage and clinical isolates

Although metagenomic analysis showed the number of each bacterial species and ARG, it is impossible to deduce which bacterial strain carries a particular ARG. Thus, whole genome sequencing was performed for 78 EPO isolates from STW0522 and STN0717, and 20 EPO clinical isolates (May 8, 2018 to July 17, 2018) for comparison of genomic sequences of EPOs from sewage tanks and clinical sources. In the STW0522 sample, bacteria such as *E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Achromobacter* were detected, and in the STN0717 sample, *E. coli*, *Klebsiella*, *Enterobacter*, and *Citrobacter* were detected (Table 1). Clinical isolates included only *E. coli* and the *Klebsiella* spp., wherein *E. coli* displayed sequence variations compared to sewage isolates (Table 2).

A pairwise *single nucleotide variation* (SNV) analysis of the core genome was conducted for all *E. coli* strains (Fig. 6). The *E. coli* sequence types (STs) included ST393 (n = 16), ST38 (n = 8), ST131 (n = 5), ST1011 (n = 4), ST12 (n = 1), ST73 (n = 1), ST9586 (n = 1), and ST224 (n = 1). *E. coli* ST12, ST73, ST131, and ST1011 were detected exclusively in clinical isolates (Table 2). Clinical EPO isolates (THO-008 and -019 from same patient; Patient-No. 8) comprised of ST393 harboring *bla*_{CTX-M-27} and there was no difference in SNVs between isolates (Fig. 6), indicating that the clone was identified from the same patient. In addition, these clinical isolates also showed no SNVs with sewage isolates (14 isolates; STN0717-1 to -11, 14, 15, and 19), suggesting that these sewage isolates may have originated from the same patient (Patient-No. 8) (Supplemental Data Set S2). Monitoring of ARB/ARGs in hospital sewage may enable detection of latent carriers or nosocomial infections.

Three ST38 clinical isolates (THO-002 identified from Patient-No. 2, THO-007 and -020 identified from Patient-No. 7) harboring *bla*_{CTX-M-14} were identified within a three-week duration, and further SNV analysis revealed marked 21-110 SNVs in the core-genome. This result suggested that all three isolates are closely related; however, there was no strong evidence of a nosocomial outbreak associated with this single clone. It is reported that molecular evolution of *E. coli* genome is possible with less than 5 SNVs within a 60-day duration [73]. In contrast, ST38 sewage isolates (STN0717-12, -13, -17, -18, and -21) harboring *bla*_{CTX-M-55} exhibited strict clonality with 3 SNVs, and showed ≥ 123 SNVs with clinical isolates (THO-002, -007, and -020) (Fig. 6). ST131 is an *E. coli* isolate responsible for a worldwide pandemic and carries a broad range of pathogenicity and antimicrobial resistance-associated genes, including a variety of β -lactamase genes (CTX-M family, TEM, SHV, and CMY β -lactamases) on a transferable plasmid [74-77].

Five ST131 isolates (THO-001, -003, -005, -015, and -016) harboring various CTX-M genes (*bla*_{CTX-M-15}, *bla*_{CTX-M-27}, *bla*_{CTX-M-44}) were identified from individual patients and 32–84 SNVs were observed between the isolates, suggesting that the isolates were closely related but not identical (Fig. 6).

Thus far, the healthy carriage rate of EPOs has been rising worldwide. In Japan, detection rate of EPO was reported at 12.2% in healthy adult volunteers [78] and at 15.6% in healthy food handlers [79]. Additionally, it is reported that 92.9% of EPOs were *bla*_{CTX-M} gene positive [80]. The CTX-M genes (*bla*_{CTX-M-14}, *bla*_{CTX-M-27}, *bla*_{CTX-M-15} and *bla*_{CTX-M-2} in descending order in size) are mainly identified in Japan and its reported sequence is similar to that observed in EPOs in the hospital sewage and clinical isolates in this study (Table 3). Prominent global trends in CTX-M epidemiology indicate that the reduced occurrence of *bla*_{CTX-M-2} and emergence of *bla*_{CTX-M-27} [75], suggesting that healthy carriers could be at risk for nosocomial infections by EPOs.

In the sewage samples, isolates possessed various β -lactamase genes; *bla*_{SHV-12}, *bla*_{CTX-M-14}, and *bla*_{CTX-M-62}, in *Enterobacter kobei*; *bla*_{SHV-12} in *Enterobacter cloacae*; *bla*_{CTX-M-9} in *Enterobacter homaechei*; *bla*_{IMP-11} in *Pseudomonas monteilli*, and *bla*_{SHV-12} in *Raoultella planticola* (Supplemental Data Set S2). Many of the potential EPOs harboring ARGs in the sewage tanks were different from the clinical isolates. It is not clear whether these EPOs were excreted by healthy carriers or were transformed by acquiring the ARGs in the sewage tank.

