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Genomic characterisation of extended-spectrum β-lactamase-producing multidrug-resistant *Escherichia coli* in Rabat, Morocco

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ABSTRACT

Objectives: Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* are an increasingly significant cause of hospital- and community-acquired infections worldwide. Whereas several reports have highlighted their increased prevalence also in North African countries, genomic data on isolates associated with these infections are still scarce. This study aimed to provide data on ESBL-producing *E. coli* isolates from patients with extraintestinal infections at the Military Teaching Hospital Mohamed V of Rabat, Morocco.

Methods: Whole-genome sequencing was carried out on 18 ESBL-producing extraintestinal pathogenic *E. coli* (ExPEC) isolates for analysis of phylogenomic evolution, virulence factors and antimicrobial resistance genes. Data were compared with ExPEC lineages from several surrounding countries using multilocus sequence typing (MLST) and single nucleotide polymorphism-based phylogenetic approaches.

Results: The majority of *E. coli* isolates were ST131 ($n = 15$), followed by ST617 ($n = 2$) and a novel sequence type (ST10703) that is closely related to the pandemic ST405 clone. All ST131 isolates belonged to the O25b-ST131 pandemic clone. They harboured more virulence genes than their non-ST131 counterparts. IncF plasmid replicons and the $bla_{CTX\text{-}M\text{-}15}$ β-lactamase gene were identified in all isolates. No ESBL-producing *E. coli* isolates carried any known carbapenemase gene.

Conclusion: Our findings underscore the pre-eminence of ST131 as the major factor driving the expansion of ExPEC in the Rabat region while highlighting the potential links with isolates circulating in other neighbouring countries.

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1. Introduction

Extraintestinal pathogenic *Escherichia coli* (ExPEC) are associated with severe infections at non-intestinal sites (e.g. urinary tract, bloodstream, cerebrospinal fluid) both in hospital and community settings [1]. ExPEC isolates carry specific virulence genes that allow them to colonise tissues and survive out of the intestinal environment and to cause extraintestinal disease [2]. Multidrug-resistant (MDR) ExPEC play a major role in the worldwide increasing prevalence of infections caused by Gram-negative bacteria [3]. Indeed, most MDR ExPEC display an extended spectrum β-lactamase (ESBL)-producing phenotype often associated with resistance to other antimicrobial agents such as fluoroquinolones, gentamicin and sulfonamides [4,5]. A number of molecular studies from different continents have shown the major role played by some major ExPEC lineages in the pandemic expansion of multidrug resistance [3,5]. While a high prevalence of MDR ESBL-

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producing Enterobacteriaceae has been reported in Morocco and other North African countries [6–8], genomic characterisation of the ExPEC lineages is still lacking. Up to now, ExPEC studies in Morocco mainly focused on molecular characterisation of genes associated with an ESBL phenotype [9] and their potential relationship with worldwide lineages, including those reported in neighbouring Spain [10–12] and Tunisia [13]. Whole-genome sequencing (WGS) of bacterial pathogens has now become a necessary tool for monitoring pathogen evolution and defining antibiotic resistance from One Health and Global Health perspectives and especially the acquisition of antimicrobial resistance (AMR) and virulence genes [14]. In this study, WGS was used to analyse non-duplicate ESBL-producing ExPEC isolates (n = 18) collected from patients at the Military Teaching Hospital Mohamed V of Rabat, Morocco, and to carry out subsequent phylogenomic analyses and characterisation of virulence and resistance genetic determinants.

2. Methods

2.1. Bacterial isolates

Between January and June 2019, Gram-negative bacteria (n = 2959) were isolated from adult patients presenting at the 700-bed Military Teaching Hospital Mohamed V of Rabat with extraintestinal infections, mainly urinary tract infections. In brief, urinary samples were directly inoculated onto CLED (cysteine–lactose–electrolyte-deficient) agar plates and were incubated at 37°C for 24–48 h under normal atmosphere. All significant colonies were identified by morphological and biochemical characteristics. Biochemical identification of suspected Enterobacteriaceae at the species level was performed using API20E ready-to-use galleries (bioMérieux SA, Marcy-l'Étoile, France) according to the manufacturer's instructions. *Escherichia coli* isolates (n = 949, i.e. 32.1% of consecutively collected Gram-negative bacteria) were selected for further analyses including antimicrobial susceptibility testing.

