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Original article

Secondary multidrug resistant bacterial pneumonia among adult COVID-19 patients: A molecular study

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ABSTRACT

Background: Secondary bacterial pneumonia especially with multidrug resistant (MDR) organisms is one of the devastating complications that can worsen COVID-19 patients' outcomes. The study aimed to evaluate the impact of secondary MDR bacterial pneumonia on COVID-19 patients' outcomes with molecular detection of genes involved in antimicrobial resistance among isolates of the most prevalent causative pathogen. **Methods:** The study included 50 critically ill patients with acute severe COVID-19 with evidence of secondary MDR bacterial pneumonia, 50 critically ill patients with acute severe COVID-19 without evidence of secondary bacterial pneumonia, and 30 clinically stable patients with acute moderate COVID-19 infections. Respiratory samples were cultured for identification and antibiotic susceptibility of the causative pathogens. MDR/XDR *A. baumannii*, the most prevalent pathogen, was screened for multiple antibiotic resistance genes using single-plex and multiplex polymerase chain reactions. **Results:** Critically ill COVID-19 patients with secondary MDR bacterial pneumonia in group I had a significantly higher mortality rate. MDR/XDR *A. baumannii* was the most prevalent pathogen (39.2%) isolated with the highest cause specific mortality rate (38%). Multiple resistance genes were detected including *bla*$_{\text{OXA-51}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{ADC}}$, *bla*$_{\text{CIT}}$, *bla*$_{\text{KPC}}$, *aacA4*, *aacC1*, *acc (6')*, *aphA1*, *aph6*, and *aadA1*. **Conclusion:** Secondary MDR bacterial pneumonia had a significant impact on critically ill COVID-19 patients with a significantly higher mortality rate. Thus, preventing secondary MDR bacterial pneumonia through infection prevention measures, including standard precautions, preventive care bundles and antimicrobial stewardship programs, should be strictly implemented to protect critically ill COVID-19 patients and help avoid its detrimental effect on patients' outcomes.

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Introduction

With the surging number of cases since 2019, the global spread of the 2019 coronavirus disease (COVID-19) pandemic became a growing health crisis [1]. Over 2.3 million new cases were reported during the second week of November, 2022, with a 2% rise from the previous week [2]. More than 640 million confirmed cases of COVID-19, including > 6.5 million deaths, have been reported to World Health Organization (WHO) as of November, 2022 [3].

The COVID-19 clinical spectrum spans from mild flu-like illness to severe, potentially fatal disease. As much as 48% of COVID-19 patients die after being admitted to the intensive care unit (ICU), largely due to acute respiratory distress syndrome [4]. Old age, male gender, pre-existing comorbidities, and racial differences are some of the known risk variables that may contribute to higher morbidity from COVID-19 in adults [1]. Also, changes in laboratory indices like creatinine and lymphocytopenia, pro-inflammatory cytokine levels and potential complications, are key risk factors for the severity and mortality of COVID-19 [5].

Bacterial coinfections may contribute to COVID-19 severity. Severe COVID-19 patients may be admitted to ICU with the need for mechanical ventilation, which may predispose them to secondary, opportunistic, and hospital-acquired infections (HAIs) [6]. Thus, they are at risk of developing MDR bacterial infections in the ICU [7]. Broad-spectrum antibiotic use, invasive procedures (mechanical ventilation, central venous access, etc.), and prolonged ICU stay are all factors that contribute to an increased MDR infection risk [8].

Patients with severe COVID-19 have significantly less CD4 and CD8 T cells, making them more vulnerable to bacterial coinfection, which is identified in over 50% of COVID-19 fatalities [9].

A high risk of MDR bacterial infections is associated with the combination of ICU-related immune suppression and SARS-CoV-2-induced immune deregulation in seriously sick COVID-19 patients [10]. The incidence of healthcare acquired MDR bacterial and fungal infections was as high as 21.7%, with pneumonia representing the most prevalent type of infection (19.9%) [11]. Bacterial coinfections complicating pulmonary viral infections were evidenced to be linked to deleterious patients' outcomes [12].

