Multiple NDM-5-Expressing *Escherichia Coli* Isolates From an Immunocompromised Pediatric Host

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Background. Genes conferring carbapenem resistance have disseminated worldwide among Gram-negative bacteria. Here we present longitudinal changes in clinically obtained *Escherichia coli* isolates from 1 immunocompromised pediatric patient. This report demonstrates potential for antibiotic resistance genes and plasmids to emerge over time in clinical isolates from patients receiving intensive anticancer chemotherapy and broad-spectrum antibiotics.

Methods. Thirty-three isolates obtained over 7 months from 1 patient were included. Clinical data were abstracted from the medical record. For each isolate, studies included phenotypic antibacterial resistance patterns, sequence typing, bacterial isolate sequencing, plasmid identification, and antibiotic resistance gene identification.

Results. Sites of isolation included blood, wound culture, and culture for surveillance purposes from the perianal area. Isolates were of 5 sequence types (STs). All were resistant to multiple classes of antibiotics; 23 (69.6%) were phenotypically resistant to all carbapenems. The *bla*NDM-5 gene was identified in 22 (67%) isolates, all of ST-167 and ST-940, and appeared to coincide with the presence of the IncFII and IncX3 plasmid.

Conclusions. We present unique microbiologic data from 33 multidrug-resistant *E. coli* isolates obtained over the course of 7 months from an individual patient in the United States. Two *E. coli* sequence types causing invasive infection in the same patient and harboring the *bla*NDM-5 gene, encoded on the IncX3 plasmid and the IncFII plasmid, were identified. This study highlights the emergence of multidrug-resistant bacteria on antibiotic therapy and the necessity of adequate neutrophil number and function in the clearance of bacteremia.

Keywords. immunocompromised; multidrug-resistant; NDM-5; pediatric.

Carbapenem-resistant Enterobacteriaceae (CRE) are now globally disseminated [1]. Carbapenemases, a group of beta-lactamases, are 1 potential mechanism of carbapenem resistance [2]. The New Delhi metallo-beta-lactamase (NDM), a carbapenemase, was first discovered in 2008 [3]. Twenty unique NDMs have since been described [4]; *bla*NDM-5 was first discovered in an *Escherichia coli* isolate from a patient who had received medical care in India [5]. Two amino acids differentiate *bla*NDM-5 from other *bla*NDM protein sequences. These substitutions provide enhanced hydrolytic activity against a variety of beta-lactams, including carbapenems [1, 5].

Plasmid-mediated transmission has facilitated spread of NDMs among Gram-negative bacteria. *bla*NDM genes have been identified on several plasmid replicon types. These include IncF, IncX3, IncL/M, IncH, and IncA/C [6]. Since its initial description in 2011, the *bla*NDM-5 gene has been identified on 5 plasmids [7]. The IncX3 *bla*NDM-5 was first identified in 2016 and has subsequently become recognized as the primary mechanism for plasmid-mediated transmission [8, 9] in humans and animals [9–13]. All *bla*NDM-1 and *bla*NDM-5 sequences on IncX3 plasmids have the same upstream and downstream genes, which makes it possible that *bla*NDM-5 developed from point mutations of *bla*NDM-1 on an IncX3 plasmid [14]. The *bla*NDM-5 gene has also been identified on the IncFII [15], IncF [5, 16, 17] and IncN plasmids [7, 18].

Importantly, plasmids reported to harbor *bla*NDM genes may also carry other antibiotic resistance genes, which further complicates patient treatment [19, 20]. IncX3 has been shown to carry other antibiotic resistance genes, including other *bla*NDM genes [9, 20–24]. IncFII has also been found to carry multiple antibiotic resistance genes, including those that confer resistance to other classes of antibiotics [6]. As these plasmid replicons may be transmitted among bacteria under
selective antibiotic pressure, bacteria may develop resistance broadly to multiple classes of antibiotics through the conjugation of 1 plasmid [12].

