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SHORT COMMUNICATION

Comparative Genomic Analysis and Phenotypic Characterization of Bronchoscope-Associated *Klebsiella aerogenes*

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Abstract

Bronchoscopes have been linked to outbreaks of nosocomial infections. The phenotypic and genomic profiles of bronchoscope-associated *Klebsiella aerogenes* isolates are largely unknown. In this work, a total of 358 isolates and 13 isolates were recovered from samples after clinical procedures and samples after decontamination procedures, respectively, over the five months. Antimicrobial susceptibility testing found seven *K. aerogenes* isolates exhibiting a low-level resistance to antimicrobial agents. Among seven *K. aerogenes* isolates, we found five sequence types (STs) clustered into three main clades. Collectively, this study described for the first time the phenotypic and genomic characteristics of bronchoscope-associated *K. aerogenes*.

K e y w o r d s: *Klebsiella aerogenes*, bronchoscope-associated, phenotypic, genomics characteristics

Flexible bronchoscopes are medical devices widely used for diagnostic and therapeutic procedures. Usually, they are heat-labile and complex, which leads to difficulty in cleaning procedures (DiazGranados et al. 2009). Patient-ready reusable, flexible bronchoscopes can be contaminated and damaged and pose a severe threat to patient safety (Zamani 2004). It is well documented in the literature that some nosocomial outbreaks have been linked to contaminated or inadequately disinfected bronchoscopes (Srinivasan et al. 2003). Moreover, recent studies determined that microbial transmission occurs even when proper cleaning and disinfection protocols are followed with standard guidelines (Galdys et al. 2019). Recently, endoscopes have been suggested as point sources of nosocomial Enterobacteriaceae infections (Gastmeier and Vonberg, 2014). Previous investigations have shown a significant reduction of microbial contaminants with cleaning alone, and recommendations require cleaning to be performed promptly following use (Galdys et al. 2019). In China, the national standard "Regulation for Cleaning and Disinfection Technique of Flexible Endoscope (WS507-2016)" was used for disinfection of endoscopes, including the selection of chemicals to ensure quality control throughout the clinical management (Gu et al. 2020). However, the microbiology data on cleaning and disinfection of endoscopes is unclear. Moreover, bronchoscope-associated microbial profiles and phenotypic characteristics are largely unknown.

K. aerogenes is a Gram-negative, rod-shaped, anaerobic bacterium, a commensal microorganism living in the mouth and gut. However, the bacterium is now

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resistant to many antimicrobial agents, including one of the latest antibiotics-carbapenems, which represents a serious challenge to public health (Zheng et al. 2020). It is an important opportunistic and multi-resistant bacterial pathogen for patients during the past decades in clinical settings (Malek et al. 2019). It is widely associated with bloodstream, skin and soft tissue, respiratory, and urinary tract infections (Shen et al. 2019). Recently, the emergence of carbapenem-resistant *K. aerogenes* and colistin-resistant *K. aerogenes* isolates in China is of concern (Liao et al. 2020). A previous study revealed that the prevalence of carbapenem-resistant *K. aerogenes* isolates in a Chinese teaching hospital was caused by clonal dissemination (Qin et al. 2014). However, the prevalence, epidemiology, resistance mechanism, and genetic background of *K. aerogenes* in China remain largely unknown due to the limited number of investigations performed to date in this field (Miao et al. 2019; Ma et al. 2020).

The aims of the current study were to evaluate the microbial profiles of endoscopes pre- and post-disinfection in the Disinfection and Sterilization Center, and investigate the phenotypic characteristics and genomic complexity of *K. aerogenes* strains isolated from bronchoscope samples.

From January 2019 to May 2019, the study was conducted in the Disinfection and Sterilization Center of the First Affiliated Hospital, School of Medicine, Zhejiang University, where both gastrointestinal and respiratory endoscopes are reprocessed. During the study period, procedures in our institution were performed using bronchoscopes (model BF260) (Olympus, Japan). The cleaning of bronchoscopes was carried out with an enzymatic detergent solution, endozyme. Manual disinfection was performed by soaking the device into 2% glutaraldehyde for 30 minutes.

