



Note

Potential *in vivo* transfer of a *bla*_{CTX-M14}-harbouring plasmid established by combining long- and short-read sequencing

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ABSTRACT

Horizontal transfer of plasmid-mediated antibiotic resistance has rarely been documented *in vivo*. Utilizing long-read (Oxford Nanopore) and short-read (Illumina) sequencing, we confirmed that a gut-colonizing *Escherichia coli* and a hypervirulent *Klebsiella pneumoniae* ST23, isolated from a surgical site culture of a patient receiving cefuroxime therapy, harboured a 100% identical *bla*_{CTX-M-14}-harbouring plasmid, indicative of a potential transfer *in vivo*.

The spread of antimicrobial resistance is of great concern for public health and undermines our ability to respond to bacterial infections. Particularly, transfer of antimicrobial resistance *via* plasmid exchange is of concern as it enables unrelated (pathogenic) bacteria to acquire resistance (Card et al., 2017). While *in vitro* experiments provide ample proof of such an event, there are very few reports documenting horizontal transfer of plasmid-mediated antibiotic resistance under antibiotic pressure in a patient (Subbiah et al., 2011). Nonetheless, recent use of long-read sequencing technology that allows delineating entire plasmid sequences (Li et al., 2018), has greatly aided in establishing and quantifying nosocomial reservoirs of multi-drug resistant plasmids and also in establishing probable transmission routes (Weingarten et al., 2018). We present here a clinical case wherein a potential transfer of a *bla*_{CTX-M-14} harbouring plasmid, identified using long-read sequencing (Oxford Nanopore), occurred in a patient receiving cefuroxime therapy.

A 80-year old woman of Italian origin was admitted to the Geneva University Hospitals (HUG), Geneva, Switzerland on 4 April 2017. She was initially hospitalized and operated in February and March 2017 for lateral and anterior lumbar interbody fusions, followed by a surgical revision due to mechanical problems in early April 2017. During those 3 interventions, she received single-shot perioperative prophylaxis with cefuroxime (1.5 g IV). One week after her last surgery, she developed

signs and symptoms of deep postoperative wound infection at the site of spondylodesis and received empiric IV meropenem therapy (5 g per 24 h) through continuous perfusion. An extended spectrum beta-lactamase (ESBL)-producing, but carbapenem-sensitive *Klebsiella pneumoniae* (n° 0671) was isolated from the deep surgical site and blood cultures (BACTEC, Becton-Dickinson) on 10 April 2017. This strain was sub-cultured on blood agar and identified by MALDI-TOF MS (Biotyper, Bruker). Antibiotic susceptibility was tested by disk diffusion, and interpreted according to the EUCAST breakpoints. Strain 0671 was resistant to amoxicillin, piperacillin, amoxicillin-clavulanic acid, cefuroxime, cefepime, cefotaxime, ceftazidime, aztreonam, and to fosfomycin. During the following days, another ESBL-producing *K. pneumoniae* (n° 0303) was isolated from intra-operative swabs of her spondylodesis. On 13 April 2017, routine rectal swab screening on ESBL chromogenic media (bioMérieux) yielded an ESBL-harbouring *Escherichia coli* (n° 0164) that was also sub-cultured on blood agar and identified by MALDI-TOF MS (Biotyper, Bruker). Antimicrobial susceptibility testing by disk-diffusion (EUCAST breakpoints) found the *E. coli* 0164 to be resistant to amoxicillin, amoxicillin-clavulanic acid, piperacillin, cefuroxime, ceftazidime, ceftriaxone, cefepime and aztreonam and was sensitive to carbapenems, fluoroquinolones, aminoglycosides, fosfomycin and cotrimoxazole. All *K. pneumoniae* isolates from various clinical samples exhibited the same antimicrobial

