ENTEROBACTERIACEAE ANTIMICROBIAL AGENTS AND RESISTANCE: RELATIONSHIP WITH THE THERAPEUTIC APPROACH

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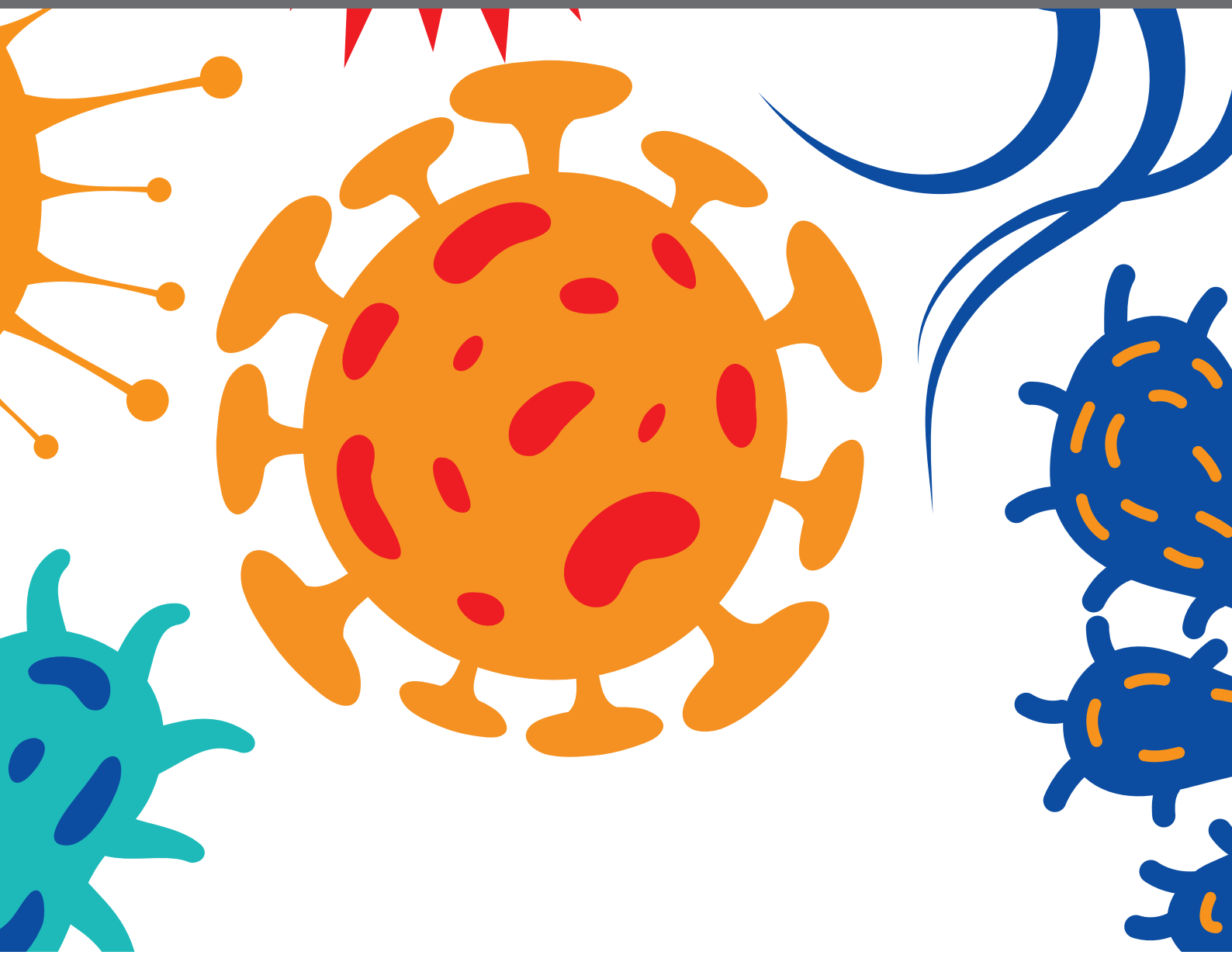

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ENTEROBACTERIACEAE ANTIMICROBIAL AGENTS AND RESISTANCE: RELATIONSHIP WITH THE THERAPEUTIC APPROACH

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EDITORIAL
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Editorial: Enterobacteriaceae Antimicrobial Agents and Resistance: Relationship With the Therapeutic Approach

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Editorial on the Research Topic

Enterobacteriaceae Antimicrobial Agents and Resistance: Relationship With the Therapeutic Approach

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In the field of infectious diseases multidrug-resistant (MDR) Gram-negative bacteria such as *Enterobacterales*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Aeromonas hydrophila* constitute an important issue for establishing correct and appropriate therapies in patients admitted to both Intensive Care Units and General Medicine centers including respiratory care wards. Carbapenem-resistant *Enterobacterales* (CRE) have undergone extensive dissemination worldwide, resulting in increased mortality and a global threat to public health. Additionally, the predominant production of *Klebsiella pneumoniae* carbapenemase (KPC) contributed to the most important mechanism of carbapenem resistance in *K. pneumoniae* (Nordmann et al., 2009).

To deal with this situation it is necessary to understand the infection epidemiology and the resistance patterns other than those that comply with guidelines for treatment (Kaye and Pogue, 2015). The development of new drugs capable of eradicating multidrug-resistant Gram-negative pathogens is highly recommended as is combination therapy (which is more effective than monotherapy) or the research into other alternatives (**Figure 1**) (Morris and Cerceo, 2020).

The topics covered by this Research Topic include contributions on the dissemination and characteristics of carbapenemases such as KPC, NDM, OXA 48, IMP, and VIM among CRE, which are crucial for detecting resistance in adult or child patients. These concepts are well analyzed by the Antimicrobial Surveillance Network (CHINET) Study Group in China (Han et al.).

In this large study, carbapenemases were found in 97.4% of CRE strains, including KPC-2 (51.6%), NDM (35.7%), and OXA-48-like carbapenemases (7.3%). The most prevalent carbapenemase genes were *blaKPC-2* among *K. pneumoniae* isolates from adult patients, and *blaNDM* among *E. coli* isolates from children. All the CRE strains were highly resistant to cephalosporins and carbapenems. The risk factors and the epidemiology for the establishment of

Abbreviations: KPC, *Klebsiella pneumoniae* carbapenemase producing; NDM, New Delhi metallo-beta-lactamase; VIM, Verona Integron-encoded Metallo-β-lactamase.

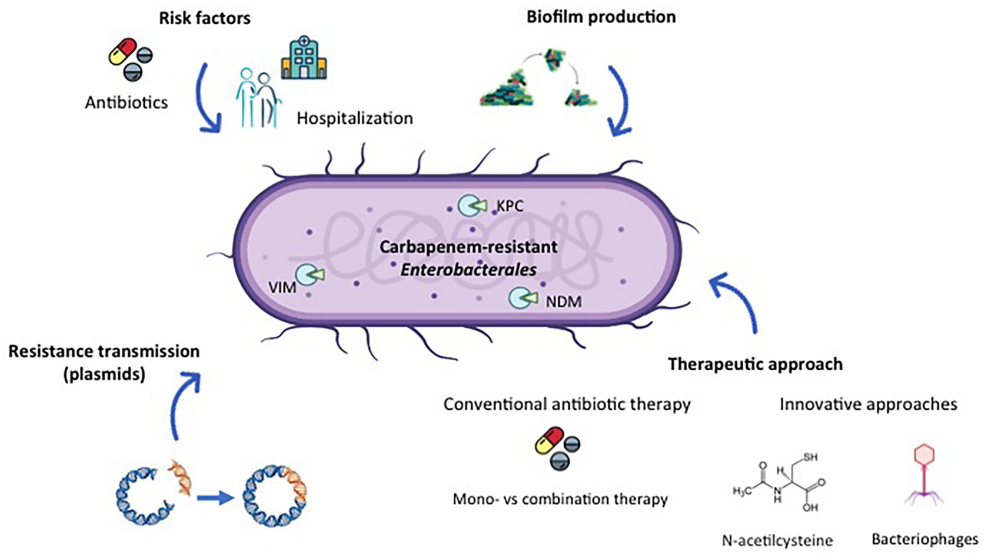

FIGURE 1 | Overview of different features of Carbapenem-Resistant *Enterobacterales (CRE)* management.

carbapenem-resistant *Klebsiella pneumoniae* (CRKP) are issues widely reported in literature on this subject (Pitout et al., 2015; Logan and Weinstein, 2017; Pin et al., 2018).

These concepts are highlighted in other studies performed in China (Fang et al.). Here, the colonization and the incidence of infections were reported to be 2.7 per 100,000 patient days, and the presence of CRKP KPC-2 was found predominantly. The MALDI-TOF of MS system was shown to be useful for detecting this prevailing serotype with the same performance as the PFGE (Pulsed-Field Gel Electrophoresis) system. The mainstream gene of CRKP in the geographic areas of China was *blaKPC*−2.

Another paper also found that there was a predominance of KPC-2. In this case, the mobile elements such as plasmids were detected and accurately studied. CRKP strains co-harboring *blaKPC-2*-carrying plasmid and pLVPK-like virulence plasmid are mostly involved in bacterial multidrug resistance, enhanced virulence, and above all, in the transfer of these mobile elements to other bacteria such as *E. coli* and other isolates (Du et al.).

A very interesting article concentrates on the presence of plasmids and concerns a new unusual non–carbapenemase-producing CRKP carrying a rare plasmid-borne inducible AmpC gene, *blaDHA-1* from an isolate belonging to blood culture. This strain showed complex susceptibility patterns. The genetic method Whole Genome Sequencing was able to detect this peculiar plasmid bearing the resistance genes of the third generation cephalosporins (Realegeno et al.).

The early detection of the third generation cephalosporins resistant *Enterobacterales* directly from positive blood cultures is very important in identifying antimicrobial resistance before the culture, strongly shortening the time for the establishment of a correct and appropriate antibiotic therapy (Durand et al.). For this purpose, two methods (electrochemical and chromogenic) were proposed by Hospital in Nimes (France) and are discussed in comparison with the traditional technology, which consequently allows for a rapid adaptation of therapy, with great benefits for patient outcome.

The production of bacterial biofilm is another big inconvenience (**Figure 1**). The microorganisms universally attach to surfaces and produce extracellular polysaccharides, resulting in biofilm formation (Donlan, 2001). This process especially occurs on medical devices (Oliva et al., 2013). The presence of biofilm together with other factors related to virulence is a significant risk factor, especially in oncological patients, as demonstrated in a study performed in a hospital in Rome (Italy). The detection of the biofilm in these high-risk patients may be of help in the management of oncological individuals (Di Domenico et al.).

Other than *Enterobacterales*, other microorganisms such as *Aeromonas hydrophila*, which lives in an aquatic environment and rarely infects individuals, are found to own chromosomally encoded carbapenem resistant genes, such as *blacphA7* (metallo-beta-lactamase). Consequently, the emerging MDR *Aeromonas* should also be taken into account. The first case of CphA-mediated carbapenem resistant *A. hydrophila* was reported in the U.S. (Hilt et al.).

Different ST (Sequence Types) have been found among resistant *Enterobacterales*, especially CRKP. Even though the mortality rate was detected and shown to be no different because of the diverse ST, other factors affected mortality, such as the treatment strategies following source control and bacterial eradication (Lim et al.). Furthermore, the mortality resulted as being increased in long-term facilities such as residences for the elderly or hospices, which achieved a rate of up to 75% (Chen et al.).

Besides phagotherapy (Principi et al., 2019) and possible antimicrobial combinations, including non-antibiotic compounds

such as N-acetylcysteine (MacNair et al., 2018: Oliva et al., 2021), there are two crucial tools for overcoming microbial multidrug resistance and consequently decreasing the great menace of this issue to public health (**Figure 1**). Bacteriophages are expected to become a potentially effective therapeutic agent for difficult-to-treat infections. In this case, a specific bacteriophage against a particular microorganism could be used to lyse and kill the bacterium infected by the phage. Unlike antibiotics, in this situation, no resistance occurs. The limit of phage therapy, inherent to bacteriophage, lies in a narrow spectrum of action. In fact, in the case reported in this Special Issue concerning a mixed infection, a failure of three consecutive phage therapies was reported. However, a set of different bacteriophages were selected against the single bacterial strains involved in the infection, leading to a successful patient outcome (Qin et al.).

Interestingly, combination therapy seems to be more beneficial than monotherapy as far as mortality rates are concerned. Many antibiotics are used in association against CRE, such as colistin-based regimens whereas data on ceftazidime/avibactam used in combination or alone are still conflicting.

Even the association of old antibiotics such as nitrofurantoin and amikacin appears to be effective against 12 clinical MDR uropathogenic *E. coli in vitro*, as shown by an article in this Research Topic (Zhong et al.).

In conclusion, the multi-resistance in *Enterobacterales* and other Gram-negative bacteria is an increasing problem that requires a drastic intervention for limiting the further spread of resistant bacteria. Alternative therapeutic programs other than antibiotics could be proposed, such as medicinal oils, antibodies, common biocides, killing factors, and phage therapy.

AUTHOR CONTRIBUTIONS

MTM conceived, organized and wrote the Editorial. SB contributed to the revision of articles concerning the epidemiology and the risk factors. AO contributed to the revision of articles concerning the production of the biofilm and the antibiotics association. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Dissemination of Carbapenemases (KPC, NDM, OXA-48, IMP, and VIM) Among Carbapenem-Resistant *Enterobacteriaceae* Isolated From Adult and Children Patients in China

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This study aimed to investigate the dissemination and characteristics of bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{IMP}, and bla_{VIM} among the carbapenem-resistant *Enterobacteriaceae* (CRE) strains isolated from adult and children patients. A total of 935 non-duplicate CRE strains were collected from 36 hospitals in 24 provinces or cities across China from 2016 to 2018. Antimicrobial susceptibility testing was performed by broth microdilution method and carbapenemase genes bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{IMP}, and bla_{VIM} were screened by PCR and confirmed by DNA sequencing. Overall, carbapenemases were produced in 97.4% (911/935) of CRE strains, including KPC-2 (51.6%, 482/935), NDM (35.7%, 334/935), and OXA-48-like carbapenemases (7.3%, 68/935). Overall, the most prevalent carbapenemase gene was $bla_{KPC\text{-}2}$ among *Klebsiella pneumoniae* (64.6%, 457/709) and the CRE strains isolated from adult patients (70.3%, 307/437), and bla_{NDM} among *Escherichia coli* (96.0%, 143/149) and the CRE strains from children (49.0%, 247/498). The $bla_{OXA\text{-}232}$-positive carbapenem-resistant *K. pneumoniae* (9.3%, 66/709) were all isolated from children. Sixteen strains were positive for bla_{IMP} and 9 strains produced multiple carbapenemases. No strain was positive for bla_{VIM}. Most of the CRE strains (>90%) were resistant to cephalosporins and carbapenems, more than half (>50%) were resistant to aminoglycosides and fluoroquinolones, but the majority (95.8 and 98.4%) were susceptible to polymyxin B and tigecycline. Ceftazidime-avibactam showed excellent *in vitro* activity against $bla_{KPC\text{-}2}$ and $bla_{OXA\text{-}48\text{-}like}$ positive strains (100% susceptible). In China, KPC-2, NDM, and OXA-48-like carbapenemases were predominant among CRE clinical isolates. The most prevalent carbapenemase gene was $bla_{KPC\text{-}2}$ among *K. pneumoniae* isolates from adult patients, and bla_{NDM} among *E. coli* isolates from children.

Keywords: carbapenem-resistant *Enterobacteriaceae*, $bla_{KPC\text{-}2}$, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{IMP}

INTRODUCTION

Enterobacteriaceae are opportunistic pathogens causing severe hospital-acquired infections (Feil, 2016). The spread of carbapenemase-producing *Enterobacteriaceae* (CPE) has been a global threat to public health. Carbapenems have conventionally been used for treating infections caused by extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*, and are still considered as last resort antibiotics to date (van Duin and Doi, 2016). According to the data from China Antimicrobial Surveillance Network (CHINET, www.chinets.com), the resistance rate of *K. pneumoniae* to meropenem and imipenem rapidly increased from 2.9 and 3.0% in 2005 to 26.3 and 25% in 2018, respectively. In Europe, carbapenem-resistant *K. pneumoniae* are most widespread in the Mediterranean and Balkan countries with a prevalence of 60% in Greece and 40% in Italy, respectively (Perez and Villegas, 2015; Feil, 2016). The production of carbapenemases including KPC, NDM, and OXA-48-like is the most common resistance mechanism among carbapenem-resistant *Enterobacteriaceae* clinical isolates (Nordmann et al., 2012; Goodman et al., 2016). The bla_{KPC}-positive *Enterobacteriaceae* were widespread in the United States, Latin America, Italy, Greece, the Middle East, and China (Albiger et al., 2015; Feil, 2016; Villegas et al., 2016; Iovleva and Doi, 2017). The bla_{NDM}-positive *Enterobacteriaceae* were widespread in India, Pakistan, Bangladesh, Italy, Poland, Denmark, Latin America, and African countries (Yong et al., 2009; Albiger et al., 2015; van Duin and Doi, 2016). The $bla_{\mathrm{OXA-48-like}}$-positive strains remained rare in the US, in contrast to the prevalence in Turkey, Spain, France, Belgium, Romania, Middle East, Africa, Asia, and South America as well (Albiger et al., 2015). These infections are usually associated with very poor prognosis and high mortality, especially in neonates or high-risk immunocompromised patients (Falagas et al., 2014; Feil, 2016). In China, the presence of bla_{KPC} and bla_{NDM} is responsible for phenotypic resistance in most of the CRE strains (Zhang et al., 2017; Wang et al., 2018). Most researches currently focus on the dissemination of carbapenemases among CRE strains isolated from adult patients, while only a few are available to investigate the distribution of carbapenemases among CRE strains isolated from children. To obtain the comprehensive characteristic of carbapenemases among CRE isolated from both adults and children patients in China, we conducted this study to characterize the dissemination and characteristics of carbapenemases (including KPC, NDM OXA-48, IMP, and VIM) among CRE clinical isolates and the susceptibility to antimicrobial agents.

MATERIALS AND METHODS

Clinical Strains

From January 2016 to December 2018, a total of 935 non-duplicate sequential CRE strains were collected from 36 hospitals in 24 provinces or cities across China (**Figure 1**), including *K. pneumoniae* (n = 709, 75.8%), *E. coli* (n = 149, 15.9%), *Enterobacter cloacae* (n = 36, 3.9%), *Citrobacter freundii* (n = 14, 1.5%), *Serratia marcescens* (n = 8, 0.9%), *Enterobacter aerogenes* (n = 7, 0.7%), *Klebsiella oxytoca* (n = 7, 0.7%), *Morganella morganii* (n = 3, 0.3%), *Proteus vulgaris* (n = 1, 0.1%), *Providencia rettgeri* (n = 1, 0.1%). In this study, 46.7% (437/935) of CRE strains were collected from adult patients and 53.3% (498/935) from children patients. The *Enterobacteriaceae* strains resistant to at least one of the carbapenem antibiotics (ertapenem, meropenem, doripenem, or imipenem) or producing a carbapenemase (an enzyme that can make them resistant to carbapenem antibiotics) were defined as CRE by Centers for Disease Control and Prevention of USA (https://www.cdc.gov/hai/organisms/cre/technical-info.html#Definition). These CRE strains were isolated from sputum (27.5%), blood (27.1%), urine (17.0%), secreta (6.9%), bile (5.0%), ascites (3.2%), catheter (2.8%), drainage (2.8%), pus (1.4%) and other aseptic body fluid (6.4%). Species identification was confirmed by MALDI-TOF/MS system (bioMérieux, France). *E. coli* ATCC 25922, *E. coli* ATCC 35218, and *K. pneumoniae* ATCC 700603 were tested as the quality control strains for antimicrobial susceptibility testing.

Antimicrobial Susceptibility Testing (AST)

AST was performed by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute. Minimum inhibitory concentrations (MICs) of piperacillin, cefoperazone-sulbactam, piperacillin-tazobactam, cefazolin, cefuroxime, ceftazidime, ceftriaxone, ceftazidime-avibactam, cefepime, cefmetazole, aztreonam, ertapenem, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole, polymyxin B, nitrofurantoin, tigecycline were determined. The MIC breakpoints for *Enterobacteriaceae* (susceptible, $\leq$2 mg/L; resistant, $\geq$8 mg/L) issued by the Food and Drug Administration were used as the breakpoints for tigecycline.

Detection of Carbapenemase and *mcr-1* Genes

All the CRE strains were tested for the presence of the most common carbapenemase genes (bla_{KPC}, bla_{NDM}, $bla_{\mathrm{OXA-48-like}}$, bla_{IMP}, and bla_{VIM}) by polymerase chain reaction (PCR) with specific primers and conditions as described previously (Poirel et al., 2011; Liu et al., 2016). The colistin resistance gene *mcr-1* was also detected by PCR, as previously described (Liu et al., 2016). The positive PCR amplicons were sequenced and compared with the reported sequences from GenBank by Blast (www.ncbi.nlm.nih.gov/blast/).

Statistical Analysis

Descriptive statistics were used to summarize the epidemiologic characteristics of CRE strains. For categorical variables, the percentage of CRE strains in each category was calculated. All analyses were performed using WHONET (version 5.6) and the IBM SPSS Statistics (version 21).

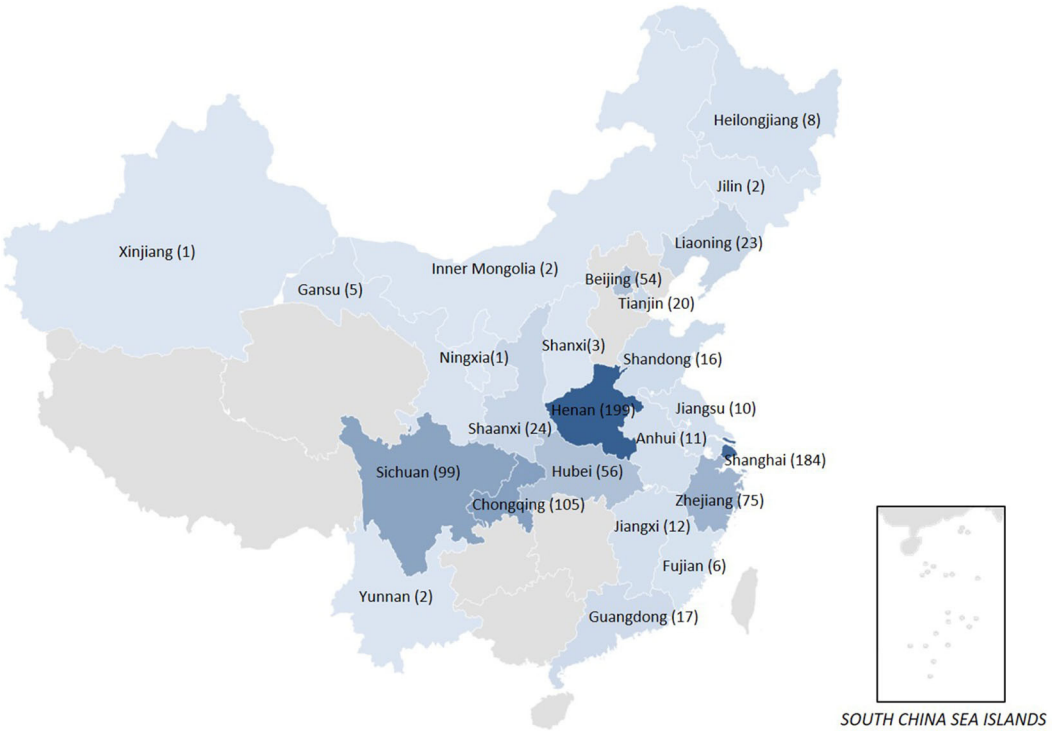

FIGURE 1 | The map of CRE clinical strains collected from 24 provinces or cities in China.

RESULTS

In vitro Antimicrobial Susceptibility

Most of the CRE strains (>90%) were resistant to cephalosporins, piperacillin, cefoperazone-sulbactam, piperacillin-tazobactam, aztreonam, and carbapenems. Overall, 61.4, 50.1, and 45.2% of the strains were susceptible to ceftazidime-avibactam, amikacin, and trimethoprim-sulfamethoxazole, respectively, followed by gentamicin (31.8%), levofloxacin (22.9%), ciprofloxacin (19%), and nitrofurantoin (18.8%). Polymyxin B and tigecycline showed excellent antibacterial activity against CRE strains (95.8 and 98.4% susceptible, respectively) (**Table 1**). Ceftazidime-avibactam had potent activity against both KPC-2-producing and OXA-48-like producing *Enterobacteriaceae* (100% susceptible) and inhibited all of $bla_{\text{KPC-2}}$-positive and $bla_{\text{OXA-48-like}}$-positive strains at 8 mg/L. However, all NDM-producing *Enterobacteriaceae* were resistant to ceftazidime-avibactam (MIC_{90} > 32 mg/L). The MICs of ceftazidime-avibactam were higher than 32 mg/L against IMP- and multi-carbapenemase producing *Enterobacteriaceae* (KPC and NDM co-producers, NDM and OXA-48 co-producer). Most of the bla_{NDM}-positive strains were susceptible to amikacin (86.2% susceptible) (**Table 1**).

Prevalence of bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$, bla_{IMP}, and bla_{VIM} Carbapenemase and *mcr-1* Genes

Carbapenemase gene was positive in 97.4% (911/935) of the CRE strains, including $bla_{\text{KPC-2}}$ in 51.6% (482/935), bla_{NDM} in 35.7% (334/935), $bla_{\text{OXA-48-like}}$ in 7.3% (68/935), bla_{IMP} in 1.7% (16/935), bla_{KPC} and bla_{NDM} in 1.0% (9/935), $bla_{\text{NDM-24}}$ and $bla_{\text{OXA-48}}$ in 0.1% (1/935), $bla_{\text{NDM-1}}$ and $bla_{\text{IMP-4}}$ in 0.1% (1/935) of the strains (**Table 2**). KPC-2 was the most prevalent carbapenemase among *K. pneumoniae* (64.5%, 457/709) and *S. marcescens* (100%, 8/8) strains. NDM-5 was the predominant type carbapenemase among *E. coli* (74.5%, 111/149), *E. cloacae* (66.7%, 24/36) and *C. freundii* (64.3%, 9/14). Among all OXA-48-like producing *K. pneumoniae*, PCR and DNA sequencing results showed the presence of $bla_{\text{OXA-232}}$ (97.1%, 66/68) and $bla_{\text{OXA-48}}$ (2.9%, 2/68) (**Table 2**).

Of the CRE strains isolated from adult patients, 70.3% (307/437) were KPC-2-producers; 20.6% (90/437) were NDM-producers (including 12.1% of NDM-1-producers, 8.2% of NDM-5-producers, and 0.2% of NDM-3-producer); and 0.5% (2/437) were OXA-48-producers (**Table 3**, **Figure 2**) ($P < 0.01$). However, of the CRE strains isolated from children, 49.0% (244/498) were NDM-producers (including 32.9% of NDM-5-producers, 15.9% of NDM-1-producers and 0.2% of NDM-3-producer); 35.1% (175/498) were KPC-2-producers and 13.3% (66/498) were OXA-232-producers (**Table 3**, **Figure 2**) ($P < 0.01$). The $bla_{\text{OXA-232}}$-positive *K. pneumoniae* were only isolated from children patients while $bla_{\text{OXA-48}}$-positive *K. pneumoniae* were isolated from adults. One polymyxin B resistant *E. coli* was positive for *mcr-1* with co-producing $bla_{\text{NDM-5}}$.

DISCUSSION

Previous studies have proved that the presence of carbapenemase genes, including $bla_{\text{KPC-2}}$ and bla_{NDM}, was the major mechanism of carbapenem resistance among CRE strains in China, which

TABLE 1 | Antimicrobial susceptibility testing results of clinical CRE strains (MICs, mg/L).

Antimicrobial agent	All CRE (n = 935)					KPC-producers (n = 482)				NDM-producers (n = 334)				OXA-48-like producers (n = 68)			
	MIC range	MIC_{50}	MIC_{90}	%R	%S	MIC_{50}	MIC_{90}	%R	%S	MIC_{50}	MIC_{90}	%R	%S	MIC_{50}	MIC_{90}	%R	%S
Piperacillin	4->256	>256	>256	98.9	0.9	>256	>256	99.4	0.2	>256	>256	99.7	0.3	>256	>256	100	0
Cefoperazone-sulbactam	1->128	>128	>128	98.3	1.2	>128	>128	98.1	1.2	>128	>128	99.4	0	>128	>128	100	0
Piperacillin-tazobactam	2->256	>256	>256	97.2	1.5	>256	>256	98.8	0.6	>256	>256	99.4	0	>256	>256	100	0
Cefazolin	32->32	>32	>32	100	0	>32	>32	100	0	>32	>32	100	0	>32	>32	100	0
Cefuroxime	2->64	>64	>64	99.9	0.1	>64	>64	100	0	>64	>64	100	0	>64	>64	100	0
Ceftazidime	0.5->32	>32	>32	98.6	0.7	>32	>32	98.1	0.8	>32	>32	99.7	0	>32	>32	100	0
Ceftriaxone	0.12-64	>32	>32	99.4	0.6	>32	>32	99.4	0.6	>32	>32	99.7	0.3	>32	>32	100	0
Ceftazidime-avibactam	0.25->32	2	>32	38.6	61.4	2	4	0	100	>32	>32	100	0	0.5	4	0	100
Cefepime	0.25->32	>32	>32	98.1	0.9	>32	>32	97.9	1	>32	>32	99.4	0	>32	>32	100	0
Cefmetazole	1->64	>64	>64	92.7	4.5	>64	>64	92.3	5.6	>64	>64	97.6	1.2	64	>64	73.5	13.2
Aztreonam	0.25->128	>128	>128	93.2	4.2	>128	>128	99	0.8	>128	>128	85.3	7.8	>128	>128	100	0
Ertapenem	0.25->32	>32	>32	98.9	1	>32	>32	99	1	>32	>32	99.7	0.3	>32	>32	100	0
Imipenem	0.12->16	>16	>16	96.1	2.1	>16	>16	99.2	0.6	16	>16	99.4	0.3	>16	>16	73.5	17.6
Meropenem	0.12->16	>16	>16	97	1.9	>16	>16	98.1	1.5	>16	>16	99.7	0.3	>16	>16	85.3	4.4
Amikacin	1->128	16	>128	49.6	50.1	>128	>128	69.7	29.9	1	>128	13.8	86.2	>128	>128	100	0
Gentamicin	1->128	128	>128	67.9	31.8	>128	>128	83.8	16	1	128	40.4	59.3	>128	>128	100	0
Ciprofloxacin	0.06->8	>8	>8	78.4	19	>8	>8	95.6	3.7	8	>8	53.6	41.3	>8	>8	100	0
Levofloxacin	0.06->16	>16	>16	76.3	22.9	>16	>16	94.6	4.8	4	>16	49.4	49.7	>16	>16	100	0
Trimethoprim- Sulfamethoxazole	0.25->32	32	>32	54.8	45.2	1	>32	47.9	52.1	>32	>32	54.5	45.5	>32	>32	100	0
Polymyxin B	0.125->16	0.25	1	4	95.8	0.25	1	4.4	95.4	0.25	1	3.6	96.1	0.5	0.5	1.5	98.5
Nitrofurantoin	4->128	>128	>128	64.1	18.8	>128	>128	92.9	4.6	64	>128	22.8	41.9	128	>128	64.7	8.8
Tigecycline	0.12–8	0.5	2	0.3	98.4	0.5	2	0.4	97.7	0.5	1	0.3	99.1	1	2	0	100

CRE, carbapenem-resistant Enterobacteriaceae; $MIC_{50/90}$, 50%/90% minimum inhibitory concentration; %R, % of isolates resistant; %S, % of isolates susceptible.

TABLE 2 | Prevalence of different carbapenemase genes among 935 CRE strains.

Species	Strains tested, *N*	bla_{KPC-2}, *n* (%)	bla_{NDM}, *n* (%)	$bla_{OXA-48-like}$, *n* (%)	bla_{IMP}, *n* (%)	Two genes, *n* (%)	Any gene, *n* (%)
K. pneumoniae	709	457 (64.5)	bla_{NDM-1}, 64 (9.0) bla_{NDM-5}, 85 (12.0) bla_{NDM-3}, 1 (0.1)	bla_{OXA-48}, 2 (0.3) $bla_{OXA-232}$, 66 (9.3)	bla_{IMP-4}, 6 (0.8) bla_{IMP-69}, 3 (0.4)	bla_{KPC-2}+bla_{NDM-1}, 6 (0.8) bla_{KPC-2}+bla_{NDM-5}, 1 (0.1) bla_{NDM-1}+bla_{IMP-4}, 1 (0.1) bla_{NDM-24}+bla_{OXA-48}, 1 (0.1)	693 (97.7)
E. coli	149	4 (2.7)	bla_{NDM-1}, 31 (20.8) bla_{NDM-5}, 111 (74.5) bla_{NDM-3}, 1 (0.7)				147 (98.7)
E. cloacae	36	3 (8.3)	bla_{NDM-1}, 24 (66.7) bla_{NDM-5}, 3 (8.3)		bla_{IMP-4}, 4 (11.1) bla_{IMP-6}, 1 (2.8)	bla_{KPC-2}+bla_{NDM-1}, 1 (2.8)	36 (100)
C. freundii	14	3 (21.4)	bla_{NDM-1}, 9 (64.3)				12 (85.7)
S. marcescens	8	8 (100)					8 (100)
E. aerogenes	7	1 (14.3)	bla_{NDM-1}, 1 (14.3) bla_{NDM-5}, 1 (14.3)				3 (42.9)
K. oxytoca	7	3 (42.9)	bla_{NDM-1}, 2 (28.6)		bla_{IMP-4}, 1 (14.3)	bla_{KPC-2}+bla_{NDM-1}, 1 (14.3)	7 (100)
M. morganii	3	2 (66.7)	bla_{NDM-1}, 1 (33.3)				3 (100)
P. vulgaris	1	1 (100)					1 (100)
P. rettgeri	1				bla_{IMP-4}, 1 (100)		1 (100)
Total	935	482 (51.6)	334 (35.7)	68 (7.3)	16 (1.7)	11 (1.2)	911 (97.4)

CRE, carbapenem-resistant Enterobacteriaceae.

TABLE 3 | Distribution of different carbapenemase genes in 935 CRE strains isolated from adults and children patients.

Carbapenemase genes	All CRE, *n* (%)		*E. coli*, *n* (%)		*K. pneumoniae*, *n* (%)	
	From children	From adults	From children	From adults	From children	From adults
bla_{KPC-2}	175 (35.1)	307 (70.3)	3 (2.8)	1 (2.3)	169 (44.7)	288 (87.0)
bla_{NDM-1}	79 (15.9)	53 (12.1)	21 (19.8)	10 (23.3)	50 (13.2)	14 (4.2)
bla_{NDM-5}	164 (32.9)	36 (8.2)	82 (77.4)	29 (67.4)	81 (21.4)	4 (1.2)
bla_{NDM-3}	1 (0.2)	1 (0.2)		1 (2.3)	1 (0.3)	
bla_{OXA-48}		2 (0.5)				2 (0.6)
$bla_{OXA-232}$	66 (13.3)				66 (17.5)	
bla_{IMP-4}	3 (0.6)	9 (2.1)			2 (0.5)	4 (1.2)
bla_{IMP-6}		1 (0.2)				
bla_{IMP-69}	2 (0.4)	1 (0.2)			2 (0.5)	1 (0.3)
bla_{KPC-2}+bla_{NDM-1}	2 (0.4)	6 (1.4)			1 (0.3)	5 (1.5)
bla_{KPC-2}+bla_{NDM-5}		1 (0.2)				1 (0.3)
bla_{NDM-1}+ bla_{IMP-4}	1 (0.2)				1 (0.3)	
bla_{NDM-24}+ bla_{OXA-48}		1 (0.2)				1 (0.3)
Others	5 (1.0)	19 (4.3)		2 (4.7)	5 (1.3)	11 (3.3)
Total	498	437	106	43	378	331

CRE, carbapenem-resistant Enterobacteriaceae.

were the most prevalent in *K. pneumoniae* and *E. coli*, respectively (Zhang et al., 2017; Wang et al., 2018). However, the researches on CRE strains isolated from children patients are limited in China. This study provided a comprehensive and updated carbapenemase profile of 935 CRE strains isolated from both adult and children patients. We found that bla_{KPC-2} (51.6%) and bla_{NDM} (35.7%) were the most common carbapenemase genes among CRE strains, while the emergence of $bla_{OXA-232}$, bla_{IMP}, and other multi-carbapenemase genes have been increasing in recent years. KPC-2 was the most frequently detected carbapenemase gene in *K. pneumoniae*, while NDM was the most prevalent one in *E. coli*. This pattern in China is significantly different from that in Europe. In Europe, the prevalence of OXA-48-like producing *Enterobacteriaceae* was 38% (333/927), next to KPC- (42%, 393/927), but higher than NDM-producing *Enterobacteriaceae* (12%, 113/927) (Grundmann et al., 2017). The distribution of carbapenemase-producers also varied with bacterial species. In *K. pneumoniae*, KPC-producers were the most prevalent, followed by OXA-48-like (37%, 310/850) and NDM-producers (11%, 93/850). In *E. coli*, OXA-48-like

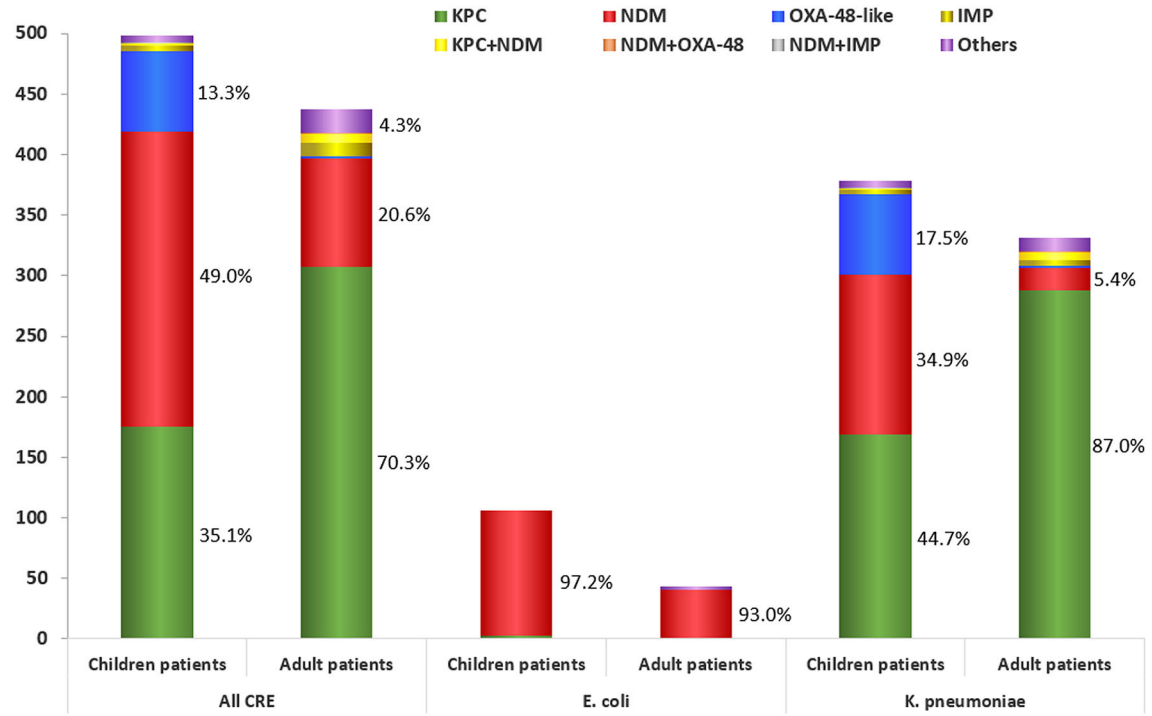

FIGURE 2 | Carbapenemase distribution among the carbapenem-resistant *Enterobacteriaceae* strains isolated from adult and children patients.

producers were the most prevalent (56%, 43/77), followed by NDM- (26%, 20/77) and KPC-producers (18%, 14/77). *K. pneumoniae* and *E. coli* were the two main species in China with a ratio of 5:1 (4:1 in children, 8:1 in adults) in this study, which differed from the prevalence trends (ratio of 11:1) in EuSCAPE (Grundmann et al., 2017).

Notably, KPC-2-producers were widespread in adult patients, followed by NDM-producers, while NDM-producers were prevalent in children patients, followed by KPC-2- and OXA-48-like producers. These findings described the different patterns of carbapenemases among CRE strains from adults and children. In contrast to the previous finding that NDM-1 was the most common carbapenemase among children patients, we have found that NDM-5-producers (32.9%) were most frequently detected CRE strains from children (Tian et al., 2018; Yin et al., 2018; Zhang et al., 2018). The outbreak of NDM-5-producing ST48 *K. pneumoniae* was first reported in Shanghai (Tian et al., 2018). We speculated that outbreak of NDM-5-producers accounted for the spread of NDM-5 among children patients in this study (Tian et al., 2018; Li et al., 2020). Further study is needed to track the type of plasmids harboring these carbapenemase genes.

Unlike the previous report that few OXA-48-like producing *Enterobacteriaceae* (0.1%, 2/1801) were detected in China from 2012 to 2016 (Wang et al., 2018), we found 7.3% (68/935) OXA-48-like producing *K. pneumoniae* between 2016 and 2018. Since the first OXA-232-producing *K. pneumoniae* isolated from neonate in 2017, the outbreaks of OXA-232-producing *Enterobacteriaceae* have been successively reported in children patients (Yin et al., 2017; Tian et al., 2018). Subsequently, 10 strains of OXA-232-producing *K. pneumoniae* were isolated from elderly patients in the intensive care unit in 2019 and the $bla_{\text{OXA-232}}$ was located in a 6.1-kb ColKP3-type non-conjugative plasmid, which was highly similar to the pkNICU5 first reported (similarity about 99%) in 2017 (Yin et al., 2017; Shu et al., 2019). We speculated that the presence of $bla_{\text{OXA-232}}$ on a mobile element and its spread among different strains were responsible for the recent dissemination of OXA-232-producing *Enterobacteriaceae*, which would make it possible to become the "third epidemic" carbapenemase after KPC-2 and NDM in China (Yin et al., 2017; Tian et al., 2018).

All of the CRE strains were highly resistant to cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones but susceptible to polymyxin B and tigecycline. Ceftazidime-avibactam, launched last year in China, showed excellent *in vitro* antibacterial activity against both KPC-2- and OXA-48-like producers, but not active against metallo-β-lactamases producers. Most (86.2%) of NDM-producers were susceptible to amikacin. In addition, we found a $bla_{\text{NDM-5}}$ and *mcr-1* co-harboring *E. coli* resistant to polymyxin B. These findings limited the utility of ceftazidime-avibactam and polymyxin B and prompted the development of novel or combinational therapies to combat CRE strains. For example, aztreonam plus meropenem-vaborbactam and aztreonam plus ceftazidime-avibactam showed good antibacterial activity against NDM- and non-OXA-48-like producing *Enterobacteriaceae* (Biagi et al., 2019). The combination of colistin and amikacin showed

consistently bactericidal against NDM-5-bearing *mcr-1*-positive *E. coli*, which might be an alternative therapeutic option for the treatment of lethal infections (Zhou et al., 2017).

CONCLUSIONS

In conclusion, KPC-2, NDM, and OXA-48-like enzymes were the most prevalent carbapenemases among CRE clinical isolates in China. The most prevalent carbapenemase gene was $bla_{\text{KPC-2}}$ among *K. pneumoniae* isolated from adult patients, and bla_{NDM} among *E. coli* isolates from both children and adult patients. The $bla_{\text{OXA-232}}$ was only detected among *K. pneumoniae* isolates from children.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study protocol was approved by the Institutional Review Board of Huashan Hospital, Fudan University (Number: 2018-408).

AUTHOR CONTRIBUTIONS

FH and RZ designed the study. RH, QS, SW, and MP performed the experimental work. RH and DY collected the data. FH analyzed the data. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Evaluation of Two Methods for the Detection of Third Generation Cephalosporins Resistant Enterobacterales Directly From Positive Blood Cultures

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Due to the importance of a rapid determination of patients infected by multidrug resistant bacteria, we evaluated two rapid diagnostic tests for the detection of third-generation cephalosporins (3GC)-resistant Enterobacterales directly from positive blood cultures within 1 h: BL-RED™ (electrochemical method) and β-LACTA™ test (chromogenic method). A panel of 150 clinical strains characterized for their resistance profiles (e.g., penicillinases, extended-spectrum beta-lactamases (ESBLs), overproduction of cephalosporinase, carbapenemases, impermeability) was tested. Approximately 100 CFU of each isolate was spiked into sterile blood culture bottles and incubated in a BD BACTEC™ FX automated system (Becton Dickinson, USA). Positive blood cultures were examined to parallel testing using the BL-RED™ and β-LACTA™ tests and conventional susceptibility method (disc diffusion following EUCAST recommendations). For all phenotypes combined, the sensitivity, specificity, positive predictive value, and negative predictive value in the detection of 3GC resistance were, respectively (i) with BL-RED™: 45.7, 100, 100, and 54.2% and (ii) with β-LACTA™ test: 52.2, 100, 100, and 56.9%. The positivity of tests allows to adapt antibiotic treatment whereas the negative result requires other tests. Moreover, these tests detect most Ambler class A-producing Enterobacterales (KPC, ESBL, extended-spectrum OXY) with sensitivities and specificities of 87.5 and 99% for BL-RED™, respectively and both 100% for β-LACTA™ test (47/47 isolates). These two rapid tests failed to detect AmpC overexpressed (sensitivities of 2.7% for BL-RED™ and 0% for β-LACTA™ test) and Ambler class B-producing Enterobacterales (sensitivities of 40% for both tests) notably strains without ESBLs associated (sensitivities of 0% for both tests). BL-RED™ and β-LACTA™ tests are easy-to-use and mainly attractive when a positive result is obtained notably to detect most of the Ambler class A-producing Enterobacterales in <1 h after the positivity of the blood culture, allowing a rapid adaptation of the antibiotic therapy in patients.

Keywords: bacteremia, BL-RED™ test, β-LACTA™ test, blood cultures, extended-spectrum beta-lactamases, multidrug resistance, third-generation cephalosporins

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INTRODUCTION

The spread of multidrug resistant (MDR) Enterobacterales is a major public health problem notably related to the misuse and overuse of antibiotics (Laxminarayan et al., 2013; World Health Organisation, 2014). Extended-spectrum cephalosporins and carbapenems are currently become the first line to treat community-acquired and nosocomial infections (Coque et al., 2008), involving a growing problem of the expansion of non-susceptibility of Enterobacterales to third-generation cephalosporins (3GC) and carbapenems. This increase is largely due to the importance of extended-spectrum β-lactamases (ESBL), but also to the overproduced chromosomal or plasmid-mediated AmpC cephalosporinases and to the emergence of carbapenemases (Peirano and Pitout, 2019). The rapid optimization of antibiotic therapy, according to the organism and its resistance profile, is a major goal both for individual patients and for public health (Roca et al., 2015). Bloodstream infections (BSI) are 30% of admissions in ICU and is responsible for an increase in length of stay and a leading cause of mortality (close to 40%) (Kang et al., 2005). The importance of the early appropriate treatment is crucial in these pathologies, given the linear increase in the risk of mortality with each hour for which administration is delayed (Ibrahim et al., 2000; Ferrer et al., 2014). However, conventional microbiological methods are time consuming requiring 12–48 h to identify the causal microorganism and provide an antimicrobial susceptibility testing (AST). Over the last couple of years, the improvement of available rapid diagnostic tests directly from positive blood cultures has changed approaches for identification and AST (Maugeri et al., 2019). These tests have the advantage of providing fast results, optimizing antibiotic therapy, improving survival, and reducing the length of hospitalization (Perez et al., 2013). These new techniques promote the proper use of antibiotics by limiting the antimicrobial resistance and decreasing the medico-economic impact by (de)-escalation of the antibiotic therapy and reduction of hospital stay (Farfour et al., 2019). They include molecular biology tests identifying the MDR encoding genes (pathogen-specific real-time or multiplex PCR), spectrometry assays detecting β-lactamase hydrolytic activity (MALDI TOF-MS), biochemical tests, β-lactamases inhibitor-based tests, chromogenic tests and electrochemical test (Buchan et al., 2013; Renvoise et al., 2013; Bogaerts et al., 2016; Salimnia et al., 2016; Faron et al., 2017; Pantel et al., 2018).

The aim of the present study was to evaluate the performance of: (i) a new electrochemical test, BL-RED^{TM} (Beta-Lactamase Rapid Electrochemical Detection, CORIS BioConcept, Belgium); and (ii) a colorimetric test, β-LACTA^{TM} test (Bio-Rad, Marnes la Coquette, France), for the detection of 3GC-resistance on a panel of Enterobacterales directly from positive blood cultures within 1 h.

MATERIALS AND METHODS

Bacterial Panel

A panel of 150 Enterobacterales isolates from our regional MDR Gram-negative Bacilli Reference Lab (CARB-LR group) in the Occitanie region was tested (Pantel et al., 2014; Robert et al., 2014). The isolates were included with the following distribution: *Escherichia coli* (n = 62), *Klebsiella pneumoniae* (n = 29), *Enterobacter cloacae* (n = 20), *Klebsiella aerogenes* (n = 9), *Proteus mirabilis* (n = 7), *Morganella morganii* (n = 6), *Klebsiella oxytoca* (n = 5), *Citrobacter freundii* (n = 5), *Citrobacter koseri* (n = 2), *Providencia rettgeri* (n = 2), *Serratia marcescens* (n = 2), and *Hafnia alvei* (n = 1).

