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PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF CARBAPENEMASES AND BIOFILM FORMATION IN CLINICALLY ISOLATED CARBAPENEM-RESISTANT KLEBSIELLA PNEUMONIAE

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ABSTRACT

Background: Carbapenem resistance and biofilm formation are important characteristics of Klebsiella pneumoniae that contribute to treatment challenges and bacterial persistence.

Objective: In response to this growing threat, the present work investigated carbapenemase production among clinical K. pneumoniae isolates and evaluated its potential relationship with biofilm formation.

Methodology: Twenty clinical K. pneumoniae isolates were evaluated for antimicrobial susceptibility using the disc diffusion technique. Carbapenemase and metallo-β-lactamase production were phenotypically evaluated using mCIM and eCIM, respectively. Biofilm production was quantitatively assessed using the crystal violet microtiter plate assay. PCR was performed to detect selected carbapenemase-associated genes (bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}) and biofilm- related genes (fimH, mrkD, and sugE).

Results: All isolates exhibited multidrug-resistant CRKP profiles. Carbapenemase activity was demonstrated in 85% of isolates, while MBL phenotype was observed in 40%. Biofilm production was detected in 70% of isolates, with weak biofilm formation being the predominant phenotype. The bla_{NDM}, bla_{OXA-48}, bla_{VIM}, and bla_{KPC} genes were detected in 70%, 50%, 30%, and 25% of isolates, respectively, whereas bla_{IMP} was not detected. Although biofilm formation was prevalent, no statistically significant association was identified between biofilm phenotype and carbapenemase production, carbapenemase type, or individual carbapenemase genes.

Conclusion: The findings demonstrate a high burden of carbapenemase-associated resistance among the investigated CRKP isolates. Despite the absence of statistically significant associations, the observed variations in biofilm phenotype across different carbapenemase types and genes suggest the need for further investigation into the multifaceted processes that control biofilm development in CRKP isolates.

KEYWORDS: Klebsiella pneumoniae, Carbapenem resistance, Biofilm.

INTRODUCTION

Klebsiella pneumoniae is a crucial Gram-negative bacterium responsible for a varied array of infections in both healthcare facilities and community environments [1]. Over time, this pathogen has acquired resistance to carbapenem antibiotics through several mechanisms. The most common mechanism involves carbapenemase production, often accompanied by additional resistance strategies for instance decreased outer membrane penetrability and increased efflux activity [2]. In K. pneumoniae, the major carbapenemases are classified into serine carbapenemases (encoded by blaKPC), metallo-β- lactamases (MBLs) (encoded by blaNDM, blaVIM, and blaIMP), and oxacillinases, particularly OXA-48-like enzymes (encoded by blaOXA−48) [3,4]. The increasing dissemination of carbapenemase producing strains has become a significant clinical and public health challenge [5], particularly because carbapenems are commonly reserved for the management of severe infections caused by resistant Gram-negative bacteria [6].

K. pneumoniae possesses several virulence determinants that participate in its pathogenicity, among which biofilm formation is considered one of the most important. Biofilms are highly organized communities of microbes embedded within a self-generated complex matrix constituted primarily of proteins, polysaccharides, and extracellular DNA [7,8]. The biofilm structure provides protection to bacterial cells, enhancing their tolerance to antimicrobial agents and reducing their susceptibility to host immunity [9]. Consequently, infections combined with biofilms are difficult to eliminate. Furthermore, the capacity of K. pneumoniae to establish biofilms on medical equipment, including urinary catheters and endotracheal tubes, considerably enhances the risk of healthcare-associated infections in patients requiring these equipment [10].

Biofilm-associated growth can favor bacterial survival during infection and may be accompanied by increased tolerance to antimicrobial treatment. At the same time, carbapenemase production provides an important mechanism of resistance to critically important β-lactam antibiotics. The co-existence of these two characteristics may therefore contribute to the persistence and multidrug-resistant phenotype of K. pneumoniae isolates [11]. Accordingly, the present investigation was designed to determine the frequency of carbapenemase-producing K. pneumoniae among clinical isolates. In addition, the study evaluated biofilm-forming capacity and examined whether biofilm formation was associated with carbapenemase production, carbapenemase type, or individual carbapenemase genes among the recovered isolates.

METHODOLOGY

Bacterial isolates

A total of twenty clinical K. pneumoniae specimens were isolated in this study from different clinical sources, including blood (n = 7), wound swap (n = 5), sputum (n = 4), urine (n = 2), and pus (n = 2). The isolates were collected between April 2023 and August 2023 from Mansoura University Hospitals, Egypt. Isolates were purified by streaking on MacConkey agar (Oxoid, UK) medium to obtain pure bacterial colonies. The purified colonies were then subjected to microscopic examination and further identification was performed using conventional IMViC (indole, methyl red, Voges-Proskauer, and citrate utilization) tests [12].