Samples were collected under aseptic conditions from bronchoscopes following clinical procedures and after usual decontamination procedures by flushing thoroughly with 10 ml of sterilized phosphate-buffered saline (PBS) and shaking for 30 seconds, as described previously (Jørgensen et al. 2016). Collected samples were put in cool boxes with ice packs (4–8°C) upon collection and transported within 4 hours to the laboratory.

All samples (100 µl) were plated on Mueller-Hinton agar plates (Oxoid, UK) using the sterile swab. The agar plates were incubated for 18–24 hours at 37°C. A single colony was selected from each species per sample. All of the positive cultures were selected for identification. Bacterial identification was conducted by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) (Bruker, Leipzig, Germany) and further checked by PCR and sequencing.

The minimum inhibitory concentrations (MICs) of seven *K. aerogenes* isolates were determined using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) standards (Zheng et al. 2015). Nineteen antimicrobials were tested as described previously (Zheng et al. 2015). Antimicrobial susceptibility testing for colistin and tigecycline was performed using the microbroth dilution method described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The MIC results were interpreted using the CLSI standards (Third Edition: M45).

WGS was performed on all *K. aerogenes* strains identified in this work. The extracted genomic DNA was evaluated by agarose gel electrophoresis. The concentration and purity of genomic DNA were determined using NanoDrop 2000 (Thermo Scientific, Waltham, USA) and Qubit® version 2.0 fluorometer (Thermo Scientific), respectively. The sequencing library was prepared with the Illumina Nextera XT kit (Illumina, San Diego, USA). A-tailed fragments were ligated with paired-end adaptors and PCR-amplified with a 500-bp insert. WGS was performed using an Illumina NovaSeq 6000 platform (Novogene Co., China). PCR adapter reads and low-quality reads from the paired-end and mate-pair library were filtered using an in-house pipeline. Paired reads were then assembled into many scaffolds using Velvet version 1.2.10 (Zerbino and Birney, 2008). Multilocus sequence typing (MLST) analysis was performed as described previously (Cerqueira et al. 2017). Antibiotics Resistance Genes (ARGs) were identified using the ResFinder 4.1 database (https://cge.cbs.dtu.dk/services/ResFinder) (Zankari et al. 2012).

To further characterize the evolutionary relationship among *K. aerogenes* isolates, we created a core genome-based phylogenetic tree using seven *K. aerogenes* genomes sequenced in this study and 51 randomly selected publicly available *K. aerogenes* genomes (Table SI). The isolate collection included strains from humans (n = 44), the environment (n = 9), and other sources (n = 5) widely distributed over time and geographical locations. All collection genomes were annotated using Prokka (https://github.com/tseemann/prokka) and the RAST tool (https://rast.nmpdr.org). The core genes in *K. aerogenes* genomes were identified using Prokka, and maximum likelihood-based phylogenetic reconstruction was performed with Roary (https://github.com/yikedou/Roary). One hundred bootstrap replicates were evaluated to determine branch support. A maximum-likelihood phylogenetic tree based on the core single nucleotide polymorphism alignments was generated using FastTree (Price et al. 2009). Phylogenetic tree visualizations were produced using the Interactive Tree of Life (https://itol.embl.de).

Over the five months, 250 bronchoscopes were sampled, and 500 samples were collected in a single cycle, including 250 samples after clinical procedures and 250 samples after usual decontamination procedures.

Table I
The Minimum Inhibitory Concentration (MIC) and isolation time of seven bronchoscope-associated *Klebsiella aerogenes* isolates.