Different β-lactam resistance profiles were selected: (i) 3GC-susceptible isolates (n = 50), (ii) ESBL producers (n = 41, including three which also overproduced AmpC), (iii) chromosomal-hyperproduced (n = 36) or plasmid-mediated (n = 10) AmpC producers, (iv) extended-spectrum OXY (n = 3), and (v) carbapenemase producers (n = 30, including 12 ESBL, 4 chromosomal AmpC, and 1 plasmid-mediated AmpC). Twenty-two isolates producing ESBL, high level AmpC or OXY were resistant to ertapenem by membrane permeability alteration. The presence of ESBLs, plasmid mediated AmpC, and carbapenemases genes was previously confirmed by specific PCR and sequence analysis (Perez-Perez and Hanson, 2002; Pitout et al., 2004, 2007; Poirel et al., 2011). All characteristics of the strains are summarized in **Table 1**.

Sample Preparation

For each isolate, ∼100 CFU was spiked into sterile blood cultures [BD BACTEC^{TM} Plus Aerobic/F and BD BACTEC^{TM} Lytic/10 Anaerobic/F (BD Diagnostics, Le Pont de Claix, France)] containing 10 mL of fresh blood. Blood cultures were incubated in a BACTEC^{TM} FX automated blood culture device until they flagged positive for microbial growth. All positive blood cultures were divided into three samples: 1 mL for the conventional comparator methods, 1 mL for the β-LACTA^{TM} test (Bio-Rad, Marnes-la-Coquette, France) and 0.5 mL for the BL-RED^{TM} test (Coris BioConcept, Gembloux, Belgium).

Conventional Comparator Method

The positive blood cultures were subcultured on blood agar (bioMérieux, Marcy l'Etoile, France) for 18–24 h at 37 ± 2°C. Species-level identification was performed on isolated colonies on blood agar by the mass spectrometry (Vitek® MS, bioMérieux). AST was determined by the agar disc-diffusion method according to the EUCAST-SFM 2019 recommendations (www.eucast.org). The results of disc diffusion were used as the comparator for the 3GC resistance of each isolate (determined by the agar disc-diffusion size), using notably: ceftazidime 10 μg and cefotaxime 5 μg discs.

BL-RED^{TM} Test V1.0

All blood cultures were tested after the blood culture flagged positive as recommended by the manufacturer. For aerobic blood culture, a 40 μL sample was mixed with 40 μL of reagent and incubated 1 h at 37 ± 2°C. For anaerobic blood culture, a pre-analysis was needed: a 500 μL sample was centrifuged one time 2 min at 6,000 g; the pellet was re-suspended into 500 μL of NaCl solution (0.9%) and re-centrifuged 2 min at 6,000 g; this second pellet was suspended into 40 μL of buffer and 40 μL of reagent; the mix was incubated 1 h at 37 ±

TABLE 1 | Characteristics of the studied Enterobacterales isolates.

Resistance profile (no. of strains)		Species (no. of strains)	β-lactamase content (no. of strains)
3GC susceptible (50)	No resistance to β-lactams (19)	*E. coli* (18)	None (18)
		P. mirabilis (1)	None (1)
	Penicillinases (22)	*E. coli* (17)	TEM-1 (10)
			Inhibitor-resistant TEM (7)
		K. pneumoniae (3)	SHV-1 (2)
			Inhibitor-resistant TEM (1)
		C. koseri (2)	CKO (2)
	Cephalosporinases (9)	*M. morganii* (3)	Low-level AmpC (3)
		P. rettgeri (2)	Low-level AmpC (2)
		K. aerogenes (1)	Low-level AmpC (1)
		E. cloacae (1)	Low-level AmpC (1)
		E. coli (1)	Low-level AmpC (1)
		S. marcescens (1)	Low-level AmpC (1)
β-lactamases conferring resistance to 3GC (70)	ESBL (29)	*E. coli* (16)	CTX-M-group 1 (7)
			CTX-M-group 1 + CTX-M-group 8 (5)
			CTX-M-group 9 (4)
		P. mirabilis (5)	CTX-M-group 1 (3)
			CTX-M-group 9 (1)
			CTX-M no group typed (1)
		K. pneumoniae (4)	CTX-M-group 1 + SHV-1 (2)
			SHV-5 (1)
			CTX-M-group 1 + CTX-M-group 8 + SHV-1 (1)
		E. cloacae (3)	CTX-M-group 1 + High-level AmpC (1)
			CTX-M-group 9 + High-level AmpC (1)
			TEM-24 + High-level AmpC (1)
		K. oxytoca (1)	CTX-M-group 8 + OXY-1 (1)
	Chromosomal overproduced cephalosporinases (29)	*E. cloacae* (9)	High-level AmpC (9)
		E. coli (6)	High-level AmpC (6)
		K. aerogenes (6)	High-level AmpC (6)
		C. freundii (4)	High-level AmpC (4)
		M. morganii (3)	High-level AmpC (3)
		H. alvei (1)	High-level AmpC (1)
	Plasmid cephalosporinases (9)	*K. pneumoniae* (9)	DHA-1 + SHV-1 (9)
	Chromosomal overproduced penicillinases (3)	*K. oxytoca* (3)	High-level OXY-1 (3)
Carbapenemases (30)	Class A carbapenemase (6)	*K. pneumoniae* (5)	KPC-2 + SHV-1 (4)
			KPC-2 + CTX-M-group 1 + SHV-1 (1)
		E. cloacae (1)	IMI-1 + Low-level AmpC (1)
	Class B carbapenemase (10)	*E. cloacae* (4)	VIM-1 + High-level AmpC (3)
			VIM-1 + CTX-M-group 9 (1)
		E. coli (2)	NDM-1 + CTX-M-group 9 (1)
			NDM-1 + DHA (1)
		K. pneumoniae (2)	NDM-1 + CTX-M-group 1 + SHV-1 (2)
		C. freundii (1)	VIM-1 + High-level AmpC (1)
		P. mirabilis (1)	NDM-1 (1)
	Class D carbapenemase (12)	*K. pneumoniae* (4)	OXA-48 + SHV-1 (2)
			OXA-48 + CTX-M-group 1 + SHV-1 (2)
		K. aerogenes (2)	OXA-48 + Low-level AmpC (2)
		E. cloacae (2)	OXA-48 + CTX-M-group 1 + Low-level AmpC (2)

(Continued)

TABLE 1 | Continued

Resistance profile (no. of strains)	Species (no. of strains)	β-lactamase content (no. of strains)
	E. coli (2)	OXA-48 (1)
		OXA-181 + CTX-M-group 1 (1)
	K. oxytoca (1)	OXA-48 + OXY-1 (1)
	S. marcescens (1)	OXA-48 + Low-level AmpC (1)
Class B + D carbapenemase (2)	*K. pneumoniae* (2)	NDM-1 + OXA-48 + CTX-M-group 1 + SHV-1 (2)

TABLE 2 | Analytical performance of assays for detection of 3GC-resistant Enterobacterales isolates in positive blood cultures.

Method	Result	3GC cephalosporins		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)	Youden
		Resistant ($n = 92$)	Susceptible ($n = 58$)						
BL-RED™	Positive	43	0	46.7% (36.9–56.9)	100% (93.8–100)	100% (91.8–10)	54.2% (44.8–63.3)	67.3%	46.7%
	Negative	49	58						
β-LACTA™	Positive	48	0	52.2% (42.1–62.1)	100% (93.8–100)	100% (92.6–100)	56.9% (47.2–66.1)	70.7%	52.2%
	Negative	44	58						

PPV, positive predictive value; NPV, negative predictive value.

2°C. After this incubation, 20 μL of the mix was placed on the ceramic electrode and after in the sensor. The reagent includes a "false" 3GC substrate which is hydrolysed when there is a 3GC β-lactamase, this frees an electro-conductive product. The intensity of the electrochemical signal [measured by DropSens (Metrohm, Villebon Courtaboeuf, France)] is posted in nanoAmpere (nA). The threshold of positivity is 80 nA for blood culture.

β-LACTA™ Test

The protocol B of the manufacturer recommendations was followed for the treatment of the positive blood cultures. Three different centrifugations and different solutions (Triton 0.01%, NaCl solution) were used to obtain the final bacterial pellet. After using the reagents provided in the kit on the bacterial pellet, we can read visually the hydrolysis of the chromogenic substrate (HMRZ-86, cephalosporin) from yellow to red in 15 min for a positive test. No change in color was considered a negative result (no hydrolysis of HMRZ-86).

Statistical Analysis

The sensitivity, specificity, negative and positive predictive value (NPV and PPV, respectively) were calculated and comparison with molecular characterization which served as the reference standard. 3GC-sensitive isolate with a negative test and 3GC-resistant isolate with a positive test are considered correct. Conversely, 3GC-sensitive isolate with a positive test and 3GC-resistant isolate with a negative test are considered incorrect. Additionally, the 95% confidence intervals (CIs), accuracy and the Youden index were calculated.

RESULTS

Evaluation on 3GC-Susceptible Isolates

Among our panel, 50 isolates had no 3GC resistance. These isolates harbored either a penicillinase (CKO, TEM-1, SHV-1, inhibitor-resistant TEM) or a naturally chromosomally-encoded inducible AmpC cephalosporinase. The two assays confirmed the absence of 3GC-resistance in all strains: 0 nA for most of the strains with BL-RED™, yellow color with β-LACTA™ test.

Of note, two isolates (one wild-type *E. coli* and one TEM-1-producing *E. coli*) presented a very low intensity using BL-RED™ at 10 nA. However, they were still considered negative because the positive threshold for blood culture was established at 80 nA.

Global Detection of 3GC-Resistance

Concerning all isolates belonging to the tested panel, the performance of the BL-RED™ and β-LACTA™ tests were, respectively: sensitivity, 46.7% (CI 36.9–56.9%) and 52.2% (CI 42.1–62.1%); specificity, both 100% (CI 93.8–100%); NPV, 54.2% (CI 44.8–63.3%), and 56.9% (CI 47.2–66.1%) and PPV, both 100% (CI 91.8–100%; 92.6–100%). The Youden index was 46.7 and 52.2%, respectively (**Table 2**).

Evaluation on Ambler Class a β-Lactamase Producers

When considering only the Ambler class A ESBL (without carbapenemase associated), 79.3% (23/29) of the isolates were positive using the BL-RED™, with a signal >80 nA [100–1,220] (**Table S1**). Six isolates were negative: two CTX-M-group 1-producing *P. mirabilis*, one CTX-M-group 9-producing *E. coli*, one CTX-M-group 1 and 8-producing *E. coli*, one CTX-M-group 9-producing *E. cloacae* with altered membrane permeability, and one SHV-5-producing *K. pneumoniae* with altered membrane

TABLE 3 | Analytical performance of assays for detection of Ambler Class A β-lactamases-producing Enterobacterales isolates in positive blood cultures.

Method	Result	Class A b-lactamase*		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)	Youden
		Resistant ($n = 37$)	Susceptible ($n = 113$)						
BL-RED™	Positive	31	1	83.8% (68.9–92.4)	99.1% (95.2–99.8)	96.9% (84.3–99.5)	94.9% (89.4–97.7)	95.3%	82.9%
	Negative	6	112						
β-LACTA™	Positive	37	0	100% (90.6–100)	100% (96.7–100)	100% (90.6–100)	100% (96.7–100)	100%	100%
	Negative	0	113						

**IMI-producing E. cloacae strain susceptible to 3GC was excluded.*
PPV, Positive Predictive Value; NPV, Negative Predictive Value.

permeability. All these isolates ($n = 29$) were positive using the β-LACTA™ test that shows a sensitivity of ESBL detection of 100%. Interestingly, the two techniques detected the OXY-hyperproducing *K. oxytoca* isolates with low outer membrane permeability ($n = 3$).

Concerning Ambler class A carbapenemases, both tests were able to detect the KPC-producing isolates ($n = 5$) whatever the presence or absence of an ESBL (**Table S1**). Negative results were obtained with IMI-1-producing *E. cloacae* strain that is resistant to carbapenems but remains susceptible to 3GC.

The analysis of the different previous results suggested that the two methods evaluated in this study had an interest in the detection of Ambler class A producers (not restricted to ESBL producers). When considering all Ambler class A producers (excluding IMI producers), the performance of BL-RED™ and β-LACTA™ were, respectively: sensitivity, 87.5% (CI 75.3–94.1%) and 100% (CI 92.6–100%); specificity, 99.0% (CI 94.7–99.8%) and 100% (CI 96.4–100%); PPV, 97.7% (CI 87.9–99.6%) and 100% (CI 92.6–100%) and NPV, 94.4% (CI 88.3–97.4%) and 100% (CI 96.4–100%) (**Table 3**).

Evaluation on Ambler Class C β-Lactamase Producers

All AmpC-overproducing isolates ($n = 29$, excluding ESBL co-producers) tested were negative for the two techniques (**Table S2**). The alteration of outer membrane permeability (seven strains) did not influence the results. One isolate (*C. freundii*) presented a high intensity using BL-RED™ at 60 nA but under the positive threshold. The antibiogram analysis performed after blood cultures confirmed the resistance to 3GC and the presence of the hyperproduction of AmpC in all isolates.

Among plasmid-mediated AmpC producers ($n = 10$), only one strain (*K. pneumoniae* DHA-1 with decreased membrane permeability) was positive with BL-RED™ test (480 nA).

Evaluation on Ambler Class B and D β-Lactamase Producers

Despite the high level of 3GC resistance, Ambler class B carbapenemase producers were only detected (6/12) in both tests when the isolates were also ESBL co-producers.

Similarly, OXA-48 carbapenemases were only detected (7/14) by the two tests when the isolates were ESBL co-producers.

DISCUSSION

Enterobacterales are the most important etiologies of community-onset and hospital-acquired BSI (Laupland and Church, 2014). The diffusion of 3GC-resistance among Enterobacterales, resulting from the spread of ESBL- and carbapenemase-producing isolates represents a serious problem worldwide. This is particularly true for life-threatening infections such as BSI, for which inappropriate first-line antibiotic therapy has a dramatic impact on short-term mortality (Kumar et al., 2009; Zilberberg et al., 2014).

Rapid and reliable identification of MDR Enterobacterales in the clinical microbiology laboratory is essential to effective infection control. In this study, we evaluated two rapid and easy-to-use methods: BL-RED™ and β-LACTA™ tests. If the β-LACTA™ test has been used for few years on bacterial strains and directly from blood cultures, the present study is the first to evaluate the recently commercialized BL-RED™ test v1.0. Both tests allowed early detection of most Ambler class A β-lactamases conferring 3GC resistance (e.g., ESBL, OXY, and KPC) to Enterobacterales (**Table 3**) in <1 h after positivity of the blood culture with interesting performance (sensitivity and specificity to 83.8 and 99.1% for BL-RED™ test and both 100% for β-LACTA™ test) suggesting that the two tests may provide useful therapeutic guidance. However, when testing challenged 3GC-resistant isolates non-ESBL producers, the two tests showed poor performance. Of note, a very low detection rate of plasmid-mediated AmpC or AmpC-overproducing isolates could be observed: 2.4% for BL-RED™ and 0% for β-LACTA™ test. This limitation has been previously published concerning β-LACTA™ test, when testing with subculture on agar plates (Renvoise et al., 2013; Morosini et al., 2014; Compain et al., 2015). The lack of sensitivity for the detection of high level AmpC must be balanced against the fact that ESBL producers are predominant among 3GC resistant Enterobacterales in European countries, notably among BSI (European Antimicrobial Resistance Surveillance Network (EARS-Net), 2018). Moreover, early adaptation by cefepime could be proposed when naturally AmpC producer was identified by MALDI-TOF MS, as previously suggested (Mizrahi et al., 2018). Another limitation of both tests was the failure to identify metallo-β-lactamase-producing isolates directly in blood cultures, whereas β-LACTA™ test carried

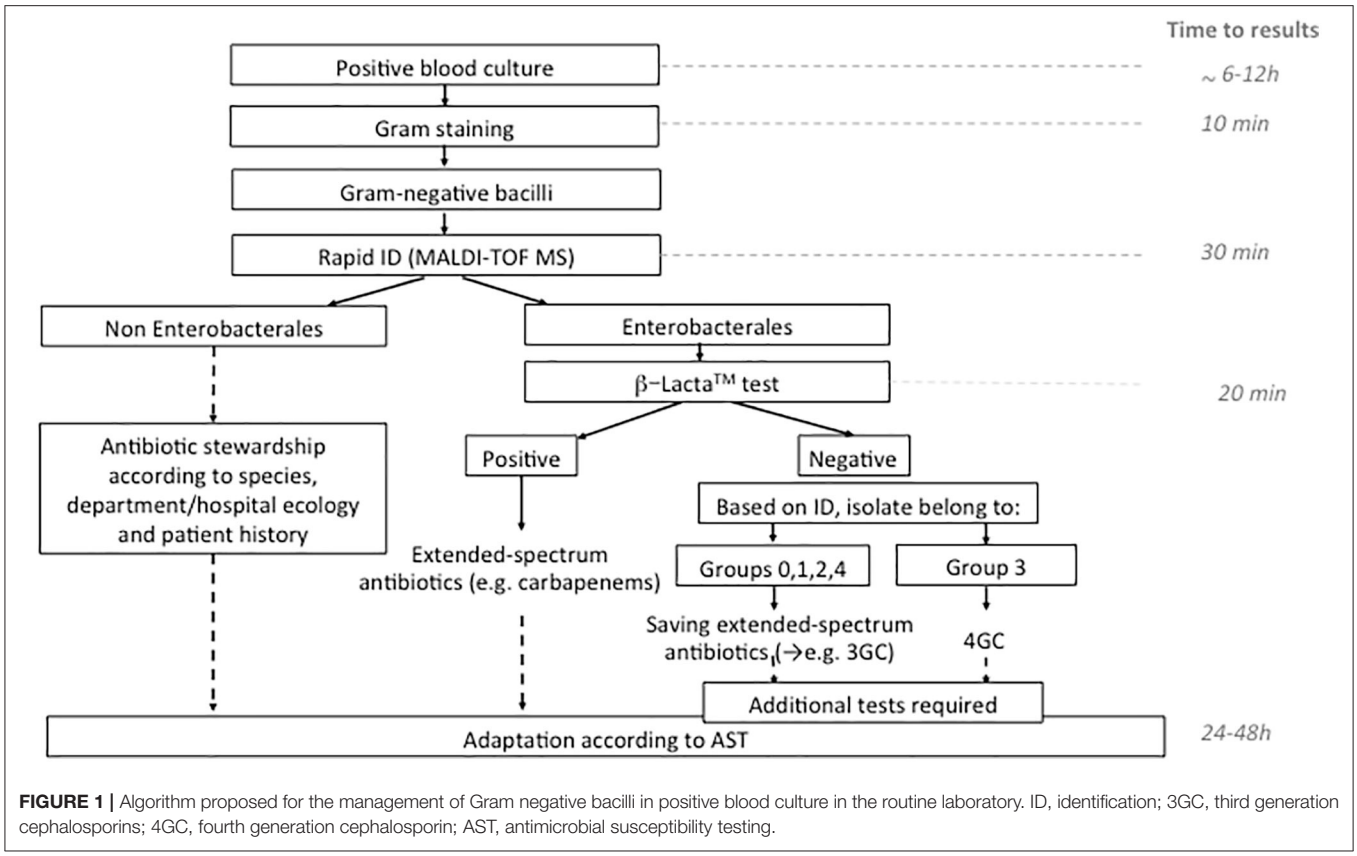

FIGURE 1 | Algorithm proposed for the management of Gram negative bacilli in positive blood culture in the routine laboratory. ID, identification; 3GC, third generation cephalosporins; 4GC, fourth generation cephalosporin; AST, antimicrobial susceptibility testing.

out with VIM-1-producers on agar plates showed acceptable sensitivity (Morosini et al., 2014). The lack of detection of metallo-β-lactamase-producing Enterobacterales isolates have been previously reported with phenotypical tests applied on blood culture, the zinc concentration in the medium being essential for an efficient detection of Ambler class B enzymes (Dortet et al., 2014; Pantel et al., 2018). Concerning class D carbapenemase producers, the isolates are frequently associated with ESBL positive producers (Bakthavatchalam et al., 2016) allowing their detection with both evaluated tests (7/7, 100%). Without the presence of ESBL-carrying plasmids, the tests failed to identify this resistance mechanism. To date, molecular methods remain the faster tool with a higher sensitivity to detect the carbapenemase-producing Enterobacterales (Rood and Li, 2017).

If no study has previously evaluated the BL-RED™ test, some previous works were performed on the use of β-LACTA™ test combined with MALDI-TOF MS from positive blood cultures (Compain et al., 2015; Walewski et al., 2015; Hasso et al., 2017; Mizrahi et al., 2018). The first study conducted in 2015 by Compain et al. evaluated the β-LACTA™ test on 3-h subcultures. 84.8% of the 33 blood culture isolates resistant to 3GC were correctly detected Compain et al. (2015). Walewski et al. (2015) used the test on bacterial pellets from the blood culture broths after treatment by saponin followed by two washes, with 95.7% of sensitivity and 100% of specificity for identifying ESBL-producing Enterobacterales. Hasso et al. (2017) evaluated the accuracy of the β-LACTA™ test for rapid detection of ESBL-producing *E. coli* and *Klebsiella* spp. from smudge plates prepared with positive blood cultures. The authors noted a sensitivity and specificity of 100 and 97.8%, respectively. Finally, the last two studies carried out in France by Mizrahi et al. (2018) and Farfour et al. (2019) obtained similar results: excellent performance regarding ESBL-producers and lack of sensitivity for Group 3 Enterobacterales. All results underlined the interest of these rapid techniques to be used effectively in daily laboratory practice.

The use of rapid phenotypic susceptibility techniques have demonstrated their interest in an optimized antimicrobial treatment (Schneider et al., 2019). Globally, our study shows excellent PPV (both 100%) but poor NPV (46.7 and 52.2%) for the BL-RED™ and β-LACTA™ tests, respectively. This reinforces the idea to only manage positive results with these assays and adapt antibiotic treatment whereas the negative results need to use additional tests. In this way, we suggest an algorithm incorporating MALDI-TOF MS combined with β-LACTA™ test for optimized Ambler class A producers detection with the advantage of being rapid, affordable, and simple (**Figure 1**). This algorithm could be accurate to give in <2 h a decisive orientation in the antibiotic management of sepsis or septic shock patients, particularly when the β-LACTA™ test is positive. An early adaptation by 4GC could be proposed when naturally AmpC producer was identified by MALDI-TOF MS with a negative β-LACTA™ test. However, its use must be debated. Recently, Dépret et al. (2018) evaluated the rapid identification

using MALDI-TOF MS results only vs. the combined MALDI-TOF MS and the β-LACTA^{TM} test in a method close to our algorithm. No impact on the current standard choice of therapy in terms of escalation/de-escalation or in reduction of carbapenems prescription was observed. Indeed, only 9% of the positive blood cultures in this study were confirmed as ESBL-producing, decreasing the interest of the techniques. This suggests that the use of a rapid test for Ambler class A-producers detection would be useful in a population with high prevalence of these enzymes and/or when treatment choices are not made by infectious disease specialists. In contrast, Mizrahi et al. (2018) have demonstrated the crucial role played by rapid detection of 3GC resistance with β-LACTA^{TM}. In this study, 75% (21/28) of the patients with BSI involving 3GC-resistant bacteria received a non-adapted first-line treatment. β-LACTA^{TM} test performed on blood culture significantly reduced the delay for treatment adaptation (28.1 h) and patient isolation (35 h). In 2019, Farfour et al. (2019) observed the same impact of rapid diagnostic tests on the management of BSI. Patients receiving a rapid strategy (rapid identification and 3GC-susceptibility testing) received more frequently an effective and appropriate antibiotic therapy than patients receiving conventional strategy on the first day of BSI diagnosis. Studies in settings where the prevalence of Ambler class A producers is high must be done to definitively conclude on the interest of the rapid technique.

In conclusion, the two evaluated assays (BL-RED^{TM} and β-LACTA^{TM} tests) showed excellent PPV, at least for the detection of the common Ambler class A family conferring 3GC-resistance and are suitable for the routine microbiology laboratory, decreasing the time to detection, and the need to refer these isolates to molecular biology confirmation. When a positive result is detected, these techniques permitted a quick and easy detection of the presence of ESBL-producing Enterobacterales in a clinical sample. They may help clinicians to guide appropriate antimicrobial therapy in septic patients and presumably improve their prognosis in a country or region where the Ambler class A family is particularly prevalent.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

CD has performed experiments, statistical analysis, and wrote the manuscript. AB has performed experiments and critically reviewed the manuscript. J-PL and AP have conceived the study, discussed the results, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Epidemiology and Risk Factors for Carbapenem-Resistant *Klebsiella Pneumoniae* and Subsequent MALDI-TOF MS as a Tool to Cluster KPC-2-Producing *Klebsiella Pneumoniae*, a Retrospective Study

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Background: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) appeared recently and now presents a particularly critical problem to hospitalized patients worldwide. We aim to investigate the epidemiology and the risk factors for CRKP colonization and infections, and to evaluate the application performance of MALDI-TOF MS in clustering CRKP.

Results: CRKP colonization and infections incidence was 2.7 (35/1,319,427) per 100,000 patient-days. Inpatients in CRKP group had higher medical expense than CSKP group. Inpatients with underlying conditions, particularly with pulmonary diseases, and with antimicrobial use prior to culture within 30 days, especially with carbapenem use, were risk factors for CRKP acquisition. All CRKP isolates were detected producing KPC-2. The MALDI-TOF MS system and PFGE system provided similar results, with a good concordance between the two methods (adjusted Rand's coefficient, 0.846) and a high probability of MALDI-TOF MS to predict PFGE results (Wallace coefficient, 0.908).

Conclusions: Underlying conditions, particularly pulmonary diseases, and antimicrobial use prior to culture within 30 days, especially carbapenem use, are risk factors for CRKP acquisition. Bla_{KPC-2} is the mainstream gene of CRKP in our geographic area of analysis. As only simple sample preparation is needed and the results can be obtained in a short time, MALDI-TOF MS may be considered a probable alternative to PFGE in clustering KPC-2-producing CRKP.

Keywords: carbapenems, resistance, *Klebsiella pneumonia*, carbapenem-resistant *Klebsiella pneumonia*, MALDI-TOF MS

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BACKGROUND

Carbapenems are widely used due to their broad spectrum of activity. Nevertheless, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) appeared and now presents a particularly critical problem to hospitalized patients worldwide (Yigit et al., 2001; Canton et al., 2012; McConville et al., 2017; Asai et al., 2018). The limited clinical options often make anti-infective therapy extremely difficult and also cause an extra financial burden on patients. Thus, it is necessary to identify the risk factors to prevent CRKP colonization and infections.

Molecular typing of bacterial isolates is the key strategy to identify clusters that are due to the transmission of clonal strains. Multilocus sequence typing (MLST), the repetitive sequence-based PCR Diversi Lab system and pulsed-field gel electrophoresis (PFGE) are good genotyping approaches, but these techniques remain time-consuming with a substantial cost. Rapid methods for molecular typing in colonization or infections with pathogens can not only provide basis for preventing cloning spread but also timely treatment. Therefore, quick methods that can be easily integrated into the routine work flow and do not cause increased costs are important (Sauget et al., 2017). Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been used as a simple tool for typing in infections with bacteria such as *Enterobacter cloacae* (Khennouchi et al., 2015). But other researcher do not recommend MALDI-TOF-based typing as a bacterial typing method given the heterogeneity in comparison to genotyping (Sachse et al., 2014). Thus, the application performance of MALDI-TOF MS as a clustering analysis method is still controversial.

Here, we set out to conduct a study for CRKP in Xiamen, a southern area in China, and we considered the following objectives: (1) study the epidemiology and risk factors for CRKP colonization and infections in this area, (2) evaluate the application performance of MALDI-TOF MS in clustering CRKP.

MATERIALS AND METHODS

Patients and Settings

With the intent of examining prevalence, the background of the patients and the risk factors of CRKP acquisition (colonization and infection), we conducted a case-controlled study. A retrospective epidemiologic surveillance study of CRKP colonization and infections was conducted within a 1900-bed academic Medical Center in the southern area of China from 1 January 2015 to 31 January 2017. Either CRKP colonization or infections cases during the inpatients' stay period in hospital were classified as the case group. Patients who were negative for CRKP but positive for carbapenem-susceptible *Klebsiella pneumoniae* (CSKP) during their stay in hospital were used as the selection pool for the control group during the same study period. Exclusion criteria were community-acquired colonization and infections, missing key data, screening samples, and subsequent episodes in the same patient. The same exclusion criteria were applied to cases and controls.

CRKP cases were selected by a review of microbiological reports. All identified inpatients were initially eligible to participate, and their medical charts were reviewed. For inpatients with multiple episodes of colonization and infection with CRKP, only data relevant to the first episode were collected and analyzed. A colonization or infection case is defined according to CDC definitions of nosocomial infections (Garner et al., 1988).

The CSKP cases as control group were randomly selected from the same units where the inpatients isolated with CRKP during the study period. Records of the control participants were cross-referenced with microbiology results to ensure that they did not have any CRKP positive cultures. Controls whose records had insufficient information were replaced by other randomly selected controls. For inpatients with multiple episodes of infection with CSKP, only data relevant to the first episode were collected and analyzed. The age ($\pm$2 years) and sexes of the patients were matched to inpatients with CRKP colonization and infections, and the ratio for the CRKP:CSKP group was 1:2. We used age ($\pm$2 years) and sexes as the matching variables because both two are strong confunders and good candidates for direct mathing (Mansournia et al., 2018). We set a ratio of 1:2 in this study for two reasons: (1) concern for sufficient numbers in a stratified analysis; and (2) the increase in power given the expected prevalence of exposure among the controls (Hennessy et al., 1999).

Both case and control groups' data were collected from a database of hospital infection monitoring system. This database drew information from numerous sources, including patients' electronic health record, laboratory, microbiology, and medication administration records.

For identifying possible risk factors of CRKP colonization and infections, patients' demographic characteristics and medical conditions were collected from the electronic sources mentioned above by comparing the CRKP and CSKP groups.

This study was approved by the local Ethics Committee of The First Affiliated Hospital of Xiamen University and complied with the Declaration of Helsinki (2008). Written and informed consent was obtained from all participants.

Definition of CRKP

A CRKP case was defined as the first clinical *Klebsiella pneumoniae* positive culture from inpatient with one or more of the following criteria, minimum inhibitory concentrations (MICs) for meropenem/imipenem $\geq$4 mg/L, MICs for ertapenem $\geq$2 mg/L according to the CLSI guidelines (CLSI, 2020).

Abbreviations: CRKP, carbapenem-resistant *Klebsiella pneumoniae*; CSKP, carbapenem-susceptible *Klebsiella pneumonia*; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; PCRs, multiplex polymerase chain reactions; NCBI, National Center of Biotechnology Information; FDA, Food and Drug Administration; MICs, minimum inhibitory concentrations; CLSI, the Clinical and Laboratory Standards Institute; IQR, interquartile range; ICU, intensive care unit; CCI, Charlson comorbidity index; COPD, pulmonary diseases included chronic obstructive pulmonary disease; OR, odds ratio; aOR, adjusted odds ratio; CI, confidence interval; SID, Simpson's index of diversity.

Microbiological Investigations

Species identification was performed with the Vitek 2 Compact automatic microbial analyzer (BioMerieux, Marcy—l'Etoile, France) and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; BioMerieux, Marcy-l'Etoile, France).

Molecular Detection

Multiplex polymerase chain reactions (PCRs) were used to detect the presence of carbapenemase genes (bla_{NDM}, bla_{KPC}, bla_{IMP}, and bla_{VIM}). PCR products were sequenced, and the nucleotide and deduced protein sequences were analyzed with software programs that were available from the National Center of Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov).

Antimicrobial Susceptibility Testing

With regard the antimicrobial susceptibility test, MICs of ceftazidime, cefepime, cefotaxime, ceftriaxone, piperacillin/tazobactam, meropenem, ertapenem, imipenem, aztreonam, amikacin, gentamycin, tobramycin, ciprofloxacin, levofloxacin, trimethopri-sulfamethoxazole, and tigecycline were determined with the Vitek 2 Compact automatic microbial analyzer (BioMrieux, Marcy-l'Etoile, France) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. In addition, MICs of ertapenem, imipenem, meropenem and colistin-polymyxin-B were determined using E-test strips (BioMerieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Ertapenem, imipenem and meropenem MICs were interpreted according to the CLSI guidelines. The interpretive criteria for colistin-polymyxin-B was based on the breakpoints of EUCAST. And the interpretive criteria for tigecycline was based on the breakpoints of the Food and Drug Administration (FDA).

Identification and Clustering of *Klebsiella Pneumoniae* Using MALDI-TOF MS

The *Klebsiella Pneumoniae* isolates were plated on Columbia blood agar (bioMérieux, Marcy l'Étoile, France) and incubated for 18 h to 24 h at 37°C. Isolated colonies of each strain were selected and used for MALDI-TOF MS identification using the MALDI-TOF MS (BioMerieux, Marcy-l'Etoile, France), as previously described (Rodel et al., 2019). The obtained spectra were manually selected in the spectra mode of SARAMIS Premium software (BioMerieux, Marcy-l'Etoile, France). Cluster analysis were performed by spectra compared to each other in SARAMIS RUO database according to the manufacturer's instructions (Vitek MS Plus SARAMIS Premium user manual, BioMerieux, Marcy-l'Etoile, France). Consensus spectra were analyzed with a single link agglomerative clustering algorithm, applying the relative taxonomy analysis tool of SARAMIS premium software to show the resulting dendrogram with differences and similarities in relative terms (percent matching masses). As a standard setting, the mass signal intensity was not considered in the cluster analysis. According to the type assignment, we defined a cut-off value was >75% similarity (Meng et al., 2019).

Typing of *Klebsiella Pneumoniae* Using Pulse-Field Gel Electrophoresis (PFGE)

The 1 day, standardized PFGE protocol (Han et al., 2013) was used for all CRKP isolates during the study periods. Cell suspensions were placed in polystyrene tubes (Falcon; 12 × 75 mm), and their optical densities were adjusted to 3.8–4.0 by a Densimat photometer (BioMérieux, Marcy l'Etoile, France). Slices of CRKP agarose plugs were digested using 50 U of XbaI (TaKaRa Bio, Dalian, China) per slice for 4 h at 37°C, and electrophoresis was performed using a CHEF-DRIII system (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was conducted with a switch time of 6 to 36 s for 18.5 h, and images were captured using a Gel Doc 2000 system (Bio-Rad) and converted to TIFF files which were analyzed by BioNumerics version 5.1 software (Applied Maths, Kortrijk, Belgium). A similarity analysis of the PFGE patterns was performed by calculating the Dice coefficients (S_D) and clustering was performed using the unweighted-pair group method with average linkages (UPGMA).

Statistical Analysis

CRKP colonization and infections incidence was reported as the number of CRKP cases per 100,000 hospital patient-days. Descriptive statistics were used to summarize the clinical and epidemiologic characteristics of CRKP colonization and infections. Continuous variables were presented as medians with the range or interquartile range. For categorical variables, the percentage of patients or isolates in each category was calculated. The Chi-square test were used to compare categorical variables. The Mann-Whitney U-test was used to compare continuous variables. To identify risk factors for isolating CRKP, the Chi-square test were performed. Factors showing $p < 0.05$, were considered candidate predictors that were significantly related to CRKP isolation and were extracted; following which, multivariate analysis was performed for these factors using the Logistic Regression model. The discriminatory power of each typing method was assessed using Simpson's index of diversity (SID), calculating the probability that two unrelated strains sampled from the test population will be placed into different typing groups (Hunter and Gaston, 1988), and the 95% confidence intervals (CI) of the SID values were calculated as described previously (Grundmann et al., 2001). The quantitative concordance between typing methods was analyzed by using adjusted Randand Wallace coefficients (Carrico et al., 2006). All analyses were performed using the IBM SPSS statistical software package version 25 (IBM Corp, Armonk, NY, USA).

RESULTS

Prevalence of CRKP Colonization and Infections

CRKP colonization and infections incidence during 1 January 2015 and 31 January 2017 was 2.7 (35/1,319,427) per 100,000 patient-days. During 1,319,427 patient-days, we found that 2,875 patients with *Enterobacteriaceae* isolates were obtained, and 36 patients with CRKP colonization and infections were eligible for

screening in this study. After application of the exclusion criteria, 35 inpatients were included. Five of 35 patients isolating CRKP had infections. All the five were bloodstream infections, all were cured. The characteristics of the inpatients are shown in **Table 1** and included 27 males and 8 females. The median age was 73 years (range 0–91 years).

Clinical and Microbiological Characteristics in CRKP Inpatients

We found that 25.7% (9/35) of inpatients had functional status deterioration seen in **Table 1**. One patient in ICU died within 30 days of admission that was not due to that of a bloodstream infection, but of multiple organ failure caused by cancer, the same reason as the one patient died in CSKP group. CRKP group patients had higher medical expense than those among CSKP group (as shown) in **Table 1** ($p = 0.015$). With regard the antimicrobial susceptibility test, colistin-polymyxin-B, and tigecycline retained excellent activity, with a susceptibility rate of more than 97%. Trimethopri-sulfamethoxazole remained quite susceptible, with susceptibility rate of 57.1%. All isolates of CRKP were detected producing KPC-2 carbapenemase. Further, no CRKP was detected producing two or more gene types of carbapenemase.

Analysis of Risk Factors for Patients Isolating CRKP

The results of univariate analysis using the Chi-square test in patients with CRKP are shown in **Table 1**. Eight parameters were associated with patients isolating CRKP, namely one or more underlying conditions ($p = 0.007$), pulmonary diseases ($p < 0.001$), gastric tube ($p < 0.001$), antifungal agents ($p = 0.021$), one or more antimicrobial use prior to culture within 30 days ($p < 0.001$), carbapenem use ($p < 0.001$), quinolone use ($p < 0.001$) and length of stay ($p < 0.001$).

Multivariate logistic regression analysis was applied to analyze the prognostic significance of these eight factors, revealing that one or more underlying conditions ($p = 0.031$, odds ratio [OR]: 3.991, 95% confidence interval [CI]: 1.132–14.068), pulmonary diseases ($p = 0.007$, odds ratio [OR]: 5.293, 95% confidence interval [CI]: 1.590–17.618), one or more antimicrobial use prior to culture within 30 days ($p = 0.009$, odds ratio [OR]: 17.358, 95% confidence interval [CI]: 2.051–146.931) and carbapenem use ($p = 0.018$, odds ratio [OR]: 5.118, 95% confidence interval [CI]: 1.321–19.829) were indeed independent risk factors for patients isolating CRKP. Four different clusters of 35 KPC-2-producing CRKP isolates were identified by PFGE and MALDI-TOF MS. Cluster I, II, and III were mainly isolated from geriatrics and respiratory wards. Cluster IV was mainly isolated from pediatrics and icu departments.

Clonal Typing KPC-2 Producing CRKP by PFGE

The PFGE system identified four different clusters of 35 KPC-2-producing CRKP isolates (**Figure 1A**). All indistinguishable isolates in four clusters presented an average genomic similarity

TABLE 1 | Comparison with patients' characteristics between CRKP and CSKP groups.

Characteristic[a]	CRKP group[b] ($n = 35$) n, %	CSKP group[b] ($n = 70$) n, %	p-value
Health care exposure during prior year			
Acute care hospitalization	5 (14.3)	9 (12.9)	0.839
Dialysis	1 (2.9)	2 (2.9)	1.000
Resident of a long-term-care facility	6 (17.1)	14 (20.0)	0.725
Transfer to ICU within 30 days	6 (17.1)	5 (7.1)	0.115
Receipt of corticosteroids	4 (11.4)	9 (12.9)	0.834
Underlying conditions			
One or more underlying conditions	17 (48.6)	16 (22.9)	0.007
Cancer[c]	4 (11.4)	14 (20.0)	0.272
Diabetes mellitus	8 (22.9)	16 (22.9)	1.000
Heart diseases[d]	4 (11.4)	6 (8.6)	0.638
Hypertension	11 (31.4)	29(41.4)	0.320
Liver diseases[e]	7 (20.0)	14 (20.0)	1.000
Neurological diseases[f]	5 (14.3)	20 (28.6)	0.105
Pulmonary diseases[g]	28 (80.0)	25 (35.7)	<0.001
Renal diseases[h]	8 (22.9)	16 (22.9)	1.000
CCI score [Median (IQR)]	2.0 (4.0)	2.0 (4.0)	1.000
CCI ≥ 3	15 (42.9)	31 (44.3)	0.889
Smoking history	6 (17.1)	4 (5.7)	0.060
Indwelling devices prior to culture			
Central venous catheter	15 (42.9)	20 (28.6)	0.143
Gastric tube	24 (68.6)	21 (30.0)	<0.001
Tracheal cannula	5 (14.3)	12 (17.4)	0.708
Tracheotomy	10 (28.6)	10 (14.3)	0.079
Urinary catheter	20 (57.1)	36 (51.4)	0.580
Laboratory findings			
White blood cells/mm^3			
Median (IQR)	12,350 (3, 900)	9,790 (6,853)	0.054
Subgroup			
<4,000	0 (0.0)	6 (8.6)	0.074
>10,000	24 (68.6)	39 (55.7)	0.205
C-reactive protein > 10 mg/liter	16 (45.7)	39 (55.7)	0.333
Procalcitonin			
0.5 to 2 ng/ml	5 (14.3)	20 (28.6)	0.105
>2 ng/ml	10 (28.6)	18 (25.7)	0.755
Use of proton pump inhibitors	8 (22.9)	18 (25.7)	0.749
Antifungal agents	8 (22.9)	5 (7.1)	0.021
Antimicrobial use prior to culture within 30 days			
One or more Antimicrobial uses	34 (97.1)	33 (47.1)	<0.001
Third- or fourth-generation cephalosporin use	6 (17.1)	15 (21.4)	0.605
Carbapenem use	16 (45.7)	5 (7.1)	<0.001
Quinolone use	14 (40.0)	6 (8.6)	<0.001

(Continued)

TABLE 1 | Continued

Characteristic[a]	CRKP group[b] ($n = 35$) n, %	CSKP group[b] ($n = 70$) n, %	p-value
Specimen isolating *Klebsiella pneumoniae*			
Respiratory specimen	13 (37.1)	31 (44.3)	0.484
Urine	9 (25.7)	16 (22.9)	0.746
Blood	6 (17.1)	13 (18.6)	0.858
Ascites	2 (5.7)	2 (2.9)	0.471
Bile	1 (2.9)	3 (4.3)	0.718
Skin	1 (2.9)	2 (2.9)	1.000
Others	3 (8.6)	3 (4.3)	0.372
Length of stay [Median (IQR)][i]	34 (38.0)	16 (20.0)	<0.001
Discharge disposition			
Recovery	7 (20.0)	13 (18.6)	
Improvement	18 (51.4)	39 (55.7)	0.375
Patients transfer to other hospital	0 (0.0)	1 (1.4)	
Functional status deterioration	9 (25.7)	16 (22.9)	
In-hospital mortality	1 (2.9)	1 (1.4)	0.015
Medical expense for admission[i] (Mean ± SD, RMB)	107,472.27 ± 110,564.67	60,738.59 ± 72,925.18	

CRKP, carbapenem-resistant Klebsiella pneumoniae; CSKP, carbapem-sensitive Klebsiella pneumoniae; CCI, Charlson comorbidity index.
[a] IQR, interquartile range; ICU, intensive care unit.
[b] Data are presented as the number/total number (%), unless otherwise indicated.
[c] Cancer includes malignancy of the lung, digestive tract, gynecology, hematological system, and neurological system.
[d] Heart diseases include congestive heart failure, coronary heart disease, valve replacement, and congenital heart disease.
[e] Liver diseases included cirrhosis, hepatitis, liver abscess, hepar adiposum (i.e., fatty liver), and hepatic injury.
[f] Neurological diseases include stroke, transient ischemic attack, cerebral palsy, and meningitis.
[g] Pulmonary diseases included chronic obstructive pulmonary disease (COPD), asthma, interstitial lung disease, history of pneumonia and tuberculosis, emphysema, respiratory failure, and infection.
[h] Renal diseases include azotemia and chronic kidney disease.
[i] Only patients admitted to hospital were evaluated.

ratio of >90.0%. The four clusters were significantly different from each other in the percentage of similarity.

Clustering CRKP Isolates Using MALDI-TOF MS

All the 35 CRKP isolates were correctly identified at the species level by MALDI-TOF MS. The hierarchical clustering of MALDI-TOF peak profiles identified four different clusters, substantially interchangeable with those obtained with the PFGE system (**Figure 1B**). The statistical analysis of the data showed that the PFGE system (Simpson's index, 0.608; 95% CI, 0.512–0.705) and MALDI-TOF MS system (Simpson's index, 0.640; 95% CI, 0.532–0.748) provided similar results, with a good concordance between the two methods (adjusted Rand's coefficient, 0.846) and a high probability of MALDI-TOF MS to predict PFGE results (Wallace coefficient, 0.908).

DISCUSSION

This present retrospective case-controlled study assessed potential risk factors for the development of colonization and infections by CRKP in hospitalized patients. In this study, it demonstrates that inpatients with one or more underlying conditions, especially pulmonary diseases, and antimicrobial use prior to culture within 30 days, particularly carbapenem use, were risk factors for CRKP acquisition. And four different clusters of KPC-2-producing CRKP isolates were identified. Cluster I, II, and III were mainly isolated from geriatrics and respiratory wards. Patients with underlying conditions, such as pulmonary diseases, often visit an outpatient clinic or transfer from icu to respiratory ward, or transfer between the two, even from one hospital to another hospital, and they are subsequently exposed to additional health care and antimicrobials, which are among the most prominent risks (Gupta et al., 2011). These patients could have poor functional status and severe clinical symptoms, which not only places them at a greater risk of an infection caused by CRKP but also results in higher medical expense. Our study demonstrated that medical expense for admission of CRKP groups were almost double higher than those of CSKP group (107,472 vs. 60,739 RMB, $p = 0.015$).

Among the four classes of β-lactamases defined by the Ambler classification system, the KPC β-lactamase, in Bush group 2f, belongs to Class A. Yigit et al. (2001) first reported KPC β-lactamases in *Klebsiella pneumoniae* strains isolated from a patient in North Carolina in the United States in 2001. After that, the KPC-producing organisms had being reported globally (Villegas et al., 2006; Wiener-Well et al., 2010; Canton et al., 2012; Mojica et al., 2012; Cuzon et al., 2013; Asai et al., 2018; Kim et al., 2018). Since in 2015, Biberg et al. (2015) reported KPC-2-producing *Klebsiella pneumoniae* in the Midwest region of Brazil, the rapid increase and dissemination of KPC-2, the primary type of β-lactamases, in CRKP from many areas, has become a significant public health challenge in the whole word (Gaiarsa et al., 2015). In this study, all CRKP isolates were detected with KPC-2 carbapenemase. The $bla_{\mathrm{KPC-2}}$ is the mainstream gene of CRKP in our geographic area of analysis.

