A Clinical Extensively-Drug Resistant (XDR) Escherichia coli and Role of Its β-Lactamase Genes

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An extensively-drug resistant (XDR) Escherichia coli W60 was isolated from the urine sample of a patient. The genetic basis for its XDR phenotype was investigated, particularly the basis for its resistance toward β-lactam/BLI (β-Lactamase Inhibitor) combinations. Following determination of the XDR phenotype, third generation genomic sequencing was performed to identify genetic structures in E. coli W60. Further cloning analysis was performed to identify determinants of β-lactam/BLI combination resistance. It was found that E. coli W60 is resistant to nearly all of the tested antibiotics including all commonly used β-lactam/BLI combinations. Analysis of the genomic structures in E. coli W60 showed two novel transferable plasmids are responsible for the resistance phenotypes. Further genetic analysis showed bla<sub>NDM-5</sub> leads to high resistance to β-lactam/BLI combinations, which was enhanced by co-expressing bla<sub>MBL</sub>. pECW602 harbors a truncated bla<sub>TEM</sub> that is not functional due to the loss of the N-terminal signal peptide coding region. Research performed in this work leads to several significant conclusions: the XDR phenotype of E. coli W60 can be attributed to the presence of transferable multidrug resistance plasmids; NDM-5 confers high resistance to β-lactam/BLI combinations; co-expression of bla<sub>MBL</sub> enhances resistance caused by NDM-5; the signal peptides of TEM type β-lactamases are essential for their secretion and function. Findings of this work show the danger of transferable multidrug resistance plasmids and metallo-β-lactamases, both of which should be given more attention in the analysis and treatment of multidrug resistant pathogens.

Keywords: antimicrobial resistance, extensively drug resistance, Escherichia coli, β-lactamase, β-lactamase inhibitor, multidrug resistant plasmid

INTRODUCTION

Escherichia coli is one of the most common clinical bacteria, of which many isolates are pathogenic. E. coli can cause enteritis, urinary tract infection and many other diseases, leading to significant morbidity and mortality (Russo, 2003). In the past few decades, following the increased use of antibiotics, the resistance of clinical E. coli to antibiotics rises, making it difficult for treatment. In particular, many E. coli strains developed multi-, extensively- or pan-drug resistance (MDR, XDR, or PDR) phenotypes, posing a great challenge to infection treatment (Magiorakos et al., 2012;
Therapeutic options to these antibiotic resistant *E. coli* strains include last-resort antibiotics such as carbapenems and tigecycline, along with those still under development (Karaiskos and Giamarelou, 2014).

β-lactam antibiotics are the most widely used antibiotics in the treatment of bacterial infection. However, antibiotic resistant bacteria often produce β-lactamase, inactivating β-lactams. To address this, β-lactamase inhibitors (BLI) were developed to reenable the use of β-lactam antibiotics. Today, the most commonly used BLIs include tazobactam, clavulanate, sulbactam, and avibactam (Ehmann et al., 2012). Effective β-lactam/BLI combinations include piperacillin–tazobactam, amoxicillin–clavulanate, ticarcillin–clavulanate, ampicillin–sulfactam, and ceftazidime–avibactam (Tooke et al., 2019). The use of these combinations has replaced other last-resort antibiotics to become the most popular option in treating β-lactam resistant bacteria infections.

Based on sequence homology, β-lactamases are divided into four classes A, B, C, and D (Ambler, 1980). Despite differing by their mechanisms, all β-lactamases deactivate β-lactams by hydrolytic opening of the β-lactam ring. TEM is one of the most prevalent and typical class A β-lactamases. It was discovered in as early as 1965 when a plasmid harboring *bla*<sub>TEM−1</sub> was found (Datta and Kontomichalou, 1965). A large number of TEM variants have been identified to date that mediate resistance to most β-lactams (Paterson and Bonomo, 2005). Among β-lactamases, metallo-β-lactamases (MBL) such as New Delhi Metallo-β-Lactamases (NDMs) rank among the most detrimental for their ability to lead to resistance against not only β-lactams but also carbapenems, and unlike other serine β-lactamases that exploit a serine active site for hydrolysis, MBLs rely on zinc ions in their active site to facilitate hydrolytic reaction (Bebrone, 2007). This different mechanism of MBLs on β-lactam hydrolysis leads to the consensus that BLIs are ineffective against MBLs. However, experimental evidence for whether all common β-lactam/BLI combinations are ineffective against MBLs is still needed. Statistics in recent years show that the prevalence of NDMs is increasing worldwide (Bush, 2018). Since the discovery of NDM-1, a total of 24 different NDM variants have been identified, the coding genes of which (*bla<sub>NDM</sub>*) are hosted by a variety of bacteria, predominantly *Enterobacteriaceae* followed by other pathogenic bacteria such as *Acinetobacter spp.* (Wu et al., 2019). Transferable plasmids play an important role in the dissemination of *bla<sub>NDM</sub>* by hosting and spreading of the gene through horizontal gene transfer (HGT) (Adamczuk et al., 2015; Wailan et al., 2015; Sugawara et al., 2017; Liu et al., 2019). This has led to the wide distribution of NDM worldwide, posing a severe threat to public health (Dortet et al., 2014; Dadashi et al., 2019; Wu et al., 2019).