Isolate	Isolation time	AMC	TZP	CXM	FOX	CAZ	CRO	CSL	FEP	ETP	IPM	AMK	LVX	TGC	SXT
05021124	post-disinfection	16(R)	≤4(S)	4(S)	≥64(R)	≤0.12(S)	≤0.12(S)	≤8(S)	≤0.12(S)	≤0.12(S)	2(I)	≤2(S)	≤0.12(S)	≤0.5(S)	≤20(S)
04304169	pre-disinfection	≥32(R)	≤4(S)	4(S)	≥64(R)	1(S)	≤0.25(S)	≤8(S)	≤0.12(S)	≤0.12(S)	1(S)	≤2(S)	≤0.12(S)	≤0.5(S)	≤20(S)
04292179	pre-disinfection	≥32(R)	≤4(S)	4(S)	≥64(R)	1(S)	≤0.25(S)	≤8(S)	≤0.12(S)	≤0.12(S)	1(S)	≤2(S)	≤0.12(S)	≤0.5(S)	≤20(S)
04251141	pre-disinfection	≥32(R)	≤4(S)	4(S)	≥64(R)	≤0.12(S)	≤0.25(S)	≤8(S)	≤0.12(S)	≤0.12(S)	2(I)	≤2(S)	≤0.12(S)	≤0.5(S)	≤20(S)
04250663	pre-disinfection	≥32(R)	≤4(S)	4(S)	≥64(R)	≤0.12(S)	≤0.25(S)	≤8(S)	≤0.12(S)	≤0.12(S)	2(I)	≤2(S)	≤0.12(S)	≤0.5(S)	≤20(S)
04161141	pre-disinfection	≥32(R)	≤4(S)	≤1(S)	≥64(R)	≤0.12(S)	≤0.25(S)	≤8(S)	≤0.12(S)	≤0.12(S)	2(I)	≤2(S)	≤0.12(S)	≤0.5(S)	≤20(S)
04160493	pre-disinfection	≥32(R)	≤4(S)	4(S)	≥64(R)	≤0.12(S)	≤0.25(S)	≤8(S)	≤0.12(S)	≤0.12(S)	2(I)	≤2(S)	≤0.12(S)	≤0.5(S)	≤20(S)

AMC – amoxicillin-clavulanic acid, TZP – piperacillin-tazobactam, CXM – cefuroxime, FOX – cefoxitin, CAZ – ceftazidime, CRO – ceftriaxone, CSL – cefoperazone-sulbactam, FEP – cefepime, ETP – ertapenem, IMP – imipenem, AMK – amikacin, LVX – levofloxacin, TGC – tigecycline, SXT – trimethoprim-sulfamethoxazole

All bronchoscope samples were tested for bacteria. A total of 358 isolates and 13 isolates were recovered from samples after clinical procedures and samples after decontamination procedures, respectively (Table SII and Table SIII). Of note, most of the detected microorganisms were Gram-positive bacteria, such as *Staphylococcus epidermidis* (n = 69), *Streptococcus salivarius* (n = 42), and *Streptococcus oralis* (n = 23). Among the Gram-negative bacilli isolates, most of them belong to the Enterobacterales group (Table SII and Table SIII). Moreover, *K. aerogenes* was found in both groups, includes six isolates before the cleaning procedure and one after disinfection. This prompted us to investigate further the phenotypic and genomic characteristics of seven *K. aerogenes* identified in this work.

Antimicrobial susceptibility of seven *K. aerogenes* isolates is detailed in Table I. The full resistance (100% isolates) was observed for amoxicillin-clavulanic acid and cefoxitin (100%). All isolates were susceptible to piperacillin-tazobactam, cefuroxime, ceftazidime, ceftriaxone, cefoperazone-sulbactam, cefepime, ertapenem, amikacin, levofloxacin, tigecycline, and trimethoprim-sulfamethoxazole. Interestingly, five isolates were intermediate to imipenem. Among seven *K. aerogenes* isolates, we found five sequence types (STs), which were ST135 (n = 2) and ST1358 (n = 2), followed by ST1357 (n = 1), ST1359 (n = 1), and ST1363 (n = 1).

The Roary matrix-based gene sequence analysis generated a large pan-genome of 18,105 gene clusters of 58 full genomes. The whole-genome phylogeny (Fig. 1) revealed a population structure that was generally concordant with MLST (data not shown). Genetic diversity was observed in our bacterial collection, which clustered into three main clades.