Among the 156 CHROMagar ESBL-positive strains from hospital sewage tanks (STW0522 and STN0717) (Supplemental Data Set S2), carbapenemase gene (*bla*_{IMP-11}) was identified only in *Pseudomonas monteilli*, but not in *E. coli* and *Klebsiella* (Table 3). The *bla*_{IMP-11} gene was originally found in clinical isolates of *E. cloacae* [81], *K. pneumoniae*, *E. coli* [82], *P. aeruginosa* [83], and *Acinetobacter* species [84] in Japan. *P. monteilli* is a Gram-negative bacteria of the *P. putida* group and occurs in the soil, garbage, and drains [85]. *P. monteilli* is less pathogenic to humans, but was isolated from clinical samples [49, 86–88] and hospital environment [85, 89]. *P. monteilli* may play a role as a Metallo- β -lactamase (MBL) reservoir and transfer of MBL genes to other species such as *Enterobacteriaceae* may be a cause of concern, especially in hospital sewage tanks [49, 87, 89, 90].

Concentration of residual antimicrobial agents in hospital sewage tanks

There is a possibility that sewage tanks provide an environment conducive to the development of novel drug resistant bacteria. Sewage tanks are reservoirs of ARGs and transfer of genes between bacteria may occur under the selective pressure of antimicrobial agents and antiseptics. Indeed, concentration of chemical contaminants in the tank (Table 4) showed that the most predominant antimicrobial agent was levofloxacin (32,500 ng/l) and clarithromycin (13500 ng/l), which may potentially exhibit inhibitory action in the sewage tank environment, although their concentrations were below MIC breakpoints.

In hospital wastewater, ciprofloxacin concentration was reported to be 22,000 - 179,000 ng/L in Germany [91], 19,110 - 428,00 ng/L in Vietnam, and clarithromycin was reported to be up to 960 ng/L in Portugal [92] and 760 - 72,800 ng/l in Singapore [27]. The present study detected an antibiotic concentration similar to the above reports. Although β -lactam antibiotics were not measured in this study, they are known to be almost undetectable in environmental samples [93, 94]. This is because unstable lactam rings increase susceptibility of β -lactam antibiotics to hydrolysis immediately after excretion [94, 95]. Hospital sewage tanks containing a large amount of residual antibiotics [96] may change the composition of tank flora and further promote the development of AMR by high selective pressure on bacteria [20, 97, 98]. Antibiotics exert selective pressure in favor of the growth resistant bacteria, even at very low concentrations [99-101]. Moreover, there are some reports that concentration of antibiotics in hospital *sewage* are often higher than the reported no-effect or minimum concentrations for resistance selection, suggesting that the selection of ARB can occur in this environment [97, 102]. We presume that selective pressure of antibiotics exists in our hospital sewage tanks; however, this will be verified in future studies.

Horizontal gene transfer is a cause of great concern as it is one of the most important mechanisms of the spread of antibiotic resistance in the environment [103, 104]. It is suggested that nutrient-rich environments such as wastewater offer optimal conditions for horizontal gene transfer, involving the transconjugation of AMR plasmids encoding ARGs [105] and transformation of non-pathogenic bacteria into reservoirs of ARGs. It is known that a microbial gut flora composed of a spectrum of bacteria functions as a reservoir for ARGs and horizontal plasmid transfer between bacteria is common [103]. This is plausible as sewage tanks consists of an accumulation of excrement and acquisition of resistance may occur frequently.

In Sweden, carbapenemase-producing *Klebsiella* was also detected from clinical specimens, in Svartån River and the rivers downstream of it [11]. In Japan, KPC-2 producing *Klebsiella* [16, 17] and NDM-5-coproducing *Escherichia coli* [17] were detected from the effluent of urban wastewater treatment plants. Effective actions should be taken including advanced wastewater treatment processes such as ozone and UV treatment [47, 106, 107], and ultrafiltration [108] to accelerate the removal of ARB in WWTP. However, even the above methods do not ensure a complete removal of resistant bacteria, therefore, treatment processes may be introduced prior to the release of hospital sewage into the main sewage to reduce ARB and residual drug concentrations. In Japan, there are a few reports of contamination of sewage with ARB and presence of ARGs in microbial flora of hospital sewage tanks [109, 110]. Nevertheless, this study is the first comprehensive description of AMR in a hospital setting using metagenomic and whole genome analysis.

Conclusions

In many countries including Japan, hospital wastewater is discharged into sewage without treatment, and ARB are detected from the drained rivers, and soil and water environment. Therefore, the frequency of ARGs in bacteria increases and consequently, there is an AMR burden on the environment. The

dissemination of ARB/ARGs in the environment can increase the risk of infectious diseases [18]; however, there are very few direct suggestive data about their epidemiological effects [111]. Our study reveals the presence of ARB/ARGs in the hospital sewage tank and suggests that every hospital patient/staff/visitor can be a potential carrier. Furthermore, the hospital sewage tank is a hot spot where various bacterial species may acquire ARGs. Monitoring of ARB/ARGs in hospital sewage is expected to identify the presence of carriers, and control nosocomial outbreaks and dissemination of ARB/ARGs in the community.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The metagenomic short-read sequences for DNA-seq were deposited in the DNA Data Bank of Japan (BioProject PRJDB9036; BioSample SAMD00195117-SAMD00195180; DRA accession DRA009310) (Supplemental Data Set S1). The NGS data for whole genome sequencing were deposited in the DNA Data Bank of Japan (BioProject PRJDB9036; BioSample SAMD00195843-SAMD00196018; DRA accession DRA009309). All complete sequences in this study are available from the DDBJ/ENA/GenBank database (accession numbers AP022380-AP022556), as shown in the supplementary data files (Supplemental Data Set S2).

