Pivmecillinam (amdinocillin pivoxil) is the recommended first choice antibiotic used to treat urinary tract infections (UTIs) in Denmark. Yet, in laboratory settings the frequency of mutation to mecillinam resistance has been found to be very high (Thulin, Sundqvist, & Andersson, 2015). The clinical relevance of in vivo resistance is unknown, and previous studies of mecillinam for treatment of UTI found good clinical response, but low bacteriological cure rates (Titelman, Iversen, Kalin, & Giske, 2012). This could imply that most *Escherichia coli* found with in vivo development of mecillinam resistance lack the ability to survive in the bladder, as previously shown in vitro (Thulin et al., 2015). The resistance mechanisms reported from in vitro studies include a large variety of mutations in genes associated with, for example, energy metabolism and lipopolysaccharides (LPS) synthesis (Thulin et al., 2015). Thus, mecillinam resistance is difficult to detect based on genomic data. A study from France found a mutation in the promotor of a plasmid-borne *bla*<sub>TEM-1</sub> gene to cause in vivo mecillinam resistance, yet, the clinical relevance was unknown and combining mecillinam with amoxicillin/clavulanate inhibited the newly developed resistance (Birgy et al., 2017). In Denmark, we have seen a low prevalence of mecillinam resistance in *E. coli* with 7% of UTIs in Danish hospitals and 6% of primary care urine isolates, in spite of many years of use (DANMAP 2016).

In this study, we describe occurrence of in vivo mecillinam resistance in a clinical isolate of extended-spectrum β-lactamase producing *E. coli* following pivmecillinam treatment. The only identified phenotypic differences in MEC-R compared to the original MEC-S isolate were full-length lipopolysaccharides with O-antigen (O25), mecillinam resistance, and a lower minimum inhibitory concentration for ceftazidime. Whether the serotype change and mutation in *fhlA* is the cause of the mecillinam resistance is unknown. Further studies are required to elucidate this possible mechanism. We continue to recommend the use of pivmecillinam as first-line treatment for UTI, yet mecillinam treatment for other foci should be investigated.

**KEYWORDS**
*Escherichia coli*, LPS, mecillinam, metabolism, mutation, O-antigen, pivmecillinam, resistance, serotype, whole-genome sequencing
resistance (MEC-R), respectively, were characterized and sampled from a previous study by Jansåker, Frimodt-Møller, Sjögren, and Dahl (2014). The isolates caused complicated lower UTI in the patient with observed resistance to mecillinam, after treatment with pivmecillinam 400 mg t.i.d during the primary infection. The patient had recurrent symptoms of UTI, and the second urine sample two weeks after the initial revealed the same ESBL-producing *E. coli*, however, now resistant to mecillinam.

To characterize the isolates, we performed a range of phenotypic and genotypic characterizations. With respect to phenotype, the isolates were subject to minimum inhibitory concentration (MIC) determination using E-test®, disk diffusion (EUCAST method), and serotyping. Additionally, the isolates were tested for synergy between mecillinam (10 µg) and ampicillin (10 µg) and amoxicillin/clavulanate (30 µg), respectively, by disk diffusion in order to evaluate the activity of mecillinam + ampicillin and mecillinam + amoxicillin/clavulanate in MEC-S and MEC-R. We performed phenotypic O-serogrouping by agglutination tests in microtiter plates using commercially available antisera against all recognized *E. coli* O-antigens (O1–O187; SSI Diagnostica, Hillerød, Denmark). The genotypes of the two isolates were investigated by whole-genome sequencing (MiSeq 2000) based on paired-end and mate-pair Illumina libraries.

