Diagnostic Microbiology and Infectious Disease 93 (2019) 380–385

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

# Interspecies plasmid transfer appears rare in sequential infections with extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae

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ARTICLE INFO

*Article history:*
Received 2 July 2018
Received in revised form 29 September 2018
Accepted 24 October 2018
Available online 1 November 2018

*Keywords:*
Extended-spectrum β-lactamase (ESBL)
Recurrent infection
Plasmid
Transfer
*E coli*
*K. pneumoniae*
Optical DNA mapping

## ABSTRACT

From a cohort of 1836 Swedish patients infected with ESBL-producing Enterobacteriaceae (EPE) during 2004–2014, 513 patients with recurrent EPE infection were identified. Only in 14 of the 513 patients was a change of species (ESBL-*E. coli* to ESBL-*K. pneumoniae* or vice versa) found between the index and subsequent infection. Eleven sequential urine isolates from 5 of the 14 patients were available for further analysis of possible transfer of ESBL-carrying plasmids. The plasmid content was studied using optical DNA mapping (ODM), PCR-based replicon typing, and ESBL gene sequencing. ODM allowed us to directly compare whole plasmids between isolates and found similar ESBL-carrying plasmids in 3 out of the 5 patients. The ODM results and the rarity in shift of species between ESBL-*E. coli* and ESBL-*K. pneumoniae* imply that in recurrent EPE infections interspecies plasmid transfer is uncommon.

## 1. Introduction

Horizontal transfer of extended-spectrum β-lactamase (ESBL) genes via mobile genetic elements, i.e., ESBL-carrying plasmids, and the emergence of successful virulent bacterial clones are key reasons for the rapid spread of antibiotic multiresistance among Enterobacteriaceae (Brolund and Sandegren, 2016; Mathers et al., 2015). The role of interspecies plasmid spread of resistance genes in polyclonal outbreaks of ESBL- or carbapenemase-producing Enterobacteriaceae is well documented (Bocanegra-Ibarias et al., 2017; Dautzenberg et al., 2014; Fernandez et al., 2015; Muller et al., 2016a; Stillwell et al., 2015). However, the extent of a change of ESBL-carrying bacterial species and possible transfer of ESBL-carrying plasmids between consecutive isolates in patients with repetitive infections with ESBL-producing Enterobacteriaceae (EPE) is, to our knowledge, largely unexplored.

The most prevalent and globally disseminated ESBL is CTX-M-15, belonging to CTX-M group 1 and generally carried by easily transmissible plasmids of the IncF family (Brolund and Sandegren, 2016; Mathers et al., 2015). An increase in CTX-M-27 from CTX-M group 9 has been noted internationally, also generally carried on IncF plasmids (Ghosh et al., 2017). The IncF plasmids are by far the most prominent plasmids in uropathogenic *E. coli* (Brolund and Sandegren, 2016; Johnson and Nolan, 2009). They are also strongly associated with the pandemic virulent ESBL-producing *E. coli* clone ST131-O25b (Banerjee and Johnson, 2014). A most worrying feature of the IncF type plasmids is their ability to integrate and transmit carbapenemase genes (Carattoli, 2009).

Transfer of plasmids carrying antibiotic resistance genes between coexisting bacteria in the gut microbiome is known to occur in vivo, especially under antibiotic pressure (Goren et al., 2010; Karami et al., 2007; Porse et al., 2017). Persistence studies of EPE colonization have identified a small number of patients with sequential isolates of different strain types carrying the same resistance gene, supporting that interspecies plasmid transfer may have occurred within the gut flora (Tham et al., 2012; Titelman et al., 2014). Such horizontal transfer suggests that plasmid analysis could be useful for risk evaluation of new potentially hard-to-treat infections with these bacteria (Ding et al., 2016; Goren et al., 2010; Martins et al., 2017). We recently reported that the frequency of subsequent EPE infections is high (28%) in previously EPE-infected patients, implicating that ESBL-plasmid characterization in these patients might be important in the risk evaluation of recurrent infections (Lindblom et al., 2018).