Antimicrobial susceptibility testing (AST)

The Kirby-Bauer disc diffusion method was adopted to assess the antimicrobial susceptibility of all the purified K. pneumoniae isolates as documented by the Clinical Laboratory Standard Institute (CLSI 2021) [13]. The surface of sterile Mueller-Hinton agar (MHA) plates were inoculated with a pure bacterial culture of each isolate matched to 0.5 McFarland standard then 12 antimicrobial agents (Himedia, India) from different classes were applied including: ampicillin (AMP, 10 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), piperacillin-tazobactam (PIT, 100/10 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CTR, 30 µg), ceftazidime (CAZ, 30 µg), cefepime (CPM, 30 µg), aztreonam (AT, 30 µg), imipenem (IPM, 10 µg), meropenem(MRP, 10 µg), ertapenem (ETP, 10 µg), and amikacin (AK, 30 µg). All plates were incubated aerobically overnight at 37°C. The inhibition zone diameter around each disc was measured and interpreted based on CLSI breakpoints. Furthermore, isolates were considered as multi-drug resistant (MDR) when showed resistance to three or more antibiotic classes based on CLSI interpretive criteria [13].

Phenotypic detection of carbapenemases and metallo-β- lactamases

Carbapenemases and MBLs activity were investigated using the modified carbapenem inactivation method (mCIM) and EDTA-modified carbapenem inactivation method (eCIM), respectively, following the CLSI recommendations [13]. Concisely, a meropenem disc (10 µg) was immersed into a 2 mL suspension of each isolate in tryptic soy broth (TSB). Another meropenem disc (10 µg) was immersed into a 2 mL suspension of each isolate in TSB with added EDTA at 5 mM final concentration. The tubes were incubated at 35°C for 4 h. After incubation, the discs were removed and placed onto MHA plates that were recently inoculated with a 0.5 McFarland suspension of a carbapenem-susceptible E. coli ATCC 29522 indicator strain and incubated at 37 °C for 24 h. Following incubation, the inhibition zones around the discs were determined in accordance with the standard disc diffusion method. For mCIM positive isolates, an increase in zone diameter for eCIM vs zone diameter for mCIM by ≥5 mm was considered as metallo-β-lactamase positive. An increase in zone diameter for eCIM vs zone diameter for mCIM by ≤ 4 mm was considered as serine carbapenemase producer.

Biofilm assay

Biofilm production was quantified employing the crystal violet plate method [14,15]. Overnight cultures in TSB were diluted to the 0.5 McFarland standard (10^8 CFU mL^{-1}). Then the microtiter plate wells were seeded in triplicate with 200 µL of each diluted bacterial isolate. A volume of 200 µL of TSB only was distributed in three wells as a sterility control. The plates were incubated overnight at 37°C. After incubation, the cultures were removed gently, and the wells were washed three times using 250 µL phosphate buffered saline (10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.0) to remove non-adherent bacterial cells. Methanol (250 µL) was added to each well for 15 min as a biofilm fixative. The fixed biofilm was stained with 250 µL of 1% w/v crystal violet (CV) for 20 min. The excess CV was discarded and the plates were washed three times with distilled water and were air dried. Subsequently, 250 µL of 33% v/v glacial acetic acid was added to resolubilize the bound dye. The optical density was measured at 490 nm using a microplate reader (Bio Tek instruments, USA). For each isolate, the mean optical density of the three replicate wells was calculated (OD). The isolates were categorized into four groups as follows: OD ≤ ODc, nonadherent; ODc < OD ≤ 2 ODc, weakly adherent; 2 ODc < OD ≤ 4 ODc, moderately adherent; and 4 ODc < OD, strongly adherent, where ODc (cut-off OD) was defined as three standard deviations above the mean OD of the negative control.

Molecular detection of some carbapenemase and biofilm-encoding genes

Genomic DNA was prepared using a rapid colony PCR extraction procedure [16]. A pure colony was suspended in 100 µL sterile water, heated at 95°C for 10 min, and centrifuged at 10,000 rpm. The supernatant was stored at −20°C until use.

PCR was utilized for detection of five carbapenemase-encoding genes (bla_{OXA-48}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{KPC}) and three biofilm-related genes (fimH, mrkD, and sugE). Each 25 µL reaction mixture consisted of 12.5 µL Cosmo PCR red Master Mix (2x), 5 µL of bacterial DNA, 1 µL of each primer (10µM), and 5.5 µL nuclease-free water. A reaction without DNA template was used as a negative control. Amplification started with denaturation at 95 °C for 2 min, followed by 35 cycles each consisting of three steps: denaturation at 95 °C for 30 sec, annealing at temperatures specified for each primer pair in **Table 1** for 30 sec, and extension at 72 °C for 30 sec, with a final extension step at 72 °C for 5 min[17]. Amplicons were resolved by running on 1.5% (w/v) agarose gels with100 bp DNA Ladder (Willowfort, UK).

Table 1: List of oligonucleotide primers employed in molecular examination.