2.2. Antimicrobial susceptibility testing

Susceptibility of the *E. coli* isolates to 14 antimicrobial agents (ampicillin, mecillinam, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefoxitin, cefixime, ceftriaxone, ertapenem, imipenem, fosfomycin, gentamicin, amikacin, norfloxacin and trimethoprim/sulfamethoxazole) was determined by the disk diffusion method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [15] as updated in January 2021. Minimum inhibitory concentrations (MIC) for colistin were determined by the broth microdilution method using a Sensititre Antimicrobial Susceptibility Testing System (Thermo Fisher Scientific, Illkirch, France) according to the manufacturer's recommendations. EUCAST breakpoints were used to interpret the susceptibility or resistance status of each isolate. An ESBL production assay was performed by the double-disk synergy method as described by Drieux et al. [16]. *Escherichia coli* ATCC 35218 and *Klebsiella pneumoniae* ATCC 700603 strains were used as ESBL-negative and -positive controls, respectively, whereas *E. coli* ATCC 25922 and *E. coli* NCTC 13846 were used as colistin-susceptible and -resistant strains, respectively. Carbapenemase detection was performed using the β-CARBA^{TM} test (Bio-Rad, Temse, Belgium) according to the manufacturer's instructions.

2.3. Whole-genome sequencing (WGS)

Whole-genome paired-end sequencing was performed using a MiSeq sequencer (Illumina Inc., San Diego, CA, USA). Accordingly, genomic DNA (gDNA) from ESBL-producing *E. coli* was isolated using an EZ1 Advanced XL Biorobot and the Tissue DNA Kit (QIAGEN, Hilden, Germany) with the Bacterial Card according to the manufacturer's instructions. For each isolate, gDNA was quantified using a Qubit® fluorometer (Thermo Fisher Scientific, Eugene, OR, USA) and was normalised to 0.333 ng/μL. A standard Nextera XT library (Illumina Inc.) was constructed for each genome with 1 ng of gDNA as recommended by the manufacturer. Briefly, gDNA was simultaneously fragmented and tagged with sequencing adapters in a single step using Nextera transposome (Nextera XT DNA Library Preparation Kit; Illumina Inc.). Tagmented DNA was then amplified (12-cycle PCR amplification) and cleaned up with 0.5 × Agencourt® AMPure® XP beads (Beckman Coulter, Indianapolis, IN, USA). Nextera libraries were quantified using a Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Eugene, OR, USA) and the size profile was analysed on a 2100 Bioanalyzer using High Sensitivity DNA Assay Kit (Agilent Technologies, Waldbronn, Germany). Fragments with sizes ranging from 373 bp to 3226 bp (mean 961 bp) were generated. Libraries selected for sequencing were normalised to 2 nM and were pooled. The 2 nM pooled library was denatured and diluted to 14 pM prior to loading on a MiSeq flow cell (MiSeq Reagent Kit v.3, 600 cycles) for a 2 × 300-bp paired-end sequencing run.

2.4. Bioinformatics analysis

Raw sequencing data of the 18 *E. coli* isolates from Rabat have been submitted to the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena) and are available under accession no. **PRJEB42611**. These data were analysed using the following bioinformatics pipeline. All reads were quality checked by FastQC v.0.11.9 [17] and were processed using Trimmomatic v.0.39 [18]. Paired-end reads from each *E. coli* isolate were assembled de novo using the SPAdes v.3.13.0 algorithm [19] to generate a draft genome sequence for each isolate, and quality assessment for genome assemblies was carried out using QUAST 5.0.2 [20]. In addition, the quality and completeness of the genome assemblies was confirmed with BUSCO v.4.1.1 algorithm and the bacteria_odb10 data set [21].

The PATRIC database [22,23] was used to identify complete or draft genomes of other *E. coli* strains isolated from patients between 2000–2020 in Morocco and other surrounding countries, e.g. Spain, Portugal, Egypt and Saudi Arabia. It is worth nothing that no complete or draft WGS genomes were available for other surrounding countries at the time of presenting the current data (e.g. Algeria, Mauritania). In silico multilocus sequence typing (MLST) typing was performed on all genomes using the *E. coli* MLST scheme developed by Achtman, the home-made EcoliGenomic R package (https://github.com/JeromeAmbroise/EcoliGenomic), and the *E. coli* allele sequences and MLST profiles available in the EnteroBase database (https://enterobase.warwick.ac.uk/species/index/ecoli) [24]. In parallel, WGS data were used to characterise *E. coli* isolates through the combination of four DNA gene markers (i.e. *arpA, chuA, yjaA* and TSPE4-C2) as described by Clermont et al. [25]. In brief, *E. coli* draft genomes were screened for the presence of these four genetic markers, a combination thereof determining the phylogenetic clustered distribution of the isolates.

