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Original article

Carbapenem resistant *Klebsiella pneumoniae* in COVID-19 patients admitted in intensive care units of Zagazig University Hospitals

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ABSTRACT

Background: Continuous monitoring is crucial to detect infection by Carbapenemase-producing *Klebsiella pneumoniae* (CPKP) in intensive care COVID-19 patients. **Aim:** to evaluate the CR-KP colonization and co- infection and evaluate the antibiotic resistance pattern. **Methods:** A cross -sectional study was done on COVID-19 patients with CPKP infection in Zagazig University Hospitals where rectal swab samples were detected using XpertCarba-R Assay(Cepheid, Sunnyvale), while isolates from sputum, urine, and blood culture were identified phenotypically by VITEK® 2 (bioMérieux SA) and modified Hodge test while genotypically by XpertCarba-R Assay(Cepheid, Sunnyvale). **Results:** CPKP was found in 60 patients out of 140 patients (42.8%), with 34 CPKP identified from rectal swabs, 12 patients infected from diverse locations (blood, respiratory secretions, and urine), and 14 COVID-19 patients developing CPKP co-infection with colonization. A widespread resistance to all antibiotic classes was found on the antibiograms of CPKP isolates and the blaNDM was the most common genotype detected by preliminary PCR testing. **Conclusion:** Regarding CPKP infection, a continues focus should be taken when dealing with it, particularly among COVID-19 patients. The incidence of multi-drug resistance due to nosocomial infection can be decreased by the rigorous monitoring of colonized patients and expedite the infection control practices.

Introduction

SARS-CoV-2, also known as COVID-19 and 2019-nCoV was discovered in Wuhan, Hubei Province, China in December 2019, causing acute respiratory syndrome. The coronavirus has eventually spread worldwide, killing many humans. As a result of its sequelae, intensive care together with mechanical ventilation were required for some severely ill patients [1].

In Egypt, COVID-19 cases were detected at the beginning of 2020. Moreover, the cases have tremendously increased to reach more than 100,000 at the end of that year [2]. In order to contain the epidemic, Egyptian authorities took rigorous actions to limit the cases and control the problem. These were social distancing and management of cases at home.

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Viruses, bacteria and fungi cause hospital acquired infection via instrument contaminations or human carriage, during the patient's stay at hospitals within two to three days after hospital admission. It can be increased in ICU patients, especially those who underwent tube insertion and had LRTI "Lower Respiratory Tract Infection" [3]. Hospital acquired infections or the nosocomial infections (NI) are frequently caused by *Klebsiella pneumoniae*. These species are prevalent opportunistic microorganisms, which cause no symptoms, and are responsible for the gene resistance exchange and spread in hospitals [3,4].

After the pandemic of COVID-19 infection, the result of the NI and antimicrobial resistance remains the greatest public health threat. These circumstances necessitate both triggering the alarm for the anti-microbial resistance and the proper practice of preventive, quarantine and infection control measures. These include washing hands, reduction of frequent internal and external visits, and social distancing [5]. Carbapenem-resistant *Klebsiella pneumoniae* (CR-KP) are types of Carbapenem-resistant species, which occur more prevalently during hospitalization.

The increased prevalence rate of both morbidity and mortality are positively linked to the CR-KP, which is resistant to all antimicrobial drugs. During the course of treatment of severe infection by Gram negative organisms, carbapenem antibiotics, administered as a last resolution intervention. On the other hand, these organisms exhibit drug resistance [6].

The highly prevalent carbapenemase genes are "*klebsiella pneumonia* carbapenemases (KPC), encoded by bla_{KPC}, and *Klebsiella pneumoniae* carbapenemases (OXA-48), encoded by bla_{OXA-48}, Verona integron metallo-beta-lactamases types (VIM), imipenemase (IMP) types, and New Delhi metallo-betalactamase-1 (NDM-1)". The silent carriers of the CR-KP were the most implicated source of spread during outbreaks at the hospitals [7]. Four to fifteen percent of CR-KP infection occurs during the hospital stay, with increased rate of mortality among COVID-19 patients [8]. The niche for the occurrence of antimicrobial resistance is the widespread of antibiotics use [9,10].