Genes	Gene name	Type	Nucleotide sequence (5` to 3`)	Amplicon size (bp)	Annealing Temp	References
Carbapenemase encoding genes	bla_{OXA-48}	Fw Rv	GCTTGATCGCCCTCGATT GATTTGCTCCGTGGCCGAAA	281	57	[18]
	bla_{NDM}	Fw Rv	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	621	52	[19]
	bla_{IMP}	Fw Rv	TTGACACTCCATTTACDG[b] GATYGAGAATTAAGCCACYCT[b]	139	55	[18]
	bla_{VIM}	Fw Rv	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	55	[20]
	bla_{KPC}	Fw Rv	CATTCAAGGGCTTTCTTGCTGC ACGACGGCATAGTCATTTGC	538	55	[18]
Biofilm-related genes	fimH	Fw Rv	CGGAAACGATCACCGACTAC GGGTAAGAGGTGCCGTTATAT T	108	56	Designed by primer quest from integrated DNA technologies
	mrkD	Fw Rv	AAGCTATCGCTG TAC TTC CGGCA GCGTTGGCGCTCAGATAG G	339	60	
	sugE	Fw Rv	ACC GGT ATT GTG CTG TTA GG TTAGTGCGCGCTGATCTT	102	56	

Fw: Forward, **Rv**: Reverse, **bp**: Base pair, [b]D=A or G or T; Y=T or C.

Data analysis

Statistical analyses were conducted using GraphPad Prism version 8.4.2 for Windows. Associations between biofilm-forming ability and carbapenemase production, carbapenemase type, and individual carbapenemase genes were examined using Fisher's exact test. Statistical significance was defined as a P value < 0.05.

Hierarchical clustering analysis was performed to assess the similarity among the 20 collected isolates based on their combined phenotypic and genotypic profiles. Binary data were coded as 1 for presence/positive and 0 for absence/negative. The clustering analysis included carbapenemase production (mCIM/ eCIM), biofilm formation, and the presence of bla_{NDM}, bla_{OXA-48}, bla_{VIM}, and bla_{KPC} genes. Variables showing identical values across all isolates including, the AST profile, bla_{IMP}, fimH, mrkD, and sugE, were excluded from the clustering analysis, as they provided no discriminatory information. Similarity was calculated using the Jaccard similarity coefficient, and dendrogram clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method.

RESULTS

Bacterial isolates collection and identification

Twenty clinical isolates of K. pneumoniae were included in this study. The isolates exhibited typical colony morphology on MacConkey agar characterized by large, circular, smooth, convex, mucoid, pink colonies due to lactose fermentation. The isolates showed negative reactions for both the indole and methyl red tests, in contrast, positive reactions were observed for the Voges–Proskauer and Simmons citrate utilization tests. The obtained cultural, microscopic, and biochemical characteristics were consistent with the standard identification criteria of K. pneumoniae.

Antimicrobial sensitivity pattern

The susceptibility profile of the 20 collected isolates was determined against 12 different antimicrobial agents. Based on the interpretive standards established by the CLSI, all isolates were resistant to all the tested antibiotics and were

therefore categorized as MDR. Additionally, all isolates demonstrated concurrent resistance to the three carbapenems evaluated, confirming their classification as carbapenem-resistant K. pneumoniae (CRKP).

Phenotypic detection of carbapenemases and metallo-β- lactamases

In the present study, out of 20 CRKP isolates, 17 (85%) isolates were confirmed to be carbapenemase producers based on the mCIM results, whereas 3 (15%) isolates were non-carbapenemase producers **(Figure 1a)**. Among the 17 carbapenemase-producing isolates, 8 (40% of total isolates; 47.1% of carbapenemase producers) demonstrated MBLs activity as determined by eCIM **(Figure 1b)**, while 9 (45% of total isolates; 52.9% of carbapenemase producers) exhibited serine carbapenemase activity **(Figure 1c).**

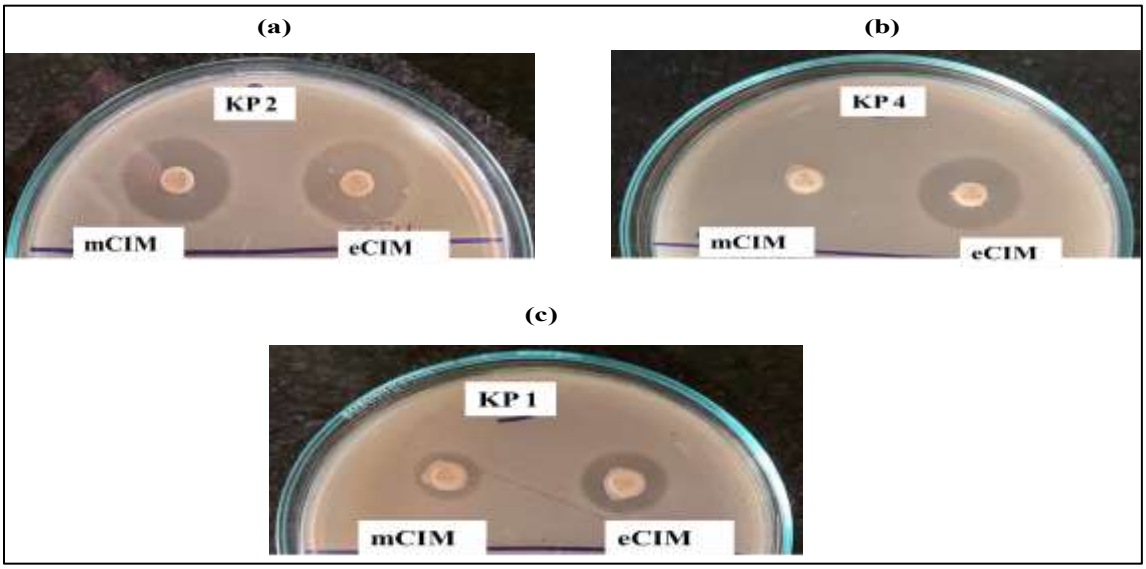

Figure 1: Phenotypic detection of carbapenemase production by modified carbapenem inactivation method (mCIM) and EDTA-modified carbapenem inactivation method (eCIM) in representative CRKP isolates.

a: non- carbapenemase producer, b: metallo-β- lactamase producer, c: serine carbapenemase producer, KP: K. pneumoniae.

