Whole genome-based characterization of multi-drug resistant *Enterobacter* and *Klebsiella aerogenes* isolates from Lebanon

Georgi Merhi¹¶, Sara Amayri¹¶, Ibrahim Bitar²³¶, George F. Araj⁴, Sima Tokajian¹*

¹Department of Natural Sciences, Lebanese American University, Byblos, Lebanon

²Department of Microbiology, Faculty of Medicine, and University Hospital in Pilsen, Charles University, Pilsen, Czech Republic

³Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic

⁴Department of Pathology & Laboratory Medicine, American University of Beirut Medical Center, Beirut, Lebanon

* Corresponding author

E-mail: stokajian@lau.edu.lb (ST)

¶These authors contributed equally to this work.
Abstract

Background: Enterobacter spp. are rod-shaped Gram-negative opportunistic pathogens belonging to Enterobacterales. This study aimed at the molecular and genomic characterization of multi-drug resistant Enterobacter spp. isolates recovered from hospitalized patients in a tertiary care hospital in Lebanon.

Materials: A total of 59 Enterobacter spp. clinical isolates consisting of 41 carbapenem-resistant and 18 susceptible by E-test were included in this study. Genotypic identification through whole-genome sequencing was performed and confirmed in silico. Resistance and plasmid profiles were studied using ResFinder4.0 and Plasmid-Finder2.1. Multi-locus sequence typing (MLST) was used to determine the isolates’ clonality.

Results: ANI identified and confirmed that 47 (80%) isolates were E. hormaechei, 11 (18%) were Klebsiella aerogenes and 1 (2%) was an E. cloacae. Carbapenem-resistance was detected among 41 isolates all showing an MIC90 of ≥ 32 µg/ml for ertapenem, imipenem, and meropenem. blaNDM-1 (58.5%), blaACT-16 (54%), and blaOXA-1 (54%) were the most common detected β-lactamases, while blaCTX-M-15 gene (68%) was the main detected extended-spectrum β-lactamase (ESBL) encoding gene. Chromosomal ampC gene, carbapenemase encoding genes, and porin modifications were among the detected carbapenem resistance determinants. The carbapenemase encoding genes were linked to three well-defined plasmid Inc groups, IncFII/IncFIB, IncX3, and IncL. MLST typing revealed the diversity within the studied isolates, with ST114 being the most common amongst the studied E. hormaechei.

Conclusion: The spread of carbapenem-resistant isolates in clinical settings in Lebanon is a serious challenge. Screening and continuous monitoring through WGS analysis could effectively limit the dissemination of drug-resistant isolates in hospitalized patients.
Importance

Drug resistance is an increasing global public health threat that involves most disease-causing organisms and antimicrobial drugs. Drug-resistant organisms spread in healthcare settings, and resistance to multiple drugs is common. Our study demonstrated the mechanisms leading to resistance against the last resort antimicrobial agents among members of the *Enterobacteriaceae* family. The spread of carbapenem-resistant bacteria in clinical settings is a serious challenge. Screening and continuous monitoring could effectively limit the dissemination of drug-resistant isolates in hospitalized patients.

Introduction

*Enterobacter cloacae* complex (ECC) members and *Klebsiella aerogenes*, belong to the family *Enterobacteriaceae* and are Gram-negative, rod-shaped, non-spore forming opportunistic pathogens causing healthcare-associated infections (HAIs) (1–3). ECC members are saprophytic microorganisms in that they inhabit and colonize diverse environments such as sewage, soil and the human GI tract (4). Detection of *K. aerogenes* is mostly linked to clinical settings and geographically widespread outbreaks (2). The emergence of ECC and *K. aerogenes* as potent nosocomial pathogens was further aggravated by the worldwide occurrence of multi-drug resistant clones (5).

Hoffmann *et al*, separated the ECC into 12 (I to XII) diverse genetic clusters based on *hsp60* single marker sequencing (6). Currently, *hsp60* typing remains the most widely used method categorizing species within the ECC (5). With the advent of whole-genome sequencing however, average nucleotide identity (ANI) analysis is becoming the gold standard for *in silico* species assignment. Using such approaches on the entire collection of RefSeq *Enterobacter* genomes allowed the distinction of 18 phylogenetic clusters (A to R) within the ECC (7).
Carbapenems are considered drugs of last resort for the treatment of extended-spectrum β-lactamase (ESBL) producing Enterobacterales isolates (8). ECC members were among the first Enterobacterales to harbor carbapenem resistance determinants and are currently the second most prevalent Carbapenem resistant Enterobacteriaceae (CRE) in the US and other major regions (3). Carbapenemase producing Enterobacter spp. (CPE) isolates in hospital acquired infections (HAIs) predominately belonged to the E. hormaechei species with its various respective subspecies (3, 9). E. hormaechei subsp. xiangfangensis (CIII) and E. hormaechei subsp. steigerwaltii (ST114, ST90 and ST93) are the most geographically prevalent CPEs (9).