The most commonly reported multidrug-resistant Gram-negative bacteria (MDR-GN) among COVID-19 patients was *Acinetobacter baumannii (A. baumannii)*, followed by *Klebsiella pneumonia (K. pneumonia)*, *Escherichia coli (E. coli)*, and *Pseudomonas aeruginosa (P. aeruginosa)* [13]. The majority of secondary coinfections were healthcare-associated infections (HAIs) since they were discovered 48 hours or more after patient's admission [14]. Therefore, with the global escalation of the crisis, it is crucial to clarify the impact of secondary MDR bacterial pneumonia on COVID-19 patients and its detrimental effect on patients' outcomes.

Aim

To evaluate the impact of secondary multidrug resistant bacterial pneumonia on COVID-19 patients' outcomes with molecular detection of genes involved in antimicrobial resistance among isolates of the most prevalent causative pathogen.

Methods

Study design

The prospective cohort study included 50 critically ill patients with acute severe COVID-19 with evidence of secondary MDR bacterial pneumonia, 50 critically ill patients with acute severe COVID-19 without evidence of secondary bacterial pneumonia, admitted to the COVID-19 ICU, and 30 clinically stable patients with acute moderate COVID-19 infections admitted to COVID-19 isolation ward, King Abdulaziz Hospital (KAAH), Jeddah from August to October 2021.

Inclusion criteria

Adult patients who were clinically and radiologically diagnosed as COVID-19 laboratory confirmed cases by positive novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real-time reverse transcriptase-polymerase chain reaction (RT-PCR) testing of nasopharyngeal swabs using BGI Real-Time Fluorescent quantitative RT-PCR Kit for Detecting SARS-CoV-2 with ORF1ab gene as a domain target (Hong Kong, China) with measuring cycle threshold (Ct) values for SARS-CoV-2 viral load estimation following manufacturer's instructions with positivity cut off value of ≤ 38 according to **Altamimi et al.** [15].

Patients were categorized according to **Sekine et al.** as cases with acute severe COVID-19 [Patients admitted to ICU, with low-flow oxygen support (3-10 L/min), high-flow oxygen support (>10 L/min),

or invasive mechanical ventilation.] and cases with acute moderate COVID-19 [Patients admitted to in isolation ward and low-flow oxygen support (0–3 L/min)] [16]. Patients were followed up for determining patients' outcome as survivor (ICU/ hospital discharge) or non-survivor (in-hospital death). Patients' groups were:

- Group I (50 patients): Critically ill patients with acute severe COVID-19 with evidence of secondary MDR bacterial pneumonia.
- Group II (50 patients): Critically ill patients with acute severe COVID-19 without evidence of secondary bacterial pneumonia.
- Group III (30 patients): Clinically stable patients with acute moderate COVID-19.

Sample size was calculated using the PASS 15 program according to the study of **Liu et al.** [17] showing that in the severe COVID-infected group the total lymphocyte counts were significantly lower compared to the mild group, assuming a medium effect size difference between the different groups regarding total lymphocyte counts (d =0.4), based on this assumption a sample size of 90 patients (at least 30 patients per group) achieves 90% power to detect differences among the means versus the alternative of equal means using an F test with a 0.050 significance level. The size of the variation in the means is represented by the effect size $f = \sigma m / \sigma$, which is 0.40. By the end of data collection time sample size included 50 patients for group I, 50 patients for group II and 30 patients for group III, which allowed comparing patients' outcomes among the two groups of critically ill patients.

Exclusion criteria

Age < 18 years old, pregnant females, repeatedly unsuitable respiratory samples for culture or critically ill COVID-19 patients with secondary bacterial pneumonia caused by antibiotic susceptible strains only.

Ethical considerations

The study was performed after approval of the Research and Studies Department – Jeddah Health Affairs Institutional Review Board (IRB) registration number with KACST, KSA: H-02-J-002 research number 1573 and in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) and Good Clinical Practice guidelines. Patients or guardians were informed, and their informed consent was obtained before specimen collection.

Data collection

Clinical data was collected from electronic patients' files including demographic data, co-morbidities, mechanical ventilation, and laboratory data including total leukocytic count, neutrophil count, lymphocytes count, eosinophil count, platelets count, aspartate transaminase (AST) serum level, alanine aminotransferase (ALT) serum level, serum creatinine (S. Cr), blood urea nitrogen (BUN) levels in the serum and C reactive protein (CRP).