Given the dynamic nature of plasmids, traditional methods for plasmid characterization, such as S1 nuclease-pulse field gel electrophoresis

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https://doi.org/10.1016/j.diagmicrobio.2018.10.014

(PFGE) or PCR-based replicon typing, may not provide sufficiently detailed information (Brolund and Sandegren, 2016). Next-generation DNA sequencing approaches are increasingly replacing these techniques but are time consuming and complicated by the demand of high knowledge of bioinformatics for the assembly of plasmids. Optical DNA mapping (ODM) offers a rapid and simple way to analyze and compare plasmid DNA (Muller et al., 2016a; Nyberg et al., 2016). In combination with CRISPR/Cas9, it provides detailed information about plasmid size, gene location, as well as a fingerprint based on the underlying sequence for plasmid detection and tracing, thus being a promising tool in plasmid analysis (Muller et al., 2016b). We have previously showed its usefulness in a hospital EPE outbreak, where early identification of possible plasmid transmission routes may be crucial (Muller et al., 2016a).

In this study, we first evaluated the frequency of change of species between ESBL-carrying *E. coli* (ESBL-*E.c*) and ESBL-carrying *K. pneumoniae (*ESBL-*K.p)* in a cohort of patients with recurrent EPE infections. Secondly, we compared the ESBL-carrying plasmid identity in available sequential isolates from the respective patients. We used ODM to address the possibility of ESBL-plasmid transfer as the underlying cause of species shift in sequential EPE infections.

## 2. Material and methods

### 2.1. Patients and bacterial isolates

Data were retrieved from a consecutively collected cohort of all patients positive for ESBL-*E.c* or ESBL-*K.p* in a diagnostic clinical culture between 2004 and 2014 (median follow-up time 3.7 years) at the Clinical Microbiology Laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden (Lindblom et al., 2018). The laboratory covered all medical care in the greater Gothenburg area during the study period which coincided with the emerge of EPE in our region from late 2003 and onwards. *E. coli* isolates detected in urine augmented from ~10,000 in 2004 to ~13,000 in 2014 and in blood from ~250 to ~620. The frequency of *K. pneumoniae* found in urine and blood did not increase and was ~1200 and ~100 per year respectively. The ESBL-*E.c* prevalences increased from 0.3% to 4.4% for *E. coli* in urine and from 0% to 6.6% in blood; the corresponding prevalences for ESBL-*K.p* were 0.1% to 3.8% in urine and 0% to 4.4% in blood.

During the study period, we found 1836 unique patients culture positive for EPE of which 94% were infected with ESBL-*E.c* and 6% with ESBL-*K.p,* in urine (94%) or blood (6%), at their first EPE infection episode. Of these, 513 patients (91% primarily positive for ESBL-*E.c* and 9% for ESBL-*K.p*) had repeated EPE-positive clinical cultures, that is, 2–23 EPE-positive cultures per patient (median 3 EPE-positive cultures/patients, altogether 2018 cultures).

Isolates were identified according to routine practice at the laboratory using conventional biochemical tests for species identification. All cephalosporin-resistant isolates were screened for ESBL phenotype using the double-disc diffusion test and were subsequently stored at −70 °C (Legrand et al., 1989).

### 2.2. DNA extraction, genotypic detection of ESBL genes, and multilocus sequence typing (MLST)

Stored isolates were retrieved, plated on blood agar medium, and verified for species identity with MALDI-TOF (VITEK MS/IVD, bioMérieux, Marcy l'Etoile, France) and for ESBL phenotype. Two to 5 bacterial colonies from overnight cultures were suspended in 100 μL EDTA, heated for 15 min at 95 °C, and centrifuged at 18,000× *g* for 5 min. Cell supernatants, containing the bacterial DNA, were separated from pelleted cell debris and used for the subsequent DNA analysis. A multiplex PCR assay for detecting CTX-M-, TEM-, OXA-, and SHV-β-lactamase genes was used both on the total DNA content and on the plasmid DNA after extraction (Fang et al., 2004). Isolates with the CTX-M gene were analyzed for CTX-M groups (Birkett et al., 2007), followed by amplification and sequencing to confirm the presence of a particular CTX-M gene in the isolates and subsequent plasmid preparation. MLST was performed according to the method of the *E. coli* MLST database website for ESBL-*E.c* (Wirth et al., 2006) and for the ESBL-*K. p* isolates as described by Diancourt et al. (2005). PFGE was performed as previously described (Muller et al., 2016a).