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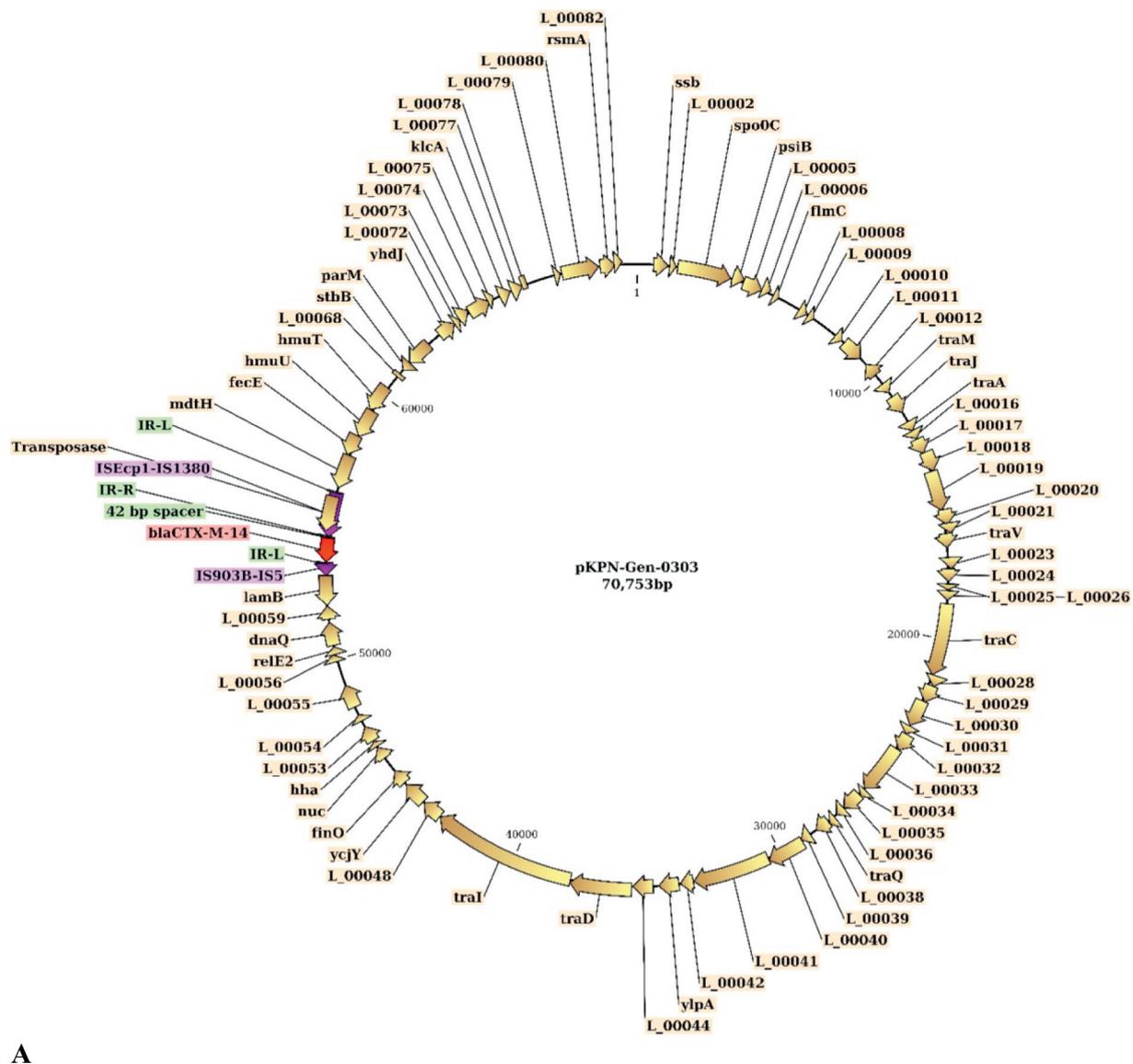
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A

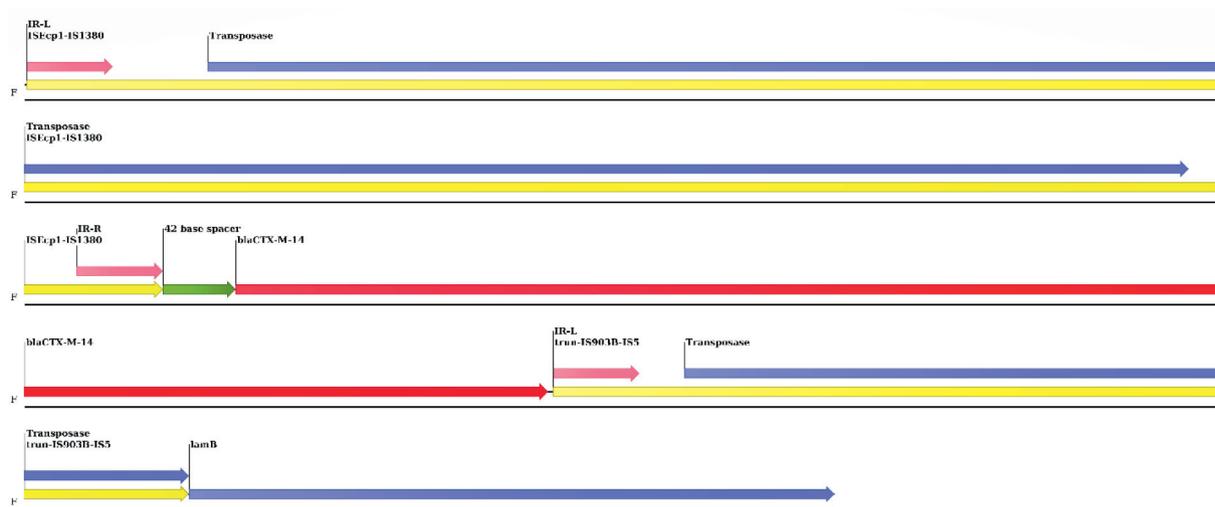
Fig. 1. A: Genetic map of the *bla*_{CTX-M14}-harbouring IncFII plasmid isolated from both *E. coli* and *K. pneumoniae*, B. Genetic association of *bla*_{CTX-M14} and IS elements, C. Comparison of time required by the two sequencing methods employed.

