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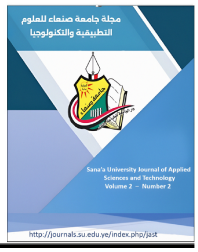

Carbapenem-Resistant *Klebsiella pneumoniae* in Yemeni Patients: Prevalence, Phenotypes, and Resistance Profile to Last-Resort Antibiotics

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Abstract

The spread of carbapenem-resistant *Klebsiella pneumoniae* (CRKp), which is one of the top pathogens in the WHO list of antibiotic-resistant bacteria, is a true threat to human life. This study aimed to determine the prevalence, carbapenemase phenotypes, and resistance to last-resort antibiotics of CRKp isolated from clinical specimens of inpatients and outpatients in 3 health-service facilities in Sana'a-Yemen between December 2020 and November 2022. The carbapenemases produced by these isolates were phenotyped by mCIM and eCIM, and the susceptibility of the isolates to last-resort antibiotics was determined using the disk diffusion method. A total of 140 CRK_p isolates were recovered in this study, mostly from urine specimens (35 %), from inpatients hospitalized in the gynecology ward (30%) and intensive care units (22.9 %). Differences in CRKp prevalence were insignificant between females and males and between age ranges but significant between urban and rural residents. Moreover, 75.7% of CRKp isolates were producers of metallo-β-lactamases and 81.4, 78.6 %, 17.1%, 15.7 %, and 7.1% of these isolates were resistant to aztreonam, ceftazidime+ avibactam, colistin, fosfomycin, and tigecycline, respectively. In conclusion, there is a considerable prevalence of CRKp isolates among Yemeni patients. These isolates are mostly metallo-β-lactamase producers and have remarkable resistance to most last-resort antibiotics. Accordingly, there is a need to establish and employ urgent control and prevention measures to diminish the spread of CRKp and limit its emergent resistance to last-resort antibiotics in our community.

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Keywords:
Carbapenem-Resistant, *Klebsiella Pneumoniae*, Prevalence, Yemen, Metallo-β-lactamases, Last-Resort Antibiotics

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1. INTRODUCTION

Klebsiella pneumoniae is a significant member of *Enterobacteriaceae*. It is considered to be one of the opportunistic pathogens causing broad spectra of diseases [1] and the second most frequent etiological agent involved in community-acquired urinary tract infections. The organism accounts for approximately one-third of all Gram-negative infections, including pneumonia, surgical wound infections, endocarditis, and septicemia [2]. The rapid emergence of resistant bacteria is occurring worldwide, thereby endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives [3]. Some strains of *K. pneumoniae*, particularly carbapenem-resistant *K. pneumoniae* (CRKp), have become increasingly resistant to antibiotics, rendering infection by these strains very challenging to treat [4]. CRKp is considered one of the top three pathogens of international concern as documented in 2017 in the World Health Organization (WHO) global priority list of antibiotic-resistant bacteria that need new antibiotics urgently [5]. The spread of CRK_p in any human community is a true threat to lives. The more troubling mechanism of resistance of CRKp to antibiotics is the expression of carbapenemases enzymes, which render bacteria resistant to almost all available-β-lactams, including car-

bapenems1. It has been reported that the carbapenemases commonly encountered in CRKp include variants of *K. pneumoniae* carbapenemases (KPCs) and the metallo-β-lactamases. The latter include is subdivided into New Delhi type (NDM-1), Verona integron-encoded type (VIM), and imepenemase (IMP) [6, 7]. Furthermore, more recent studies have reported the existence of other types of other carbapenemase in these bacteria, such as oxacillinase- like carbapenemase e.g. OXA-48 [8, 9]. Rapid and easy determination of carbapenemases in Enterobacteriaceae is required for effective practices and infection control measures [10]. Last-resort antibiotics are defined as the remaining antibiotics reserved for the treatment of infections caused by drug-resistant bacteria, particularly multidrug- resistant bacteria [4, 11]. With regard to the treatment of infections caused by CRKp and other carbapenem-resistant Enterobacteriacea, few last-resort antibiotics are available, including polymyxins (polymyxin-B, colistin), tigecycline, fosfomycin, aztreonam, and beta-lactam/beta-lactamase inhibitors (βLβLI) such as ceftazidime +avibactam [12–15]. The prevalence of CRKp among *K. pneumonia* isolated from patients was reported to be within the ranges of (53-100%) [8, 9, 16] and (24.6-42.5)% in Arabian and East Asian countries, respectively [17–19]. This study was undertaken to determine the prevalence, phenotypes of carbapenemases, and resistance profile to last-resort antibiotics of CRKp isolated from clinical specimens of Yemeni patients.

