Characteristics of Carbapenemase-Producing Klebsiella pneumoniae Isolated in the Intensive Care Unit of the Largest Tertiary Hospital in Bangladesh

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For addressing the issue of antimicrobial drug resistance in developing countries, it is important to investigate the characteristics of carbapenemase-producing organisms. We aimed to genetically characterize a carbapenemase-producing Klebsiella pneumoniae (CPKP) isolated in the intensive care unit of a tertiary hospital in Bangladesh. The number of CPKP isolates were 43/145 (30%), of which pandrug-resistant (PDR) strains were 14%. These carbapenemases were New Delhi metallo-beta-lactamase (NDM)-1 (53%), NDM-5 (14%), oxacillinase (OXA)-181 (12%), OXA-232 (10%), NDM-5 + OXA-181 (5%), and NDM-5 + OXA-232 (2%). Many CPKP isolates harbored a variety of resistance genes, and the prevalence of 16S rRNA methyltransferase was particularly high (91%). The 43 CPKP isolates were classified into 14 different sequence types (STs), and the common STs were ST34 (26%), ST147 (16%), ST11 (9%), ST14 (9%), ST25 (7%), and ST231 (7%). In this study, PDR strains were of three types, ST147, ST231, and ST14, and their PDR rates were 57, 33, and 25%, respectively. The spread of the antimicrobial drug resistance of CPKP in Bangladesh was identified. In particular, the emergence of PDR is problem, and there may be its spread as a superbug of antimicrobial treatment.

Keywords: PDR, ST147, NDM-1, NDM-5, OXA-181, OXA-232

INTRODUCTION

The worldwide spread of carbapenemase-producing organisms (CPOs) is increasing (Bonomo et al., 2009). Carbapenem resistance in Klebsiella pneumoniae is frequently associated with antimicrobial resistance to other antibiotic classes, making treatment of infections challenging (Fournier et al., 2018). In North America, South America, and Europe, the main carbapenemase-producing genes are the Klebsiella pneumoniae carbapenemase (KPC) types; in the Middle East and Africa, they are the oxacillinase-48-like (OXA-48-like) types; and in the Indian subcontinent, they are of the New Delhi metallo-beta-lactamase (NDM) and OXA-48-like types. Many of these multidrug-resistant (MDR) strains are sequence type (ST) 11, ST14, ST37, ST147, and ST258 and are spreading worldwide as international MDR high-risk clones (Mathers et al., 2015).
The Center for Disease Control and Prevention (CDC) and the European Centre for Disease Prevention and Control have jointly developed definitions for MDR, extensively drug-resistant (XDR), and pandrug-resistant (PDR) bacteria (Magiorakos et al., 2012). *K. pneumoniae* strains classified as XDR are rapidly emerging due to the dissemination of resistance toward aminoglycosides, β-lactams, fluoroquinolones, and carbapenems (Al-Marzooq et al., 2015). Recently, XDR strains have evolved to become PDR by acquiring resistance to tigecycline and polymyxin antibiotics (Papadimitriou-Olivergeris et al., 2018). The spread of such strains is associated with high mortality rates, limited treatment options, and rapid dissemination of successful bacterial clones in the hospital setting (Guo et al., 2016; Protonotariou et al., 2018).

Antimicrobial resistance (AMR) is a major problem in Asia (O’neill, 2016). Bangladesh is a developing country surrounded by India and is known to have a high prevalence of carbapenem-resistant organisms. The CPOs detected are mainly NDM-producing strains, but in recent years, OXA-48-like-producing strains have also been detected. However, no surveillance system has been established in developing countries in this region, and there are few reports on molecular epidemiology (Hasan and Rabbani, 2019). We started the short-term prospective study of MDR bacteria with the ultimate goal of building a resistant surveillance system in Bangladesh (Okanda et al., 2018). In this study, we performed an analysis of acquired drug resistance mechanism and molecular epidemiology of carbapenemase-producing *Klebsiella pneumoniae* (CPKP) isolated in a tertiary hospital in Dhaka, Bangladesh.