susceptibility profile as the gut colonizing *E. coli* 0164, except for fosfomycin resistance. We performed Nanopore sequencing (Oxford Nanopore) on the ESBL harbouring *E. coli* 0164 from the rectal swab and the *K. pneumoniae* 0303 from intra-operative swabs of spondylodis to obtain the entire plasmid backbone in order to establish potential horizontal transfer of the ESBL harbouring plasmid.

Plasmid DNA from the both ESBL-harbouring strains was isolated (PureLink HiPure Plasmid Miniprep Kit, Invitrogen, Carlsbad, USA), and sequenced using long read sequencing (MinION, Oxford Nanopore) utilizing the MinION flow cell (R9 flow cell chemistry, 24 h protocol) https://community.nanoporetech.com/protocols/rapid-barcoding-sequencing-sqk-rbk004/v/rbk_9054_v2_rev_d23jan2018 as described (Ashton et al., 2015). The nanopore raw reads statistics were with average read length of 9557 bp, longest read length 84,665 bp and N50 17,250 bp. In addition, to polishing the nanopore assembly we also utilized Illumina (2 × 250 bp, Nextera XT sample preparation kit, MiSeq) sequencing and the reads used to perform hybrid assembly using SPAdes v3.12.0 (Bankevich et al., 2012) by combining nanopore reads. Further sequence analysis was done using Prokka (Seemann, 2014) for annotating scaffolds and Resfinder 3.1 (<https://cge.cbs.dtu>

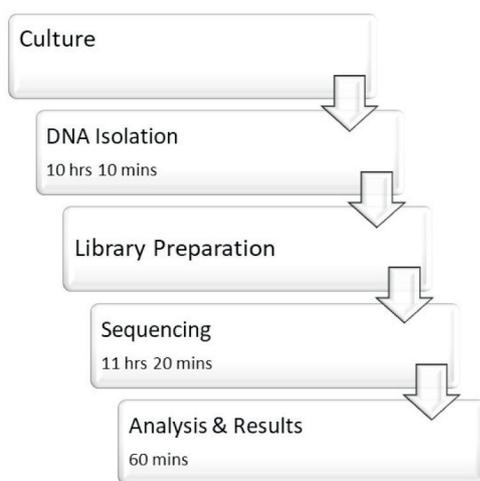
<https://cge.cbs.dtu>) for screening for antibiotic resistance genes.; insertion elements (IS) were identified using IS finder <https://www-is.biotoul.fr/>. The plasmid incompatibility type was identified using PlasmidFinder, and pMLST was determined using <https://cge.cbs.dtu.dk/services/PlasmidFinder/> and <https://cge.cbs.dtu.dk/services/pMLST/>, respectively. Of note, when we utilized contigs assembled from only the nanopore reads, the *bla*_{CTX-M} gene could not be identified as *bla*_{CTX-M-14}, Illumina reads based polishing was required to obtain the correct type although the nanopore reads helped us to obtain the complete plasmid backbone.