Bacterial typing is an important method to identify the route of pathogen transmission. Currently, the main method for bacterial typing is the time-consuming and expensive molecular biology technique like Pulsed Field Gel Electrophoresis (PFGE) or Multilocus sequence typing (MLST). Nevertheless, with the application to cultured microorganism identification, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry MS (MALDI-TOF MS) presents incomparable advantages. However, as a new method of bacteria clustering, the application performance of mass spectrometry is controversial. Some studies reported that MALDI-TOF MS could be a good bacterial typing method in several kinds of bacteria, such as extended-Spectrum-β-Lactamase- and *armA* methyltransferase-producing *Enterobacter cloacae* clinical isolates, methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, *Serratia marcescens,* and *Citrobacter freundii* (Mencacci et al., 2013; Khennouchi et al., 2015; Steensels et al., 2017; Rodel et al., 2019). But, Jiang et al. (2019) employed

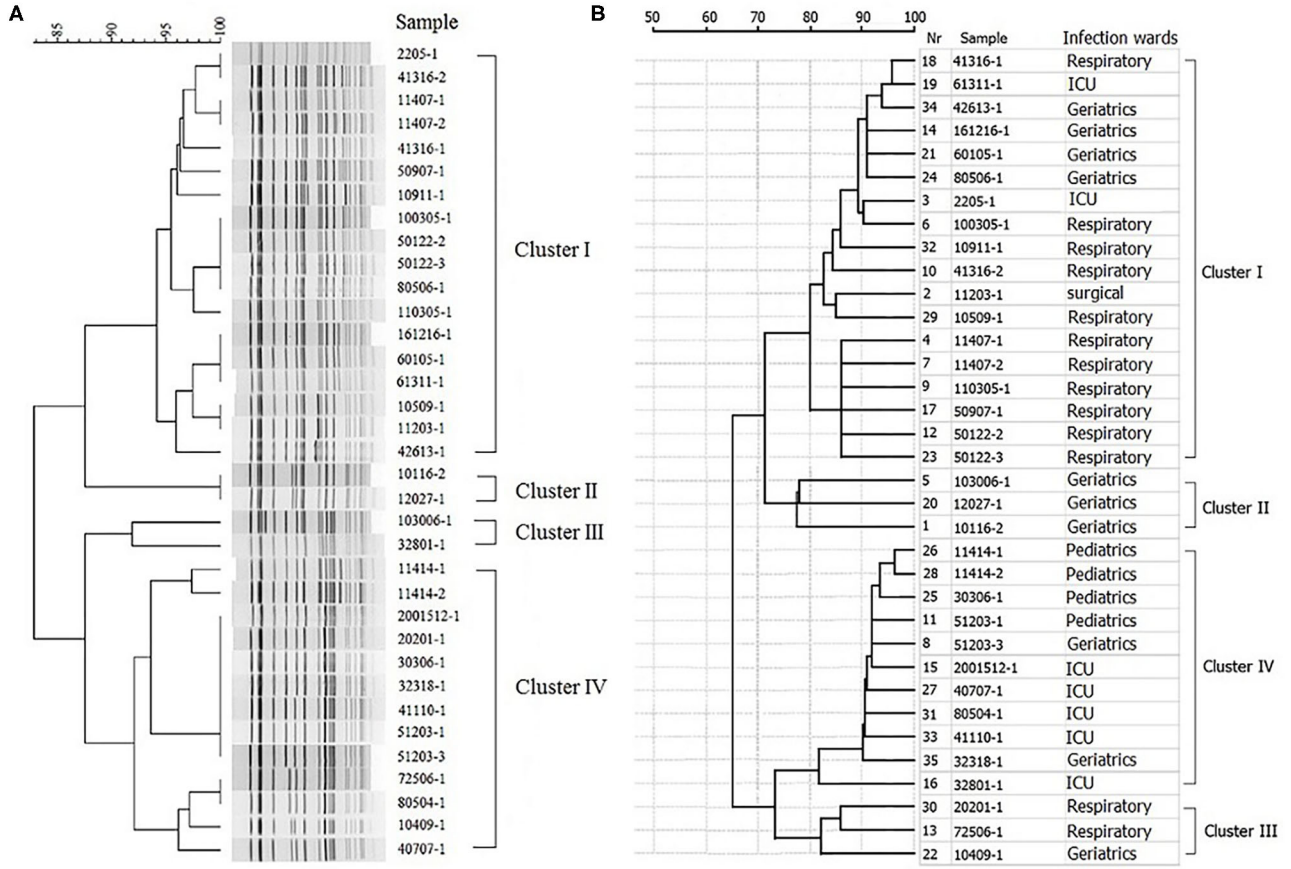

FIGURE 1 | Hierarchical clustering of KPC-2-producing carbapenem-resistant *Klebsiella pneumoniae* isolates by PFGE and MALDI-TOF MS. **(A)** Hierarchical cluster analysis provided by PFGE. **(B)** Hierarchical cluster analysis provided by MALDI-TOF MS.

44 CRKP isolates of 15 STs covering divere carbapenemases and they demonstrated that MALDI-TOF MS had a lower predictive power than PFGE. And Sachse et al. (2014) did not recommend MALDI-TOF-based typing as a bacterial typing method given the heterogeneity in comparison to genotyping.

In this study, all 35 CRKP isolates were correctly identified at the species level by MALDI-TOF MS. The hierarchical clustering of MALDI-TOF peak profiles identified four different clusters, substantially interchangeable with those obtained with the PFGE system. The statistical analysis of the data showed that the PFGE system and MALDI-TOF MS system provided similar results, with a good concordance between the two methods and a high probability of MALDI-TOF MS to predict PFGE results. Since rapid microorganism identification using MADI-TOF MS not only can lead to more effective antimicrobial use and reduced patient care costs (Galar et al., 2012; Tan et al., 2012; Huang et al., 2013; Perez et al., 2013), but also include the high through put, low reagent costs and ease of use, the usage of MALDI-TOF MS in clustering the CRKP of epidemic KPC-2 type was an agreeable practice and the subsequent clinical application would be meaningful to both hospital infection control and patients. It could be one of the choices to rapidly reveal the routes of transmission of infectious diseases. However, because of the small size of sample, further studies are needed to confirm our observations.

There were three limitations of this study. Firstly, information on the clinical characteristics and outcomes could not be completely acquired because of the limitations that are inherent in a retrospective clinical study. Second, this is a retrospective study with a relatively small study population. Furthermore, this study was a case-controlled design in which the level of risk factors were not equal to the expected level commonly seen in the population.

CONCLUSIONS

One or more underlying conditions, especially pulmonary diseases, and one or more antimicrobial use prior to culture within 30 days, particularly carbapenem use, are risk factors for CRKP acquisition. The $bla_{\mathrm{KPC-2}}$ is the mainstream gene of CRKP in our geographic area of analysis. As only simple sample preparation is needed and the results can be obtained in a short time, MALDI-TOF MS may be considered a probable alternative to PFGE in clustering KPC-2-producing CRKP.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local Ethics Committee of The First Affiliated Hospital of Xiamen University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LF, HX, and XR designed the study. XLi, XM, and HZ analyzed data. LF and XLia drafted the manuscript. GH and XLia contributed to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00462/full#supplementary-material

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Treatment and Outcomes of Infections Caused by Diverse Carbapenemase-Producing Carbapenem-Resistant *Enterobacterales*

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Background: Diverse sequence types (ST) and various carbapenemase-producing carbapenem-resistant *Enterobacterales* (CP-CRE) infections, which complicate treatment strategies, have emerged in Singapore. We aim to describe these CP-CRE infections and clinical outcomes according to their carbapenemase types and determine the hierarchy of predictors for mortality that are translatable to clinical practice.

Methods: Clinically significant CP-CRE infections were identified in Singapore General Hospital between 2013 and 2016. Retrospectively, all clinically relevant data were retrieved from electronic medical records from the hospital. Univariate analysis was performed. To further explore the relationship between the variables and mortality in different subsets of patients with CP-CRE, we conducted recursive partitioning analysis on all study variables using the "rpart" package in R.

Results: One hundred and fifty five patients were included in the study. Among them, 169 unique CP-CRE were isolated. Thirty-day all-cause in-hospital mortality was 35.5% ($n = 55$). There was no difference in the severity of illness, or any clinical outcomes exhibited by patients between the various carbapenemases. Root node began with patients with Acute Physical and Chronic Health Evaluation (APACHEII) score ≥ 15 ($n = 98$; mortality risk = 52.0%) and <15 ($n = 57$; mortality risk = 9.0%). Patients with APACHEII score ≥ 15 are further classified based on presence ($n = 27$; mortality risk = 23.0%) and absence ($n = 71$, mortality risk = 62.0%) of bacterial eradication. Without bacterial eradication, absence ($n = 54$) and presence ($n = 17$) of active source control yielded 70.0 and 35.0% mortality risk, respectively. Without active source control, the mortality risk was higher for the patients with non-receipt of definite combination therapy ($n = 36$, mortality risk = 83.0%) when compared to those who received ($n = 18$, mortality risk = 47.0%). Overall, the classification tree has an area under receiver operating characteristic curve of 0.92, with a sensitivity of 0.87 and specificity of 0.91.

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Conclusion: Different mortality risks were observed with different treatment strategies. Effective source control and microbial eradication were associated with a lower mortality rate but not active empiric therapy for CP-CRE infection. When source control was impossible, definitive antibiotic combination appeared to be associated with a reduction in mortality.

Keywords: treatment, outcomes, infections, carbapenemase-producing, carbapenem-resistance, *Enterobacterales*

INTRODUCTION

The growing incidence of carbapenem-resistant Gram-negative bacilli (CRGNB) is an urgent global healthcare challenge today (Paterson and Doi, 2007). Prevalence of carbapenem resistance among GNB in South and Southeast Asian countries, including Singapore, is potentially driven by extensive carbapenem use (Hsu et al., 2017). The number of carbapenem-resistant *Enterobacterales* (CRE) colonization cases reported swelled on the back of ramp-up screening within both public and private hospitals in Singapore (Marimuthu et al., 2017). High counts of carbapenemase-producing (CP) CRE were also recovered from the sewage systems of four main hospitals locally (Koh et al., 2015). Heightened surveillance of these organisms is crucial to their management as CRE is associated with increased mortality and limited treatment options (Molton et al., 2013).

Unlike countries in the United States and Europe, where a predominant *Klebsiella pneumoniae* clonal (ST-258) and resistance type (KPC) is observed, there is greater diversity in Singapore (Teo et al., 2016). This greatly complicates management strategies, including the selection of effective combinations for clinical use. Our local CRE is associated with a variety of resistance mechanisms (e.g., various carbapenemases production in IMPs, KPCs, NDMs, OXA-48, OXA-181, OXA-232, and dual-carbapenemase production with or without porin downregulation) and at least 16 sequence types (ST) (Teo et al., 2016).

To date, there is a lack of consensus on treatment recommendations for CRE infections and data is scanty for patients infected with CP-CRE of varied ST and mechanisms of resistance, especially in the types of carbapenemases production (Molton et al., 2013; Tzouvelekis et al., 2014). Our institution, which is the largest tertiary care hospital in Singapore and an international health hub with a diverse CP-CRE landscape (Teo et al., 2016; Marimuthu et al., 2017), has the highest number of CP-CRE infections.

This retrospective cohort study aims to describe our CP-CRE infections, the various treatment strategies, and the outcomes of CP-CRE infections according to their carbapenemase types and determines the hierarchy of predictors for mortality that are translatable to clinical practice.

METHODS

The retrospective cohort study was conducted in Singapore General Hospital (SGH), a 1,700-bed tertiary care hospital in Singapore. SingHealth Institutional Review Board provided approval with waiver of informed consent (CIRB number: 2014/912/F). Hospitalized adult patients ($\geq$18 years old) with clinical CP-CRE infections from 1st January 2013 to 31st December 2016 were included in the study. These patients were identified from a hospital microbiology database. Criteria for inclusion were as follows: (i) documented CP-CRE culture from clinically relevant sterile sites, with exclusion of positive cultures from the urinary system as it was difficult to retrospectively ascertain if the patient had a true infection; (ii) patient exhibited clinical signs and symptoms of sepsis with systemic inflammatory response from a documented or suspected site of infection, as defined in Society of Critical Care Medicine and European Society of Intensive Care Medicine Surviving Sepsis guidelines (Rhodes et al., 2017); (iii) patient received treatment of CP-CRE infection with at least two consecutive doses of antimicrobial, with the exception of single-dose aminoglycosides. In patients with more than one episode of CP-CRE infection, only the first infection was documented and analyzed.

Data was retrieved from electronic hospital medical records. Data includes demographic information, admission details, past medical history, comorbid conditions (according to Charlson Comorbidity Index, CCI; Charlson et al., 1987), past invasive procedure, prior antibiotic or immunosuppressive therapy, infection characteristics, severity on onset according to Acute Physical and Chronic Health Evaluation (APACHE II) (Knaus et al., 1985) and sepsis-related organ failure assessment (SOFA) (Jones et al., 2009), and treatment regimen. Primary outcome measured was 30-day all-cause in-hospital mortality. Secondary outcomes include time to clinical response, microbiological eradication, and occurrence of reinfection in 1 year.

The following terms were defined prior to data collection and analysis. Onset of infection was determined as the day of sampling of the positive CP-CRE culture. Baseline characteristics of patients and infection details were documented on the day of index culture. Infections were categorized according to the European Center for Disease Prevention and Control guidelines (European Centre for Disease Prevention Control, 2016). Prior hospital exposure was dated for the preceding 1 year, while antibiotic use and surgical operations or invasive procedures at bedside were dated 3 months from index culture. Septic shock was defined as sepsis, a life-threatening organ dysfunction caused by dysregulated host response to infection, with circulatory and metabolic dysfunction (Rhodes et al., 2017). Source control includes any intervention that physically removes the infectious source. Empiric and definitive antimicrobial treatments were

defined as regimens given prior to or after susceptibility of index culture was available, respectively. Adequate empirical treatment was defined as receipt of an empirical agent that is active *in vitro* against the pathogen and was administered for at least 48 h. Effective combination therapy was the concurrent use of at least one antimicrobial agent that was still active against the CP-CRE. Clinical improvement was defined as complete or partial response as determined by downtrending inflammatory markers and associated resolution of infective symptoms. Microbiological eradication involved a repeat negative culture of the CP-CRE from the same site after the index culture. Reinfection was defined as clinically significant CP-CRE infections occurring after clinical improvement or microbiological clearance of infection, 7-days after and within 1 year of index culture.

Carbapenem-resistant isolates were identified from the hospital microbiological database. The genus was determined using Vitek 2 ID-GN cards (bioMerieux Inc. Hazelwood, MO). Carbapenem susceptibility was determined using disk diffusion and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Polymerase chain reaction was conducted to identify the type of carbapenemase production (Hammoudi et al., 2014). Susceptibility of the following antibiotics were tested: amikacin, aztreonam, cefepime, doripenem, ertapenem, imipenem, levofloxacin, meropenem, piperacillin-tazobactam, polymyxin, tigecycline. Susceptibility of tigecycline and polymyxin was determined using Food and Drug Administration breakpoints (Pillar et al., 2008) and interpreted from CLSI breakpoints against *Enterobacterales* (Lat et al., 2011), respectively. Extensive drug resistance is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two antimicrobial categories) (Magiorakos et al., 2012).

Statistical Analysis

All statistical analyses were performed with SPSS (IBM Corp, Version 20.0) and R (Version 3.6.0). Continuous variables were presented as mean and standard deviation for normal distributed data and as median and interquartile range for non-normal distributed data. Categorical variables were presented as number and percentages. For non-normal distributed data, three-group and two-group comparisons were analyzed via Kruskal–Wallis H test and Kruskal–Wallis test, respectively. Categorical variables were analyzed using Pearson's chi-squared test or Fisher's exact test. To determine the independent factors associated with mortality in CP-CRE patients, lasso regression was first used to identify the factors that best predicted mortality ("glmnet" package in R) (Tibshirani, 1996). Once the final model was identified, traditional multivariable logistic regression was performed, and a final two-tailed $p < 0.05$ was 5% level considered to be statistically significant. To further explore the relationship between the variables and mortality in different subset of patients with CP-CRE, we conducted recursive partitioning analysis on all study variables using the "rpart" package in R (Therneau and Atkinson, 1997). To avoid overfitting, the decision tree was pruned based on the complexity parameter associated with minimal error (i.e., when no additional variables achieve further reductions in node impurity). The sensitivity, specificity, and area under the receiver operating characteristic curve (ROC) was tabulated to assess the performance of the final decision tree.

RESULTS

CP-CRE Isolates

Two hundred and three patients with CP-CRE-positive culture were identified during the study period; 48 did not harbor clinically significant infection. One hundred and fifty-five patients met the inclusion criteria and were included in the study (**Figure 1**). From these patients, a total of 169 CP-CRE isolates were identified.

Study Population

The baseline characteristics of the patients and their infections were compared between the various carbapenemases in **Table 1**. The median age of these patients was 65 years (IQR 56.5–74 years), and the age-adjusted CCI score was 6 (IQR4–8); 43.2% had malignancies. One hundred and thirty-one patients (84.5%) were hospitalized in the preceding 1 year, with 23 (14.8% of the recruited patients) in a foreign hospital. Prior antibiotic exposure was prevalent among 95.5% ($n = 148$) of the patients. Patients, who were infected with KPC-producing isolates, were also more likely to have received surgery compared to patients infected with OXA- and MBL-producing isolates ($p = 0.035$). There was no difference in the severity of illness or any clinical outcomes exhibited by patients between the various carbapenemases. Each eligible patient, whose infection was caused by more than one carbapenem-resistant isolates but with similar carbapenemase production was counted once, under the respective carbapenemase type. However, if any eligible patient had infection involving 2 carbapenemase types, this patient would be included twice, under the respective carbapenemase types for analysis.

Types of CP-CRE Isolates and Their Susceptibilities

The characteristics of CP-CRE isolates are described in **Table 2**. Among the 174 carbapenemases observed in the 169 isolates, there were 95 (54.6%) KPC, 51 (29.3%) MBL, and 28 (16.1%) OXA carbapenemases. Five out of 169 isolates were CP-CRE carbapenemase co-producers. Four *K. pneumoniae* and 1 *Citrobacter freundii* isolates produced a combination of MBL (specifically NDM) and OXA carbapenemases, each. *K. pneumoniae* was the most commonly encountered *Enterobacterales* ($n = 86$, 50.9%), followed by *Enterobacter* spp. ($n = 42$, 24.9%), *Escherichia coli* ($n = 32$, 18.9%), *Citrobacter* spp. ($n = 8$, 4.7%), and *Serratia marcescens* ($n = 1$, 0.6%). The proportion of extensive drug-resistant KPC-producing isolates (54.7%) was significantly lower ($p = 0.001$) than those of OXA- (82.1%) and MBL- (80.4%) producing isolates. MBL- (OR = 3.39, 95% CI, 1.52–7.55) and OXA-producing (OR = 3.80, 95% CI, 1.33–10.85) isolates demonstrated significantly higher proportion of extensive drug resistance when compared to KPC. The most common infection sites were skin and soft

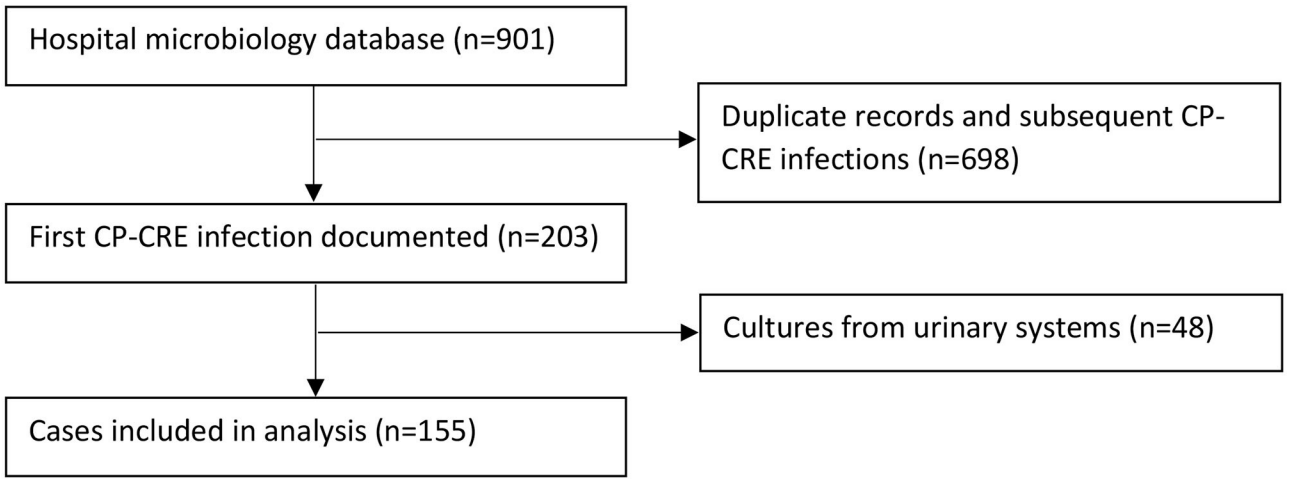

FIGURE 1 | Flowchart of patient selection.

tissue ($n = 47$, 27.8%), followed by intra-abdominal infection ($n = 43$, 25.4%).

The susceptibilities of the isolates are described in **Table 3**. The isolates were highly resistant to all carbapenem and cefepime, with 65.7% ($n = 111$) demonstrating extensive drug resistance. Susceptibilities to tigecycline and polymyxin B were only performed in some isolates upon request. As some patients died, or were transferred out of hospital, the susceptibilities of polymyxin B and tigecycline were not requested for. However, among those tested, susceptibilities remained high for tigecycline at 78.1% (100/128 tested) and polymyxin B at 90.6% (115/127 tested). Susceptibilities of MBL-producing isolates remained high toward polymyxin B (88.1%, 37/42 tested) and tigecycline (70.7%, 29/41 tested). The susceptibilities of polymyxin B, tigecycline, and amikacin for KPC-producing isolates remained above 85%, while levofloxacin's susceptibility was notably at 67.9% (38/56 tested). Only 1 KPC-producing isolate demonstrated pan-drug resistance while the 5 carbapenemase co-producers were all extensive drug resistant.

Treatment Regimens

One hundred and fifty-two (98.1%) patients received empiric antibiotic therapy, of which 106 (68.4%) had monotherapy. Of the 3 patients not empirically treated, 1 demised within 24 h of index culture, another was terminally discharged, while the last patient was initiated on antibiotics only after culture results were released. When culture and susceptibilities results were back, only 43 (27.7%) patients were found to be receiving adequate empirical therapy, and out of which, 18 (41.9%) and 25 (58.1%) patients received adequate empirical monotherapy and combination therapy, respectively.

Definitive treatment regimens are described in **Table 4**. Seventy-one percent of patients ($n = 110$) received definitive treatment based on culture results. Definitive monotherapy and combination therapy were administered to 45 (29.0%) and 65 (41.9%) patients, respectively. Tigecycline and polymyxin-containing combination therapy were equally common in the treatment of skin and soft tissue infections while polymyxin-containing combination therapy were most frequently administered for intra-abdominal infections. Only 4 and 9 patients received polymyxin and tigecycline monotherapy, respectively. Among these 13 patients, 6 underwent source control interventions, while the rest (7 patients) demised within 30 days of index culture. There were 3 patients who were treated with meropenem as their CP-CRE exhibited ertapenem resistance but remained susceptible or intermediately susceptible to meropenem. Out of these 3 patients, only 1 patient had surgical debridement and survived, while the other 2 died at 14- and 15-days of infection, respectively. Out of the 15 patients who were treated appropriately with culture-directed monotherapy with fluoroquinolones, 4 patients demised. Only 1 patient, out of these 4 demised, had active surgical source control, while 9 out of the 11 patients who survived had active source control.

Forty-five (29.0%) patients did not receive definitive antibiotic therapy. Of which, 11 (24.4%) patients underwent source control interventions and survived; 6 (13.3%) patients, with CP-CRE as part of their polymicrobial cultures, were continued with their empiric non-active antibiotic therapy as they had demonstrated clinical improvement by the time their microbiological cultures and their susceptibilities were known. The remaining 28 (62.2%) were patients who had demised prior to culture results being made available, patients who were transferred to another hospital, or patients on palliative care who were managed expectantly.

Outcomes

During the onset of infection, 58 (37.4%) required ICU admission with a median APACHE II score of 17 (IQR 12–22.5), and 32 (20.6%) presented with septic shock. The overall 30-day all-cause in-hospital mortality rate was 35.5% ($n = 55$). Of these 55 cases, 24 patients succumbed to the infection. The overall clinical response rate was 60.6% ($n = 94$), after a median duration of 7-days (IQR 5–11-days) from infection onset (when index

TABLE 1 | Baseline characteristics of study population and their infections.

	Total patients	Carbapenemase $n = 163$			*P*-value
	$n = 155$	*KPC $n = 86$	*MBL $n = 49$	*OXA $n = 28$	
Demographic					
Age, median (IQR)	65 (56.5–74)	67 (61–76)	62 (46–69.5)	62.5 (49–71)	0.001
Male (%)	91 (58.7)	48 (55.8)	30 (61.2)	18 (64.3)	0.676
Duration from admission to index culture, median days (IQR)	16 (1–38)	21 (7–40)	12 (1–37)	7 (0.5–26.5)	0.079
Comorbidities					
Charlson comorbidity index, median (IQR)	6 (4–8)	6 (5–8)	6 (2–8)	7 (4–8.5)	0.292
Malignancies (%)	67 (43.2)	40 (46.5)	19 (38.8)	11 (39.3)	0.623
Receiving immunosuppressive therapy (%)	29 (18.7)	13 (15.1)	13 (26.5)	5 (17.9)	0.263
Prior healthcare exposure					
Hospitalization (%)	131 (84.5)	68 (79.1)	45 (91.8)	26 (92.9)	0.061
Foreign hospitalization (%)	23 (14.8)	3 (3.5)	14 (28.6)	8 (28.6)	<0.001
Antibiotic use (%)	148 (95.5)	84 (97.7)	44 (89.8)	28 (100.0)	0.091
Surgery (%)	65 (41.9)	44 (51.2)	15 (30.6)	9 (32.1)	0.035
Invasive procedure at bedside (%)	138 (89.0)	81 (94.2)	42 (85.7)	22 (78.6)	0.050
Severity of infection					
SOFA score, median (IQR)	5 (2–8)	4 (2–8)	5 (2–9)	5 (3.5–7)	0.822
APACHE II score, median (IQR)	17 (12–22.5)	17 (12–25)	15 (12–21.5)	18 (14–20.5)	0.714
Septic shock on infection onset (%)	32 (20.6)	19 (22.1)	10 (20.4)	4 (14.3)	0.671
ICU admission (%)	58 (37.4)	31 (36.0)	19 (38.8)	10 (35.7)	0.943
Outcomes					
Length of hospital stay, median (IQR)	48.5 (21.5–74.5)	53 (24–75)	43 (20.5–78)	39.5 (11.5–66)	0.179
Receipt of source control (%)	57 (36.8)	28 (32.6)	22 (44.9)	12 (42.9)	0.309
Clinical response (%)	94 (60.6)	54 (62.8)	30 (61.2)	14 (50.0)	0.478
Time to clinical response, median (IQR)	7 (5–11)	7 (5–10)	9 (3–15)	7 (5–15)	0.529
Microbiological eradication (%)	43 (27.7)	22 (25.6)	14 (28.6)	11 (39.3)	0.380
30-day-all-cause mortality (%)	55 (35.5)	32 (37.2)	13 (26.5)	11 (39.3)	0.379

**Each eligible patient, whose infection was caused by more than one carbapenem-resistant isolate, but with similar carbapenemase production was counted once, under the respective carbapenemase type. However, if any eligible patient had infection involving 2 carbapenemase types, this patient would be included twice, under the respective carbapenemase types for analysis.*

culture was sent). Microbiological clearance was achieved in 43 patients (27.7%) while reinfection with CP-CRE occurred in 15 (9.7%) patients. There were no significant differences in 30-day all-cause mortality rates among the patients who were infected with different carbapenemases producing *Enterobacterales*. From **Table 5**, 30-day all-cause in-hospital mortality was significantly associated with older patients, ICU admission, septic shock, higher CCI score, APACHE II score or SOFA score, pneumonia infection, patients not receiving definitive combination therapy and those without clinical response, microbiological clearance, or source control interventions.

Risk Factors for Mortality

The results of the multivariable analysis are shown in **Table 6**. As shown, the presence of source control (OR, 0.258; 95% CI, 0.093–0.661), presence of microbial eradication (OR, 0.176, 95% CI, 0.053–0.504), and receipt of definitive combination therapy (OR, 0.391, 95% CI, 0.162–0.906) were associated with a significant lower risk for 30-day all-cause in-hospital mortality, while APACHE II score ≥15 (OR, 8.755, 95% CI, 3.573–31.997) was a significant predictor for mortality. Using binary recursive partitioning, the final classification tree for mortality in CP-CRE patients included 4 study variables and is shown in **Figure 2**. The root node classified the patients with APACHEII score ≥15 ($n = 98$; mortality risk = 52.0%) and <15 ($n = 57$; mortality risk = 9.0%). Patients with APACHEII score ≥15 are further classified based on presence ($n = 27$; mortality risk = 23.0%) and absence ($n = 71$, mortality risk = 62.0%) of bacterial eradication; in the subset of patients without bacterial eradication, patients were further subdivided based on presence ($n = 17$, mortality risk = 35.0%) and absence ($n = 54$, mortality risk = 70.0%) of active source control. In patients who did not achieve active source control, the risk of mortality was higher for the patients that did not receive definite combination therapy ($n = 36$, mortality risk = 83.0%) when compared to those who received definite combination therapy ($n = 18$, mortality risk = 47.0%).

TABLE 2 | Characteristics of carbapenemase-producing carbapenem-resistant *Enterobacterales* isolates.

	Total isolates	Carbapenemase *n* = 174			*P*-value
	n = 169*	KPC *n* = 95	MBL *n* = 51	OXA *n* = 28	
Species					
Klebsiella pneumoniae (%)	86 (50.9)	49 (51.6)	22 (43.1)	19 (67.9)	0.109
Enterobacter spp. (%)	42 (24.9)	29 (30.5)	11 (21.6)	2 (7.1)	0.035
Escherichia coli (%)	32 (18.9)	15 (15.8)	11 (21.6)	6 (21.4)	0.624
Others (%)	9 (5.3)	2 (2.1)	7 (13.7)	1 (3.6)	0.013
Primary site of infection					
Skin and soft tissue infection (%)	47 (27.8)	22 (23.2)	16 (31.4)	11 (39.3)	0.207
Intra-abdominal infection (%)	43 (25.4)	28 (29.5)	11 (21.6)	6 (21.4)	0.491
Bloodstream (%)	36 (21.3)	18 (18.9)	12 (23.5)	6 (21.4)	0.804
Pneumonia (%)	28 (16.6)	18 (18.9)	7 (13.7)	4 (14.3)	0.674
Others (%)	15 (8.9)	9 (9.5)	5 (9.8)	1 (3.6)	0.662
Resistance					
Extensive drug resistance (%)	111 (65.7)	52 (54.7)	41 (80.4)	23 (82.1)	0.001

**Of 169 isolates, there were 5 co-producers for carbapenemases, with two carbapenemases in each isolate.*

TABLE 3 | Antimicrobial susceptibility of carbapenemase-producing carbapenem-resistant *Enterobacterales* isolates.

Antibiotic	Overall susceptibility (%)	Carbapenemase (%)		
		KPC	MBL	OXA
Levofloxacin	46.9	67.9	21.4	12.5
Cefepime	7.7	8.5	2.0	14.3
Ertapenem	1.2	1.1	0.0	3.6
Imipenem	3.1	1.4	0.0	15.0
Meropenem	6.1	7.5	0.0	12.0
Doripenem	5.0	3.6	3.2	12.5
Tigecycline	78.1	86.8	70.7	62.5
Polymyxin B	90.6	91.0	88.1	95.7
Amikacin	74.5	92.5	55.6	40.0
Piperacillin-tazobactam	1.9	1.6	3.7	0.0
Aztreonam	5.4	0.0	14.8	11.1

TABLE 4 | Definitive antibiotic regimen prescribed for patients in this study.

Antibiotic regimens	Overall (%) *n* = 155
Monotherapy	45 (29.0)
Fluoroquinolone	15 (9.7)
Aminoglycoside	13 (8.4)
Tigecycline	9 (5.8)
Polymyxin B	4 (2.6)
Carbapenem	3 (1.9)
Cefepime	1 (0.6)
Combination therapy	65 (41.9)
Polymyxin B containing	48 (31.0)
Polymyxin B + carbapenem	30 (19.4)
Polymyxin B + tigecycline	6 (3.9)
Polymyxin B + carbapenem + aminoglycoside/fluoroquinolone	4 (2.6)
Polymyxin B + tigecycline + aminoglycoside/fluoroquinolone	4 (2.6)
Polymyxin B + tigecycline + carbapenem	2 (1.3)
Polymyxin B + aminoglycoside/fluoroquinolone	2 (1.3)
Non-polymyxin B containing	17 (11.0)
Tigecycline + aminoglycoside/fluoroquinolone	6 (2.6)
Carbapenem + aminoglycoside/fluoroquinolone	3 (1.9)
Tigecycline + carbapenem	3 (1.9)
Aminoglycoside + fluoroquinolone	3 (1.9)
Cephalosporin + aminoglycoside	2 (1.3)
No definitive antibiotic therapy	45 (29.0)

Overall, the classification tree has an area under ROC of 0.92, with a sensitivity of 0.87 and specificity of 0.91.

DISCUSSION

Heterogeneous CP-CRE Infections in Singapore

Unlike countries with a predominant carbapenemase reported (van Duin and Doi, 2016), Singapore, an international health hub, has to deal with a more diverse range of CP-CREs at our healthcare institutions (Molton et al., 2013; Marimuthu et al., 2017). There are 3 predominant carbapenemases (KPC, MBL, OXA-producing) observed in the isolates from our center. Majority of our patients with CRE infections were infected with KPC-producing strains (54.6%) (Hsien Koh et al., 2013), and this was usually associated with prior surgical procedure. However, there was also a significant proportion of MBL isolates

(29.3%). Approximately one-sixth of the study population had prior foreign hospital exposure which was significantly associated with MBL infections, and half of these patients were from India or Bangladesh where MBL carbapenemase predominates (Lascols et al., 2011; Snyder et al., 2016; Islam et al., 2017). Among the patients with OXA isolates, ∼64.3 and 28.6% had prior local and foreign hospitalization, respectively. Surgery compromises the protective barriers and has been well-established to be associated with infections by CP-CRE (Di Carlo et al., 2013; da Silva et al., 2016; Hilliquin et al., 2018). Locally, we have observed that patients infected with KPC isolates were also more likely to have received prior surgical operations, when compared to the OXA or MBL isolates.

Given the heterogeneity of CP-CRE infections and its associated morbidities and mortality, we applied recursive partitioning to identify predictors of outcomes in CP-CRE infections in a simple and intuitive manner. Translating this to clinical practice, we found that in severely ill (i.e., APACHEII score $\geq$ 15) patients who could not achieve bacterial eradication and did not receive active source control, the use of definitive antibiotic combinations appeared to improve clinical outcomes with a reduction in mortality. In an environment of diverse mechanisms of resistance, individualized and target therapy for CP-CRE infections, guided by antimicrobial combination testing, is the way forward.

Degree of Resistance

Depending on the type of carbapenemase production, the antimicrobial susceptibility profile of the various CP-CRE isolates may differ. MBL-producing CP-CRE isolates were at more than 3 times more likely to be extensively drug resistant compared to KPC-producing isolates, likely attributed by the wider array of resistant genes present compared to other carbapenemases (Nordmann et al., 2011; Tzouvelekis et al., 2014). Similarly, 82.1% OXA-producing CP-CRE was extensive-drug resistant; this was at more than 3 times than KPC-producing isolates. This could be of clinical significance, given the mortality associated with OXA-48 producing CP-CRE pan-drug resistance infections (Stewart et al., 2018; Sah et al., 2019). The treatment armamentarium shrinks considerably, and the medical team is left with polymyxin, tigecycline, and to a lesser extent aminoglycoside as our only treatment options.

Interestingly in our study, more than 67% of our KPC-producing isolates retained susceptibility to levofloxacin, which is surprising considering that fluoroquinolone resistance globally is frequently mediated by prevalent plasmid or chromosomal mutations (Endimiani et al., 2008; Morrill et al., 2015; Muggeo et al., 2018). Having said this, there are also reports of fluoroquinolone activity against KPC-producing *Enterobacterales* and they provide an additional treatment option (Vasoo et al., 2015).

Antibiotic Treatment

In our cohort, approximately only one-quarter of the cohort received adequate empirical treatment, which is comparatively lower than other CP-CRE studies (Nordmann et al., 2011; Tzouvelekis et al., 2014). One of the possible reasons is that although CP-CRE infections have increased in prevalence over the years (Teo et al., 2016), CP-CRE infections are still uncommon due to heightened infection control measures and ongoing surveillance nationwide; the rates of CP-CRE infection stand at 7% (Cai et al., 2017). By far, infections with extended spectrum beta-lactamase-producing pathogens are more common at approximately 38% (Cai et al., 2017). Polymyxin and tigecycline are not used upfront as our first line antibiotics in managing severe infections.

Treatment Regimens

In general, culture-directed monotherapy with polymyxin or tigecycline was not frequently practiced at our center, despite their susceptibilities toward isolates. This practice is driven by reports of higher treatment failure rates and increased risk of further resistance with monotherapy when compared to combination therapy (Sun et al., 2013; Tumbarello et al., 2015). Similarly, mortality with monotherapy appears to be high. In addition, we had observed that 7 out of 13 patients, with appropriate monotherapy (with polymyxin or tigecycline) administered and no source control interventions, had demised. Surgical removable of infection source where possible should be the primary treatment.

The notable use of either intravenous or oral formulations of levofloxacin and ciprofloxacin as monotherapy or part of the combination treatment in our institution which was based on the high rate of fluoroquinolone susceptibility was observed, especially among our KPC-producing isolates. This is in contrast to observations elsewhere that CP-CRE isolates commonly exhibit resistance to fluoroquinolones (Morrill et al., 2015).

Regardless of the type of infection, the most common combination utilized was carbapenem paired with polymyxins, supported by data, which supports its use to reduce mortality and increase treatment success (Falagas et al., 2011; Tumbarello et al., 2012). The synergistic effect of polymyxins and carbapenem has been demonstrated *in vitro* (Qureshi et al., 2012). We have also found that definitive combination therapy was associated with a lower 30-day all-cause in-hospital mortality rate if active source control was not possible, similar to previous observation elsewhere (Lee and Burgess, 2012). This highlights the importance of individualized and targeted therapy for CP-CRE infections and the need for combination testing, especially in healthcare institutions who are treating a diverse range of CP-CRE infections. Of note, use of definitive carbapenem monotherapy to treat carbapenemase-producing *Enterobacterales*, which remains carbapenem-susceptible during testing, is concerning. Tzouvelekis et al. reported that using carbapenem monotherapy to treat such *Enterobacterales* (especially OXA-48 producing isolates) are often associated with treatment failures (Tzouvelekis et al., 2014).

Source Control

Source control is vital for the treatment of CP-CRE infections. It was found to be significantly associated with reducing 30-day mortality and clinical improvement in approximately one-quarter of patients not receiving definitive antibiotics therapy (n = 11, 24.4%). Higher rates of source control

TABLE 5 | Comparison of characteristics between survivors and non-survivors.

	Survivor $n = 100$	Non-survivor $n = 55$	*P*-value
Demographics			
Age, median (IQR)	63 (53.5–74)	68 (62–77)	0.003
Male (%)	63 (63.0)	28 (50.9)	0.144
Comorbidities			
Charlson comorbidity index, median (IQR)	5 (4–8)	7 (6–9)	0.001
Malignancies (%)	46 (46.0)	21 (38.2)	0.347
Receiving immunosuppressive therapy (%)	19 (19.0)	10 (18.2)	0.901
Prior healthcare exposure			
Hospitalization (%)	86 (86.0)	45 (81.8)	0.491
Foreign hospitalization (%)	16 (16.0)	7 (12.7)	0.583
Antibiotic use (%)	96 (96.0)	52 (94.5)	0.699
Surgery (%)	47 (47.0)	18 (32.7)	0.085
Invasive procedure at bedside (%)	89 (89.0)	49 (89.1)	0.986
Primary site of infection			
Bloodstream	17 (17.0)	16 (29.1)	0.079
Pneumonia	12 (12.0)	15 (27.3)	0.016
Skin and soft tissue	31 (31.0)	12 (21.8)	0.222
Intra-abdominal	29 (29.0)	10 (18.2)	0.138
Others	11 (11.0)	2 (3.6)	0.114
Severity of infection			
SOFA score, median (IQR)	4 (1–5)	8 (5–13)	<0.001
APACHE II score, median (IQR)	14 (10–18)	28 (17–33)	<0.001
Septic shock on infection onset (%)	8 (8.0)	24 (43.6)	<0.001
ICU admission (%)	28 (28.0)	30 (54.5)	0.001
Infection characteristics			
Carbapenemase			
KPC (%)	54 (50.5)	32 (57.2)	0.418
MBL (%)	36 (33.6)	13 (23.2)	0.168
OXA (%)	17 (15.9)	11 (19.6)	0.546
Extensive drug resistance (%)	65 (65.0)	36 (65.5)	0.955
Treatment received			
Active empiric therapy (%)	23 (23.0)	20 (36.4)	0.075
Definitive monotherapy (%)	30 (30.0)	15 (27.3)	0.720
Definitive combination therapy (%)	49 (49.0)	16 (29.1)	0.016
Source control (%)	49 (49.0)	8 (14.5)	<0.001
Outcomes			
Length of hospital stay, median (IQR)	57.5 (34–87)	28 (11–59)	<0.001
Clinical response (%)	88 (88.0)	6 (10.9)	<0.001
Time to clinical response, median (IQR)	7 (5–11)	9 (7–11)	0.675
Microbiological eradication (%)	36 (36.0)	7 (12.7)	0.002

(39.3% vs. 30.2%) among patients on inadequate empiric therapy might have contributed to better clinical rates of improvement (66.1% vs. 46.5%) when compared to patients on adequate empiric treatment. This seems to further reiterate that source control ought to be primary modality of treatment if possible. The inability to perform any form of source control for patients with pneumonia could have contributed to a higher 30-day all-cause mortality (55.6%, $p = 0.016$) compared to other types of infection studied.

30-Day All-Cause Mortality

The 30-day all-cause mortality rate of 35.5% within our study was comparable to previous studies on CRE infections (Falagas et al., 2014; Martin et al., 2018). The predictors found to be independently associated with 30-day all-cause mortality include presence of source control, microbial eradication, definitive combination therapy, and APACHE II score, which are consistent with findings from relevant studies (Morrill et al., 2015; Gutiérrez-Gutiérrez et al., 2016). Interestingly, neither presence of extensive drug-resistant phenotype or type of carbapenemases

TABLE 6 | Multivariable logistic regression of predictors for 30-day all-cause mortality in CP-CRE infections.

Variable	Odds ratio (95% confidence interval)	*P*-value
Presence of active source control	0.258 (0.093–0.661)	0.022
APACHEII score ≥ 15	8.755 (3.573–31.997)	0.006
Presence of microbial eradication	0.176 (0.053–0.504)	<0.001
Use of definitive combination therapy	0.391 (0.162–0.906)	0.031
Presence of bloodstream infection	2.295 (0.762–7.693)	0.154

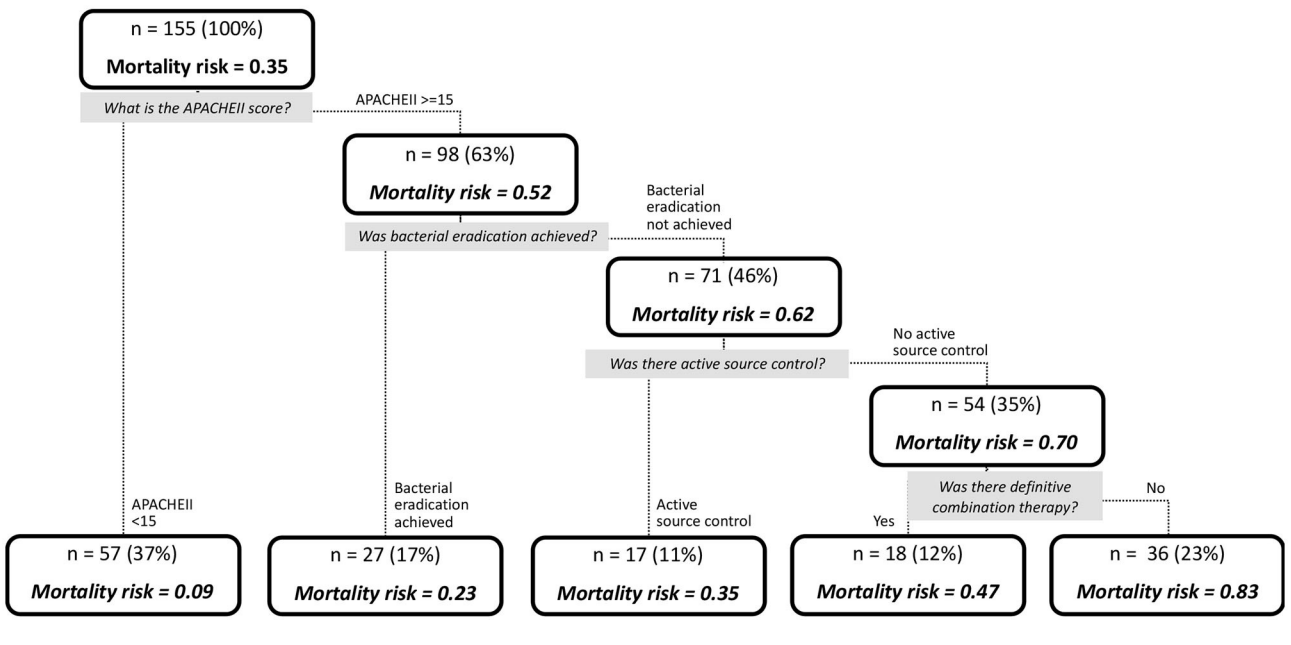

FIGURE 2 | Tree diagram depicting the likelihood of mortality in patients with CP-CRE infections, determined by recursive partitioning.

was found to be associated with higher mortality. Using recursive partitioning, we were able to further determine the hierarchy of predictors in a simple and intuitive manner that is easily translatable to clinical practice; most notably, we found that in severely ill (i.e., APACHEII score ≥ 15) patients who could not achieve bacterial eradication and did not receive active source control, use of definitive antibiotic combinations appeared to be associated with a reduction in mortality.

In medical decision-making (classification, diagnosing, etc.), there are many heterogeneous clinical situations, where decision must be made effectively and reliably. Conceptual simple decision-making models with the possibility of automatic learning are the most appropriate for performing such tasks. Decision trees are a reliable and effective decision-making technique that provides high classification accuracy with a simple representation of gathered knowledge, and they have been used in different areas of medical decision-making.

Limitation

Our study has certain limitations that must be acknowledged. Firstly, this was a retrospective cohort evaluation of treatment outcomes of diverse CP-CRE infections. Secondly, the relatively small representation, from a single tertiary care institution, large academic hospital, may further limit the applicability of our results. Thirdly, the frequent changes within the antibiotic regimens throughout the duration of treatment make systematic assessment of specific treatment regimen difficult. Lastly, with a significant portion of foreign patients, our study is prone to being lost to follow-up as patients get transferred back to their country.

CONCLUSION

Our CP-CRE are diverse, resulting in infections that require individualized antibiotic treatment strategies. Effective source control and microbial eradication were associated with a lower rate of 30-day all-cause mortality but not active empiric therapy for CP-CRE infection. If adequate source control could not be implemented safely, definitive use of antibiotic combinations appeared to be associated with a reduction in mortality.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by SingHealth Institutional Review Board provided approval with waiver of informed consent (CIRB number: 2014/912/F). The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

AK conceived the idea and helped in editing and data analysis. FL and WLa collected patient related data and wrote the manuscript. YL and WLe compiled, analyzed the data, and wrote the manuscript. JC, JT, and YC are involved in microbiological data collection, data analysis, and writing of manuscript. All authors read, vetted, and approved the manuscript.

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Carbapenem Resistant *Aeromonas hydrophila* Carrying bla_{cphA7} Isolated From Two Solid Organ Transplant Patients

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Aeromonas hydrophila resides in a variety of aquatic environments. Infections with *A. hydrophila* mainly occur after contact with fresh or brackish water. Nosocomial infections with *A. hydrophila* can also occur. *A. hydrophila* infections can be difficult to treat due to both intrinsic and acquired antimicrobial resistance (AMR) mechanisms. In 2018–19, we isolated multi-drug resistant (MDR) *A. hyrodphila* from two solid organ transplant patients with intra-abdominal infections. We aimed to characterize their AMR mechanisms and to determine their genetic relatedness to aid epidemiological investigation. We performed whole genome sequencing (WGS) using Illumina MiSeq and Nanopore MinIon on 3 *A. hydrophila* isolates, with one isolate from Patient A (blood) and two isolates from Patient B (abdominal and T-tube fluid, isolated 2 weeks apart). Phenotypic assays included: Broth Microdilution (BMD), Modified Hodge Test (MHT), Modified Carbapenem Inactivation Method (mCIM), and EDTA Carbapenem Inactivation Method (eCIM). Data analyses were performed using CLCbio and Geneious. AMR genomic analysis revealed that all three isolates possess chromosomally encoded genes including bla_{OXA-12}(oxacillinase), bla_{cepS}(AmpC), and bla_{cphA7}(metallo-beta-lactamase). All isolates tested strongly positive by MHT and mCIM, but only Patient B's second isolate (after 2 weeks of meropenem treatment) tested positive by eCIM. More intriguingly, Patient B's first isolate (before meropenem treatment) tested falsely susceptible to carbapenems by BMD, suggesting bla_{cphA7} gene was not expressed constitutively. Phylogenetic analysis showed the two isolates from Patient B were highly similar with only 1 SNP difference. The isolate from Patient A only differed from Patient B's isolates by 35 and 36 SNPs, respectively, suggesting close genetic relatedness. Further epidemiological investigation is undergoing. We report the first cases of CphA-mediated carbapenem resistant *A. hydrophila* in the U.S. It is concerning that 1 out of 3 isolates tested falsely susceptible to carbapenems by BMD despite clear carbapenemase production shown by strongly positive MHT and mCIM. In both cases, meropenem was initially used to treat the patients. Clinicians and microbiologists in the US should be aware of the emerging MDR *Aeromonas* nosocomial infections and the potential false carbapenem susceptible results due to CphA-type carbapenemase, which may be induced during treatment.

Keywords: carbapenem resistant, *Aeromonas hydrophila*, CphA7, carbapenemase, metallo- beta-lactamase

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INTRODUCTION

Aeromonas hydrophila is a Gram-negative bacillus that resides in a variety of aquatic environments (Hazen and Fliermans, 1979). Infections with *A. hydrophila* mainly occur after contact with fresh or brackish water. These infections can range from mild illness such as cellulitis or gastrointestinal disease, to serious disease such as sepsis and necrotizing fasciitis (Lee et al., 2008; Wu et al., 2009; Janda and Abbott, 2010). Nosocomial infections with *A. hydrophila* can occur and in some cases these infections are associated with contaminated medical devices such as catheters used in hemodialysis treatment (Lin et al., 1996; Khalil et al., 2013).