### 2.3. Plasmid extraction and replicon typing

Plasmid DNA was prepared from overnight culture with the Qiagen Midi kit (Qiagen, Germany) according to the manufacturer's instructions for low-copy plasmids. Eluted DNA was precipitated with isopropanol, washed with 70% ethanol, and suspended in TE buffer, pH 8.0. Incompatibility typing of the plasmids was performed by PCR-based replicon typing (Carattoli et al., 2005).

### 2.4. Optical DNA mapping

The optical DNA mapping (ODM) assay used is shown schematically in Fig. 1. Resistance genes were targeted on the plasmid DNA by adding Cas9 loaded with a gRNA, targeting the resistance gene of interest (listed in Table 1). gRNA was obtained by incubating equimolar amounts of crRNA (0.5 nmol) and tracrRNA (0.5 nmol) (Dharmacon Inc.) in 1× NEB-Buffer 3 (New England Biolabs) and 1× (0.1 μg/ml) bovine serum albumin (BSA, New England Biolabs) for 30 min at 4 °C. For targeting the CTX-M group 1, the crRNA sequence was designed as 5′ CCGUCGCGAUGUAUUAGCGU 3′, and for targeting CTX-M group 9, 5′ AACAAAUUGAUUGCCCAGCU 3′. A total of 10 μM (0.05 nmol) of gRNA was then incubated with 600 ng of Cas9 (PNA Bio Inc.) in 1× NEB-Buffer 3 and 1× BSA in a final sample volume of 5 μL at 37 °C for 15 min. The gRNA-Cas9 complex was subsequently mixed with 60 ng of plasmid DNA in 1× NEB-Buffer3 and 1X BSA, with a total volume adjusted to 15 μL using 10 mM RNase free Tris-EDTA buffer (Sigma-Aldrich), and incubated at 37 °C for 1 h.

Next, the DNA was stained with YOYO and Netropsin to generate the barcode pattern based on the underlying DNA sequence. Equal amounts of plasmid DNA and λ-DNA (New England Biolabs), used as an internal size reference, were stained with YOYO-1 (YOYO, Invitrogen) at a molar ratio of 1:3.3 (bp) and netropsin (Sigma Aldrich) at a molar ratio of 60:1 (bp) in 0.5× Tris-Borate-EDTA buffer (TBE, Medicago). The mixture was incubated at 50 °C for 30 min and subsequently diluted with MilliQ water containing 3% (v/v) β-mercaptoethanol (BME, Sigma-Aldrich) to obtain a final solution with 0.05× TBE, 0.2 μM (bp) DNA, and a total volume of 100 μL.

Nanochannels with dimensions of 100 × 150 nm$^2$ (depth × width) and 500 μm in length, connected by 2 separate microchannels, each containing a sample loading reservoir at both ends, were used in the study. Details on the fabrication process can be found elsewhere (Persson and Tegenfeldt, 2010). The nanochannels were prewetted using 0.05× TBE and 2% (v/v) BME prior to loading 10 μL of sample. The DNA was moved inside the nanofluidic chip using pressure-driven $N_2$ flow. Pressure was applied to drive the DNA molecules from the reservoir to the nanochannel entrance. By subsequently applying pressure to both sides of a microchannel, the DNA was forced into the nanochannels. Once the DNA molecules entered the nanochannels, they were allowed to relax for a few seconds prior imaging. Fluorescence imaging was done with an inverted fluorescence microscope (Zeiss AxioObserver.Z1) equipped with a 100× oil immersion objective (Zeiss, NA = 1.46), an FITC filter (488 nm exc. / 530 nm em.), and an EMCCD camera (Photometrix Evolve). Each DNA molecule was imaged for up to 200 frames, with an exposure time of 100 ms.