There is a wide data schism regarding the epidemiology and dissemination of clinically significant K. aerogenes and It’s only lately that interest in this pathogen is rising. However, data related to the global burden of resistant subtypes of this pathogen are still scarce, especially in the Middle East (10). Well documented clinical cases of carbapenem resistant K. aerogenes (CR-KE) in the US, Europe and Asia were linked to plasmid encoding carbapenemases (2). Carbapenem resistance mechanism in CR-KE were mainly linked to the constitutive overexpression of chromosomal AmpC along with changes in membrane permeability through porin regulation (10).

The ominous antimicrobial resistance (AMR) phenomenon has become a global issue in general and Lebanon in particular (11). Various studies in Lebanon described alarming cases of carbapenem resistance in members of the family Enterobacteriaceae. Arabaghian et al, reported a 70.6% rate of total carbapenem resistance in a Klebsiella pneumoniae isolate set from a tertiary care hospital (12), and Alousi et al, reported the first ST-405 Escherichia coli single isolate carrying the blaoXA-48 carbapenem resistance determinant (13). Despite their notoriety as highly resistant nosocomial pathogens, members of Enterobacter spp. were not well studied. The
sporadic studies that tackled their dissemination and resistance patterns, were based on phenotypic screening and PCR assays (14, 15). A recently published comprehensive statistical report about ESBL and carbapenemase carriage in Lebanon for ESKAPE group pathogens also lacked detailed molecular characterization (16).

We aimed in this study to setup a workflow for the accurate processing of ECC and K. *aerogenes* using isolates recovered from Lebanon. To that end, we performed a detailed molecular characterization of 50 multi-drug resistant (MDR) *E. cloacae* complex and *K. aerogenes* clinical isolates. We relied on whole-genome sequencing (WGS) to elucidate their correct nomenclature, clonal relatedness, and resistance/virulence profiles. The sequenced genomes were subjected to both intra- and inter-analysis which allowed us to build an in-depth genomic understanding of ECC and *K. aerogenes* isolates recovered from Lebanon and in positioning them within a larger more comprehensive global context. This study also included detailed analysis pertaining to the pathogenesis and dissemination of the re-emerging pathogen *K. aerogenes* in Lebanon.

**Materials and Methods**

**Ethical approval**

Ethical approval for this project was not required as the study isolates were collected and stored as part of routine clinical screening. Patients’ records and information were anonymized, and clinical isolates data were de-identified before undertaking any form of analysis.

**Bacterial Isolates**

A total of 59 ECC and *K. aerogenes* isolates were collected from the 350-bed tertiary care teaching hospital AUBMC as part of routine clinical care screening. Preliminary species
identification was performed through the Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) system (Bruker Daltonik, GmbH, Bremen, Germany) following the manufacturer’s instructions. 81% (48/59) were identified as members of the EC complex whereas 19% (11/59) belonged to the *K. aerogenes* species and accordingly were designated as ENM1-48 and KAM1-11, respectively. Isolates were recovered from different infection sites sources: skin, urine, wounds and abdominal fluids. The mean patients’ age was 47 ± 26 years old with the range being 20 days to 90 years old.

**Antibiotic susceptibility testing**

Antibiotic susceptibility testing was performed through the Kirby-Bauer disk diffusion test (DDT) on Muller-Hinton agar including an assortment of 18 antibiotic disks representing 8 categories of regularly used antimicrobial agents in clinical settings. The minimum inhibitory concentration (MIC) was determined for 8 selected antibiotics. All results were interpreted following CLSI recommendations (17, 18).

**Bacterial DNA extraction**

Colonies were grown overnight on TSA agar plates. The Nucleospin® Tissue kit (Macherey-Nagel, Germany) was used for bacterial DNA extraction according to the manufacturer’s instructions. The obtained DNA was used for downstream PCR assays and whole-genome sequencing.

**Hsp60 gene amplification and sequencing**

PCR amplification and sequencing were performed with the *hsp60*-F and *hsp60*-R primers as previously described (6). *hsp60* sequences were aligned with references representing the 12 Hoffmann clusters (I→ XII) using Clustal-Omega alignment tool,
(https://www.ebi.ac.uk/Tools/msa/clustalo/) (19). The Hoffman cluster neighbor-joining (NJ) tree was visualized using the interactive tree of life (iTOl) tool (20).

Pulse Field Gel Electrophoresis (PFGE)

PFGE was carried out using \textit{XbaI} restriction enzyme (ThermoScientific, Waltham, MA, USA), according to the pulseNet standard protocol (http://www.pulsenetinternational.org; https://www.cdc.gov/pulsenet/). Fingerprint analysis was performed using BioNumerics software version 7.6.1 (Applied Maths, Sint-Martens-Latem, Belgium) and PFGE profiles were categorized as pulsotypes based on the criteria of a minimal difference of three or more bands between select isolates (21). Fingerprint clusters were inferred with the BioNumerics software based on the Dice correlation with optimization and tolerance values set at 1.5%.

Multi-Locus sequence typing (MLST)

MLST was carried out for ECC members as previously described by Miyoshi-Akiyama \textit{et al.} (22) and resulting sequences were curated versus the public database for \textit{E. cloacae} complex isolates (https://pubmlst.org/ecloacae/). \textit{K. aerogenes} isolates sequence types (ST) were also determined using the publicly available database for the molecular typing of \textit{K. aerogenes} (https://pubmlst.org/kaerogenes/).