All genomes were submitted to kSNP3.0 with default settings except for a k-mer size of 19 and the '-core' option. This software performs single nucleotide polymorphism (SNP) identification without genome alignment or a requirement for reference genomes [26]. The kSNP3 parsimony phylogenetic tree based on the core genome was rooted on the Egypt_2000_1102 isolate using Dendroscope 3 v.3.7.5 [27] and was visualised using the ggtree R package [28]. The EcoliGenomic R package was also used to screen all genomes for the virulence factor genes described in ExPEC [28–30], or available in the VirulenceFinder database (https://cge.cbs.dtu.dk/services/VirulenceFinder/) [29], or in the Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/VFs/) [30]. The cut-

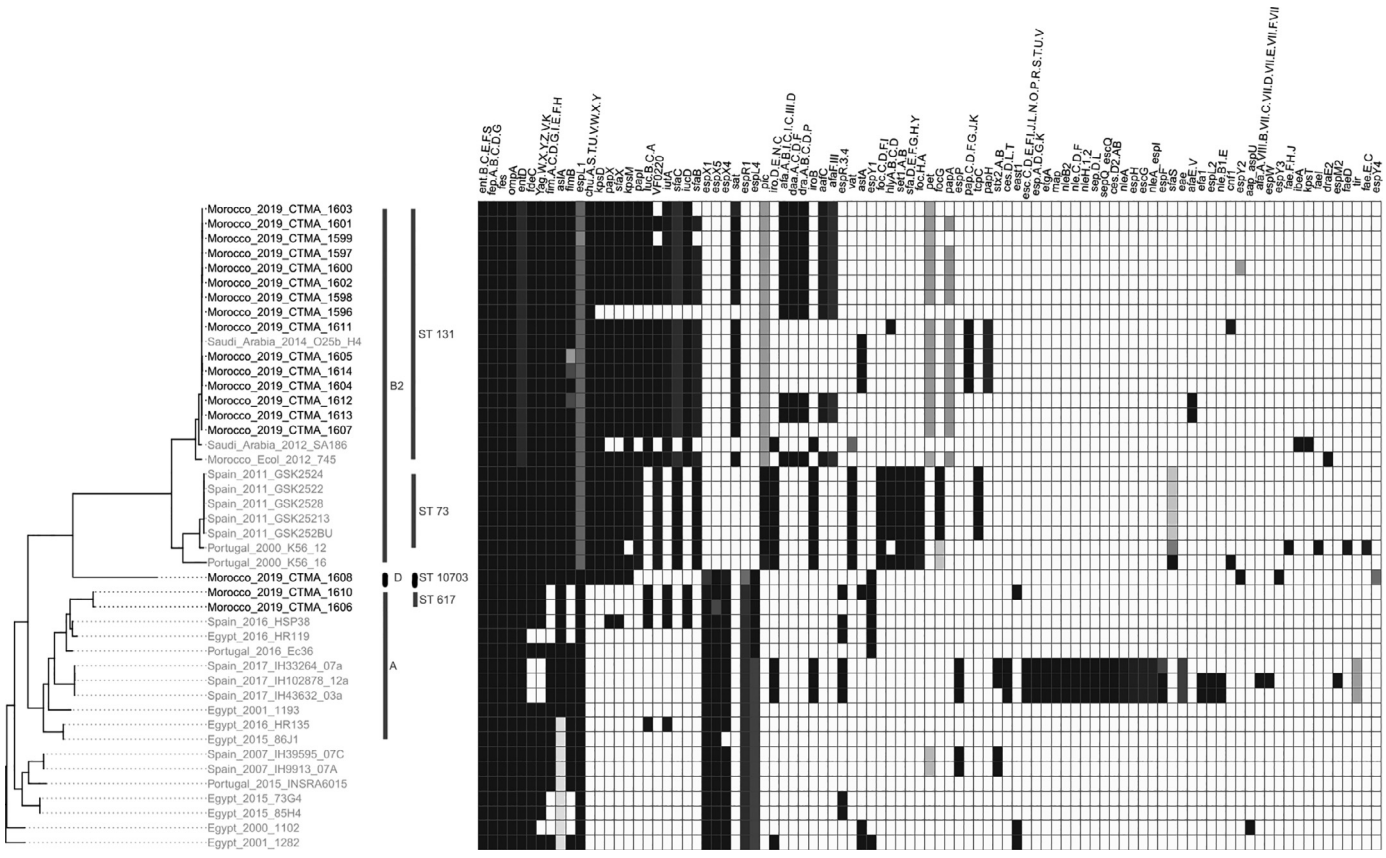


Fig. 1. Phylogenetic tree of *Escherichia coli* isolates from Rabat, Morocco, along with other isolates from neighbouring countries, and corresponding heatmap of virulence genes from the Virulence Factor Database (VFDB). The kSNP3 parsimony tree is based on single nucleotide polymorphisms (SNPs) within the core genomes of *E. coli* isolates from Rabat (in black) along with other isolates from neighbouring countries including Spain, Portugal, Egypt and Saudi Arabia and extracted from the PATRIC database. Multilocus sequence typing (MLST) and Clermont phylogroups derived from genome assemblies are indicated with vertical black lines. In the heatmap, black cells indicate fully covered genes, while grey and white cells indicate partially covered and non-covered genes, respectively. Large deletions are therefore observed for several genes including *entD* and *espL1*. The functions of the virulence genes listed in VFDB are reported in Supplementary file 4.

off value of sequence identity was set to 80%. The ggtree R package was used to display the coverage percentage of each gene within grayscale heatmaps.

Concurrently, each draft genome was screened for the presence of AMR genes and SNP-based AMR chromosomal determinants using the AMRFinder tool developed by the National Center for Biotechnology Information (NCBI) [31]. Draft genomes were screened for the presence of plasmids incompatibility (Inc) groups, especially the IncF group that is widespread in ExPEC [32]. The replicon scheme typing (RST) developed by Villa et al. [33] was used for IncF plasmid classification according to the FAB (FII, FIA, FIB) formula.