Quantitative detection of biofilm formation

Overall, 14 of the 20 isolates (70%) were identified as biofilm producers, whereas 6 (30%) isolates were classified as non-biofilm producers, irrespective of their clinical source. Out of the 14 CRKP isolates which were biofilm producers, 10 (50%) isolates exhibited weak biofilm production, 3 (15%) isolates were moderate producers, and 1 (5%) isolate was a strong producer **(Figure 2)**.

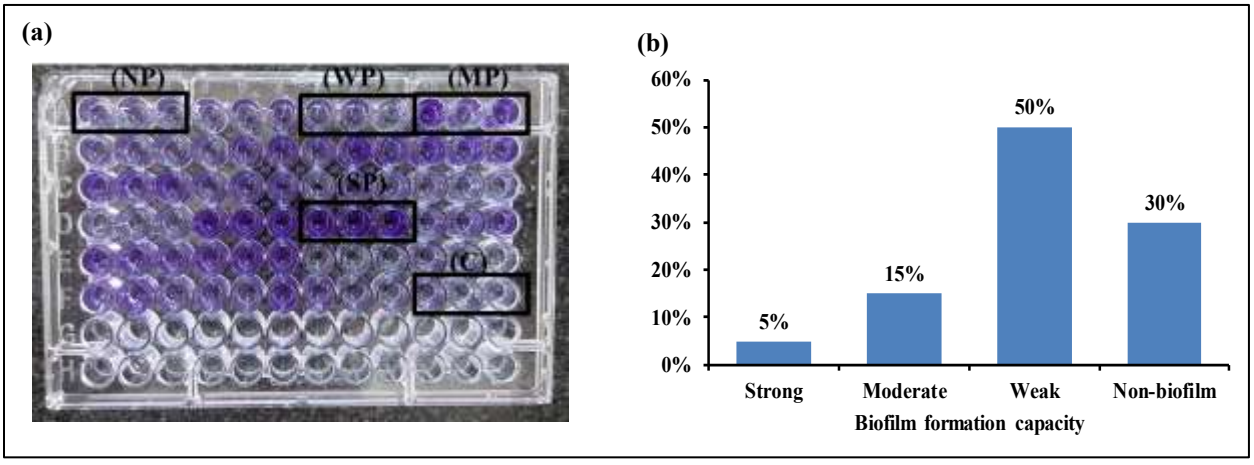

Figure 2: Biofilm formation among CRKP isolates.

a: representative crystal violet-stained 96-well microtiter plate showing different levels of biofilm formation, b: percentage distribution of isolates according to biofilm-forming capacity. SP: strong producer, MP: moderate producer, WP: weak producer, NP: non- producer, C: negative control.

Association between biofilm formation and carbapenemase production and its type

The biofilm formation assessed among the 20 CRKP isolates was categorized according to carbapenemase type. Among the 9 serine carbapenemase-producing isolates, 3 (33.3%) showed moderate biofilm formation, 4 (44.4%) were weak biofilm producers, and 2 (22.2%) were biofilm non-producers. Regarding the 8 MBLs- producing isolates, 1 (12.5%) demonstrated strong biofilm formation, 5 (62.5%) showed weak formation, and 2 (25%) lacked biofilm **Table** 2).

Fisher's exact test showed a statistically significant association ($p = 0.0\%$) between carbapenemase production and biofilm formation ($P < 0.05$). Of the 3 non-carbapenemase-producing isolates, 1 (33.3%) showed weak biofilm formation, while 2 (66.7%) were non- biofilm producers **(** ($P > 0.05$).

Table 2: Biofilm formation capacity among CRKP isolates according to carbapenemase type.

Carbapenemase type	Biofilm formation category			Total
	Strong/moderate (n, %)	Weak (n, %)	Non-biofilm (n, %)	
Serine Carbapenemase	3 (33.3%)	4 (44.4%)	2 (22.2%)	9
Metallo β-lactamase	1 (12.5%)	5 (62.5%)	2 (25%)	8
Non-producer	0 (0%)	1 (33.3%)	2 (66.7%)	3
Total	4	10	6	20

Molecular detection of carbapenemases and biofilm-associated genes

PCR screening showed that bla_{NDM} **(Figure 3a)** and bla_{OXA-48} **(Figure 3b)** were harbored by 14 (70%) and 10 (50%) of the isolates, respectively. The bla_{VIM} **(Figure 3c)** and bla_{KPC} **(Figure 3d)**genes were detected in lower percentage among the tested isolates [6 (30%) and 5 (25%) isolates, respectively]. bla_{IMP} gene was not detected **(Table 3)**.