In this unique case study, we describe phenotypic and genotypic changes occurring over time in 33 clinical isolates obtained from 1 patient receiving medical care at St. Jude Children’s Research Hospital (St. Jude) in Memphis, Tennessee. Five sequence types from 3 culture sites over 7 months are described. Each isolate carried multiple antibiotic resistance genes, including bla\textsubscript{NDM-5}.

**METHODS**

**Study Design**

The primary aim of this study was to further analyze the genetic determinants of antibiotic resistance that emerged over time in clinical isolates obtained from an individual patient with significant and prolonged antibiotic exposure over the course of several months of cancer therapy at St. Jude Children’s Research Hospital in Memphis, Tennessee. Preserved clinical isolates from this individual patient were used to perform the additional sequencing analysis, detailed below. Clinical data were abstracted directly from the patient’s electronic medical record. The St. Jude Institutional Review Board approved this study.

**Culture Methods, Bacterial Identification, and Susceptibility Testing**

Blood cultures were collected using BACTEC liquid media bottles (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated on the BACTEC FX (Becton, Dickinson and Company) blood culture system. Positive blood cultures were subcultured to the MacConkey agar plates. MacConkey culture subculture plates were examined (at 24 and 48 hours) for the presence of lactose-fermenting Gram-negative bacilli. Isolated lactose-fermenting Gram-negative bacilli were identified and tested for the presence of extended-spectrum beta-lactamase (ESBL) and carbapenem resistance using the VITEK2 Compact (bioMérieux, Marcy l’Etoile, France).

Perianal swabs were obtained for infection control purposes per institutional guidelines: at initial arrival at the institution, following infection with a resistant organism, and weekly while admitted to the inpatient transplant unit. Swabs were used to inoculate blood agar and MacConkey agar plates (Becton, Dickinson and Company) and 5 mL of trypticase soy broth (TSB; Becton, Dickinson and Company) containing a 10-µg meropenem disc (Becton, Dickinson and Company) containing. After overnight incubation, the tripticase soy broth (TSB) was subcultured to the MacConkey agar plates. MacConkey culture and subculture plates were examined (at 24 and 48 hours) for the presence of lactose-fermenting Gram-negative bacilli. Isolated lactose-fermenting Gram-negative bacilli were identified and tested for the presence of extended-spectrum beta-lactamase (ESBL) and carbapenem resistance using the VITEK2 Compact (bioMérieux).

**Isolate Preparation**

Isolates were cultured on blood agar plates (Becton, Dickinson, and Company) at 37°C and 5% CO\textsubscript{2} for 18–24 hours. McFarland suspensions of 1.5 were made using commercially prepared TSB (Becton, Dickinson, and Company) and used for downstream DNA extraction.

**Microarray Analysis**

DNA for the Checkpoints microarray was extracted from 200 µL of 1.5 McFarland suspension with the bioMérieux nucliSENS easyMAG nucleic acid extractor (bioMérieux) and stored at –20°C. To obtain quality DNA for sequencing, 600 µL of 1.5 McFarland suspension was first homogenized in BioLysing Matrix B Tubes (MP Biomedicals, Santa Ana, CA, USA) twice using the FastPrep System (MP Biomedicals, Santa Ana, CA, USA) with a 5-minute incubation on ice after each 30-second homogenization. Samples were centrifuged, supernatant was removed, and downstream sample preparation and DNA extraction was performed on the Qiagen EZ1 XL using the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany). Purified DNA was stored at –20°C until library preparation. The Check-Points Check-MDR CT103XL assay was performed according to manufacturer recommendations (Check-Points BV, Wageningen, the Netherlands). Briefly, 2 µL of extracted DNA was ligated to probes specific to beta-lactamase genes, amplified using multiplex polymerase chain reaction, hybridized to microarray tubes, and visualized and analyzed using the Check Points Tube Reader (Check-Points). Isolates were identified as carrying variants to ESBL, AmpC, and carbapenemase genes.