### TABLE 1 Pheno- and genotypic data of MEC-S and MEC-R

| Phenotype                  | MIC-S | Interpretation<sup>b</sup> | MIC-R | Interpretation<sup>b</sup> |
|----------------------------|-------|-----------------------------|-------|-----------------------------|
| Ampicillin                 | >256  | R                           | >256  | R                           |
| Mecillinam                 | 4     | S                           | 4     | S                           |
| Cefpodoxime                | >256  | R                           | >256  | R                           |
| Ceftazidime                | >128  | R                           | >128  | R                           |
| Meropenem                  | 0.032 | S                           | 0.032 | S                           |
| Ciprofloxacin              | >32   | R                           | >32   | R                           |
| Gentamicin                 | 0.5   | S                           | 0.5   | S                           |
| Tetracycline               | 4     | S                           | 4     | S                           |
| Sulfamethoxazole           | 64    | S                           | 64    | S                           |
| Trimethoprim               | >32   | R                           | >32   | R                           |
| Nitrofurantoin             | 16    | S                           | 16    | S                           |
| Piperacillin-tazobactam    | 23<sup>c</sup> | S                     | 25<sup>c</sup> | S                     |

| Genotype                  | MEC-S | MEC-R |
|----------------------------|-------|-------|
| Phylogroup                 | B2    | B2    |
| MLST                       | ST131 | ST131 |
| SeroTypeFinder             | O25K:H2 | O25K:H2 |
| O-serotyping               | Rough | O25   |
| Phage content              | PHAGE_Entero_Arya_NC_031048 | PHAGE_Entero_Arya_NC_031048 |
|                           | PHAGE_Entero_BP_4795_NC_004813 | PHAGE_Entero_BP_4795_NC_004813 |
|                           | PHAGE_Entero_lambda_NC_001416 | PHAGE_Entero_lambda_NC_001416 |
|                           | PHAGE_Burkho_phiE255_NC_009237 | PHAGE_Burkho_phiE255_NC_009237 |
|                           | PHAGE_Entero_P88_NC_026014 | PHAGE_Entero_P88_NC_026014 |
|                           | PHAGE_Salmon_Fels_2_NC_010463 | PHAGE_Salmon_Fels_2_NC_010463 |
| Genome                     |       |       |
| Scaffolds                  | 21    | 21    |
| Average coverage           | 170   | 208   |
| Plasmid Inc-groups         | IncFIA, IncFIB, IncFII, IncX4, IncN | IncFIA, IncFIB, IncFII, IncX4, IncN |
| Resistance genes           | bla<sub>CTX-M-15</sub>, bla<sub>TEM-1B</sub>, bla<sub>AP-2<sup>d</sup></sub>, QnrS1, dfrA14 | bla<sub>CTX-M-15</sub>, bla<sub>TEM-1B</sub>, bla<sub>AP-2<sup>d</sup></sub>, QnrS1, dfrA14 |

<sup>a</sup>MIC, minimum inhibitory concentration. <sup>b</sup>Interpretation based on EUCAST break points with the exception of tetracycline and sulfamethoxazole where ECOFFs have been applied. <sup>c</sup>Zone diameter reported. <sup>d</sup>Not in ResFinder database, but identified by BLAST.
SNP differences between the two isolates were identified with Geneious R9 using MEC-S as reference for the SNP call followed by verification by inspection of reference mapping with parameters previously described (Nielsen et al., 2016). Whole-genome alignments were analyzed with Mauve in Geneious followed by verification with reference mapping (>10x coverage with unique mapping). For further depiction of the isolates, we used the following genomic tools: PHAST (phage identification), PlasmidFinder, ResFinder, MLST typing, SerotypeFinder (O:H-antigen).

The phenotypic analyses proved identical MIC values between the two isolates for all tested antimicrobials, apart from mecillinam and ceftazidime (Table 1). The synergy test revealed that a combination of mecillinam + amoxicillin/clavulanate abolished resistance in MEC-R, similar to the results from Birgy et al. (2017).

The genomic analyses revealed no differences between the two isolates with respect to MLST (ST131) and phylotype (B2), prophage content, and plasmid Inc-groups (Table 1). Regarding serotype, SeroTypeFinder identified O25:K-:H2 in both MEC-S and MEC-R. However, from the phenotypic serotype it was evident that MEC-S was a rough isolate that did not produce the O25 antigen, but genetically, it belonged to the O25:K-:H2 group. SeroTypeFinder does not identify rough phenotypes.