A. hydrophila infections can be difficult to treat due to both intrinsic and acquired antimicrobial resistance (AMR) mechanisms. The main mechanism of intrinsic resistance is chromosomally encoded ß-lactamases including Ambler class C cephalosporinases, class D penicillinases and class B metallo-ß-lactamases (MBLs) (Janda and Abbott, 2010). The most common MBL found in *A. hydrophila* is CphA that has a very specific substrate profile: highly active on carbapenems but not penicillin and cephalosporins (Segatore et al., 1993; Wu et al., 2012). CphA is also found in other clinically relevant *Aeromonas* species: *A. bestiarum, A. caviae, A. sobria, A. veronii,* and *A. jandaei* (Rossolini et al., 1995). Disseminated infections by CphA carrying *A. hydrophila*, primarily bacteremia, have mainly been reported in Asian and South American countries including Taiwan (Wu et al., 2007, 2011, 2012), Australia (Sinclair et al., 2016), and Colombia (Rosso et al., 2019).

CphA carrying *A. hydrophila* are resistant to extended-spectrum cephalosporins but are susceptible to monobactams such as aztreonam (Janda and Abbott, 2010). Reports have shown that carbapenemase-mediated resistance due to CphA is not easily detected by common *in vitro* susceptibility methods (Rossolini et al., 1995). Susceptibility tests with a large inoculum such as the Modified Hodge Test (MHT) have been shown to accurately detect carbapenem resistance and carbapnemase activity in CphA carrying strains of *Aeromonas* (Wu et al., 2011, 2012).

In 2018–19, we isolated several multi-drug resistant (MDR) *A. hydrophila* isolates from two solid organ transplant patients both presenting with intra-abdominal infections and subsequent bacteremia. We aimed to characterize their AMR mechanisms and to determine their genetic relatedness to aid epidemiological investigation.

MATERIALS AND METHODS

Bacterial Isolates, Antimicrobial Susceptibility, and Phenotypic Testing

A total of three *Aeromonas* isolates were included in this study. The first (A-1) was from a patient's blood culture (Patient A), while the other two were isolated 2 weeks apart from the intra-abdominal abscess (B-1) and T-tube fluid (B-2) from Patient B. These three isolates were identified as *Aeromonas hydrophila* from the original culture plates using Matrix Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (Biomerieux). The antimicrobial susceptibility testing was performed using Broth Microdilution (BMD) following guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2016). The Modified Carbapenem Inactivation Method (mCIM) and the EDTA-Carbapenem Inactivation Method (eCIM) were performed following the CLSI of the M100 guidelines (29th edition) (Clinical Laboratory Standards Institute, 2019). Modified Hodge Test (MHT) was also performed (Amjad et al., 2011). A positive control (MHT positive Klebsiella pneumoniae ATCC1705) and a negative control (MHT negative Klebsiella pneumoniae ATCC1706) were included in all the three phenotypic carbapenemase tests.

Whole Genome Sequencing (WGS)

The three *Aeromonas* isolates were sequenced with both short-read and long-read sequencing technologies. In brief, for short-read sequencing, extraction of the DNA from the isolates was done using the QIAGEN EZ1 DNA Tissue Kit (Germantown, MD) and the EZ1 Advanced XL (Germantown, MD). The libraries were prepared for sequencing using the Nextera DNA Flex Library Prep (Illumina; San Diego, CA). These prepared libraries were then loaded onto the Illumina MiSeq (San Diego, CA) with the 2 × 250 pair-read protocol. A positive control using *Escherichia coli* ATCC® 25922 DNA and a negative control of just water were run alongside the three *Aeromonas* isolates. For long-read sequencing, genomic DNA was extracted using Qiagen AllPrep Mini Kits (Germantown, MD) on the QIAcube automated system (Germantown, MD). Library preparation for sequencing on the Oxford Nanopore Technologies system was performed using the Native Barcoding Kit 1D and Ligation Sequencing kits, and run using FLO-MIN107 flow cells on the MinION system, following the manufacturer's instructions (Wick et al., 2017a). Base calling for Nanopore reads were completed using Guppy (v2.1.3, Oxford nanopore Technologies), and sequence reads were demultiplexed using Porechop (v0.2.4, https://github.com/rrwick/Porechop). Hybrid genome assembly was completed using Unicycler (v0.4.8-beta) (Wick et al., 2017b). Genomes were submitted to NCBI and can be found under BioProject PRJNA648413.

Bioinformatics

Mapping of the three *Aeromonas* isolates to reference strains were performed using both CLCbio Workbench Version 12 (Qiagen, Germany) and Geneious Prime 2.1 (Biomatters, New Zealand). The Kmer-based phylogenetic tree and single nucleotide polymorphism (SNP)-based analyses were done using CLCbio Workbench Version 12 (Qiagen, Germany). Specifically, the Kmer-based phylogenetic tree was performed using Feature Frequency Profile which is an alignment free genome comparison (Sims et al., 2009). The AMR prediction analyses were performed using Nucleotide Database (DB) with QIAGEN Microbial Insight—Antimicrobial Resistance (QMI-AR) as the reference database and the parameters of a minimum percent identity and percent length of 90% on CLCbio Workbench Version 12 (Qiagen, Germany). Center of Genomic Epidemiology tools, including KmerFinder (for closely related strain) (Hasman et al., 2014; Larsen et al., 2014) and PlasmidFinder (for plasmid types)

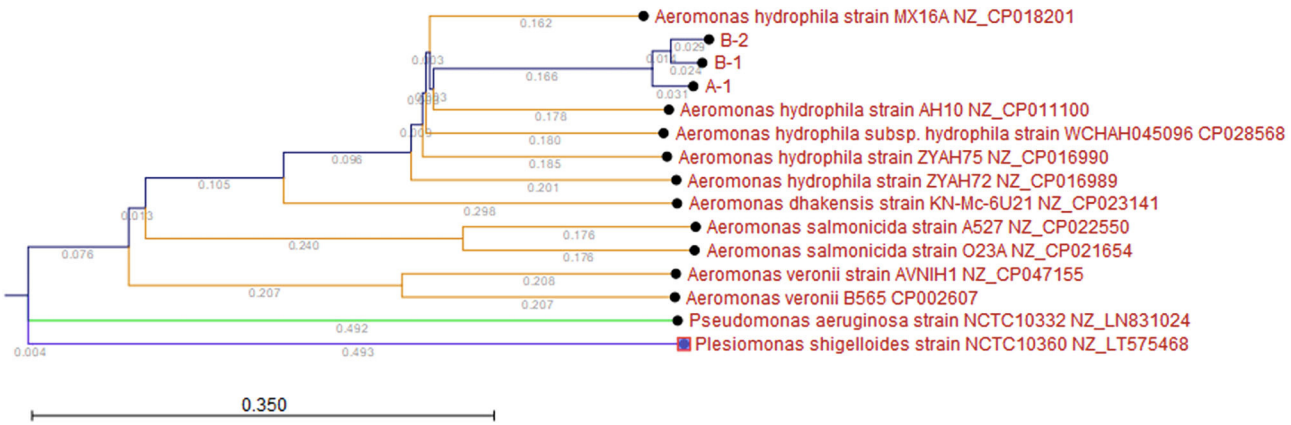


FIGURE 1 | Kmer Tree. Kmer analysis was performed with the three *A. hydrophila* isolates (A-1, B-1, and B-2) and five reference *A. hydrophila* (Genbank IDs: NZ_CP018201, NZ_CP011100, NZ_CP016990, NZ_CP028568, NZ_CP016989), two reference *Aeromonas salmonicida* (Genbank IDs: NZ_CP022550, NZ_CP021654), two reference *Aeromonas veronii* (Genbank IDs: NZ_CP046155, NZ_CP002607), one reference *Aeromonas dhakensis* (Genbank ID: NX_CP023141), one reference *Pseudomonas aeruginosa* (Genbank ID: NZ_LN831024), and one *Plesiomonas shigelloides* (Genbank ID: NZ_LT575468). Branch lengths are shown to show the relatedness.

TABLE 1 | Mapping statistics of the three *A. hydrophila* Isolates to NZ_CP018201.

Isolate	Pairwise identity	Mean coverage	Ref-seq percentage
2018–1	98.5%	63.4	94.0%
2019–1	98.6%	48.8	94.5%
2019–2	98.6%	54.3	94.6%
Mean	98.6%	55.5	94.4%

(Carattoli et al., 2014) were also used. Plasmids were annotated using RAST (Aziz et al., 2008).

RESULTS

Clinical History

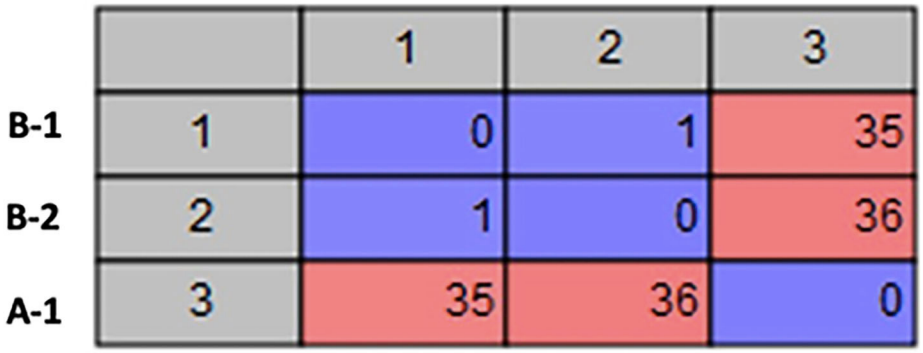
Patient A had a history of megacystitis, hypoperistalsis syndrome, renal failure requiring hemodialysis, multiple drug allergies, and a history of small bowel transplant complicated by rejection and subsequent ex-plantation. The patient developed end-stage liver disease due to chronic exposure to total parental nutrition. Patient A was initially admitted to the hospital for a foot infection and received treatment with broad spectrum antibiotics including meropenem. During to hospitalization, the patient had a wound culture from the site of a previous catheter insertion that grew multidrug resistant *Aeromonas* classified as *Aeromonas hydrophila group*, however this isolate was not able to be sequenced. Two weeks later the patient had a fever and a blood culture was obtained which grew multidrug resistant *Aeromonas* (A-1). Patient A was treated with broad spectrum antibiotics initially and ultimately completed a course of amikacin. The patient's hospital course was complicated by a gastrointestinal bleed, bacteremia, and fungemia. Patient A died several months later after transitioning to comfort care.

Patient B had a history of hypertension, polycystic liver-kidney disease who was initially admitted to our medical center for liver transplant. The patient's post-transplant course was complicated by bacteremia, cardiac arrest, and cerebral infarcts. Patient B received multiple courses of broad-spectrum antibiotics including meropenem during hospitalization. On 9/23/19, the patient had a surgical drain with milky drainage which grew multidrug resistant *Aeromonas* (B-1). Again, on 10/10/19 there was further drainage from a surgical drain which grew multidrug resistant *Aeromonas* (B-2). Patient B was treated with ceftazidime/avibactam and polymyxin/colistin. Patient B died 2 months later after transitioning to comfort care.

The Infection Prevention team conducted a thorough investigation to determine whether there was a correlation between Patient A and B's infection. The patients were admitted to the hospital during different time periods separated by over 6 months. No procedural items (e.g., duodenoscope) were shared between the two patients. Cleaning protocols for the rooms and point of care filter replacements for faucets were reviewed. Surveillance culture results of the dialysis machines were reviewed and had no evidence of contamination.

Phylogenetic Analysis

The Kmer Tree analysis revealed that the three *Aeromonas* isolates are all closely related to an *Aeromonas hydrophila* MX16A strain (NZ_CP018201) isolated from a water source in China in 2012 (**Figure 1**). Mapping of the three isolates to this reference strain showed an average Pairwise identity of 98.6% with a coverage of 94.4% (**Table 1**). We performed a SNP analysis and found that the two isolates from Patient B were highly similar with only 1 SNP difference detected (**Figure 2**). This SNP difference was in the gene that encodes for the protein RodA and appeared to cause a neutral amino acid change (**Supplementary Table 1**). RodA is a peptidoglycan glycosyltransferase important for cell wall elongation (Henriques



	1	2	3
B-1	1	0	1	35
B-2	2	1	0	36
A-1	3	35	36	0

FIGURE 2 | SNP Analysis. Matrix showing the number of SNP differences between each of the *A. hydrophila* isolates.

et al., 1998). The isolate (A-1) from Patient A differed from B-1 and B-2 by 35 and 36 SNPs, respectively, suggesting a close genetic relatedness among these bacteria. A complete list of the SNPs among the 3 isolates are shown in **Supplementary Table 1**.

AMR Genotypic Analysis

AMR genotypic analysis revealed that all three isolates possess three different chromosomally encoded ß-lactamases: bla_{OXA-12}(oxacillinase), bla_{cepS}(AmpC), and bla_{cphA7}(metallo-beta-lactamase) (**Table 2**). The two isolates from the Patient B (B-1 and B-2) had additional AMR genes detected conferring resistance to the following antibiotic classes: aminoglycosides, fluoroquinolones, phenicols, macrolides, and trimethoprim-sulfonamide (**Table 2**). Hybrid sequencing revealed that these additional AMR genes were on plasmids (**Table 3**). We identified a novel plasmid (Plasmid 1) present in both isolates from Patient B that has 123,554 bp and carries 185 genes (**Supplementary Tables 2, 3**). BLAST analysis showed only 20% of the sequences in this plasmid matched to any known plasmids (**Table 3**). These plasmids have many hypothetical proteins based on RAST data (**Supplementary Tables 2**, **3**); AMR gene analysis of this plasmids identified genes conferring to resistance to beta-lactams (bla_{SHV-5}), aminoglycosides (*aadA2* and *aph(3′)-la*), fluoroquinolones (*qacH*), macrolides (*mphA*) and trimethoprim-sulfonamide (*dfrA12* and *sul1*) (**Tables 2**, **3**).

The second isolate from the Patient B (B-2) appeared to have additional AMR genes detected compared to the first isolate (B-1) (**Table 2**). PlasmidFinder revealed an IncC-type plasmid present in isolate B-2 but not in the other two isolates. Hybrid assembly of both Illumina and NanoPore sequencing data confirmed that B-2 has a 129,067 bp plasmid (Plasmid 2) which carries 183 genes (**Supplementary Table 4**). BLAST analysis showed that this second plasmid in B-2 is 94% similar to a plasmid found in *Klebsiella pneumoniae* known as pHM881QN. It is a IncA/C plasmid isolated in Japan (GenBank ID: LC055503.1). The additional AMR genes on this plasmid in B-2 include the ß-lactamase bla_{OXO-10}, aminoglycoside resistance genes *ant(3″)-la, aac(6′)-lld* and *aadA13*, phenicol resistance genes *catB3* and *floR,* and trimethoprim/sulfonamide resistance gene *sul2* (**Tables 2**, **3**). The additional plasmid found in B-2 suggested that either this isolate gained a plasmid or that the antibiotic treatment of the patient with meropenem had selected this sub-population from a mixed bacteria population. Further analysis is undergoing to polish and finalize the complete genomes of these two plasmids.

Correlation Between AMR Genotypes and Phenotypic Susceptibility and Carbapenemase Profiles

The phenotypic antimicrobial susceptibility results of the three *A. hydrophila* isolates demonstrated several interesting patterns (**Table 4**): (1) all isolates are resistant to cefazolin, piperacillin/tazobactam, and all 3rd generation cephalosporins, which is consistent with the presence of the oxacillinase gene bla_{OXA-12} and the AmpC gene bla_{cepS}; (2) Isolate B-1 had elevated cefepime MIC due to the additional ESBL gene bla_{SHV-5} (Gutmann et al., 1989); (3) Isolate B-2 (after meropenem treatment) exhibits full resistance to all beta-lactams except ceftazidime/avibactam due to the additional beta-lactamase genes bla_{SHV-5} and bla_{OXA-10}(Gutmann et al., 1989), as well as the apparently induced MBL gene bla_{cphA7} expression, which is shown by the only positive eCIM result. Interestingly, although all isolates tested strongly positive by MHT and mCIM, Isolate B-1 demonstrated false susceptibility results to imipenem and meropenem by BMD (**Table 4** and **Figure 3**). In addition, the two eCIM negative isolates showed much lower minimum inhibitory concentration (MIC) for cefepime, suggesting no or low level of bla_{cphA7} gene expression in these two isolates.

DISCUSSION

Here we report the first cases of CphA-mediated carbapenem resistant *A. hydrophila* in the United States. The majority of CphA-mediated carbapenem resistance has been detected in soil or water environments in Asia and Europe (Walsh et al., 1997; Balsalobre et al., 2009; Piotrowska et al., 2017; Piccirilli et al., 2019), but also found in human infections as severe as bacteremia reported from Taiwan (Wu et al., 2007, 2011, 2012), Australia (Sinclair et al., 2016), and Colombia (Rosso et al., 2019). The idea that MDR organisms from the environment cause human infection is not novel; however, what is highlighted in this case is that current phenotypic methods are limited in correctly

TABLE 2 | Antimicrobial genotypic prediction of the three *Aeromonas hydrophila* isolates.

Antibiotic class	Antibiotic gene	A-1	B-1		B-2		
		Chromosome	Chromosome	Plasmid_1	Chromosome	Plasmid_1	Plasmid_2
Beta-Lactams	bla_{OXA-12}	✓	✓		✓		
	bla_{cepS}	✓	✓		✓		
	bla_{cphA7}	✓	✓		✓		
	bla_{OXA-10}						✓
	bla_{SHV-5}			✓		✓	✓
Aminoglycoside	aadA2			✓		✓	
	aph(3′)-Ia			✓		✓	
	ant(3″)-Ia						✓
	aac(6′)-Iid						✓
	aadA13						✓
Trimethoprim-Sulfonamide	dfrA12			✓		✓	
	sul1			✓		✓	✓
	sul2						✓
Fluoroquinolone	qacH			✓		✓	✓
Phenicol	catB3						✓
	floR						✓
Macrolide	mphA			✓		✓	

TABLE 3 | Hybrid sequence plasmid results.

Isolate	Plasmid number	Length (base pairs)	Genes*	BLAST results			
				Plasmid description	Query	Percent identity	Accession
B-1	Plasmid 1	123,554	184	*K. pneumoniae* AR_0120 plasmid tig00000500 pilon	19%	99.55%	CP021834.1
B-2	Plasmid 1	123,554	185	*K. pneumoniae* AR_0120 plasmid tig00000500 pilon	19%	99.56%	CP021834.1
	Plasmid 2	129,067	183	*K. pneumoniae* plasmid pHM881QN	94%	99.98%	LC055503.1

**Based on RAST annotation results.*

detecting carbapenem resistance in *Aeromonas* species. We demonstrated that using WGS and phenotypic carbapenemase assays can detect wider spectrum of resistance mechanisms that may be missed otherwise in highly resistant strains.

It is concerning that 1 out of 3 *A. hydrophila* isolates tested falsely susceptible to imipenem and meropenem by the conventional BMD method despite clear carbapenemase production shown by strongly positive MHT and mCIM results. These false susceptible results are defined by CLSI as very major errors (VMEs) (Humphries et al., 2018). VMEs are serious because they mislead clinicians to falsely believe that an ineffective antibiotic therapy is appropriate to administer. Current guidelines recommend ciprofloxacin or levofloxacin as first-line therapies for the infection with *Aeromonas* (Gilbert et al., 2019). Carbapenems are usually used empirically in treating Gram-negative bacteremia, as demonstrated in our cases. The carbapenem treatment in Patient B may have resulted in the selection of a sub-population of *A. hydrophila* that had an additional plasmid with antimicrobial resistance genes, or an induction of the carbapenemase gene CphA, which led to extended resistance profile (**Table 3**).

This work demonstrated the strength of using both mCIM and MHT phenotypic tests to detect the CphA-type carbapenemases in *A. hydrophila*. Currently the CLSI guidelines for the interpretation of mCIM results are limited in *Enterobacterales* and *Pseudomonas aeruginosa* while eCIM results are limited to only *Enterobacterales* (Pierce et al., 2017; Clinical Laboratory Standards Institute, 2019; Sfeir et al., 2019). MHT results are no longer endorsed by CLSI to be used for any species. Our study suggests that these phenotypic tests are useful for the detection of carbapenemase in *A. hydrophila.* The intriguing part is that we do not have an explanation for why the B-2 isolate tested positive for eCIM only after meropenem treatment. One possible explanation is that positive eCIM requires hyperproduced CphA enzyme, but MHT and mCIM do not. Further studies are needed to solve this puzzle.

The detection of chromosomally encoded β-lactamase genes in our study is consistent with previously published literature on *Aeromonas* species isolated from human infections (Janda and Abbott, 2010). However, the two Patient B isolates demonstrated a substantial increase in the number of antimicrobial genes detected genotypically with AMR prediction (**Table 2**). This

TABLE 4 | Summary of phenotypic results of the three *Aeromonas hydrophila* isolates.

Antibiotic class	Antibiotic	*Aeromonas hydrophila* (11/20/18) A-1		*Aeromonas hydrophila* (9/23/19) B-1		*Aeromonas hydrophila* (10/10/19) B-2	
		MIC (MCG/mL)	Interpretation	MIC (MCG/mL)	Interpretation	MIC (MCG/mL)	Interpretation
Beta-Lactams	Piperacillin/Tazobactam	>128	Resistant	>128	Resistant	>128	Resistant
	Ceftriaxone	>32	Resistant	>64	Resistant	>64	Resistant
	Ceftazidime	32	Resistant	>32	Resistant	>32	Resistant
	Ceftolozane/Tazobactam	16	No Inter Criteria	8	No Inter Criteria	16	No Inter Criteria
	Cefepime	8	Susceptible	4	Intermediate	>32	Resistant
	Imipenem	8	Resistant	1	Susceptible	>16	Resistant
	Meropenem	4	Resistant	1	Susceptible	>16	Resistant
	Ertapenem	>4	Resistant	>4	Resistant	>4	Resistant
	Ceftazidime/Avibactam	<=2	No Inter Criteria	<=2	No Inter Criteria	4	No Inter Criteria
Aminoglycoside	Gentamicin	1	Susceptible	<=1	Susceptible	>16	Resistant
	Tobramycin	8	No Inter Criteria	4	No Inter Criteria	>16	No Inter Criteria
	Amikacin	2	Susceptible	16	Susceptible	32	Intermediate
Trimethoprim-Sulfonamide	Trimethoprim-Sulfonamide	<=1/20	Susceptible	>4/80	Resistant	>4/80	Resistant
Colistin	Colistin	>4	No Inter Criteria	<=2	No Inter Criteria	>4	No Inter Criteria
Fluoroquinolone	Ciprofloxacin	>2	Resistant	>4	Resistant	>4	Resistant
	Levofloxacin	4	Intermediate	4	Intermediate	2	Susceptible
Modified Carbapenem Inactivation Method (mCIM)		Positive		Positive		Positive	
EDTA Carbapenem Inactivation Method (eCIM)		Negative		Negative		Positive	
Modified Hodge Test (MHT)		Positive		Positive		Positive	

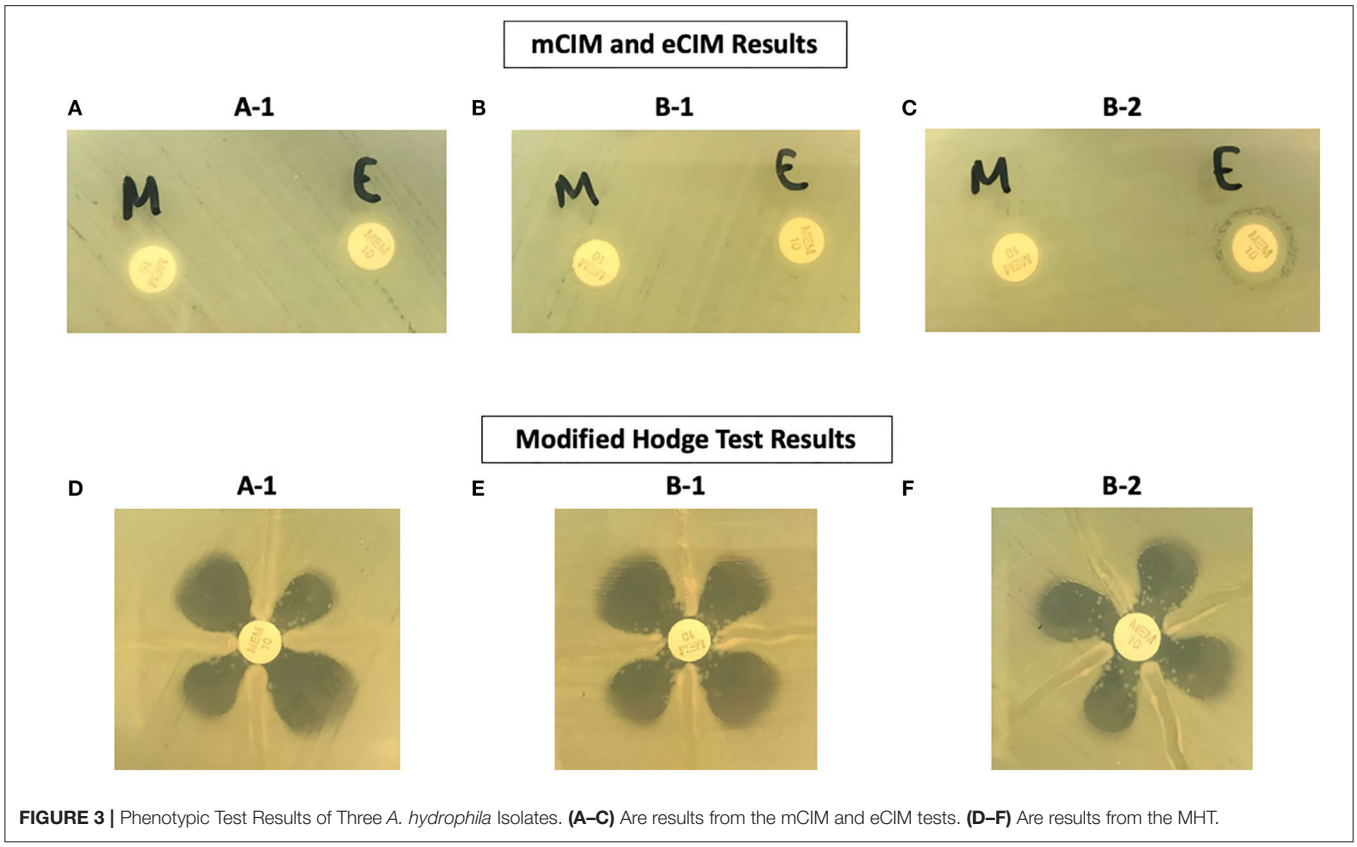


FIGURE 3 | Phenotypic Test Results of Three *A. hydrophila* Isolates. **(A–C)** Are results from the mCIM and eCIM tests. **(D–F)** Are results from the MHT.

increase in the number of antimicrobial genes detected genotypically is attributed to the presence of plasmids with one novel plasmid in both isolates and an additional plasmid in B-2 (**Table 3**). The ability of *Aeromonas* to exchange and gain plasmids is well-known within the literature and all species within *Aeromonas* are known to have an open pan-genome with extensive genomic variability (Bello-López et al., 2019; Zhong et al., 2019). Therefore, there is a greater ability for *Aeromonas* spp. to acquire AMR genes. Of note, in a recent pan-genome analysis, *A. aquatica* MX16A which was just recently re-classified as *A. hydrophila* MX16A (NZ_CP018201), was shown to harbor the greatest number of AMR genes among 29 species of *Aeromonas*. All three *A. hydrophila* isolates in our study were closely related to this strain. There was no known travel history or exposure history for these two patients. The epidemiological investigation revealed no common source or mode of transmission for the multidrug resistant *Aeromonas* strains isolated from these two patients. In addition, no other multidrug resistant *Aeromonas* were isolated or identified from sources or patients in the time period between the two patients. As of writing this manuscript, the sources of these *Aeromonas* were still unknown.

One major limitation of this study is that we did not perform further investigation to check if the bla_{cphA7} gene was derepressed in Patient B's Isolate B-2, which could provide more clear explanation for its much higher MICs for the carbapenems and cefepime compared to Isolate B-1. We did, however, identify a non-synonymous point mutation (AC) resulting in an Asp17Tyr substitution in the beta-lactam response regulator transporter gene *blrA* in both Isolate B1 and B2 (**Supplementary Table 1**). The gene *blrA* had been shown to regulate three inducible beta-lactamases encoded by bla_{ampH}, bla_{cepH}, and bla_{imiH} in an *A. hydrophila* strain T429125 (Niumsup et al., 2003). Further studies are required to elucidate how this mutation in the *blrA* gene affect the gene expression of various beta-lactamases in the isolates from Patient B.

Clinicians and clinical microbiologists in the US should be aware of the emerging MDR *Aeromonas* infections and the potential false carbapenem susceptible results due to CphA-type carbapenemase. The inability to accurately detect this type of carbapenemase using the conventional methods is concerning because carbapenem is a common drug choice for treating MDRO. Workflows within the clinical microbiology lab will need to adapt to detecting these type of resistance mechanisms in the future. We suggest one good strategy is combining both genomic analysis by WGS and phenotypic characterization by MHT, mCIM, and eCIM. Genomic analysis by WGS is not yet accessible for all clinical laboratories; therefore, we propose further studies be performed to establish a reliable and fast algorithm to detect these types of resistance mechanisms in *Aeromonas*. In addition, we suggest the susceptible carbapenem MIC results on *Aeromonas* species isolated from the sterile site

in patients with severe infections should always be carefully evaluated before being finalized and reported.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

EH conceived of the presented idea, performed the experiments, analyzed the data, and drafted the manuscript. SF performed the experiments and analyzed the data. AM reviewed clinical history, and reviewed and revised the manuscript. KW performed the experiments. OG supervised the project, and reviewed and revised the manuscript. SY conceived of the presented idea, supervised the project, analyzed the data, and reviewed and revised the manuscript. 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/fcimb.2020.563482/full#supplementary-material

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Biofilm Production by Carbapenem-Resistant *Klebsiella pneumoniae* Significantly Increases the Risk of Death in Oncological Patients

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Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is a prominent cause of nosocomial infections associated with high rates of morbidity and mortality, particularly in oncological patients. The hypermucoviscous (HMV) phenotype and biofilm production are key factors for CRKP colonization and persistence in the host. This study aims at exploring the impact of CRKP virulence factors on morbidity and mortality in oncological patients. A total of 86 CRKP were collected between January 2015 and December 2019. Carbapenem resistance-associated genes, antibiotic susceptibility, the HMV phenotype, and biofilm production were evaluated. The median age of the patients was 71 years (range 40–96 years). Clinically infected patients were 53 (61.6%), while CRKP colonized individuals were 33 (38.4%). The most common infectious manifestations were sepsis (43.4%) and pneumonia (18.9%), while rectal surveillance swabs were the most common site of CRKP isolation (81.8%) in colonized patients. The leading mechanism of carbapenem resistance was sustained by the KPC gene (96.5%), followed by OXA-48 (2.3%) and VIM (1.2%). Phenotypic CRKP characterization indicated that 55.8% of the isolates were strong biofilm-producers equally distributed between infected (54.2%) and colonized (45.8%) patients. The HMV phenotype was found in 22.1% of the isolates, which showed a significant (P<0.0001) decrease in biofilm production as compared to non-HMV strains. The overall mortality rate calculated on the group of infected patients was 35.8%. In univariate analysis, pneumoniae significantly correlated with death (OR 5.09; CI 95% 1.08–24.02; P=0.04). The non-HMV phenotype (OR 4.67; CI 95% 1.13–19.24; P=0.03) and strong biofilm-producing strains (OR 5.04; CI95% 1.39–18.25; P=0.01) were also

associated with increased CRKP infection-related mortality. Notably, the multivariate analysis showed that infection with strong biofilm-producing CRKP was an independent predictor of mortality (OR 6.30; CI 95% 1.392–18.248; P=0.004). CRKP infection presents a high risk of death among oncological patients, particularly when pneumoniae and sepsis are present. In infected patients, the presence of strong biofilm-producing CRKP significantly increases the risk of death. Thus, the assessment of biofilm production may provide a key element in supporting the clinical management of high-risk oncological patients with CRKP infection.

Keywords: biofilm, *Klebsiella*, carbapenem, skin colonization, cancer

INTRODUCTION

Klebsiella pneumoniae is a major human pathogen with mortality rates up to 50%, particularly in immune-compromised individuals (Kanj and Kanafani, 2011; David et al., 2019). It causes a broad spectrum of diseases including pneumonia, urinary tract infections, bloodstream infections, skin and soft tissue infections (Melot et al., 2015; Pitout et al., 2015; Paczosa and Mecsas, 2016; David et al., 2019). Carbapenems are often considered the last line therapy for the treatment of multidrug-resistant *K. pneumoniae* (Tzouvelekis et al., 2012; David et al., 2019). However, global surveillance studies indicate that a significant fraction of nosocomial *K. pneumoniae* isolates display extended-spectrum β-lactamases (ESBLs) and carbapenemases activities (Molton et al., 2013; Morrissey et al., 2013; Pitout et al., 2015; Brescini et al., 2019). The endemic distribution of carbapenem-resistant *K. pneumoniae* (CRKP) has been reported worldwide (Munoz-Price et al., 2013). In European countries, the population-weighted mean percentage of CRKP is 7.2%. Greece, Italy, and Romania had the highest rates of CRKP as compared to the rest of Europe (Cassini et al., 2019). Dissemination of CRKP is primarily sustained by the horizontal transfer of carbapenemase genes on mobile elements (Mathers et al., 2011; Martin et al., 2017; Partridge et al., 2018). *K. pneumoniae* carbapenemase (KPC), imipenemase metallo β-lactamase (IMP), New Delhi metallo β-lactamase (NDM), Verona integron metallo β-lactamase (VIM), and oxacillinase-48 (OXA-48) are the most common carbapenemases in CRKP (Meletis, 2016; Partridge et al., 2018). Treatment for CRKP infections is often limited to colistin, which represents, in many cases, a last-resort option due to its nephrotoxicity and neurotoxicity (Karaiskos et al., 2017). More recently, novel combinations of β-lactam- β-lactamase inhibitors, such as ceftazidime-avibactam and meropenem-vaborbactam, have been found effective against CRKP producing KPC-type and OXA-48-like enzymes, but not for those strains producing metallo carbapenemases (Bassetti et al., 2018).

The production of capsular polysaccharide is the prominent virulence factor of *K. pneumoniae* that allow this bacterium to overcome innate host immunity (Zhang et al., 2016). Currently, more than 130 different capsule types have been recognized for *Klebsiella* (Follador et al., 2016). A recent study demonstrated that *K. pneumoniae* can enhance its pathogenicity by adopting two opposing strategies based on the capsule biosynthesis. The first is related to hypercapsule production, which confers phagocytosis resistance, enhanced dissemination, and higher mortality in animal models (Ernst et al., 2020). Alternatively, *K. pneumoniae* can acquire mutations impairing capsule production, thus allowing enhanced epithelial cell invasion, increased persistence in urinary tract infections, and biofilm formation (Ernst et al., 2020). Hypervirulent strains of *K. pneumoniae* can be identified by a hypermucoviscous (HMV) phenotype on agar plates, as a result of a positive string test (Compain et al., 2014). HMV subtypes, initially described in 1986, are characterized by increased production of a capsular substance compared with classic *K. pneumoniae*, which confers a HMV phenotype (Casanova et al., 1989). Mutations in genes reducing capsule production affect the HMV phenotype and correlate with a substantial reduction in virulence when tested in mice (Walker and Miller, 2020). Thus, the HMV phenotype is directly linked with the amount of capsule production. However, a recent study demonstrated that a mutation in a gene encoding a transcriptional regulator of the mucoid phenotype (RmpC) reduces capsule production but does not affect the HMV phenotype (Walker et al., 2019). This finding suggests that HMV is dependent on the presence of the capsule, but HMV and capsule have to be considered independently (Walker and Miller, 2020). HMV, isolates showed an increased ability to cause both severe community-acquired infections such as pneumonia, liver abscesses, and meningitis in young, healthy individuals, and healthcare-associated invasive infections (Fang et al., 2007; Turton et al., 2010; Decre et al., 2011; Liu and Guo, 2019). Most HMV *K pneumoniae* strains have been related to the capsular type K1, and, in a lower fraction, with the serotype K2 (Alcantar-Curiel and Giron, 2015; Gu et al., 2018; Cubero et al., 2019) both reported as antibiotic-sensitive (Yeh et al., 2007; Gu et al., 2018). However, in recent years, carbapenem-resistant HMV strains have been reported worldwide (Gu et al., 2018; Huang et al., 2018; Lev et al., 2018; Ferreira et al., 2019; Wang et al., 2020).

Biofilm production is also important to the virulence of *K. pneumoniae* because the biofilm matrix facilitates the transfer of antibiotic-resistance mobile elements while physically protecting bacteria, thus increasing microbial tolerance to antibiotics, bacterial persistence, and dissemination (Clegg and Murphy, 2016; Ribeiro et al., 2016; Cubero et al., 2019). Biofilm eradication requires high antimicrobial concentrations, which are often impossible to achieve due to drug-related toxicity. Thus, relapses are frequent even after targeted and prolonged

therapies (Clegg and Murphy, 2016; Di Domenico et al., 2019). Despite its role in microbial virulence, biofilm is not routinely assessed in clinical microbiology, and diagnosis of biofilm-related infection, in most cases, can only be presumed based on clinical signs and symptoms (Di Domenico et al., 2016).

This study analyzes the impact of different CRKP virulence determinants to assess their predictivity in supporting clinical decision-making in high-risk oncological patients.

MATERIALS AND METHODS

This retrospective study was performed at the San Gallicano Dermatological Institute and Regina Elena National Cancer Institute, Rome, Italy, between January 2015 and December 2019.

The Central Ethics Committee I.R.C.C.S. Lazio, approved the study (Prot. CE/1016/15—15 December 2015, trials registry N. 730/15).

Microbiology

The samples were collected from a total of 86 oncological patients colonized or infected with CRKP. Bacterial identification was performed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) system (Bruker Daltonik, Bremen, Germany). The antimicrobial susceptibility was assessed by the VITEK® 2 system (bioMérieux, Marcy l'Étoile, France) (Lucarelli et al., 2017). Susceptibility for colistin and ceftazidime/avibactam was determined by the Sensititre broth microdilution method (Thermo Scientific, New Jersey, USA), and results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (http://www.eucast.org/clinical_breakpoints). The presence of bla_{KPC}, bla_{VIM}, bla_{OXA-48}, bla_{IMP-1}, bla_{NDM} types was determined by the Cepheid Xpert® Carba-R assay and the GeneXpert® device (Cepheid, Sunnyvale, USA).

Biofilm Formation

Biofilm production was assessed by the clinical BioFilm Ring Test (cBRT) (Biofilm Control, Saint Beauzire, France), as described in Di Domenico et al., 2016. Briefly, an overnight culture of *K. pneumoniae* grown on a blood agar plate was used to inoculate 2 ml of 0.45% saline solution to 1.0 ± 0.3 McFarland turbidity standard. The bacterial suspension was used to inoculate a 96-well polystyrene plate with 200 µl/well. The test was performed using the toner solution (TON004) containing magnetic beads 1% (*v*/*v*) mixed in the Brain Heart Infusion medium. Ten-fold serial dilutions were performed in a volume of 200 µl BHI/TON mix. *K. pneumoniae* ATCC700603 and *K. pneumoniae* ATCC 13883 were included in each plate as standard reference and internal control. After 5 h of incubation at 37°C in a static condition, wells were covered with contrast liquid, placed for 1 min on the block carrying 96 mini-magnets, and scanned with a plate reader (Pack BIOFILM, Biofilm Control, Saint Beauzire, France). The adhesion strength of each strain was expressed as BioFilm Index (BFI). Each *K. pneumoniae* strain was classified as weak moderate and high biofilm producers (Di Domenico et al., 2016; Di Domenico et al., 2017). Besides, moderate and high biofilm producers were grouped and classified as strong biofilm producers (Di Domenico et al., 2019). Each *K. pneumoniae* isolate was analyzed in duplicate, and experiments were repeated three times.

String Test

The HMV phenotype of the CRKP isolates was revealed by the string test as described previously (Zhan et al., 2017; Liu et al., 2019).

Sedimentation Assay

Overnight cultures were pelleted by centrifugation at 9,000×g and resuspended in PBS to an OD600 of 1. The suspensions were centrifuged at 1,000×g for 5 min, and the OD600 of the supernatants was measured. Readings were normalized to the OD600 of the strains before centrifugation (Bachman et al., 2015; Walker et al., 2019).

Statistics

Continuous variables were compared by Student's t-test for normally distributed variables and the Mann-Whitney U test for non-normally distributed variables. Categorical variables were evaluated using the $\chi 2$ or two-tailed Fisher's exact test. Univariate and multivariate analyses were carried by a logistic regression model to identify independent risk factors for 30-days mortality. Statistical analyses were carried out using IBM SPSS v.21 statistics software.

RESULTS

From January 2015 to December 2019, 86 consecutive patients infected or colonized with CRKP were included in the study. Patients' demographic and clinical characteristics are described in **Table 1**. The most represented underlying malignancy was hepato-bilio-pancreatic cancer (27.9%), urinary tract cancer (24.4%), hematologic malignancy (12.8%), and gastrointestinal cancer (12.8%) (**Table 1**). Infected patients were 61.6% (N53), while colonized patients accounted for 38.4% (N33). A concomitant fungal infection was detected in 5.8% (N5) of patients. Among infected patients, the most frequent manifestation caused by CRKP was sepsis (N23; 43.4%) followed by pneumoniae (N10; 18.9%), urinary tract infections (N7; 13.2%) and intra-abdominal infection (N5; 9.4%). CRKP caused 9 cases of catheter-related bloodstream infections and one case of catheter-acquired urinary infection. Among colonized patients, rectal surveillance swabs (RSS) were the most common site of CRKP isolation (N27; 81.8%) followed by urine samples (N4; 12.1%).

Based on genotypic characterization, the leading mechanism of carbapenem resistance was related to the KPC gene (N83, 96.5%), followed by OXA-48 (N2, 2.3%) and VIM (N1, 1.2%). None of the strains analyzed were positive for the class B metallo-β-lactamases IMP and NDM. The OXA-48 and VIM were only isolated from

TABLE 1 | Demographic and clinical characteristics of patients at enrollment.

Clinical Characteristics	N	%
Female	46	53.5
Male	40	46.5
Median age (range)	71	40–96
Primary Cancer		
Hepato-bilio-pancreatic cancers	24	27.9
Urinary tract cancers	21	24.4
Hematologic malignancies	11	12.8
Gastro-intestinal cancers	11	12.8
Others	19	22.1
Infected patients	53	61.6
Sepsis	23	43.4
Pneumoniae	10	18.9
Urinary tract infections	7	13.2
Intra-abdominal infection	5	9.4
Other	8	15.1
Colonized patients	33	38.4
Rectal swab	27	81.8
Urine	4	12.1
Other	2	6.1
Genotipic characterization		
KPC	83	96.5
OXA-48	2	2.3
VIM	1	1.2
NDM	0	0
IMP	0	0
Phenotype		
HMV	19	22.1
Non-HMV	67	77.9
Biofilm Production		
Weak	38	44.2
Strong	48	55.8
Clinical outcome		
Infection-related mortality	19	35.8

KPC, Klebsiella pneumoniae carbapenemase; IMP, imipenemase metallo β-lactamase; NDM, New Delhi metallo β-lactamase; VIM, Verona integron metallo β-lactamase; OXA-48, oxacillinase-48. Hypermucoviscous (HMV) phenotype.

RSS in colonized patients, while all CRKP from infected patients were KPC-producing *K. pneumoniae* strains. The antimicrobial susceptibility profile confirmed that almost all the CRKP strains were resistant to three carbapenems with a high level of resistance to all tested beta-lactams (**Table 2**). Among the CRKP strains, 10.5% (N9) were also resistant to colistin. Notably, only one strain were found resistant to ceftazidime-avibactam. The only CRKP isolate resistant to ceftazidime-avibactam was the VIM-positive strain. In the colistin-resistant group, seven strains were isolated from infected patients and two from colonized individuals. Among aminoglycosides, 25.6% (N22) of CRKP strains were susceptible to amikacin, and 17.4% (N15) were susceptible to gentamycin. Trimethoprim/sulfamethoxazole-susceptible isolates were 23.3% (N20), while fosfomycin and tigecycline were below the breakpoints in only 13.9% (N12) and 12.7% (N11) of cases, respectively. Notably, only 2.3% (N2) of the CRKP strains were found to be susceptible to ciprofloxacin.

Phenotypic CRKP characterization indicated that 22.1% (N19) of the isolates were HMV, and 77.9% (N67) were classified as non-HMV. The HMV isolates showed a positive string test result (**Figure 1A**). The median length of the string was 7 mm (ranging from 5–25 mm). The mucoviscosisty levels were determined by the sedimentation assay. HMV strains do not sediment properly during low-speed centrifugation, and the supernatant remains turbid, while the non-HMV strains produce compact pellets with clear supernatants. The turbidity of supernatant can be measured by the optical density at 600 nm (OD600) (Walker and Miller, 2020). The OD600 of HMV strains was 0.32±0.12 and, non-HMV was 0.13±0.08 ($P<0.001$) (**Figure 1B**).

TABLE 2 | Antibiotic susceptibility profile of carbapenem-resistant *K. pneumoniae* clinical isolates.

Antibiotic	N	%
Amikacin	22	25.6
Amoxicillin/clavulanic acid	1	1.2
Cefepime	0	0
Cefotaxime	0	0
Ceftazidime	0	0
Ceftazidime/avibactam	85	98.8
Ciprofloxacin	2	2.3
Colistin	77	89.5
Ertapenem	1	1.2
Fosfomycin	12	13.9
Gentamycin	15	17.4
Imipenem	0	0
Meropenem	0	0
Piperacillin/Tazobactam	0	0
Tigecycline	11	12.7
TMP-SMX	20	23.3

N, number of strains susceptible for the indicated antibiotic; TMP-SMX, Trimethoprim/sulfamethoxazole.

One HMV isolate was found colistin-resistant and eight were non-HMV. Due to the low number of colistin-resistant compared to colistin-susceptible strains the difference was no statistically significant. Besides, HMV and non-HMV isolates did not show any significant association to infected or colonized patients as well as to a specific site of isolation.

Among the 86 CRKP isolates, 55.8% (N48) were classified as strong biofilm producers, while 44.2% (N38) showed a weak production (**Table 1**). Strong biofilm-producing CRKP were equally distributed in both infected (N26) and colonized (N22) patients, while weak biofilm-producing strains were more abundant in infected (N27) as compared to colonized patients (N11). Although the level of biofilm was not significantly related to the site of isolation, strong biofilm producers were detected in 80% of BAL from patients with pneumoniae, 63% of urine samples, 63% of RSS, and 43.5% of blood cultures of septic patients (**Figure 2**). Among the colistin-resistant isolates, six were classified as strong and three as weak biofilm producers. The degree of biofilm was not significantly associated with colistin resistance. Noteworthy, biofilm production was significantly different in HMV and non-HMV strains ($P=0.0002$), with the former being mostly weak biofilm producers (88.2%) as compared to non-HMV (33.3%) isolates (**Figure 2**). Confocal microscopy analysis of the biofilms was performed after 24 h of incubation (**Figure 3**). The strong biofilm-producing CRKP isolates (**Figure 3A**) formed a compact 15–25 μm thick multi-layered structure. Conversely, weak biofilm-producing strains, including non-HMV (**Figure 3B**) and HMV (**Figure 3C**) isolates, were scattered over the polystyrene slide surface and no three-dimensional structure could be observed.

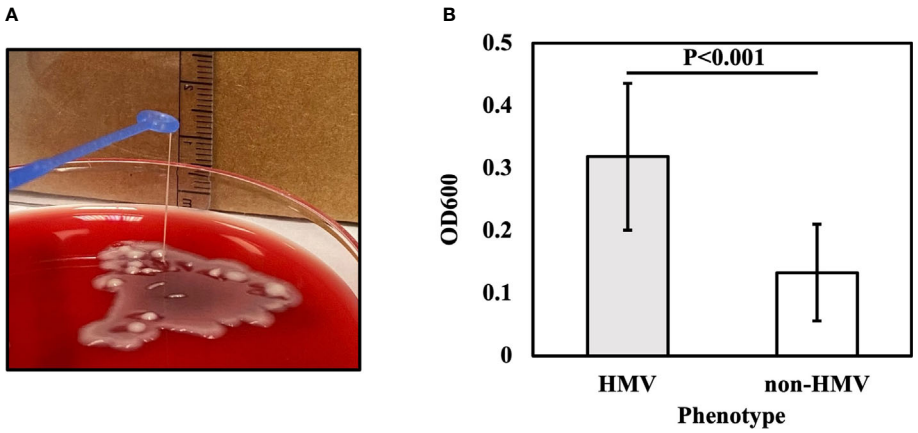

FIGURE 1 | String test for identification of the HMV phenotype. A positive string test **(A)** is defined as the formation of viscous strings of >5 mm in length on an agar plate. **(B)** Sedimentation assay for HMV and non-HMV isolates.