The analysis and statistical methods used in this study are the same as in our previous work (Muller et al., 2016b; Nyberg et al., 2016), except for here using a bin size of 5 pixels for gene detection. The sizes of the plasmid molecules were obtained from imaging the plasmids in their intact circular form (Frykholm et al., 2015) using λ-DNA (Roche Life Science) as an internal size reference and a conversion factor of

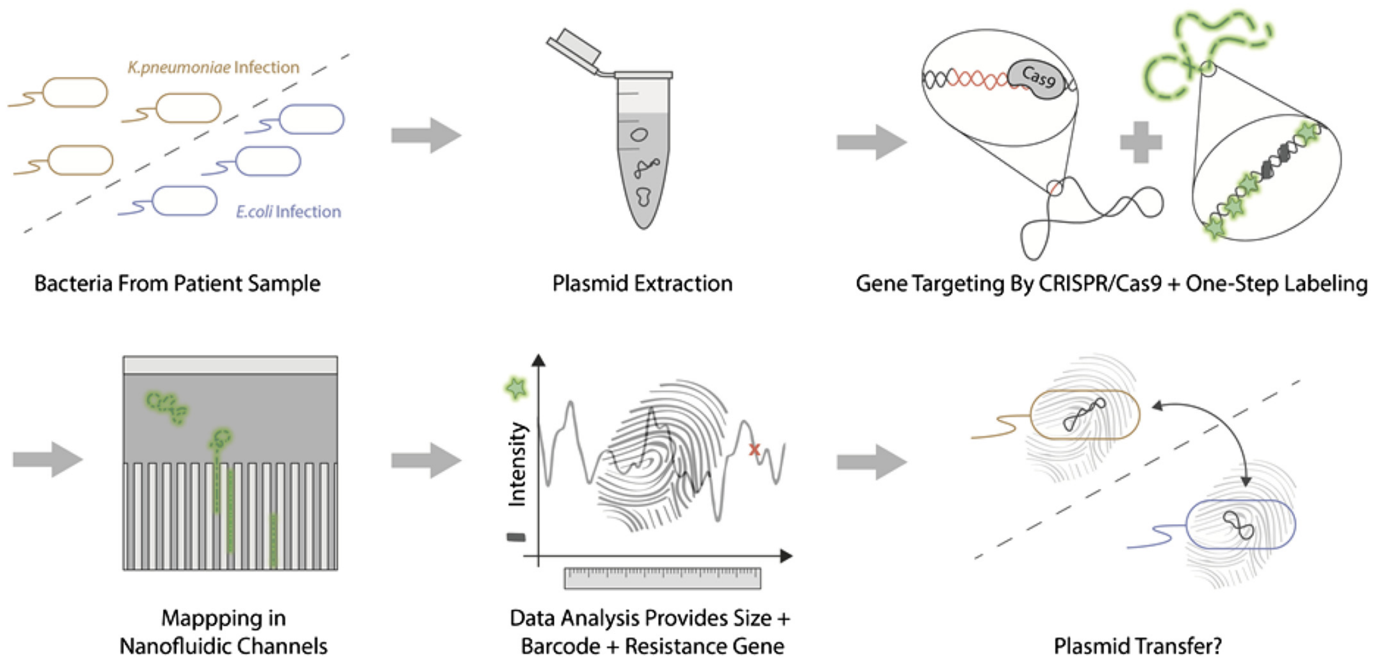

**Fig. 1.** Schematic overview of the optical DNA mapping assay. Plasmids and the ESBL gene of interest are targeted by Cas9. The plasmids are stained and imaged using a fluorescence microscope when stretched in nanofluidic channels, creating an emission intensity profile (barcode) along the contour of the entire plasmid molecule. From the measurements, it is possible to determine the size of a plasmid, obtain a sequence specific barcode, as well as detect the ESBL gene.

1.8 between circular and linear DNA (Alizadehheidari et al., 2015). The linear DNA molecules were analyzed in order to find any resistance gene on the detected plasmids. Moreover, the kymographs of the linear DNA were used to obtain a barcode of the resistance carrying plasmid for comparison between isolates in order to evaluate if plasmid transfer could have occurred. The significance of this analysis is determined using a $P$ value, where $P > 0.01$ indicates “no match” and $P < 0.01$ indicates a “match” (Muller et al., 2016a).

## 3. Results and discussion

In our cohort of 1836 patients with EPE-positive clinical diagnostic cultures, a shift of species from ESBL-*E.c* to ESBL-*K.p* or vice versa was only observed in 14 of the 513 (2.7%) patients with recurrent EPE infection. The 14 patients had a heterogeneous age distribution (median 65 years, range 3 months–90 years), where women and men were equally represented; most (11/14) had recurrent UTIs.