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

MK2, MW, and SK designed the study. MK1 contributed to the sampling of waste water samples. MK2 conducted bacterial isolation and performed genome sequencing. TS performed the comparative genome analysis. NN conducted a measurement of concentration of chemical compounds. MK1 and MK2 wrote the manuscript.

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Tables

Table 1. Bacteria isolates growing on CHROMagar ESBL

Bacteria species	Hospital sewage tank		Clinical isolate ^c
	STW0522 ^a	STN0717 ^b	
<i>Escherichia coli</i>	1	20	16
<i>Klebsiella pneumoniae</i>	2		3
<i>Klebsiella aerogenes</i>		1	
<i>Klebsiella michiganensis</i>			1
<i>Klebsiella quasipneumoniae</i>	1		
<i>Klebsiella variicola</i>	1		
<i>Klebsiella sp.</i>	2		
<i>Enterobacter kobei</i>	21	2	
<i>Enterobacter bugandensis</i>		12	
<i>Enterobacter hormaechei</i>	3	1	
<i>Enterobacter cloacae</i>		3	
<i>Enterobacter roggenkampii</i>	1		
<i>Citrobacter portucalensis</i>	16	39	
<i>Citrobacter freundii</i>	20		
<i>Achromobacter xylosoxidans</i>	5		
<i>Pseudomonas monteilii</i>	3		
<i>Raoultella planticola</i>	1		
<i>Stenotrophomonas maltophilia</i>	1		

^aSTW0522: sewage tank of west building at 22, May, 2018

^bSTN0717: sewage tank of new building at 17, July, 2018.

^cClinical isolate: patients from 8, May 2018 to 17, July 2018

Table 2. Serotype of ESBL-producing *E.coli* in sewage and clinical isolate

Sequence type	Serotype	Hospital sewage tank		Clinical isolate ^c
		STW 0522 ^a	STN 0717 ^b	
ST12	O4:H1			1
ST38	O86:H18		5	3
ST73	O6:H1			1
ST131	O25:H4			5
ST224	O8:H23	1		
ST393	O15:H1		14	2
ST1011	O130:H10			4
ST9586	O8:H10		1	

^aSTW0522; sewage tank of west building at 22, May 2018

^bSTN0717; sewage tank of new building at 17, July, 2018.

^cClinically isolate; clinically isolate from 8, May 2018 to 17, July 2018

Table 3. ESBL-producer in hospital sewage and clinical isolate

Bacteria	Antimicrobial resistant gene (ARG)	Hospital sewage tank		Clinical isolate ^c
		STW 0522 ^a	STN 0717 ^b	
<i>Escherichia coli</i>				
	blaCTX-M-14	1		4
	blaCTX-M-15			2
	blaCTX-M-27		14	6
	blaCTX-M-44			1
	blaCTX-M-55		6	4
<i>Klebsiella pneumoniae</i>				
	blaCTX-M-14			2
	blaCTX-M-15			1
	blaCTX-M-2	2		
	blaSHV-26	2		
	blaSHV-77			1
	blaSHV-78			2
<i>Klebsiella quasipneumoniae</i>				
	blaCTX-M-2	1		
<i>Klebsiella variicola</i>				
	blaCTX-M-62	1		
<i>Klebsiella michiganensis</i>				
	blaCTX-M-8			1
<i>Klebsiella sp.</i>				
	blaCTX-M-5	2		
<i>Enterobacter cloacae</i>				
	blaSHV-12		1	
<i>Enterobacter kobei</i>				
	blaCTX-M-14	5		
	blaCTX-M-62	1		
	blaSHV-12		2	

Enterobacter hormaechei

blaCTX-M-9	3	1
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Pseudomonas monteilii

blaIMP-11	3	
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Raoultella planticola

blaSHV-12	1	
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^aSTW0522; sewage tank of west building at 22, May 2018

^bSTN0717; sewage tank of new building at 17, July, 2018.