In this study, an extensively-drug resistant (XDR) *E. coli* W60 was isolated from the urine sample of a patient following his bladder tumor surgery. This strain was found resistant to all tested antibiotics except tigecycline. In particular, *E. coli* W60 was found resistant to all commonly available β-lactam/BLI combinations. Whole-genome sequencing revealed that W60 hosts two novel transferable plasmids, the IncFIB-type plasmid pECW601 and the IncFII-type plasmid pECW602, and showed that the two multidrug resistance plasmids carry the main genetic determinants of antimicrobial resistance for *E. coli* W60. pECW601 contains the *bla<sub>NDM−5</sub>* gene, which encodes the metallo-β-lactamase NDM-5. pECW602 contains a truncated *bla<sub>TEM</sub>* gene. Further genetic analysis provides experimental evidence that NDM5 leads to resistance to β-lactam/BLI combinations and that the N-terminal 28 amino acids containing signal peptide appear essential for the functionality of TEM. This work provides a detailed insight into the resistance mechanisms of a clinical XDR *E. coli* strain, and provides evidence on the role of β-lactamase genes. In particular, this work demonstrates MBLs indeed renders BLIs ineffective, further stressing the danger of these now widespread β-lactamase genes.

**Materials and Methods**

**Bacterial Strains**

The strain *E. coli* W60 used in this study was isolated from a urine sample of a patient from the Second Hospital of Shandong University who had an infection after bladder tumor resection. The preliminary identification results of the hospital showed that the bacterium was resistant to multiple antibiotics, so further research was needed to develop a treatment plan for the patient. The handling and experiments of the studied bacteria followed security and safety guidelines of Shandong University and the Second Hospital of Shandong University. All procedures were approved by the Scientific Ethics Committee of the Second Hospital of Shandong University with Approval No. KYLL-2020(LW)-044.

**Susceptibility Tests**

Drug susceptibility testing was carried out by the disk diffusion method, and the standard for inhibition zones followed the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2018b). Minimum Inhibition Concentrations (MICs) for all antibiotics (ampicillin, amoxicillin-clavulanate, ceftazidime-avibactam, piperacillin-tazobactam, ampicillin-sulbactam, ticarcillin-clavulanate, cefoperazone, cefotaxime, ceftazidime, cefoxitin, cefepime, cefazolin, imipenem, meropenem, kanamycin, ciprofloxacin, gatifloxacin, nalidixic acid, chloramphenicol, trimethoprim, and tetracycline) but tigecycline was determined with the agar dilution method following CLSI guidelines (Clinical and Laboratory Standards Institute, 2019). For tigecycline, MIC was determined with the broth microdilution method following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Marchaim et al., 2014). *E. coli* ATCC 25922 was used as the control strain for most antibiotics. *E. coli* ATCC 27853 was used as the control strain for carbapenems. For resistance against β-lactam/BLI combinations, *E. coli* ATCC 35218 was used as the control strain as instructed by the CLSI guidelines (Clinical and Laboratory Standards Institute, 2018a,b, 2019).
Whole Genome Sequencing and Sequence Analyses

The genomic DNA of *E. coli* W60 was extracted with the SDS method (Natarajan et al., 2016). Libraries for single-molecule real-time (SMRT) sequencing was constructed with an insert size of 10 kb using the SMRTbell™ Template kit, version 1.0. Sequencing libraries were generated using NEBNext™ Ultra® DNA Library Prep Kit for Illumina (NEB, United States) following manufacturer’s recommendations and index codes were added to attribute sequences to each sample. The whole genome of *E. coli* W60 was sequenced using PacBio Sequel platform and Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd., SMRT Link v5.1.0 software was used for read assembly (Ardui et al., 2018; Reiner et al., 2018), which was further optimized by the Arrow software (part of SMRT Link v5.1.0). General function annotation databases GO (Gene Ontology, 2015), KEGG (Kanehisa and Goto, 2000), COG (Natale et al., 2000), NR (Li et al., 2002), Pfam (El-Gebali et al., 2019), TCDB (Saier et al., 2006), and Swiss-Prot (Boeckmann et al., 2003) were used for functional annotation of genes, and the Comprehensive Antibiotic Resistance Database (CARD) was used to manually annotate antimicrobial resistance genes (ARGs) (Jia et al., 2017). PlasmidFinder 2.1 was used to analyze plasmid types (Carattoli et al., 2014).