A species shift between ESBL-producing isolates of other species than *E. coli* and *K. pneumoniae* was only seen occasionally, and these patients were not further analyzed due to scarcity.

Our study covers all patients with culture samples in a large geographic area including all types of healthcare over a very long time. Moreover, the setting with a low risk of constant exposure to new EPE strains is a prerequisite in understanding the natural history in patients with recurrent EPE infection. Considering the large patient material, our results reflect a low frequency of species change. The findings suggest that interspecies plasmid transfer is a less frequent underlying cause of a new EPE infection with another species and, from the clinical

**Table 1**
Detailed characteristics of 13 ESBL-producing isolates analyzed for plasmid identity from six different patients.

| Patients | Species | Duration (days)[a] | ST | CTX-M group | CTX-M gene | Replicon type (Inc) | Size of plasmids (kb)[b] | Plasmid similarity[c] |
|---|---|---|---|---|---|---|---|---|
| P1 | *E. coli* | 91 | 394 | 1 | CTX-M-15 | FII, K | 30, 76, **95**, 118 | Yes |
| | *K. pneumoniae* | | 2329 | 1 | CTX-M-15 | -[d] | 33, 78, **99**, 189 | |
| P2 | *K. pneumoniae* | 97 | 37 | 1 | CTX-M-15 | FII, A/C | 61, **98**, 174 | No |
| | *E. coli* | | 131 | 1 | CTX-M-15 | FII, FIA, FIB, I1 | 59, **87**, 152 | |
| P3 | *K. pneumoniae* | 51 | 14 | 1 | CTX-M-15 | -[d] | **69**, 135, 222, 237 | No |
| | *E. coli* | | 4038 | 1 | CTX-M-3 | I1 | **99** | |
| P4 | *E. coli* | 874 | 10 | 9 | CTX-M-27 | FII | **92** | Yes |
| | *K. pneumoniae* | | 1228 | 9 | CTX-M-27 | FII | 128, **170** | |
| P5 | *E. coli* | 16 | 167[f] | 1 | CTX-M-15 | FII, FIA, FIB | 60, **151**, 201 | Yes |
| | *E. coli*[e] | | 167[f] | 1 | CTX-M-15 | FII, FIA, FIB | 46, 101, **158** | |
| | *K. pneumoniae*[e] | | 309 | 1 | CTX-M-15 | FII, FIA, FIB | 107, **162**, 201 | |
| P6 | *K. pneumoniae*[g] | 28 | 15 | 1 | CTX-M-15 | N | **93**, 112 | Yes |
| | *E. coli* | | 88 | 1 | CTX-M-15 | N | **79**, 202 | |

Eleven isolates from 5 patients (P1–P5) with sequential urine isolates and 2 isolates from 1 patient (P6) with a fecal screen isolate followed by a urine isolate.

[a] Time between the initial and sequential EPE infection.

[b] Plasmid size indicated by optical DNA mapping. Plasmids containing the CTX-M gene are indicated in bold.

[c] Possible interspecies plasmid transfer indicated by optical DNA mapping.

[d] No known replicon types were detected by conventional PCR-based replicon typing.

[e] Isolates found simultaneously in a urine sample.

[f] The PFGE type differed by >80%, indicating lack of strain identity despite same sequence type (ST).

[g] Fecal screen isolate.

point of view, question the need for plasmid typing for risk evaluation in an unselected patient population.

In order to investigate the role of possible plasmid transfer in more detail, isolates from the patients displaying a shift of species were further analyzed. Eleven urine isolates from five patients were retrieved, including isolates from initial and subsequent infection episodes (Table 1, Fig. 2). The same CTX-M group was identified in both isolates in all 5 cases, indicating that ESBL-plasmid transfer could have occurred. Isolates from 9 patients (1–9 missing isolates/patient) were not available due to lack of storage. We also added a sixth patient (P6) with an ESBL-*K.p.* isolate from a fecal screen sample followed by an ESBL-*E.c* urine isolate.