**Isolate Sequencing**

Isolates were prepared for sequencing on a single MiSeq instrument (Illumina, San Diego, CA, USA) using the NexteraXT DNA sample preparation kit (Illumina) and paired-end sequenced with the MiSeq Reagent Kit v3 150 bp (Illumina). The resulting sequence files (fastq format) were imported into SeqSphere +, version 4.1.9 (Ridom GmbH, Muenster, Germany). The SeqSphere + pipeline performed automated quality trimming and assembly. To determine the cgMLST gene set, a genome-wide comparison was done by the MLST target definer within SeqSphere + using the default parameters, as previously described by others [25]. The strain 042 (NC_017626.1) was used as the reference genome, and the task template included 2906 targets defined for cgMLST and 1855 accessory targets. Default Query Genome BLAST search settings were used. The resulting alleles for each gene were called and assigned by SeqSphere + as described by others [26]. The allelic profile was used to generate minimum spanning trees, and relatedness between the isolates was visualized. Draft assemblies were interrogated against CosmosISD antibiotic resistance and virulence gene databases using the BLASTN (version 2.7) tool. The best-matching genes were identified using a threshold of >98% identity and >60% alignment coverage of the reference gene. Finally, the best-identified antibiotic resistance and virulence genes were grouped into different antibiotic resistance
and virulence classes, respectively, and the sample-specific frequency of the identified genes belonging to each class was plotted on a heatmap. To find the plasmid in each of the samples, the PlasmidFinder tool [27] was adopted for searching the plasmid sequence against the E. coli plasmid reference database.

RESULTS

Case Presentation

The patient was an adolescent referred for treatment of relapsed Philadelphia Chromosome–positive acute lymphoblastic leukemia (Ph + ALL). He had first been diagnosed with and treated for ALL in November 2014 in Lebanon. Documentation of infectious complications during his initial treatment in Lebanon is unavailable. After relapse in May 2016, the patient was started on anticancer chemotherapy at St. Jude in July 2016.

The patient was enrolled in a relapsed ALL protocol and received 3 blocks of induction therapy, which resulted in prolonged profound neutropenia. A perianal surveillance culture (PASC) obtained before chemotherapy did not identify colonization with CRE. Two weeks after the first block of chemotherapy, he developed bacteremia due to ESBL-positive E. coli (sample 1), which was treated with a 14-day course of meropenem. A PASC obtained before the third block of chemotherapy and after the exposure to meropenem and levofloxacin (for prophylaxis, per protocol for relapsed leukemia treatment, while neutropenic) revealed colonization with a multidrug-resistant (MDR) E. coli. (sample 2). When this result returned, he was placed on contact isolation precautions. He remained in contact isolation until his death. Contact isolation precautions were maintained during all outpatient visits as well as during inpatient hospitalizations. After the third block of chemotherapy, he was diagnosed with a viridans group streptococci bacteremia. Two weeks later, he developed septic shock; MDR E. coli was isolated from a new perineal abscess (sample 3). During the first 3 cycles of chemotherapy, his absolute neutrophil count (ANC) remained <500 for 50 days, and he received a total of 88 days of cumulative antibiotic exposure, which included meropenem (33 days), cephalexins (including cefepime and ceftazidime; 19 days), metronidazole (10 days), aminoglycosides (19 days), and levofloxacin (7 days) (Figure 2).

The patient was then referred for a second allogeneic hematopoietic cell transplant (HCT). HCT conditioning included melphalan, thiotepa, fludarabine, and antithymocyte globulin. The PASC before transplant and 1 week after hospitalization confirmed colonization with MDR E. coli (samples 5, 6, 6B). He developed an ESBL-positive E. coli bloodstream infection on the day of HCT. Ten days after HCT, while on treatment with meropenem, he developed MDR E. coli sepsis associated with multisystem organ failure. His transplant never engrafted. Blood cultures remained positive despite antibiotic therapy (samples 15–31). Twelve days after onset of CRE bacteremia, a CT of the abdomen demonstrated changes suspicious for gangrenous cholecystitis. Due to his critical condition, he was not deemed a suitable candidate for surgical intervention. Removal of his central catheter was also considered but was not possible due to ongoing need for significant pressor support. Treatment for this infection initially included prolonged infusion meropenem in combination with amikacin, followed by colistin in combination with meropenem and amikacin, and finally triple therapy consisting of colistin, tigecycline, and amikacin. In total, he received 14 days of meropenem, 23 days of amikacin, 20 days of colistin, and 14 days of tigecycline. Granulocyte infusions were given with transient increase in absolute neutrophil count, but without clearance of peripheral blood cultures. The patient died 24 days later of multisystem organ failure.