### MATERIALS AND METHODS

#### Collection of Isolates

Bangabandhu Sheikh Mujib Medical University Hospital is the premier Postgraduate Medical Institution of Bangladesh and a major tertiary hospital with 1,900 beds. The intensive care unit (ICU) at this hospital accepts critically ill patients not only throughout Dhaka but also from neighboring countries. In this study, collection of isolates was originally scheduled for only between August and October 2015, but because of observed subsequent changes in AMR at ICU, isolates were also collected between August and September 2017. A total of 145 third-generation cephalosporin-resistant *Klebsiella pneumoniae* were isolated from blood, sputum or tracheal aspirate, urine, and pus in ICU patients (82 in 2015 and 63 in 2017). These were all consecutive isolates selected on the CHROMagar ESBL medium (Kanto Chemical Co., Inc., Japan), eliminating duplication by patient. These isolates were inoculated into casitone medium and transported to Tokyo Medical University in Japan, where they were incubated on modified Drigalski agar (EIKEN Chemical Co., Ltd) at 35°C for 18 h. The resulting cultures were re-identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using MALDI Biotyper (library ver. 9.0.0.0) and stored at −80°C in heart infusion broth containing 20% glycerol until use.

#### Screening of Carbapenemase-Producer and Detection of Carbapenemase-Encoding Genes

Phenotypic detection of carbapenemase was performed using the modified carbapenem inactivation method (mCIM) and Carba NP test. *Escherichia coli* ATCC 25922 was used as an indicator organism and negative control, whereas *K. pneumoniae* KPP 127 was used as a positive control. All isolates that showed positive results and intermediate results were detected of carbapenemase-encoding genes by polymerase chain reaction (PCR) assay. DNA was extracted using the Cica Genes DNA Extraction Reagent (Kanto Chemical Co., Inc., Japan). The examined carbapenemase-encoding genes were *bla*KPC, *bla*GES, *bla*IMP, *bla*VIM, *bla*NDM, *bla*OXA−48-like, *bla*BIC, *bla*AIM, *bla*GIM, *bla*SIM, *bla*DIM, and *bla*SPM (Supplementary Table S1).

#### Genotypic Detection of Carbapenemase-Encoding Genes and Other Resistance Genes

The confirmed CPKP isolates were tested for extended-spectrum β-lactamases (ESBLs)-producing genes, plasmid-mediated AmpC β-lactamase (PABLS)-producing genes, mobilized colistin resistance (*mcr*) gene, 16S rRNA methyltransferase (16S MRTase) genes, tetracycline resistance genes, plasmid-mediated quinolone resistance (PMQR) genes, fosfomycin resistance genes, sulfonamide resistance genes, and dihydrofolate reductase (DHFR)-encoding genes (Supplementary Table S1). Target genes were confirmed via single PCR, and PCR fragments were then purified from PCR product or agarose gels using Cica Genes® PCR & Gel Prep Kit (Kanto Chemical Co., Inc., Japan). Following sequencing, the similarity search was performed with the sequenced data using BLAST analysis1.

#### Antimicrobial Susceptibility Testing

Carbapenemase-producing *Klebsiella pneumoniae* isolates were tested for their susceptibility to piperacillin/tazobactam (TZP), ceferone (FEP), cefmetazole (CMZ), moxalactam (MOX), flomoxef (FOX), aztreonam (ATM), imipenem (IPM), meropenem (MEM), doripenem (DOR), biapenem (BPM), colistin (CST), gentamicin (GEN), amikacin (AMK), minocycline (MIN), tigecycline (TGC), ciprofloxacin (CIP), levofloxacin (LVX), fosfomycin (FOF), and trimethoprim/sulfamethoxazole (SXT). Microdilution susceptibility testing was performed and minimum inhibitory concentrations (MIC) determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute (CLSI), 2020). In this edition, CST breakpoints were defined as resistant ≥4 mg/l and intermediate ≤2 mg/l, with no susceptible category defined. In addition, no TGC and FOF breakpoints were defined for *K. pneumoniae*. Thus, CST, TGC, and FOF breakpoints were performed according to the European Committee on Antimicrobial Susceptibility Testing