2. MATERIALS AND METHODS

2.1. Study Design

The design of this study was descriptive and cross-sectional. Specimen investigation, isolation of *K. pneumoniae*, and detection of CRKp were conducted in Sana'a-Yemen at the microbiology departments of Al-Thawra hospital, Al-Awalqi specialized medical laboratory, and New-Lab specialized medical laboratory. Confirmation of CRKp identity, phenotyping, and antimicrobial susceptibility tests were conducted at the microbiology department of the National Center of Public Health Laboratories (NCPHL), Sana'a-Yemen. The study was conducted between December 2020 and November 2022.

2.2. Sample size and inclusion /exclusion criteria

A total of 140 CRKp isolates out of 242 *K. pneumonia* isolates were recovered from the specimens of Yemeni patients. The isolates were detected in specimens of different types including urine, blood, vaginal swabs, cerebrospinal fluid, pus, wound swabs, and lower respiratory tract specimens (including sputum, bronchoalveolar (BAL) fluid and tracheal aspirates) and from both male and female patients who resided in rural (villages) and urban areas. Children ($<$ 18 years), relatives, and those who had traveled to any foreign country within 6 months of specimen collection were excluded. All bacterial isolates other than CRKp were excluded. All CRKp isolates were non-duplicate, *i.e.* every isolate was obtained from a different patient and a single type of the patient's specimens. Patients, specimens, and isolates that didn't match the inclusion criteria were excluded.

2.3. Microbiological Investigations

All microbiological tests employed in this study were conducted in accordance with the guidelines of the Clinical & Laboratory Standards Institute (CLSI), USA [20]. *E. coli* ATCC 25922, which was a gift from NCPHL, was also investigated as a control strain.

2.3.1. Isolation and identification of bacteria

Spread plate method was used for isolation of *K. pneumoniae* from the collected human samples. The samples were streaked on MacConkey agar plates and incubated at 37 °C for 24 hr. The bacteria growth was distinguished by its mucoid growth, appear as pink color. The obtained isolates were purified on MacConkey agar as well and sub-cultured on blood agar plates using the streaking method. Gram stain and lactose fermintation tests were then conducted. Colonies that were mucoid on blood agar, appear as Gram-negative rods under the light microscope after staining, and were lactose-fermenting mucoid colonies in MacConkey's were identified as *K. pneumoniae* [21].

2.3.2. Detection of CRKp

The disk diffusion technique (Kirby-Bauer) on Muller–Hinton agar (MHA) was used to differentiate between carbapenem-resistant and-sensitive isolates of *K. pneumoniae*. Standard carbapenem disks (Cosmos biomedical, UK) were used in this study. The strain of *K. pneumoniae* that showed inhibition zones of $\leq$ 19 mm (vs. imipenem 10 μg), $\leq$ 19 mm (vs. ertapenem 10 μg) or $\leq$ 18 mm (vs. meropenem 10 μg) was considered CRKp [20].

2.3.3. Phenotyping of CRKp

i. *Modified carbapenem inactivation method (mCIM)*

This method was used to test the ability of CRKp to produce carbapenemase (of any class) among Enterobacteracea. The concept of this test is that when carbapenem antibiotic (meropenem) is mixed and incubated with an isolate of *K.pneumonia* that produces carbapenem enzyme, meropenem is inactivated by hydrolysis. Hence, when the inactivated meropenem is then incubated with meropenem-susceptible E. coli ATCC 25922, the bacteria becomes resistant to meropenem and the inhibition zone produced becomes small (6-15 mm). According to CLSI [20], 1 μl of the *K. pneumonia* suspension (which was prepared to possess the same

optical density of 0.5 McFarland) was placed in a sterile test tube containing 2 ml of tryptic soy broth (TSB) and vortexed for 15 s. Then, a standard disk of meropenem 10 μg was added to the tube, which was incubated for 4 hr. at 37 °C. Subsequently, the suspended meropenem disk was removed and placed on MHA previously inoculated with E. coli ATCC 25922 and incubated for 24 hr. at 37 °C. The inhibition zone around the tested disk was then measured and interpreted. The presence of an inhibition zone of 6-15 mm was interpreted as carbapenemase +ve, whereas an inhibition zone of $\leq$ 19 mm indicated carbapenemase – ve. The carbapenemase intermediate was interpreted when the inhibition zone diameter was between 16 and 18 mm or when it was $\leq$ 19 mm but pinpoint colonies were observed within the zone. Intermediate results were considered non-conclusive, and the absence/presence of carbapenemase was not confirmed [20].