Plasmid Based Replicon Typing (PBRT)

Plasmid incompatibility (Inc) types were elucidated using the DIATHEVA PBRT kit (Diatheva, Fano, Italy) through a PCR-based replicon typing method consisting of eight multiplex PCR assays for the amplification of 25 replicons. All reactions were carried out according to the manufacturer’s instructions, including positive controls for all assays.
Conjugative Transfer

The transfer of resistant markers \(\text{bla}_{\text{NDM-1}}, \text{bla}_{\text{NDM-5}}, \text{bla}_{\text{OXA-48}}, \text{and bla}_{\text{OXA-181}}\) was determined by conjugation using a plasmid-free recipient azide resistant strain \(E. \text{coli J53}\) and the study isolates as donors. Single colonies of both the donor and recipient were inoculated on Luria-bertani (LB) broth and incubated overnight at 37°C. Equal volumes of the donor and the recipient cultures were mixed and incubated overnight at 37°C, followed by plating on LB agar supplemented with meropenem. To ensure the effectiveness of the selected plates or the used antibiotics, positive and negative controls were run in parallel by separately plating the donor and recipient. Presence or absence of carbapenemases was determined through a PCR assay on the trans-conjugants followed by PCR-Based Replicon Typing (PBRT).

Whole-Genome sequencing and Quality Control (QC)

Genomic libraries of all 59 study isolates were created using the Nextera XT DNA library preparation kit with dual indexing (Illumina). The resulting libraries were sequenced on an Illumina MiSeq with forward and reverse reads with 300 bp length. Long read sequencing technology was applied on the PacBio Sequel I platform (Pacific Biosciences, Menlo Park, CA, USA) for three representative isolates ENM17, ENM30 and KAM9 as previously described (23). Quality assessment for the raw reads was done using FastQC version 0.11.8 and QUAST version 5.0.2 (24, 25). Adapter removal and trimming was performed through Trimmomatic v0.39 (26).

Assembly, identification, annotation and genome analysis.
Long read sequencing data were assembled using the HGAP4 De Novo Assembly Application (Pacific Biosciences) with a minimum seed coverage of 30 X. Short read trimmed sequences were assembled with SPAdes version 3.14 with read error correction enabled (27).

Accurate species identification was performed through Average Nucleotide Identity (ANI) testing. FastANI tool was used to generate a distance matrix of ECC isolates against Refseq complete E. hormaechei and E. cloacae genomes (supplementary table S2) (28). Subsequently, the assembled genomes were uploaded and annotated with the RASTtk (http://rast.nmpdr.org). ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and the comprehensive antibiotic resistance database (CARD) were used to assess resistance determinants in all the isolates (29, 30). Sequences of the omp35 and omp36 porin genes were extracted from the K. aerogenes genomes and sequences were translated to detect non-synonymous mutations, using the translate webtool available at the Bioinformatics resource portal (https://web.expasy.org/translate/). Putative K-types and virulence systems (yersiniabactin and colibactin) were inferred with the Kleborate and Kaptive (31).

**Plasmid studies**

PlasmidFinder 2.1 was used to detect and confirm the plasmid Inc types found in the study isolates (32). Potential plasmidic contigs resulting from long read sequencing were extracted and the closest matching reference was detected through the BLASTn tool on NCBI (www.ncbi.nlm.nih.gov/BLAST). PLACNETw was also used to identify plasmid sequences from short read sequencing data (33). Sequences were aligned and overlapping bases were trimmed and contiguous sequences were manually assembled. Open Reading Frames (ORFs) were inferred with Prodigal v2.6.1 with the closed option enabled (34). All coding sequences
were investigated and manually annotated through a BLASTp strategy and the IS finder database for insertion sequences ([https://isfinder.biotoul.fr/](https://isfinder.biotoul.fr/)).

**Phylogenetic Analysis**

The GToTree pipeline was used to determine the phylogenetic relationship between the study isolates (ECC and *K. aerogenes*). The pipeline relies on the analysis of 172 single-copy genes (SCGs) unique to Gamma-proteobacteria and outputs a phylogenetic tree based on the comparison of the selected SCG set within the specified input files (35). All ECC and *K. aerogenes* complete Refseq genomes were downloaded from NCBI with the “complete genome” and “latest Refseq” filters. The final tree was generated and visualized on iTOL (20).

**Sequence Data**

All 59 WGS sequences were deposited at DDBJ/EMBL/GenBank under the BioProject number **PRJNA551102**. Plasmid sequences of pLAU_ENM30_NDM1, pLAU_ENM17_OXA181 and pLAU_KAM9_OXA48 were deposited at DDBJ/EMBL/GenBank under the following accession numbers: **MN792917, MN792918** and **MN792919**.