Description of the Isolates

Of the 33 isolates, 23 (69.6%) were obtained from blood cultures, 9 (27.2%) were obtained from PASCs, and 1 (3%) was obtained from a wound culture (Figure 1). In 2 PASCs, 2 separate morphotypes of E. coli were isolated. The 33 isolates belonged to 1 of 4 identifiable sequence types (STs): ST-167 (22 isolates), ST-940 (3 isolates), ST-405 (3 isolates), and ST-648 (4 isolates); 1 isolate did not have an identifiable ST. Cultures of E. coli ST-167 were obtained from blood (18 cultures), PASC (3 cultures), and perianal abscess fluid (1 culture). The ST-940 and ST-405 isolates were each isolated from blood cultures (2 cultures) and PASC (1 culture). All 4 of the ST-648 isolates were from PASCs. The unknown sequence type was cultured from blood and was most similar genotypically to ST-167.

Phenotypic Resistance

Over time, phenotypic antibiotic resistance changed (Figure 1). Except for the first ST-940 isolate, which was susceptible to tobramycin, phenotypic resistance to penicillins, cephalexins, beta-lactam/beta-lactamase inhibitors, fluoroquinolones, trimethoprim-sulfamethoxazole, and aminoglycosides (except amikacin) was present in all isolates from all strains. ST-167 and ST-648 both developed phenotypic resistance to carbapenems over time; ST-940 and the unknown strain type were both resistant when first cultured. ST-405 never demonstrated carbapenem resistance. There was intermediate phenotypic resistance to meropenem detected in 1 isolate of ST-167, accompanied by resistance to doripenem without the blaNDM-5 gene present. A similar observation was made for an ST-648 isolate identified in PASC, which was found to be phenotypically resistant to both meropenem and doripenem, but without carriage of the blaNDM-5 gene. The amikacin minimum inhibitory concentration (MIC) varied over time for sequential isolates, notably for ST-167; 2 isolates from ST-167 eventually demonstrated phenotypic amikacin resistance. Although the measured MIC varied, phenotypic resistance to colistin or tigecycline was not detected.
Resistance Genes

Genes associated with resistance to multiple classes of antibiotics, including amino-glycosides, beta-lactams, fluoroquinolones, phenicols, sulphonamides, tetracyclines, and trimethoprim, were identified in these 33 E. coli isolates (Figure 2).

At least 1 gene associated with resistance to beta-lactam antibiotics was found in all isolates. *bla*<sub>OXA-1</sub> was the most common and was identified in 33 (100%) isolates. *bla*<sub>Cam-42</sub> was found in 30 (90.9%) isolates, *bla*<sub>CTX-M-15</sub> in 24 (72.7%) isolates, *bla*<sub>TEM-1β</sub> in 11 (33.3%) isolates, *bla*<sub>CTX-M-3</sub> and *bla*<sub>CTX-M-11</sub> in 8 (24.2%) isolates, and *bla*<sub>CTX-M-14</sub> in 3 (9.1%) isolates. Both *bla*<sub>CTX-M-3</sub> and *bla*<sub>CTX-M-11</sub> were present in the 8 (24.2%) isolates. ST-940 was the only strain type to not carry the *bla*<sub>CMY-42</sub> gene.