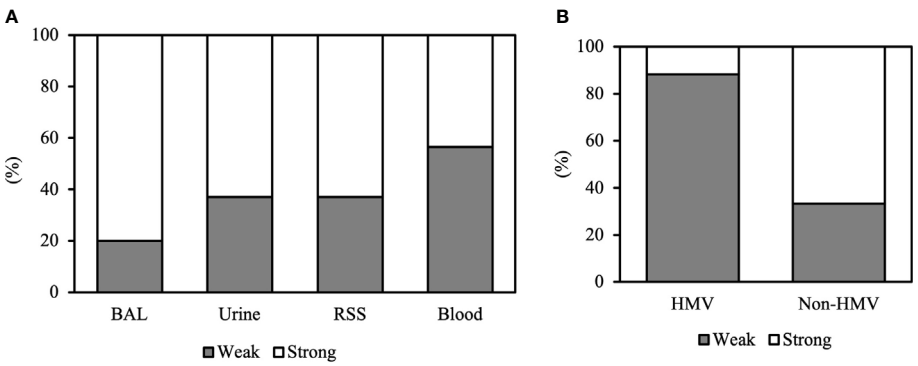

FIGURE 2 | Biofilm formation of CRKP clinical isolates according to **(A)** the site of isolation and **(B)** the HMV and non-HMV phenotype.

None of the patients colonized with CRKP died in the study period. Therefore, the 30-day mortality rate was calculated on the group of infected patients (**Table 3**). Infection-related mortality in this group was 35.8% (N19). In univariate analysis, a significantly high proportion of patients dying within 30 days had pneumoniae (OR 5.09; CI 95% 1.08–24.02; P=0.04). The presence of colistin resistance was not significantly related to increased attributable mortality in this group of patients (OR 3.08; CI 95% 0.33–28.77; P=0.32). Likewise, a concomitant fungal infection was not correlated with increased 30-day mortality (OR 4.00; CI 95% 0.33–47.73; P=0.27). Among the CRKP virulence factors, either the presence of a non-HMV phenotype (OR 4.67; CI 95% 1.13–19.24; P=0.03) or the presence of strong biofilm-producing isolates (OR 5.04; CI 95% 1.39–18.25; P=0.01) represents a significant predictive element for 30-day mortality. Further assessment of CRKP virulence factors by multivariate analysis gave a strong biofilm-producing phenotype as the only independent predictor of mortality (OR 6.30; CI 95% 1.78–19.24; P=0.004).

DISCUSSION

Infections caused by CRKP represent a considerable clinical challenge, often burdened by a delay in the introduction of appropriate antimicrobial therapy, prolonged hospitalization, and considerable mortality rates (Gasink et al., 2009; Mouloudi et al., 2010; Freire et al., 2015; David et al., 2019). Therefore, understanding the impact of microbial infection/colonization factors on the outcome of CRKP-induced diseases may help improve patient management and prognosis.

This study analyzed data from 86 oncological patients with an infection or colonization sustained by CRKP. We found that the leading mechanism of carbapenem resistance was due to the expression of the KPC gene, present in 96.5% of the isolates, followed by OXA-48 and VIM, found in 2.3 and 1.2% of cases, respectively. These data, though from a limited group of strains, are consistent with previous epidemiological studies. Indeed, in Italy, approximately 90% of the CRKP isolates carry the KPC gene, followed by VIM (9.2%) and, in a small percentage, by

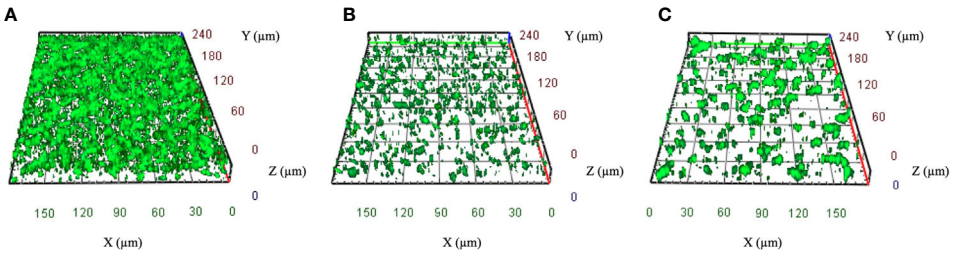

FIGURE 3 | Representative confocal microscopy images of CRKP biofilms developed on polystyrene slides for 24 h at 37°C. **(A)** Strong biofilm-producing non-HMV isolates. **(B)** Weak biofilm-producing non-HMV and **(C)** Weak biofilm-producing HMV strain. Orthogonal sections displaying horizontal (z) and side views (x and y) of reconstructed 3D biofilm images are shown.

TABLE 3 | Univariate and multivariate analyses of factors associated with for 30-day mortality in 53 patients infected with carbapenem-resistant *K. pneumoniae*.

Variables	Univariate Analysis		Multivariate Analysis	
	OR (CI 95%)	*P* value	OR (CI 95%)	*P* value
Biofilm (strong vs weak)	5.04 (1.39–18.25)	0.01	6.30 (1.78–19.24)	0.004
Colistin resistance (no vs. yes)	3.08 (0.33–28.77)	0.32	–	ns
Fungal infection (yes vs. no)	4.00 (0.33–47.73)	0.27	–	ns
Phenotype (non-HMV vs. HMV)	4.67 (1.13–19.24)	0.03	–	ns
Site (respiratory vs. other)	5.09 (1.08–24.02)	0.04	–	ns

Hypermucoviscous (HMV) phenotype.

OXA-48 (1.3%) (Giani et al., 2015; Navon-Venezia et al., 2017; Ripabelli et al., 2018; Di Tella et al., 2019). Different classes of carbapenemases exhibit specific functional properties and susceptibilities, which may be clinically relevant (Cassini et al., 2019). Therefore, information regarding the molecular mechanism leading to carbapenem resistance may also provide a guide in antibiotic selection and administration upon suspicion of infection (Giannella et al., 2014).

The antimicrobial susceptibility profile confirmed that almost all the CRKP strains assessed in this study were resistant to three carbapenems with high resistance levels against all the β-lactams tested (**Table 2**). Novel β-lactam/β-lactamase inhibitor combinations have been recently introduced as new treatment options against infections caused by carbapenem-resistant Enterobacteriaceae (Karaiskos et al., 2019; Sheu et al., 2019). Recent evidence indicates that ceftazidime-avibactam may represent an effective treatment for CRKP infections (Shields et al., 2016; van Duin and Bonomo, 2016; Krapp et al., 2017; Caston et al., 2017; Tumbarello et al., 2019). Indeed, ceftazidime-avibactam inhibits KPC and OXA-48 enzymes, but it is not active against the metallo-β-lactamases (ECDC EARS-NET report, 2017; Shirley, 2018; Sousa et al., 2018; Ambretti et al., 2019). Consistently with these observations, our results show that ceftazidime-avibactam is effective against KPC and OXA-48 but not against CRKP strains harboring the VIM gene (Shirley, 2018; García-Castillo et al., 2018).

Colistin is considered as an antibiotic of last resort for treating severe CRKP infections, because of increasing microbial resistance and associated toxicity (Elnahriry et al., 2016; Schwarz and Johnson, 2016; Rojas et al., 2017; Wang et al., 2018; Ghirga et al., 2020). In this study, we found a 10.5% of CRKP resistant to colistin. This result is consistent with studies performed worldwide, which confirm a colistin-resistant rate not exceeding 8.8%–13% among CRKP isolates, as assessed by broth microdilution (Goel et al., 2014; Olaitan et al., 2014; Rojas et al., 2017; Zafer et al., 2019). Previous colistin therapy was considered an independent risk factor for colistin resistance among CRKP (Giacobbe et al., 2015). In this study, the prevalence of colistin-resistant clinical CRKP isolates was relatively low. Such colistin resistance rates may indicate that infection prevention procedures and antimicrobial stewardship adopted in our institution have reduced the selective pressure, limiting the spread of colistin resistance. In our colistin-resistant group, seven strains were isolated from infected patients and two from a colonized individual. Notably, we did not observe a statistically significant difference in mortality rates between patients infected with colistin-resistant and colistin-susceptible isolates. This observation is consistent with a recent study showing that the patient's conditions and not the presence of colistin-resistant strains have the most significant impact on the clinical outcome (Brescini et al., 2019). However, other studies pointed to a direct association between colistin-resistant strains and mortality (Giacobbe et al., 2015; Rojas et al., 2017). In particular, results from a multicenter study conducted in Italy, in which a 20% colistin resistance was found, reported a mortality rate significantly higher than that observed in patients infected with colistin-susceptible strains (Giacobbe et al., 2015).

The HMV strains represent a serious health threat, causing severe infections in both immune-compromised and healthy

individuals (Shon and Russo, 2012; Shon et al., 2013; Gu et al., 2018; Liu and Guo, 2019). In critically ill patients, such as those from intensive care units, HMV *K. pneumoniae* can induce invasive infection and syndromes (Lee et al., 2010; Liu and Guo, 2019). Thus, the assessment of the HMV phenotype by the string test has been proposed as a necessary addition into the daily practice of microbiological surveillance in ICU (Hagiya et al., 2014). Globally, the prevalence of HMV strains in *K. pneumoniae* isolates is reported in the range of 17%–45% (Yu et al., 2006; Liu and Guo, 2019). The HMV strains are usually highly susceptible to antibiotics, and infections can be generally treated with success using carbapenems (Shon and Russo, 2012; Holt et al., 2015). Nevertheless, sporadic reports of isolation of carbapenemase-producing HMV strains are emerging worldwide, mostly occurring in hospitalized patients (Arena et al., 2017; Gu et al., 2018; Simner et al., 2018). In our study, CRKP-HMV strains accounted for 22.1% of the total isolates. This result is in contrast with previously reported epidemiological data showing a prevalence of about 1% (Gu et al., 2018; Simner et al., 2018; Liu and Guo, 2019). An important concern when considering the highly susceptible HMV strains is their ability to became resistant to carbapenems when subjected to a meropenem regimen (Simner et al., 2018). The carbapenem resistance in HMV appears to be maintained only in the presence of meropenem and is lost after antibiotic removal (Huang et al., 2013; Simner et al., 2018). This suggests that the presence of carbapenemase-encoding plasmids in HMV strains may someway harm bacterial fitness and is dispensable in the absence of selective pressure (Huang et al., 2013; Simner et al., 2018). Such instability may recognize several possible causes and associated factors, including the specific *K. pneumoniae* strains, the type of plasmid incompatibility groups and/or the acquisition of different carbapenemase genes (Simner et al., 2018). The exposure to multiple cycles of prolonged antibiotic treatment in our group of hospitalized patients might have exerted the selective pressure necessary to acquire and preserve carbapenemase genes in such a high number of strains. If true, this further emphasizes the judicious use of antibiotics to limit the development and spread of antibiotic resistance in hypervirulent strains of *K. pneumoniae*. Of importance, we found that non-HMV strains were associated with a significant increase in infection-related mortality. This is in contrast with a previous study describing high mortality rates caused by HMV *K. pneumoniae* strains (Shon and Russo, 2012). However, some controversies exist regarding the HMV classification and its putative virulence (Lin et al., 2011; Zhang et al., 2015). In animal models, HMV strains did not show more severe infections or higher mortality rates as compared to non-HMV (Zhang et al., 2016; Catalan-Najera et al., 2017). Besides, CRKP with an HMV phenotype were found to produce a significantly lower amount of biofilm compared to non-HMV isolates, suggesting that exopolysaccharides production has a negative impact on CRKP fitness (Cubero et al., 2019). This further confirms that the presence of the capsular polysaccharides reduces bacterial adhesion probably by the shielding of the fimbrial adhesins (Wang et al., 2015; Wang et al., 2017). However, in this reduced ability of adhesion may reside an advantage of the HMV strains. Indeed, capsule allows tighter bacterial packing, as compared to capsule-deficient cells, promoting an increased ability to disseminate to distant sites, including the lung, eye, soft tissue and central nervous system (Dzul et al., 2011; Choby et al., 2020). On the other hand, biofilm production may explain, at least in part, the association of non-HMV strains with a significant increase in infection-related mortality, since most non-HMV strains (55.8%) were strong biofilm producers, being equally distributed between infected and colonized patients. Notably, in infected patients, the presence of strong biofilm-producing CRKP significantly (P=0.01) correlated with increased mortality. Strong biofilm producers were detected in 80% of pneumonia cases, 63% of urine samples, 63% of RSS, and 43.5% of blood cultures. The fraction of strong biofilm-producing CRKP observed in this study is consistent with previous reports (Di Domenico et al., 2017; Vuotto et al., 2017; Nirwati et al., 2019; Ramos-Vivas et al., 2019). Studies directed at assessing carbapenem-susceptible *K. pneumoniae* isolated from blood, respiratory specimens, urine, and wounds, found strong biofilm producers in percentages ranging from 65% to 85% (Yang and Zhang, 2008; Hassan et al., 2011; Seifi et al., 2016; Cepas et al., 2019). The analysis of biofilm production *in vitro* showed a large variation among *K. pneumoniae* isolates according to the microenvironment, the surface where the biofilm adheres, temperature, pH, and the physicochemical characteristic of the isolate. A number of reports have pointed to an association between higher level of biofilm formation and the acquisition of a multidrug-resistant phenotype in *K. pneumoniae* (Yang and Zhang, 2008; Subramanian et al., 2012; Sanchez et al., 2013; Vuotto et al., 2017; Bocanegra-Ibarias et al., 2017; Cepas et al., 2019; Nirwati et al., 2019). In particular, an increased rate of horizontal gene transfer among bacteria growing in close contact within the biofilm matrix is deemed responsible for the rapid acquisition of antibiotic resistance, both at the single and multispecies levels (Ghigo, 2001; Madsen et al., 2012; Lebeaux et al., 2014). Despite these findings, the association between antibiotic resistance and biofilm formation is still debated (De Campos et al., 2016; Di Domenico et al., 2017; Cepas et al., 2019).

The overall CRKP infection-related mortality rate observed in the present study was 35.8%. This figure is consistent with recent studies reporting mortality rates of approximately 40% in Italy and other European countries (Hoxha et al., 2016; Xu et al., 2017; Ramos-Castañeda et al., 2018). However, geographic variations, as well as co-morbidities, should be considered. Studies in South America gave figures of 51.0% of CRKP-related mortality while in North America, a 33.2% mortality rate was reported (Rossi Gonçalves et al., 2016; GBD 2015, 2017; Xu et al., 2017). In immune-compromised patients, CRKP infection gave mortality rates higher than those observed in our study, particularly when considering patients undergoing liver transplantation (78%), or patients with hematologic malignancies and solid tumors (56%–73%) (Lübbert et al., 2013; Satlin et al., 2013; Freire et al., 2015; Ramos-Castañeda et al., 2018). We found the highest rate of mortality in patients with pneumoniae and sepsis. Similar results, in association with additional factors, including a high APACHE

score, inappropriate initial antimicrobial therapy, advanced age and shock, were previously found among cancer patients infected with multidrug-resistant agents (Gasink et al., 2009; Souli et al., 2010; Zarkotou et al., 2011; Tumbarello et al., 2012; Bodro et al., 2014; Xu et al., 2017).

Assessment of these data by univariate analysis indicated that both a Non-HMV phenotype (P=0.001) and a strong biofilm-producing strain (P=0.01) are predictive of an increased CRKP infection-related mortality. Besides, multivariate analysis indicated that the presence of strong biofilm-producing CRKP strains was the only microbial factor independently associated with death (95% CI, 1.78-19.24; P=0.004) in oncological patients infected with CRKP. This result is also supported by previous study demonstrating that biofilm formation contributes to increased *K. pneumoniae* pathogenicity (Wu et al., 2011; Ernst et al., 2020). These data further support the notion that biofilm production represents a key CRKP virulence factor, which protects bacteria from physical and chemical insults, including antimicrobials, supporting microbial persistence and dissemination The effective antibiotic concentration required for biofilm eradication *in vivo* is, in most cases, impossible to reach due to drug toxicity and side effects (Ciofu et al., 2015). Therefore, the diagnosis of a biofilm-associated infection represents an area of serious concern for the clinical management of patients. The timely recognition of a strong biofilm producer, before the development of a mature biofilm matrix, may provide key decision-making elements for most appropriate targeting of either medical or surgical intervention, including type, doses, duration of antimicrobial therapy or removal of medical devices, respectively. However, conventional antimicrobial susceptibility testing performed on planktonic cells does not detect the additional resistance mechanism provided by biofilm. Thus, the introduction of reliable microbiological platforms for the diagnosis of biofilm-associated infections and the determination of biofilm-induced antibiotic tolerance represents a desirable addition in clinical microbiology.

Although bringing relevant information, this study has a few limitations. Being a retrospective study performed in a single oncological Hospital, our epidemiology findings might differ from those emerging from other experiences. Nevertheless, data from this study indicated that the mortality rate among oncological patients infected with CRKP is high (35.8%). The infection-related mortality rate did not correlate with the presence of HMV strains but, conversely, was significantly associated with non-HMV, strong biofilm-producing isolates, the latter representing an independent risk factor of death in oncological patients infected with CRKP. A more in-depth exploration of the mechanisms promoting biofilm formation in *K. pneumoniae* will help identify specific virulence markers. Nevertheless, the timely recognition of biofilm-associated infections and biofilm-induced drug tolerance still represents an unmet need in clinical microbiology.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Central Ethics Committee I.R.C.C.S. Lazio, approved the study (Prot. CE/1016/15—15 December 2015, trials registry N. 730/15). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ED designed the study and wrote the paper. FM, GP, FP, IS, LP, FA, LT, AL, AM, and FE, discussed the results and implications and wrote the manuscript. ICa, FS, and TK performed the phenotypic and genomics experiments. FDS, ICe, and SP analyzed the presence of the carbapenemase genes. TK and CP collected and interpreted the clinical data. All authors contributed to the article and approved the submitted version.

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Nitrofurantoin Combined With Amikacin: A Promising Alternative Strategy for Combating MDR Uropathogenic *Escherichia coli*

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Urinary tract infections (UTI) are common infections that can be mild to life threatening. However, increased bacterial resistance and poor patient compliance rates have limited the effectiveness of conventional antibiotic therapies. Here, we investigated the relationship between nitrofurantoin and amikacin against 12 clinical MDR uropathogenic *Escherichia coli* (UPEC) strains both *in vitro* and in an experimental *Galleria mellonella* model. *In vitro* synergistic effects were observed in all 12 test strains by standard checkerboard and time-kill assays. Importantly, amikacin or nitrofurantoin at half of the clinical doses were not effective in the treatment of UPEC infections in the *G. mellonella* model but the combination therapy significantly increased *G. mellonella* survival from infections caused by all 12 study UPEC strains. Taken together, these results demonstrated synergy effects between nitrofurantoin and amikacin against MDR UPEC.

Keywords: MDR UPEC, nitrofurantoin, amikacin, antibiotic combination, *G. mellonella* model

INTRODUCTION

Urinary tract infections (UTI) are defined microbiologically as the inflammatory response of the urothelial to microbial pathogens and are some of the most common bacterial infections affecting 150 million people each year worldwide (Klein and Hultgren, 2020). UTIs are most commonly associated with uropathogenic *Escherichia coli* (UPEC) and *E. coli* ST131 is the globally dominant multiple drug-resistant (MDR) UPEC clone that causes infections associated with limited treatment options (Daoud et al., 2015; Phan et al., 2020). These infections can also be highly recurrent and following antibiotic therapy, 20–30% of women with acute UTIs will have a recurrent episode within six months and half of these recurrences are caused by the same UPEC strain that caused the initial infection (Godaly et al., 2015). The increases in bacterial resistance as well as poor patient compliance rates have limited the effectiveness of conventional antibiotic therapies for UTIs especially in developing countries (Ayukekbong et al., 2017; Goodlet et al., 2018). Therefore,

there is a great need for alternative strategies to treat these infections and one important approach is combination therapy using pre-existing antibiotics (Brochado et al., 2018).

Carbapenems have been recommended for treating UTIs caused by extended spectrum β-lactamase (ESBL) producing bacteria but currently are being restricted due to increased resistance (Han et al., 2015). However, non-carbapenem antibiotics are also important for UTI treatment. Trimethoprim-sulfamethoxazole (TMP-SMX) was the preferred antibiotic for UTI treatment for many years due to its efficacy and low cost. However, the development of TMP-SMX resistance among uropathogens has altered this strategy and fluoroquinolones are now preferred because they are highly concentrated in urine and have excellent activity against most uropathogens (Johnson and Stamm, 1987). The US Food and Drug Administration (FDA) approved nitrofurantoin in 1953 and was the standard UTI treatment until the late 1970s when other antibiotics became available (Muller et al., 2017). In 2011 nitrofurantoin was again recommended as first-line therapy for lower UTI due to increasing resistance to newer antibiotics such as the fluoroquinolones (Gupta et al., 2011). Antibiotic resistance is spreading rapidly in UPEC, which may be related to the genetic modulate, own pathogenicity and drug resistance of themself. The use of fluoroquinolones can induce the UPEC to partial or total loss of the pathogenicity islands and lead to cross-resistance of β-lactam drugs (Soto et al., 2006; Rohde et al., 2018; Adamus-Bialek et al., 2019; Tchesnokova et al., 2019). On the other hand, the high prevalence of integrons and plasmids also leads to high levels of antibiotic resistance and virulence genes in clinical urogenic bacteria, such as resistance of extended spectrum β-lactamase (ESBL) producing and to quinolones (Raeispour and Ranjbar, 2018; Abbasi and Ranjbar, 2018; Farajzadah Sheikh et al., 2019; Halaji et al., 2020a; Halaji et al., 2020b). However, the use of nitrofurantoin is contraindicated in patients with renal failure due to metabolites that may cause peripheral neuropathy (Spring et al., 2001). Similarly, nitrofurantoin is not recommended for the treatment of complicated UTIs because these infections often compromise the kidney and lead to renal dysfunction (Ingalsbe et al., 2015). In contrast, amikacin is a first-line drug used for Gram-negative infections other than UTIs that is economical and convenient to administer. Its potential ototoxicity and nephrotoxicity is dose related so that it could be efficacious and safe for the treatment of pyelonephritis and sepsis if managed properly (Leibovici et al., 2009).

Interestingly, amikacin and nitrofurantoin can synergize against *E. coli in vitro* (Yeh et al., 2006), and these two antibiotics are used separately to treat or prevent UTIs caused by MDR *E. coli*. In this study, we examined whether the co-administration of amikacin and nitrofurantoin could provide a new strategy for UTI treatment and evaluated the synergistic effects of these two drugs *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Twelve clinical isolates were obtained from the urine of hospitalized UTI patients at the Third Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China). All tested strains were identified to the species level by MALDI-TOF MS (Axima-Assurance-Shimadzu) and *E. coli* ATCC 25922 was used as a quality control strain (**Table 1**).

Genomic DNA from the 12 clinical isolates was subjected to 250 bp paired-end whole genome sequencing (WGS) using the Illumina MiSeq system (Illumina, San Diego, CA, USA) and the reads were assembled using SPAdes v3.6.2. (Bankevich et al., 2012) MLST and antibiotic resistance genes (ARG) were analyzed using the CGE server (https://cge.cbs.dtu.dk/services/) and ABRicate (https://github.com/tseemann/abricate).

Antimicrobial Agents

Amikacin (AMK), nitrofurantoin (NIF), meropenem (MEM), cefotaxime (CTX), tigecycline (TIG) and tetracycline (TET) were

TABLE 1 | *In vitro* antimicrobial susceptibility profiles for clinical strains.

E. coli strain	Relevant genotype	MIC (mg/L)										
		NIF	AMK	MEM	AMP	CTX	FOS	CST	TIG	TET	CIP	SXT
ATCC 25922	ST73	16	4	0.03	4	0.125	2	2	0.06	1	0.015	1/19
E7102	ST131	16	2	0.015	>256	256	0.25	2	0.06	64	128	>16/304
E6929	ST131	16	4	0.25	>256	>256	0.5	1	2	1	128	>16/304
E4396	ST1193	16	2	0.015	>256	>256	2	4	0.06	64	32	>16/304
E3759	ST101	16	4	0.03	>256	>256	64	0.5	0.25	128	16	>16/304
E7088	ST1426	16	2	0.015	>256	>256	4	2	0.125	256	1	>16/304
E4181	ST53	16	4	0.015	>256	256	8	2	0.06	2	32	>16/304
E68071	ST3177	8	2	0.015	>256	256	16	2	0.03	64	128	>16/304
E67991	ST3177	16	2	0.25	>256	>256	0.25	2	2	1	64	>16/304
E3966	ST354	8	4	0.015	>256	>256	>256	0.5	0.03	128	>256	>16/304
E4740	ST53	16	4	0.015	>256	>256	8	1	0.06	64	32	>16/304
E68317	ST1249	16	16	0.015	>256	>256	256	2	0.125	64	2	>16/304
E62603	ST1196	32	4	0.125	>256	>256	8	0.5	4	128	64	>16/304

NIF, nitrofurantoin; AMK, amikacin; MEM, meropenem; AMP, ampicillin; CTX, cefotaxime; FOS, fosfomycin; CST, colistin; TIG, tigecycline; TET, tetracycline; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim.

purchased from Yuan Ye Biological Technology (Shanghai, China). Ampicillin (AMP), fosfomycin (FOS), colistin (CST), ciprofloxacin (CIP) and sulfamethoxazole/trimethoprim (SXT) were purchased from Xiang Bo Biotechnology (Guangzhou, China). Antibiotic stocks solutions were prepared according to the manufacturer's recommendations.

MIC Determinations

Antimicrobial susceptibility assays were performed and interpreted according to CLSI guidelines (CLSI, 2018) using the microdilution broth method except for fosfomycin. The MIC of Fosfomycin was tested using the agar dilution method in agar media supplemented with 25 mg/L glucose-6-phosphate. *E. coli* strain ATCC 25922 was used for quality control.

In Vitro Fractional Inhibitory Concentration Index (FICI) Assay

The checkerboard technique was employed to determine the Fractional Inhibitory Concentration Index (FICI) of nitrofurantoin/amikacin combinations as previously described (White et al., 1996). Briefly, 96 well plates containing serial dilutions of nitrofurantoin and amikacin (range 0.125 to 32 mg/L) were inoculated with 5 × 10 (Goodlet et al., 2018) cfu/mL of test bacteria and incubated for 18 h at 37°C. Plates were screened for growth by spectrometry at 600 nm. Control wells did not receive any drugs. The FICI was calculated by the following equation: FICI= (MIC of agent A in combination/MIC of agent A alone) + (MIC of agent B in combination/MIC of agent B alone) (Odds, 2003). Synergy was defined as FICI ≤0.5, antagonism as FICI ≥4 and no interaction as 0.5 < FICI < 4. All FICI assays were carried out three times on three different days. FICIs were calculated as the mean values from three independent experiments.

In Vitro Time–Kill Curves

Time–kill experiments were conducted to further characterize the synergistic activity of the nitrofurantoin and amikacin combination as previously described (Dong et al., 2017). In brief, an initial inoculum of ~10^4 cfu/larva logarithmic-phase cells were incubated with amikacin in the presence and absence of nitrofurantoin and time–kill curves were compared to assess efficacy. Serial samples were obtained at 0, 3, 6, 9, and 24 h after incubation at 37°C. Bacterial counts were determined based on the quantitative cultures on MHA plates. Synergy was defined as achieving a ≥ 2 $\log_{10}$ cfu/mL reduction in bacterial growth at 24 h with the combination compared with the most active individual drug concentration used on its own (Gomara and Ramon-Garcia, 2019). Three independent experimental runs were performed.

Antibiotic Resistance Evolution Under Nitrofurantoin and Amikacin Single or Combination Stress

After time–kill experiments, five clones used for viable count enumeration in 24 h were randomly selected for each experimental group from MHA plates. MIC values were measured for these clones to compare whether drug resistance developed under nitrofurantoin and amikacin selection pressure when used alone and in combination.

Galleria mellonella Infection Model

A well-characterized *G. mellonella* model was used in this study as previously described publication (Dong et al., 2017). The *G. mellonella* larvae were obtained from Kaide Ruixin (Tianjin, China). The optimal infection doses of the study test strains were determined using *G. mellonella* larvae that were randomly distributed into six experimental groups (n=10/group or ~250 mg). These were then infected by injection of 10 μL of logarithmic phase *E. coli* cells (~10^4 cfu/larva) into the last left proleg. After injection, the larvae were incubated in plastic Petri dishes at 37°C for 72 h and scored for survival daily. In all experiments, PBS injections were used as negative controls.

The *in vivo* efficacy of nitrofurantoin and amikacin alone and in combination were assessed in the same *G. mellonella* model caused by our study *E. coli* strains using the optimal infection doses as determined above (~10^4 cfu/larva). At 2 h post-infection, animals were randomized to receive no therapy or nitrofurantoin and amikacin alone, and in combination (n = 10/group) (Seed and Dennis, 2008; Ahmad et al., 2010; Dong et al., 2017). The antibiotics were administered only once (10 μL) into the last right proleg with nitrofurantoin at 3.75 mg/kg, amikacin at 7.5 mg/kg alone or in combination at half doses (Beaucaire et al., 1991; Amabile-Cuevas and Arredondo-Garcia, 2011). Larvae were observed daily for 3 days and percent of larvae survival was calculated for each group (**Figure 3A**).

Statistical Analysis

Bacterial counts were transformed to $\log_{10}$ values and the data were analyzed using Graphpad Prism 7.0 (GraphPad Software, San Diego, CA, USA). P values were determined using a two-sided, Mann–Whitney U-test. A P-value of ≤ 0.05 was considered significant. All data were presented as means ± SD.

RESULTS

In Vitro Susceptibility and Interaction Assessment

The MICs of 11 antibiotics were determined against our collection of clinical isolates. The MICs for amikacin ranged from 2 to 16 mg/L and all strains were susceptible. The MICs for nitrofurantoin ranged from 8 to 32 mg/L and all 12 UPEC strains were susceptible. These 12 clinical UPEC strains were classified as MDR *E. coli* (**Table 1** and **Table S1**). *In vitro* testing of amikacin/nitrofurantoin combinations indicated a synergistic action against all 12 UPEC strains with FICI values ranging from 0.292 ± 0.072 to 0.500 ± 0.125 (**Figure 1**). These data indicated that combination of amikacin and nitrofurantoin can synergize to combat MDR UPEC strains.

In Vitro Time–Kill Curves

We then performed kinetic time–kill assays for all test strains to better evaluate the pharmacodynamics of the amikacin and

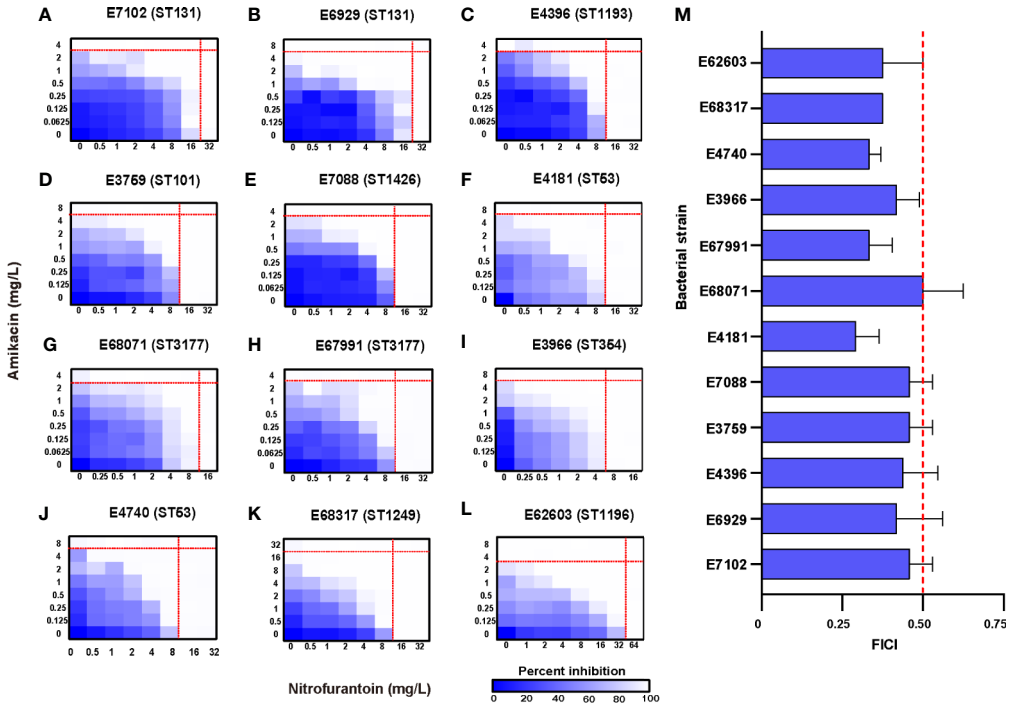

FIGURE 1 | Potentiation of amikacin partnered with nitrofurantoin against 12 test strains. **(A–L)** Microdilution chequerboard assays are shown as 8×8 matrix heat map graphs. The blue colour gradient represents the bacterial cell density estimated by OD_{600}. AMK, amikacin; NIF, nitrofurantoin. **(M)** FICI of the test strains where synergy is defined as a FIC index of ≤ 0.5. The thin red line represents the (MICs) for antibiotics used separately and the think one represents the FIC index of 0.5.

nitrofurantoin interaction. We examined time–kill curves representing $\log_{10}$ changes in bacterial burden using the ST131 UPEC strains E7102 and E6929 over 24 h following exposure to amikacin (1×MIC) in the presence of increasing amikacin concentrations (1/4–1×MIC). The addition of nitrofurantoin to amikacin increased *in vitro* bactericidal activity compared with nitrofurantoin alone. Similarly, the addition of amikacin to nitrofurantoin also significantly increased *in vitro* bactericidal activity (**Figures 2A, B**). We then tested amikacin at 1/2 MIC alone and in combination with 1/2 MIC nitrofurantoin, to observe whether they could have a good bactericidal effect under the sub-inhibition concentration. The combination therapy resulted in synergistic effects against all 12 clinical UPEC strains. For instance, the combination therapy caused more than a 2 $\log_{10}$ cfu/mL reduction for all 12 UPEC strains as compared to the most active antibiotic alone (**Figure 2C** and **Figure S1**). The amikacin/nitrofurantoin combination significantly increased *in vitro* antimicrobial activity and resulted in a rapid killing of the bacterial test strains for 9 combination groups caused reductions as compared with the most active antibiotic alone that ranged from 4.055 ± 1.050 to 8.714 ± 0.131 cfu/mL (**Table S2**). The combination group against *E. coli* strain E67991 was obtained most weakly synergistic effects, but also caused more than 4 log10 cfu/mL reductions and showing bactericidal action. Against *E. coli* strain E4740 and E62603, the combination group almost completely elimination the bacteria at 24 h of incubation, and the bacterial burden less than 1 $\log_{10}$ cfu/mL (**Figure 2D**).

Antibiotic Resistance Evolution Under Nitrofurantoin and Amikacin Single or Combination Stress

Considering the pronounced effects that the combination therapy had on *in vitro* bacterial growth, five clones were selected for MIC determinations. In the 12 nitrofurantoin groups, 3 MICs were increased while the other 9 were unchanged. In contrast, 5 amikacin MICs were decreased, 5 were unchanged and 2 groups displayed MIC increases. The 12 amikacin groups displayed 10 MICs that were increased and 2 unchanged while 4 nitrofurantoin MICs were decreased and 8 remained unchanged. When the amikacin groups were compared with the combination group, the amikacin MICs were decreased in 10/12 of the combination groups and in the other two groups, one group was unchanged and one increased. Compared to the nitrofurantoin groups, the nitrofurantoin MICs had decreased in 3/12 of the combination groups while the remaining 9 groups were unchanged (**Figure S2**).

In Vivo Synergistic Efficacy

The amikacin/nitrofurantoin combination showed significant synergistic effects *in vitro* so we investigated whether these effects in the *in vivo G. mellonella* model using amikacin and

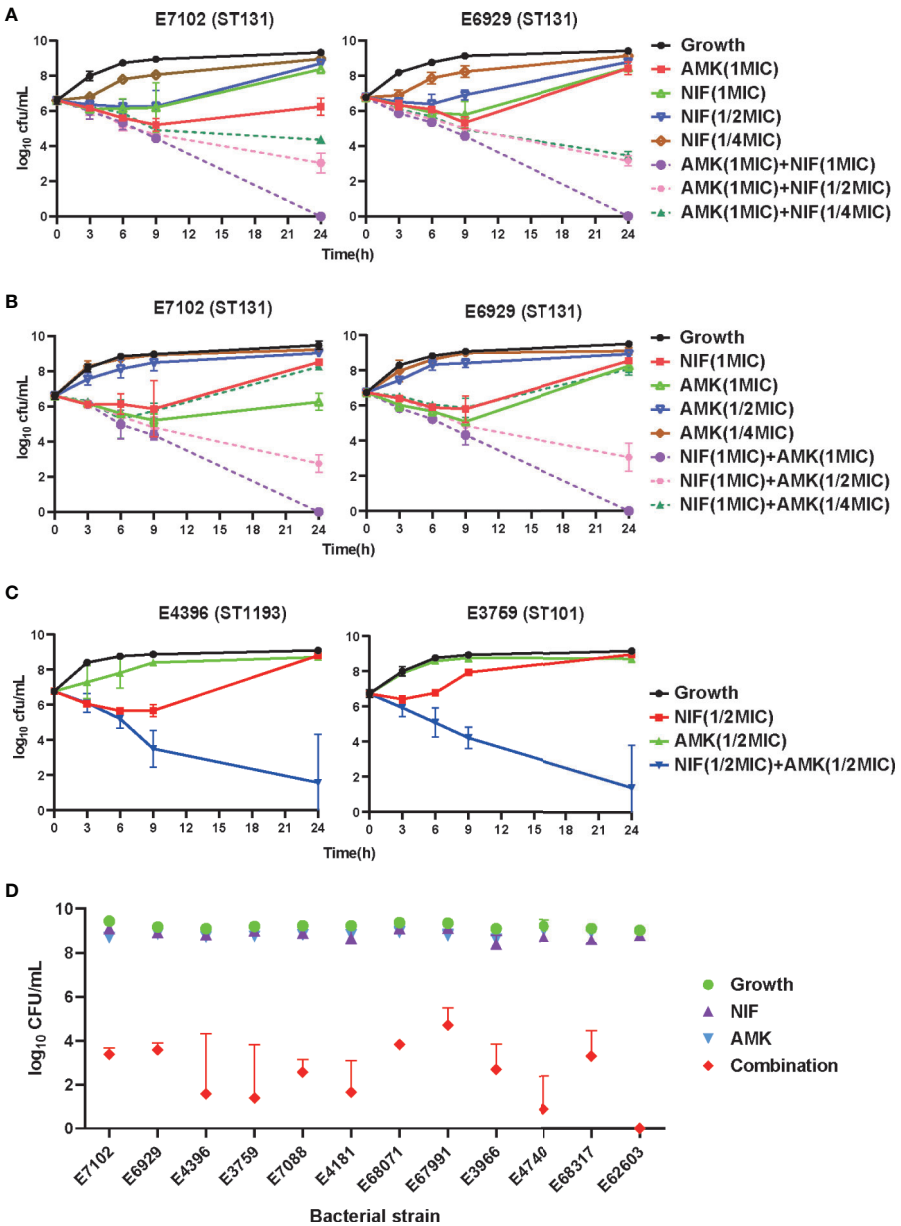

FIGURE 2 | *In vitro* time–kill curves using amikacin and nitrofurantoin alone and in combination against the indicated test strains. **(A–C)** Combinatorial bactericidal activity of amikacin and nitrofurantoin against ST131 UPEC strains. Mean ± standard error from three independent experiments are shown. AMK, amikacin; NIF, nitrofurantoin. **(D)** Growth after 24 h for all the test strains using amikacin and nitrofurantoin alone and in combinations compared to the control.

nitrofurantoin at half clinical dosages. The amikacin and nitrofurantoin monotherapies were ineffective against our two ST131 UPEC strains (E7102 and E6929) but the combination therapy resulted in 80–90% survival after 72 h. The combination therapy also significantly increased survival from infections with the E4396 and E3759 strains (**Figure 3B**). The use of amikacin monotherapy significantly increased *G. mellonella* survival from infections in only a single UPEC strain (E4181). Similarly, nitrofurantoin monotherapy significantly increased *G. mellonella* survival from infections caused by two strains; E4181 and E68071. Therefore overall, amikacin or nitrofurantoin given at subinhibitory concentrations was not effective in the treatment of UPEC infections in the *G. mellonella* model. In contrast, the amikacin/nitrofurantoin combination significantly increased survival from infections caused by all 12 study UPEC strains. More importantly, the combination therapy significantly increased survival compared with amikacin or nitrofurantoin monotherapies with 11/12 of the test strains ($p < 0.05$). Only one UPEC strain E68071 generated a non-significant P value (0.0957). Overall, we found increases in survival with the

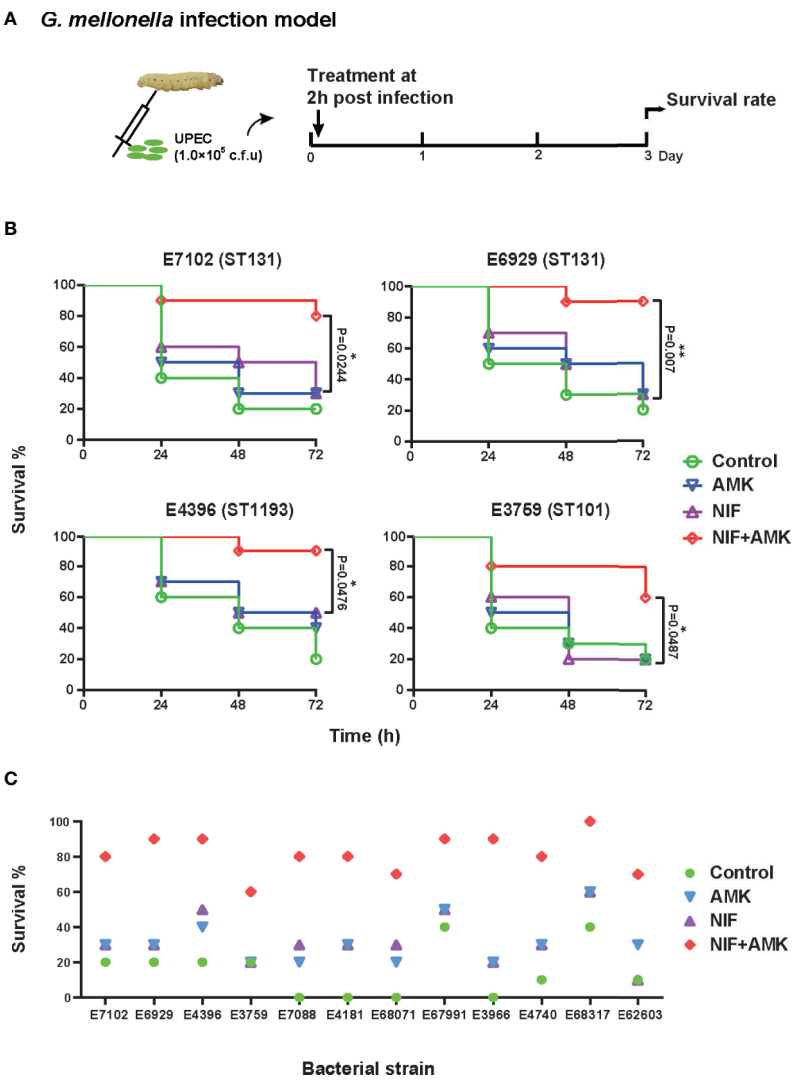

FIGURE 3 | Therapeutic effects of amikacin combined with nitrofurantoin in the *G. mellonella* model. **(A)** Scheme of the experimental protocol for the *G. mellonella* model. **(B)** Survival rates of amikacin and nitrofurantoin alone (most effective) and in combination treatment in an experimental *G. mellonella* model caused by the indicated UPEC strains. **(C)** Survival rates after 72 h caused by the indicated UPEC strains. (∗) $p < 0.05$ and (∗∗) $p < 0.01$.

combination therapies that increased survival against challenge by our UPEC test strains from 40 to 70% (**Figure 3C** and **Figure S3**).

DISCUSSION

Almost all patients with UTI are treated with antibiotics that generates annual costs estimated for the United States at $2.14 billion (Brown et al., 2005). The antimicrobial agents most commonly used to treat uncomplicated UTI include the combination trimethoprim and sulfamethoxazole, trimethoprim, β-lactams, fluoroquinolones, nitrofurantoin, and fosfomycin, third-generation cephalosporins, aminoglycosides and carbapenems (Jancel and Dudas, 2002; Koningstein et al., 2014) and this wide range of treatment options belies the serious threat that these MDR organisms pose. The increasing prevalence of antibiotic-resistant uropathogens has begun to limit the effectiveness of our existing antibiotic arsenal (Barber et al., 2013). Combinations of antibiotics are commonly used in medicine to broaden the antimicrobial spectrum and generate synergistic effects and this therapy has proven effective against MDR bacteria (Gomara and Ramon-Garcia, 2019). For example, the use of oral cephalosporin and β-lactamase inhibitor combinations for ESBL-producing Enterobacteriaceae UTI (Stewart et al., 2020).

Nitrofurantoin and amikacin are both used for the treatment and prevention of UTIs. To the best of our knowledge, we are the

first to report of the synergistic effect of amikacin and nitrofurantoin against UPEC in the *G. mellonella* model. This provides a new strategy for the treatment of UTIs caused by UPEC. It is worth noting that aminoglycoside drugs (such as gentamicin and tobramycin) previously been reported can improve the sensitivity of UPEC to Nitrofurantoin after treatment (Adamus-Bialek et al., 2019). One challenge presented by drug combinations is the requirement to determine coincident pharmacological properties such as tissue distribution and penetration (Ejim et al., 2011). The nitrofurantoin/amikacin combination can avoid this problem because these two antibiotics are in current used for UTI treatment and prevention. In general, maximum urine concentrations of nitrofurantoin vary from 15 mg/L to 230 mg/L and were found between ~ 3 and 10 h after dosing, depending on the crystal size, formulation of the nitrofurantoin product and the fasting status of the subject (Wijma et al., 2018). Conventional amikacin is almost entirely excreted unchanged in the urine within hours after administration in all species studied (Fielding et al., 1999).

The synergistic mechanism of nitrofurantoin and amikacin is not clear because nitrofurantoin possesses several mechanisms of antimicrobial action that involve damage to DNA and ribosomes (Woody-Karrer and Greenberg, 1963; Jenkins and Bennett, 1976; Huttner et al., 2015). Amikacin targets the bacterial ribosome and inhibits translation by causing misreading and hindering translocation (Taber et al., 1987; Allison et al., 2011). Both these drugs target the ribosome and this is the most likely site of action for the combination. In addition, nitrofurantoin stimulates the production of reactive oxygen species (ROS) (Garcia Martinez et al., 1995) that can facilitate the entry of aminoglycosides and subsequent bacterial killing (Ezraty et al., 2013). Furthermore, aminoglycosides have collateral sensitivity with many antibiotics, nitrofurantoin has also been reported to be collateral sensitivity with tigecycline, mecillinam and protamine, therefore, whether nitrofurantoin has synergistic sensitivity with amikacin is also very much studied (Suzuki et al., 2014; Pal et al., 2015; Roemhild et al., 2020).

The results of the time-kill assays in this study demonstrated that nitrofurantoin/amikacin at 1/2 MIC concentration displayed synergistic bactericidal effects and indicated that combination therapy can reduce antibiotic dosage. In addition, at half of the clinically recommended dose, the combined treatment group significantly increased the survival rate of larva compared with the single treatment group. After 24 h under nitrofurantoin or amikacin stress, the MIC values of the corresponding drugs increased to different degrees, especially in the case of amikacin. However, the MIC values for both nitrofurantoin and amikacin were decreased in the combination group compared with the single drug group, although the decrease was less than 2-fold. This suggests drug combination strategies can be effective against MDR bacteria while slowing down the development of antibiotic resistance.

In the *G. mellonella* infection model, the combination treatment significantly improved larvae survival compared with the most active antibiotic alone. These results were consistent with the *in vitro* time kill assays except for a single UPEC strain, 68071. These results indicated that the *G. mellonella* model is useful for assessing the *in vivo* efficacy of anti-UPEC agents. Previous studies have indicated that *in vivo* synergism results were not always directly related to the *in vitro* results (Thieme et al., 2020). This may be due to strain differences and the test antibiotics. The *G. mellonella* model enables a rapid, economical and reproducible model to assess the synergistic effects of antimicrobials in an *in vivo* setting (Cools et al., 2019).

There are several limitations to this study that should be noted. For example, we did not have a full complement of strains with low level resistance to both nitrofurantoin and amikacin. In addition, we only tested UPEC strains in the *G. mellonella* model rather than the murine urinary tract infection model and future will address this concern. There was a certain gap in the model construction compare with the natural infection, and we did not take into account factors such as the formation of biofilm by UPEC in animal, these require further study. Moreover, based on our current findings, further investigations are necessary to examine the effectiveness of this combination in PK/PD models to optimize the dose regimen. The tests of synergy for this drug combination must also be linked to patient outcome (Doern, 2014).

In summary, we confirmed that the combination of nitrofurantoin and amikacin possesses a significantly synergistic effect on MDR UPEC *in vitro*. In addition, we demonstrated for the first time that this drug combination was significantly synergistic effect on MDR UPEC in the *G. mellonella* model. Our findings constitute an alternative and promising therapeutic option for the treatment of UTIs caused by MDR UPEC.

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: PRJNA678682].

ETHICS STATEMENT

Clinical strains isolated from humans in this study were provided by the Third Affiliated Hospital of Sun Yat-sen University. This study was carried out in accordance with the recommendations of ethical guidelines of South China Agricultural University. SCAU Institutional Ethics Committee did not require the study to be reviewed or approved by an ethics committee because we are not involved in the isolation of bacteria.