^cClinical isolate; clinical isolate from 8, May 2018 to 17, July 2018

Table 4. Concentrations of chemical compounds in the hospital sewage tank

Antimicrobials and others	Concentration (ng/L)		Note
	STN0717 ^{ab}	Limit of quantity	
Quinolone			
Levofloxacin	32500	5.00	clinical
Norfloxacin	n.d.	1.00	clinical
Enrofloxacin	n.d.	2.00	veterinary
Macrolide/lincosamide			
Azithromycin	390	0.01	clinical
Clarithromycin	13500	0.10	clinical
Lincomycin	370	0.05	clinical
Roxithromycin	n.d.	10.00	clinical
Sulfonamides			
Sulfamethoxazole	1360	20.00	clinical
Sulfadimethoxine	n.d.	0.04	veterinary
Trimethoprim			
Trimethoprim	1160	5.00	clinical
Tetracycline			
Tetracycline	n.d.	0.70	clinical
Oxytetracycline	n.d.	3.00	veterinary
Other clinicals			
Antihypertensive			
Valsartan	6670	20.00	angiotensin \square receptor antagonist
Telmisartan	6010	100.00	angiotensin \square receptor antagonist
Olmesartan	1890	0.10	angiotensin \square receptor antagonist
Irbesartan	1470	10.00	angiotensin \square receptor antagonist
Losartan	1350	0.15	angiotensin \square receptor antagonist
Candesartan	681	5.00	angiotensin \square receptor antagonist
Azilsartan	167	20.00	angiotensin \square receptor antagonist
Valsartan acid	140	5.00	angiotensin \square receptor antagonist
Atenolol	1020	5.00	β -blocker
Atenolol acid	280	0.82	β -blocker
Metoprolol	30	5.00	Adrenergic effect blocker
Diltiazem	210	0.03	Vasodilators
Anti-allergic drug			
Fexofenadine	7440	5.00	H1 receptor antagonist
Diphenhydramine	340	0.06	H1 receptor antagonist

Epinastine	27	10.00	H1 receptor antagonist
Caffeine			
Caffeine	38100	10.00	xanthine derivative
Bronchodilator			
Theophylline	14200	0.10	xanthine derivative
Non-steroidal anti-inflammatory analgesic			
Ibuprofen	2520	39.00	propionic acid
Ketoprofen	2180	10.00	propionic acid
Ethenzamide	40	8.24	Salicylic acid
Gastric secretion inhibitor			
Pirenzepine	70	0.03	Muscarinic receptor antagonist
Antipsychotic drug			
Quetiapine fumarate	20	0.05	Dibenzothiazepine
Sulpiride	2820	0.05	Benzamide
Hyperlipemia drug			
Bezafibrate	9760	10.00	Hyperlipidemic agent
Antiarrhythmic drug			
Disopyramide	2650	0.09	Antiarrhythmic drug
Vasodilator drug			
Dipyridamole	190	5.00	Antiplatelet drug
Diuretic			
Furosemide	7510	20.00	Diuretic