ii. *EDTA-modified carbapenem inactivation method (eCIM)*

This method was used only to test the isolate that showed +ve mCIM. The method was employed to test whether CRKp is a producer of metallo-β-lactamases or not. When meropenem, EDTA and carbapenemase – producing *K. pneumonia* are mixed together, EDTA inhibits metallo–β-lactamase (a type of carbapenemase enzymes) produced by *K. pneumonia*. Therefore, when meropenem is then incubated with meropenem-susceptible E. coli ATCC 25922, the antibiotics produces same inhibition zone ($\geq$20 mm) and hence the difference between inhibition zones in eCIM and mCIM is $\geq$5 mm. The test was conducted as follows: 20 μl of 0.5 M EDTA was added to a sterile tube containing 2 ml TSB. Then, 1μl of *K. pneumonia* suspension was added to the tube, which was vortexed for 15 s. Next, the same procedures described previously for the mCIM method were performed. A difference of $\geq$ 5 mm between inhibition zone diameters for eCIM and that for mCIM indicated metallo-β-lactamases +ve, whereas a difference of $\leq$ 4 mm between inhibition zones for the two tests was interpreted as metallo-β-lactamases –ve. When the results of mCIM and eCIM were both +ve, the existence of metallo-β-lactamases was confirmed, whereas when the result of mCIM was +ve and that of eCIM was - ve, this indicated the absence of metallo-β-lactamase and presence of serine carbapenemases. The results of mCIM–ve and eCIM +ve were considered invalid and therefore ignored [20].

2.3.4. Susceptibility of CRKp to last-resort antibiotics

The tests were conducted using the method of disk diffusion (Kirby-Bauer) on MHA inoculated with a bacterial suspension (which was prepared to have the same optical density of 0.5 McFarland) and incubated for 24 hr at 37 °C. Standard disks (Cosmos biomedical, UK) of ceftazidime + avibactam (30/20 μg), colistin (10 μg), aztreonam (30 µg), tigecycline (10 µg), and fosfomycin (200 μg) were used. Following incubation, inhibition zones were measured and interpreted [20].

2.4. Data Analysis

SPSS (Statistical Package for the social sciences) v28 was used to perform statistical analysis in this study. Every experiment in this study was conducted in at least three runs, and the outcomes reported in this study were averages of those results. Furthermore, comparative statistical tests, such as t-test, Chi-square, and ANOVA, were used whenever applicable with p value $<$ 0.05 indicating significant difference.

3. RESULTS AND DISCUSSION

The results revealed that 140 non-duplicates and consecutive CRKp isolates were detected out of 242 K. pneumonia isolates. This prevalence (57.9 %) was lower than that reported in Djibouti (100 %) [16] , Egypt (93 %) [8] and Saudi Arabia (53-100 %) [9], but much higher than that reported in some East-Asian countries, e.g., Pakistan (29 %) [17] and India (24.6 -42.5%)[18, 19].

These findings could be attributed to the differences in the applied infection control and prevention measures and the awareness of healthcare professionals in each country. With respect to the prevalence of CRKp isolates, as shown in Table 1 , recovered from clinical specimens of Yemeni specimens of Yemeni patients, most of these isolates (54.3%) (Table 1) were recovered from female specimens, but the difference between males and females was insignificant (p $>$0.05). This finding is probably because women are 27% more likely to receive an antibiotic prescription in their lifetime than men [21]. This might indicate that physicians tend to prescribe classical antibiotics to older patients, which leads to fewer encounters of those patients with last-resort antibiotics and hence less opportunity for the bacteria to develop resistance to the last-resort antibiotics. Nevertheless, further investigations are still required to test this hypothesis. In another respect , it was found that most clinical specimens (Table 1) from which CRKp were recovered were urine specimens (35 %), followed by lower respiratory tract clinical specimens (22.1 %) and blood (21.4 %) which was in agreement with those reported in the literature [22–24]. Concerning carbapenemase phenotypes of CRKp isolates, as shown in (table 2), it was found that 9.3 % of CRKp isolates were carbapenemase –ve while 75.7% and 15% of CRKp isolates were producers of metallo-β-lactamases (MBLs) and serine carbapenemases, respectively. This predominant prevalence of MBLs was in accordance with those reported in other countries such as Egypt (81.1 %) [8].