There is high prevalence of the MDR colonization in the GIT, in particular carbapenem-resistant bacteria, among the intensive care patients comparable to other hospital units. This accounts as a crucial predisposing factor for the secondary bacterial infection [11,12].

The risk of the GIT colonization is highly increased by "dysbiosis of the gut microbiota" among severely ill patients and this can be contributed to many factors as competition for key nutrients, production of inhibitory bile acids, short chain fatty acids production, lowering the luminal PH and production of bacteriocins. Morbidity and mortality can be dramatically dropped by the early prevention, diagnosis and MDR HAIs treatment [13,14].

In Egyptian hospital, the situation is quite different due to lack of the data about antibiotic resistance, and lack of the clinical trials about it among COVID-19 patients. As a consequence, the aim of our study is to evaluate the CR-KP colonization and co- infection and evaluate the antibiotic resistance pattern.

Methods

Study Design

A cross-sectional study was carried out at Zagazig University Hospitals among COVID -19 patients in ICU and Clinical Pathology Department, in the period of 6 months from June 2022 to December 2022. During this period all patients admitted to the ICU wards in our hospital for SARS-CoV-2 infection and having positive culture for CPKP were enrolled in the present study. The etiological diagnosis was performed by a real-time polymerase chain reaction (RT-PCR) via a nasopharyngeal swab according to the WHO and Egyptian Ministry of Health and Population (MOH) regulations [15].

Laboratory tests such as (complete blood count, C-reactive protein (CRP), D-dimer, clotting tests, lactic dehydrogenase (LDH), interleukin 6, ferritin and procalcitonin) and Thoracic Computed Tomography (CT) were performed in all patients with confirmed COVID-19-induced pneumonia.

The inclusion criteria:

(i) Patients hospitalized in the ICU>48 h.

The exclusion criteria:

(i) Admission to the ICU <24 h.

(ii) All positive tests with the same strains of CRE identified in the same patient.

(iii) Patients who refused to participate in the study.

Ethical Approval

The study was approved by the Institutional Review Board (IRB) committee of Zagazig University (no.#9970/11-10-2022). The World Medical Association's Code of Ethics (Declaration of Helsinki) for studies involving human subjects and human specimens has been followed in the execution of this work. Informed consent was obtained and data were collected from electronic medical records.

Case diagnosis

All male and female patients who met the inclusion criteria were accepted to participate in the study. Blood stream infection (Patient has at least one positive blood culture for a recognized pathogen or patient has at least one of the following signs or symptoms: fever (> 38 °C), chills, or hypotension and two positive blood cultures for a common skin contaminant (from two separate blood samples, usually within 48 hours) and ventilator-associated pneumonia (A pneumonia where the patient is on mechanical ventilation for >2 consecutive calendar days on the date of event, with day of ventilator placement being day 1 and the ventilator was in place on the date of event or the day before) were defined according to the European Centre for Disease Prevention and Control (ECDC) [16].

CRKP isolates detection

CRKP positive cultures were found in sputum, urine, blood samples and rectum swabs. Bacteriological screening using rectal swabs was performed for the detection of carbapenemases among ICU patients at the time they were admitted to the ICU and at 7 days after hospitalization regarding the national protocol.

CRKP infection was detected in the lungs, urinary tract and blood as regards the bacteriological and lab investigations. Also, CRKP colonization was evaluated in the rectum. All samples identified as *K. pneumoniae* by “Gram stain and VITEK 2 (bioMérieux SA)” identification system.

CRKP from rectal swab samples were detected using XpertCarba-R Assay(Cepheid, Sunnyvale), while isolates from sputum, urine culture and blood culture phenotypically identified by VITEK® 2 (bioMérieux SA), modified Hodge test, and genotypically by Xpert Carba-R Assay(Cepheid, Sunnyvale).

Detection of CPKP rectal colonization using XpertCarba-R Assay (Cepheid, Sunnyvale)

The Cepheid XpertCarba-R Assay, performed on the GeneXpert® Instrument Systems, which is a "qualitative in vitro diagnostic test designed for rapid detection of carbapenem-non-susceptible *Klebsiella pneumoniae* obtained from rectal swab specimens from patients at risk for intestinal colonization with carbapenem-non-susceptible bacteria by using Cepheid Sample Collection Device (P/N 900-0370)."