^aSTN0717; sewage tank of new building at 17, July 2018

^bn.d; not detected

Figures

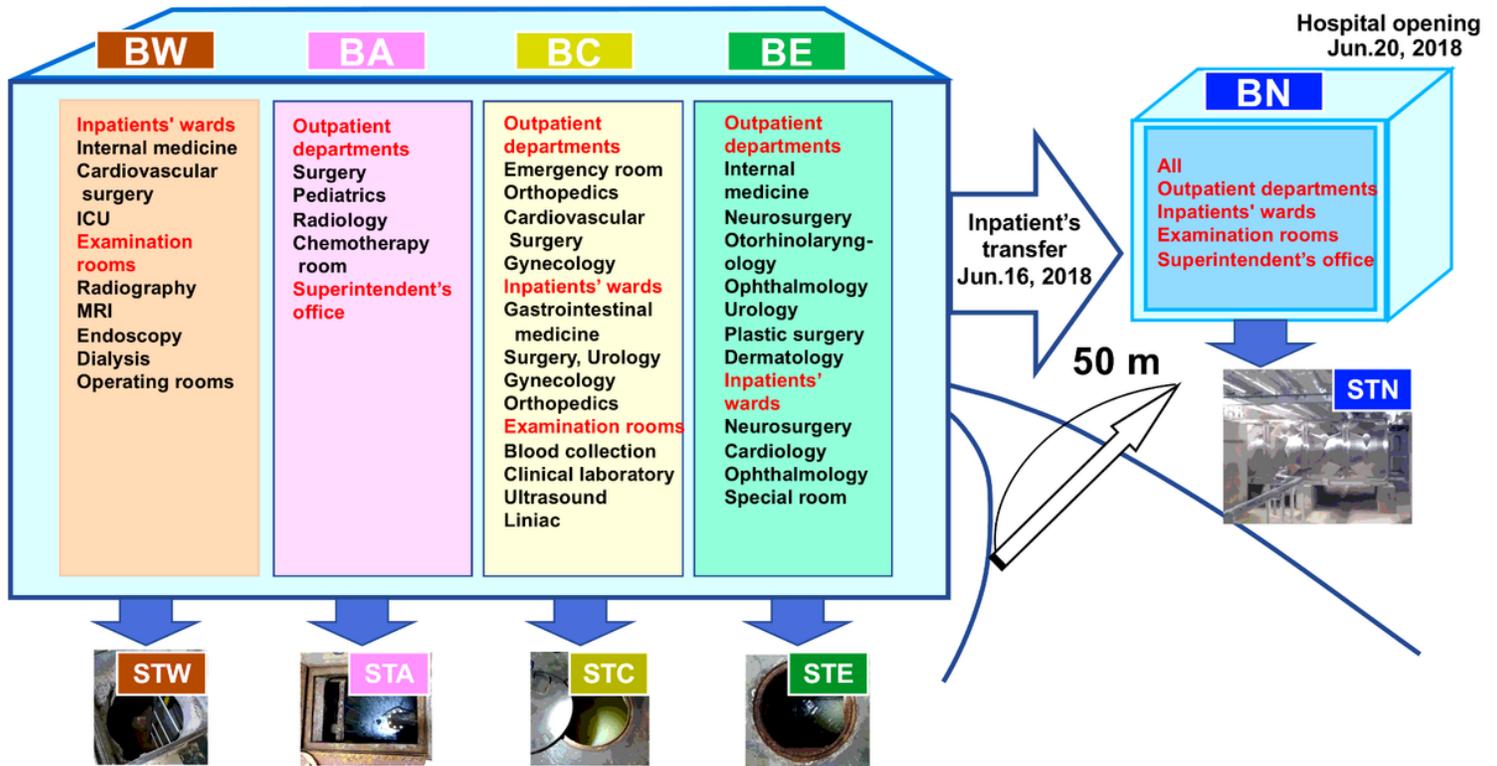


Figure 1

Outline of the Ohashi Medical Center, Toho University. Prior to relocation to the new building (BN), the hospital consisted of four buildings: West building (BW), Administration building (BA), Central building (BC), and East building (BE).