3. Results

3.1. Antimicrobial susceptibility patterns

From the collection of *E. coli* isolates ($n = 949$), 10.6% ($n = 101$) were associated with an ESBL-producing phenotype. Among these, 18 isolates (17.8%) were randomly selected for WGS analysis. All 18 ESBL isolates exhibited resistance to ampicillin, cefixime, ceftriaxone and norfloxacin, while 6 isolates were resistant to gentamicin (Supplementary file 1). Three isolates were susceptible to trimethoprim/sulfamethoxazole. In contrast, all isolates were susceptible to cefoxitin, mecillinam, ertapenem, imipenem and colistin. All 18 isolates were negative by the β CARBA^{TM} assay.

3.2. Whole-genome sequencing (WGS) and assembly

Computation of the total number of reads and quality metrics of the genome assemblies (Supplementary file 2) showed homogeneous results with high-quality metrics for all isolates from Morocco. In addition, a completeness score of 100% with the BUSCO algorithm was obtained for each genome.

3.3. Multilocus sequence typing (MLST) and phylogenomic analysis

As shown in Fig. 1, the overwhelming majority of Moroccan ExPEC isolates (15/18) belonged to ST131 by MLST. All of the ST131 isolates belonged to the same phylogenetic group B2 according to the Clermont classification [25].

A cluster of ST131 isolates from Rabat was composed by 7 isolates (from CTMA_1597 to CTMA_1603) displaying very low (0–2) SNP pairwise differences in their core genomes. The SNP pairwise distance between this cluster and other ST131 isolates from Rabat ranged around 50–100 in the core genomes. The SNP pairwise distance between this cluster and ST131 isolates from Saudi Arabia was equal to 61 and 887 for O25b_H4 and SA186, respectively.

Two isolates (CTMA_1606 and CTMA_1610) belonged to ST617 and Clermont phylogenetic group A. One isolate (CTMA_1608) displayed a novel sequence type, reported here for the first time, and was assigned the new ST10703 profile. This sequence type is closely related to ST405 with a single point mutation in the *fumC* allele compared with the seven gene-based MLST typing (Warwick