We identified a total of 43 antimicrobial resistance genes in the *K. aerogenes* core genomes (Fig. 2). The resistome of *K. aerogenes* comprises a high number of antibiotic efflux pumps as well as narrow and extended-spectrum β-lactamases. As expected, human isolates encoded more antimicrobial resistance genes than environmental isolates. Of note, three isolates from this work possessed only one resistance gene, *fosA*, which is consistent with their phenotypic characteristics.

This study assessed the phenotypic characteristics and genomic complexity of *K. aerogenes* strains isolated from bronchoscope samples. It is worthy to note that 13 strains were isolated after cleaning procedures. It might indicate their low-level contamination with environmental and skin bacteria since bronchoscope samples were collected after the clinical procedures without disinfection or cleaning processes.

It is well known that outbreaks and pseudo-outbreaks may be associated with bronchoscopes (Guy et al. 2016). These nosocomial infections are commonly associated with *Mycobacterium* spp. and Enterobacteriaceae

isolates (Kirschke et al. 2003). In this work, we recovered seven *K. aerogenes* isolates from bronchoscope samples. *K. aerogenes* is associated with nosocomial infections and display multidrug resistance (Shen et al. 2019). The most prevalent STs were ST93 and ST4 (Passarelli-Araujo et al. 2019a). However, we did not detect any multidrug-resistant *K. aerogenes* in this work. Furthermore, STs of *K. aerogenes* found in this work have not been described in the literature. The 2 ST1358 strains identified in this study were aggregated in 1 clade with 1 ST1364 human isolate from Spain, which suggested that ST1358 and ST1364 might originate from the same ancestor. The results also indicate that 11 ST93 isolates and 9 ST56 were clustered into one separate sub-cluster, respectively, which exhibited a slight difference in the core genome sequence. Recent studies found that ST93 was the most prevalent clone in the global *K. aerogenes* genome database, indicating that ST93 might be the dominant global clone sequence in clinical settings (Malek et al. 2019; Passarelli-Araujo et al. 2019b). Furthermore, the emergence of fecal carriage and human infection of s *K. aerogenes* isolates resistant to multiple antibiotics, especially resistant to carbapenems, is considered a substantial threat to public health (Liu et al. 2019; Tian et al. 2020).

Although phenotypic and genomic evidence from the current study revealed that isolates recovered in this work are not multi-resistant. Active surveillance of bronchoscope-associated *K. aerogenes* isolates would improve our understanding of the population structure of this species. Of note, all isolates recovered from this study have a close relation to environmental or human isolates. A recent study investigated the population structure, virulence, and antimicrobial resistance in *K. aerogenes* (Passarelli-Araujo et al. 2019a). Their findings showed that *K. aerogenes* has an open pangenome and a large effective population size, which is in line with our results.

This study described the phenotypic and genomic characteristics of bronchoscope-associated *K. aerogenes*, although the relatively small number of not multi-resistant strains identified limits this finding. The detection of seven isolates of *K. aerogenes* in the surveyed Disinfection and Sterilization Center further indicates that this opportunistic pathogen may be a source of nosocomial infections without proper disinfection protocols. These results may lead to a better understanding of the genetic background and population structure of *K. aerogenes* in clinical settings.

Availability of data and materials

All genome assemblies of *K. aerogenes* isolates were deposited in GenBank and are registered under BioProject accession no. PRJNA633774. The datasets generated during and/or analyzed during the current study are available in the NCBI repository, https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA633774. Accesion Number: JABKDA000000000-JABKDC000000000, JABKCW000000000- JABKCZ000000000.