Co-carriage of carbapenemase genes was also observed. Five (25%) isolates simultaneously harbored bla_{NDM} and bla_{OXA-48}, while bla_{NDM} and bla_{VIM} as well as bla_{OXA-48} and bla_{VIM} co-occurred in one (5%) isolate. Co-existence of bla_{NDM}, bla_{VIM}, and bla_{KPC} was detected in 3 (15%) isolates, whereas bla_{NDM}, bla_{OXA-48}, and bla_{VIM} co-existed in only one (5%) isolate.

All the 20 CRKP isolates harbored the three investigated biofilm-related genes fimH, mrkD, and sugE **(Figure 3e-g respectively, Table 3).**

Table 3: Detection of biofilm and carbapenemase related genes in CRKP isolates.

No. of isolates (%)	Code of isolates	Biofilm related genes			Carbapenemase encoding genes
		fimH	mrkD	sugE	
5 (25%)	KP1, KP6, KP8, KP11, KP14	+	+	+	$bla_{NDM} + bla_{OXA-48}$
4 (20%)	KP4, KP15, KP18, KP20	+	+	+	bla_{NDM}
3 (15%)	KP5, KP7, KP10	+	+	+	bla_{OXA-48}
3 (15%)	KP13, KP17, KP19	+	+	+	$bla_{NDM} + bla_{VIM} + bla_{KPC}$
2 (10%)	KP2, KP3	+	+	+	bla_{KPC}
1 (5%)	KP9	+	+	+	$bla_{NDM} + bla_{VIM}$
1 (5%)	KP12	+	+	+	$bla_{OXA-48} + bla_{VIM}$
1 (5%)	KP16	+	+	+	$bla_{NDM} + bla_{OXA-48} + bla_{VIM}$

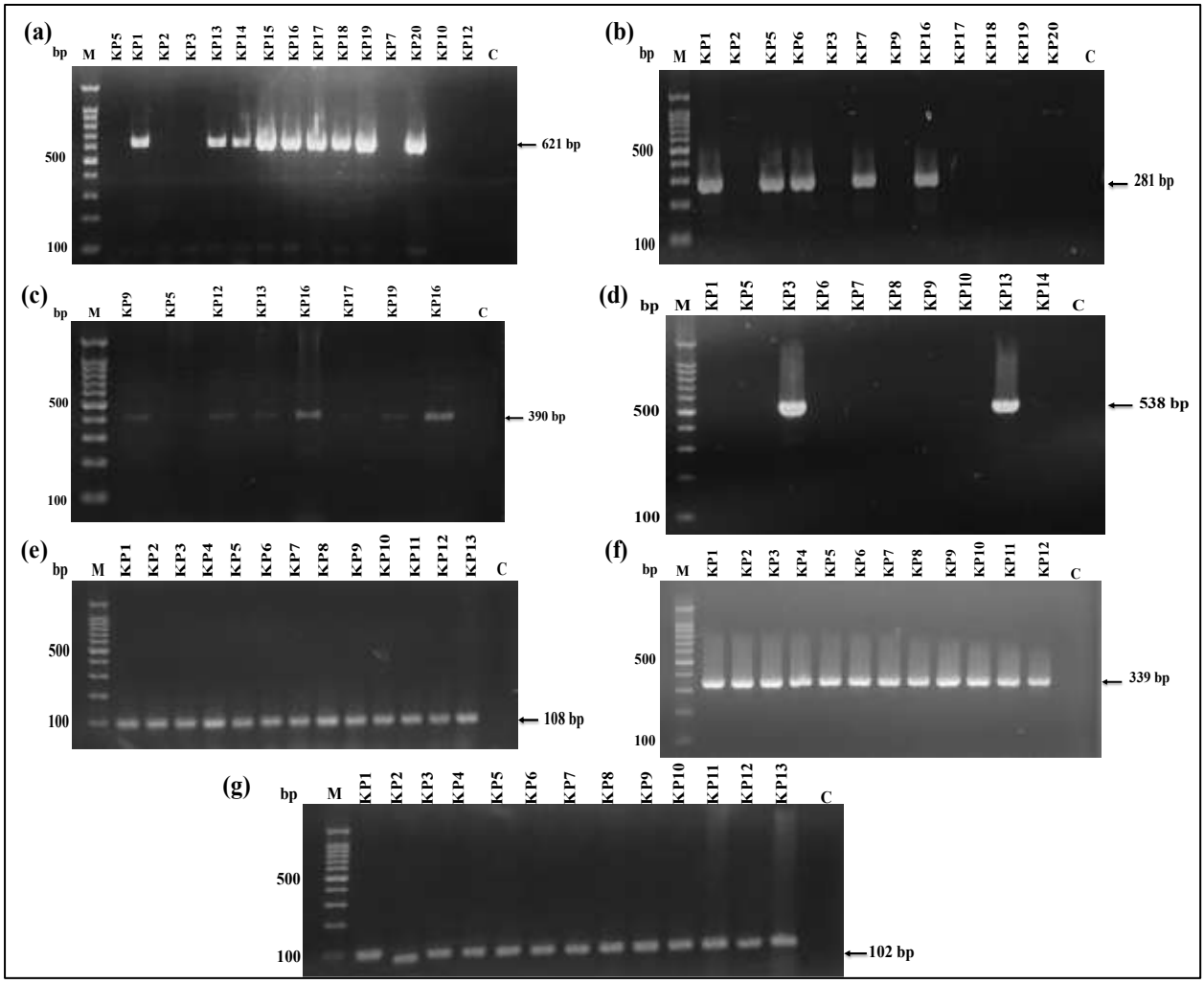

Figure 3: Agarose gel electrophoresis of PCR amplification of carbapenemases and biofilm-associated genes.

a: bla_{NDM} gene, b: bla_{OXA-48} gene, c: bla_{VIM} gene, d: bla_{KPC} gene, e: fimH gene, f: mrkD gene, g: sugE gene. M: 100 bp DNA ladder, Lane C: negative control, bp: base pair, KP: K. pneumoniae.