Secondary bacterial pneumonia diagnosis and respiratory sampling

Secondary bacterial pneumonia was suspected with clinical worsening condition and progressive radiological changes characteristic to bacterial pneumonia through serial chest radiographs (CXRs) or chest computerized tomography scan (CTS) **(Figure 1)** including lobar consolidation with air bronchogram or areas of consolidation particularly if unilateral, associated with necrosis with or without cavitation, pleural effusion or empyema (thickened pleura, mottled air lucencies), extensive new onset unilateral or bilateral consolidations, often in non-dependent areas according to **Naranje et al.** [18]. Respiratory samples were collected from all patients including sputum samples and endotracheal tube aspirate (ETT) samples from mechanically ventilated patients. Respiratory samples were examined based on modified Barilett's criteria according to **Zakuan et al.** [19]. Briefly, samples were evaluated macroscopically followed by microscopic examination for scoring the number of pus cells and squamous epithelial cells per high power field (HPF). Suitable samples were cultured on standard culture media.

Pathogens identification and antimicrobial susceptibility testing

Isolated pathogens were identified based on colonial morphology, Gram's staining, catalase and oxidase test. Full identification of bacterial isolates and minimal inhibitory concentration (MIC) for different antibiotics were determined using the automated systems BD Phoenix (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) and MicroScan WalkAway (Dade Behring INC. West Sacramento, CA, USA). Quality control strains included *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus (S. aureus)* ATCC 29213 as recommended by Clinical and Laboratory Standards Institute (CLSI), 2021 [20].

Multidrug resistant (MDR) organisms were defined as organisms with acquired non-susceptibility to at least one agent in three or more antimicrobial categories, extendeddrug resistant (XDR) was defined as non-susceptibility to at least one agent in all except two or less antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) [21]. Ventilator associated pneumonia (VAP) was defined as early-onset and late onset VAP as pneumonia diagnosed before and after 5 days of mechanical ventilation respectively according to **Sadigov et al.** [22].

Antimicrobial sensitivity testing for *A. baumannii* isolates was done using Kirby–Bauer disc diffusion method according to CLSI 2021 [20], using the following antibiotic discs (Mast Group, UK): ceftazidime (CAZ-30 µg), ceftriaxone (CRO-30 µg), cefepime (CPM-30 µg), imipenem (IMI-10 µg), meropenem (MEM-10 µg), piperacillin–tazobactam (PTZ-100/10 µg), ciprofloxacin (CIP-5 µg), levofloxacin (LEV-5 µg), gentamicin (GM-10 µg), amikacin (AK-30 µg), and trimethoprim/sulfamethoxazole (TS-1.25/23.75 µg).

Single-plex and Multiplex polymerase chain reaction (PCR) analysis for genotypic identification and detection of aminoglycosides resistance genes and β-lactamases genes among MDR/XDR *A. baumannii* isolates, the most prevalent isolated pathogen

Isolates were sub-cultured and stored in 40% sterile glycerol-broth medium at −80 °C in preparation for subsequent analysis. DNA extraction was done using QIAamp DNA Micro Kit, (Qiagen, Hilden, Germany). Primers were manufactured by Macrogen Genomics (Seoul, South Korea). Primers, annealing temperatures, expected amplicon sizes are given in **table(1).** The PCR assay was performed using the GoTaq® Green Master Mix (M712) (Promega, USA) and Veriti™ 96-Well Fast Thermal Cycler (Thermo Fisher Scientific, USA). Gel electrophoresis for PCR products was done on 2% agarose gel for amplicons visualization against 100-bp ladder (Promega, USA). For molecular studies, *K. pneumoniae* ATCC 2146, *K. pneumoniae* ATCC 1705, *K. pneumoniae* NCTC 13442 were used as positive controls and *K. pneumoniae* ATCC 25955 was used as negative control.

Acinetobacter baumannii species identification was genotypically confirmed through detection of the naturally occurring carbapenemase gene ($bla_{\text{OXA-51}}$) intrinsic to *A. baumannii* using Single-plex PCR (S-PCR) according to **Handal et al.** [23]. The 25-µL final reaction mixture consisted of 12 µL 2 X GoTaq® Green Master Mix (M712), with l µL for each primer (10 pmol/ml), 8 µL nuclease-free water and a volume of 3 µL of the extracted DNA template. The cycling conditions were followed according to **Turton et al.** [24] with denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 45 sec., followed by annealing at 58 °C for 45 sec., extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min.