The genomic investigations revealed that the two isolates differed by a single nonsynonymous SNP. MEC-S had a premature stop codon in fhlA, encoding a transcriptional activator required for expression of formate dehydrogenase H and hydrogenase-3 and -4 structural genes (Skibinski et al., 2002). MEC-R on the other hand has the fully translational variant of this gene. The assembled genome of MEC-R lacks one copy of blaTEM-1B compared to MEC-S and reference mapping verified that this was not an assembly artifact. We did not find any of the mutations in the 60 genes associated with mecillinam resistance in E. coli as previously reported (Birgy et al., 2017; Thulin et al., 2015; Titelman et al., 2012). Furthermore, we did not identify any hyper-producing alterations in the promoter of blaTEM-1B.

We describe occurrence of in vivo mecillinam resistance in a clinical isolate of ESBL-producing E. coli following pivmecillinam treatment. The only identified phenotypic differences in MEC-R compared to the original MEC-S isolate were a full-length LPS with O-antigen (O25), mecillinam resistance, and a lower MIC for ceftazidime. Whether the serotype change and mutation in fhlA is the cause of the mecillinam resistance and changed MIC for ceftazidime is unknown. Yet, a rough serotype is caused by mutations in E. coli which prevent the bacteria from producing O-antigens due to nonfunctional production of full-length LPS (Reyes, González, Jimenénez, Herrera, & Andrade, 2012). A study described that LPS mutations affect mecillinam susceptibility in combination with other mutations and could therefore be a possible explanation for the increased MIC of MEC-R (Antón, 1995). In this perspective, it could be hypothesized that the reversion of the premature stop codon in fhlA to the fully translational fhlA of MEC-R could be correlated with the LPS production and mecillinam resistance by a serotype change from rough to O25, through yet unknown mechanisms.

Whether the blaTEM-1B deletion contributed to changed MIC for ceftazidime is not conclusive from these results, however should be investigated further. Alternatively, the changed ceftazidime MIC could be caused by minority variants of TEM, which cannot be detected by ResFinder but could differ undetected between the two isolates.

In this paper, we describe the clinical occurrence of mecillinam resistance, which has rarely been described with respect to genomic analyses on resistance in a patient. The observed mecillinam resistance is in accordance with previous laboratory findings (Thulin et al., 2015). The data indicate that a spontaneous mutation rather than horizontal gene transfer caused the resistance, contributing to the previously described mutations identified in the laboratory. The clinical significance of such resistance is unknown; we are planning further studies to investigate the in vivo fitness in mice as well as susceptibility in urine for MEC-R to answer these questions. We continue to recommend the use of pivmecillinam as first-line treatment for UTI; yet mecillinam treatment for other foci should be investigated.

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**CONFLICT OF INTEREST**

All authors declare no conflict of interests.

**AUTHORS CONTRIBUTION**

FJ was the primary investigator of the original clinical study. KLN, FBH, KHH, JDK, NFM, and FJ involved in the concept and design of the current study. KLN, KHH, and FBH involved in laboratory analyses. KLN, KHH, FBH, NFM, and JDK involved in analysis or interpretation of data. KLN, KHH, and FBH involved in drafting of the manuscript. FJ, NFM, and JDK involved in critical revision of the manuscript for important intellectual content. JDK and NFM involved in supervision. All authors take responsibility for the reliability and accuracy of data, and data analyses and approval of final version of the manuscript.

**ETHICS STATEMENT**

The study was approved by the Danish Data Protection Agency (ID 2014-58-4175 and a revised ID 2014-58-0075). Included patients were asked to participate and gave written consent prior to inclusion.
DATA ACCESSIBILITY
The genomes are deposited in Figshare (https://figshare.com/s/232d5fcd5c0ec85017c4)

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