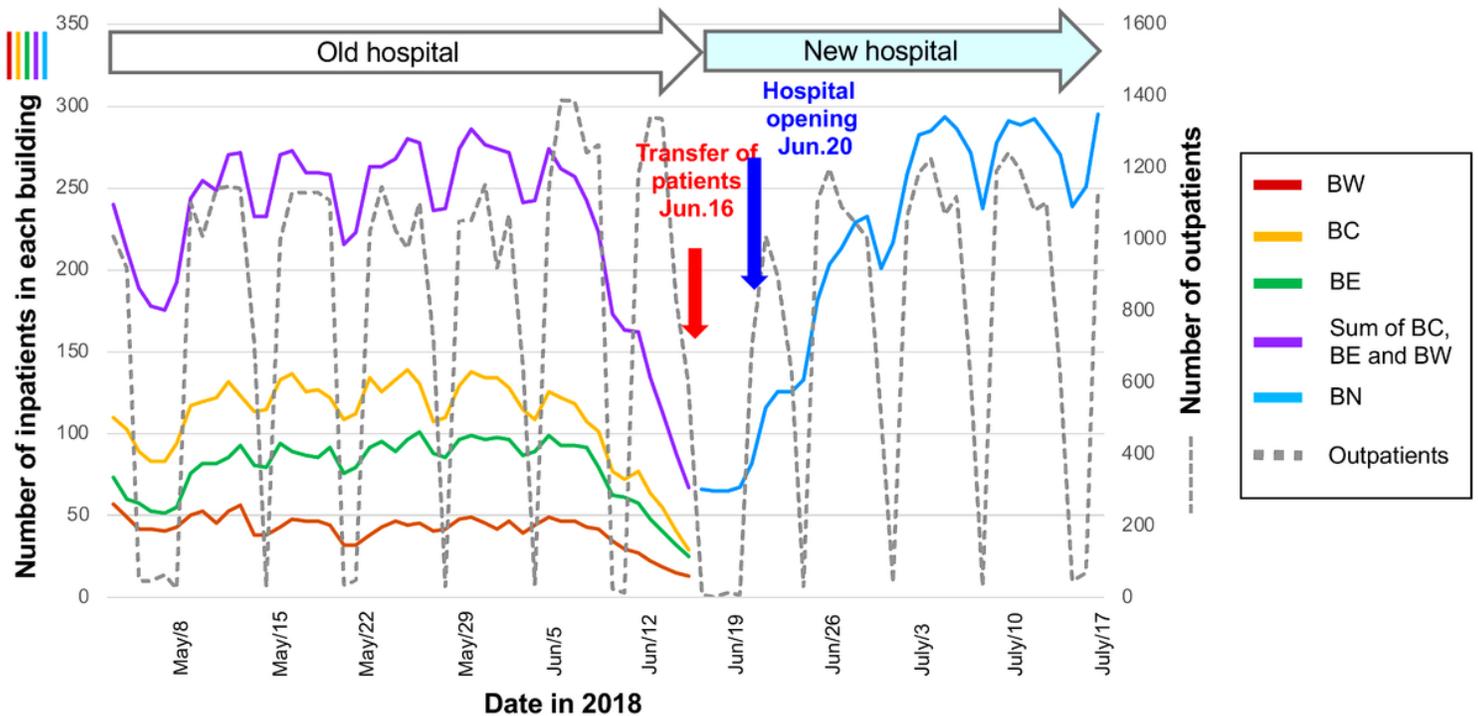


Figure 2

Graph represents the transition of the number of outpatients and hospitalized patients from May 1 to July 17, 2018. The number of beds were 65, 0, 148, 106, and 319 for BW, BA, BC, BE, and BN, respectively. In June 2018, due to the inauguration of the new hospital, the number of hospitalized patients gradually decreased, and 67 hospitalized patients (BC: 29, BE: 25, BW: 13 patients) were transferred to the BN on June 16, 2018. The number of hospitalized patients recovered to as same as the number of patients in old hospital two weeks after the new hospital opening. The number of outpatients at the old hospital was approximately 1000-1200 on weekdays. Outpatient treatment was suspended from June 16 to 19 before opening of the new hospital, subsequently the number of patients gradually recovered to the number.

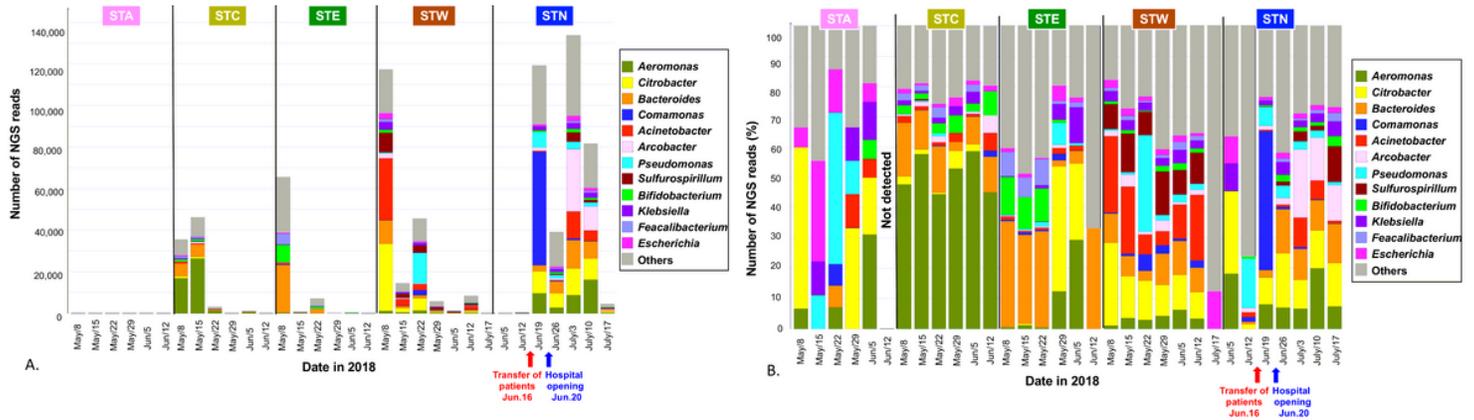


Figure 3

Metagenomic analysis of sewage samples in each building. NGS reads were classified based on the bacterial genus taxonomy and the graph displayed actual NGS read counts (a) and relative proportion (b) of each bacterial genus. In sewage tank of BA (STA), actual NGS read counts were very low compared with other sewage samples.