**Results**

**Characterization of the Study Isolates**

Isolates were collected over a period of five years (2013-2018) as part of routine microbiological screening at the American University of Beirut Medical Center (AUBMC). Primary criterion for selection was based on non-susceptibility (intermediate/resistant) to one or more of the three clinically used carbapenems (meropenem, ertapenem and imipenem). Secondary inclusion criterion was any isolate recovered from an infection displaying a susceptible phenotype to the
clinically tested antibiotics. Most of the isolates (72%; n=43/59) displayed resistance patterns to at least two and more out of the three tested carbapenems and 9% (4/43) of sub-selected isolates showed non-susceptibility to two tested carbapenems. Only 7% (3/43) were resistant to only one. All non-susceptible isolates (n=43) were resistant to ertapenem out of which four additionally were resistant to meropenem (KAM8/9-ENM28/29). KAM1, KAM4 and ENM48 were resistant only to ertapenem. Additionally, more than 60% of the isolates showed resistance to other antibiotic classes; 75% (44/59) were resistant to gentamycin and 61% (36/59) to Trimethoprim-sulfamethoxazole.

ResFinder, used for the in silico analysis of antibiotic resistant determinants (ARD), uncovered the presence of 60 different unique resistance genes. The detected genes represented the nine clinically used antibacterial classes and which included: β-lactams, aminoglycosides, fluoroquinolones, trimethoprim, sulfonamide, Fosfomycin, tetracyclines, macrolides and chloramphenicol (Figure 1). Chromosomal ampC genes were detected in all the ECC isolates.

The prevalence of carbapenem resistance determinants (CRDs), shown in Figure 1, was validated using in silico whole-genome sequence analysis. blaNDM-1 was dominant and detected in 41% (24/59) of the isolates. Second-most dominant gene was the class D oxacillinase blaOXA-181 at 8% (5/59) followed closely by blaOXA-48 at 5% (3/59). blaNDM-5 was only detected in one ECC isolate (2%; ENM23). blaACT-16 and blaACT-7 were the most common ampC genes at 39% (23/59) and 22% (13/59), respectively. Amongst the genes conferring the ESBL phenotype, blaCTX-M-15 and blaOXA-1 were the most observed variants in this study, with blaCTX-M-15 being present in 49% (29/59) of isolates and blaOXA-1 in 41% (24/59), and their presence was not mutually exclusive.

Seven isolates (KAM5, KAM6, KAM8, ENM25, ENM28/29 and ENM39) did not harbor any carbapenemase encoding gene. ENM-25 and ENM-39 only harbored their respective
chromosomal \textit{ampC} determinant suggesting a revertant wild type phenotype. In contrast, both ENM-28 and ENM-29 harbored ESBL encoding genes (Figure 1).

Analysis of outer membrane protein encoding genes against functional references (CP017179.1, CP017179.1, CP017179.1, AF335467.1) showed that only KAM-5 and KAM-6 had a mutated form of the \textit{omp}36 gene with a premature insertion of an Amber stop codon. The remaining six isolates however, had functional \textit{omp} genes with the absence of any potential non-synonymous mutation.

All three \textit{K. aerogenes} isolates were devoid of any \textit{β}-lactamases encoding genes.

\textit{Hsp60} Typing and Average nucleotide identity analysis.

MALDI-TOF identification placed 81\% (48/59) of the isolates under the \textit{E. cloacae} complex. Subsequently, ECC isolates were subjected to \textit{hsp60} typing. The \textit{hsp60} sequences of the 48 ECC members were aligned with type strain sequences, from the NCBI gene database (AJ417141-3, AJ543870, AJ543878, AJ543807, AJ417110, AJ546761, AJ417134, AJ567879, AJ417115 and AJ862866). 15\% (7/48) of ECC isolates matched with the \textit{E. cloacae} cluster III, whereas only one isolate matched to the \textit{E. cloacae} subsp. \textit{cloacae} mini clade (Figure 2). It is noteworthy that 84\% (40/48) of the isolates clustered with \textit{E. hormaechei} type strain sequences (AJ567884.1, AJ567885.1, AJ567878.1), whereas none was typed as subsp. \textit{hormaechei}. The remaining 40 were distributed between the \textit{steigerwaltii} (12/48, 25\%) or the \textit{ohtae} subspecies (28/48, 58\%) (Figure 2).

Average nucleotide identity (ANI) testing was employed as a confirmatory and higher sensitivity test for species and sub-species identification. Eight refseq complete genomes (Supplementary Table S1) were used as references representing \textit{K. aerogenes}, \textit{E. cloacae} spp. \textit{cloacae/dissolvens}
and the five subspecies of *E. hormaechei* (*xiangfangensis*, *hoffmannii*, *oharae*, *steigerwaltii*, *hormaechei*). ANI values shown in Supplementary Table S2, confirmed the identification of the 11 *K. aerogenes* isolates. As for members of the ECC, results obtained revealed significant discrepancies between *hsp*60 typing and ANI testing. According to the results of this study, the predominant *hormaechei* subsp. was *xiangfangensis* at 54% (26/48) followed by 27% (13/48) and 15% (7/48) for *steigerwaltii* and *hoffmannii*, respectively. Only one *E. hormaechei* isolate (ENM-48) belonged to the subsp. *oharae* and, similarly, only one isolate was *E. cloacae* subsp. *cloaca* (ENM-1).

**Typing**

Twenty-four different sequence types (STs) were detected among the EC complex. Fourteen of the isolates were of ST114 (29.2%), five of ST182 (10.4%), and three of ST190 (6.25%). With *K. aerogenes*, seven distinct sequence types (STs) were detected including: ST143 (18.2%; 2/11), ST93 (18.2%; 2/11), ST209 (18.2%; 2/11) and ST210 (18.2%; 2/11) (Figure 3).