This predominant prevalence of MBLs was in accor-

Table 1. Distribution of CRKp isolates (n=140) on patient`s data, medical wards, and clinical specimens

Item			CRKp n (%)	p value
Patient`s Data	Gender	Males	64 (45.7 %)	0.071◘
		Females	76 (54.3%)	
	Age (years)	18–38	47 (33.6%)	0.106 ◘
		39–59	54 (38.6 %)	
		60-80	39 (27.9 %)	
	Residence	Urban	88 (62.9 %)	< 0.0001▲
		Rural	52 (37.1%)	
	Recurrence of infections (Episodes / year)	> 3	80 (57.1 %)	< 0.0001▲
		2- 3	48 (34.3 %)	
		0-1	12 (8.6 %)	
Medical wards	Outpatients		26 (18.6 %)	< 0.001▲
	Inpatients	Gynecology	54 (38.6 %)	
		Male-Medical	19 (13.6 %)	
		Surgical Recovery	12 (8.6 %)	
		Neurology	4 (2.9 %)	
		Intensive care units	25 (17.9 %)	
Clinical specimens	Urine		49 (35 %)	< 0.00001▲
	Lower respiratory tract specimens ★		31 (22.1 %)	
	Blood		30 (21.4 %)	
	Pus		18 (12.9 %)	
	wound swab		8 (5.7 %)	
	Vaginal swab		2 (1.4 %)	
	CSF		2 (1.4 %)	
Total CRKp isolates; n (%)			140 (57.9% #)	
Total *number of K. pneumonia* isolates			242	

CRKp, carbapenem-resistant *K. pneumoniae* ; ◘: insignificant difference (p > 0.05); ▲: significant difference (p < 0.05); ★: including sputum, bronchoalveolar fluid (BAL), and tracheal aspirates ; #, percentage of the total number of *K. pneumoniae* isolates ; CSF, cerebrospina fluid

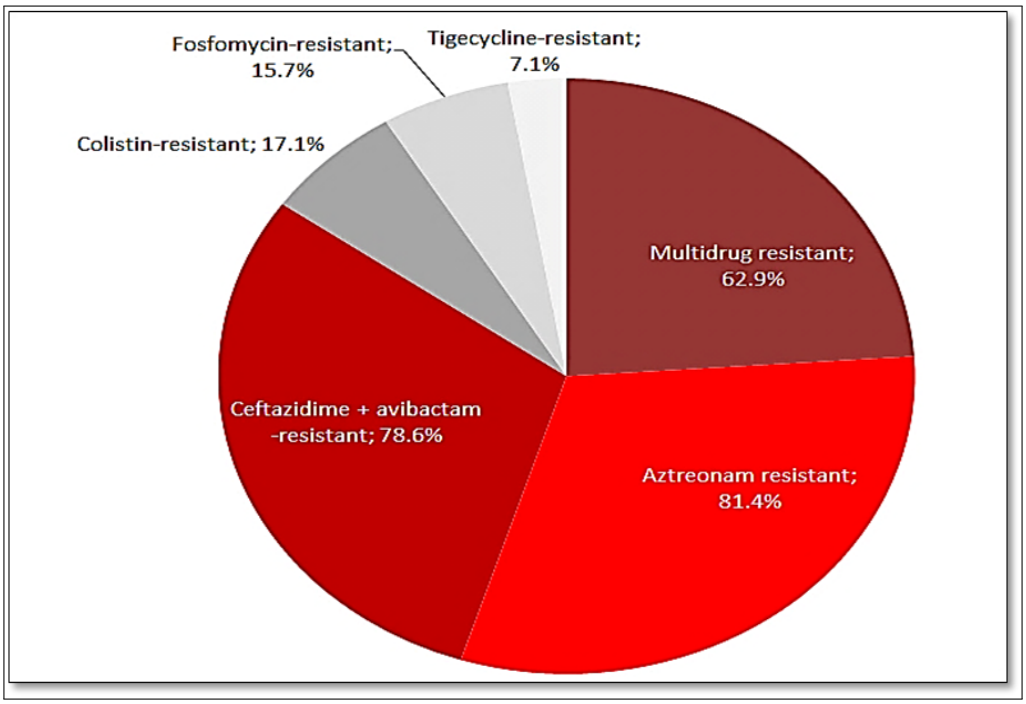

Figure 1. Normalized intensity and the phase distribution with different values of $w_{0x} = w_{0y}$.