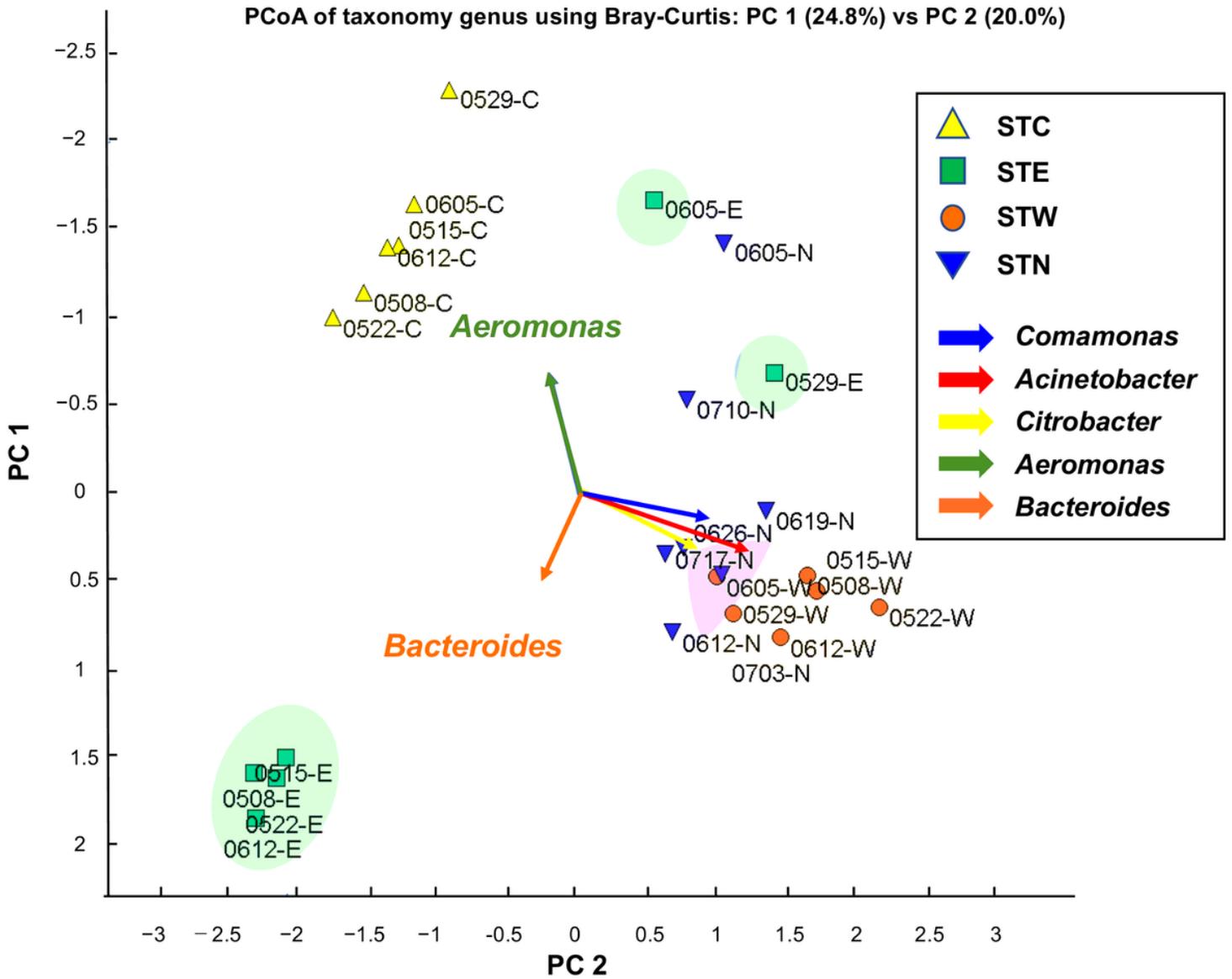


Figure 4

PCoA plot using detected NGS read counts by metagenome DNA-Seq. PCoA was performed according to Bray Curtis distance (the average linkage). The genera, *Acinetobacter*, *Citrobacter*, and *Comamonas* were common present in STW and STN samples. Most severely ill inpatients were treated in the BW and BN, thus their excretion may have a major impact on the bacterial content of the sewage tanks.

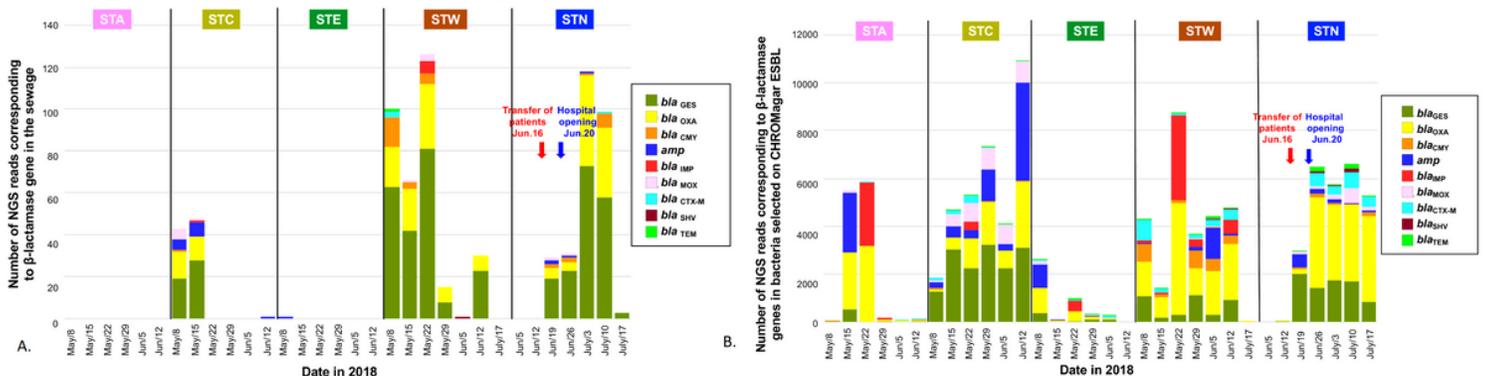


Figure 5

NGS read counts corresponding to β -lactamase genes in each sewage tank. The metagenome read counts corresponding to β -lactamase gene of (a) the sewage samples and (b) that of the grown bacteria on CHROMagar ESBL is displayed. The read counts of blaCTX-M gene in original sewages were not significant (panel a), but selection on CHROMagar ESBL resulted in increasing detection of CTX-M-positive organisms in STN, after relocation to new building (June 20, 2018).