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1http://blast.ncbi.nlm.nih.gov/Blast.cgi
Molecular Epidemiology Typing
Pulsed-field gel electrophoresis (PFGE) was carried out by the following method. After digestion of the genomic DNA by XhoI (New England Biolabs), the restriction fragments were separated using a temperature-controlled CHEF-DR III system (Bio-Rad) for 19.5 h at 14°C under the following conditions: two state modes, initial switch time of 5.3 s, final switch time of 60.0 s, a gradient of 6 V/cm, and switch angle of 120°. After staining with ethidium bromide, the fragments were visualized using an ultraviolet transilluminator. Percent similarities were determined by generating a dendrogram by the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were both set at 1.0%. A similarity coefficient of 80.0% was selected to define the 0.0 similarity coefficient. By sequence. Therefore, 40 (93%) of the 22 CPKP isolates were co-harboring ESBLs and PABLs. In addition, the prevalence of 16SRMTase, tetracycline resistance of ST11, and ST147 strains was serious, all of which were ST147, ST11, and ST231, respectively. On the other hand, the second most common cluster A was all ST34 strain. All ST34s were isolated from the ICU in 2015, and their antimicrobial susceptibility and acquisition resistance genes were almost the same. This suggested that there was an outbreak of ST34 strains in 2015. The STs of clusters A and L were ST147 and ST11, respectively. On the other hand, the second most common cluster A was all ST147, but it may be a different clone due to different banding patterns and separation dates. The band pattern of ST147 was significantly different between 2015 and 2017, forming cluster A and B, respectively. Therefore, it was considered that the transmission route was different between the ST147 strain in 2015 and in 2017.

The MLST classified the 43 isolates into 14 different STs (Figure 1). The common STs were ST34 (11, 26%), ST147 (7, 16%), ST11 (4, 9%), ST14 (4, 9%), ST25 (3, 7%), and ST231 (3, 7%). ST147, ST11, and ST231 strains were isolated in both 2015 and 2017. The eBURST analysis, based on the MLST database, found that 14 different STs were clustered into four different CGs (Figure 3). The founder of each CG was ST11, ST147, ST231, and 232 L (4, 9%), and the most common cluster R was all ST34 strain. All ST34s were isolated from the ICU in 2015, and their antimicrobial susceptibility and acquisition resistance genes were almost the same. This suggested that there was an outbreak of ST34 strains in 2015. The STs of clusters A and L were ST147 and ST11, respectively. On the other hand, the second most common cluster A was all ST147, but it may be a different clone due to different banding patterns and separation dates. The band pattern of ST147 was significantly different between 2015 and 2017, forming cluster A and B, respectively. Therefore, it was considered that the transmission route was different between the ST147 strain in 2015 and in 2017.

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RESULTS
CPKP Isolates
By phenotypic testing and sequencing, carbapenemase-producing genes were detected from 43 of the 145 isolates (30%). These 43 carbapenemase-producing genes were identified as blaNDM-1 (23, 53%), blaNDM-5 (6, 14%), blaOXA-181 (5, 12%), blaOXA-232 (4, 10%), 2 blaNDM-5 + blaOXA-181 (2, 5%), blaNDM-5 + blaOXA-232 (1, 2%), blaNDM-7 (1, 2%), and blaOXA-48 (1, 2%) by sequence. Therefore, 40 (93%) of the 43 CPKP isolates were single-harboring blaNDM (30, 70%) or blaOXA-48-like (10, 23%), while the remaining 3 (7%) were co-harboring both genes. CPKP isolates were co-harboring various AMR genes (Figure 1). ESBL- and PABL-encoding genes were detected in 43 (100%) and 22 (51%) CPKP isolates, respectively. Therefore, 22 CPKP isolates (51%) were co-harboring ESBLs and PABLS. In addition, the prevalence of 16SRMTase, tetracycline resistance, PMQR, fosfomycin resistance, sulfonamide resistance, and DHFR-encoding genes in CPKP isolates were 91% (39), 30% (13), 77% (33), 49% (21), 60% (26), and 60% (26), respectively. However, the mcr gene reported to date was not detected from colistin-resistant isolates.