Mating Experiment

The ability of plasmids to transfer was determined by mating experiment, and the *E. coli* J53 was used as recipient strain. Transconjugants were selected on LB agar supplemented with different antibiotic agents: pECW601 was selected by LB agar containing NaN_3 with different antibiotic agents: pECW601 was selected by Transconjugants were selected on LB agar supplemented and following manufacturer’s recommendations and index codes were added to attribute sequences to each sample. The whole genome of *E. coli* W60 was sequenced using PacBio Sequel platform and Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd., SMRT Link v5.1.0 software was used for read assembly (Ardui et al., 2018; Reiner et al., 2018), which was further optimized by the Arrow software (part of SMRT Link v5.1.0). General function annotation databases GO (Gene Ontology, 2015), KEGG (Kanehisa and Goto, 2000), COG (Natale et al., 2000), NR (Li et al., 2002), Pfam (El-Gebali et al., 2019), TCDB (Saier et al., 2006), and Swiss-Prot (Boeckmann et al., 2003) were used for functional annotation of genes, and the Comprehensive Antibiotic Resistance Database (CARD) was used to manually annotate antimicrobial resistance genes (ARGs) (Jia et al., 2017). PlasmidFinder 2.1 was used to analyze plasmid types (Carattoli et al., 2014).

Cloning of bla_NDM–5 and bla_TEM–W60

The bla_NDM–5 and bla_TEM–W60 genes were amplified and cloned into pBCKS(+) genes, followed by sequencing for final confirmation (Supplementary Figure S1). The sequences of primers for transconjugant confirmation are shown in Supplementary Table S1.

Accession Numbers

The nucleotide sequences of *E. coli* W60 genome and its plasmids can be found on NCBI under accession numbers CP058342, CP058343 and CP058344.

Bioinformatics

SerotypeFinder 2.0^1 was used for serotype prediction (Joensen et al., 2015), followed by serum aggregation reaction experiment for confirmation. Prediction of signal peptides was performed using SignalP 5.0^2 (Armenteros et al., 2019). Sequence alignment was performed using ESPript 3.0^3 with the Maximum Likelihood method and Poisson correction model (Robert and Gouet, 2014). Phylogenetic analysis was done by MEGA-X (Kumar et al., 2018). Protein structure analysis was performed using PyMol (Seeliger and de Groot, 2010).

Ethics

All experiments in this work were performed adhering to the Declaration of Helsinki and were approved by the Scientific Ethics Committee of the Second Hospital of Shandong University with Approval No. KYLL-2020(LW)-044.

RESULTS

Isolation and Resistance Properties of a Clinical XDR *E. coli* W60 Strain

*Escherichia coli* strain W60 was isolated from the urine sample of a bladder tumor patient from the Second Hospital of Shandong University. *In silico* prediction of the serotype was performed for *E. coli* W60, suggesting it was either serotype O101, O8, or H9. Further serum aggregation reaction assay was performed, showing that *E. coli* W60 belongs to serotype O101 (Supplementary Figure S2). O101 is a common enterotoxigenic *E. coli* (ETEC) serotype originated from pigs and cattle (Staaf et al., 1997). The antibiotic resistance profiles were determined for this strain by testing its resistance against major classes of antibiotics including β-lactams, quinolones, carbapenems, aminoglycosides, chloramphenicol, trimethoprim, tigecycline, macrolides, and polymyxins. *E. coli* W60 was found resistant to all antibiotics tested except for tigecycline (Table 1). Of particular interest, *E. coli* W60 is highly resistant to all commonly available β-lactam/BLI combinations with MIC values much higher than the resistance breakpoint.

Genome Characteristics and Genotypic Basis for Antibiotic Resistance of *E. coli* W60

Whole genome sequencing was performed with PacBio and Illumina sequencing on *E. coli* W60. *E. coli* W60 has a chromosome at the size of 4,808,792 bp and GC content of 50.8% (Figure 1). Two circular plasmids were identified from *E. coli* W60, respectively, named pECW601 and pECW602. BLAST analysis of both plasmids found no known plasmids that share both high sequence identity and coverage. pECW601 has a size of 140,410 bp. Its closest known relative is a *E. coli*-harboring unnamed plasmid from that has a size of

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^1<https://cge.cbs.dtu.dk/services/SerotypeFinder/>

^2<http://www.cbs.dtu.dk/services/SignalP/>

^3<http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>
286,854 bp (GenBank accession number CP025329.1). Plasmid replication analysis showed that pECW601 is an IncFIB type plasmid. Comparison of pECW601 and CP025329.1 show that both plasmids share similar conjugation genes, iron oxidase related genes and IS26 transposase genes but their MDR regions are fundamentally different (Figure 2A). This comparison suggests that the two plasmids have little in common, along with other minor different features between the two plasmids (Figure 2B). This comparison suggests that pECW601 is a new multidrug resistance plasmid.