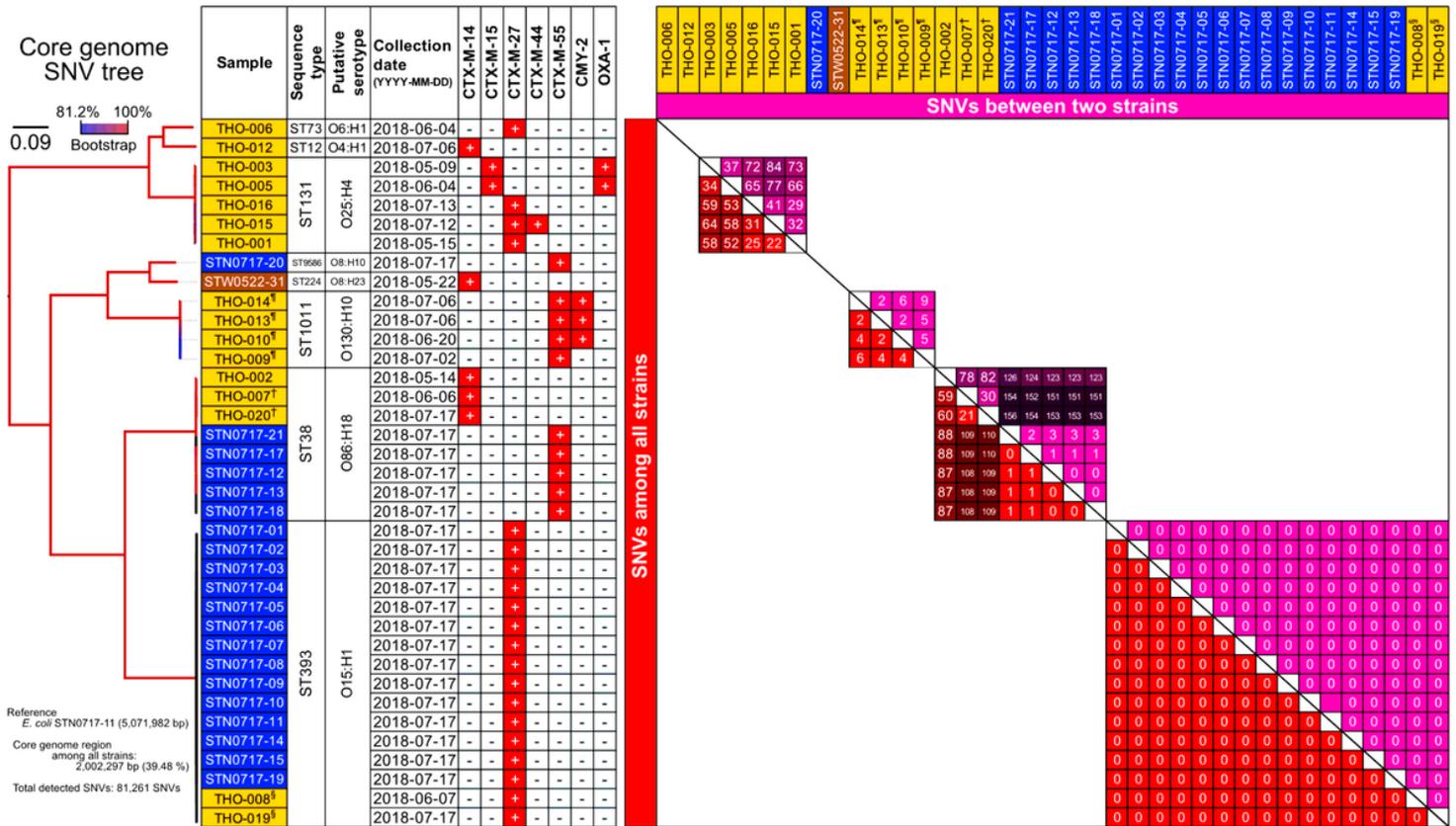


Figure 6

Core genome phylogeny using single-nucleotide variations (SNVs) of ESBL-producing *E. coli* isolates. Core genome phylogeny was constructed using ESBL-producing *E. coli* isolates; 20 clinical isolates (THO-number, orange highlighted), one sewage isolate from STW0522 (brown highlighted), and 20 sewage isolates from STN0717 (blue highlighted). Complete genome sequence of STN0717-11 was used as a genome reference and 39.48% of the genome sequence was used as core-genome regions among all tested strains. Few clinical isolates were obtained from same patient (§, patient No.8; ¶, patient No.9; †, patient No.7 in supplement Data Set S1). Heatmap of pairwise differences of core genome SNVs are shown using a color gradient with pink and red. The lower half part indicates core genome SNVs among all strains, and the upper half part shows core genome SNVs between indicated two strains. THO-008 and -019 from Patient No. 8 showed no SNVs with sewage isolates (14 isolates; STN0717-1 to -11, 14, 15, and 19). There were no identical clones between different patients.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [DataSetS1Metagenome.xlsx](#)
- [DataSetS2Isolates.xlsx](#)