The ODM results are summarized in Fig. 2. For the first patient (P1), both isolates contained multiple plasmids (Table 1, Fig. 2). Both the ESBL-*E.c* isolate and the subsequent ESBL-*K.p* isolate harbored a plasmid of approximately the same size that had overlapping barcodes. Moreover, the 2 plasmids of similar size carried the CTX-M gene at the same location along the barcode, further strengthening the evidence of possible plasmid transfer. For the second patient (P2), several plasmids were also found in each isolate, and the CTX-M gene was identified on 1 plasmid in each isolate. Interestingly, even if the size of the 2 plasmids harboring the CTX-M gene was similar, the barcodes showed a low degree of similarity. Moreover, at the best overlap of the 2 barcodes, the position of the CTX-M gene was different, further supporting that

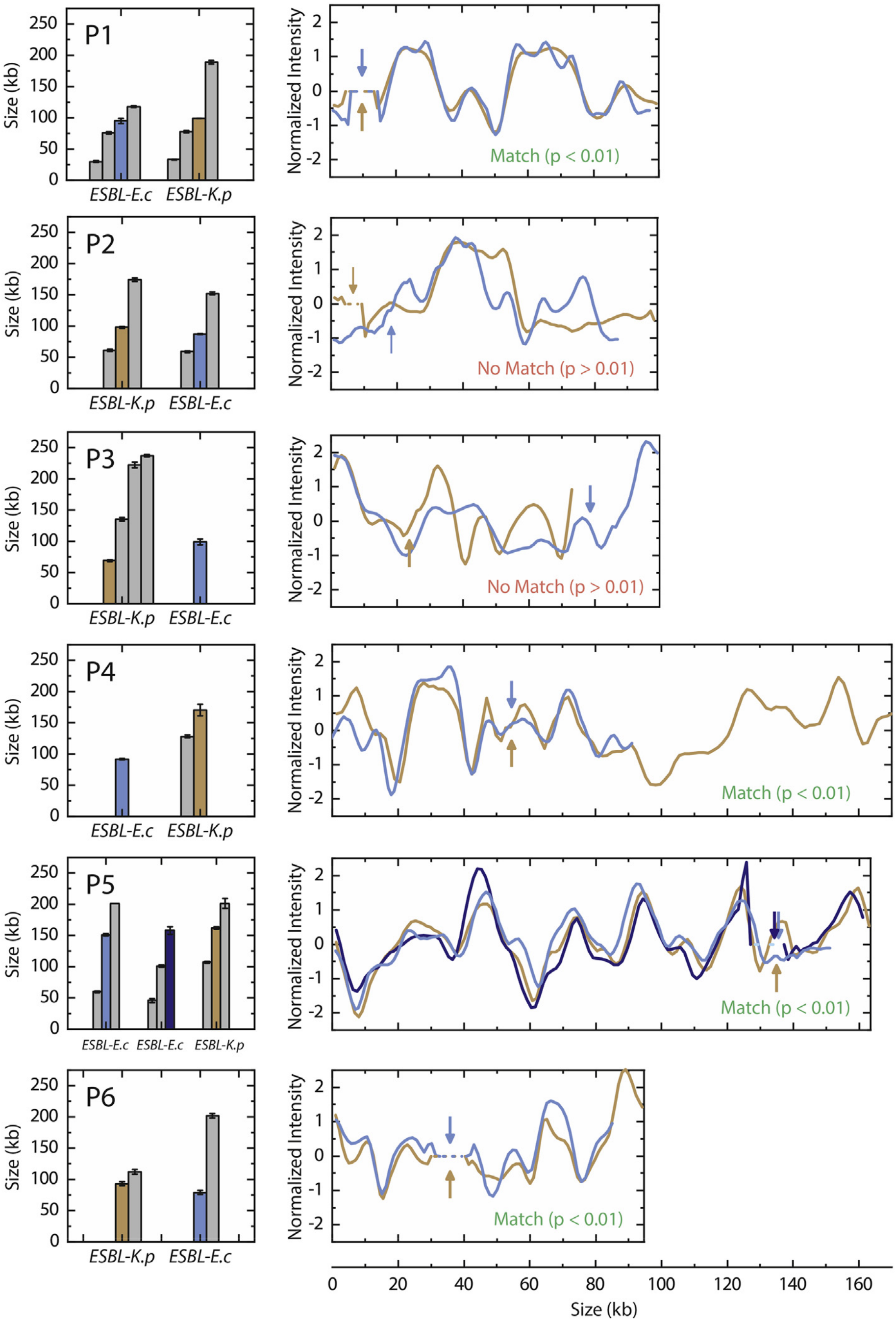

**Fig. 2.** Overview of the results obtained from optical DNA mapping. Left: The number of plasmids and their corresponding size are shown for all patients (P1–P6) with plasmids harboring the resistance gene marked in brown (ESBL-*K. p*) or blue (ESBL-*E. c*). Right: Comparison between barcodes of resistance encoding plasmids for each patient. ESBL-*K.p* barcodes are shown in brown and ESBL-*E. c* barcodes in blue with corresponding arrows to indicate the position of the CTX-M gene. The barcodes have been compared based on similarity, rendering a *P* value either below or above a threshold of 1%.

the plasmids were different. It should nevertheless be noted that even if ODM can rule out a direct transfer of the resistance carrying plasmid between the isolates, it cannot be excluded that CTX-M gene mobilization had occurred via transposition.

Also, for P3, the plasmids that carried the CTX-M group 1 gene could be directly detected using the ODM assay. When comparing the barcodes, we found no evidence that plasmid transfer had occurred since neither the barcode nor gene position overlapped. For P4, the size of the plasmids that encoded the CTX-M group 9 gene differed drastically between the ESBL-*E.c* and the subsequent ESBL-*K.p*. However, when comparing the barcode and the position of the ESBL-gene, the initial plasmid found in the *E. coli* isolate matched perfectly onto a region of the plasmid found in the *K. pneumoniae* isolate. The subsequent plasmid thus seemed to have acquired an additional DNA segment (Fig. 2).