Pulse-field Gel electrophoresis was performed to study the genetic relatedness between the isolates. A total of 36 distinct pulsotypes were identified. ENM-37 and ENM-40 showed a similarity of 93% based on the banding patterns (Figure 3). These two isolates are of the same subsp. (*E. hormaechei* subsp. *xiangfangensis*) belonging to the sequence type ST182, and with mostly identical resistance and plasmid profiles. ST114 isolates however, had seven different pulsotypes where the PFGE banding patterns did not match fully with the MLST typing. All ST114 isolates shared ≥80% PFGE similarity patterns with identical resistance patterns and determinants, except for ENM-24 which had a distinct PFGE pattern compared to all other ST114 isolates. ENM-16 was untypable even after treatment with secondary enzyme.
Seven different pulsotypes were determined based on clustering and banding patterns for *K. aerogenes* (Figure 4). Some of the banding patterns matched with the MLST. KAM-9 and KAM-10 are of ST143 and shared high degree of clonal relatedness with only one band difference. KAM-2 and KAM-11 on the other hand, were typed as ST93 and distinct banding patterns.

We used Kaptive, a web-based tool for the *in silico* k-type determination through targeting the complete *cps* locus, and accordingly we detected three different K-types among the eleven *K. aerogenes* isolates. All the *K. aerogenes* were identified as having the KL68 K-type except for KAM-3 and KAM-8, which respectively were identified as KL107 and KL26 (Table S3).

**Phylogenetic Analysis**

The primary phylogenetic placement of the isolates was performed through the GToTree pipeline. The analysis of 172 SCGs for all the isolates and complete RefSeq genomes clustered all into two major clades (I & II) (Figure 5). Clade I (red block) distinctively harbored all the *K. aerogenes* (11/59) and at a large distance from the remaining Enterobacter spp. isolates (Figure 5). All the ENM isolates (ENM1-48) clustered within clade II. ECC members, except the *E. hormaechei* species, clustered in a sub-clade within clade II (green block) (Figure 5). ENM1 (blue block) was the only isolate identified as closely related to the type strain *E. cloacae* subsp. *cloacae* ATCC13047 (CP001918.1). The remaining of ECC isolates were all grouped within one sub-clade (Figure 5). Four *E. hormaechei* subspecies were detected, of which 55% (26/47) belonged to the subspecies *xiangfangensis*. Fourteen had the sequence type ST114 (Figure 5).

**Virulence of *K. aerogenes***
We looked for virulence loci in *K. aerogenes* using Kleborate, a tool to screen for ICE*Kp* (virulence-associated integrative conjugative element of *K. pneumoniae*) associated virulence loci including yersiniabactin (*ybt*), colibactin (*clb*) (Supplementary Table S3). The yersiniabactin locus (*ybtS, ybtX, ybtQ, ybtP, ybtA, ybtU, ybtT, ybtE*) encoding the yersiniabactin siderophore was detected in 36.4% (4/11) of *K. aerogenes* isolates. KAM-2, KAM-3 and KAM-11 harboured the *ybt* locus on ICE*Kp10* isoform, whereas KAM-8 harboured a novel variant of the *ybt* locus. As for the bacterial toxin colibactin, all *K. aerogenes* were negative except for KAM2, KAM3, and KAM-11, which harboured the *clb3* colibactin locus.

**Plasmid identification and characterization**

We used PlasmidFinder v2.1 and PCR-Based Replicon Typing (PBRT) to identify the incompatibility groups for the recovered plasmids (Figure 6). Distinct Inc groups were detected. IncFII was the most common present in 56% (n=33) of the ECC and *K. aerogenes* isolates, followed by IncFIB (33.9%; 20/59), IncX3 (22%; 13/59), IncHI1A (18.6%; 11/59), IncL (5.1%; 3/59), and IncA/C2 (1.7%; 1/59).

IncFII (pKPX1) was primarily associated with *bla*<sub>NDM-1</sub>, IncL plasmid with *bla*<sub>OXA-48</sub> gene and was detected in three of the isolates (ENM-22, KAM-9, and KAM-10), and IncC which was found in a *bla*<sub>NDM-1</sub> positive isolate (ENM-42) lacking all IncF groups. All *bla*<sub>OXA-181</sub> harbouring isolates (ENM-15, ENM-16, ENM-17, ENM-21, and ENM-34) had the IncX3 plasmid in common and a single occurrence of *bla*<sub>NDM-5</sub> was also linked to IncX3.

Three isolates, harboring the most prevalent CRDs, were selected for long read sequencing (ENM17, ENM30 and KAM9). As a result, three complete plasmids were extracted, identified
and named as follows: pLAU_ENM17_OXA181, pLAU_ENM30_NDM1 and pLAU_KAM9_OXA48.