For additional confirmatory diagnosis of carbapenem-non-susceptible bacteria, concomitant cultures to find organisms for antimicrobial susceptibility testing are a necessity. CRKP colonization screening (culture and real-time PCR assay on rectal swab at hospital admission and weekly during hospitalization) was applied during the 2 periods.

The decision to interpret a CRE result as an infection or colonization was made by the infectious diseases specialist after analyzing the patient's clinical and para-clinical data. In isolates that were determined to be bacterial colonization, patients showed no clinical signs of infection and serum inflammatory parameters were within normal limits.

CDC (Center for Diseases control and prevention in US) has defined CRKP infection clinically by fever (>38 °C), leukocytosis, an increased ESR (erythrocyte sedimentation rate), C-reactive protein (CRP) and increased pro-calcitonin values [17].

Bacterial strains & microbiological identification

Bact/ALERT3D System (bioMérieux) in a 7-14 days IP (incubation period) were used to detect the bacterial growth in blood cultures. In agar plates, +ve results cultures were relocated again. In standard agar plates, all the remaining clinical collections were collected and allow growing in sterile containers, while positive results were defined by the CFU cut-off value regulations [17]. Isolates from bacteria were let to grow the whole night at 35°C–37°C in microbiology labs for sensitivity testing according to the standard guidelines and were assessed by the VITEK® 2 compact identification system (BioMérieux, Marcy L'Etoile, France) according to manufacturer's instructions.

Antimicrobial susceptibility testing

For only isolates assessed in the UT (urinary Tract), sputum and blood, performed on

VITEK® 2 compact system, the antibiogram were done. The interpretation from the antibiogram were in line with test antimicrobial susceptibility MIC consistent with resistance to carbapenems defined by cutoff values on CLSI M100-ED31:2014 [18] and the VITEK® 2 compact system (BioMérieux, Marcy L'Etoile, France).

Confirmation of carbapenemases production

Modified Hodge Test (MHT):

MHT confirm the carbapenemases synthesis. After adding 0.5-4.5 mL of saline (45%), a 1:10 dilution of a 0.5 McFarland standard E. coli strain ATCC 25922 suspensions was streaked onto Muller-Hinton agar plates. After that, meropenem (10 g) was added to the mixture and it was centered on the plate. The sample was spread directly from the disc to the plate's rim. The plates were stored in a 35–37^{0}C environment whole night. CLSI standards were followed in the positive and negative results interpretation [18].

Molecular detection of carbapenemase genes

Carbapenem resistance genes were assessed using an XpertCarba-R assay (Cepheid, Sunnyvale, CA, USA), in order to find the 5 important carbapenemase-encoding genes in *K.pneumoniae* (*bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *and bla*$_{OXA-48}$) [19].

Statistical analysis: All data were collected and analyzed by SPSS (version 25) for windows (SPSS Inc., Chicago, IL, USA). Data were expressed as number and percentage for qualitative variables.

Results

190 samples were collected from 140 patients confirmed with COVID-19, urine samples were 45, respiratory samples were 65, blood samples were 80 and rectal swabs were taken from all patients at time of admission and at 7 days after hospitalization.

Out of 140 COVID-19 patients, only 60 had CRKP positive results, out of them 34 had a positive rectal swab for CPKP, 12 patients had infections and 14 patients were colonized and infected by CPKP during their ICU stay (three patients were positive for CPKP at ICU admission) as described in **Table 1**.

Among 60 COVID-19 patients, 48 (80%) had rectal colonization, three (5%) had urinary tract infections, five (8.3%) had lung infections and four (6.7%) had blood infections. Associations between colonization and infection with CRKP were found in patients with COVID-19. For example, rectal colonization and lung infection were detected in eight of 60 patients (16%), rectal colonization and a blood infection were detected in four patients (8%) while rectal colonization and urinary tract infection were two (4%) as shown in **Figure 1**.