Multiplex PCR (M-PCR) analysis was customized for the detection of the OXA-type class D carbapenemases $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, and $bla_{\text{OXA-58}}$ according to **Handal et al.** [23] and for the detection of the AmpC β-lactamases according to **Liu et al**. [25]. For the OXA-type genes the cycling conditions consisted of an initial denaturation step of 95˚C for 15 min, followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 52˚C; and extension at 72˚C for 90 sec, followed by the final extension step at 70˚C for 10 min [26]. For AmpC genes the cycling conditions consisted of an initial denaturation step at 94˚C for 3 min, followed by 28 cycles of denaturation at 94˚C for 30 sec, annealing at 56˚C for 30 sec, and extension at 72˚C for 1 min. followed by a final extension step at 72˚C for 7 min [25].

Single-plex PCR analyses were performed for the detection of class A carbapenemase bla_{KPC}, class B carbapenemase bla_{NDM}, class D carbapenemases $bla_{\text{OXA-143}}$ and $bla_{\text{OXA-235}}$ and Aminoglycoside-modifying enzymes (AMEs), the acetyltransferases *aacC1* and *aacA4* genes according to **Handal et al.** [23]. Single-plex PCR for $bla_{\text{OXA-48}}$ gene was performed as described by **Asadian et al.** [27] with cycles programmed as denaturation at 94°C for 60 sec and 30 cycles of 94°C for 30 sec, annealing at 55°C for 40s in, extension at 72°C for 60 sec for extension, and the final extension at 72°C for 7 min. Single-plex PCR for AMEs genes including *acc(6')*, *aph(3')-IIb*, *aphA1*, *aph6*, and *aadA1*was performed according to **Tahbaz et al.** [28].

Data Management and Analysis: Data was processed using Statistical package for Social Science (SPSS 25). Descriptive statistics included mean, standard deviation (± SD) and range for parametric numerical data, median and interquartile range (IQR) for non-parametric numerical data and frequency and percentage of non-numerical data. Student t test for comparing two groups means and ANOVA test for comparing more than two groups

means. Post Hoc Test for comparisons of all possible pairs of group means. Chi-Square test was used to compare between two qualitative variables. Fisher's exact test was used to compare between two qualitative variables when the expected count is less than 5 in more than 20% of cells. The Kruskal-Wallis test was used for more than two study groups ordinal variables. $p>0.05$: Non significant (NS), $p< 0.05$: Significant (S).

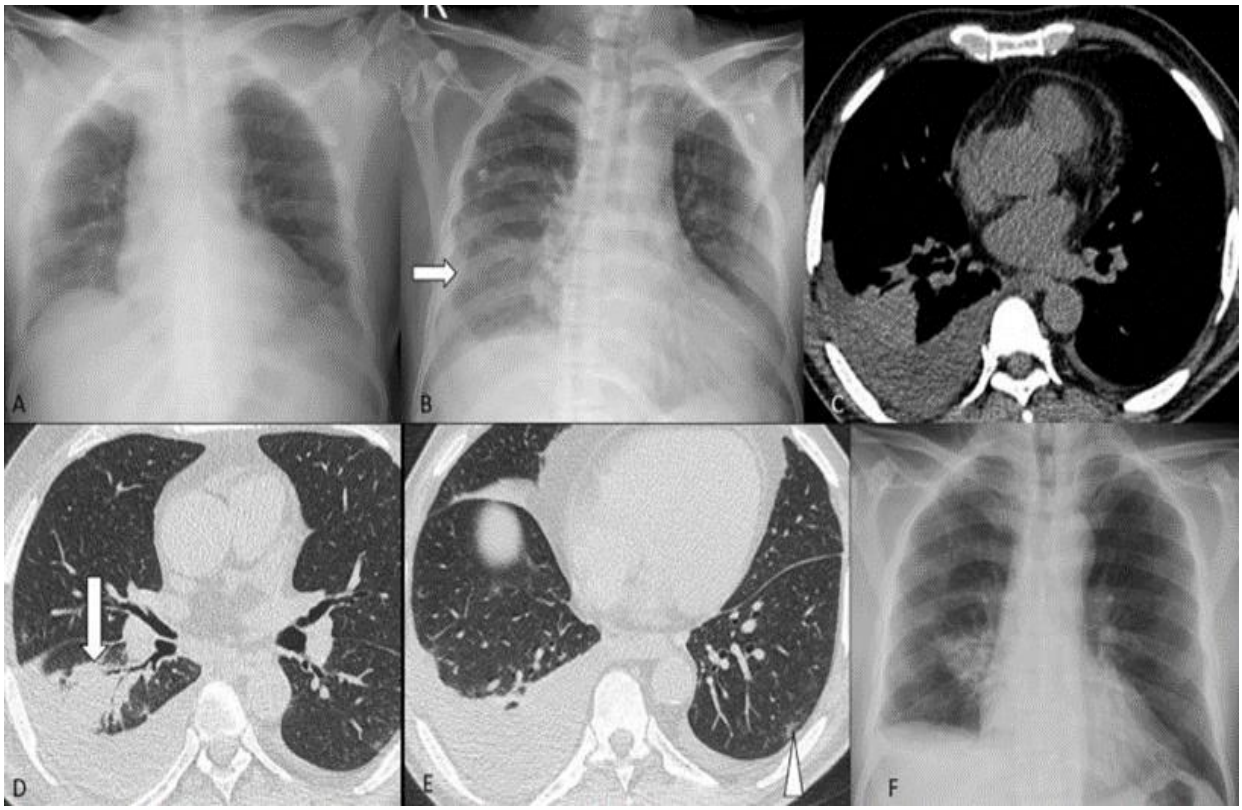
Table 1. Single-plex and multiplex PCR primers.