Association between carbapenemase genes and biofilm formation

The relationship between the presence of individual carbapenemase genes and the biofilm phenotype was examined using Fisher's exact test (**Table 4**). bla_{IMP} was not included in the association as it was not detected in any isolate. Biofilm production was detected among isolates carrying each of the investigated carbapenemase genes, however, none of individual carbapenemase gene showed a statistically significant association with biofilm formation ($P > 0.05$ for all comparisons). Thus, the presence of a specific carbapenemase gene alone was not significantly related to the biofilm-forming phenotype among the studied CRKP isolates.

Table 4: Association between carbapenemase genes and biofilm formation among CRKP isolates.

Gene		Biofilm producer (n, %)	Biofilm non-producer (n, %)	P value
bla_{NDM}	Positive	11 (78.6%)	3 (21.4%)	0.3027
	Negative	3 (50%)	3 (50%)	
bla_{OXA-48}	Positive	9 (90%)	1 (10%)	0.1409
	Negative	5 (50%)	5 (50%)	
bla_{VIM}	Positive	4 (66.7%)	2 (33.3%)	0.9999
	Negative	10 (71.4%)	4 (28.6%)	
bla_{KPC}	Positive	2 (40%)	3 (60%)	0.1313
	Negative	12 (80%)	3 (20%)	

Hierarchical clustering analysis

UPGMA hierarchical clustering based on the Jaccard similarity coefficient was performed to assess the relatedness among the 20 CRKP isolates according to their binary phenotypic and genotypic profiles. At an 80% similarity cutoff, the isolates were grouped into eight clusters (A to H) **(Figure 4)**. The largest cluster (A) comprised eight isolates (KP1, KP6, KP11, KP8, KP14, KP16, KP7, and KP10), within which three groups showed 100% similarity, KP1/KP6/KP11, KP8/KP14, and KP7/KP10. Another related cluster (B) included four isolates (KP4, KP15, KP18, and KP20), among which KP4/KP15/KP18 exhibited 100% similarity. KP17, and KP19 clustered together with a high degree of similarity (>90%), whereas KP2 and KP3 exhibited identical profiles, showing 100% similarity. The remaining isolates (KP5, KP9, KP12, and KP13) each formed an individual cluster.

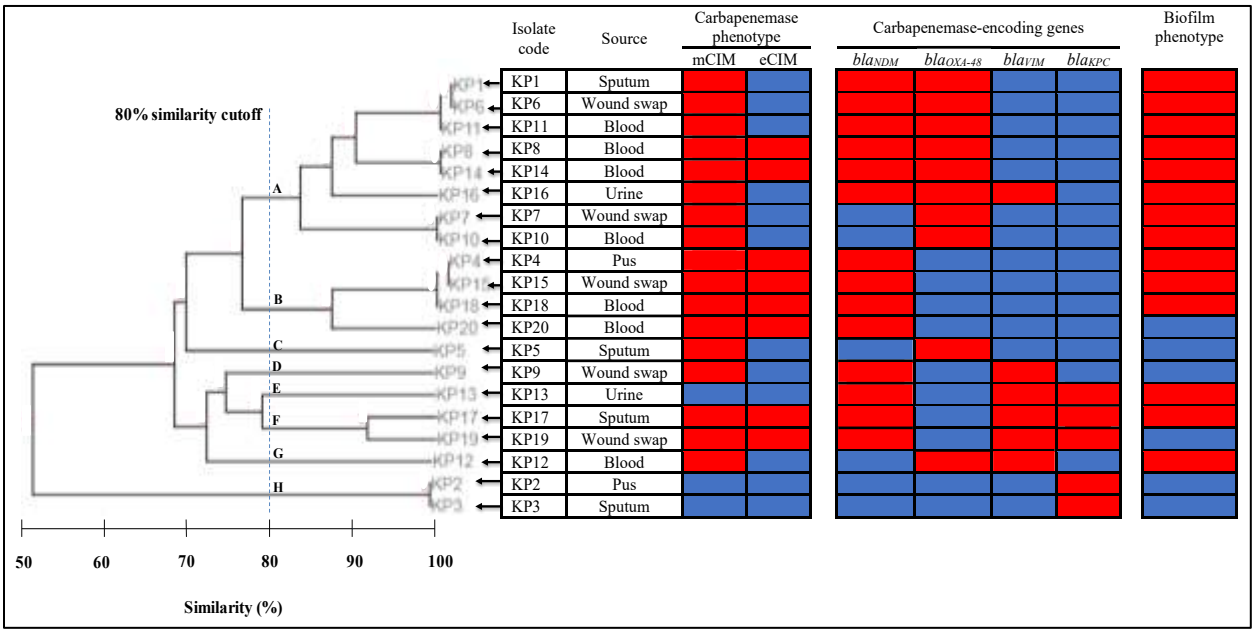

Figure 4: UPGMA hierarchical clustering dendrogram of the 20 isolates based on Jaccard similarity of binary phenotypic and genotypic profiles, including carbapenemase production (mCIM/ eCIM).