Analysis of the genomic sequence of E. coli W60 reveals resistance determinants putatively responsible for the antimicrobial resistance phenotype of this strain. Two AmpC-type and one AmpH-type β-lactamas are encoded by the chromosome that are potentially responsible for resistance against β-lactams (Supplementary Table S3). The chromosome harbors a gyrA gene that encodes a D87N/S83L variant and a pacC gene that encodes a S80I variant (Supplementary Table S3). Both variants are responsible for resistance to quinolones (Yoshida et al., 1990). Respectively, 6 and 8 antimicrobial resistance genes (ARGs) were found on pECW601 and pECW602 (Table 2 and Supplementary Tables S4, S5). ARGs responsible for the resistance to aminoglycosides, β-lactams and sulfonamides are found on both plasmids. pECW601 harbors ARGs responsible for the resistance to trimethoprim, tetracycline and glycopeptides, while pECW602 harbors ARGs responsible for the resistance to chloramphenicol and fosfomycin. Therefore, genetic features were found for all the major resistant antibiotic classes investigated in the antimicrobial resistance phenotype analysis (Table 1), and the two multidrug resistance plasmids account for the resistance to most of these antibiotics.

Genetic analysis of the E. coli W60 genome leads to two interesting observations: E. coli W60 is resistant to all the β-lactam/BLI combinations tested, while the genetic basis for this observation remains unclear; pECW602 harbors a truncated version of blaTEM−1, leading us to wonder its role in mediating β-lactam resistance. Both these observations were further investigated in this work.

Transferability of pECW601, pECW602, and β-Lactam/BLI Combination Resistance Phenotypes

Conjugation assays between E. coli W60 and the recipient E. coli J53 strain show that both pECW601 and pECW602 are transferable plasmids. Analysis of the antimicrobial resistance phenotypes of both transconjugants leads to the finding that the pECW601-harboring transconjugant showed nearly the same high level of resistance to β-lactam/BLI combination as E. coli W60 (Table 3). Considering the only β-lactamase-coding gene on pECW601 is blaNDM−5, a hypothesis is raised that blaNDM−5 can lead to high level resistance to β-lactam/BLI combinations in E. coli.

β-Lactam/BLI Combination Resistance of blaNDM−5-Harboring E. coli Strain

To further explore the role of blaNDM−5 in the resistance of β-lactam related antibiotics, we cloned blaNDM−5 into pBCKS(+) plasmid and transformed the resulting construct to E. coli DH5α. An empty pBCKS(+) vector does not increase the resistance of E. coli DH5α to β-lactams or β-lactam/BLI combinations. However, blaNDM−5-containing pBCKS(+) increased the resistance of E. coli DH5α to β-lactams by 4–256-fold, and increased the resistance of E. coli DH5α to β-lactam/BLI combinations by 8–1,024-fold (Table 4). The finding that blaNDM−5 leads to high resistance to β-lactam/BLI combinations in E. coli DH5α, together with the observation that pECW601-containing E. coli J53 transconjugant is highly resistant to β-lactam/BLI combinations, experimentally confirm that blaNDM−5 leads to resistance to β-lactam/BLI combinations, and is the reason for the high level of resistance to β-lactam/BLI combinations of E. coli W60.