Antimicrobial Resistance
The susceptibility of 43 CPKP isolates are shown in Table 1. Forty-three CPKP isolates were resistant to many of the antibiotics tested, with 10 isolates (23%) classified as XDR and 6 isolates (14%) classified as PDR. Interestingly, these CPKP isolates were highly resistant to aminoglycosides, and all AMK-resistant isolates showed MIC ≥ 512. From 2015 to 2017, the resistant rates of BPM, MIN, TGC, LVX, FOF, and SXT were increased, while the CST susceptibility was almost unchanged. In addition, the MIC value of six PDR isolates are shown in Table 2. PDR isolates showed high-level resistance to many antimicrobials, but low-level resistance to CST and TGC.

Molecular Epidemiological Characteristics of CPKP
Pulsed-field gel electrophoresis identified 29 different banding patterns from 43 CPKP isolates. These banding patterns were classified into 18 clusters (A to R) by more than 80% genetic associations (Figure 1). Common clusters were R (11, 26%), A (4, 9%), and L (4, 9%), and the most common cluster R was all ST34 strain. All ST34s were isolated from the ICU in 2015, and their antimicrobial susceptibility and acquisition resistance genes were almost the same. This suggested that there was an outbreak of ST34 strains in 2015. The STs of clusters A and L were ST147 and ST11, respectively. On the other hand, the second most common cluster A was all ST147, but it may be a different clone due to different banding patterns and separation dates. The band pattern of ST147 was significantly different between 2015 and 2017, forming cluster A and B, respectively. Therefore, it was considered that the transmission route was different between the ST147 strain in 2015 and in 2017.

The MLST classified the 43 isolates into 14 different STs (Figure 1). The common STs were ST34 (11, 26%), ST147 (7, 16%), ST11 (4, 9%), ST14 (4, 9%), ST25 (3, 7%), and ST231 (3, 7%). ST147, ST11, and ST231 strains were isolated in both 2015 and 2017. The eBURST analysis, based on the MLST database, found that 14 different STs were clustered into four different CGs (Figure 3). The founder of each CG was ST11, ST147, ST231, and ST43, while ST893 did not form a cluster and was a singleton. CG11 was the most common group, but those STs were diverse, and only ST11 strain was isolated in both years. The multidrug resistance of ST147 and ST231 strains was serious, all of which were XDR or PDR.
FIGURE 1 | Genetic and antibiogram profiles of 43 carbapenemase-producing Klebsiella pneumoniae isolates. A similarity coefficient of 80% was selected to define the clusters after reviewing the epidemiological data associated with each of the clusters of isolates. The presence or absence of plasmid-mediated antimicrobial resistance genes is indicated by black filled square or gray filled square, respectively. Antimicrobial susceptibility: susceptible (S) and resistant/intermediate (+). TZP, piperacillin/tazobactam; FEP, cefepime; CMZ, cefmetazole; MOX, moxalactam; FOX, flomoxef; ATM, aztreonam; IPM, imipenem; MEM, meropenem; DOR, doripenem; BPM, blipenem; CST, colistin; GEN, gentamicin; AMK, amikacin; MIN, minocycline; TGC, tigecycline; CIP, ciprofloxacin; LVX, levofloxacin; FOF, fosfomycin; SXT, trimethoprim/sulfamethoxazole.
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TABLE 1 | Susceptibility of carbapenemase-producing Klebsiella pneumoniae in Bangladesh.

|                  | 2015      | 2017      | Total     |
|------------------|-----------|-----------|-----------|
| Number of isolates| 21/82 (26%)| 22/63 (35%)| 43/145 (30%) |