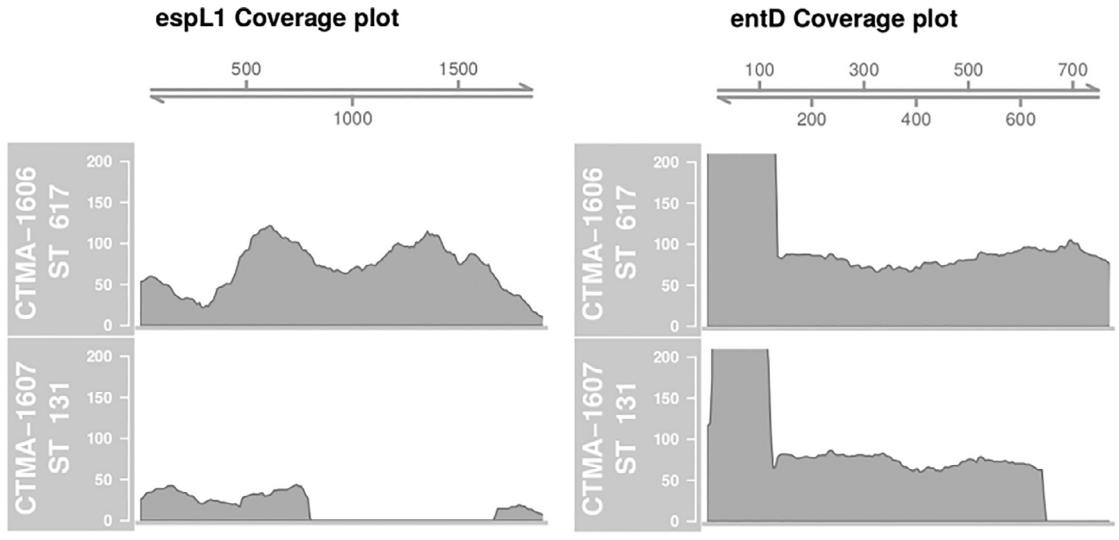


Fig. 2. Sequence reads coverage plots of *espL1* and *entD* virulence genes. This figure compares a ST131 isolate (CTMA_1607) with a non-ST131 isolate (CTMA_1606). In the ST131 isolate (CTM-1607), there is a deletion extending from 798–1670 in *espL1* and from 643–756 in *entD*.

scheme). This isolate was the single representative isolate of Clermont phylogenetic group D.

3.4. Detection of virulence factor genes

Comparison of Moroccan ST131 and non-ST131 (i.e. ST617 and ST10703) ExPEC isolates showed more virulence genes in the former group (Fig. 1; Supplementary file 3). Several deletions were observed specifically in all ST131 isolates (e.g. *entD, sfaC, sfaB, pic*) (Fig. 1) but other deletions (*espL1*) were also observed in several STs (ST131 and ST617). A 873-bp and 114-bp deletion were observed in the *espL1* and *entD* virulence genes (Fig. 2). In contrast, ST131 ExPEC strain CTMA_1596 harboured fewer virulence factor genes, while especially lacking a cluster of *iucA, iucB, iucC, iucD, iutA, kpsD, kpsM, papX, sfaX, papI, sat, sfaB, sfaC, iha* and *traT* genes.

3.5. Detection of antimicrobial resistance (AMR) determinants

Each draft genome sequence of ExPEC isolates from Rabat harboured AMR genetic determinants. They consisted of either chromosomal SNP-based determinants (Fig. 3) or plasmid-mediated AMR genes (Fig. 4), both mechanisms being involved in resistance against various classes of critically-important antibiotics.

SNP-based determinants of AMR found in Moroccan *E. coli* isolates conferred resistance to three distinct classes of antibiotics, including fosfomycin (V25I in *ptsI* and E350Q in *uhpT*), fosmidomycin (S352T in *cyaA*) and quinolones (D87N and S83L in *gyrA*, E84V and S80I in *parC*, and I529L and S458A in *parE*).

Plasmid-mediated AMR genes conferred resistance to eight different classes of antibiotics, including aminoglycosides, aminoglycoside/quinolone, β-lactams, macrolides, phenicols, sulfonamides, tetracycline and trimethoprim. It is of note that the $bla_{\text{CTX-M-15}}$ gene was identified in all (18/18) Moroccan ExPEC isolates.

WGS-based analysis identified IncF plasmid replicons in all isolates. Seven different RST profiles belonging to the IncF group were characterised in the 18 plasmid replicons: F2:A1:B- (CTMA_1596, CTMA_1597, CTMA_1598, CTMA_1599, CTMA_1600, CTMA_1601, CTMA_1602, CTMA_1603); F1:A1:B- (CTMA_1605); F31:A4:B1 (CTMA_1606, CTMA_1607); F1:A1:B16 (CTMA_1604, CTMA_1611, CTMA_1614); F1:A-:B23 (CTMA_1612, CTMA_1613); F31:A4:B10 (CTMA_1608); and F31:A4:B8 (CTMA_1610). Except for IncF, no other incompatibility plasmid replicon types (i.e. IncA/C, IncH1, IncH2, IncI1, and IncN) were characterised in these isolates.