### Table 1 | Antimicrobial resistance of Escherichia coli W60.

| Antibiotic class | Antibiotics | Antimicrobial resistance1 | MIC (mg/L) |
|------------------|-------------|---------------------------|------------|
|                  |             | Inhibition zone (mm)      |            |
| β-lactam         | Ampicillin (AMP) | R(0)                      | R(>512)    |
|                  | Amoxicillin-clavulinate (AMC) | R(0) | R(64/32) |
|                  | Cefazidime-avibactam (CAZ-AVI) | R(13) | R(>512/4) |
|                  | Piperacillin-tazobactam (TZP) | R(0) | R(>512/4) |
|                  | Ampicillin-sulbactam (SAM) | R(0) | R(>256/128) |
|                  | Ticarcillin-clavulinate (TIM) | R(7) | R(>25B/2) |
|                  | Cefoperazone (CIP) | R(0) | R(>512) |
|                  | Cefotaxime (CTX) | R(0) | R(>512) |
|                  | Cefazidime (CAZ) | R(0) | R(>512) |
|                  | Cefoxitin (FOX) | R(0) | R(>512) |
|                  | Cefepime (FEF) | R(0) | R(512) |
|                  | Cefazolin (CF2) | R(0) | R(>512) |
| Carbapenem       | Imipenem (IPM) | R(14) | I(2) |
|                  | Meropenem (MEM) | R(0) | R(4) |
| Aminoglycoside   | Kanamycin (KAN) | R(12) | R(>64) |
| Quinolone        | Ciprofloxacin (CIP) | R(0) | R(32) |
|                  | Gatifloxacin (GAT) | R(0) | R(8) |
|                  | Nalidixic acid (NAL) | R(0) | R(512) |
| Phenicol         | Chloramphenicol (CHL) | R(8) | R(256) |
| Diaminopimydine  | Trimethoprim (TMP) | R(0) | R(>512) |
| Glycopeptide     | Tigecycline (TGC) | S(18) | S(0,5) |
| Tetracycline     | Tetracycline (TET) | R(10) | R(128) |

1, R, resistant; I, intermediate; S, sensitive.
A bleomycin resistance-conferring bleMBL gene is located downstream of blaNDM-5 under the control of the same promoter on pECW601. The potential impact of this gene on the function of blaNDM-5 was probed by cloning both blaNDM-5 and bleMBL to pBCKS(+), transforming the construct to E. coli DH5α, and comparing the resistance of the transformant to β-lactams and β-lactam/BLI combinations (Table 4). Increased resistance, although by only two–fourfold, was found for ampicillin, ceftazidime, cefoxitin, and ampicillin-sulbactam. This finding suggests a potential function of bleMBL in enhancing the role of blaNDM-5 in β-lactam resistance.

**Presence and Function of a Truncated blaTEM Gene on pECW602**

A truncated blaTEM gene that encodes a TEM β-lactamase missing the N-terminal 28 amino acids, termed blaTEM−W60, was found on pECW602. Sequence comparison of TEM-W60
with TEM-1 and TEM-2 showed that other than missing the N-terminal 28 amino acids, TEM-W60 also has two mutations V29L and L38A (Figure 3). Phylogenetic analysis suggest that TEM-W60 does not cluster with known TEM β-lactamases (Supplementary Figure S3). The function of blaTEM−W60 was analyzed by cloning it into pBCKS(+), transforming the construct into E. coli DH5α, and analyzing the resistance of the transformant to β-lactams and β-lactam/BLI combinations (Table 5). It was found that blaTEM−W60 has little impact on resistance to β-lactams and β-lactam/BLI combinations. Structural analysis showed the two mutated amino acids reside on the N-terminal α-helix of TEM β-lactamase, far away from the active site and all other sites important for the activity of β-lactamase (Figure 4) (Jelsch et al., 1993; Minasov et al., 2002). It is therefore unlikely that these two substitutions significantly impact β-lactamase activity. Further sequence analysis predicted that the first 23 amino acids of TEM-1 form the signal peptide that is critical for secretion (Supplementary Figure S4). As

FIGURE 2 | Linear schematic of sequence comparison between plasmids found in this work and their closest relatives. (A) Comparison between pECW601 and Escherichia coli strain ExPEC XM plasmid unnamed; (B) comparison between pECW602 and E. coli strain Eco889 plasmid pECO-fce. Different colors represent gene clusters with different functions. Arrows indicate the direction of genes. The light gray indicates high similarity between sequences.
Fosfomycin
fosA3
Tetracycline
Phenicols
floR sul1 sul2
Sulfonamides
dfrA12
Diaminopyrimidine

β
inhibit

> CTX

DISCUSSION

MDR and XDR E. coli and β-Lactamases

β-lactamases are extracellular or periplasmic proteins and need to be secreted for their function (Livermore, 1995), it strongly suggests that the loss of function for TEM-W60 is due to the loss of a signal peptide and subsequent inability for secretion.