The *bla*<sub>NDM-5</sub> gene was found in 24 (72.7%) isolates. All ST-940 isolates carried the *bla*<sub>NDM-5</sub> gene, and ST-940 was the first sequence type to harbor the *bla*<sub>NDM-5</sub> gene. The ST-940 isolate also carried the *bla*<sub>NDM-5</sub> gene. The *bla*<sub>NDM-5</sub> gene was not detected in any isolates of ST-405 or -648.

Plasmids

Eighteen different plasmids were identified in the 33 E. coli isolates (Figure 2). The IncFIB plasmid was present in all 33 (100%) isolates, and IncFIA was present in 32 (96.9%) of the isolates. The p0111 plasmid was only present in the 3 ST-648 isolates. These were also the only isolates found to harbor the *bla*<sub>NDM-5</sub> gene. The IncFII plasmid was present in the first clinical isolate, which was ST-167; however, the *bla*<sub>NDM-5</sub> gene was not found, and there was no phenotypic resistance to carbapenems detected. The next E. coli isolated (ST-940) carried the IncFII
plasmid, harbored the bla<sub>NDM-5</sub> gene, and was phenotypically resistant to carbapenems. The IncX3 plasmid was first detected in the third ST-167 isolate, which was found to be phenotypically resistant to carbapenems. This isolate also carried the IncFII plasmid. IncX3 and phenotypic carbapenem resistance were detected in all subsequent ST-167 isolates; IncFII was present in 19 (86%) of these isolates. Subsequent ST-940 isolates were IncX3-positive but lacked IncFII, which had been present initially.

**DISCUSSION**

This unique study was undertaken to better understand the phenotypic and genotypic correlates of antibacterial drug resistance in a series of multidrug-resistant *E. coli* isolates obtained from a single patient. Ultimately, the patient died from complications of medically refractory septicemia from carbapenem-resistant *E. coli* ST-167 carrying bla<sub>NDM-5</sub> and...
the IncX3 plasmid after several months of anticancer chemotherapy and antibiotic exposure.

In our series, ST-167, ST-940, and the isolate with an unidentifiable sequence type were cultured from blood and carried the blaNDM-5 gene, conferring phenotypic carbapenem resistance. Our patient received initial treatment in Lebanon before being transferred for further care in the United States at St. Jude in Memphis, Tennessee. NDM-producing Enterobacteriaceae are most common in the Indian subcontinent and in portions of the Middle East [28]. NDM-producing Gram-negative bacteria have been identified in Lebanon [29, 30], though isolates harboring blaNDM-5 have not previously been documented there. NDM-5 has been found in an E. coli isolate from a patient receiving medical care in the United States [31].

The first sequence type resistant to all carbapenems was ST-940 (isolate 2), which carried the blaNDM-5 gene as well as the IncFII plasmid. Although the IncFII plasmid had been identified in the first E. coli isolate in this series (of ST-167), the blaNDM-5 gene and phenotypic carbapenem resistance were not present. Subsequently, following prolonged carbapenem exposure for treatment of a perianal abscess, an ST-167 isolate identified in a PASC was found to be carbapenem-resistant and carried the blaNDM-5 gene. This was the first isolate that carried the IncX3 plasmid. All subsequent ST-167 isolates harbored the IncX3 plasmid and the blaNDM-5 gene. Subsequent ST-940 isolates were also found to have the IncX3 plasmid as well as the blaNDM-5 gene; the IncFII plasmid was no longer present in these ST-940 isolates but was variably present in the ST-167 isolates. Based on these observed patterns, we infer that the IncII and IncX3 plasmids were responsible for carriage of the blaNDM-5 gene in these isolates. The IncX3 plasmid appeared to be more stable over time compared with the IncFII plasmid. Similar in vitro observations have been made regarding these plasmids in E. coli strains. The IncX3 plasmid is highly stable over time in serial passage studies and confers a fitness advantage in the presence of selective antibiotic pressure [32]. Conversely, the IncFII plasmid carrying the blaNDM-5 gene has previously been shown to be less stable in serial passage studies than other blaNDM-5—carrying plasmids [33].