Regarding antimicrobial resistance performed by VITEK® 2 compact system**,** antibiograms of CPKP isolates show an extensive resistance to almost all the classes of antibiotics. CRKP isolates were 100% resistant to Piperacillin/Tazobactam, Piperacillin, ceftazidime, cefepime, imipenem, ticarcillin and meropenem. Also, they were highly resistant to aminoglycosides, quinolones, minocycline and sulfamethoxazole/trimethoprim. With 11% of these isolates were resistant to colistin as described in **Table 2**.

Carbapenemase activity was phenotypically assessed by the MHT method as described in **Figure 2**.

According to the Ambler classification, carbapenemase encoding genes were screened by the XpertCarba-R Assay to detect the most prevalent ones :*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$, *and bla*$_{OXA-48}$. CRKP isolates were positive for one or more carbapenemase encoding gene, with *bla*$_{NDM}$ was predimonant in both colonized and infected cases (47%,46.1%) respectively, with no statistical significant difference between them as described in **Table 3**.

Demographic characteristics for patients showed median patient age was 60 years (range 46–69 years). Most of the patients were males (61.7%).

Comorbidities were present in more than half of the patients, with hypertension being the most prevalent (40%), followed by obesity (38.3%) and type 2 diabetes mellitus (30%). The increase in PCT levels mostly occurred on the sixth day of hospitalization, with the levels ranging from 0.67 ng/mL to 4.39 ng/mL (median: 1.27 ng/mL). About (38%) of COVID-19 patients received empirical antibiotics, while the remaining 31 patients (62%) did not. The length of hospital stays for COVID-19 patients with CRKP positive isolates, ranged from 9

to 18 days, with a median of 13 days as described in **Table 4**.

Table 1. Percentage of CRKP isolates in patients with COVID-19

Isolates with CRKP strains n=60	(%)
CRKP colonization	34(56.7%)
CRKP infection	12(20%)
CRKP colonization +infection	14(23.3%)

Table 2. Resistant pattern of CRKP isolates to Antibacterial Drugs

Antibiotic	Resistant pattern n= 26 (52%)
Piperacillin/Tazobactam	26(100%)
Piperacillin	26(100%)
Tobramycin	24(92.3%)
Cefepime	26(100%)
Imipenem	26(100%)
Meropenem	26(100%)
Gentamicin	21(82.5%)
Ceftazidime	26(100%)
Amikacin	23(87.5%)
Sulfamethoxazole-Trimethoprim	20(77.5%)
Azithromycin	26(100%)
Minocycline	23(89.2%)
Ticarcillin	26(100%)
Pefloxacin	25(98.3%)
Ciprofloxacin	25(98.3%)
Colistin	3(11%)

Table 3. Frequency distribution of carbapenemases genes in CRKP isolates among patients with COVID-19

Carbapenemase -encoding genes	Colonization (n=34) n (%)	Infection (n=26) n (%)	Chi-square	P value
***bla*$_{OXA-48}$**	11 (32.5%)	8 (30.8%)	0.1544	0.9845 (NS)
***bla*$_{KPC}$**	3 (8.8%)	2 (7.7%)		
***bla*$_{NDM}$**	16 (47%)	12 (46.1%)		
***bla*$_{NDM}$ + *bla*$_{OXA-48}$**	4 (11.7%)	4 (15.4%)		

Chi-square statistic is 0.1544. The p-value is .98459. The result is not significant at $p < .05$

Table 4. Patient characteristics of COVID-19 patients with CPKP positive results.

Characteristics	Total (n = 60)
Age, median (IQR), years	60 (46–69)
Age group, n (%), years	
≥60	31(51.7%)
<60	29 (48.3%)
Sex, n (%)	
Male	37(61.7%)
Female	23(38.3%)
Comorbidities, n (%)	
Hypertension	24 (40%)
Obesity	23 (38.3%)
Type 2 DM	18 (30%)
Chronic kidney disease	16 (26.7%)
Cardiovascular diseases	14 (23.3%)
Stroke	3 (5%)
No comorbidities	12 (20%)
Time from illness onset to hospital admission, median (IQR), days	3 (2–5)
Duration of hospitalization, median (IQR), days	13 (9–18)
<14 days	24 (40%)
>14 days	26 (43.3%)
Oxygen support (%)	
Nasal cannula Non-rebreathing mask Ventilator High-flow nasal cannula Without oxygen support Simple mask	18(30%) 14(23.3%) 10(16.7%) 8(13.3%) 6(10%) 4(6.7%)
Antibiotics history, n (%) No Yes	 **36(60%)** **24(40%)**