The plasmid map was generated using CLC Genomics Workbench v9.5.3. Simultaneously, we also performed variant calling of IncFII plasmids between *E. coli* 0164 and *K. pneumoniae* 0303 that showed no differences, indicating that both organisms harboured the same plasmid with the following characteristics: pMLST F2:A-B- and incompatibility type IncFII, 70,753 bp size and 80 protein coding regions (Fig. 1A). This plasmid harboured *bla*_{CTX-M-14} embedded between *ISEcp1* (IS1380 superfamily) upstream and a truncated IS903B (IS5 family) downstream. The genetic distance between upstream *ISEcp1* and *bla*_{CTX-M-14} was 42 bp (Fig. 1B). MLST showed that *E. coli* 0164 belonged to ST1 and *K.*

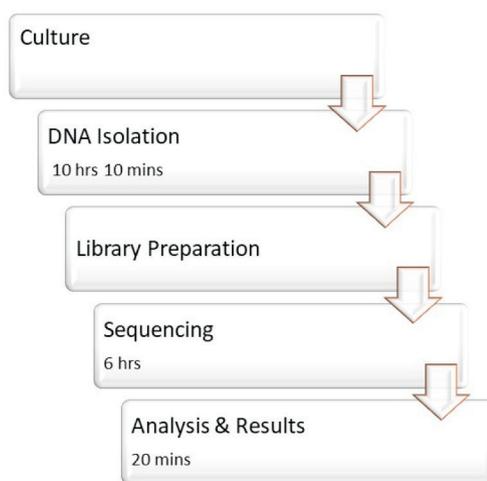


B

Illumina (MiSeq)
23 hrs 30 mins



Oxford Nanopore (MinION)
16 hrs 30 mins



C

Fig. 1. (continued)

pneumoniae 0303 to ST23, a hyper-virulent clone of the K1 serotype primarily found in the Asia-Pacific region (Shon et al., 2013). The patient had reported visits to Sicily (Italy) and Moscow (Russia) in 2016, but without any medical contact.

Also, both *E. coli* 0164 and *K. pneumoniae* 0303 harboured other unrelated plasmids and resistance genes: *E. coli* 0164 showed presence of *bla*_{TEM-1C}, *strA*, *strB* (both streptomycin resistance encoding genes) and of IncB/O/K/Z and Col156 plasmids. Similarly, *K. pneumoniae* 0303 harboured an IncHIB plasmid with 228,916 bp size and 211 protein-coding sequences. This is a ‘virulence’ plasmid, responsible for the hypervirulent phenotype of this ST type, and carries a host of virulence gene clusters including the aerobactin siderophore biosynthesis cluster and receptor proteins, enterobactin siderophore receptor protein, yersiniabactin cluster and salmochelin gene (Holt et al., 2015; Schlüter et al., 2014). Also, the plasmid harbors two copies of the *rmpA* gene that is known to confer hypermucoidity (Struve et al., 2015). Interestingly, we also found *bla*_{SHV-11} integrated in the chromosome of the *K. pneumoniae* 0303 ST23. Previous *in vitro* selection experiments have shown

that this beta-lactamase can easily mutate to become an ESBL under cephalosporin pressure and hence its presence signals a predisposition or priming of a *K. pneumoniae* strain to develop resistance to extended-spectrum beta-lactams (Hammond et al., 2008). However, our data indicates that in the human environment, an ESBL-harboring plasmid might be preferentially acquired under cephalosporin (cefuroxime) pressure despite the presence of *bla*_{SHV-11} in *K. pneumoniae* 0303, which remained unmodified.

Taken together, these findings reiterate the importance of screening patients for presence of ESBLs in the colonizing flora irrespective of travel history of the patient. The hyper virulent ST23 clone is a prevalent cause of liver abscesses and meningitis in Asia and treatment of choice is a broad-spectrum cephalosporin (Ku et al., 2017). ESBL genes have been rarely detected in this well-known, community-acquired, hyper-virulent and invasive clone. Infections due to ST23 remain as yet rare in Europe (Gundestrup et al., 2014). While conjugation events and transfer of ESBL harbouring plasmids occur with a high frequency between *E. coli* and *K. pneumoniae* *in vitro* and in the mouse gut under

cephalosporin selection pressure (Schjørring et al., 2008), here we report evidence of a potential *in vivo* transfer of an ESBL-harboring plasmid under cephalosporin pressure. Acquisition of an ESBL-harboring plasmid by the hyper-virulent *K. pneumoniae* ST23 is likely to further complicate treatment options for severe infections caused by this clone. Finally, we demonstrate the utility of long-read sequencing that presently needs to be combined with short-read sequencing, to obtain a high quality and complete plasmid backbone in order to unequivocally establish plasmid transmission (Fig. 1C).

Conflict of interest

The authors declare no conflict of interest.

Data access

All sequencing data is available under accession number Bioproject ID PRJNA509549.

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