Reaction	Primer name	Sequence (5' to 3')	Annealing temperature* (℃)	Amplicon size (bp)	Reference
Single-plex PCR for $bla_{\text{OXA-51}}$ *A. baumannii* identification	**OXA-51**	F: 5'- TAATGCTTTGATCGGCCTTG -3'	58℃	353 bp	19
		R: 5'- TGGATTGCACTTCATCTTGC -3'			
Multiplex PCR for OXA-type class D carbapenemases	**OXA-23**	F: 5'- GATCGGATTGGAGAACCAGA-3'	52℃	501 bp	22
		R: 5'- ATTTCTGACCGCATTTCCAT-3'			
	OXA-24	F: 5'- GGTTAGTTGGCCCCCTTAAA -3'	52℃	246 bp	22
		R: 5'- AGTTGAGCGAAAAGGGGATT -3'			
	OXA-58	F: 5'- AAGTATTGGGGCTTGTGCTG -3'	52℃	599 bp	22
		R: 5'- CCCCTCTGCGCTCTACATAC -3'			
Multiplex PCR for AmpC β-lactamases	**ADC**	F: 5' TAAACACCACATATGTTCCG -3'	56℃	663 bp	21
		R: 5'- ACTTACTTCAACTCGCGACG -3'			
	MOX	F: 5' GCTGCTCAAGGAGCACAGGAT -3'	56℃	520 bp	21
		R: 5'- CACATTGACATAGGTGTGGTGC -3'			
	CIT	F: 5' TGGCCAGAACTGACAGGCAAA -3'	56℃	462 bp	21
		R: 5'- TTTCTCCTGAACGTGGCTGGC -3'			
	DHA	F: 5' AACTTTCACAGGTGTGCTGGGT -3'	56℃	405 bp	21
		R: 5'- CCGTACGCTTACTGGCTTTGC -3'			
	ACC	F: 5' AACAGCCTCAGCAGCCGGTTA -3'	56℃	346 bp	21
		R: 5'- TTCGCCGCAATCATCCCTAGC -3'			
	EBC	F: 5' TCGGTAAAGCCGATGTTGCGG -3'	56℃	302 bp	21
		R: 5'- CTTCCACTGCGGCTGCCAGTT -3'			
	FOX	F: 5' AACATGGGGTATCAGGGAGATG -3'	56℃	190 bp	21
		R: 5'- CAAAGCGCGTAACCGGATTGG -3'			
Single-plex PCR for class A carbapenemase bla_{KPC}	**KPC**	F: 5'- ATGTCACTGTATCGCCGTCT-3'	58℃	538 bp	19
		R: 5'- TTTTCAGAGCCTTACTGCCC-3'			
Single-plex PCR for class B carbapenemase bla_{NDM}	**NDM**	F: 5'- CCAATATTATGCACCCGGTCG-3'	58℃	812 bp	19
		R: 5'- ATGCGGGCCGTATGAGTGATTG-3'			
Single-plex PCR for class D carbapenemases $bla_{\text{OXA-143}}$	**OXA-143**	F: 5'- TGGCACTTTCAGCAGTTCCT -3'	58℃	180 bp	19
		R: 5'- TAATCTTGAGGGGGCCAACC -3'			
Single-plex PCR for class D carbapenemases $bla_{\text{OXA-235}}$	**OXA-235**	F: 5'- TTGTTGCCTTTACTTAGTTGC -3'	58℃	700 bp	19
		R: 5'- CAAAATTTTAAGACGGATCG -3'			
		F: 5'- CCAAGCATTTTTACCCGCATCKACC-3'			