For P5, 3 isolates were available. Initially, ESBL-*E.c* was detected followed by ESBL-*E.c* and ESBL-*K.p* simultaneously present in a subsequent urine sample. The first and second ESBL-*E.c* isolates were of the same sequence types (ST) but not of similar strain type by PFGE (Table 1). ODM identified a plasmid with the same CTX-M gene and similar barcodes in all 3 isolates, indicating possible plasmid transfer from the index isolate to the subsequent isolates (Fig. 2). The plasmids carrying the ESBL gene in the 2 subsequent isolates were slightly larger than the plasmid in the first *E.c*-isolate (Table 1), and the barcode analyses revealed that they possessed an extra piece of DNA compared to the plasmid found in the initial isolate (Fig. 2). This may imply that a second shift could have occurred between the 2 subsequent *E.c* and *K.p* isolates.

For the final patient, P6, with an ESBL- *K.p.* isolate from a fecal screen sample followed by an ESBL-*E.c* urine isolate, plasmid similarity was also indicated by the ODM results. As in the case for P4, the plasmids did not display similar size; however, both barcode and gene position indicated a significant match.

The results of the ODM assay were compared with conventional PCR-based replicon typing and sequencing of CTX-M genes (Table 1). For P1, where ODM indicated plasmid transfer, no corresponding replicon types could be identified between the isolates. For P2, on the other hand, the results from the PCR based analysis, in contrast to the ODM assay, indicated a potential plasmid transfer with the same CTX-M-15 gene and common replicon types (IncFII) identified. Adding to this, the similar size of the 2 plasmids in P2 may easily lead to the incorrect conclusion that plasmid transfer had occurred. In patient P3, we found different CTX-M genes, CTX-M-15 and CTX-M-3, by sequencing and moreover no shared replicon types, i.e., in good agreement with the ODM-results. This case also demonstrates the differential potential of ODM, where there is no need for sequencing to confirm noncorresponding CTX-M genes. In patients P4–P6, the sequential isolates carried the same CTX-M genes and replicon types as shown in the table. Interestingly, the ODM could demonstrate the dynamics of the sequential plasmids in showing identity despite considerable plasmid size differences in both P4 and P6 and possibly 2 sequential plasmid transfer episodes for P5.

Our plasmid analysis shows that even when sequential ESBL-*E.c* or *K. p*-isolates from a patient carry the same CTX-M gene by group, this does not mean that they share the same CTX-M gene, and even if they do, they may not share the same plasmid despite harboring plasmids of similar size and conventional replicon types. On the other hand, plasmids of different sizes or replicon types were found to share common genetic elements.