The blaCTX-M-14 gene was present in only 3 isolates, all ST-648 from PASCs. ST-648 was again identified in a later PASC, but without the blaCTX-M-14 gene. The p0011 plasmid was present in the first 3 isolates harboring the blaCTX-M-14 gene, but not in the later ST-648 isolate. In contrast to blaCTX-M-15, the blaCTX-M-14 gene may have a more confined geographic distribution, including Spain and Japan [34]. blaCTX-M-14 has been associated with plasmid-mediated transmission, but potentially more commonly of the IncK plasmid [35]. To our knowledge, the p0011 plasmid has not previously been associated with the blaCTX-M-14 gene. However, other mechanisms of resistance, including chromosomal encoding of the blaCTX-M-14 gene, which has been described previously in E. coli isolates [36], as well as non-plasmid-mediated transposable genetic elements, may offer equally plausible explanations for this observation [34]. We observed 2 isolates with phenotypic resistance to carbapenems without carriage of a carbapenemase gene. We hypothesize that these findings are related to other mechanisms of acquired antibiotic resistance, most notably changes in cell membrane structure such as porins or drug efflux pumps [37].

Despite not having phenotypic resistance to colistin or tigecycline, the patient died despite apparently appropriate therapy with both antibiotics. The patient’s second HCT failed to engraft, which led to profound and prolonged neutropenia while attempting to control the septicemia. Granulocyte infusions produced short-lived increases in ANC without sterilization of peripheral blood cultures. We presume the treatment failure resulted from prolonged profound immune suppression, which highlights the importance of host immune competence in the treatment of severe infection.

This study was limited in that we did not include a comprehensive assessment of all mechanisms of antibacterial resistance. Additionally, the use only of short-read sequencing limited our ability to prove which plasmids carried each resistance locus in each isolate. Finally, it is possible that undetected small subpopulations of isolates in early cultures may have already carried the blaNDM-5 gene and could have been responsible for its emergence in clinical isolates. These weaknesses prevent a definitive conclusion that phenotypic carbapenem resistance was related to the blaNDM-5 gene carried on the IncX3 and IncFII plasmids in this isolate series and definitive differentiation between new acquisition and emergence of new characteristics. However, there is prior literature to support these observations. The future addition of long-read sequencing may also help confirm the hypothesis that plasmid-mediated transmission was responsible for blaNDM-5 transmission among the isolates identified in this individual patient. The reason for treatment failure in this case could not be determined, as the patient received appropriate dosing of antibiotics that appeared, based on phenotypic testing, to be adequate (as no phenotypic colistin or tigecycline resistance was detected in vitro). It is possible that other mechanisms of resistance not detected by phenotypic testing led to clinical failure; however, it is more likely that a high bacterial burden in the context of multisystem organ dysfunction following prolonged absolute neutropenia after failed HCT engraftment explains the clinical treatment failure witnessed in this case.

Despite these remaining questions, the clinical and microbiologic data presented here represent a novel, unusual, and longitudinal look at clinical isolates obtained over the course of several months from a single immunocompromised pediatric patient.

**CONCLUSIONS**

Colonization and invasive infection with multidrug-resistant Gram-negative bacteria occur in patients receiving anticancer chemotherapy. This study highlights the longitudinal changes...
in the genotypic correlates of phenotypic resistance that may potentially develop in patients exposed to multiple classes of antibiotics for prolonged periods of time. In this case, 2 E. coli sequence types causing invasive infection and harboring the blaNDM-5 gene, presumably encoded on the IncFII plasmid and the IncFII plasmid, were identified. Multiple antibiotic resistance genes causing phenotypic resistance to nearly all classes of antibiotics were also identified in these isolates. Despite apparently appropriate antimicrobial therapy on the basis of phenotypic and genotypic susceptibility testing that suggested susceptibility to colistin and tigecycline, the outcome in this case demonstrates that adequate neutrophil number and function are likely required for recovery from severe invasive bacterial infection.

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