Single-plex PCR for class D carbapenemases $bla_{\text{OXA-48}}$	**OXA-48**	R: 5'- GYTTGACCATACGCTGRCTGCG -3'	55℃	389 bp	23
Single-plex PCR assays for AMEs genes	**aacC1**	F:5'ATGGGCATCATTCGCACATGTAGG-3'	65℃	456 bp	19
		R: 5'-TTAGGTGGCGGTACTTGGGTC-3'			
	aacA4	F: 5'-ATGACTGAGCATGACCTTGCG-3'	65℃	518 bp	19
		R: 5'-TTAGGCATCACTGCGTGTTCG-3'			
	acc(6')	F:5'-TTGCGATGCTCTATGAGTGGCTA-3'	63℃	482 bp	24
		R: 5'-CTCGAATGCCTGGCGTGTTT-3'			
	aph6	F: 5'-GAGCGCACCTTCGACTATGC-3'	63℃	248 bp	24
		R: 5'-GCCATGGCGTTTACGGCCAG-3'			
	aph(3')-IIb	F: 5'-ATGCATGATGCAGCCACCTCC-3'	64℃	807 bp	24
		R: 5'-CTAGAAGAACTCGTCCAATAGCCT-3'			
	aphA1	F: 5'-AAACGTCTTGCTCGAGGC-3'	56℃	461 bp	24
		R: 5'-CAAACCGTTATTCATTCGTGA-3'			
	aadA1	F: 5'-GTGGATGGCGGCCTGAAGCC-3'	63℃	527 bp	24
		R: 5'-AATGCCCAGTCGGCAGCG-3'			

∗Annealing temperature (℃) used in this study.

Figure 1. CXRs and chest CTS findings suggestive of secondary bacterial pneumonia in COVID-19 patients, **Naranje et al.** [16].



Results

The study included 50 patients with acute severe COVID-19 with evidence of secondary MDR bacterial pneumonia (group I), 50 patients with acute severe COVID-19 without evidence of secondary bacterial pneumonia (group II), and 30 patients with acute moderate COVID-19 infections (group III). **Table 2** shows the demographic data and co-morbidities among the studied patients' groups with a non-significant difference as regards age and co-morbidities distribution among the studied groups. As regards sex distribution, a significant difference is shown between critically ill patients in group I and II compared to stable patients in group III. The majority of patients in group I and II were males (82% and 80% respictively).

Table 3 shows that critically ill patients with evidence of secondary MDR bacterial pneumonia in group I had a significantly higher percentage of mechanically ventilated patients (56%) and higher mortality rate (90%) compared to critically ill patients without evidence of secondary bacterial pneumonia in group II. None of the clinically stable patients in group III was mechanically ventilated with 100% survival rate. Among mechanically ventilated critically ill patients with evidence of secondary MDR bacterial pneumonia in group I (N = 28), 4 (14.3%) patients had early onset VAP and 24 (85.7%) patients had late onset VAP. No VAP was detected in group II.

Tables 4 shows the comparison of laboratory data between the study groups. A significant difference is observed between group I patients compared to groups II and III as regards all data except ALT serum level.

Table 5 shows that a significantly lower mean Ct value was detected among patients of group I compared to patients of group II and group III. Also, a significant difference is shown between survivors and non survivors as regards mean Ct value among patients of group I and group II.

Table 6 and **figure (2)** show that, among patients of group I, the most prevalent MDR pathogen was MDR/XDR *A. baumannii* with the highest cause specific mortality rate (38%). Coinfection was detected in 7 patients in group I; 6 patients showed MDR isolates mixed with 6 antibiotic susceptible isolates including 3 *E. coli*, 2 *P. aeruginosa*, and 1 *S. aureus*, and 1 patient was coinfected by 2 MDR isolates (*E. coli and S. aureus*). Among stable patients in group III one patient had secondary bacterial pneumonia caused by antibiotic susceptible *P. aeruginosa.* No MDR isolates were detected among patients of group II and group III.