In this study, we identified an XDR E. coli W60 strain in the urine sample of a patient with postoperative infection. E. coli W60 belongs to serotype O101 after analysis by agglutination reaction assay. Unlike the enterohaemorrhagic E. coli O157, which is highly pathogenic and virulent, although serotype O101 has been shown to be related to diarrhea and urinary tract infection (Mandal et al., 2001; Sun et al., 2019), it is only a risk factor and has no direct connection with human disease. This strain shows resistance to almost all common antibiotic agents, including β-lactams, aminoglycosides, carbapenems, quinolones and etc. Whole genome sequencing shows that E. coli W60 contains two new multidrug resistance plasmids, pECW601 and pECW602. Analysis of the ARGs harbored by these two plasmids suggested that these plasmids are the primary reason for the extensively-drug resistance phenotype, while resistance genes located into the chromosomes are presumably responsible for only β-lactam and quinolone resistance. Further conjugation assays show both plasmids are transferable. These findings again confirm the danger of multidrug resistance plasmids: the concentration of different multidrug resistance plasmids into one bacterium can lead to the generation of highly resistant pathogens as demonstrated in this work and the work of others (Zhao et al., 2010; Guo et al., 2017; Li et al., 2019). Because transfer of plasmids is way more efficient than the evolution of new antibiotic resistance genotypes, we suspect this is the primary route for the generation of extensively- or pan-drug resistant pathogens. The danger of multidrug resistance plasmids should therefore be given high attention. The fact that both multidrug resistance plasmids found in this work are new rings a bell for us: there could be many more such plasmids out there waiting to be found. We therefore would like to call upon scientists and doctors in the field of antimicrobial resistance to perform more surveillance studies on clinical multidrug resistance plasmids and have a better understanding on the types and structures of these mobile genetic elements.

A particularly interesting and troubling feature of E. coli W60 is that it is resistant to all the β-lactam/BLI combinations tested. Further genetic analysis shows that the blaNDM−5 gene harbored by pECW601 is the reason for the resistance of β-lactam/BLI combinations. β-lactams are by far the most important antibiotics for their high efficiency to both Gram-positive and Gram-negative bacteria, and for their relatively better safety to human in comparison with other more recently introduced last-resort antibiotics (Bush and Bradford, 2016). Therefore, reusing β-lactams that already develop widespread resistance by combining BLIs is a great strategy and the first choice when treating infections of β-lactam resistant pathogens. It has been long suspected that this strategy does not work well with MBLs for their different resistance mechanism (Bebrone, 2007). This work provides solid microbiological and genetic evidence that NDM-5 can lead to high β-lactam/BLI resistance against all commonly available β-lactam/BLI combinations, and it is already causing strong resistance in a clinical pathogen. The mechanism behind this phenotype is likely that these commonly used BLIs (tazobactam, clavulanate, sulbactam, and avibactam) are serine-β-lactamases inhibitors that inhibit the serine active site of

### TABLE 2 | Presence of ARGs on plasmids.

| Targeted antibiotic class | pECW601 | pECW602 |
|---------------------------|---------|---------|
| Aminoglycosides            | aadA2   | APH(3’)-Ia |
|                           |         | APH(3’)-Ib |
|                           |         | APH(6’)-Id |
| β-lactam                  | blaTEM-5 | b12TX-M-55 |
|                           |         | b12TEM-W60 |
| Diaminopyrimidine          | dfrA12  |          |
| Sulfonylides               | su1     | su2     |
| Phenicols                 | flbR    |          |
| Tetracyclines              | tetA    | fomA3   |
| Fosfomycin                 |         | blsVEBL |

### TABLE 3 | Antibiotic sensitivity of pECW601 and pECW602-containing transconjugants.

| Antibiotics | W60† (mg/L) | JS3‡ (mg/L) | JS3/pECW601¶ (mg/L) | JS3/pECW602∥ (mg/L) |
|-------------|-------------|-------------|---------------------|--------------------|
| AMP         | >512        | 2           | >512                | 128                |
| CFP         | >512<0.125  | 16          | >512<0.125          | 16                 |
| CTX         | >512<0.125  | 12          | >512<0.125          | 12                 |
| CAZ         | >512        | 1           | >512                | 16                 |
| FOX         | >512        | 1           | >512                | 16                 |
| FEP         | 512<0.125   | 1           | >512                | 16                 |
| CFZ         | 64/32       | 4/2         | 64/32               | 4/2                |
| AMC         | 64/32       | 4/2         | 64/32               | 4/2                |
| CAZ-AVI     | >512/4      | 0.25/4      | >256/4              | 0.125/4            |
| TZP         | >512/4      | 4/4         | >256/4              | 4/4                |
| SAM         | >256/128    | 4/2         | >256/128            | 16/8               |
| TIM         | >256/2      | 2/2         | >256/2              | 16/2               |

†AMP, ampicillin; AMC, amoxicillin-clavulanate; CAZ-AVI, ceftazidime-avibactam; TZR, piperacillin-tazobactam; SAM, ampicillin-sulbactam; TIM, ticarcillin-clavulanate; CFP, ceftoperazone; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; CFZ, cefazolin.
‡W60, E. coli W60.
§JS3, E. coli JS3.
¶JS3/pECW601, E. coli JS3 transconjugant harboring pECW601.
∥JS3/pECW602, E. coli JS3 transconjugant harboring pECW602.