Backbone analysis of pLAU_ENM17_OXA181 (MN792919) showed that it is an IncX3 self-conjugative plasmid, harboring a \textit{bla}_OXA-181 class D oxacillinase, with an overall size of 51,084 bp. The backbone components of pLAU_ENM17_OXA181 were consistent with a self-conjugative plasmid, harboring the necessary genes for propagation, stability, and replication. The \textit{bla}_OXA-181 gene was flanked upstream by an IS\textit{Kpn19}/transposase, followed by a replicase gene. Downstream, the class D oxacillinase was flanked by three consecutive truncated transposases \textit{\DeltaIS}_{3000}/transposase-\textit{\DeltaISEc63}-\textit{\DeltaIS}_{26}. Moreover, the \textit{qnrS1} fluoroquinolone resistance determinant was also identified on the plasmid and flanked upstream and downstream by IS\textit{26} and Tn3-like resolvase, respectively. BLAST analysis showed 99% similarity between pLAU_ENM17_OXA181 and pSTIB_IncX3_OXA_181 (MG570092) isolated in Lebanon (36).

Based on the same analysis workflow, pLAU_KAM9_OXA48 was a 61,913 bp IncL conjugative plasmid. The IncL replicon identity was confirmed through PBRT and \textit{in silico} BLAST analysis against publicly available IncL \textit{repA} gene reference sequence in NCBI (NC_021488.1). Backbone screening of pLAU_KAM9_OXA48 identified conjugative transfer proteins (\textit{tra} genes), \textit{relB/relE} toxin-antitoxin system and stability related proteins. \textit{bla}_OXA-48 was directly bracketed by a \textit{lysR} gene upstream and a \textit{\DeltaIS}_{1R} downstream. However, analysis of the overall genetic environment identified two IS\textit{1O4} upstream and downstream of the class D oxacillinase as follows: IS\textit{1O4}-\textit{lysR}-\textit{bla}_OXA-48-\textit{\DeltaIS}_{1R}-IS\textit{1O4}. Interestingly, this cassette is similar to Tn\textit{1999}, but has different insertion sequences bracketing the cassette. IS\textit{1O4} was found to be 99% identical to IS\textit{1999} differing in the position of two SNPs. BLAST analysis of pLAU_KAM9_OXA48 showed also showed close relatedness to other plasmids.
pLAU_ENM30_NDM1 was linked to an IncFII plasmid through in silico scaffolding. Detailed analysis showed the presence of a copy of blaNDM-1 directly bracketed upstream by a ∆ISaba125 and downstream by a bleMBL also known as a bleomycin gene. Further analysis of blaNDM-1 genetic environment showed that it falls within a large genomic cassette harboring, from upstream to downstream: IS3000- ∆ISaba125-blaNDM-1-bleMBL-//-Tn5403. BLAST analysis revealed high sequence similarity (>99%) between pLAU_ENM30_NDM1, pLAU_ENC1 (MN688131.1) (37), pAR_0128 (CP021720) and pKXP-1 (AP012055). Despite the high sequence similarity however, they were different in terms of size being larger than pLAU_ENM30_NDM1 by at least 90 Kbp.

Discussion

In this study, we performed an in-depth molecular characterization of 48 ECC and 11 K. aerogenes, known previously as E. aerogenes, clinical isolates from Lebanon. We used molecular techniques backed by in silico whole-genome based characterization to resolve the molecular identity of the clinical isolates and determined and compared the phylogenetic relatedness, resistomes and virulomes. The distribution of resistance determinants was not uniform, the blaNDM-1 gene was commonly detected and associated with the IncF plasmid incompatibility group, class D oxacillinases, blaOXA-48 and blaOXA-181, were also prevalent and were associated with IncL and IncX3 plasmid types, respectively.

ECC members and K. aerogenes remain largely understudied and the focus often shifts towards E. coli and K. pneumoniae. Moghnieh et al., (2019) showed the antimicrobial susceptibility data gathered from 13 local hospitals in Lebanon. Data revealed the constant increase in carbapenemase and ESBL carriage among E. coli and K. pneumoniae (16). Moreover, a study in 2018 identified 27 carbapenem resistant E. coli clinical isolates in a Lebanese hospital, mediated
by the dissemination of the class D oxacillinases \textit{bla}\textsuperscript{OXA-48}, \textit{bla}\textsuperscript{OXA-181} and the ESBL gene CTX-M-15 coupled with outer membrane porin alterations (38). Arabaghian et al. (2019) also characterized 34 multi-drug resistant \textit{K. pneumoniae} clinical isolates with all being at least resistant to ertapenem (12).

Our results illustrated a predominant presence of NDM-1 (41%; 24/59), especially within the ECC population and three of the isolates harbored the \textit{bla}\textsuperscript{OXA-48} gene two being \textit{K. aerogenes} (KAM9 and KAM10). Previous reports revealed that the Middle-East and the Mediterranean regions were endemic to \textit{bla}\textsuperscript{OXA-48} (12, 38, 39), and which according to our results seem to be also attributed to the prevalence of \textit{K. aerogenes}. \textit{bla}\textsuperscript{OXA-181}, a single base pair mutant analog of \textit{bla}\textsuperscript{OXA-48}, was also detected in 8% (5/59) of the isolates all of which were ECC members. Both NDM-1 and OXA-181 originated from the Indian sub-continent suggesting that the driving force for carbapenem resistance within ECC in Lebanon was similar to that seen in the Balkans and the South-Eastern regions of Asia (9, 39).