Though restricted to a few examples, we demonstrate the future need of more advanced methods allowing detailed analysis, such as extended replicon typing methods, plasmid sequence typing, or combined methods to confirm identity of the entire or part of the plasmid carrying the ESBL gene, as proposed by others (Brolund and Sandegren, 2016). This is particularly important in nonoutbreak situations when plasmid similarity may be less likely. In our hands, ODM with the addition of CRISPR/Cas9 to pinpoint the exact position of the CTX-M gene provided valuable information, and the simplicity and rapidity of the method are true advantages.

For the overall study, some important limitations must be mentioned. Because of the retrospective design, missing patients due to cultures not taken cannot be excluded. Also, tested isolates were few, limited to those available from storage.

The most likely place of ESBL-plasmid transfer is within the gut flora. Such transfer may very well occur without the result of a new virulent EPE strain causing infection (Titelman et al., 2014; Vading et al., 2016). Thus, we can by no means exclude that plasmid transfer would have been much more common if fecal ESBL-*E.c*/*K.p* isolates would have been examined. In our recent study, we found that subsequent EPE infections were very rare in patients with fecal EPE colonization (Lindblom et al., 2018). However, our aim in the present study was to investigate possible ESBL-plasmid transfer in cases with recurrent EPE infections and not plasmid transfer per se.

A requirement for interspecies plasmid transfer is the concomitant presence of both species in sufficient quantities. The literature is scarce in this respect, other than fecal colonization studies of EPE, in for instance healthy travelers (Lubbert et al., 2015; Vading et al., 2016). In these studies, simultaneous carriage of several EPE species seems to be less common.

We have not investigated within-species ESBL-plasmid transfer, for instance, between isolates causing sequential ESBL-*E.c* infections. However, in UTI caused by non-ESBL-producing *E.coli*, it has previously been demonstrated that certain virulent clones are in abundance in the fecal flora in patients suffering from UTI and that the same bacterial strain often causes the recurrences (Ejrnaes et al., 2006; Moreno et al., 2008; Skjot-Rasmussen et al., 2011). This might also be the case for ESBL-*E.c* recurrences — leaving less room for new EPE to cause infection. Secondly, a change of ESBL-carrying plasmids within the same bacterial strain from one infection episode to the next appears less likely, especially bearing in mind the high prevalence of certain CTX-M types in prevalent bacterial clones (Banerjee and Johnson, 2014; Mathers et al., 2015).

## 4. Conclusion

In patients with recurrent EPE infection, a shift between the 2 most common ESBL-producing bacterial species causing UTI appears rare over time and thus also interspecies plasmid transmission of ESBL genes between sequential isolates that cause disease. Although important in understanding the epidemiology in polyclonal EPE outbreak situations, the clinical value of ESBL-plasmid characterization in addition to strain typing and resistance determination for evaluating recurrent EPE infections in the general EPE-infected patient appears low. Our findings also imply that investigating possible plasmid transfer requires advanced methods. In our hands, ODM proved to be a valuable tool for plasmid analysis.

## Acknowledgments

We thank Shora Yazdanshenas and Beatriz Piñeiro Iglesias for valuable technical assistance. We also thank Prof. Alessandra Carattoli for kindly providing us the control strains for plasmid replicon typing. Finally, we thank Tobias Ambjörnsson and his research group for providing the data analysis software for the analysis of the optical DNA mapping data.

## Funding

C.Å. obtained support from the Centre for Antibiotic Resistance Research (CARe), University of Gothenburg, and Region Västra Götaland, Sweden (VGFOUREG-660231) and N.K. from FoU-rådet i Göteborg and Södra Bohuslän, Sweden (VGFOUGSB-578851). F.W. acknowledges funding from Åke Wibergs Stiftelse and the EU Horizon 2020 program BeyondSeq (no. 634890).

## Declarations of interest

None.

## Compliance with ethical standards

This study was conducted in accordance with the Declaration of Helsinki and national and institutional regulations.

## Ethical approval

The study was approved by the Regional Ethical Review Board in Gothenburg, Sweden (recordal: 170-17).

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