carbapenemase-encoding genes (bla_{NDM}, bla_{OXA-48}, bla_{VIM}, and bla_{KPC}) genes, and biofilm phenotype. A presence/absence table supported the Jaccard similarity index estimation and UPGMA clustering analysis. Red and blue colors in the table refer to phenotypic/gene presence and absence, respectively. mCIM: modified carbapenem inactivation method, eCIM: EDTA-modified carbapenem inactivation method.

DISCUSSION

Recently, the prevalence of CRKP has escalated substantially in healthcare settings worldwide. A number of twenty K. pneumoniae isolates were analyzed in the current research, with blood samples representing the most common source of isolation. Previous studies conducted in Egypt reported similar findings [21,22] as well as in Italy [23] where bloodstream specimens were the primary source of K. pneumoniae isolates.

The antimicrobial susceptibility testing demonstrated that all isolates exhibited resistance to the three carbapenem agents examined in this study (imipenem, meropenem, and ertapenem). Based on their resistance patterns, all isolates were classified as MDR-CRKP. El-kholy and coauthors also observed similar results [22].

Phenotypic testing using mCIM/eCIM identified carbapenemase activity in 85% of the isolates, with 40% carrying MBL phenotypes. The proportion observed in the present study is broadly consistent with the findings reported by Ramadan and coauthors as well as Abou-Dobara and colleagues, who documented similar percentages among CRKP isolates [24,25]. Interestingly, mCIM/eCIM assay did not demonstrate carbapenemase activity in three isolates. Two of these isolates carried blaKPC gene, whereas the third simultaneously harbored blaNDM, blaVIM, and blaKPC. The absence of detectable carbapenemase activity despite the presence of carbapenemase genes could reflect limited gene expression or genetic alterations which could result in the production of nonfunctional enzymes [22].

Furthermore, the most frequently reported carbapenemase-encoding genes in K. pneumoniae were investigated among the CRKP isolates. Molecular screening showed that all CRKP isolates possessed at least one of the investigated carbapenemase-associated genes. This finding is consistent with previous studies conducted in Egypt, which also detected carbapenemase genes in all investigated isolates [22,26,27]. In contrast, blaIMP was not detected in any isolate

in the present collection, supporting previous Egyptian studies observations that this gene occurs at a relatively low frequency among CRKP isolates [22,24,27].

Among the carbapenemase genes examined, blaNDM showed the highest detection rate (70%) afterward blaOXA-48, which was detected in 50% of isolates. The high prevalence of blaNDM observed in our isolates is in agreement with findings from several Egyptian studies published in recent years [22,27,28]. Nevertheless, other studies conducted in Egypt have identified blaOXA-48 as the prevalent carbapenemase determinant among CRKP isolates [29,30]. The predominance of blaNDM in the current study may be related to its efficient horizontal dissemination. This gene is frequently associated with mobile conjugative plasmids, which can facilitate its transfer both among strains of the same bacterial species and across different bacterial species [28].

It is worth noting that blaNDM and blaOXA-48 co-occurred in 25% of the isolates, while blaNDM and blaVIM were co-harbored by 5% of isolates as well as blaOXA-48 and blaVIM. Furthermore, 20% of isolates carried more than two carbapenemase-associated genes at the same time. Similar co-occurrence patterns have been documented previously [22,28]. The presence of multiple carbapenemase determinants within a single isolate can lead to a broader spectrum of β-lactam hydrolytic activity, thereby conferring greater resistance to antimicrobial therapy and making effective antibiotic treatment more difficult [28].

Biofilm formation is recognized as a key virulence determinant that promotes CRKP pathogenicity and persistence [31]. In the current research, 70% of isolates demonstrated the ability to produce biofilms, with weak biofilm formation representing the predominant phenotype. Similar observations have been recently reported by Sabença and coauthors as well as El Naggar and coauthors [28,32]. In contrast, Khodadadian et al. documented a considerably higher biofilm production rate, reporting that 91.2% of the investigated isolates were biofilm producers33. The high prevalence of biofilm-forming isolates observed in the present and previous studies emphasizes the potential contribution of biofilm formation to CRKP persistence and colonization.

Moreover, biofilm-related genes were examined in the collected isolates. FimH, mrkD and sugE genes were harbored by all isolates. These results are in agreement with many studies [22,34]. Type 1 and type 3 fimbrial structures, together with the bacterial capsule, are important virulence factors that facilitate bacterial adhesion, colonization, and biofilm development especially during urinary tract infections. Because of their important contribution to biofilm development and bacterial pathogenicity, fimbriae may serve as promising targets for controlling infections caused by CRKP [34].