β-lactamase resistance plasmids found in this work are new rings a bell for us: there could be many more such plasmids out there waiting to be found. We therefore would like to call upon scientists and doctors in the field of antimicrobial resistance to perform more surveillance studies on clinical multidrug resistance plasmids and have a better understanding on the types and structures of these mobile genetic elements.

A particularly interesting and troubling feature of E. coli W60 is that it is resistant to all the β-lactam/BLI combinations tested. Further genetic analysis shows that the blaNDM−5 gene harbored by pECW601 is the reason for the resistance of β-lactam/BLI combinations. β-lactams are by far the most important antibiotics for their high efficiency to both Gram-positive and Gram-negative bacteria, and for their relatively better safety to human in comparison with other more recently introduced last-resort antibiotics (Bush and Bradford, 2016). Therefore, reusing β-lactams that already develop widespread resistance by combining BLIs is a great strategy and the first choice when treating infections of β-lactam resistant pathogens. It has been long suspected that this strategy does not work well with MBLs for their different resistance mechanism (Bebrone, 2007). This work provides solid microbiological and genetic evidence that NDM-5 can lead to high β-lactam/BLI resistance against all commonly available β-lactam/BLI combinations, and it is already causing strong resistance in a clinical pathogen. The mechanism behind this phenotype is likely that these commonly used BLIs (tazobactam, clavulanate, sulbactam, and avibactam) are serine-β-lactamases inhibitors that inhibit the serine active site of
TABLE 4 | Antibiotic sensitivity of \( \text{bla}_{\text{TEM-5}} \) and \( \text{bla}_{\text{NDM-5}/\text{BLE}} \)-harboring strains.

| Antibiotics | DH5α (mg/L) | DH5α/pBCKS (+) (mg/L) | DH5α/pBCKS (+)-NDM5 (mg/L) | DH5α/pBCKS (+)-NDM5+BLE (mg/L) |
|-------------|-------------|------------------------|-----------------------------|-------------------------------|
| AMP         | 1           | 2                      | 64                          | 128                           |
| CFP         | <0.125      | <0.125                 | 8                           | 8                             |
| CTX         | <0.125      | <0.125                 | 32                          | 32                            |
| CAZ         | <0.125      | <0.125                 | 64                          | 128                           |
| FOX         | 16          | 16                     | 64                          | 256                           |
| FEP         | <0.125      | <0.125                 | 1                           | 1                             |
| CFZ         | 2           | 1                      | 256                         | 256                           |
| AMC         | 2/1         | 4/2                    | 64/32                       | 64/32                         |
| CAZ-AVI     | <0.125/4    | <0.125/4               | 128/4                       | 128/4                         |
| TIP         | 2/4         | 4/4                    | 16/4                        | 16/4                          |
| SAM         | 2/1         | 2/1                    | 64/32                       | 128/64                        |
| TIM         | 1/2         | 2/2                    | 256/2                       | 256/2                         |

1. AMP, ampicillin; AMC, amoxicillin-clavulanate; CAZ-AVI, ceftazidime-avibactam; T2P, piperacillin-tazobactam; SAM, ampicillin-sulbactam; TIM, ticarcillin-clavulanate; CFP, cefoperazone; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; CFZ, cefazolin.
2. DH5α, E. coli DH5α.
3. DH5α/pBCKS (+), E. coli DH5α containing empty pBCKS (+) vector.
4. DH5α/pBCKS (+)-NDM5, \( \text{bla}_{\text{TEM-5}} \)-harboring E. coli DH5α strain.
5. DH5α/pBCKS (+)-NDM5 + BLE, \( \text{bla}_{\text{NDM-5}/\text{BLE}} \)-harboring E. coli DH5α strain.