AUTHOR CONTRIBUTIONS

Z-XZ and Z-HC contributed equally in this study. JS, Y-HL, and X-PL designed the study. Z-HC, Z-XZ, TT, X-JL, Z-JZ, and W-NN carried out the experiments. JS, Z-HC, YY, and Y-FZ

analyzed the data. JS, Z-HC, L-XF, and Z-XZ wrote the draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.608547/full#supplementary-material

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Heterogeneous *Klebsiella pneumoniae* Co-infections Complicate Personalized Bacteriophage Therapy

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Multidrug-resistant (MDR) organisms have increased worldwide, posing a major challenge for the clinical management of infection. Bacteriophage is expected as potential effective therapeutic agents for difficult-to-treat infections. When performing bacteriophage therapy, the susceptibility of lytic bacteriophage to the target bacteria is selected by laboratory isolate from patients. The presence of a subpopulation in a main population of tested cells, coupled with the rapid development of phage-resistant populations, will make bacteriophage therapy ineffective. We aimed to treat a man with multifocal urinary tract infections of MDR *Klebsiella pneumoniae* by phage therapy. However, the presence of polyclonal co-infectious cells in his renal pelvis and bladder led to the failure of three consecutive phage therapies. After analysis, the patient was performed with percutaneous nephrostomy (PCN). A cocktail of bacteriophages was selected for activity against all 21 heterogeneous isolates and irrigated simultaneously *via* the kidney and bladder to eradicate multifocal colonization, combined with antibiotic treatment. Finally, the patient recovered with an obviously improved bladder. The success of this case provides valuable treatment ideas and solutions for phage treatment of complex infections.

Clinical Trial Registration: www.chictr.org.cn, identifier ChiCTR1900020989.

Keywords: urinary tract infection, phage therapy, percutaneous nephrostomy, heterogeneous cells, multidrug-resistant *Klebsiella pneumoniae*

INTRODUCTION

Urinary tract infections (UTIs) are among the most prevalent microbial diseases in both men and women during their lifespan and cause a major burden worldwide (Sihra et al., 2018). Administration of antibiotics is one of the key means for the management of infectious pathogens. However, MDR organisms have increased worldwide, posing a major challenge for the clinical management of infection (Moellering, 2010). One of the most urgent areas is the rapid evolution of antibiotic resistance among *Enterobacteriaceae* e.g., *Klebsiella pneumoniae* (*K. pneumoniae*) (Tacconelli et al., 2018). MDR *K. pneumoniae* is known to cause various bodily infections, including UTIs, pneumoniae, bloodstream infections, and sepsis. It is also a threat to individuals with weak immune systems and hospitalized patients following invasive surgical procedures. Infections caused by these organisms are not only difficult to treat but are also known to cause significant mortality (Marr and Russo, 2019). Bacteriophage is expected as potential effective therapeutic agents for difficult-to-treat infections, with some successful case reports supported by a large fundamental knowledge base (Schooley et al., 2017; Watts, 2017; Corbellino et al., 2019; Dedrick et al., 2019; Schmidt, 2019).

Heteroresistance phenomena was first described in the 1940s (Alexander and Leidy, 1947), which refers to seemingly identical bacterial cells in a population with one subpopulation or several subpopulations that exhibit increased levels of antibiotic resistance compared with the main population (Andersson et al., 2019). Such populations are often difficult to detect and cause antibiotic treatment failure (Nicoloff et al., 2019). Phage therapy also requires phage screening with clinically isolated strains to select the appropriate phage for treatment. Due to the high specificity of bacteriophage, the presence of bacterial heterogeneity in a population can also lead to the failure of bacteriophage therapy. Thus, a phages that will be selected for treatment should have a broad range of activity. In the present study, we report a case of polyclonal heterogenous bacterial UTI treated by personalized bacteriophage cocktails. After four round phage screenings against clinical isolates, bacteriophage cocktail with combination of antibiotics and PCN eventually cured the patient of long-term MDR *K. pneumoniae* UTI.

MATERIALS AND METHODS

Bacteriological Studies

Bacterial isolates were obtained from routine microbiological cultures from patient. Twenty-one *K. pneumoniae* strains were recovered from the patient's urine, renal pelvis effusion and proximal ureteral stent tip, as detailed in **Table 1**. Identification of isolates at the species level was obtained by MALDI-TOF Biotyper (Bruker, Germany).

Minimum inhibitory concentrations (MICs) for carbapenems (imipenem and ertapenem) and aminoglycosides (gentamicin, amikacin and tobramycin) were determined using VITEK 2 COMPACT (bioMérieux), and tigecycline was determined using E-test strips (Oxoid) on Mueller-Hinton agar plates (Oxoid). The MICs for colistin were determined by broth culture microdilution. Meropenem (Oxoid) was determined using the disc diffusion test on Mueller-Hinton agar plates (Oxoid). The results were interpreted according to the CLSI2018 (Clinical and Laboratory Standards Institute).

TABLE 1 | *Klebsiella pneumoniae* strains and their phage susceptibilities.

Strain	Date	Origin	Phage sensitive				
			ΦJD902	ΦJD905	ΦJD907	ΦJD908	ΦJD910
4137	25 Nov 2017	Urine	+	+	+	+	+
0344	8 Jan 2018	Urine	+	+	+	+	+
1231	26 Jan 2018	Urine	+	+	+	+	+
1280	28 Jan 2018	Urine	+	+	+	+	+
1439	30 Jan 2018	kidney[r]	−	+	−	+	+
1440	30 Jan 2018	kidney[l]	+	+	+	+	+
1469	31 Jan 2018	Urine	+	+	+	+	+
1518	1 Feb 2018	Urine	−	+	+	+	+
1532	2 Feb 2018	Urine	−	+	+	+	+
1591	3 Feb 2018	Urine	+	+	+	+	+
1639	4 Feb 2018	Urine	+	+	+	+	+
1667	5 Feb 2018	Urine	−	+	+	+	+
1769	6 Feb 2018	Urine	−	+	−	+	+
1789	7 Feb 2018	Urine	−	+	+	+	+
3549	21 Mar 2018	Double J [b]	+	+	−	+	+
3637	21 Mar 2018	Double J [k]	+	+	−	+	+
3837	26 Mar 2018	Urine	+	+	−	+	+
4078	28 Mar 2018	Urine	+	+	+	+	+
4163	1 Apr 2018	Urine	+	+	+	+	+
4247	3 Apr 2018	Urine	+	+	−	+	−
4321	4 Apr 2018	Urine	+	+	+	+	+

Kidney[r] represents right renal pelvis effusion; kidney[l] represents left renal pelvis effusion; Double J [b] represents stent tip from bladder; Double J [k] represents stent tip from right renal pelvis. "+" represents lytic activity; "-" represents non-lytic.

Illumina WGS and Phylogenetic Analysis

The clonal relationship of the isolates was analysed by whole-genome sequencing. Briefly, total DNA from the *K. pneumoniae* isolates was extracted and sequenced with the Illumina X10 (Illumina, San Diego, CA, USA). Genome assembly was performed using the Velvet 1.0.15 program (Zerbino and Birney, 2008). The sequence of *K. pneumoniae* was deposited in the GenBank databases with accession number (SAMN13324145, SAMN13324146, SAMN13324147, SAMN13324148, SAMN13324149, SAMN13324150, SAMN1332415, SAMN13324152, SAMN13324153, SAMN13324154, SAMN13324155, SAMN13324156, SAMN13324157, SAMN13324158, SAMN13324159, SAMN13324160, SAMN13324161, SAMN13324162, SAMN13324163, SAMN13324164, SAMN13324165). Genome-wide single nucleotide polymorphism (SNP) calling and phylogenetic analysis were performed by using kSNP v3 (Gardner et al., 2015). The genome sequences of 4 K. *pneumoniae* ST15 isolates, including PMK1, BR, Kp-Geo-39795 and Kp36, were downloaded from GenBank. The phylogeny scheme was generated from the kSNP3-detected SNP sites for all the genome sequences under analysis with k = 21, as determined by Kchooser. A parsimony tree was generated by kSNP3 based on an extended majority rule consensus of the equally most parsimonious trees from a sample of 100 trees. The tree was displayed with iTOL with midpoint rooting (Letunic and Bork, 2016).

Bacteriophage Studies

Bacteriophages were purified by caesium chloride (CsCl) density-gradient ultracentrifugation. Transmission electron microscopy (Hitachi 700, Tokyo, Japan) studies were performed on the bacteriophage preparation after staining with 2% phosphotungstic acid. Bacteriophage DNA was extracted with the Phage DNA Isolation Kit (Aidlab Biotech, Beijing). PacBio single-molecule real-time (SMRT) sequencing was performed using a PacBio RSII sequencer with C4 chemistry. De novo assembly was conducted using the Hierarchical Genome Assembly Process (HGAP) method based on the SMRT Analysis package 2.0. All of the ORFs predicted by Prokka (Seemann, 2014). Using BLAST at the NCBI, comparative genome analysis of phage was carried out. The prediction of the conserved protein domain was conducted using BLASTP and the NCBI Conserved Domain Database. The sequence of bacteriophages has been deposited in the GenBank databases with accession number (SAMN13324166, SAMN13324167, SAMN13324168, SAMN13324169, SAMN13324170).

The MDR *K. pneumoniae* Infection and Pathophysiology of the Patient

A 66-year-old man whose cancerous bladder was partially excised in 2002 was enrolled. He had UTIs since 2006. MDR *K. pneumoniae* was the causative agent that led to frequent and urgent urination and dysuria over the past dozen years. Antibiotics that have *in vitro* activity against the isolates have been used for conventional treatment since then. However, none of those antibiotics or their combination worked to eradicate the pathogen. The UTIs with *K. pneumoniae* reappeared immediately post drug withdrawal and were continuously susceptible to the previous antibiotic panels. A cystoscope scan showed that his bladder mucosa was hyperemic with local ulceration and pseudomembrane attachment; bilateral ureteral openings were not clearly observed (**Figure 1A**). He was recruited with hospital admission to receive bacteriophage treatment at the Shanghai Public Health Clinical Center (ChiCTR1900020989), Shanghai, China.

Selection and Preparation of Therapeutic Bacteriophages

Bacteriophage collections were stored at Shanghai Public Health Clinical Center, which was isolated from various environmental samples by using routine isolation techniques (Wommack et al., 2009). Bacteriophages used for this treatment were screened from bacteriophage collection. The lytic activity of the bacteriophage was screened *via* spot testing against successive patient isolates as those isolates became available. To evaluate the killing efficacy of each phage on clinical isolates, 1 µl dilution aliquots of 10-fold serial dilutions of each bacteriophage was spotted on a bacterial lawn to observe plaque formation. The bacteriophage candidates that showed the strongest antibacterial activity and broad spectrum against available isolates as measured by this assay were selected for inclusion in the therapy. Bacteriophages were generated using solid media and recovered by diffusion into SM buffer (5.8 g/L NaCl, 20 mM Tris HCl pH 7.5, 2 g/L mM $MgSO4.7H_2O$), yielding lysates with titres of $>1x10^{10}$ pfu/ml. These lysates were concentrated using CIM® Anion-exchange column QA (BIA Separations, Slovenia) according to the protocol. The concentration was dialysed against 0.9% sodium chloride physiological solution (Shandong Qidu Pharmaceutical Co., Ltd.). The resulting lysate was further sterilized through 0.22 µm filters. The final bacteriophage preparation was used for therapeutic application, with a titre estimated at $>5x10^9$ pfu/ml.

Bacteriophage Therapy

The entire treatment process for the patient is shown in **Figure 2A**. Strain *Kp*0344 were used as host to amplify bacteriophage Φ902, ΦJD908, and ΦJD910. Strain *Kp*1440 were used as host to amplify bacteriophage Φ905 and ΦJD907. Fifty ml of the bacteriophage preparation containing $5×10^8$ pfu/ml was irrigated *via* bladder every 48 h for 2 weeks at a time. For the fourth bacteriophage treatment, in addition to irrigation *via* the bladder, 10 ml of the phage preparation containing $5x10^8$ pfu/ml was also irrigated *via* the kidney every 48 h for 2 weeks. Prior to the fourth phage treatment, we performed a bilateral PCN on the patient (**Figure S1**). The patient was hospitalized during therapy. The clinical examination and urine culture were performed throughout the study (**Figure 2B**). After the treatment, the patient visited every week in the following 2 months. Urine cultures and blood tests were obtained on each visit.

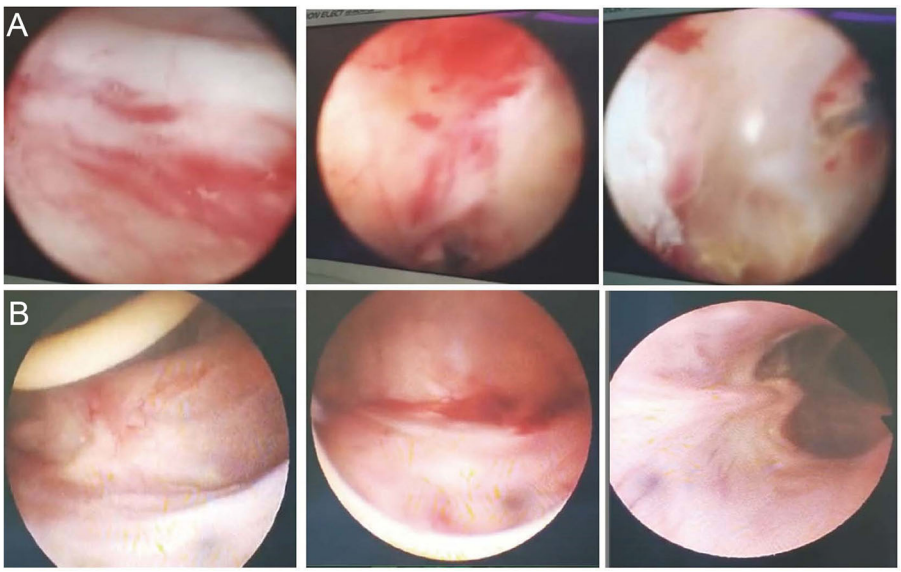

FIGURE 1 | Cystoscope of the inner wall of the bladder. **(A)** The inner wall of the bladder before phage treatment. The bladder mucosa was hyperemic with local ulceration and pseudomembrane attachment; bilateral ureteral openings were not clearly observed. **(B)** The inner wall of the bladder post phage treatment. Bladder mucosa was smooth and complete; bilateral ureter openings were clear.

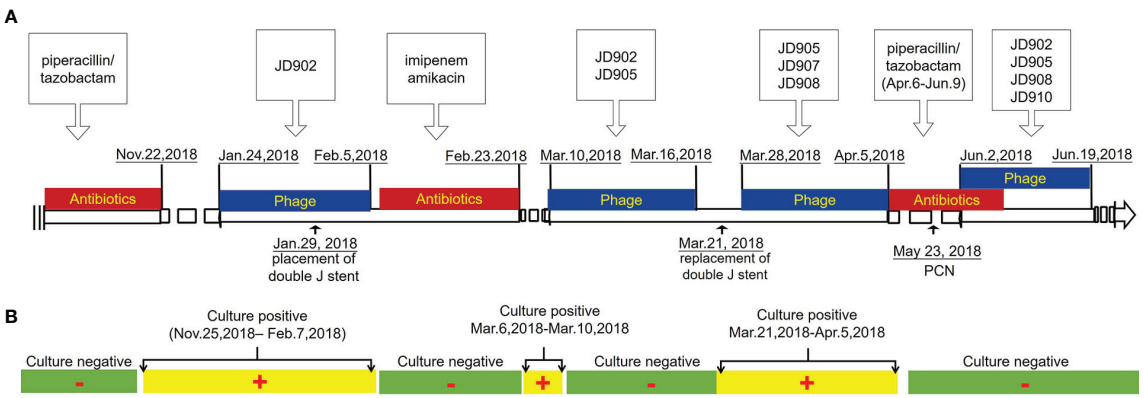

FIGURE 2 | Time course of clinical treatment. **(A)** Timeline beginning with patient hospitalization and ending with recovery. Major treatment during the course is indicated above the line. Surgery during the course is indicated below the line. **(B)** Laboratory culture event and culture result during the course.

RESULTS

Morphological Characteristics and Genome Sequence of Bacteriophages

Bacteriophage was screened against successive patient isolates as those isolates became available. A total of five bacteriophages Φ902, ΦJD905, ΦJD907, ΦJD908, and ΦJD910 were selected for therapy. Photograph of five bacteriophages was obtained by transmission electron microscopy, as shown in **Figure 3**. Morphologically, Φ902, ΦJD907, ΦJD908, and ΦJD910 belong to the podoviridae family while ΦJD905 belong to the myoviridae family. Φ902, ΦJD907, ΦJD908, and ΦJD910 have genome sizes of 43,274, 39,465, 40,777, and 38,834 bp, respectively. ΦJD905 has genome sizes of 147,174 bp. These five bacteriophages encoded ORFs were searched by blast in the database of virulence and antibiotic resistance genes (http://www.genomicepidemiology.org/), with no identifiable virulence or antibiotic resistance genes found in their genome.

Heterogeneity of Bacterial Isolates

A total of 21 K. *pneumoniae* strains were isolated from the patients during treatment. Whole genome sequence analysis showed that they all belong to ST15. The genome-wide detection of these 21 sequenced isolates and 4 completely sequenced ST15 *K. pneumoniae* currently available at GenBank (PMK1, BR, Kp-Geo-39795, and Kp36) generated 9,170 SNPs. The SNP-based phylogenetic tree analysis showed that these 21 isolates are from the same clone (**Figure 4A**).

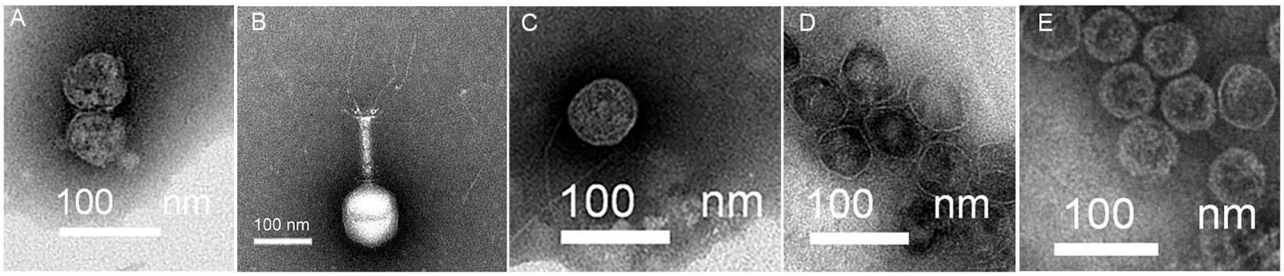

FIGURE 3 | Transmission electron microscopy image of phages. **(A)** phage ΦJD902. **(B)** phage ΦJD905. **(C)** phage ΦJD907. **(D)** phage Φ JD908. **(E)**, phage ΦJD910.

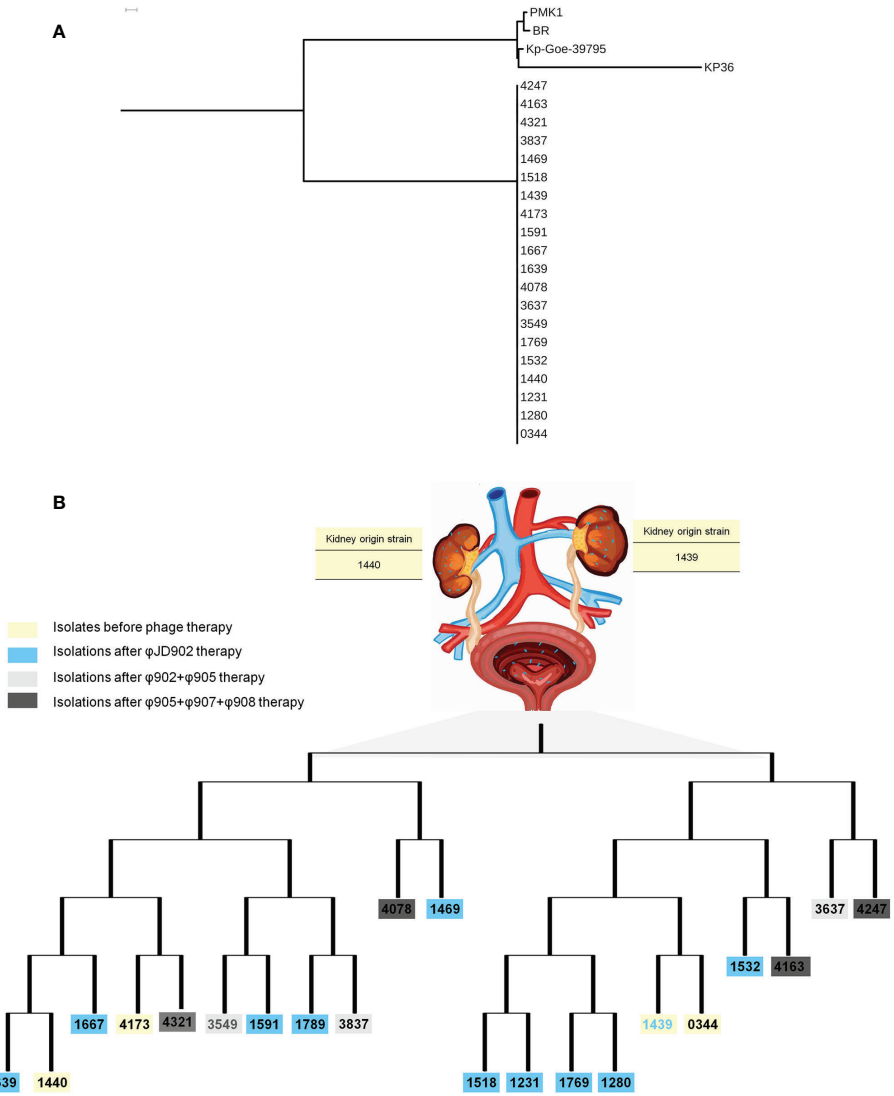

FIGURE 4 | Phylogenetic trees of *K. pneumoniae* isolates. **(A)** The genome-wide SNP-based phylogenetic trees of 21 sequenced *K. pneumoniae* ST15 isolates and 4 completely sequenced ST15 *K. pneumoniae* isolates currently available at GenBank (PMK1, BR, Kp-Geo-39795, and Kp36). **(B)** The genome-wide SNP-based phylogenetic trees of 21 sequenced *K. pneumoniae* isolates by this study. The phylogeny scheme based on parsimony was generated from 9,170 SNPs for all genomes and 2,795 SNPs for 21 genomes by this study using kSNP3 with k = 21 and displayed by iTOL with midpoint rooting.

Further genome-wide detection of these 21 isolates generated 2,795 SNPs. The SNPs-based phylogenetic tree analysis showed the polyclonal strains (Kp4137, Kp0344, Kp1439, Kp1440) cloned in the patient before phage treatment (**Figure 4B**). The sensitivity of these strains to the phage 902, ΦJD905, ΦJD907, ΦJD908, and ΦJD910 was also different (**Figure 5A**). These results suggested that the patient was infected with polyclonal *K. pneumonia* for long time. These results suggest that the colonized *K. pneumoniae* has evolved into polyclone as a result of the patient's chronic infection.

K. pneumoniae continued to be isolated in the urine of patients during bacteriophage ΦJD902 treatment (**Table 1** and **Figure 2B**). Analysis of phage lytic spectrum of these 10 successively isolates showed that five strains (Kp1231, Kp1280, Kp1469, Kp1591, and Kp1639) were still sensitive to ΦJD902, whereas the remaining five strains (Kp1518, Kp1639, Kp1667, Kp1769, Kp1789) developed resistance to ΦJD902 (**Figure 5B**). Phylogenetic tree analysis showed that the five bacteriophage-sensitive strains are located in different branches. Similarly, the

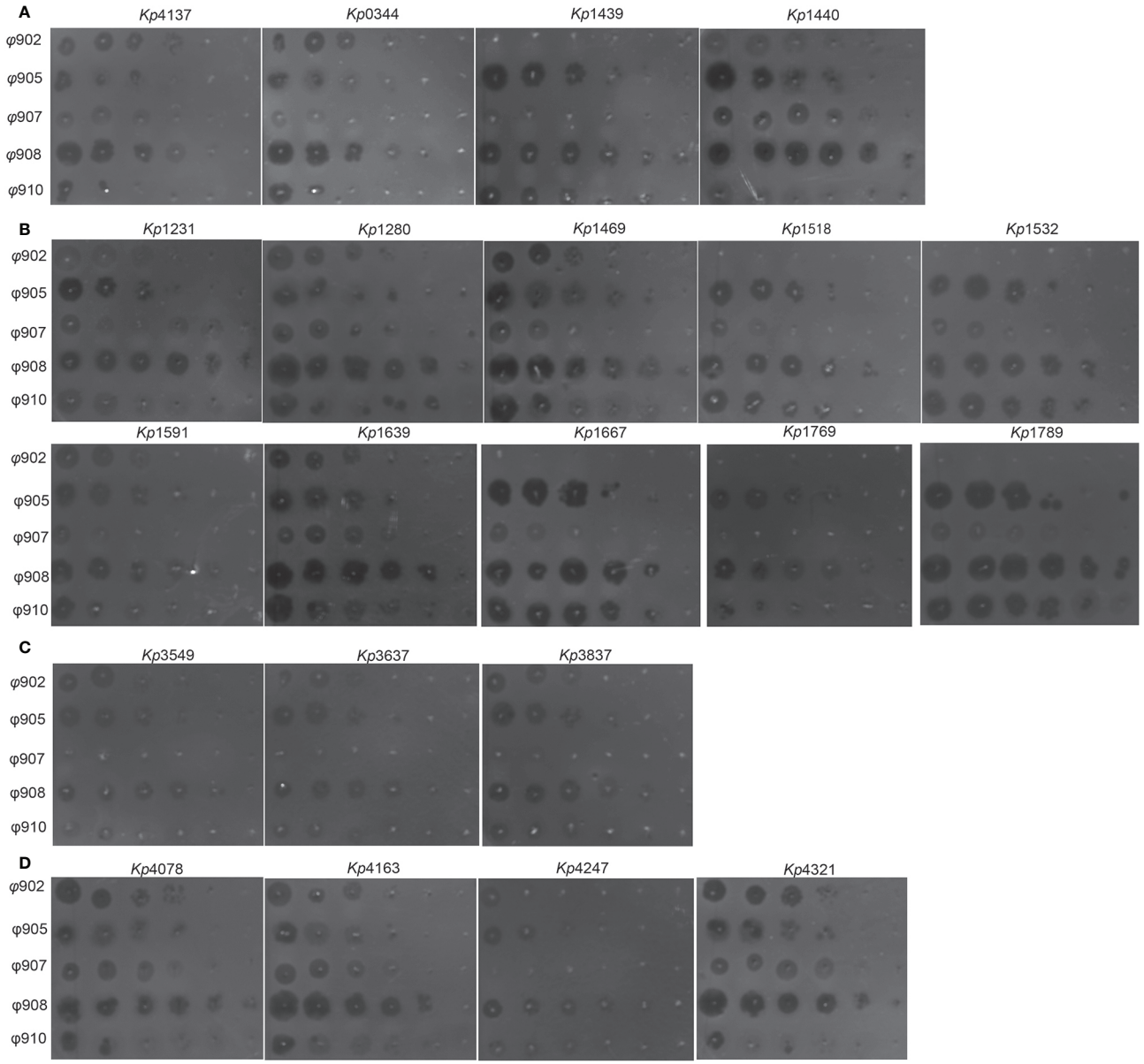

FIGURE 5 | Phage susceptibilities of clinical isolates. **(A)** *K. pneumoniae* strains isolated before phage therapy. **(B)** *K. pneumoniae* strains isolated post ΦJD902 therapy. **(C)** *K. pneumoniae* strains isolated post Φ902+Φ905 therapy. **(D)** *K. pneumoniae* strains isolated post Φ905+Φ907+Φ908 therapy. Phages were serially diluted 10-fold and spotted onto 21 *K. pneumoniae* as indicated. These assays were repeated three times with similar results and a representative experiment is shown.

five bacteriophage-resistant strains are in different branches. It has been reported that bacteria mutate rapidly to develop phage resistance when a bacteriophage infects a bacterium (Hesse et al., 2020). It was speculated that the five bacteriophage-resistant strains may come from the mutation during bacteriophage therapy. However, it cannot be ruled out that it may be the heterogeneous strain colonized in the patient.

By screening for lytic bacteriophages against previous isolates, a two-phage cocktail (ΦJD902+ΦJD905) and a three-phage cocktail (ΦJD905+ΦJD907+ΦJD908) was used to the continued treatment. However, after the phage cocktail was administered, the patient still had *K. pneumoniae* in his urine. As shown in **Figures 5C**, **D**, these isolates are still sensitive to the phage cocktail, suggesting that the phage cocktail did not function in some degree. It is speculated that heterogeneous bacteria colonized in the renal pelvis cannot be effectively cleared because the phage cannot reach them.

Phage Therapy Outcome

According to the isolates (strain Kp4173 and Kp0344) from the patient urine, lytic phage ΦJD902 against them was selected for his first therapy (**Figure 2A**). We performed a regimen of antibiotic withdrawal and a two-week phage administration by bladder irrigation. During the time, we placed a "Double J" stent to dredge the connection between the pelvis and bladder, and the phage was irrigated to the renal pelvis once during the placement process. Renal pelvis effusion was submitted to culture. Despite the ongoing phage therapy, urine cultures were positive (**Figure 2B**). Five isolates recovered from urine developed resistance to ΦJD902 (**Figure 3B**). Bilateral pelvis effusion cultures were positive.

Thus, a phage cocktail containing ΦJD902 and ΦJD905 lytic to all previous isolates was administrated for second therapy *via* bladder irrigation. The patient felt relief of his symptoms with negative urine culture during therapy. Considering that the double J stent was in place for two months, we felt it necessary to replace it with a new one (**Figure 2A**). The right stent was successfully removed, while the left stent was missing. Unexpectedly, his urine culture became positive again (**Figures 2B**, **3C**). Following screening, an adapted phage cocktail containing ΦJD905, ΦJD907, and ΦJD908 for the third therapy *via* bladder irrigation were continued. However, the patient's urine culture remained positive with *K. pneumoniae*. We had to halt the phage therapy and replaced it with antimicrobial therapy with piperacillin/tazobactam (**Figure 2A**).

According to the renal pelvis culture, *K. pneumoniae* colonized the kidney. We hypothesized that the heterogeneous pathogens in the renal pelvis were unreachable by phage cocktails *via* bladder irrigation. Thus, they could be released to the bladder continuously. To remove pathogens colonizing the bladder and renal pelvis, phage should be irrigated both *via* kidney and bladder. After obtaining informed consent from the patient, we performed PCN on the patient prior to phage therapy (**Figure 2A** and **Figure S1**). A phage cocktail containing ΦJD902, ΦJD905, ΦJD908, and ΦJD910 was irrigated *via* the pelvises and subsequently the bladder (**Figure 2A**). At the same time, the administration of piperacillin/tazobactam continued to enhance the eradication of minority subpopulations of phage-resistance variants. Then, phage therapy continued for another 10 days without antibiotic treatment (**Figure 2A**). Finally, the patient recovered with an obviously improved bladder with smooth mucosa (**Figure 1B**). MDR *K. pneumonia* infection did not recur after two months of follow-up as determined by culture growth from the patient's urine.

DISCUSSION

Although UTIs are normally not considered life-threatening, these recalcitrant infections lead to unbearable symptoms of urinary irritation and diminished quality of life (Portsmouth et al., 2018). The bactericidal mechanism of bacteriophages is completely different from that of antibiotics. When bacteriophage therapy is administered, it is necessary to screen for highly lytic activity bacteriophages directly to the bacterial pathogen that is causing a clinically relevant infection (Schooley et al., 2017; Corbellino et al., 2019; Dedrick et al., 2019). Therefore, the clinical isolates are of great significance for guiding clinicians in choosing optimal bacteriophage therapy. Bacterial heterogeneity means that a patient may have polyclone bacterial infections, and generally we can only diagnose the main population and miss the subpopulations with low densities (Andersson et al., 2019; Nicoloff et al., 2019).

In this case, heterogeneous *K. pneumoniae* colonized renal pelvis and bladder prior to phage therapy. However, for our first phage therapy, we were able to isolate *K. pneumoniae* strain only from the urine and performed phage screening against these isolates. The patient's urine culture still had phage sensitive and phage-resistant strains during phage therapy. It was later discovered that isolates from the renal pelvis and the bladder were different clones, and that *K. pneumoniae* colonized in the renal pelvis was not sensitive to therapeutic bacteriophage ΦJD902. Even with the phage cocktail treatment, phage-resistant strains were rapidly isolated from the patient's urine. These resistant strains could be the result of minor undetected resistant populations or from mutations. In this case, the patient's renal pelvis is also colonized by pathogenic bacteria, which makes it difficult for phages to reach *via* bladder irrigation. We speculate that these are the two main causes of treatment failures.

In the following treatment, we performed a PCN on the patient so that phages could be irrigated *via* the kidney. A cocktail of bacteriophages was selected for activity against all previously isolates and irrigated simultaneously *via* the kidney and bladder, using antibiotics in combination. It is reported that bacterial mutation to bacteriophage resistance has also been associated with significant fitness costs of the reduction of antibiotic resistance or virulence (Ofir and Sorek, 2018; Gordillo Altamirano and Barr, 2019). Therefore, we hypothesized that the eventual successful clearance of *K. pneumoniae* was due to the synergistic effect of bacteriophage and antibiotics.

CONCLUSION

In recent years, phage is expected as potential effective therapeutic agents for the untreatable infections (Gordillo Altamirano and Barr, 2019; Monteiro et al., 2019; Schmidt, 2019). In this case, as a patient with urinary tract infection, we designed phage treatment *via* bladder effusion for first therapy since it was easy to perform and less invasive. However, *K. pneumoniae* infection did not clear due to bacterial colonization of the renal pelvis and bacterial heterogeneity. Thus, we performed simultaneous bladder and renal pelvis perfusion using bacteriophage cocktails with activity against a range of pathogen. We developed a personalized approach for the patient, including phage cocktail-made and administration. The success of this case provides valuable ideas and solutions for personalized phage therapy of complex infection.

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 in the article/**Supplementary Material**.

ETHICS STATEMENT

This trial was registered at the Chinese Clinical Trial Registry (www.chictr.org.cn) (ChiCTR1900020989). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JQ, XG, TZ, JB, NW, and XS contributed to the study design. JQ, JB, NW, XS, SY, WZ, ZW, JC, LL, MG, JW, HL, DT, JZ, QH, ZZ, YS, and CH contributed to participant recruitment and data collection. JQ, NW, XS, and HO did the data analyses. JQ, NW, JB, XG, and TZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.608402/full#supplementary-material

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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
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Prevalence of Carbapenem-Resistant *Klebsiella pneumoniae* Co-Harboring blaKPC-Carrying Plasmid and pLVPK-Like Virulence Plasmid in Bloodstream Infections

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This study aimed to characterize carbapenem-resistant *Klebsiella pneumoniae* (CR-KP) co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid. Between December 2017 and April 2018, 24 CR-KP isolates were recovered from 24 patients with bacteremia. The mortality was 66.7%. Pulsed-field gel electrophoresis and multilocus sequence typing results indicated four clusters, of which cluster A (n = 21, 87.5%) belonged to ST11 and the three remaining isolates (ST412, ST65, ST23) had different pulsotypes (cluster B, C, D). The bla_{KPC-2}-carrying plasmids all belonged to $IncFII_K$ type, and the size ranged from 100 to 390 kb. Nineteen strains (79.2%) had a 219-kb virulence plasmid possessed high similarity to pLVPK from CG43 with serotype K2. Two strains had a 224-kb virulence plasmid resembled plasmid pK2044 from *K. pneumoniae* NTUH-K2044(ST23). Moreover, three strains carried three different hybrid resistance- and virulence-encoding plasmids. Conjugation assays showed that both bla_{KPC-2} and *rmpA2* genes could be successfully transferred to *E. coli* J53 in 62.5% of the strains at frequencies of 4.5×10^{-6} to 2.4×10^{-4}, of which three co-transferred bla_{KPC-2} along with *rmpA2* in large plasmids. Infection assays in the *Galleria mellonella* model demonstrated the virulence level of these isolates was found to be consistently higher than that of classic *Klebsiella pneumoniae*. In conclusion, CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid were characterized by multi-drug resistance, enhanced virulence, and transferability, and should, therefore, be regarded as a real superbug that could pose a serious threat to public health. Hence, heightened efforts are urgently needed to avoid its co-transmission of the virulent plasmid (gene) and resistant plasmid (gene) in clinical isolates.

Keywords: *Klebsiella pneumonia*, bloodstream infections, pLVPK-like virulence plasmid, KPC-2, carbapenem-resistant

INTRODUCTION

Carbapenem-resistant *Klebsiella pneumoniae* (CR-KP) has emerged as one of the most challenging pathogens in the latest years (Holt et al., 2015). CR-KP showed resistant to almost all available antibiotics and was related to limited treatment options and high mortality rates. CR-KP has been listed as a "critical priority" by the World Health Organization (WHO). For pathogen survival, the acquisition of virulent traits is necessary (Vila et al., 2011), and some reports suggest that the virulence of carbapenem-resistant *Klebsiella pneumoniae* is enhanced (Ferreira et al., 2018).

The virulence plasmid carrying major virulence genes such as capsular polysaccharides regulator genes (*rmpA* and *rmpA2*) and those encoding siderophores (eg, *iroBCDN*, *iucABCD*, *iutA*) were recognized as essential contributors to the virulence of hypervirulent *Klebsiella pneumoniae* (hvKP), and might serve as potential biomarkers for hvKP. The loss of this pLVPK-derived virulence plasmid significantly decreased virulence. Danxia Gu and colleagues (Gu et al., 2018) reported that CR-KP strains could further evolve to become carbapenem-resistant hvKP (CR-hvKP) through the acquisition of a pLVPK-like virulence plasmid. Meanwhile, CR-hvKP strains may emerge as a result of the acquisition of a carbapenemase-encoding plasmid by K1 or K2 hypervirulent *Klebsiella pneumoniae* (Zhang et al., 2016a). The emergence of carbapenem-resistant hypervirulent *Klebsiella pneumonia*(CR-hvKP) was due to the convergence of virulence and resistance. An increasing number of cases have also been observed worldwide. The high prevalence of carbapenem-resistant *K pneumoniae* (average 9.0% in 2017 and 15.4% in Jiangxi) and hypervirulent *K pneumoniae* (about 30–50%) (Zhang et al., 2016b; Liu and Guo, 2019) in Chinese hospitals may have contributed to the emergence of carbapenem-resistant and hypervirulent microorganism.

In the present study, we characterize clinical characteristics, clonal relationships, virulence and resistance potential of CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid in bloodstream infections. The findings of this study provide insight into the current prevalence and features of CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid in a Chinese hospital.

MATERIALS AND METHODS

Bacterial Isolates and Antimicrobial Susceptibility Tests

Between December 2017 and April 2018, 24 CR-KP strains, which were identified by the VITEK 2 system (bioMérieux) and confirmed by 16S rRNA gene sequencing, were isolated from blood cultures of 24 patients hospitalized in the First Affiliated Hospital of Nanchang university (Nanchang), Southern China. Antimicrobial susceptibility testing was done for all isolates using Vitek 2 automated systems. Results were interpreted according to the Clinical and Laboratory Standards Institute (document M100-S27). Furthermore, antimicrobial susceptibility of tigecycline was performed by the broth microdilution method and interpreted by the recommendation of the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints (http://www.eucast.org). Patient information was queried from the medical records. This study was approved by the ethical committee of the First Affiliated Hospital of Nanchang University. Informed consent was also obtained from all of the study patients.

Antimicrobial Resistance Genes

Polymerase chain reaction was used to detect carbapenemase-encoding genes (bla_{KPC}, bla_{VIM}, bla_{NDM}, bla_{IMP}, and $bla_{OXA-48-like}$), β-lactamase genes (bla_{CTX-M}, bla_{TEM}, and bla_{SHV}), plasmid-mediated quinolone resistance determinants (*qnrA, qnrB, qnrS, aac(6')-Ib-cr*) and 16S rRNA methylase genes(*armA*, *rmtB*) as described previously (Liu et al., 2019). The positive PCR products were purified and sequenced, and the sequences alignments were compared to those in the NCBI database using BLAST.

Capsular Serotyping and Virulence-Associated Genes Detection

The capsular type of *K. pneumoniae* was determined by PCR and sequencing of *wzi* loci as previously described (Brisse et al., 2013). The sequences of products were compared to the *wzi* sequences deposited in the database of Institute Pasteur to identify the corresponding capsular types using BLAST program (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). Isolates were screened for the presence of 14 virulence-associated genes, including *rmpA, rmpA2,terW, iutA, silS, mrkD, fimH, ybtS, entB, kpn, aerobactin, kfu, magA*, and *wcaG* (Turton et al., 2018). Primers used for PCR are shown in **Table S2**.

Plasmid Analysis and Plasmid Transfer

S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE) and southern blotting hybridization were performed to determine the plasmid location of bla_{KPC-2}-carrying plasmid and virulence plasmid (Xu et al., 2019). Briefly, total DNA was embedded in agarosegel plugs. The plugs were digested with S1 nuclease (TaKaRa) for 30 min at 37°C and then separated by eletrophoresis. Labeling of the probes (**Table S1**) and hybridization were performed with the DIG-High Prime DNA Labeling and Detection Starter Kit II, according to the manufacturer's instructions (Roche, Basle, Switzerland).

Conjugal transfer experiment was performed using broth-based methods with *Escherichia coli* J53 as the recipient strain. Donor and recipient cells were mixed at 2:1 donor-to-recipient ratio. Transconjugants were selected using 2 or 8 μg/ml potassium tellurite or 2 μg/ml meropenem plus 150 μg/ml sodium azide. Successful conjugation and transformation were confirmed by antimicrobial susceptibility and PCR detection of the bla_{KPC-2} gene and pLVPK-derived gene (*rmpA*, *rmpA2*, *terW*, *iutA*, *silS*). S1-PFGE was performed as described previously to confirm acquisition of this plasmid by the recipient strain.

Galleria Mellonella Infection Model

For virulence testing, the *Galleria mellonella* model was used to investigate toxicity. Ten larvae weighing between 250 and 350 mg (purchased from Tianjin Huiyude Biotech Company, Tianjin, China) were used for the assessment of the virulence level of

each isolate. The insects were inoculated by injecting 1×10^6 CFU per 10 μl aliquot into the hemocoel *via* the rear left proleg using methods described previously (Mclaughlin et al., 2014), followed by a recording of survival rate every 12 h for 2 days. All experiments were performed in triplicates. The recent assessment of a range of *K. pneumoniae* isolates suggests the parameters for the *Galleria* model to define hypervirulence, based on a calculation of LD_{50} value (Shi et al., 2018). The hvKP strain NTUH-K2044 and *K. pneumoniae* strain ATCC700603 were used as controls of high and low virulence strains, respectively. Statistical analyses were performed and visualized with GraphPad Prism 7.00.

Multilocus Sequence Typing (MLST) and Pulsed-Field Gel Electrophoresis (PFGE)

MLST was performed by amplifying and sequencing the seven conserved housekeeping loci including *gapA, infB, mdh, pgi, phoE, rpoB*, and *tonB* (Diancourt et al., 2005), according to protocols on the Pasteur Institute MLST website (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

Clonal relatedness was established using XbaI-PFGE (Taraka). DNA fragments were separated with a CHEF DR III apparatus (Bio-Rad; Richmond, CA, USA). The molecular marker was *Salmonella* serotype *Braenderup* strain H9812. The isolates sharing >80% similarity were defined as the same PFGE cluster (Tenover et al., 1995).

RESULTS

Patients and Bacterial Isolates

The clinical characteristics of the 24 patients with *K. pneumoniae* bacteremia are shown in **Table 1**. These patients were mainly from the ICU (58.3%, n = 14). The mean age of the patients was 61.9 ± 16.6 years (range, 25–87 years) and 79.2% of these patients were males. The mean time of hospitalization from admission to the identification of CR-KP was 27.5 ± 16.5 days (range, 3–58 days). Hyperglycemia was found among eight cases (33.3%) and seven patients (29.2%) had hypertension. The majority of patients used various invasive procedures and devices, of which the usage rate of mechanical ventilation and tracheal intubation were highest (70.8%). All cases had received a wide variety of antibiotics in combination. The incidence rate of septic shock was 41.7%, and the mortality was 66.7%.

TABLE 1 | Clinical characteristics of patients with carbapenem-resistant *K. pneumoniae* bacteremia.

Demographics		Prior antibiotic exposure	
Age (mean ± SD), years	61.9 ± 16.6	Carbapenem	24 (100.0)
Gender, male	19(79.2)	cephalosporin	3 (12.5)
Length of stay (mean ± SD), days	27.5 ± 16.5	β-lactam and β-lactamase inhibitor	11(45.8)
Underlying disease		Fluoroquinolone	9 (37.5)
Diabetes mellitus	8(33.3)	Aminoglycoside	8 (33.3)
Hypertension	7(29.2)	Tigecycline	11 (45.8)
Invasive procedures and devices		Glycopeptide	14(58.3)
Central venous catheter	13(54.2)	**Clinical outcomes**	
Urinary catheter	14(58.3)	Septic shock	10(41.7)
Endotracheal tube	17(70.8)	30-day Mortality	16(66.7)
Mechanical ventilation	17(70.8)		
Surgical drainage	11(45.8)		
Tracheostomy	5(20.8)		
Surgery	16(66.67)		

Antimicrobial Susceptibility and Antimicrobial Resistance Genes

The detailed antimicrobial resistance profiles are shown in **Table 2**. The antibiotic susceptibility test showed that all 24 isolates were resistant to ceftriaxone, cefotaxime, aztreonam, ertapenem, imipenem, and meropenem. The percentage of bacteria resistant to gentamicin (16.7%, n = 4), tobramycin (16.7%, n = 4), amikacin (12.5%, n = 3) is low. Resistant to ceftazidime (95.8%, n = 23), cefepime (95.8%, n = 23), piperacillin/tazobactam (91.7%, n = 22), levofloxacin (87.5%, n = 21), ciprofloxacin (87.5%, n = 21), and sulfamethoxazole-trimethoprim (95.8%, n = 23) was high. However, all isolates were sensitive to tigecycline.

All the 24 isolates were positive for bla_{KPC-2} gene. The β-lactamase genes were detected, including bla_{TEM-1} (95.8%, n = 23), $bla_{CTX-M-15}$ (95.8%, n = 23), and bla_{SHV-11} (79.2%, n = 19). In addition, 20 isolates (83.3%) carried *qnrS1*, 17 isolates carried *aac (6')-Ib-cr* (70.8%) and 2 isolates (8.3%) carried *qnrB4*. However, only one isolate carried plasmid-mediated 16S rRNA methylase gene *rmtB*. All isolates were negative for bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{OXA-48}, *armA*, and *qnrS*.

Plasmid Profiles

Plasmid location of bla_{KPC}-carrying plasmid and pLVPK-like virulence plasmid was determined by S1-PFGE and Southern blot analysis. The results demonstrated that the plasmid size carrying bla_{KPC-2} ranged from 100 to 390 kb (**Figures 1**, **S1**). Furthermore, two isolates had two different plasmids harboring bla_{KPC-2} gene. Nineteen strains (79.2%) had a 219-kb virulence plasmid possessed high similarity to previously reported pLVPK from *Klebsiella pneumoniae* CG43 with serotype K2. Two strains had a 224-kb virulence plasmid resembled plasmid pK2044 from *K. pneumoniae* NTUH-K2044 belonged to sequence type 23. Moreover, there were three isolates (KP3, KP5, KP6) carrying a hybrid resistance- and virulence-encoding plasmid, which harbored both the carbapenemase gene bla_{KPC-2} and the virulence gene *rmpA2*.

Conjugation assays showed that both bla_{KPC-2} and *rmpA2* genes could be successfully transferred to *E. coli* J53 in 62.5% (15/24) of the strains at frequencies of 4.5×10^{-6} to 2.4×10^{-4} (transconjugant/recipient), of which three co-transferred bla_{KPC-2} along with *rmpA2* in large plasmids. KP3 isolate transferred a hybrid resistance- and virulence-encoding plasmid of 390 kb to *E. coli* J53 at a frequency of 3.5×10^{-5} (transconjugant/recipient) by mating. In addition, KP10 and KP24 isolates co-transferred the bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid to *E. coli* J53 at a frequency of 7.4×10^{-6} (transconjugant/recipient) by mating (**Table S3**).

Virulence-Associated Features

The prevalence and distribution of virulence factors are shown in **Figure 2A**. The virulence-related genes detected in 24 isolates

TABLE 2 | Resistance genes and antibiotic susceptibilities of 24 CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid.