Our results also highlighted the extensive occurrence of \textit{bla}\textsuperscript{CTX-M-15} (49% of the isolates; 29/59), and its co-existence with other CRDs. This conforms with previous reports showing that \textit{bla}\textsuperscript{CTX-M-15} was one of the most prevalent ESBL enzymes within global members of the ECC (40, 41). Haenni et al, (2016) showed that having \textit{bla}\textsuperscript{CTX-M-15} among high-risk \textit{E. cloacae} clones in companion animals, namely ST114, was alarmingly consistent (42). \textit{bla}\textsuperscript{CTX-M-15} was mainly linked to mobile genetic elements but was chromosomal in ENM1. Analysis of its genetic environment revealed an \textit{ISEcp1} upstream and a disrupted \textit{ΔTn2} downstream of the gene. In ENM1 the cassette was integrated downstream of the aerobactin operon which could contribute to heightening the expression of virulence and resistance determinants during host infection (43–45).
ECC members and *K. aerogenes* are both intrinsic carriers of chromosomal *ampC* genes. *ampC* genes are inducible and prone to acquire mutations leading to their constitutive expression. When coupled with outer membrane porin alteration, primarily in *K. aerogenes*, these organisms exhibited carbapenamase independent resistance (2, 4). Both KAM5 and KAM6 were devoid of any CRDs and displayed phenotypic carbapenem resistance. Analysis of *omp36* genes showed that non-synonymous mutations were introduced through a single point mutation (C→T), introducing as a result a premature stop-codon within the gene. This is in line with the tendency of *K. aerogenes* to adapt when exposed to antimicrobial agents and promote changes in porin permeability further complicating treatment regimens (2, 10).

ST114 is an epidemic clone belonging to clonal complex CC114, and was linked to *bla*<sub>CTX-M-15</sub> and recently to *bla*<sub>NDM-1</sub> in Palestine, Italy, Japan, France, Spain, Greece, Latvia and other countries (9, 46). In our study, all ST114 isolates were NDM-1 carbapenemase positive highlighting the dissemination pattern of the carbapenem-resistant ECC from south-eastern Asia to Midwestern United States to the Middle Eastern countries. On the other hand, ST78 MDR *E. cloacae* was globally characterized as being a dominant ESBL clone linked to *bla*KPC harboring plasmids (47). In this study two ST78 (3.4%) *E. hormaechei subsp. hoffmanni* isolates harbouring either *bla*<sub>NDM-1</sub> or *bla*<sub>OXA-48</sub> were detected further confirming the potential spread of high-risk clones associated with different ECC sub lineages.

The yersiniabactin and colibactin siderophores were detected in 4/11 (36.4%) and 3/11 (27.3%) of *K. aerogenes*, respectively. KAM-2, KAM-3, and KAM-11 were found to carry a conjugative and integrative isoform ICE<sub>Kp10</sub>. This element is associated with the mobilization of the yersiniabactin locus and cohabours the colibactin (*clb3*) and yersinbactin (*ybt17*) siderophores (48). A diverse set of ICE<sub>Kp</sub> isoforms were detected in carbapenem-resistant *K. pneumoniae*.
isolated from clinical settings in Lebanon (12). None of the multidrug resistant isolates cohaboured *clb* or *ybt* proposing the positive selection for *K. aerogenes* ICE*Kp* isoform enhancing invasiveness (hypervirulence) over the resistance (12, 48).

In this study, we identified three main CRDs, *bla*<sub>NDM-1</sub>, *bla*<sub>OXA-48</sub> and *bla*<sub>OXA-181</sub> on three separate plasmids. *bla*<sub>OXA-48</sub> was detected in three isolates and associated with a 61,913 bp self-conjugative plasmid pLAU<KAT> KAM9_OXA48. The plasmid being >99% similar to the reference OXA-48 plasmid pOXA-48a (JN626286.1) implicated its close relevance to the one originally recovered from *K. pneumoniae* isolated in Turkey in 2001(49). Multiple studies reported *bla*<sub>OXA-48</sub> dissemination locally and globally and its association with the ~60 Kbp widespread IncL plasmid (12, 13, 38, 50, 51). However, our study revealed a cassette-linked diversion. CRD on pLAU<KAT> KAM9_OXA48 was bracketed by IS<sub>10A</sub>, an isoform of IS<sub>1999</sub>.

OXA-181, on the other hand, a *bla*<sub>OXA-48</sub>-like variant, was first detected in New-Delhi in 2007 and is currently considered the second most common OXA-48 like globally. The first report of *bla*<sub>OXA-181</sub> in Lebanon was from an *E. coli* (36). pLAU<sub>ENM17</sub> OXA181 showed >99% similarity to pSTIB<sub>IncX3</sub> OXA181 (recovered from Lebanon; MG570092) and pOXA181 (KP400525.1) indicating that IncX3 plasmid was highly conserved despite its wide geographic spread (36, 49, 52).