Figure 3 and **table (7)** show the confirmatory molecular identification of the 20 *A. baumannii* isolates through detection of $bla_{\text{OXA-51}}$ gene, detected in 100% of the *A. baumannii* isolates and the antimicrobial susceptibility testing with highest resistance rates observed with carbapenems (90%), amikacin (90%) and gentamicin (85%-). The lowest resistance rate is observed with colistin followed by trimethoprim/sulfamethoxazole (5%, 58% respectively).

Figure 4 shows the detected OXA-type class D carbapenemases among 20 isolates of *A. baumannii*. The $bla_{\text{OXA-48}}$ gene was detected in 8 isolates (40%) and the $bla_{\text{OXA-24}}$ gene was detected in 4 isolates (20%). **Figure 5** shows the detected AmpC genes including bla_{ADC} gene and bla_{CIT} gene, simultaneously detected in 3 isolates (15%). Also, the bla_{KPC} gene was detected in 2 (10%) of the *A. baumannii* isolates. **Figure 6** shows the detected AMEs genes including *aacA4* gene detected in 6 isolates (30%), *aacC1* gene detected in 5 isolates (25%), *acc(6')* gene detected in 5 isolates (25%), *aphA1* gene detected in 5 isolates (25%), *aph6* gene detected in 3 isolates (15%), and *aadA1* gene detected in 3 isolates (15%). **Table 8** shows isolates that co-harbored multiple resistance genes.

Table 2. Demographic data and co-morbidities among the studied groups.

		Groups			Test of significance		
		Group I: Severe COVID-19 with pneumonia N=50	Group II: Severe COVID-19 without pneumonia N=50	Group III: Stable COVI-19 N=30			
		Mean ± SD N (%)	Mean ± SD N (%)	Mean ± SD N (%)	Value	*p*-value	Significance
Age		58.78 ± 11.3	56.96 ± 12.8	57.6 ± 15.08	f= 0.257	0.774	NS
Gender	Male	41 (82%) [a]	40 (80%) [a]	15 (50%) [b]	X^2= 11.534	0.003	S
	Female	9 (18%) [a]	10 (20%) [a]	15 (50%) [b]			
Co-morbidities	DM	23 (46%)	16 (32%)	6 (20%)	χ^2 = 5.85	0.054	NS
	Hypertension	23 (46%)	19 (38%)	13 (43.3%)	χ^2 = 0.67	0.715	NS
	COPD	14 (28%)	8 (16%)	5 (16.6%)	χ^2= 2.59	0.274	NS

* One Way ANOVA test of significance (f)
* Chi-Square test of significance (X^2).
* Each subscript letter denotes a subset of Group categories whose column proportions do not differ significantly from each other at the .05 level. * DM: Diabetes Mellitus, COPD: chronic obstructive airway disease

Table 3. Need for ventilation and patients' outcomes among critically ill patients in group I and group II.

		Groups		Chi-Square test		
		Group I: Severe COVID-19 with pneumonia	Group II: Severe COVID-19 without pneumonia			
		N (%)	N (%)	X^2	*p*-value	Significance
Ventilation	No	22 (44%)	41 (82%)	15.487	<0.001	S
	Yes	28 (56%)	9 (18%)			
Outcome	Survivor	5 (10%)	41 (82%)	52.174	<0.001	S
	Non Survivor	45 (90%)	9 (18%)			

Table 4. Comparison of laboratory data between studied groups.