4. Discussion

Despite several reports on infections in Morocco caused by MDR ESBL-producing Enterobacteriaceae, including *E. coli* [9,34], genomic ExPEC lineages were hitherto lacking. Our study contributes to this needed molecular characterisation. While the number of ExPEC isolates analysed (n = 18) is limited, this WGS study suggests that ESBL-producing *E. coli* O25b-ST131 carrying the $bla_{\text{CTX-M-15}}$ gene is one of the main ExPEC lineage representative in Rabat. This finding is in line with studies from other continents, showing that E. coli ST131 is a pandemic MDR ExPEC lineage associated both with urinary tract infections and bloodstream infections [35–37]. This pandemic lineage is a real concern considering the successful combination of its MDR profile and high virulence gene content, an observation that appears to contradict the common view that a high MDR phenotypic pattern is associated with a fitness cost leading to decreases pathogenicity [38]. ST131 strains have been shown to exhibit high levels of genomic plasticity, including frequent recombination and plasmid flux, particularly involving IncF-type plasmids that facilitate the spread of antibiotic resistance and virulence genes [39]. Not surprisingly, nearly all ST131 isolates were associated with a large number of virulence genes. However, it worth noting that the virulence gene content somewhat differed from that observed in a previous study by our team on ExPEC in eastern Democratic Republic of Congo [36]. Interestingly, one ST131 isolate from Rabat (CTMA_1596) displayed fewer virulence factors as it lacked a cluster of genes including *iucA, iucB, iucC, iucD, iutA, kpsD, kpsM, papX, sfaX, papI, sat, sfaB, sfaC, iha* and *traT*, which are virulence genes usually identified in ST131 isolates [40]. Our results also highlight the close relationship of Moroccan ST131 isolates to isolates from other regions of the world, including Saudi Arabia, a country primed for dissemination of MDR ExPEC as a natural consequence of the Hajj, the Mecca pilgrimage that is the largest annual gatherings in the Islamic world. One of the inherent limitations of this study is the low number of ExPEC isolates, which could possibly explain why no ST405 isolate was identified despite this sequence type being reported as one of the widespread clones in Africa [6,28,33]. Interestingly enough, a novel ST (ST10703) with a high similarity to ST405 was identified making it probably an offshoot of the latter. While this apparent lack of ST405 isolates needs to be addressed in complementary WGS studies, it will also be interesting to monitor