Isolates	Resistance profile of *K. pneumoniae*	Carbapenemase	β-lactamase genes	16S rRNA methylase gene	PMQR genes
Kp1	CRO,CAZ,CTX,FEP,TZP,ATM,GEN,TOB,AMK,LVX,CIP, ETP,IMP,MEM,SXT	KPC-2	TEM-1	–	qnrB4
Kp2	CRO,CAZ,CTX,FEP,TZP,ATM,TOB,LVX,CIP,ETP,IMP, MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14	rmtB	qnrS1,acc6-Ib-cr
Kp3	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM	KPC-2	SHV,TEM-1,CTX-M-14	–	acc6-Ib-cr
Kp4	CRO,CAZ,CTX,FEP,TZP,ATM,AMK,LVX,CIP,ETP,IMP, MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14,15	–	qnrS1,acc6-Ib-cr
Kp5	CRO,CAZ,CTX,FEP,TZP,ATM,ETP,IMP,MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14	–	–
Kp6	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM	KPC-2	SHV-11,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp7	CRO,CAZ,CTX,FEP,TZP,ATM,GEN,TOB,AMK,LVX,CIP, ETP,IMP,MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp8	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp9	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp10	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	SHV-11,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp11	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp12	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14	–	qnrB4,qnrS1, acc6-Ib-cr
Kp13	CRO,CAZ,CTX,FEP,TZP,ATM,GEN,LVX,CIP,ETP,IMP, MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp14	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp15	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	TEM-1,CTX-M-14	–	qnrS1
Kp16	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	SHV-11,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp17	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	SHV-11,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp18	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14,-15	–	qnrS1,acc6-Ib-cr
Kp19	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp20	CRO,CAZ,CTX,FEP,ATM,LVX,CIP,ETP,IMP,MEM	KPC-2	SHV-11,TEM-1,CTX-M-14	–	qnrS1,acc6-Ib-cr
Kp21	CRO,CAZ,CTX,FEP,TZP,ATM,ETP,IMP,MEM	KPC-2	SHV-12,TEM-1,CTX-M-14	–	qnrS1
Kp22	CRO,CAZ,CTX,FEP,TZP,ATM,GEN,TOB,AMK,LVX,CIP, ETP,IMP,MEM,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14,-15	–	qnrS1
Kp23	CRO,CAZ,CTX,FEP,TZP,ATM,LVX,CIP,ETP,IMP,MEM, TGC,SXT	KPC-2	SHV-12,TEM-1,CTX-M-14	–	qnrS1
Kp24	CRO,CTX,ATM,ETP,IMP,MEM	KPC-2	CTX-M-14	–	–

CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; TZP, piperacillin/tazobactam; ATM, Aztreonam; GEN, gentamicin; TOB, Tobramycin; AMK, amikacin; LVX, Levofloxacin; CIP, ciprofloxacin; ETP, Ertapenem; IMP, imipenem; MEM, meropenem; SXT, trimethoprim-sulfamethoxazole.

included *fimH-1* (100%), *mrkD* (100%), *ybtS* (91.7%), *entB* (83.3%), *kpn* (83.3%), *aerobactin* (62.5%), *kfu* (20.8%), *magA* (12.5%), and *wcaG* (8.3%) (**Figure S2**). Moreover, all the five pLVPK-derived locus, *rmpA, rmpA2, terW, iutA, silS*, were detected in all 24 isolates.

The *G. mellonella* larvae infection model was used to assess the potential virulence of these isolates (**Figure 2B**). After 48 h of infection, the mortality of the larvae infected with CR-KP isolates co-carrying virulence plasmid and KPC-2 plasmid were consistently higher than that infected with cKP ($P < 0.05$) (**Table S2**). Among the 24 strains, the virulence level of 15 isolates is similar to hvKP previously reported (P>0.05), but nine isolates are less virulent (P < 0.05) (**Table S2**).

Clonal Relationship

Among the 24 isolates, four STs were identified, including ST11 (14 wzi47-K47 isolates, five wzi64-K64 isolates, and two wzi125-K1 isolates), ST23 (1 wzi1-K1 isolate), ST65 (1 wzi2-K2 isolate), ST412 (1 wzi206-K57 isolate). PFGE (**Figure 1**) identified one major pulsotype (cluster A), encompassing 21 of the 24 isolates, all belonging to ST11 (**Figure 3**). The three remaining isolates (Kp20, Kp21, and Kp24) had different pulsotypes.

DISCUSSION

In our study, we reported the prevalence of carbapenem-resistant *K. pneumoniae* co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid in patients with bacteremia. *Klebsiella pneumoniae* is the second most common pathogen in *Enterobacteriaceae* bloodstream infections (Meatherall et al., 2009). In this study, the overall 30-day mortality rate was 66.7%, which was higher than in those with KPC-producing *K. pneumoniae* bloodstream infections (44.2%) (Xu et al., 2018). Ten patients (41.7%) developed septic shock, which was the recognized reason for increased mortality (Falcone et al., 2016). In addition, 33.3% of the patients had hyperglycemia, which was considered to be a significant risk factor for hypervirulent *Klebsiella pneumoniae* infection (Zhang et al., 2016b). There are many possible contributing factors to the emergence, rise, and spread of antibiotic resistance, including ICU admission, antibiotics exposure, using invasive devices and procedures (Li et al., 2019). These risk factors may have contributed to the high rates of antibiotic resistance found in our study.

Although all the 24 CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid were multi-drug-

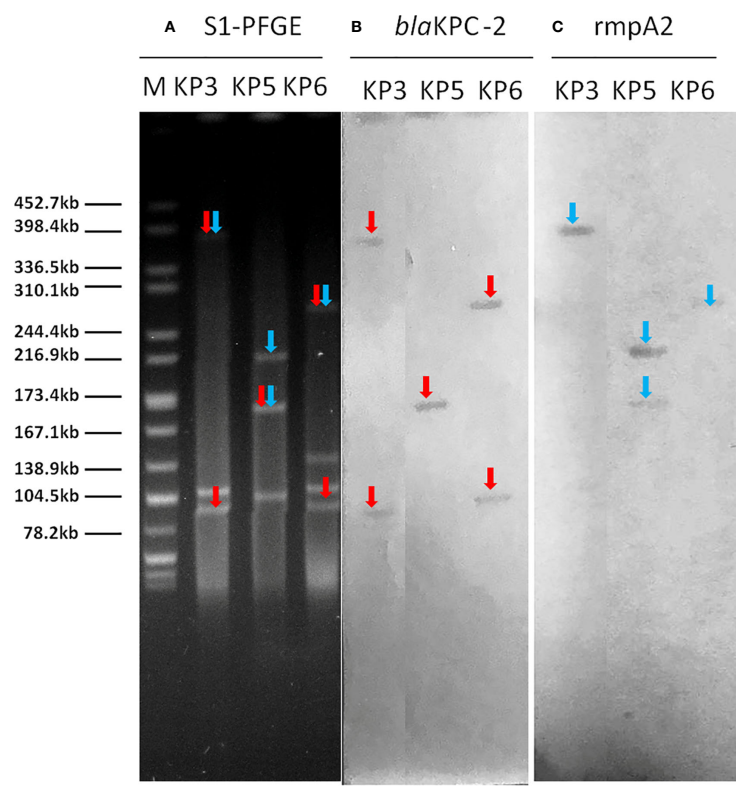


FIGURE 1 | The S1-PFGE and Southern hybridization analysis of 3 strains hybrid resistance- and virulence-encoding plasmids. Notes: **(A)** S1 nuclease digestion of genomic DNA of *K. pneumoniae* strains was followed by PFGE. Plasmid bands are shown as linearized fragment on the gel. **(B)** Southern blot hybridization of bla_{KPC-2} gene. Mark it with a red arrow. **(C)** Southern blot hybridization of the marker gene (rmpA2) of the virulence plasmid. Mark it with a blue arrow. Lane M, reference standard strain *Salmonella* serotype Braenderup H9812 restricted with Xbal. M, marker; S1-PFGE, S1 nuclease pulsed-field gel electrophoresis.

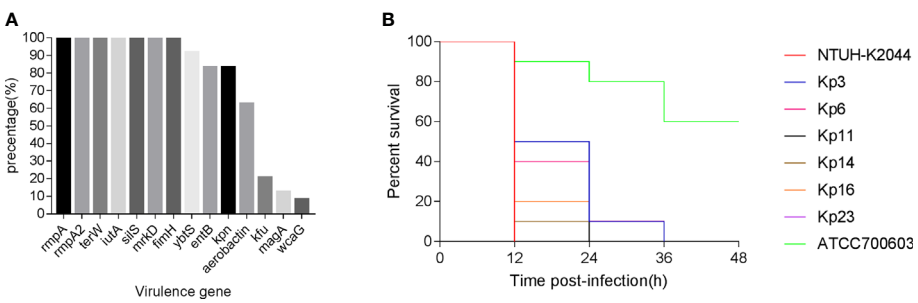
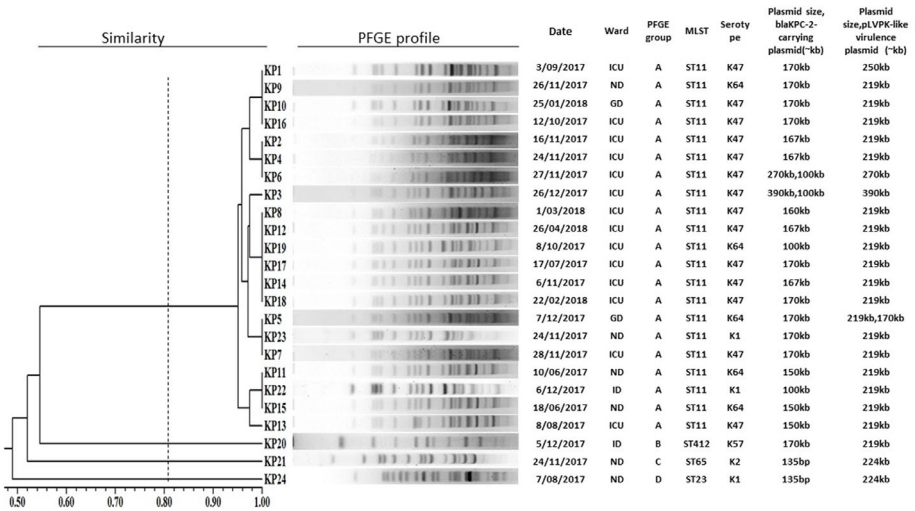


FIGURE 2 | **(A)** The distributions of virulence-associated genes among CR-KP co-harboring blaKPC-2-carrying plasmid and pLVPK-like virulence plasmid. **(B)** Virulence potential of representative CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid in a *Galleria mellonella* infection model. CR-KP, carbapenem-resistant *Klebsiella pneumoniae*.

resistant, amikacin, gentamicin, and tigecycline still had efficient antimicrobial activity *in vitro* against these isolates, indicating that they could be valuable treatment choices. The production of *Klebsiella pneumoniae* carbapenemase (KPC) is the most prevalent mechanism of resistance to carbapenems (Munoz-Price et al., 2013). In China, the first detection of the plasmid-mediated class A carbapenemase KPC-2 gene was located on an approximately 60-kb plasmid in 2007 (Wei et al., 2007). In this study, the bla_{KPC-2} carrying plasmids all belonged to $IncFII_K$ type, and the size ranged from 100-kb to 390-kb.

Virulence plasmids were associated with hypervirulent serotypes of *Klebsiella pneumoniae* and predisposed patients to



	Date	Ward	PFGE group	MLST	Seroty pe	Plasmid size, blaKPC-2-carrying plasmid(~kb)	Plasmid size,pLVPK-like virulence plasmid (~kb)
KP1	3/09/2017	ICU	A	ST11	K47	170kb	250kb
KP9	26/11/2017	ND	A	ST11	K64	170kb	219kb
KP10	25/01/2018	GD	A	ST11	K47	170kb	219kb
KP16	12/10/2017	ICU	A	ST11	K47	170kb	219kb
KP2	16/11/2017	ICU	A	ST11	K47	167kb	219kb
KP4	24/11/2017	ICU	A	ST11	K47	167kb	219kb
KP6	27/11/2017	ICU	A	ST11	K47	270kb,100kb	270kb
KP3	26/12/2017	ICU	A	ST11	K47	390kb,100kb	390kb
KP8	1/03/2018	ICU	A	ST11	K47	160kb	219kb
KP12	26/04/2018	ICU	A	ST11	K47	167kb	219kb
KP19	8/10/2017	ICU	A	ST11	K64	100kb	219kb
KP17	17/07/2017	ICU	A	ST11	K47	170kb	219kb
KP14	6/11/2017	ICU	A	ST11	K47	167kb	219kb
KP18	22/02/2018	ICU	A	ST11	K47	170kb	219kb
KP5	7/12/2017	GD	A	ST11	K64	170kb	219kb,170kb
KP23	24/11/2017	ND	A	ST11	K1	170kb	219kb
KP7	28/11/2017	ICU	A	ST11	K47	170kb	219kb
KP11	10/06/2017	ND	A	ST11	K64	150kb	219kb
KP22	6/12/2017	ID	A	ST11	K1	100kb	219kb
KP15	18/06/2017	ND	A	ST11	K64	150kb	219kb
KP13	8/08/2017	ICU	A	ST11	K47	150kb	219kb
KP20	5/12/2017	ID	B	ST412	K57	170kb	219kb
KP21	24/11/2017	ND	C	ST65	K2	135bp	224kb
KP24	7/08/2017	ND	D	ST23	K1	135bp	224kb

FIGURE 3 | PFGE dendrogram of 24 carbapenem-resistant *Klebsiella pneumoniae* co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid. ICU, intensive care unit; ND, neurosurgery department; GD, gastroenterology department; ID, infectious department.

abscess formation (Tang et al., 2010). In the present study, nineteen strains (79.2%) carry a 219-kb virulence plasmid similar to pLVPK plasmid from serotype K2, *K. pneumoniae* CG43 (Chen et al., 2004). Two strains (8.3%) carry a 224-kb virulence plasmid similar to the pK2044 plasmid from serotype K1, sequence type (ST) 23 strain NTUH-K2044 (Wu et al., 2009). The pLVPK-like virulence plasmids in *K. pneumoniae* are very large and would, therefore, be regarded as non-conjugative. This would explain their strong association with particular hypervirulent serotypes (Struve et al., 2015). Nevertheless, it is obvious that virulence plasmids have been reported in several serotypes of *Klebsiella*, indicating that conjugation is occurring, albeit at a low frequency. In this study, three strains CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid can transfer virulence plasmids to *E. coli* J53. The conjugative transfer of this virulence plasmid increased the virulence level of such strain.

Carbapenem-resistant *K. pneumoniae* rarely carry virulence plasmids and hypervirulent *K. pneumoniae* generally do not carry antibiotic resistance genes. Nevertheless, in the current study, 24 strains *Klebsiella pneumoniae* co- harbored bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid. Most recently, Dong et al. (2018) reported that a bla_{KPC-2}-encoding element can be integrated into a virulence plasmid, which then possesses the ability to mediate expression of both hypervirulence and hyper-resistance phenotype in K1 hypervirulent *Klebsiella pneumoniae*. Similarly, we found three strains ST11 *K. pneumoniae* carrying a bla_{KPC-2}-harboring virulence plasmid, which were approximately 390, 270, and 170 kb, respectively. The convergence of virulence and MDR in a single plasmid vector enables simultaneous transfer and potentially rapid emergence of hypervirulence-MDR *K. pneumoniae* clones.

The presence of *mrkD* and *fimH* has previously been related to KPC-positive *K. pneumoniae* (De Cassia Andrade Melo et al., 2014). However, previous studies (Yeh et al., 2007) reported that *magA* was characteristic of the K1 capsular operon, which was associated with the hypermucoviscosity phenotype of *K. pneumoniae*. Siderophore-associated genes, such as *entB*, *ybtS*, and *iutA*, were critical for bacterial growth, replication, and virulence (Holden and Bachman, 2015). *entB* was only characterized for virulence when it occurs in association with *iutA* or *ybtS* (Daehre et al., 2018). By analyzing virulence genes, all *K. pneumoniae* isolates carried both *mrkD a*nd *fimH* genes in our study. Moreover, the *entB, iutA* or *ybtS* genes were present from three-quarters of all isolates, all of which serve as high mark of virulence.

Capsule, lipopolysaccharide (LPS), fimbriae (types 1 and 3), siderophores, and pLVPK-like virulence plasmid are virulence factors that contribute to the pathogenicity of *K. pneumoniae*. Nevertheless, Shu et al. (2019) reported OXA-232-producing ST15 carbapenem-resistant *K. pneumoniae* were not hypervirulent despite harboring a virulence plasmid. In the current study, the virulence level of CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid was found to be consistently higher than that of cKP. But we also found nine strains CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid were less virulent than hvKP. Further studies are required to establish the relationship between the hypervirulence phenotype and the carriage of the virulence plasmid in *K. pneumoniae*.

In our study, 87.5% of CR-KP co-harboring bla_{KPC-2}-carrying plasmid and pLVPK-like virulence plasmid belonged to ST11, in accordance with the report by Qi et al. (2011), which described that ST11 was the dominant clone of KPC-2-producing *K. pneumoniae* in China. Nineteen out of twenty-one ST11 isolates were wzi47-K47 or wzi64-K64 by the capsular serotyping. Two ST11 isolates belonged to wzi125-K1, which was rarely reported in a previous study (Wei et al., 2016). One wzi1-K1 strain belonged to ST23, was strongly correlated with liver abscess (Shon et al., 2013); one wzi2-K2 strain belonged to

ST65, which is in accordance with the previous study that ST65 was the most common ST associated with K2 serotype in *K. pneumoniae* (Liao et al., 2014); one wzi206-K57 belonged to ST412, which was hypermucoviscous.

In conclusion, all isolates were characterized by multi-drug resistance, enhanced virulence, and transferability, and should, therefore, be regarded as a real superbug that could pose a serious threat to public health. Moreover, three strains carried 3 different hybrid resistance- and virulence-encoding plasmids. We should strengthen the ability of anti-infective prophylaxis and management to avoid its co-transmission of the virulent plasmid (gene) and resistant plasmid (gene) in clinical isolates.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the ethical committee of the First Affiliated Hospital of Nanchang University. Informed consent was also obtained from all of the study patients.

AUTHOR CONTRIBUTIONS

F-lD, Q-sH, D-dW, and YL conceived and designed the experiments. F-lD, Q-sH, D-dW, DL, and W-jL designed and performed the experiments. F-lD, L-gW, and WZ analyzed the data. F-lD and YL wrote the manuscript. YL contributed to review on data analysis and the interpretation of the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.556654/full#supplementary-material

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Deceiving Phenotypic Susceptibility Results on a *Klebsiella pneumoniae* Blood Isolate Carrying Plasmid-Mediated AmpC Gene bla_{DHA-1}

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Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) frequently causes hospital-acquired infections and is associated with high morbidity and mortality. CRKP can have multiple resistance mechanisms and only a few can be routinely detected by commercial molecular or phenotypic assays making surveillance for CRKP particularly challenging. In this report, we identified and characterized an unusual non–carbapenemase-producing CRKP carrying a rare plasmid-borne inducible AmpC gene, bla_{DHA-1}. The isolate was recovered from blood culture of a 67-year-old female presenting with sepsis post bladder surgery and ureteral stent removal. The primary isolate displayed an indeterminate susceptibility pattern for ceftriaxone by broth microdilution, but was susceptible by disk diffusion with one colony growing within the zone of inhibition. The ceftriaxone resistant colony was sub-cultured and had a minimum inhibitory concentration (MIC) of 2 ug/ml for imipenem (intermediate) and a zone size of 18 mm for ertapenem (resistant), but remained susceptible to cefepime and meropenem. Further phenotypic characterization of this sub-cultured isolate showed carbapenemase activity. Whole genome sequencing (WGS) revealed the presence of two subpopulations of a *K. pneumoniae* (MLST sequence type 11) from the primary blood culture isolate: one pan-susceptible to beta-lactams tested and the other resistant to the 3rd generation cephalosporins and ertapenem. WGS analysis identified the resistant *K. pneumoniae* harboring IncFIB(K) and IncR plasmids and the presence of plasmid-borne beta-lactam resistance genes bla_{OXA-1} and bla_{DHA-1}, an inducible AmpC gene. Additional resistance genes against quinolones (*aac(6')-Ib-cr, oqxA, oqB*), aminoglycoside (*aph(3')-Ia*), sulfonamide (*sul1*), and tetracycline (*tet*(A)) were also identified. DHA-1 positive *K. pneumoniae* have been previously identified outside the US, particularly in Asia and Europe, but limited cases have been reported in the United States and may be underrecognized. Our study highlights the importance of using both extended phenotypic testing and WGS to identify emerging resistance mechanisms in clinical Enterobacterales isolates with unusual antimicrobial resistance patterns.

Keywords: carbapenem resistance, whole-genome sequence analysis, DHA-1, plasmid-mediated AmpC, *Klebsiella pneumoniae*

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INTRODUCTION

Klebsiella pneumoniae is a gram-negative rod and a member of the Enterobacterales family. These organisms are known to cause significant nosocomial infections with a wide range of clinical presentations including pneumonia, bacteremia, and urinary tract infections. One of the most concerning aspects of infection with *K. pneumoniae* is the high prevalence of drug resistance that can limit treatment options. Beta-lactamases are one of the most significant mechanisms of resistance in *K. pneumoniae*, including extended spectrum beta-lactamase (ESBLs) and carbapenemases, which are capable of hydrolyzing penicillins, cephalosporins, and carbapenems. From 1998 to 2010, *K. pneumoniae* surveillance isolates in the United States (US) showed a significant increase in antimicrobial resistance to drugs of all classes, except tetracyclines (Sanchez et al., 2013).

One under-recognized resistance mechanism of particular concern is plasmid encoded AmpC-type beta-lactamase. AmpC type beta-lactamases are part of the Ambler class C group of beta-lactamases that display resistance to penicillins, first, second, and third generation cephalosporins, cephamycins, and monobactams but are not susceptible to commonly used beta-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam (Jacoby, 2009). Inducible AmpC beta-lactamase activity is typically chromosomally encoded and is characteristic in a group of Enterobacterales species, commonly referred to as the "SPICE" group, which include *Serratia marcescens*, *Pseudomonas aeruginosa*, indole-positive *Proteus*, *Citrobacter freundii*, and *Enterobacter cloacae*. *Klebsiella* species do not have chromosomally encoded AmpC, but can acquire the resistance gene through plasmids. Plasmid-borne AmpC gene usually lacks genetic components that regulate AmpC expression and is therefore frequently found to be constitutively expressed. One exception is plasmid encoded bla_{DHA-1} which is usually adjacent to *ampR*, the transcriptional regulator gene for activation or repression of AmpC (Barnaud et al., 1998; Verdet et al., 2006; Compain et al., 2014; Luan et al., 2015), making it similar to inducible chromosomal AmpC enzymes.

In this report, we identified a clinical carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolate with inducible bla_{DHA-1} AmpC in a mixed bacterial population that initially showed inconsistent and confusing phenotypic susceptibility results which prompted further investigation. There are currently no established guidelines for the detection of plasmid-mediated AmpC expression in the clinical microbiology laboratory. WGS was used to identify resistance genes in this isolate, demonstrating that conventional methods are limited in detection of this type of resistance mechanism, which may lead to a vast under-recognition of its prevalence in the community (Jacoby, 2009).

MATERIALS AND METHODS

Antimicrobial Susceptibility and Molecular Testing

A *K. pneumoniae* isolate was recovered from a positive aerobic blood culture bottle and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) using the Vitek MS (BioMerieux, Marcy l'Etoile, France). Initial antimicrobial susceptibility testing was performed using in-house prepared broth microdilution (BMD) trays according to the CLSI guidelines (M07 and M100 29th edition, 2019). Disk diffusion (DD), modified carbapenem inactivation method (mCIM), and modified Hodge-test (MHT) were also performed to further characterize phenotypic resistance mechanisms (CLSI M100 29th edition, 2019 & M07, 11th edition, 2018). A total of 3 isolates are described: the primary isolate (Isolate 1^0) initially recovered from the blood culture and two isolates representing subpopulations of Isolate 1^0 separated based on the DD method using a ceftriaxone disk: Isolate 1A was susceptible and Isolate 1B grew inside the inhibition zone. The Xpert CarbaR (Cephid, Sunnyvale, CA) was also performed for detection of specific carbapenemase genes including KPC, NDM, VIM, IMP, and OXA-48-group genes.

Whole Genome Sequencing

DNA was extracted from bacterial isolates using the Qiagen EZ1 tissue kit (Qiagen, Hilden, Germany) extraction method according to manufacturer's instructions. Sequencing libraries were prepared using the Illumina DNA Flex kit (Illumina, San Diego, CA) and sequencing was performed on the Illumina MiSeq instrument (Illumina, San Diego, CA) using 2 x 250 protocol. Genomic analysis was done using the KmerFinder, ResFinder, Multi Locus Sequence Typing (MLST), and PlasmidFinder tools provided by the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). Additional analyses of sequence data were performed using CLC Genomics Workbench v12.0.3 (Qiagen, Hilden, Germany) and Geneious Prime software (Biomatters, Auckland, New Zealand), including mapping, *de novo* assembly, and variant analysis. Sequence data was mapped to the following references: *Klebsiella pneumoniae* strain KP38731, complete genome (Genbank NZ_CP014294.1), and *Klebsiella pneumoniae* plasmid pKPS30, complete sequence (Genbank NC_023314.1).

RESULTS

Patient History and Antibiotic Susceptibility Results

A 67-year-old woman with a history of rectal cancer and recently diagnosed bladder cancer experiencing anuria presented to the clinic for ureteral stent removal approximately 1 month post bladder surgery, cystectomy and ileal conduit (**Figure 1**). She was referred to the ER for evaluation and was admitted due to hematuria, chills, nausea, abdominal pain and severe sepsis on the same day. A primary bacterial culture (Isolate 1^0) was recovered from the aerobic blood culture bottle and identified as *Klebsiella pneumoniae* by MALDI-TOF. Antimicrobial susceptibility testing was performed using BMD but wells for ceftriaxone and ceftolozane-tazobactam displayed an indeterminate growth pattern in which the MIC could not be interpreted accurately. Upon repeat testing by DD, a single

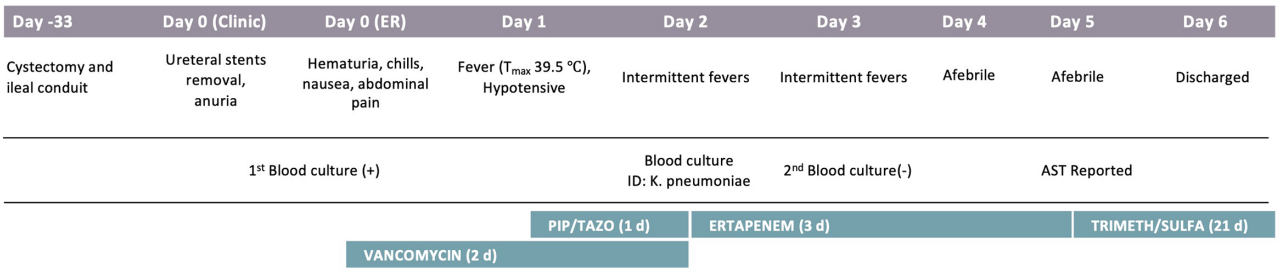

FIGURE 1 | Patient Clinical Course. Significant events are noted under each hospitalization day, including procedures, symptoms, and antibiotics course. AST, Antimicrobial susceptibility testing.

colony (Isolate 1B) was noted within the zone of inhibition for ceftriaxone and was sub-cultured for further workup.

Further phenotypic testing revealed two sub-populations in the primary culture, one susceptible to ceftriaxone and all other 3rd generation cephalosporins (Isolate 1A) and the other resistant to most cephalosporins and ertapenem (Isolate 1B) (**Table 1**). Isolate 1A showed pan susceptibility to all antibiotics tested except fluoroquinolones. Isolate 1B was resistant to most beta-lactams tested, except ceftazidime-avibactam, cefepime, and meropenem. The contrasting differences in beta-lactam susceptibility results between the two sub-populations in the primary culture prompted us to perform carbapenemase assays and WGS for further investigation.

Phenotypic Characterization of Resistance Mechanisms

Carbapenemase was tested by MHT, mCIM, and CarbaR PCR assays. No carbapenemase activity was detected in any isolate by MHT. However, Isolate 1B was mCIM positive, while Isolate 1A was negative. CarbaR PCR test, which detects KPC, NDM, VIM, OXA-48-group and IMP genes, was negative in all isolates. Further phenotypic testing was performed to determine

TABLE 1 | Antimicrobial susceptibility testing results.

Antibiotic Class	Antibiotic	Isolate 1^{0}				Isolate 1A				Isolate 1B			
		BMD		DD		BMD		DD		BMD		DD	
Beta-lactam	Ampicillin-Sulbactam					8	S			>32	R		
	Amoxicillin-Clavulanate					4	S			>32	R		
	Piperacillin-Tazobactam	64	I	18	I	≤8	S	25	S	>128	R	6	R
	Cefazolin	>32	R	6	R	2	S	23	S	>32	R	6	R
	Cefoxitin			6	R			19	S			6	R
	Ceftriaxone	Indeterminate		25	S	≤1	S	30	S	64	R	10	R
	Cefotaxime			24	S			31	S			6	R
	Ceftazidime	16	R	18	I	≤0.5	S	28	S	>32	R	6	R
	Ceftazidime-Avibactam	≤2	S			≤2	S			≤ 2	S		
	Ceftolozane-Tazobactam	≤0.5	S			≤0.5	S			>32	R		
	Cefepime	≤0.5	S	26	S	≤0.5	S	32	S	≤ 0.5	S	24	SDD
	Aztreonam					≤0.5	S			>32	R		
	Ertapenem	≤0.25	S	20	I	≤0.25	S	30	S	0.5	S	18	R
	Imipenem	1	S	24	S	≤0.25	S	26	S	2	I	26	S
	Meropenem	≤0.25	S	25	S	≤0.25	S	29	S	≤0.25	S	26	S
	Meropenem-Vabrobactam					≤0.6	S			≤0.6	S		
Aminoglycosides	Amikacin	≤4	S	23	S	≤4	S	26	S	≤4	S	23	S
	Gentamicin	≤1	S	22	S	≤1	S	26	S	1	S	23	S
	Tobramycin	4	S	25	S	≤1	S	24	S	4	S	14	I
	Streptomycin						S				S		
Macrolides	Azithromycin					16	S			>32	R		
Tetracyclines	Minocycline					2	S			8	I		
	Tigecycline					0.5				1			
Fluroquinolones	Ciprofloxacin	>4	R	6	R	>4	R	6	R	>4	R	6	R
	Levofloxacin	>4	R			>8	R			>8	R		
	Moxifloxacin					>8				>8			
Polymixins	Colistin	≤2	WT			≤2	WT			≤2	WT		
Folate Pathway antagonists	Trimethaprim/Sulfamethoazole	≤1/20	S	12	I	≤1/20	S	14	I	≤1/20	S	6	R

Broth microdilution (BMD) MIC reported in µg/ml; Disk Diffusion (DD) zone size reported in mm; R, Resistant; I, Intermediate; S, Sensitive, SDD, Susceptible-dose dependent.

possible AmpC activity with cefotaxime adjacent to imipenem or cefoxitin disks on Isolate 1A and 1B (**Figure 2**). Cefoxitin and imipenem were both able to induce resistance against cefotaxime in Isolate 1B but not in Isolate 1A as noted by truncation of the zone, suggesting a possible inducible AmpC resistance mechanism.

Genetic Determinants of Resistance

Whole genome sequencing was performed on the two sub-populations and the primary mixed culture for comparison to determine genetic relatedness and to identify drug resistance genetic elements. All isolates were identified as Sequence Type (ST) 11 based on MLST analysis and all closely related to the same strain *Klebsiella pneumoniae* strain KP38731 by KmerFinder analysis. Variant analysis was performed using KP38731 as the reference genome and showed no single nucleotide polymorphisms (SNPs) among the four isolates, indicating they are of the same bacterial lineage. Drug resistance genetic analysis using ResFinder identified numerous genes conferring resistance to beta-lactams, aminoglycosides, fluroquinolones, rifamycins, tetracycline, phenicol, macrolides, and fosfomycin in Isolate 1^0, 1B (**Table 2**). In contrast, 1A had much fewer resistance genes identified (**Table 2**), with only $bla_{SHV\text{-}182}$, *oqxA*, *oqB*, and *fosA*, which are known to be chromosomally encoded in *K. pneumoniae* (Fu et al., 2007; Ito et al., 2017). In addition, *in silico* plasmid identification analysis by PlasmidFinder detected the presence of two plasmid types, IncFIB(K) and IncR in primary Isolate 1^0 and Isolate 1B. The InR-type plasmid was not detected in Isolate1A, which was the isolate that demonstrated susceptibility to most antibiotics, further supporting that the primary culture was mixed with two-subpopulations.

TABLE 2 | Resistance genes identified by WGS.

Antibiotic Class Targeted	AMR Gene	Isolate 1^0	Isolate 1A	Isolate 1B
Beta-lactam	blaDHA-1	✓		✓
	blaOXA-1	✓		✓
	blaSHV-182	✓	✓	✓
Aminoglycoside	aac(6′)-Ib-cr	✓		✓
	aph(3′)-Ia	✓		✓
Folate Pathway	sul1	✓		✓
Quinolone	aac(6′)-Ib-cr	✓		✓
	oqxA	✓	✓	✓
	oqxB	✓	✓	✓
	qnrB4	✓		✓
Rifamycins	arr-3	✓		✓
Tetracycline	tet(A)	✓		✓
Phenicol	catB3	✓		✓
Macrolide	mph(A)	✓		✓
Fosfomycin	fosA	✓	✓	✓
Plasmids	IncFIB(K)	✓	✓	✓
	IncR	✓		✓

Further BLAST analysis identified the InR-plasmid in Isolate 1^0 and 1B to be closely related to a previously published InR pKPS30 plasmid (NC_023314.1) with 98.3% pairwise identity and 100% coverage. This 61,288-bp plasmid was initially described in a *K. pneumoniae* ST11 strain isolated in France (Compain et al., 2014). It carries several mobile genetic elements (integron and transposons) with multiple resistance genes, including $bla_{DHA\text{-}1}$, $bla_{OXA\text{-}30}$, *aac(6′))-Ib-cr*, *aphA1*, *arr-3*,

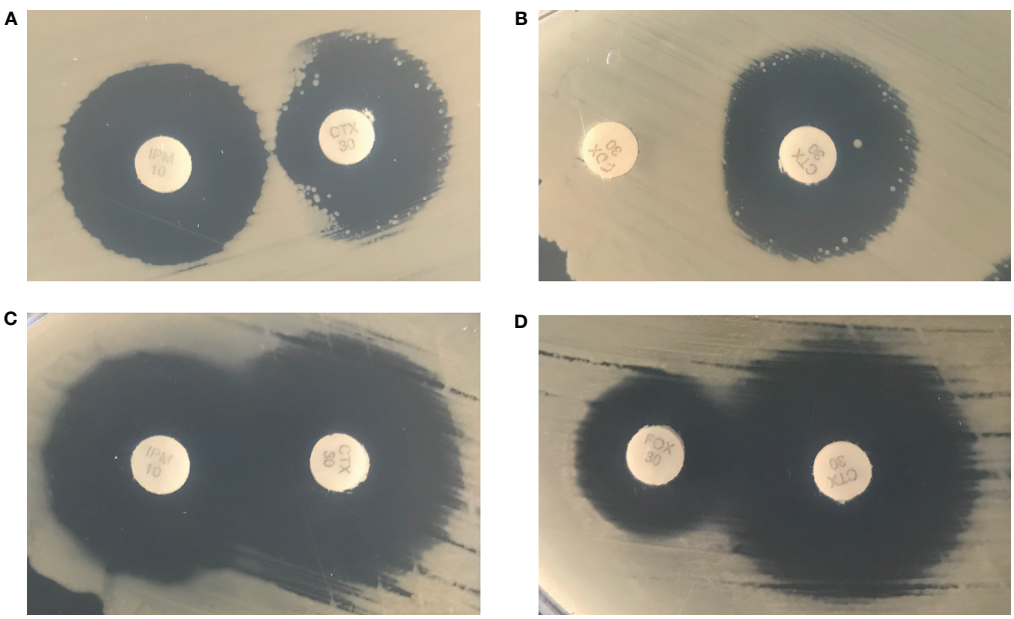

FIGURE 2 | Isolate 1B was cultured in the presence of an imipenem disk (left) placed adjacent to a cefotaxime disk (right) **(A)** and a cefoxitin disk (left) placed adjacent to a cefotaxime disk (right) **(B)**. Isolate 1A was cultured in the presence of an imipenem disk (left) placed adjacent to a cefotaxime disk (right) **(C)** and a cefoxitin disk (left) placed adjacent to a cefotaxime disk (right) **(D)**.

catB3, mph(A), qnrB4, aac(6′))-Ib-cr, sul1, and *tet(A)*, all of which were also identified in Isolate 1^0 and 1B. Notably, both bla_{DHA-1} and *ampR*, along with *sul1*, are within a class-1 integron that was originally discovered in a DHA-1-Producing *Klebsiella* spp. in France over 15 years ago (Verdet et al., 2006).

DISCUSSION

In this report, we describe the detection of AmpC expression in a subpopulation of *K. pneumoniae* recovered from a blood culture using phenotypic antimicrobial susceptibility testing, carbapenemase assays and WGS. From the primary culture isolate, we uncovered two subpopulations: one was ceftriaxone resistant with inducible AmpC and the other was ceftriaxone susceptible without AmpC. The AmpC encoding gene, bla_{DHA-1}, was detected in Isolate 1B but not in 1A, which is consistent with phenotypic susceptibility results and carbapnemase test results. No carbapenemase encoding genes were detected, suggesting the elevated ertapenem & imipenem MIC and positive mCIM might be due to the hyperproduction of AmpC after induction by cephalosporins or carbapenems.

The patient was admitted due to sepsis-like symptoms and was empirically treated with vancomycin for 2 days and piperacillin-tazobactam for 1 day, both of which would not be effective in treating the *K. pneumonia* isolate. The antibiotic regime was quickly switched to ertapenem on Hospital Day 2 pending susceptibility results, blood cultures were negative by Hospital Day 3, even though the final susceptibility results (reported on Hospital Day 5) showed the bacteria were not susceptible to ertapenem. The short duration of sepsis-like symptoms and suboptimal drug treatment suggested transient bacteremia post ureteral sent removal procedure and antibiotic regime would likely not change the clinical outcome in this case. However, in the context of more severe infection, treatment options are expected to be more challenging due to the limited selection of susceptible beta-lactams, such as imipenem, meropenem, meropenem-vaborbactam, and ceftazidime-avibactam.

Chromosomally encoded AmpC-type beta-lactamases are known to be readily induced by cephamycins and carbapenems (Jacoby, 2009). Non-SPICE group organisms, such as *Escherichia coli* and *K. pneumoniae*, can obtain plasmid-mediated AmpC resistance that is constitutively expressed due to the lack of regulatory genes. However, there is an emerging threat of organisms carrying plasmid mediated inducible AmpC, such as $bla_{\text{DHA-1,}}$ as identified in this report. Plasmid-mediated bla_{DHA-1} was first identified in 1992 from a stool isolate of *Salmonella enterica* serovar Enteritidis in Saudi Arabi (Gaillot et al., 1997). Two subsequent DHA-1 producing *K. pneumoniae* isolates were identified in California and Florida (Moland et al., 2002; Alvarez et al., 2004) the same year and additional isolates were later identified in France in 1998 (Verdet et al., 2006). The DHA-1 carrying plasmid in this study is closely related with the plasmid pKPS30 first identified in a ST11 type *K. pneumoniae* in 2008 in a urine isolate from a patient in France (Compain et al., 2014). Plasmid KPS30 contains a 12,391-bp backbone with an IncR replicon and a 44,944-bp MDR region including $bla_{\text{DHA-1}}$ and *ampR*, insertion sequences, complete class 1 integron, and several transposons. *K. pneumoniae* carrying DHA-1 AmpC have been increasingly reported worldwide, particularly in Europe and Asia (Yan et al., 2002; Lee et al., 2006; Song et al., 2006; Verdet et al., 2006; Vanwynsberghe et al., 2009; Hennequin et al., 2012; Luan et al., 2015) but rarely in the US. In a recent study examining 482 ceftriaxone not susceptible in Enterobacterales isolates in a US medical center, 17% of isolates were found to have a plasmid mediated AmpC and 5% of those were bla_{DHA} positive, including one *K. pneumoniae* isolate (Tamma et al., 2019). Although there have been few documented instances of bla_{DHA-1} carrying *K. pneumoniae* in the US, there are no routine testing methods that can detect this gene and therefore the actual prevalence may be underestimated. Importantly, we demonstrated cefoxitin and imipenem were able to induce resistance to cefotaxime in an isolate harboring the plasmid-borne bla_{DHA-1}. This would have been concerning if the patient continued to be treated with ertapenem due to the potential for AmpC derepression and therefore carbapenem resistance.

Interestingly, the mCIM test was able to demonstrate carbapenemase activity in our isolate. The mCIM test generally has high sensitivity and specificity for carpabenemase producing organisms but does not typically detect carbapenemase activity in non-carbapenemase producing organisms (Pierce et al., 2017; Zhou et al., 2018; Thomson et al., 2019). Previous studies have shown few positive mCIM results in non-cabapenemase producing organisms, including AmpC producing *Enterobacter* spp. (Pierce et al., 2017; Thomson et al., 2019). Here we demonstrate a bla_{DHA-1} encoding *K. pneuominae* isolate as another instance of a non–carbapenemase-producing organisms that could result in a positive mCIM. The MHT in all isolates were negative, which contrasts previous studies reporting weakly false positive MHT results in non-carbapenemase, plasmid mediated bla_{DHA-1} encoded AmpC in *Enterobacter* spp. and *K. pneumoniae* isolates with mCIM negative results (Zhou et al., 2018). In this case, hyperproduction of AmpC is likely causing the hydrolysis of carbapenem which resulted in positive mCIM. According to the current Council of State and Territorial Epidemiologists (CSTE) position statement (Centers for Disease Control and Prevention, 2018), this isolate qualifies as a CRE based on the mCIM positive result alone or the ertapenem resistance seen by disk diffusion. Identification of a CRE is critical for infection control precautions and accurate antibiotic susceptibility testing is important for determining CRE status. However, our study has shown that it is increasingly challenging to detect emerging resistance mechanisms due to limitations of current methods used by many clinical microbiology laboratories.

In summary, we described a case of unusual *K. pneumoniae* bacteremia with a mixed subpopulations of bacteria with or without AmpC gene bla_{DHA-1}, causing an initial false and discrepant susceptibility profile. Performing disk diffusion to resolve the ceftriaxone susceptibility result was the key in revealing the hidden bacterial subpopulation expressing AmpC. We used WGS to show the mix of bacterial populations were all genetically related except for the presence of an additional plasmid carrying bla_{DHA-1}. This case highlights the need for guidelines that include both molecular testing and phenotypic screening for inducible AmpC producing organisms outside the typical

"SPICE" group. Although plasmid-borne bla_{DHA-1} is seldom reported in the US, it is possible that organisms with this gene are under detected due to the lack of effective screening methods. WGS is instrumental in revealing inducible resistance mechanisms that are difficult to identify by routine methods. Further molecular epidemiological investigation is required to fully understand the true prevalence of this resistance mechanism in the community.

DATA AVAILABILITY STATEMENT

Whole genome sequence data presented in this study has been deposited at DDBJ/ENA/GenBank under the accession numbers JADGMK000000000 and JADGMJ000000000.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SR, KW, and SY participated in data collection, analysis, interpretation, and investigational design. SR wrote the manuscript. All authors reviewed the manuscripts for edits. SY provided project management. OG provided funding, resources, and oversight. All authors contributed to the article and approved the submitted version.

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REVIEW
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Carbapenem-Resistant Enterobacterales in Long-Term Care Facilities: A Global and Narrative Review

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The emergence of carbapenem-resistant Enterobacterales (CRE) has become a major public health concern. Moreover, its colonization among residents of long-term care facilities (LTCFs) is associated with subsequent infections and mortality. To further explore the various aspects concerning CRE in LTCFs, we conducted a literature review on CRE colonization and/or infections in long-term care facilities. The prevalence and incidence of CRE acquisition among residents of LTCFs, especially in California, central Italy, Spain, Japan, and Taiwan, were determined. There was a significant predominance of CRE in LTCFs, especially in high-acuity LTCFs with mechanical ventilation, and thus may serve as outbreak centers. The prevalence rate of CRE in LTCFs was significantly higher than that in acute care settings and the community, which indicated that LTCFs are a vital reservoir for CRE. The detailed species and genomic analyses of CRE among LTCFs reported that *Klebsiella pneumoniae* is the primary species in the LTCFs in the United States, Spain, and Taiwan. KPC-2-containing *K. pneumoniae* strains with sequence type 258 is the most common sequence type of KPC-producing *K. pneumoniae* in the LTCFs in the United States. IMP-11- and IMP-6-producing CRE were commonly reported among LTCFs in Japan. OXA-48 was the predominant carbapenemase among LTCFs in Spain. Multiple risk factors associated with the increased risk for CRE acquisition in LTCFs were found, such as comorbidities, immunosuppressive status, dependent functional status, usage of gastrointestinal devices or indwelling catheters, mechanical ventilation, prior antibiotic exposures, and previous culture reports. A high CRE acquisition rate and prolonged CRE

carriage duration after colonization were found among residents in LTCFs. Moreover, the patients from LTCFs who were colonized or infected with CRE had poor clinical outcomes, with a mortality rate of up to 75% in infected patients. Infection prevention and control measures to reduce CRE in LTCFs is important, and could possibly be controlled *via* active surveillance, contact precautions, cohort staffing, daily chlorhexidine bathing, healthcare-worker education, and hand-hygiene adherence.

Keywords: Enterobacteriaceae, long-term care facilities, oxacillinase, carbapenemases, metallo-beta-lactamase

INTRODUCTION

The emergence of antimicrobial resistance has become a major public health concern. Since the identification of carbapenem-resistant Enterobacterales (CRE) in the 1990s, CRE has spread worldwide during the past two decades (Centers for Disease Control and Prevention, 2013). The threat is not only confined to tertiary referral hospitals or academic health science centers. In a network of community hospitals in the southeastern United States, a CRE incidence of 0.26 per 100,000 patient-days in 2008 and 1.4 per 100,000 patient-days in 2012 was reported (Thaden et al., 2014). In a population-based study in seven states in the United States, CRE incidence of up to 2.93 per 100,000 persons (95% Confident Interval 2.65–3.23) was also reported (Guh et al., 2015). In addition, previous studies had reported high mortality rates among CRE-infected patients, ranging from 32.1%–48% (Patel et al., 2008; Gasink et al., 2009), which could even increase to 71% in one year among liver transplant patients (Kalpoe et al., 2012). Regarding the median cost of CRE infections with an incidence of 2.93 per 100,000 persons in the United States, it would cost hospitals $275 million, third party payers $147 million, and the society $553 million (Bartsch et al., 2017), indicating a high economic burden caused by CRE infections. Collectively, considering the rapid worldwide spreading of CRE, the association of CRE infections with poor clinical outcomes, high economic burden, and relatively limited antimicrobial treatment for CRE in the current era, the Centers of Disease Control and Prevention (CDC) announced CRE as the most urgent public health threat in 2013.

During the past decade, the demand for chronic rehabilitation and skilled nursing care after an acute illness has increased as the population ages. At the same time, a high prevalence of colonization by multi-drug resistant organisms (MDRO) among residents in long-term care facilities (LTCFs) was reported. The SHIELD Orange County Project demonstrated an MDRO prevalence of 65% among nursing homes (NHs) residents and 80% among long-term acute care hospital (LTACs) residents (Mckinnell et al., 2019). Furthermore, Guh et al. reported that most CRE isolates came from individuals with a history of health care exposure or hospitalization within one year, and most CRE hospitalized cases resulted in a discharge directly to a long-term care facility or LTAC hospitals (Guh et al., 2015). Another case-control study using the Illinois hospital discharge database reported that the CRE carriage rate at the time of admission was highly associated with prior health-care facility exposure (particularly LTACs) (Lin et al., 2019). That is, compared with the relatively low prevalence rate in the community (Prabaker et al., 2012) and in the acute care hospitals (ACHs), the high prevalence of CRE in LTCFs is an important public health issue and a critical component of large-scale antibiotic stewardship programs.

Tracing back, a systematic review and meta-analysis was conducted to highlight the importance of CRE in hospital settings (Van Loon et al., 2018). In this article, we conducted a literature review to summarize the current understanding of CRE in LTCF settings, and to demonstrate that the CRE colonization and/or infection rates are truly higher among LTCFs in different geographic regions.

We searched the English-language medical literature using PubMed/MEDLINE from 1990 to 2020, using the following keywords: carbapenem-resistant Enterobacteriaceae, carbapenem-resistant Enterobacterales, long-term care facilities, nursing home, long-term acute care hospitals. The references of articles found using this search were also reviewed to identify other potential studies that were not located using the search terms. Studies reporting CRE carriage and/or infection among elderly residents or patients who lived in or being admitted from long term care facilities were reviewed. Studies that provided data on previous long term care facilities exposure in the setting of acute care hospital or community or outbreak were also included. Exclusion criteria included studies that only documenting CRE carriage and/or infection in acute care hospital, tertiary medical centers, ICU or community; studies that tested for multiple-drug organism carriage and/or infection but did not evaluate for CRE; or studies that evaluated CRE carriage and/or infection among pediatric patients or health care workers. In total, 33 studies were reviewed in full. Other 12 articles involved in this study were searched while reviewing the similar articles in above mentioned papers.

Prevalence and Incidence of CRE Among Residents in LTCFs

An increasing number of studies have evaluated CRE in LTCFs between 2012 and 2020. In most studies, LTCF were facilities that provide long-term rehabilitation and skilled nursing care, such as long-term acute care hospitals or facilities (LTACs), skilled nursing facilities (SNFs) and nursing homes (NHs) in the United States, and residential care homes for the elderly (RCHE) in Hong-Kong. The study designs for CRE acquisition among

LTCF residents could be broadly classified into four types: point prevalence survey (identifying the number of people with CRE at a specific point in time), incidence surveillance (determining the rate of CRE in LTCF for a specific period), outbreak investigations (reports of investigation during CRE outbreak), and network model formulation. CRE colonization and/or infection in LTCFs shared similar but not identical characteristics in different geographic regions; therefore, we separated the studies geographically, that is, the United States, Europe (including Turkey, Israel, and North Lebanon) and Asia.