**Antimicrobial resistance types**

| Resistance type                  | 2015 (14%) | 2017 (23%) | Total (23%) |
|----------------------------------|------------|------------|-------------|
| Multidrug resistant              | 16/21 (76%)| 11/22 (50%)| 27/43 (63%) |
| Extensively drug resistant        | 7/22 (32%) | 10/43 (23%)|             |
| Pandrug resistant                 | 2/21 (10%) | 4/22 (18%) | 6/43 (14%)  |

**Resistant rate**

| Antimicrobial                       | 2015 | 2017 | Total |
|-------------------------------------|------|------|-------|
| Piperacillin–tazobactam             | 100% | 100% | 100%  |
| Cefepime                            | 100% | 100% | 100%  |
| Cefmetazole                         | 100% | 100% | 100%  |
| Moxalactam                          | 100% | 100% | 100%  |
| Flomoxef                            | 100% | 100% | 100%  |
| Aztreonam                           | 100% | 100% | 100%  |
| Imipenem                            | 100% | 100% | 100%  |
| Meropenem                           | 100% | 100% | 100%  |
| Doripenem                           | 100% | 100% | 100%  |
| Biapenem                            | 62%  | 95%  | 79%   |
| Colistin                            | 24%  | 27%  | 26%   |
| Gentamicin                          | 100% | 100% | 100%  |
| Amikacin                            | 95%  | 86%  | 91%   |
| Minocycline                         | 33%  | 88%  | 51%   |
| Tigecycline                         | 19%  | 36%  | 28%   |
| Ciprofloxacin                       | 100% | 100% | 100%  |
| Levofloxacin                        | 62%  | 100% | 81%   |
| Fosfomycin                          | 29%  | 68%  | 49%   |
| Trimethoprim–sulfamethoxazole       | 56%  | 56%  | 56%   |

Breakpoints are performed according to the Clinical and Laboratory Standards Institute guideline M100-30 edition and according to the European Committee on Antimicrobial Susceptibility Testing guideline ver.10.0 for colistin, tigecycline, and fosfomycin.

**DISCUSSION**

The global spread of CPOs is a major issue in AMR measures, and the emergence of PDR strains makes antimicrobial treatment more difficult. The worsening of resistance of *K. pneumoniae* is remarkable, and some international MDR high-risk clones are reported (Mathers et al., 2015). These clones have a significant burden on global public health. Therefore, it is necessary to conduct intensive studies to monitor *K. pneumoniae* international MDR high-risk clones, to mitigate this issue. In the present study, we report for the molecular epidemiological characteristics of CPKP isolates collected at the largest tertiary hospital in Bangladesh. It is worth noting that PDR strains are ST147, ST231, and ST14, which harbored various resistance genes in addition to *bla*NDM*-* and *bla*OXA*-*48*-*like* encoding genes.

Antimicrobial resistance is globally increasing via the spread of carbapenemase, and treatment options are increasingly limited (Bonomo et al., 2009). In several cases, CPE are also resistant to colistin and tigecycline, and our data agrees with this observation (Table 2). In our study, the colistin resistance rate of CPKP isolates was 26% (MIC = 4 mg/l). Colistin resistance is caused by a decrease in negative charge due to modification of the lipid A structure of the outer membrane LPS. Modifications...
include those by the acquired mcr gene and those by the two-component regulatory system caused by chromosomal gene mutations (Macesic et al., 2020). However, the mcr gene was not detected from CST-resistant isolates. Thus, it is possible that the colistin resistance mechanism in this study is due to mutations in chromosomal resistance genes. Although the mechanism of tigecycline resistance has not been fully elucidated, the tigecycline resistance rate increased from 19% to 36% with the increase in the tet(A) gene. This result suggests that the tetracycline resistance protein is involved in tigecycline resistance of CPKP isolates. Resistance to CST and TGC has been on the rise because these antibiotics are regularly prescribed due to the scarcity of treatment options for CPE (5, 42–44). A report stating that 45% of 298 K. pneumoniae isolated at an Indian tertiary hospital between 2013 and 2017 were XDR and 9% were PDR is alarming (Mohapatra et al., 2018). Unfortunately, Mohapatra et al. (2018) do not mention the molecular epidemiology of XDR and PDR strains, but the possibility that these isolates are spreading from India and neighboring developing countries is plausible and worth investigating further.