FIGURE 3 | Multiple sequence alignment of TEM-W60, TEM1, and TEM2. Red box indicates the missing N-terminal region for TEM-W60. Blue box indicates mutations.
β-lactamase, but are ineffective against the zinc ion-containing active sites for MBLs (Docquier and Mangani, 2018). It needs to be pointed out that the β-lactamases besides blaNDM–5 encoded by E. coli W60 also contribute to this β-lactam/BLI combination resistance phenotype, as E. coli DH5α harboring only blaNDM–5 showed a weaker resistance in comparison with E. coli W60 or pECW601-containing E. coli J53. The finding that blaNDM–5 confers widespread resistance to β-lactam/BLI combinations again confirms the danger of MBLs, as they render β-lactams, β-lactam/BLIs, and carbapenems (in other words all β-lactam related antibiotics) ineffective. Susceptible testing showed that E. coli DH5α containing both blaNDM–5 and blEMBL is slightly more resistant to ampicillin, cefazidime, cefoxitin, and ampicillin-sulbactam than E. coli DH5α containing only blaNDM–5 (Table 4). blEMBL encodes a BRP protein that exerts resistance to bleomycin by specifically binding to bleomycin family antibiotics, but does not appear to interact with β-lactams or β-lactamases. Previous research reported that blEMBL and blaNDM genes are often co-transcribed, and suggested that BRP influences E. coli mutation rates to stabilize NDM resistance traits (Dortet et al., 2012). An earlier report suggested that the existence of bleomycin resistance phenotype protects bacteria from external DNA damage through the DNA repair system, thereby conferring better fitness of bacteria and facilitating the inheritance of genetic characteristics (Blot et al., 1991). The enhancement of β-lactam resistance by blEMBL found in this work could be for the same reason, and this new role of blEMBL in β-lactam resistance makes more sense for the frequently observed co-transcription of blEMBL and blaNDM–5.

A survey of other β-lactamase genes leads to the finding of a truncated blaTEM gene on pECW602 that encode a TEM β-lactamase with 28 amino acids deleted at the N-terminus. This gene was found unfunctional presumably due to the loss of the signal peptide coding region in comparison with other blaTEM genes. This finding confirms the importance of the signal peptide for TEM β-lactamase, understandably for its critical role in β-lactamase secretion.

**CONCLUSION**

Combining genomic, microbiological and genetic approaches, we identified the genetic basis for the extensively-drug resistance phenotype of the clinical E. coli W60 strain. Two new conjugative multi-resistance plasmids pECW601 and pECW602 were found in E. coli W60, and were confirmed to be the primary determinants of the extensively drug resistance phenotype. Resistance phenotype analysis showed that E. coli W60 is resistant to all commonly available β-lactam/BLI combinations. Further genetic analysis showed that the NDM-5 β-lactamase coded on pECW601 is responsible for this phenotype, which is further enhanced by co-expressing BRP. A new unfunctional

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**TABLE 5 | Antibiotic sensitivity of blaTEM-W60-harboring strains.**

| Antibiotics1 | DH5α2 (mg/L) | DH5α/pBCKS(+3) (mg/L) | DH5α/pBCKS(+)-TEM-W604 (mg/L) |
|--------------|--------------|------------------------|--------------------------------|
| AMP          | 1            | 2                      | 2                              |
| CFP          | <0.125       | <0.125                 | <0.125                         |
| CTX          | <0.125       | <0.125                 | <0.125                         |
| CAZ          | <0.125       | <0.125                 | <0.125                         |
| FOX          | 16           | 16                     | 32                             |
| FEP          | <0.125       | <0.125                 | <0.125                         |
| CFZ          | 2            | 1                      | 1                              |
| AMC          | 2/1          | 4/2                    | 4/2                            |
| CAZ-AVI      | <0.125/4     | <0.125/4               | <0.125/4                       |
| TZP          | 2/4          | 4/4                    | 4/4                            |
| SAM          | 2/1          | 2/1                    | 2/1                            |
| TIM          | 1/2          | 2/2                    | 2/2                            |

1 AMP, ampicillin; AMC, amoxicillin-clavulanate; CAZ-AVI, cefazidime-avibactam; T Zap, piperacillin-tazobactam; SAM, ampicillin-sulbactam; TIM, ticarcillin-clavulanate; CFP, cefoperazone; CTX, cefotaxime; CAZ, cefazidime; FOX, cefoxitin; FEP, cefepime; CFZ, cefazolin. 
2 DH5α, E. coli DH5α. 
3 DH5α/pBCKS(+), E. coli DH5α containing empty pBCKS(+) vector. 
4 DH5α/pBCKS(+)-TEM-W60, blaTEM-W60-harboring E. coli DH5α strain.
truncated TEM $\beta$-lactamase that lacks the signal peptide-containing N-terminus is encoded by pECW602, suggesting the critical role of the signal peptide on the function of $\beta$-lactamases. Findings in this work shows the danger of transferable multidrug resistance plasmids and metallo-$\beta$-lactamases. We hope with this work these dangers are given enough attention in further developing methods for containing antimicrobial resistance.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject (accession: PRJNA642190).

**AUTHOR CONTRIBUTIONS**

WW, LL, MZ, ZL, WS, and FL performed microbial and genetic experiments. YN, TL, and XZ isolated bacteria. MW and WW performed bioinformatic analysis. MW, WW, XZ, and HX analyzed the data. MW, WW, XZ, and HX wrote the manuscript. MW, XZ, and HX conceived of the study and oversaw the project. All authors read and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2020.590357/full#supplementary-material

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