The prevalence rate of CRE colonization among LTCFs in the United States varied widely, as demonstrated in **Table 1**, ranging from 1%–30.4% (Prabaker et al., 2012; Lin et al., 2013; Johnson et al., 2014; Van Duin et al., 2014; Cunha et al., 2016; Mckinnell et al., 2016; Prasad et al., 2016; Han et al., 2017; Reuben et al., 2017; Mckinnell et al., 2019). The prevalence rate of Carbapenem-resistant *K. pneumoniae* (CRKP) among residents of LTCFs in the United States demonstrated a high geographical variation, with the highest prevalence in the West (42.2%), followed by the South (12.2%), Midwest (7.3%), and Northeast (9.9%) (Han et al., 2017). The highest CRKP prevalence was found in California (45.5%, 701/1540) (Han et al., 2017). Among the CRKP endemic West region, the CRKP prevalence rate changed significantly across the study period: 48.5% in quarter 1, 2014; 40.4% in quarter 2, 2014; 49.5% in quarter 3, 2014; 32.5% in quarter 4, 2014; and 39.0% in quarter 1, 2015 ($p<0.01$, test for trend) (Han et al., 2017). The CRE incidence among residents of LTCFs in the United States varied from 1.07–6.83 cases per 10000 patient-days (Brennan et al., 2014; Chopra et al., 2018). The pooled mean incidence rate of CRE in the United States was 0.46 per 1000 patient-days (Marquez et al., 2013).

In Europe (including Turkey, Israel, and North Lebanon), CRE in LTCFs also demonstrated a high geographic variance (**Table 2**). Recent studies about CRE in Europe were few and most of them reported low CRE prevalence rates among residents of LTCFs. There was a 0.3% CRE prevalence rate in LTCFs in Switzerland (37/12423) (Kohler et al., 2018), and only one resident with CRE carriage was found in each LTCF in Belgium and the Netherlands (Latour et al., 2019; Van Dulm et al., 2019). Even though a low prevalence rate was noted, the high association of CRE colonization with LTCF was still noted from the hospital admission data in Spain, reporting that about 37% of cases were health-care associated, of which 42% were nursing home residents (Palacios-Baena et al., 2016). Different from mainland Europe, a higher prevalence of CRE carriage was found among residents in LTCFs in Israel (12%) (Ben-David et al., 2011) and North Lebanon (1.7%) (Dandachi et al., 2016). Though a relatively low prevalence rate of CRE colonization in LTCFs was found in Belgium, the Netherlands, and Switzerland, high CRE carriage rates (28.4%) were reported among a LTAC rehabilitation facility (LTACRF) in central Italy, which is a CRE endemic region since 2010 (Ambretti et al., 2019).

As shown in **Table 3**, the prevalence rate of CRE colonization among LTCFs in the Asia region ranged from 13%–22.7% (Dandachi et al., 2016; Lee et al., 2017; Chen et al., 2018; Hagiya et al., 2018; Jean et al., 2018; Mao et al., 2018; Le et al., 2020). The two-point prevalence studies in Japan showed a 13% prevalence rate of CRE colonization in LTCFs in Hiroshima and a 19.3% prevalence rate in Osaka hospitals (one of which had approximately 200 long-term care beds) (Hagiya et al., 2018; Le et al., 2020). The CRE prevalence among the LTCFs in Taiwan was 22.7% (Lee et al., 2017). In Korea, a 10-month active surveillance survey *via* rectal specimens among LTCFs reported a low carbapenemase-producing Enterobacterales (CPE) prevalence rate (1.4%, 4/282) (Hwang et al., 2018). In Hong-Kong, no CPE fecal carriage was found in residential care homes for the elderly (RCHEs) (Dandachi et al., 2016), compatible with a previous study of 28 RCHEs in Hong-Kong by (Cheng et al. (2016).

Collectively, the high predominance of CRE in LTCFs was observed worldwide, especially in LTCFs in the West region of the United States (California), Spain, Italy, Japan, and Taiwan. Since very few studies have been conducted to evaluate the clinical epidemiology of CRE in LTCFs in some regions in Europe (Russia, Arabia) and Asia (such as the mainland China, and the southern east countries), we could not determine the clinical epidemiology of CRE colonization and/or infections in these countries or regions.

High-Acuity LTCFs Were an Important Reservoir of CRE

The high predominance of CRE in LTCFs reflects the local clinical epidemiology in the community or hospital settings, especially the ACHs or intensive care units (ICUs). In the following section, we compared recent studies evaluating CRE in LTCFs with previous studies about CRE in hospital settings and/or the community to demonstrate the issue.

In the United States, there were many studies supporting that the CRE acquisition and/or infections among residents of LTCFs was much more than that in acute care settings and/or the community. A population-based incidence study of CRKP among ACHs, LTACs, and SNHs in the Los Angeles County between 2010 and 2011 reported a higher CRE incidence rate in LTACs (2.54 per 1000 patient-days) than that in ACHs (0.31 per 1000 patient-days, $p<0.001$) (Marquez et al., 2013). Another outbreak investigation conducted among ACHs and LTACs in Indiana and Illinois in 2008 reported that one of the LTACs was the main locus of the outbreak, which accommodated 60% (24/60) of the cases, and only 10% (4/60) of the patients definitely had CRE colonization in ACHs (Gohil et al., 2017). A previous systematic review of CRE in the United States between 2000 and 2016 reported higher infection rates in LTACs than in ACHs and community settings (Livorsi et al., 2018), and community-onset cases mostly had health care exposure within the previous 90 days (Brennan et al., 2014). The multihospital case-control study in Chicago during an early KPC epidemic reported a higher prevalence of CRE carriage among LTCFs patients (8.3%, n=15) compared with patients admitted from the community (0%, n=0) ($p<0.001$) (Prabaker et al., 2012). Additionally, the HARP-DC studies (one of the first study to measure the prevalence of CRE colonization in a region aligning with CDC's recommendation of collaborative approach), highlighted that the CRE prevalence in

TABLE 1 | Studies of carbapenem-resistant Enterobacterales colonization in long-term care facilities in the United States.

Reference	Study type	Sites	Study period	Study populations	Specimen sources	Sample size	Prevalence/ incidence	LTCF percentage	Molecular studies	Risk factors
Lautenbach et al., 2009	Point prevalence study	New Jersey, Pennsylvania Delaware	2008/01/15 -2008/11/15	63 LTCFs across 3 US states	Urine culture	1,805 isolates	6% CR-KP	1,653 isolates from 44 SNF 152 isolates from 19 ALF	NA	NA
Lin et al., 2013	Point Prevalence study	Chicago	2010/07-2011/06	24 ACHs 7 LTACs	rectal, inguinal swab, or urine	391 patients	30.4% (119 of 391) of LTACHs with KPC-producing Enterobacterales, compared to 3.3% (30 of 910) of ACHs ICU patients [prevalence ratio 9.2, 95% CI 6.3-13.5]	All LTACHs had KPC, prevalence range, 10%–54%) 15 of 24 ACHs had KPC (prevalence range, 0%–29%).	NA	LTACH facility type, mechanical ventilation, and length of stay
Johnson et al., 2014	Point Prevalence study	Maryland	2010/07-2010/08	30 (67%) ACHs and 10 (83%) LTCFs	peri-anal and sputum	390 patients, total 358 samples	6% of patients (ACH/LTCF) with KPC-producing Enterobacterales.	55% (n=11) in LTCF 45% (n=9) in ACH	15 *K. pneumoniae* (60% ST258) 6 *E. Coli* (50% ST131) 1 *E. Cloacae*. Of all 19 KPC-2 3 KPC-3.	Mechanically ventilated
Han et al., 2017	Point Prevalence study	USA	2014/01-2015/03	3,470 patients across 64 LTACs	Blood, respiratory, urine	3,846 unique quarterly *K. pneumoniae* clinical cultures in 3,470 patients	24.6% CRKP (946/3846)	NA	NA	Geographic variation
Prasad et al., 2016	retrospective Point Prevalence study	New York	chart review	a single center–affiliated LTCF	rectal swab	301 residents (80-bed ventilator unit)	18.9% asymptomatic rectal CRE colonization (n=57 patients 61 isolates)	CR-KP n=46 CR *E. coli* n=15	CR-KP (n=46) 30 KPC-3 16 KPC-2 CR-*E coli* 11 KPC-3 4 KPC-2 10 additional CTX-M. [9 KP KPC-3 1 E.Coli KPC-2)	Recent CDI Tracheostomy collar mechanical ventilation
Mckinnell et al., 2016	Retrospective point prevalence study	Southern California	2015/06 - 2015/08	605 residents in 3 NHs	axilla/groin swabs	1,800 swabs from 605 residents	1% CRE	NA	NA	history of MDRO, care needs, incontinence, and catheters.

(Continued)

TABLE 1 | Continued

Reference	Study type	Sites	Study period	Study populations	Specimen sources	Sample size	Prevalence/ incidence	LTCF percentage	Molecular studies	Risk factors
Reuben et al., 2017	Retrospective prevalence study	Washington, District of Columbia	2016/01/11-2016/04/14	8 ACHs, 2 LTACs 5 SNFs 1 inpatient rehabilitation facility	perianal swab	1,022 completed tests	5.2% CRE (n=53, 95% CI, 3.9%–6.8%)	ACH 5.0% LTCF 7.0% inpatient rehabilitation facility 0%.	4.3%(n=44) KPC 1 NDM 1 both KPC and OXA48.	NA
Mckinnell et al., 2019	Retrospective point prevalence study	Southern California	2016/09-2017/03	18 NHs and 3 LTACHs [SHIELD Orange County Project]	bilateral axilla/groin and peri-rectal swabs	50 adults in 18 NHs and 3 LTACHs	67% (n=701) MDROs 3% (n=31) CRE	NA	NA	Gastrointestinal device (OR 19.7, 95% CI 3.5-109.4, p<0.001) Prior MDROs
Prabaker et al., 2012	Hospital admission Case control	Chicago	NA	Hospitalized adults from 4 hospitals with an early KPC epidemic.	Rectal swab	180 patients from LTCF	8.3% (n=15) of LTCF had KPC-producing Enterobacterales colonization	0 (0%) of the community patients (P<.001).	NA	LTCF subtype
Van Duin et al., 2014	Hospital admission	Northeastern Ohio	2011/12/24 -2013/03/01	Hospitalized patients from LTCF and community (28%)	NA	251 patients admitted to 18 hospitals	CRKP infection in 45% patients	NA	88 CRKP isolates belong to ST258	NA
Cunha et al., 2016	Hospital admission Case-Control	Providence, Rhode Island	2012/07-2012/09	hospital admission from NHs	Fecal carriage (rectal swab PCR)	404 patients with 500 hospital admissions	4.6% CPE fecal carriage rate (n=23),	NA	2 KPC producing *Citrobacter freundii*	Gastrostomy (p=0.04)
Brennan et al., 2014	Incidence surveillance	Michigan	2012/09/01-2013/02/28	17 ACHs 4 LTAC	NA	102 cases over 957220 patient days	1.07 cases per 10,000 patient days	5% hospital onset, 65% community onset (75% had health care exposure within 90d)	89 cases *K. pneumoniae* (87.2%) 13 cases *E. coli* (12.7%)	surgery in 90 days, recent infection/ colonization with a multidrug-resistant organism, recent exposures to antimicrobials
Chopra et al., 2018	Incidence surveillance Retrospective Cohort	Detroit	2011/01/01-2012/07/31	A 77-bed LTAC in Detroit	NA	30 patients with CRE 24(80%) infection, 6 (20%) colonization	Incidence 6.83 episodes per 10,000 inpatient-days (30/351112)	23 (77%) patients had CRE following LTAC admission	8 CRE isolates had bla_{KPC-3}, and belonged to ST258	
Bower et al., 2020	point incident survey	8-county Atlanta metropolitan area	2016	Georgia Emerging Infections Program (EIP), Facility-specific Connectivity Using Medicare Data	NA	NA	283 incident CRE cases	50% in ACH (n=141), 40% in SNFs (n=113) 10% in LTACHs (n=29)	CRE infections originate from almost all ACHs and half of SNFs.	Medicare patient transfers strongly correlated with CRE case-transfer data in ACHs (r=0.75; P<0.01) and LTACHs (r=0.77; P=0.03), but not in SNFs (r=0.02; P=0.85).
Marquez et al., 2013	Incidence and outbreak surveillance	NA	2010/06/01-2011/05/31	ACH, LTAC, NH outbreak in 1 LTAC	NA	814 reports (CRKP positive)	ACH (57%, n=387), LTAC (34%, n=231), SNH (8%, n=57)	pooled mean incidence rate in ACHs and LTACs was 0.46 per 1000 patient-days the rate in LTACs (2.54 per 1000 patient-days) was higher than that in ACHs (0.31 per 1000 patient-days, p<0.001)		

(Continued)

TABLE 1 | Continued

Reference	Study type	Sites	Study period	Study populations	Specimen sources	Sample size	Prevalence/ incidence	LTCF percentage	Molecular studies	Risk factors
Endimiani et al., 2009	Outbreak surveillance	South Florida	2008/03/21 -2008/04/20	1 LTAC	NA	10 KPC-KP in 241 KP isolates (4.1%)	NA	7 KPC-KP from a LTAC (3 from hospitals)	3 KPC-KP belong to ST258	NA
Dubendris et al., 2020	Outbreak surveillance (Case control)	North Carolina (USA)	2016/10/22- 2017/11/30	3 LTCF during outbreak	rectal swab	83 isolates from 76 patients	7 CROs (8.4%), 4 CRE (4.8%)	6 in LTCF (7.2%)	4 IMP (in same LTCF)	

ACHs, acute care hospitals; ALF, assisted living facility; CDI, Clostridium difficile Infection; CRO, carbapenem-resistant organisms; CPE, carbapenemase producing Enterobacterales; CRE, carbapenem-resistant Enterobacteriaceae; CRKP, carbapenem-resistant Klebsiella pneumoniae; ICU, intensive care units; KP, Klebsiella pneumoniae; KPC, Klebsiella pneumoniae carbapenemase; LTACs, long-term acute care hospitals; LTCFs, long-term care facilities; MDROs, Multidrug-Resistant Organisms; NA, not available, NDM, New Delhi metallo-β-lactamase; NHs, nursing homes; SNF, Skilled nursing facility.

LTCFs was even higher than that in the ACHs (7.0% in LTCFs vs 5.0% in ACHs), with a relative prevalence ratio of 0.9% [0.5-1.5] in LTCFs and 1.5% [0.9-2.6] in ACHs) (Reuben et al., 2017). Furthermore, up to 30.4% prevalence rate of KPC-producing Enterobacterales among LTCFs was even reported (prevalence range 10–54%), compared with the relatively low prevalence rate in short-stay hospital ICU patients (3.3%, prevalence rate 0–29%) (Lin et al., 2013). That is, a 9-fold greater risk of KPC-producing Enterobacterales was found in the LTCF patients compared to the ACH patients (Lin et al., 2013). However, conflicting results of CRE prevalence between LTCFs and ACHs remain. A population based study conducted in Atlanta reported different results, of which CRE incidence was attributed mostly to the ACHs (n=141, 50%) and skilled nursing facilities (SNFs; n=113, 40%), rather than the LTACs (n=29, 10%) (Bower et al., 2020). A CRE incidence study by Guh et al. reported that most cases were collected from ACHs (33.9%) rather than from LTCFs (26.9%) or a LTAC (7.5%) (Guh et al., 2015).

In Europe, the European survey of carbapenemase-producing Enterobacterales (EuSCAPE study) evaluating CRE colonization and/or infections in hospitalized patients reported that CRE prevalence varied geographically, with the highest rate in the Mediterranean and Balkan countries (Grundmann et al., 2017). High incidence countries included Greece patients (5.78 per 10000 hospital admissions), Italy (5.96 per 10000 hospital admissions), Montenegro (5.65 per 10000 hospital admissions), Spain (4.01 per 10000 hospital admissions), and Serbia (3 per 10000 hospital admissions) (Grundmann et al., 2017). The high predominance of CRE in Spain and Italy were similar between ACHs and LTCFs, but the difference of CRE prevalence and/or incidence among LTCFs in other countries and/or regions in Europe had not been clarified in previous studies.

In Asia region, Kayoko Hayakawa et al. reported a 30% prevalence rate of carbapenemase-producing Enterobacterales (CPE) among tertiary hospitalized patients (Hayakawa et al., 2020), which was much higher than the CRE prevalence rate in LTCFs in another study in Japan (Le et al., 2020). However, Kayoko Hayakawa highlighted that patients with CPE were more likely to be residents in the nursing homes or the LTCFs prior to hospital admission (Hayakawa et al., 2020). Dokyun Kim et al. reported a low carbapenem resistance rate among *K. pneumoniae* in Korean secondary and tertiary hospitals (less than 0.1–2%), but an increasing trend of CRE (CRKP and *E. coli*) was reported in recent years (the carbapenem susceptibility rates of *E. coli* were 100% in 2011 and 99.3% in 2015; the carbapenem susceptibility rates of *K. pneumoniae* were 99.0% in 2011 and 97.0% in 2015) (Kim et al., 2017). In China, Qi Wang et al. conducted a longitudinal large scale CRE study between 2012 and 2016 among hospitals in China, and a high prevalence rate of carbapenem resistance among Enterobacterales was found (91% in *K. pneumoniae*, 80% in *E. coli*, and 72% in *E. cloacae*). However, there were few data about CRE prevalence in LTCFs in mainland China and other countries in Europe. Though little is known about the accurate difference of CRE prevalence between ACHs and LTCFs in the Asia region and Europe, studies in the United States, Italy, and Japan highlighted the threat of high prevalence of CRE among LTCFs, of which the prevalence

TABLE 2 | Studies carbapenem-resistant Enterobacterales colonization in long-term care facilities in the Europe.

Authors	Study type	Sites	Study period	Study populations	Specimen sources	Sample size	Prevalence/ incidence	LTCF percentage	Molecular studies	Risk factors
Ben-David et al., 2011	Point prevalence study	Israel	NA	1,144 patients in 12 PACFs	Rectal swab	CRKP carriage in 1,044 patients	12% CRKP (1004/ 1144)	NA	NA	Prolonged length of stay, sharing a room with known carrier, antibiotic use in prior 3 months, prior culture grew CRKP
Kohler et al., 2018	Point prevalence study	Switzerland	2007/01-2017/10	NH	Urogenital, skin, other	16,804 samples from 9,940 residents	0.3% CRE (37/12,423)	NA	NA	Non-urogenital isolates, geographic
Latour et al., 2019	Point prevalence study	Belgium	2015/06 -2015/10	51 randomly selected residents per NH	Rectal swab	1,447 residents from 29 NHs	CRE carriage in only 1 resident	NA	NA	NA
Van Dulm et al., 2019	Point prevalence study	Netherlands	2014/11 -2015/08	12 LTCFs	Rectal swab	385 residents from 12 LTCFs	CRE carriage in only 1 resident	MDR-GNB carriage rate 18.2% (range 0-47%)	NA	NA
Dandachi et al., 2016	Point prevalence study	North Lebanon	2013/12-2014/04	2 NHs	Fecal swab	178 isolates from 68 NH residents	1.7% CRE (3/178)	NA	1.7% co-producers of OXA-48 and ESBL	Recent antibiotic use
Palacios-Baena et al., 2016	Hospital admission	Spain	2013/02/01- 2013/05/01	in 34 hospitals	NA	NA	379 isolates from 245 patients had CPE 164(66.9%) infection 81 colonized 23 asymptomatic bacteremia	Healthcare associated in 91 cases (37%), of which 42% NH residents	Of 35 NH cases 32 *K. pneumoniae* (31 OXA-48, 1 VIM-1) 3 *E. coli* (OXA-48)	NA
Legeay et al., 2019	Outbreak surveillance	Western France	2014/05-2017/07	10 isolates from 3 intra-NH outbreak	Urine Rectal swab	NA	10 CRE in 3 outbreaks	NA	10/10 OXA-48 *K. pneumoniae* 3/10 OXA-48 *E. coli*	Antibiotic consumption, high frailty, incontinence

CRKP, carbapenem resistant Klebsiella pneumoniae; PACF, post-acute-care facilities; CPE, carbapenemase-producing Enterobacterales; CRE, carbapenem-resistant Enterobacterales; LTCFs, long-term care facilities; MDR-GNB, multidrug-resistant micro-organisms; NA, not available; NHs, nursing homes.

TABLE 3 | Studies of carbapenem-resistant Enterobacterales colonization in long-term care facilities in the Asia.

Authors	Study type	Sites	Study period	Study populations	Specimen sources	Sample size	Prevalence/incidence	LTCF percentage	Molecular studies	Risk factors
Le et al., 2020	Point prevalence study	Hiroshima (Japan)	2017/02 -2018/01	Residents in a LTCF	Oropharyngeal swab	98 residents in a LTCF	13% CRE	NA	1 MDR-*P. aeruginosa.* was a bla_{IMP-1} positive ST235	NA
Hagiya et al., 2018	Hospital admission	Northern Osaka (Japan)	2015/12 -2016/01	Admission to 43 hospitals	stool	140 patients from 43 hospital	19.3% CRE (27/140)	One hospital had 200 long-term care beds	IMP-6	Longer hospital stay, lower Norton scales
Lee et al., 2017	Point prevalence study	Taiwan	2015/01 -2015/12	Residents in 6 LTCFs	Rectal swab	313 residents in 6 LTCFs	22.7% CRE 11.7% CR-*K. pneumoniae* 5.2% CR-*E. coli*	NA	NA	Functional status, dementia
Chen et al., 2018	Point prevalence study	Hong-Kong	2015/09 -2015/12	20 RCHE	Nasal, axillary, rectal swab or stool	1,028 residents	0 in 373 stool and 654 rectal swab for CRE screening	NA	NA	NA
Mao et al., 2018)	Outbreak surveillance	Taiwan	2013/05 -2014/06	1 LTCF	Case 1 abscess Case 2 blood Case 3 blood, urine	4 isolates from 3 patients in a LTCF	NA	NA	4/4 bla_{CMY-2}	NA

CRE, carbapenem-resistant Enterobacterales; IMP, imipenemase; LTCFs, long-term care facilities; NA, not available; RCHE, residential care homes for the elderly.

and/or incidence of CRE acquisition and infections in LTCFs was much higher than that in ACHs, ICUs, and the community. Our review suggests that LTCFs are vital reservoirs for CRE and are important in regional outbreaks and/or dissemination of CRE (Chitnis et al., 2012; Prabaker et al., 2012; Lin et al., 2013; Guh et al., 2015; Gohil et al., 2017).

The CRE prevalence varied among different subtypes of LTCFs. One study reported a higher CRE prevalence in facilities that managed ventilated LTAC patients and ventilator-capable nursing homes (vNH) residents (8% *vs* < 1%, p<.001) and was rare in NHs that did not offer mechanical ventilation (NH <1%, vNH median 10% [0-12%], LTACs median 8%[8-10%]) (Mckinnell et al., 2019). Predominant CRE carriage among skilled nursing facilities with ventilator care (VSNFs) (27.3%) and LTACs (33.3%) (p <0.001) was found (Prabaker et al., 2012). Furthermore, patients from VSNFs and LTACs had a 7.0-fold greater odds ratio of KPC-producing Enterobacterales colonization (95% CI 1.3-42, p=0.022) than patients from SNFs (Prabaker et al., 2012). Collectively, high-acuity LTCFs that provided mechanical ventilation, such as ventilated LTAC, vNHs, and VSNFs, were particularly important for regional CRE spread.

Similar CRE Species Distribution Between LTCFs and ACHs

The species distribution of KPC-producing Enterobacterales was similar between the ACHs and LTCFs in the United States, of which *K. pneumoniae* was the prominent species (Brennan et al., 2014; Guh et al., 2015; Cunha et al., 2016; Satlin et al., 2017; Jean et al., 2018; Hayakawa et al., 2020). The KPC-producing *K. pneumoniae* was the predominant species (87%, n=129), followed by *Enterobacter aerogenes* (6%), *E. coli* (4%), *E. cloacae* (0.7%), and co-colonization with *K. pneumoniae* plus either *E. coli* or *E. cloacae* (2.7%) (Lin et al., 2013). Consistent with the CRE incidence study from the United States communities in 2012–2013, a high prevalence (58.6%, n=351) of *K. pneumoniae* among CRE isolates was found among the United States population, followed by *E. coli* (13.2%, n=79) and *E. cloacae* (12.5%, n=75) (Guh et al., 2015). A high percentage of *K. pneumoniae* (87.2%, n=89) was also noted in a state-wide surveillance study in Michigan (Brennan et al., 2014).

In Europe, the predominant CRE species were also similar between the ACHs and the LTCFs. A hospital admission survey demonstrated *K. pneumoniae* as the primarily species in Spain, of which 37% of cases were health-care related (42% were NH residents) (Palacios-Baena et al., 2016). The EuSCPAPE study reported predominant *K. pneumoniae* among CRE species, followed by *E. coli*. Wide geographic variations in CRE prevalence existed in Europe, with a high prevalence in Italy, Romania, Turkey, and Spain (Grundmann et al., 2017).

In the Asian region, among CPE in Japan, the most common species were *E. cloacae* (30%), followed by *K. pneumoniae* (22%), *E. coli* (14.8%), *Citrobacter freundii* (11.1%), *Klebsiella oxytoca* (7.4%), *E. aerogenes* (3.7%), and *Serratia marcescens* (3.7%) (Hayakawa et al., 2020). The prominent CRE strains identified among LTCFs in Taiwan were *K. pneumoniae*, compatible with previous studies (Jean et al., 2018).

Risk Factors for CRE Colonization and/or Infections Among Residents in LTCFs

Multiple risk factors were reported to be associated with the increased vulnerability for colonization and/or infections with CRE or specifically KPC-producing Enterobacterales. The identified risk factors could be broadly classified into four groups: patient characteristics, environmental factors (including facility subtypes and the use of medical devices) and previous microbiology status or antibiotic exposures (including previous hospital stay and co-infection with other pathogens) (**Table 4**).

The identified patient characteristics associated with a significant risk factors for CRE colonization or infection among residents in LTCFs were fecal incontinence (OR 5.78, 95% CI 1.52 to 22.0, p=0.01) (Mills et al., 2016), solid organ or stem cell transplantation (OR 5.05, 95% CI 1.23 to 20.8, p=0.03) (Mills et al., 2016), comorbidity status with Charlson's score greater than three (OR 4.85, 95% CI 1.64 to 14.41) (Bhargava et al., 2014), strokes (Le et al., 2020), dementia (Lee et al., 2017), residents in dependent functional status (Hagiya et al., 2018; Hayakawa et al., 2020), and immunosuppressive status (OR 3.92, 95% CI, 1.08 to 1.28) (Bhargava et al., 2014).

The environmental factors associated with a significant risk for CRE colonization or infection among residents of LTCFs were usage of gastrointestinal devices (OR 19.7, P <0.001) (Cunha et al., 2016; Mckinnell et al., 2019), mechanical ventilation (OR 3.56, 95% CI 1.24 to 5.28, p=0.01) (Mills et al., 2016), the presence of indwelling devices, such as central venous catheters or urinary catheters (OR 5.21, 95% CI 1.09 to 2.96), LTAC facility subtype (Lin et al., 2013), particularly high acuity facilities with mechanical ventilation, prolonged length of stay (Ben-David et al., 2011; Lin et al., 2013), and sharing a room with a known carrier (Chitnis et al., 2012).

Prior antimicrobial carriage status and associated antibiotics exposure were also associated with increased risk of CRE colonization or infection among residents in LTCFs (Ben-David et al., 2012). A high prevalence (41%) of residents in LTCFs had body cultures consistent with their prior CRE colonization status (Reuben et al., 2017). Prior antibiotic exposures (OR 3.89; 95% CI 0.71 to 21.47) (Chitnis et al., 2012; Bhargava et al., 2014; Brennan et al., 2014) and previous culture growing CRKP within 90 days were identified as independent risk factors for continued CRKP carriage (Chitnis et al., 2012). Furthermore, even short-term antimicrobial exposure in the prior one month was significantly associated with the increased risk of CRE colonization and infection among residents in LTCFs (meropenem OR 3.55, 95% CI 1.04–12.1, p=0.04; vancomycin OR 2.94, 95% CI 1.18–7.32, p=0.02; metronidazole OR 4.22, 95% CI 1.28–14.0, p=0.02) (Mills et al., 2016). In addition, recent *Clostridium difficile* infection was associated with increased risk of *C. difficile* and CRE colonization (Prasad et al., 2016), which indicated that prior or co-infection with other bacteria may increase CRE colonization risk in residents of LTCFs.

In the ACHs, the risk factors for CRE acquisition were exposure to antibiotics, high comorbidity indexes, deteriorated functional status and/or cognition at baseline, recent LTCF stay, and recent invasive procedures or permanent foreign devices (Lin et al., 2019). Our review of the risk factors associated with increased risk of CRE colonization and/or infection among residents in LTCFs was similar but not identical to the previous systematic review and meta-analysis (Van Loon et al., 2018), of which the systemic review identified risk factors associated with CRE acquisition among hospitalized patients between 2005 and 2017 in Europe, Asia, America, Australia, and Africa (Van Loon et al., 2018). Risk factors identified by Karlijn Van Loon et al. for CRE acquisition among hospitalized patients were patient's underlying disease or condition (pooled OR 2.54; 95% CI 2.08 to 3.09, p <0.05), usage of medical devices (pooled OR 5.09; 95% CI, 3.38 to 7.67, p <0.05), mechanical ventilation (pooled OR 1.96; 95% CI 1.42 to 2.69, p<0.05), ICU admission (pooled OR 4.62; 95% CI, 2.46 to 8.69, p <0.05), antibiotic exposures (particularly carbapenem OR range 1.83 to 29.17 and cephalosporin OR 2.24 to 49.56), and CRE exposures (pooled OR 4.10; 96% CI, 1.46 to 11.52) (Van Loon et al., 2018).

Resistance Mechanisms of CRE From LTCFs Were Similar to ACHs

KPC is the major resistance determinant of CRE from LTCFs in the United States (**Figure 1**). Molecular study for CRE from

TABLE 4 | Risk factors for CRE acquisition in LTCFs.

Types of factors	Odds ratio or relative risks documented in studies
Patient characteristics	Fecal incontinence (OR 5.78) (Mills et al., 2016)
	Solid organ or stem cell transplantation (OR 5.05) (Mills et al., 2016)
	Immunosuppressive status (OR 3.92) (Bhargava et al., 2014)
	Comorbidities (Charlson's score > 3; OR 4.85) (Bhargava et al., 2014)
	Strokes (Le et al., 2020), Dementia (Lee et al., 2017), Dependent functional status (Hagiya et al., 2018; Hayakawa et al., 2020)
Environmental factors	Usage of gastrointestinal devices (OR 19.7) (Cunha et al., 2016; Mckinnell et al., 2019)
	Indwelling devices (e.g. CVC or urinary catheters) (OR 5.21) (Lin et al., 2013)
	Mechanical ventilation (OR 3.56) (Mills et al., 2016)
	LTAC facility subtypes, esp. high-acuity facility with mechanical ventilation (Lin et al., 2013)
	Prolonged length of stay (Ben-David et al., 2011; Lin et al., 2013)
	Sharing a room with known carriers or increased prevalence of known carriers in the same ward (Chitnis et al., 2012)
Microbiology status	Prior antibiotic exposures (OR 3.89) (Chitnis et al., 2012; Bhargava et al., 2014; Brennan et al., 2014)
	Previous culture growing CRKP within 90 days (Chitnis et al., 2012)
	Recent *Clostridium difficile* infection (Prasad et al., 2016)

CVC, central venous catheter; CRKP, carbapenem-resistant Klebsiella pneumoniae; LTAC, long-term acute care hospitals; OR, odds ratio.

LTCFs in the United States demonstrated a high prevalence of KPC-producing Enterobacterales (55%) (Johnson et al., 2014). Among KPC-producing Enterobacterales, the prominent species were KPC-producing *K. pneumoniae* (87%), followed by *E. aerogenes* (6%), *E. coli* (4%), and *E. cloacae* (0.7%) (Lin et al., 2013). Among LTCFs in the United States, most KPC-producing *K. pneumoniae* strains carried KPC-2 (19/21) and mostly belonged to the strain ST258 (60%) (Johnson et al., 2014). Regarding KPC-producing *E. coli*, half of the tested strains belong to ST131 (Johnson et al., 2014). Another report showed a higher carriage rate of KPC-3 in *K. pneumoniae* and *E. coli*, whereas other carbapenemase (NDM, IMP, VIM, OXA-48) were uncommon among CRE colonized in LTCF residents in the United States (Prasad et al., 2016; Reuben et al., 2017; Dubendris et al., 2020). Among these studies, the carriage of CTX-M (Reuben et al., 2017) or OXA-48 (Reuben et al., 2017) could be found in KPC-producing Enterobacterales. The predominance of KPC-producing CRE isolates was supported by previous CRE incidence study, of which 90% KPC was identified from CRE (79.3% were *K. pneumoniae*) (Guh et al., 2015); and previous outbreak investigation study (Gohil et al., 2017). Furthermore, clustering of the ST258 was also noted in the outbreak investigation study in Indiana and Illinois (Gohil et al., 2017). Similarly, in the ACHs, the predominance of KPC-producing CRE (KPC-3 48%, KPC-2 44%) and co-existence of NDM-1 and OXA-48 were identified in a multicenter prevalence study in the United States (Satlin et al., 2017). In addition, the prominent ST258 strain among CRKP was also identified in ACHs (Satlin et al., 2017).

There are only few studies concerning the resistance mechanisms of CRE from LTCFs in Asia. Molecular analyses showed that nearly all CRE harbored IMP-type carbapenemase in LTCFs in Japan, of which IMP-11 was the most prominent type (40.7% IMP-11, 22.2% IMP-42, 14.8% IMP-6, 11.1% IMP-10, 11.1% IMP-1) (Hayakawa et al., 2020), though another study reported IMP-6 as the most common carbapenemase in Japan (Hagiya et al., 2018). Besides, efflux pump genes (*oqxA* and *oqxB*) were mostly observed in the CP-CRE group compared with the non-CP-CRE group (Hayakawa et al., 2020). A study in Taiwan revealed $bla_{\text{CMY-2}}$ in imipenem-resistant *Providencia stuartii* isolates associated with the outbreak in a LTCF, yet the true determinant remained unidentified (Mao et al., 2018).

OXA-48 has been reported in CPE isolates from LTCFs in Europe. In a hospital admission survey of 379 CPE isolates from 245 patients in Spain, OXA-48 was the predominant carbapenemase, followed by VIM-1, IMP, and KPC (74% OXA-48, 22% VIM-1, 2% IMP, 2% KPC) (Palacios-Baena et al., 2016). In the 35 isolates from NHs, 32 of them were *K. pneumoniae*, of which 31 isolates were OXA-48 and one isolate was VIM-1. On the contrary, among hospitalized patients in Europe, KPCs remained the predominant carbapenemase (42%, 393/927), followed by OXA-48-like enzymes (38%, 353/927), NDM-1 (12%, 113/927), and VIM (7%, 68/927) (Grundmann et al., 2017). At country level, KPC were predominantly detected in Italy (96%, 187/195), Israel (80%, 31/39), Greece (65%, 56/86), and Portugal (59%, 36/61) (Grundmann et al., 2017). OXA-48-like carbapenemases were common in Turkey (79%, 98/124), Romania (74%, 50/68), Spain (70%, 81/116), Belgium (38%, 18/48), France (37%, 10/27), and Germany (33%, 12/36) (Grundmann et al., 2017).

Outcome of CRE Colonization in LTCFs and ACHs

Acquisition of CRE is associated with a high economic burden and poor clinical outcomes. Among patients colonized or infected with CRE in LTCFs, the 30-day mortality rate was 10%–25% (Tischendorf et al., 2016; Chopra et al., 2018; Igbinosa et al., 2020). It is worth noting that the mortality rates in specific subgroups of patients with clinical CRE infections were as high as 30%–75% (Borer et al., 2012; Papadimitriou-Olivgeris et al., 2013; Lubbert et al., 2014). The condition is complicated by the prolonged CRE colonization which has been documented in various studies. Manon R. Haverkate et al. demonstrated that the duration of KPC-producing Enterobacterales colonization could be more than 9 months in KPC-positive patients (Haverkate et al., 2016).

The risk of infection after colonization with CRE varied in different studies. The cumulative risk of infection after CRE colonization was 16.5% in a systemic review (Tischendorf et al., 2016). The risk of infection varied, ranging from 7.6%–44.4% (Tischendorf et al., 2016), of which the most common site of infection was the lung (50% of the patients), followed by the urinary tract (20%), primary bloodstream, and skin and soft tissue infections (including surgical sites) (Tischendorf et al., 2016).

Regarding the hospitalized patients, a systemic review and meta-analysis revealed that the pooled risk ratio of CRE infection and mortality rate was 2.85 [95% CI, 1.88 to 4.30] (Soontaros and Leelakanok, 2019). Dickstein et al. conducted a matched cohort study in the ICU, and reported that colonization with CRE was independently associated with Enterobacterales infection (cause-specific hazard ratio was 2.06, 95% CI 1.31 to 8.43) (Dickstein et al., 2016). Zilberberg et al. conducted a retrospective cohort study among the hospitals in the United States, and reported that the presence of CRE was significantly associated with increased inappropriate empirical treatment than the absence of CRE (46.5% *vs*. 11.8%, p <0.001) (adjusted relative risk ratio 3.95, 95% CI 3.5 to 4.5, p <0.001) (Zilberberg et al., 2017). In addition, increased mortality rate (adjusted mortality 12%, 95% CI 3% to 23%) and prolonged length of hospital stay (an excess of 5.2 days, 95% CI 4.8 to 5.6, p <0.001) were found (Zilberberg et al., 2017).

Colonization with CRE poses an increasing threat to other residents in the same facility. CRE are mostly transmitted *via* patient-to-patient contact, and interestingly, the CRE transmission in the environment follows the 20/80 rule. That is, 20% super-spreaders are responsible for 80% bacterial transmission, which indicated that the super-spreaders play an important role in CRE transmission (Lerner et al., 2015). CRE super-spreaders were associated with high rectal CRE concentrations (modelled as bla_{KPC} copies/16s rDNA copies ratio, OR 14.5, 95% CI, 1.09 to 192.0, p=0.042) and respiratory

illnesses on admission (OR 20.5, 95% CI, 1.41 to 297.6, p=0.027) (Lerner et al., 2015).

The Consideration of Active Surveillance for CRE in LTCFs

In 2016, with the worsening threat of CRE, the European Centre for Disease Prevention and Control (ECDC) recommended infection prevention and control measures in hospitals and other healthcare settings, active screening in the epidemic region, and active surveillance in the endemic region. Even though active surveillance is one of the infection control measures for the prevention of CPE transmission and spread (Ambretti et al., 2019), and the 2013 European Society of Clinical Microbiology and Infectious Disease (ESCMID) strongly recommended contact precautions, using alert codes to identify known colonized patients at admission, pre-emptive contact precautions, isolation in a single room for infected or colonized patients, cohort staffing, antimicrobial stewardship and education, monitoring cleaning performance and active surveillance (Ambretti et al., 2019); there are limited national or international guidelines for optimal measures for active surveillance and management of CRE colonized patients in LTCFs (Ambretti et al., 2019). Some experts suggested that screening all hospital admission from the LTCFs for CRE may not be cost-effective (Hwang et al., 2018).

For CRE, targeting patients at high risk of CRE carriage is very important, and these high risk patients should be screened for digestive tract carriage, with concomitant pre-emptive contact precaution and followed isolation if colonization was confirmed (Magiorakos et al., 2017). Lau A. F. et al. recommended that the universal nucleic acid amplification technology (NAAT)-based method of CRE screening may not be universally affordable, but molecular rapid methods may be applicable for high-risk patients (e.g. from endemic region, LTCFs, extensive exposure to carbapenems), followed by susceptible culture-based method if the screening is negative, and considering to perform the carbapenemase confirmatory test if suspicious species are detected (Lau et al., 2015). Other than molecular and/or genomic analyses, Vincent J. LaBombardi et al. suggested that Remel Spectra CRE agar plates could provide faster (18 hours for Remel Spectra CRE agar versus 36 hours for the CDC method) and reliable results for KPC-type CRE detection (Labombardi et al., 2015). The sensitivity for common commercial chromogenic media were also reported, including chromID Carba media 96.5%, Remel Spectra CRE 97.8%, CHROMagar KPC 76.6%, and Direct ertapenem disk method 83% (Labombardi et al., 2015).

The CDC recommended perirectal swab for targeted CRE screening at hospital admission, especially for those admitted directly from LTACs, other LTCFs with known endemicity, or patients who are transferred directly from another ACHs (Tischendorf et al., 2016). Although KPC-producing Enterobacterales were mostly identified in perianal swab specimens (Johnson et al., 2014) or rectal swab culture (Cunha et al., 2016)), axilla/groin screening samples also detected 1% CRE prevalence in nursing home facilities (Mckinnell et al., 2016). CRE could be found in a variety of samples. Various studies reported urine as the most common source of CRE (Brennan et al., 2014; Guh et al., 2015), and CP-CRE were prominently isolated from sputum (40.7%) (Hayakawa et al., 2020). In a study conducted in Hiroshima, CRE were detected in oropharyngeal swab specimen (Le et al., 2020), and in a study in Osaka, CRE were detected from blood, sputum, urine, and intra-abdominal samples (Hayakawa et al., 2020).

Interventions to Reduce CRE Colonization and Infection in LTCFs

The importance of interventions to reduce the CRE carriage rate in LTCFs has already been documented. An outbreak investigation of CRE colonization among patients in LTAC between 2009 and 2011 revealed that surveillance testing and targeted interventions resulted in significant reductions in CRE prevalence (49% *vs* 8%), CRE

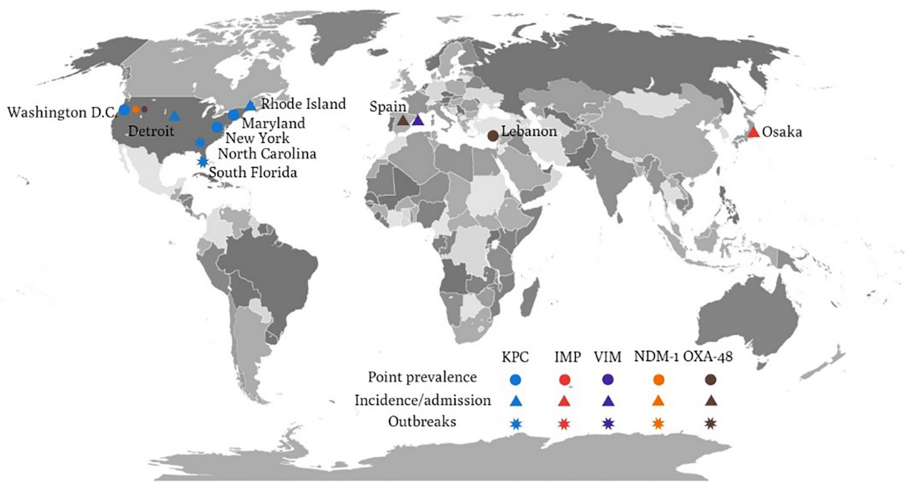

FIGURE 1 | The global distribution of various carbapenemase-producing *Enterobacteriaceae* related to long-term care facilities.

incidence (44% *vs* 0%) and CRE bacteremia (2.5 *vs* 0.0 per 1000 patient-days) (Chitnis et al., 2012). In addition, the prevention measures for CRE in LTCFs from a nation-wide coordinated protocol in Israel between 2007 and 2008 successfully reduced nosocomial CRE cases from 55.5 cases per 100000 patient-years to 11.7 cases per 100000 patient-years (Schwaber et al., 2011). The infection prevention measures in Israel were contact isolation, self-contained nursing units (single room or cohorts), and re-isolation of known carriers when they are encountered for subsequent hospital admission (Schwaber et al., 2011). Furthermore, the subsequent nation-wide intervention (alcohol-based hand rub, appropriate use of gloves, and a policy of CRE surveillance at hospital admission) in 13 post-acute hospitals in Israel between 2008 and 2011 resulted in the reduction of the overall CRE carriage rate (16.8% vs 12.5%, p=0.013) (Ben-David et al., 2014). Collectively, the Israeli guidelines for CRE prevention were isolation or cohorting of CRE carriers, cohorting of nursing staff (mandatory only for ACHs), barrier precautions on room entrance, admission CRE screening for high-risk patients, screening of patients' contacts, standard protocol for discontinuation of contact isolation, daily reporting of new cases to the Israeli National Center for Infection Control in the Ministry of Health, and periodic auditing of health care facilities' compliance with national guidelines by the National Center for Infection Control in the Ministry of Health (Ben-David et al., 2014).

In the United States, Mary K. Hayden et al. (Hayden et al., 2015) examined a bundled intervention in four LTACs in a high endemic KPC-producing Enterobacterales region (Chicago, Illinois) (Hayden et al., 2015). They adopted a rectal swab culture for active surveillance and preemptive contact isolation while awaiting the culture report. KPC intervention bundle was as follows: biweekly rectal culture surveillance, contact isolation, geographic separation of KPC-positive patients with ward cohort or single room, universal contact isolation of all patients in a high-acuity ward, daily 2% chlorhexidine gluconate (CHG) bathing for patients, healthcare-worker education, and adherence monitoring (particularly hand hygiene) (Hayden et al., 2015). During the pre-intervention period, the prevalence rate of KPC-producing Enterobacterales remained unchanged (average 45.8%, 95% CI 42.1% to 49.5%) (Hayden et al., 2015). During the intervention period, the prevalence rate of KPC-producing Enterobacterales declined significantly, then reached a plateau (34.3%, 95% CI 32.4 to 36.2%, p <0.001) (Hayden et al., 2015). The incidence of KPC-producing Enterobacterales also declined significantly during the intervention (from 4 to 2 acquisition per 100 patient-weeks, p=0.004) (Hayden et al., 2015). Other important clinical outcome indicators, including KPC identified in any clinical culture (32% reduction) and KPC bacteremia (56% reduction) also decreased (Hayden et al., 2015).

Similar to Hayden et al.' s study (Hayden et al., 2015), Toth et al. designed an agent-based simulation model for CRE transmission in a single LTAC within a regional network of ten health-care facilities (including one LTAC, three ACHs, and six NHs). The CRE prevalence rate was 45.8% and clinical detection incidence was 3.7 per 1000 patient-days (Toth et al., 2017). Two models with different transmission rates were created, and significant reduction in CRE transmission (range 79% to 93%) and prevalence (decrease from 21% to 6% in model A, decrease from 9% to 0.5% in model B) in a five-year period were reported (Toth et al., 2017). Even when the intervention was delayed until the 20th CRE detection, CRE transmission was still reduced by 60%–79% over five years (Toth et al., 2017). Furthermore, among the infection control measures, Toth et al. (Toth et al., 2019) designed a model-based assessment in Chicago, and reported that contact precautions by itself could potentially explain the decline in CPE colonization among surveillance-detected carriers (Toth et al., 2019). Other interventions, including CHG bathing, hand hygiene, and adherence monitoring may play a role in the slowing down of colonization (Toth et al., 2019). Therefore, focusing infection control and prevention measures in LTCFs is an effective strategy to reduce CRE acquisition and transmission (Toth et al., 2017).

Moreover, the importance of controlling CRE in LTCFs could not be over emphasized, since it may become a tragedy without interventions. Bruce Y Lee et al. conducted a prediction model and reported that if infection control and prevention measures were not implemented properly, CRE would become endemic in almost all health-care facilities (in Orange County) within 10 years (Lee et al., 2016). Although benefits of CRE decolonization include reduced CRE-related infection incidence and all-cause mortality (Tacconelli et al., 2019), the potential of increased antimicrobial resistance to decolonizing agents was reported in nearly all studies (Tacconelli et al., 2019). Consequently, the ESCMID-EUCIC clinical guidelines do not recommend routine decolonization of CRE (Tacconelli et al., 2019).

Antimicrobial use in LTCFs remains a critical issue in long-term care (Ricchizzi et al., 2018). Antimicrobial stewardship programs are needed to control the spreading of multidrug-resistant organisms (Oliva et al., 2018). Current data suggested effective antimicrobial stewardship strategies in LTCF reduced antimicrobial use (Jump et al., 2012). The implementation strategies vary considerably in different setting and warrant more studies to define appropriate and quality appraisal tools (Wu et al., 2019).

Summary

The emergence of CRE has become a major public health concern. Moreover, its colonization among residents of LTCFs is associated with subsequent infections and mortality. LTCFs are also an important reservoir of CRE. There are increasing studies concerning the high predominance of CRE in LTCFs. Further studies are needed to develop effective control measures.

AUTHOR CONTRIBUTIONS

H-YC, P-YL, S-SJ, Y-LL, M-CL, W-CK and P-RH collected and analyzed the data. S-SJ, Y-LL, M-CL, and W-CK conducted validation and supervision. H-YC, S-SJ, Y-LL, M-CL, W-CK and P-YL participated in the writing of the manuscript. H-YC, P-YL, S-SJ, Y-LL, M-CL, W-CK and P-RH read and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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