Outbreaks involving the ST147 clone have been reported worldwide (Guo et al., 2016; Hamzaoui et al., 2018; Protonotariou et al., 2018). In Greece, the ST147 clone co-producing KPC-2 and VIM-1 was isolated from 25 patients at a tertiary teaching hospital, and 17 of these patients developed bloodstream infections (mortality rate = 48%) (Protonotariou et al., 2018). Nahid et al. (2017) provided insight into the emergence of ST147 PDR clone and the rapid global spreading of this high-risk clone. ST147 has been commonly reported among Indian isolates and has been frequently associated with NDM-1 and OXA48-like
carbapenemases as observed from the present study (Pragasam et al., 2017). Indeed, we showed that all ST147 clones were XDR and PDR strains harboring various resistance genes in Bangladesh. In addition, the ST147 clone was previously only isolated in the hospital environment, but a strain of ST147 that co-produces NDM-9 and CTX-M-15 has been isolated from wastewater in Switzerland (Nüesch-Inderbinen et al., 2018). This suggests that these PDR strains may already be present in our public health and are increasingly spreading under the surface. Therefore, ST147 will increasingly spread as a One Health infectious disease, and its dissemination may carry higher risks than other international high-risk clones such as ST11, ST15, and ST258.

In recent years, new antibacterial agents against carbapenemase-producing bacteria have been developed one after another and are expected to greatly contribute to the treatment of CPO infection (Ito et al., 2016). However, these new antimicrobials are expensive for some time after approval and are difficult to be readily available in developing countries. As another treatment option for CPO infections, a combination therapy by existing antibiotics is expected. We reported the efficacy of 136 pattern of combination therapies for five types of CPO and non-CPO (Okanda and Matsumoto, 2020). This report mainly suggests that the combined use of protein synthesis inhibitors and cell wall synthesis inhibitor is useful. However, CPOs isolated in South Asia often carry 16SRMTase,
and there is concern that combinations using aminoglycosides are ineffective due to their high MIC values for aminoglycosides. Since 91% of CPKP isolates are AMK-high-resistant strains (MIC ≥ 512 μg/ml) harboring 16S RMTase in this study, the effect of combination therapy using aminoglycosides cannot be expected. In our report, it is suggested that the combined use of BPM or IPM with MIN or TGC is effective to AMK-high-resistant strains (Okanda and Matsumoto, 2020). This combination therapy may be effective for some PDR-CPKP isolates in Bangladesh, which are not highly resistant to these antimicrobials. However, as the MIC of the antibiotics used in combination increases, the effect of the combination is limited. Therefore, the increase in PDR strains and in MIC value are particularly serious problems for developing countries.

CONCLUSION

Spread of CPKP isolates and PDR strain in Bangladesh warrants the need for the intensive surveillance of AMR and the implementation of an efficient infection control program in developing countries for the management of such infections.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: [https://www.ddbj.nig.ac.jp/index.html/ accession numbers: JN676828, KF240808, JN676878, JN676879, AF299299, KP050489, KF055402, KP772120, KP772206, KY418157, EF406115, KU985243, HQ267531, JX442976, AF227505, KJ511462, MK469978, MK412918, MK105834, AY220558, KY402263, JQ808129, CP052141, CP052141, EF682133, GQ438249, KX580704, MK043322, KU603636, KU603660, KY658724, JN108890, and AB759690].

AUTHOR CONTRIBUTIONS

TO: conceptualization, data curation, methodology, software, supervision, validation, visualization, writing – original draft, and writing – reviewing and editing. TO and TK: formal analysis. AH, HT, TM, and SN: funding acquisition. TO, AH, AI, and QH: investigation. TO and TM: project administration. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.612020/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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