Figure 1. Distribution of CR-KP isolates

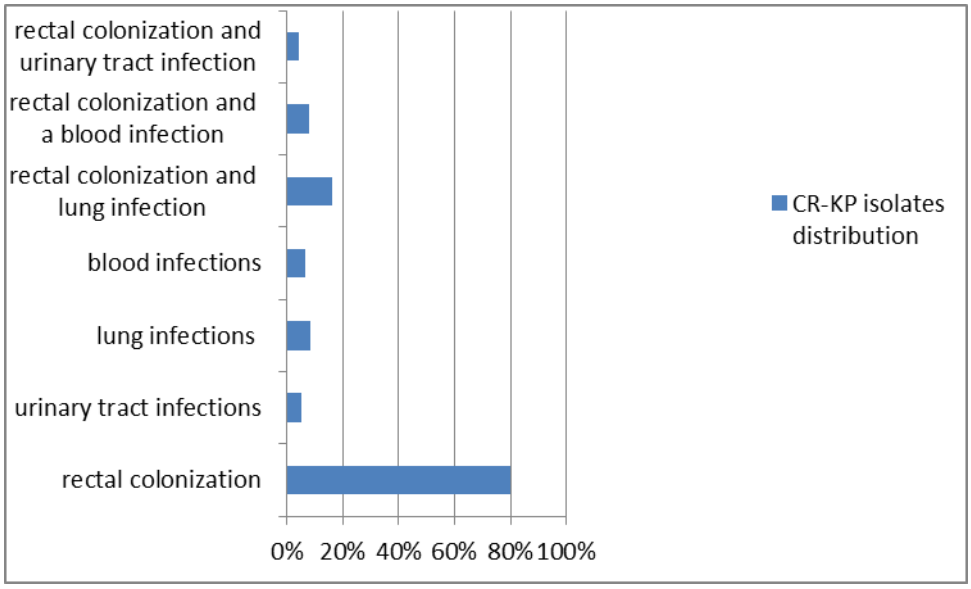
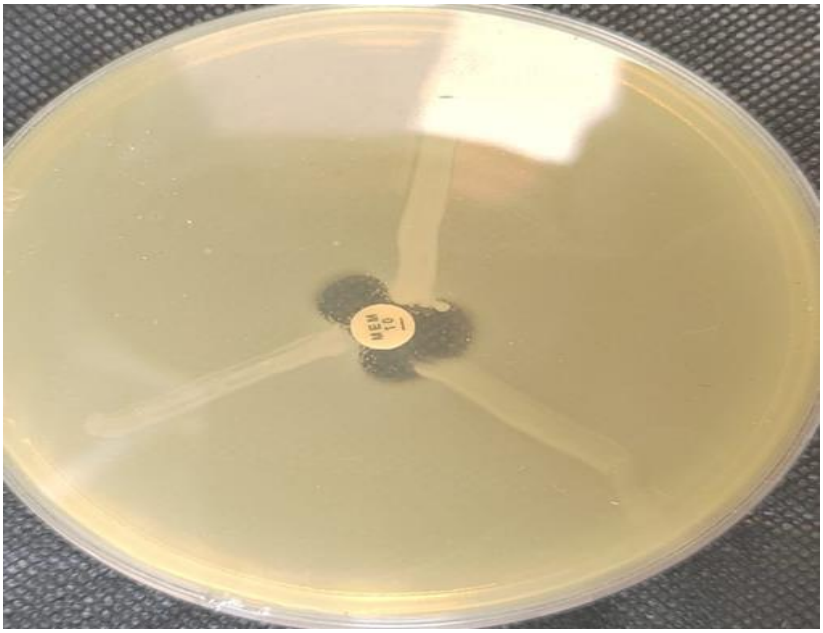


Figure 2. Modified Hodge test (MHT) A: clinical KP isolate with positive result



Discussion

COVID-19 had a high prevalence rate with worldwide affection. The worldwide situation is not away from Egypt where the incidence rate of COVID-19 was devastatingly increasing [20].