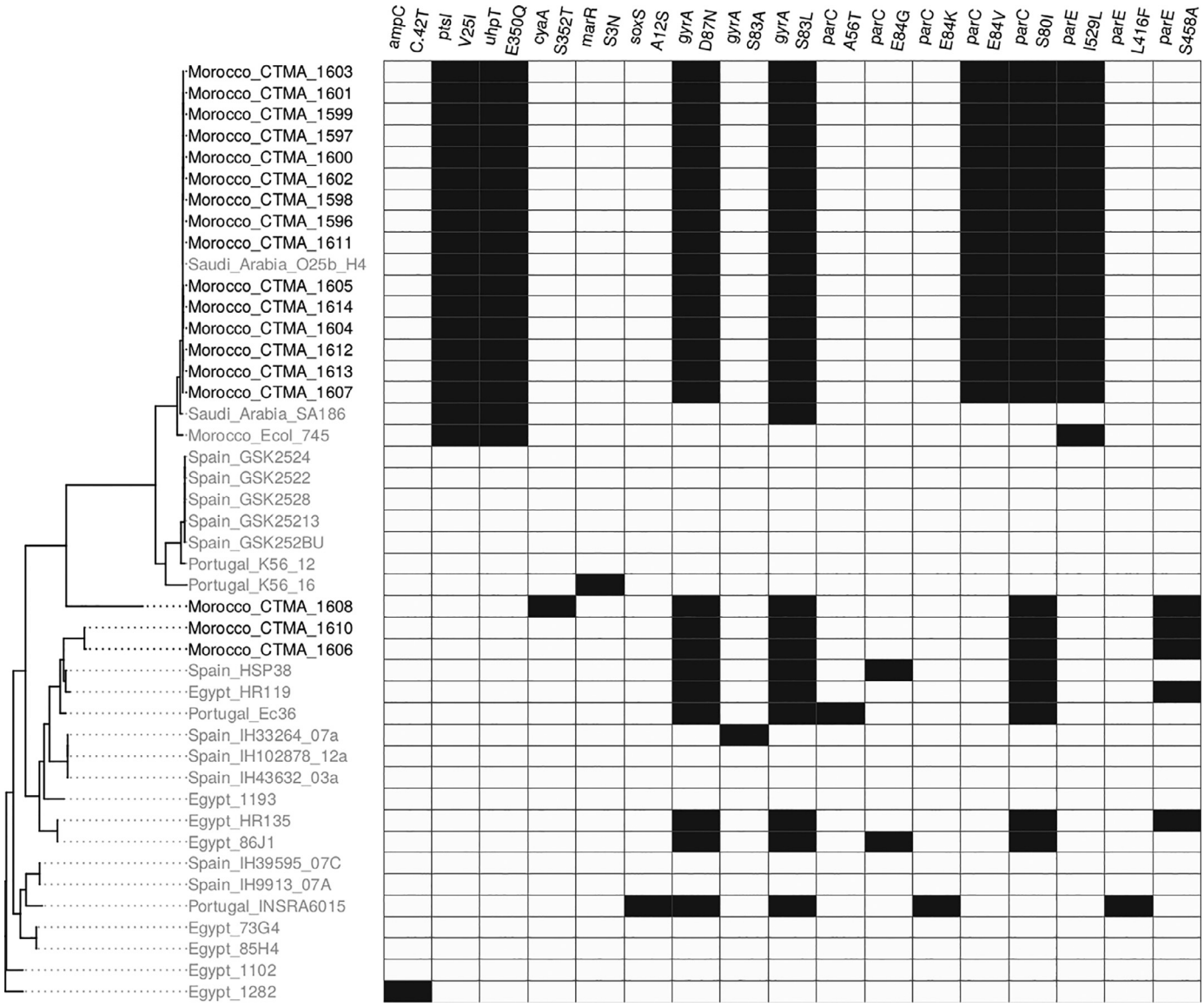


Fig. 3. Heatmap of single nucleotide polymorphism (SNP)-based determinants of antimicrobial resistance (AMR). AMR determinants were identified using the AMRFinder tool. Black cells indicate the presence of the corresponding SNP determinant.

the potential spread of this novel ST10703 and see how its potential future spread compares both with ST131 and ST405 lineages.

Inferring the resistance phenotype from genomic analysis of AMR remains challenging for most micro-organisms as the number of resistance mechanisms involved is extremely large and a range of genes most probably interact in a complex manner to give rise to resistance. On the other hand, culture-based assays for drugs can be unreliable in some species and depend on the quality of reagents used and the experience of the operator. Despite these limitations in phenotypic inference, our study showed that resistance to β-lactams (ampicillin, amoxicillin/clavulanic acid and third-generation cephalosporins) exactly matched the presence of $bla_{\text{CTX-M}}$ in ST131 *E. coli*, as confirmed by several authors [41,42]. Likewise, previous reports have shown that phenotypic resistance to quinolones (ciprofloxacin and norfloxacin) and amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of *gyrA* (S83L and D87N) and *parC* (S80I and E84V) correlated 100% [43–45]. In this study, another gene associated with low phenotypic quinolone resistance is the plasmid-mediated *aac(6′)-Ib*-cr5 [46].

Regarding trimethoprim/sulfamethoxazole, a very good correlation was found between phenotypic and genotypic resistance (i.e. *sul1* and *dfr17* genes), except for one isolate (CTMA_1606) that showed a major discrepant result; indeed, the latter isolate harboured three resistance genes (*sul1, sul2* and *dfr17*) but remained susceptible to trimethoprim/sulfamethoxazole. Another major phenotype–genotype discrepancy was observed with isolate CTMA_1613 that lacked these three aforementioned AMR genes while being resistant to trimethoprim/sulfamethoxazole. Moreover, no phenotype–genotype correlation was seen with *aac(3)* and *aadA* aminoglycoside resistance genes. Whereas current data confirm previous reports on β-lactam and quinolone resistance, these data are too limited to infer strong conclusions on AMR genes and phenotypic resistance correlation, especially regarding aminoglycosides and trimethoprim/sulfamethoxazole. Currently, algorithms are being developed to help improve the inference of phenotype from genotype [47]. These algorithms depend on data collected in many countries on large collections of bacteria. In this respect, genome characterisation of ESBL-producing MDR bacteria in Morocco where such data