Species and sub-species within the ECC are often incorrectly identified as *E. cloacae* (53, 54). In this study only one *E. cloacae* isolate (ENM1; 2%; 1/48) was detected while the remaining were identified as being *E. hormaechei* (98%; 47/48). The predominant subspecies was the *E. hormaechei* subsp. *xiangfangensis* and was in accordance with previous reports (9, 55, 56).
To our knowledge, this is the first in-depth comprehensive whole-genome-based characterization of carbapenem resistant ECC members and *K. aerogenes* in Lebanon and the region. In this study, genome-based workflow was coupled with other more traditional approaches to build a better accurate understanding of the carbapenem-resistant population within the ECC members and *K. aerogenes* recovered from clinical settings in Lebanon. Our results revealed that the predominant sequence type among all the studied isolates was the epidemic global clone ST114 (9). The prevalent ECC identified was *E. hormaechei* subsp. *xiangfangensis* and the *bla*NDM-1 was the dominant detected carbapenemase. Collectively the accurate identification and targeted genome sequencing could help in reducing ineffective treatment regimens, preventing outbreaks, and limiting the spread, mobilization, and transmission of resistance determinants.

**Acknowledgments**

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All authors declare that they have no conflict of interest.

**References**


Figures

Figure 1. Isolates’ information and detected antibiotic resistance genes. In the susceptibility column, R: Resistant to Carbapenem; S: susceptible to Carbapenem. In the Age column, d: days; m: months; w: weeks. In the gender column, F: Female; M: Male. In the specimen isolation column, A: Abscess; Ab: Abdominal fluid; B: Blood; D: DTA; S: Skin; Sp: Sputum; U: Urine; W: Wound.

Figure 2. Neighbor-Joining (NJ) tree based on multisequence alignment of partial hsp60 gene for all ECC isolates. Isolates clustering with reference hsp60 genes were highlight within colored ranges. Hormaechei subspecies were demarcated through colored strips. Isolates ENM12-14-22-43 were highlighted in purple as each has clustered as a singleton on the NJ tree.
Figure 3. PFGE dendrogram of ECC isolates. Dendrogram was generated by BioNumerics software version 7.6.1 showing isolate clonality based on their banding patterns generated by Xbal restriction digestion. The results were inferred using the dice correlation with optimization and tolerance values set at 1.5% Isolates’ STs, and pulsotypes (PT) are also shown.

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Figure 5. SCGs based maximum-likelihood phylogenetic tree of all study isolates and complete RefSeq ECC and K. aerogenes reference genomes. Colored ranges were used to differentiate between K. aerogenes and Enterobacter sp isolates. Sub-species of E. hormaechei are indicated by the outer rim-colored bands. Golden stars were used to highlight ST114 isolates, while the yellow dot was used to show the only E. cloacae subsp cloacae isolate (ENM1).

Figure 6. In silico detected plasmid incompatibility groups.
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Susceptibility</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Outcome</th>
<th>Isolation</th>
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<td>65</td>
<td>M</td>
<td>R</td>
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<td>R</td>
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<tr>
<td>bi50CT2-M-14</td>
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<td>M</td>
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<td>bi50K4-12</td>
<td>S</td>
<td>70</td>
<td>M</td>
<td>R</td>
<td>Pt</td>
</tr>
</tbody>
</table>

**Detected Resistance Genes:**
- floA
- catB1
- catB3
- floR
- catB4
- qnrB1
- acrC3-26-cr
- qepA
- quinol
- tetA
- tetB
- tetD
- mphA
- mphB
- mphC
- mphD
- mphE
- mphF
- mphG
- mphH
- mphI
- mphJ
- mphK
- mphL
- mphM
- mphN
- mphO
- mphP
- mphQ
- mphR
- mphS
- mphT
- mphU
- mphV
- mphW
- mphX
- mphY
- mphZ
- sul1
- sul2
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Colored ranges

- E. hormaechei
- E. cloacae CIII
- E. cloacae subsp. cloacae

E. hormaechei subsp steigerwaltii
E. hormaechei subsp oharae
E. hormaechei subsp hormaechei
E. cloacae CXIII
E. cloacae subsp dissolvens
E. cloacae subsp cloacae
E. cloacae CIV
E. cloacae CIX
E. hormaechei subps oharae
E. ludwigii
E. asburiae
E. kobei

Tree scale: 0.01
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ENM14 78 01
ENM46 1422 02
ENM1 1421 03
ENM29 182 04
ENM37 182 05
ENM40 182 05
ENM27 182 06
ENM21 190 07
ENM15 190 08
ENM17 190 08
ENM48 270 09
ENM13 182 10
ENM42 1325 11
ENM28 124 12
ENM10 114 13
ENM11 114 13
ENM18 114 14
ENM20 114 15
ENM30 114 16
ENM31 114 16
ENM33 114 17
ENM35 114 17
ENM32 114 17
ENM36 114 17
ENM41 114 17
ENM2 114 18
ENM26 114 19
ENM19 171 20
ENM34 295 21
ENM12 104 22
ENM24 114 23
ENM23 93 24
ENM38 544 25
ENM39 544 26
ENM43 1418 27
ENM45 1418 27
ENM6 113 28
ENM8 113 28
ENM44 1420 29
ENM47 306 30
ENM3 198 31
ENM7 50 32
ENM22 78 33
ENM25 98 34
ENM4 1417 35
ENM9 1417 35
ENM5 1419 36
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Enterobacter cloacae subsp. cloacae

Other ECC members

Klebsiella aerogenes

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