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Author contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Conflict of interest

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

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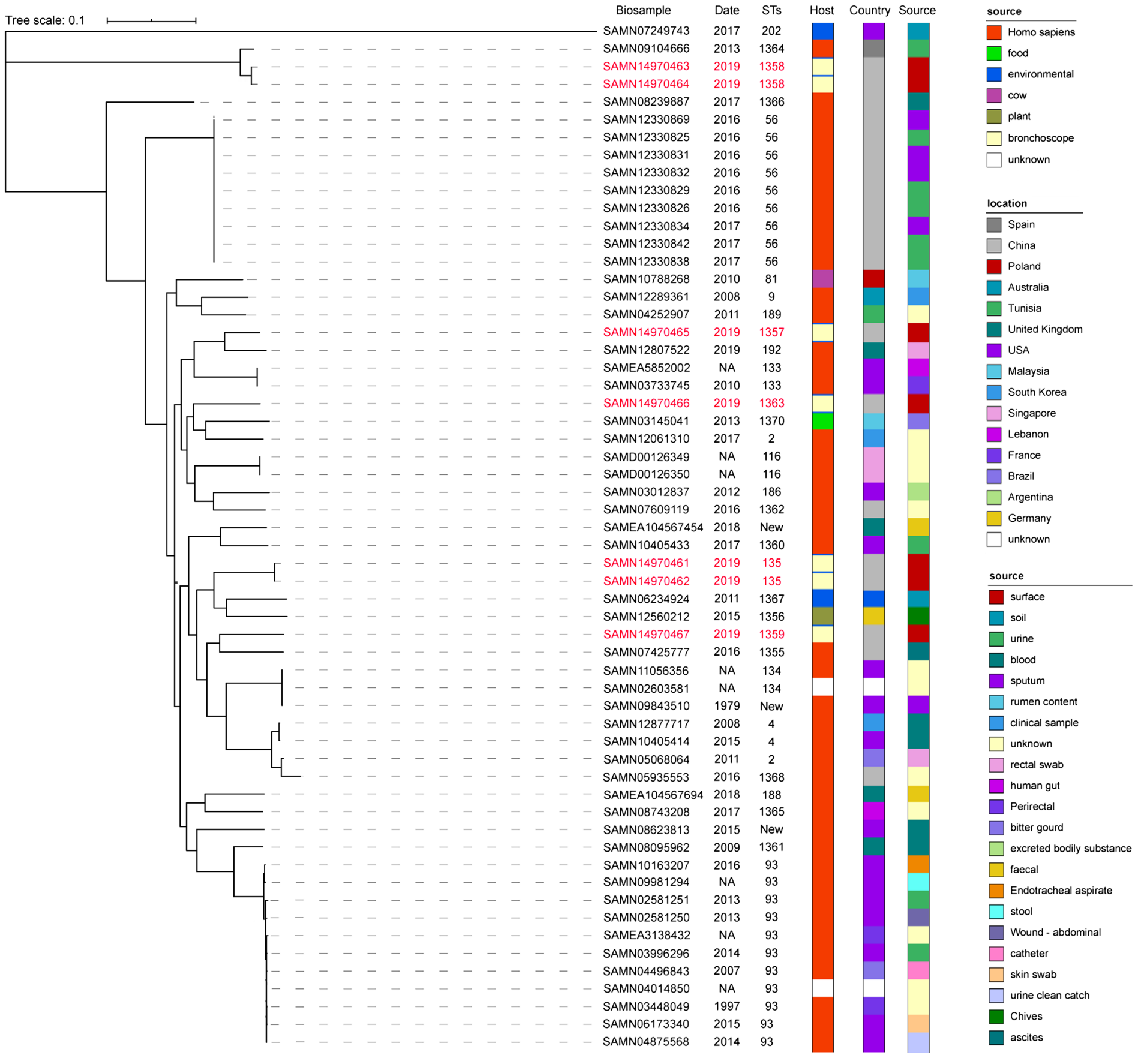

Fig. 1. Maximum-likelihood phylogeny of 58 representative global *Klebsiella aerogenes* isolates.
The trees were constructed using Roary software.
The tips of branches are colored according to hosts, countries, and sources. Red words indicate the strains in this study.