Table 2. Carbapenemase phenotypes and resistance to last resort antibiotics of CRKp (n=140) isolates

Item	Category		n (%)	p value
Carbapenemase phenotypes	Carbapenemase - ve		13 (9.3 %)	< 0.00001▲# ; < 0.0001▲*
	Carbapenemase +ve	Metallo-β-lactamases producers	106 (75.7 %)	
		Serine carbapenemase producers	21 (15 %)	
CRKp resistance to antibiotics (n =140)	Multidrug resistant		88 (62.9 %)	< 0.001▲
	Non-multidrug resistant		52 (81.4 %)	
	Aztreonam resistant		114 (81.4 %)	0.000001▲
	Ceftazidime + avibactam resistance		110 (78.6 %)	
	Colistin-resistant		24 (17.1 %)	
	Fosfomycin-resistant		22 (15.7 %)	
	Tigecycline-resistant		10 (7.1 %)	

CRKp : carbapenem-resistant *K. pneumoniae*; ▲: significant difference ($p < 0.05$); #: between carbapenemase –ve and +ve ; *: between the types of carbapenemase +ve

dance with those reported in other countries such as Egypt (81.1 %) [8], Djibouti (100 %) [16] and India (86.3 - 87.5% [18, 19], but different from that reported in other countries e.g. Saudi Arabia MBL (13-33 % patients, most of these isolates (54.3%) (table 1) were recovered from female specimens, but the difference between males and females was insignificant ($p > 0.05$). This finding is probably because women are 27% more likely to receive an antibiotic as NDM-1) [9]. Regarding prevalence of CRKp resistance to last-resort antibiotics (Fig.1, Table 2), it was found that the prevalence (62.9 %) of multidrug resistant (MDR) was higher and significant than that of non-MDR isolates. Moreover, the prevalence of CRKp resistance to last-resort antibiotics, in descending manner, was to aztreonam, ceftazidime +avibactam, colistin, fosfomycin and tigecycline. The extreme resistance of CRKp to aztreonam (80.4%) agreed with those reported in the in Egypt 88.9% [25] and India 98.3 % [19]. The remarkable resistance of CRKp to the combination of ceftazidime and avibactam (78.6%) could be attributed to the predominance of MBL type of carbapenemases phenotypes among CRKp isolates since avibactam is ineffective against CRKp isolates that produced MBL [26]. In the contrary, the lower, but still serious, prevalence of CRKp resistance to fosfomycin (15.7%) and colistin (17.1%) was comparable with those reported in the literature to fosfomycin (18.7%) [26] and (43%)[27] and to colistin (13 % among CRKp with predominant KPC [28] , (15 % among all types of carbapenem-resistant Enterobacterales isolates) [29] and (25.8 % among all CRKp isolates of which 66% and 76.6% isolates produce NDM and OXA48, respectively) [30]. Concerning tigecycline, the prevalence of CRKp resistance in this study of to this antibiotic was the lowest (7.1 %) among other last-resort antibiotics and this was within the range reported in the literature (6.7 – 37.8 %) [26, 31, 32].

The focus, objectives and results of our study were quite different from those of a resembling study conducted in Yemen in 2019 [33]. Although our study focused exclusively on CRKp and the relevant study focused on all species of carbapenem-resistant *Enterobacteriaceae* (CRE), 140 CRKp isolates were detected in our study compared to only 18 CRKp isolates detected in the mentioned study. Moreover, in the contrary to our study, neither the total number of samples nor the prevalence of CRKp among isolates of *Klebsiella pneumoniae* was revealed in the relevant study. Furthermore, despite the resistance profile of CRKp demonstrated in our study, the relevant study showed zero resistance of CRKp isolates to some last-resort antibiotics such as colistin or aztreonam. The reason of the differences between the two studies could be probably due to larger sample size, longer period of sampling and more expanded study area employed in our study compared to

the relevant one. Nevertheless, our study as well as the relevant study is appreciated because they both highlighted on the threat of carbapenem-resistant bacteria to our community.

4. CONCLUSIONS

The results reveal a considerable prevalence of carbapenem-resistant *K. pneumoniae* (CRKp) isolated from clinical specimens of Yemeni patients, which indicates the substantial spread of infections caused by these multidrug-resistant bacteria in the Yemeni population. There is a predominance of metallo-β-lactamase type of carb- apenemases produced by CRKp in the Yemeni population, which is similar to that observed in populations of other countries. What makes this health-threat of CRKp worse in Yemen is the significant resistance of the bacteria to last-resort antibiotics, particularly to aztreonam and ceftazidime + avibactam, compared with the relatively lower, but still menacing, prevalence of resistance to colistin, fosfomycin, and tigecycline. Accordingly, there is an urgent need to establish and employ urgent control and prevention measures to diminish the spread of CRKp and limit its emergent resistance to last-resort antibiotics in our community.

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