A potential risk of bacterial infection, especially MDR, is related to COVID-19 pandemic among critically ill patients in ICUs [21].

CPKP researches were scarce among these patients, while the most prevalent enterobacterales among COVID-19 patients were *Klebsiella* species [22,23]. Similar to Upper Egypt, where research on Gram-ve bacteria found that the most prevalent was *Klebsiella pneumoniae*. On the other hand, there was a report about a low incidence of the CPKP infections in New York, US [24]. A higher incidence of CPKP infection among COVID-19 patients, notably in ICUs, has been discovered in other research from Italy, the USA, and China, suffering severe infections [25-27].

A framework for evaluating the significant risk of CPKP colonization and hospital-acquired infections (HAIs) in COVID-19 ICUs was the goal of this research. The prevalence of CRE colonization in hospitalized patients has been reported, among high-risk populations like ICU patients and those taking several antibiotics in recent years, which might help these bacteria spread across healthcare facilities. This could be a result of widespread CRE contamination of the environment, especially in drinking water and waste water, as previously shown in Indian study [28].

During the pandemic, antimicrobial stewardship strategies were tested, revealing that while minimizing patient contact and hand washing may reduce HAI spread, prioritizing quarantine measures and placing COVID-19 patients in special wards or ICUs could have spread CPE [29].

Colonization was shown to be prevalent in this study (56.7%) that was seen in a Korean ICU, where pneumonia/chronic pulmonary disease, prior use of fluoroquinolones, and prior use of a nasogastric tube were risk factors for CPE infection or colonization [30]. While a study conducted in Vietnamese hospitals found that carbapenem therapy and prolonged hospitalization were risk factors for CRE colonization [31,32].

Microbial co-infection notably increases among patients of COVID-19 disease and these microorganisms showed MDR phenotype. Among large pandemics of the viral respiratory infections, the role of co-infection is understudied [33]. Our results showed that co-infections with extensively resistant *K. pneumonia* were found in 20% of COVID-19 patients, supporting Noviello and Huang's hypothesis that reported many viral respiratory pathogens being associated with other bacterial co-infections due to viral agents which decrease the mucociliary clearance of bacteria in lungs [34].

In this study, 14 colonized patients were infected with MDR-KP, which could be problematic for a number of reasons. First, the previously mentioned immune system dysfunction in these patients is due to the disease itself and to the immune modulatory therapeutic approach taken; second, it is due to factors such as their often-advanced age, comorbidities, long hospital stays, and the numerous invasive procedures they are subjected to, all of which are risk factors for MDR-bacteria colonization- infection [35].

In COVID-19 patients, we found a greater prevalence of NDM-producing *K. pneumonia* (47% in colonization and 46.1% in infection) as a sign of our hospital's current epidemiological situation, which is similar to observations made in series from US and Egypt, which declared that NDM1 was the most frequently found gene [36,37]. This distribution is consistent with the pre-pandemic ICU scenario in our hospital, as NDM and OXA-48 were the common genes detected in *K.pnumoniae* species [38,39].

A study published by García-Meniño et al. revealed that the most commonly carbapenemase was OXA-48 among COVID-19 [26]. Another study published in Italy by Arcari et al. showed that bla_{KPC} and bla_{OXA-48} were the most prevalent ones [40]. In this study, bla_{OXA-48} was found in 32.5% of colonized patients, 30.8% of infected patients, and 8.8% and 7.7% of patients with bla_{KPC} in colonization and infections, respectively.

The New Delhi metallo-β-lactamase, discovered in 2008, has significantly spread globally since its isolation from a Swedish patient in New Delhi [41].

This may be explained by NDM-1 and OXA-48, are carried on plasmid replicon type Inc L/M, Tn1999, IS1999 and rarely IncA/C in OXA-48-like. A mix of IncR, IncFII, were responsible for a spread of NDM-1. These plasmids are mobile elements and facilitate the horizontal transmission of these resistance mechanisms among patients in hospitals, especially those with a risk factor for colonization and infection with CRE [42].

Despite a lack of evidence of bacterial co-infections, international studies show that approximately 70% of hospitalized COVID-19 patients receive antibiotics, most often with broad spectrums, and that a high proportion of them (72–100%) received antibiotics during the course of disease, increasing the risk of antimicrobial resistance [43-45].