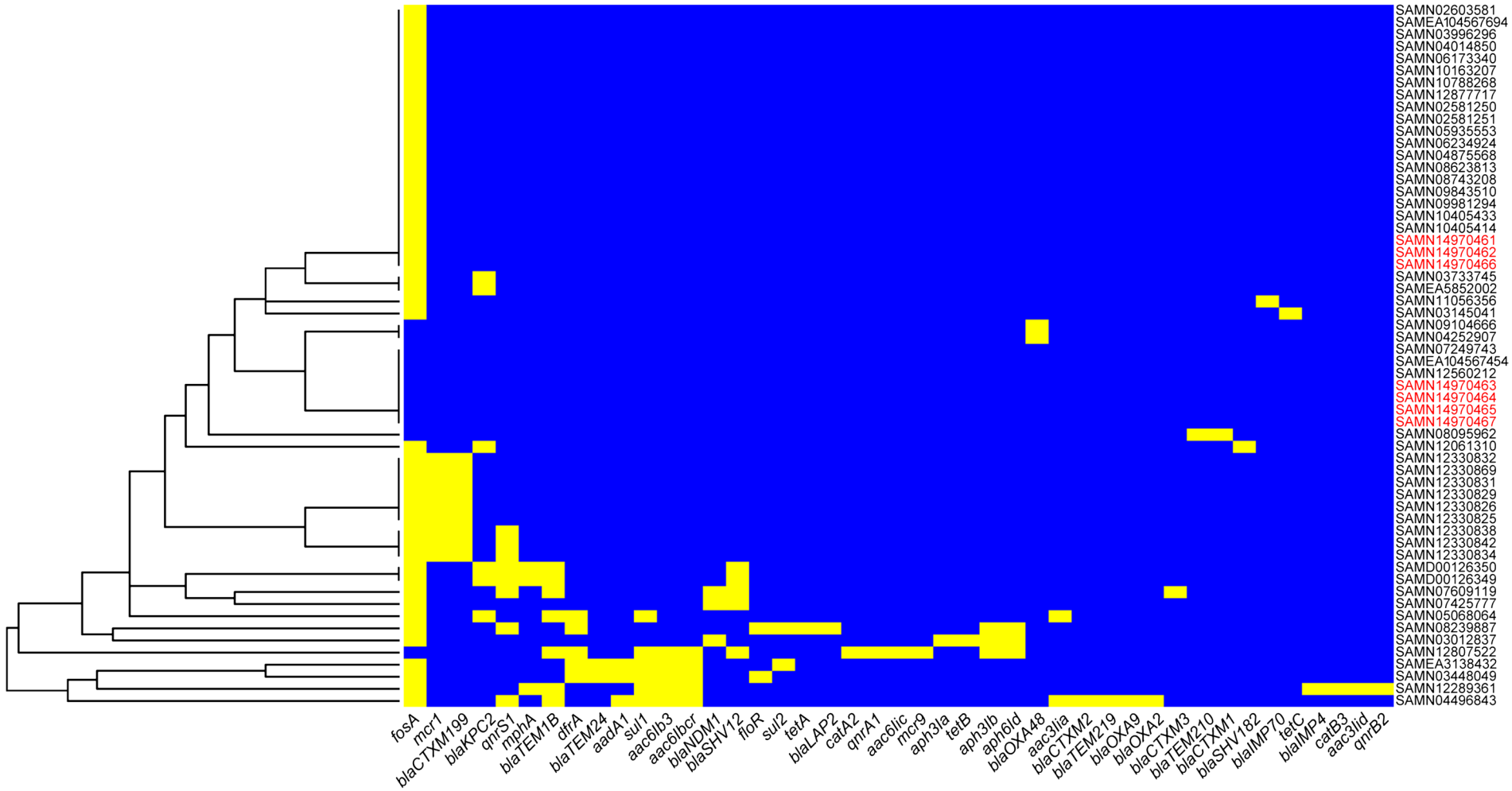

Fig. 2. Antimicrobial resistance genes were identified in the genomes of *Klebsiella aerogenes* isolates by analyzing the WGS data.
The antimicrobial resistance genes (ARGs) are shown on the bottom.
Yellow indicates the presence of the ARGs, and blue indicates the absence of the ARGs.

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Supplementary materials are available on the journal's website.