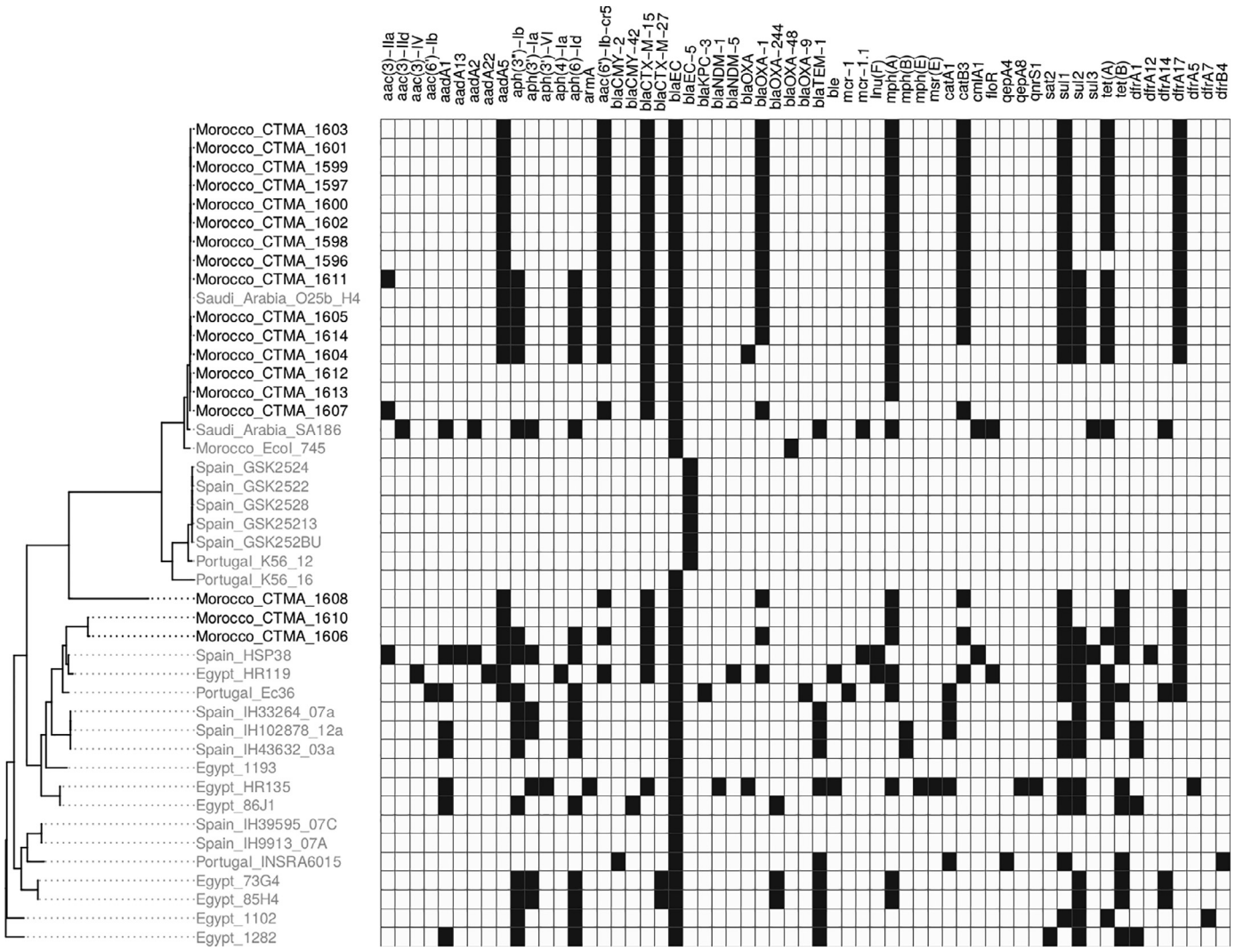


Fig. 4. Heatmap of plasmid-mediated antimicrobial resistance (AMR) genes. AMR genes were identified using the AMRFinder tool. Black cells indicate the presence of the corresponding AMR gene.

were up to now lacking somehow contributes to enrich these databases.

In conclusion, our findings underscore the pre-eminence of ST131 as the major factor driving expansion of ExPEC in Rabat region while highlighting the potential links with isolates circulating in other neighbouring countries.

Appendix A. Supplementary data: Supplementary material related to this article can be found, in the online version.

TS: add in link

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Competing interests

None declared.

Ethical approval

All data were analysed anonymously to ensure confidentiality. The study complied with Moroccan law for which ethical approval is not required for a retrospective study based on laboratory data and no consent from patients is necessary to carry out further tests on samples collected for antimicrobial research. The study also complied with the World Health Organization and international guidelines on antibiotic surveillance for which no recommendation for an informed consent has been issued.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2021.07.008.

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