This study confirmed this conclusion as broad-spectrum antimicrobials were more commonly prescribed in Egyptian environment during the pandemic, which was exacerbated by deterioration in hygiene measures in quarantine ICUs. We were plagued by drug-resistant *K. pneumonia* clinical isolates. In keeping with our findings, research conducted at Minia University Hospitals in Upper Egypt [37] that found a similar

picture of resistance, with CR-KP being resistant to almost all antibiotic classes [46].

Which is worse, the presence of co-existing resistance mechanisms leaves few therapeutic options. As polymyxin which has unfavorable side effects, emerging resistance and poor outcomes [47]. Certain NDM-producing isolates also possess 16S rRNA methylases, rendering aminoglycosides ineffective [48].

The fact that bla_{NDM} are encoded on a variety of highly mobile conjugative plasmids, allowing for horizontal inter and intra-species transfer between bacteria rather than clonal spread, may be the cause of their predominance. Since plasmids are dominant in NDM-producing isolates, this is a critical situation. So the bla_{NDM} frequently carries genes that confer pan-drug resistance to the majority of antibiotics [49].

According to our findings, a study conducted at Assiut University Hospitals found a predominance of Gram-negative co-infections in COVID-19 patients [37], which may be attributed to the administration of azithromycin in the regimen of COVID-19 therapy, which acts primarily against Gram-positive bacteria [50]. Males were infected more frequently than females in the current study, which is consistent with a recent meta-analysis [51].

Where females had lower viral infection prevalence than males because they have higher immune responses, which could be due to sex hormones, which play an important role in innate and adaptive immunity [52].

The most prevalent reported co-morbidities in this study were hypertension, diabetes, and obesity. Similarly, prior studies revealed that the most prevalent co morbidities in the recruited participants were hypertension, then diabetes mellitus and finally obesity [53].

In line with the previous studies done on COVID-19, older age was associated with a higher risk of mortality as in this investigation because older people typically have a more difficult illness that prolongs hospitalization length . It makes them more susceptible to subsequent bacterial infections [54,55].

A meta-analysis in Indonesia showed that longer hospital stays and ICU admission were risk factors for carbapenem-resistant *Klebsiella pneumoniae* infection; the current study revealed that the length of hospital stays for COVID-19 patients with CR-KP ranged from 9 to 18 days, with a median of 13 days [46].

Conclusion

Given that coronaviruses will be a constant challenge in the coming years and that antibiotic administration has implicitly increased the rate of microbial resistance, it is critical to establish a strict antibiotic administration program in intensive care units, as well as adherence to universal precautions that aid in limiting germ spread. Expanding these features through new clinical-epidemiological studies will aid in the development of appropriate solutions to these challenges.

Furthermore, we believe that the microbiota in the gut may be a potential therapeutic target for specific therapies (such as probiotic treatment, decolonization procedures, and so on) that may help to lower the risk of MDR bacteria colonizing the digestive system. Alternative therapeutic techniques, such as antimicrobial peptide or phage therapy, which are now being studied with promising results, should also be studied.

Recommendations

This current study aimed to highlight the challenges associated with colonization and secondary bacterial infections with CRKP in severely ill COVID-19 patients. Managing colonization and avoiding infection development in colonized patients is crucial, and advocating for conservative antibiotic therapy guided by microbiology data in this group of patients. Given the wide range of clinical phenotypes in COVID-19 and the high rate of resistance confirmed by our study, we recommend that their use be re-evaluated on a regular basis for discontinuation or de-escalation, particularly given the long durations of critical illness, prolonged positive cultures, and high antibiotic exposures. We appreciate the challenging responsibilities that clinicians face while caring for critically ill patients of COVID-19.

Limitation

This is a preliminary observational research to identify the most common CR-KP types in COVID-19 patients in Egypt. Future studies should cover the molecular sequencing in order to assess the ST and plasmids involved in the spread.

Conflict of interests

The authors report no conflicts of interest.

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Author contributions

All authors contributed to the study conception and design. They have all participated in material preparation, data collection and analysis. All authors have contributed in drafting and/or revising the previous versions of the manuscript. All authors read and approved the final manuscript.

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