Although the universal detection of biofilm-related genes (fimH, mrkD, and sugE), 30% of the isolates did not exhibit a biofilm-producing phenotype. Comparable findings have been reported by Naga (2021) and Shamkhali et al. (2025), who detected the fimH and mrkD genes in all isolates despite observing that some isolates did not exhibit a biofilm-producing phenotype [34,35]. The presence of these genes therefore does not necessarily indicate their functional expression or sufficient activity to produce a detectable biofilm phenotype. Phenotypic biofilm production can be affected by regulatory mechanisms, environmental conditions, and other genetic determinants, which may explain the difference between genotypic carriage and the observed phenotype [34,36].

The relationship between biofilm-forming capacity and carbapenemase production was further investigated. Carbapenemase-producing isolates exhibited a higher tendency toward biofilm formation compared with non-producers. This observation may be explained by the co-existence of resistance and virulence determinants on transferable genetic elements, which can enhance bacterial adaptation and persistence. However, no statistically significant association was detected, indicating that biofilm formation is not uniquely dependent on carbapenemase production and may instead be influenced by multiple virulence determinants, regulatory pathways, and environmental factors. These results differ from those reported by El Naggar and coauthors who identified a statistically significant association between biofilm formation and carbapenemase production in CRKP isolates [28].

When biofilm formation was analyzed according to carbapenemase type, differences in the distribution of biofilm phenotypes were observed. Weak biofilm formation was more common among both serine-carbapenemase and MBLs producers. Moderate biofilm formation was only detected among serine-carbapenemase producers, whereas strong biofilm phenotype was limited to MBLs producers. Nevertheless, these differences failed to achieve statistical significance. Therefore, the observed variations should be interpreted cautiously and cannot be considered evidence of a true association between carbapenemase type and biofilm-forming capacity. Similar heterogeneity in biofilm-forming capacity among different carbapenemase genotypes has been reported previously [28].

Moreover, no significant relationship was identified between the presence of individual carbapenemase-encoding genes and biofilm formation among the studied CRKP isolates. Although biofilm production was detected among isolates harboring these carbapenemase genes, no significant relatedness between them was observed. Notably, isolates simultaneously carrying blaNDM and blaVIM or blaNDM, blaOXA-48, and blaVIM were biofilm non-producers. In contrast, weak biofilm formation was the most frequent phenotype among isolates carrying both blaNDM and blaOXA-48 (60%), followed by moderate and strong phenotype (20% each). On the other hand, El Naggar and coauthors (2024) reported that isolates co- harboring blaNDM and blaKPC were biofilm non-producers and most isolates co-harboring blaNDM and blaOXA-48 demonstrated a moderate biofilm-forming phenotype [28].

The absence of significant associations in the present study may partly reflect the limited number of isolates included. Moreover, biofilm formation is a complicated multifactorial procedure controlled by numerous genes and

environmental conditions that may act independently of carbapenemase production or presence of carbapenemase genes. Consequently, the existence of carbapenemase genes or the type of carbapenemases may not necessarily predict the biofilm phenotype of CRKP isolates [36].

The clustering analysis demonstrated heterogeneity among the CRKP isolates despite their uniformly resistant phenotype to the tested antibiotics. A similar approach was previously used for K. pneumoniae isolates, where phenotypic and genotypic characteristics were converted into binary presence/absence data and analyzed using the Jaccard similarity index and UPGMA clustering [37]. The close clustering of isolates sharing similar carbapenemase, biofilm, and carbapenemase gene profiles suggests that these isolates had comparable phenotypic and genotypic characteristics. In contrast, the separation of other isolates reflects differences in the distribution of the investigated determinants. These findings highlight a common antimicrobial resistance phenotype may coexist with variations in the investigated phenotypic and genotypic characteristics.

Interestingly, several CRKP isolates displayed 100% similarity despite being recovered from different clinical sources. This high level of similarity suggests that isolates obtained from distinct infection sites may share highly comparable phenotypic and genotypic characteristics. Such a pattern could reflect the persistence or dissemination of closely related CRKP strains among patients and clinical settings. Notably, KP8 and KP14, both recovered from blood samples, showed 100% similarity, suggesting particularly strong relatedness between these two isolates. Nevertheless, identical clustering based on the assessed phenotypic and genotypic markers does not necessarily confirm clonality. Additional high-resolution molecular approaches would therefore be required to establish the genetic relatedness and possible epidemiological linkage among these isolates [38,39].

CONCLUSION

In summary, the present findings demonstrate a high prevalence of carbapenem resistance and carbapenemase associated determinants among the studied CRKP isolates, highlighting their clinical and public health significance. Although biofilm formation was common, no statistically significant relationship was demonstrated between biofilm-forming capacity and carbapenemase production, carbapenemase type or the presence of carbapenemase genes. Nevertheless, the observed variations in biofilm phenotype across different carbapenemase types and genes suggest the need for further investigation into the multifaceted processes that control biofilm development in CRKP isolates. Further studies involving larger collections of isolates are warranted to clarify these relationships.

Ethical considerations

This research work was approved by the Research Ethics Committee of Faculty of Pharmacy, Mansoura University and certified under the code number (2026 – 95).

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Conflict of interest

All authors declare that there are no conflicts of interest.

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