	Groups			Test of significance		
	Group I: Severe COVID-19 with pneumonia	Group II: Severe COVID-19 without pneumonia	Group III: Stable COVI-19			
	Median (IQR)	Median (IQR)	Median (IQR)	H	*p*-value	Significance
WBCs K/UL	14.5 (10.5 - 22.2)	11.2 (8.9 - 13.7)	6.85 (4.5 - 10.5)	34.777	<0.001$^{(K1)}$	S
Nu K/UL	12.55 (10.1 - 20.6)	9.7 (6.5 - 12.3)	5 (2.8 - 9.1)	40.088	<0.001$^{(K1)}$	S
LY K/UL	0.8 (0.3 - 1.3)	0.9 (0.7 - 1.5)	1.05 (0.9 - 1.5)	8.381	0.015$^{(K2)}$	S
EO K/UL	0 (0 - 0.1)	0.1 (0 - 0.1)	0.1 (0.1 - 0.2)	20.900	<0.001$^{(K2)}$	S
Platelets K/UL	166 (52 - 237)	305 (213 - 404)	265.5 (225 - 336)	24.448	<0.001$^{(K2)}$	S
AST U/L	63 (44 - 84)	37.5 (30 - 58)	41 (28 - 49)	18.015	<0.001$^{(K2)}$	S
ALT U/L	51 (30 - 78)	40 (27 - 70)	40 (27 - 48)	3.191	0.203	NS
S. Cr Umol/L	157.5 (99 - 275)	80.5 (66 - 125)	67.5 (53 - 80)	36.849	<0.001$^{(K2)}$	S
BUN mmol/L	17.4 (10.6 - 31)	8.25 (6.1 - 12.7)	4.95 (3.8 - 6.3)	50.705	<0.001$^{(K1)}$	S
CRP mg/L	165.4 (79.8 - 231)	63.5 (36.9 - 132)	20.2 (15.7 - 28)	59.995	<0.001$^{(K1)}$	S

$^{(K)}$ Kruskal Wallis test of significance (H).
*Post-hoc test was significant between: $^{(K1)}$ Between all groups. $^{(K2)}$ Group I Vs. (group II and group III).

Table 5. Comparison of Ct value between studied groups and between survivors and non survivors in groups I and II.

Groups	**Group I: Severe COVID-19 with pneumonia N=50**		**Group II: Severe COVID-19 without pneumonia N=50**		**Group III: Stable COVI-19 N=30**	
	Mean ± SD		**Mean ± SD**		**Mean ± SD**	
Ct value Mean ± SD	22.4 ± 3.5		28.5 ± 6.1		30.3 ± 3.5	
	***One Way ANOVA**					
Significance	**f** =34.001		**p-Value** <0.001		**Significant**	
Outcome	**Survivor** N=5	**Non Survivor** N=45	**Survivor** N=41	**Non Survivor** N=9	**Survivor** N=30	**Non Survivor** N=0
Ct value Mean ± SD	24.2 ± 1.8	21.02 ± 1.9	30.2 ± 4.9	20.3 ± 2.3	30.3 ± 3.5	-
Student t-test	**t** = 3.464 ***p*-value** <0.001 **S**		**t** = 8.971 ***p*-value** <0.001 **S**		NA	

*Post-hoc Bonferroni test was significant between: Group I Vs. (group II and group III).

Table 6. Detected MDR organisms and cause specific mortality rate within group I.

	Secondary Pneumonia Causative Pathogen/ Mortality Rate			
	MDR Organisms	**N of isolates (%) (Total 51)**	**Non survivors (Total 45)**	**Cause Specific Mortality Rate**
Group I: Severe COVID-19 with secondary pneumonia	*A. baumannii*	20 (39.2%)	19	38%
	*C. koseri**	5 (9.8%)	5	10%
	E. coli	5 (9.8%)	3	6%
	*E.cloacae**	4 (7.8%)	4	8%
	K. pneumonia	6 (11.7%)	4	8%
	*P. mirabilis**	4 (7.8%)	4	8%
	S. aureus	7 (13.7%)	6	12%

** Citrobacter koseri (C. koseri), Enterobacter cloacae (E.cloacae), and Proteus mirabilis (P. mirabilis).*

Table 7. *Acinetobacter baumannii* antimicrobial susceptibility testing results.

Antibiotics			**CAZ**	**CRO**	**CPM**	**PTZ**	**IMI**	**MEM**	**GM**	**AK**	**CIP**	**LEV**	**TS**	**Colistin**
A. baumannii **Total = 20 isolates**	**S**	**N/ %**	3 (15%)	2 (10%)	4 (20%)	5 (25%)	2 (10%)	1 (5%)	2 (10%)	2 (10%)	2 (10%)	1 (5%)	5 (25%)	19 (95%)
	I	**N/ %**	1 (5%)	2 (10%)	1 (5%)	1 (5%)	0 (0%)	1 (5%)	1 (5%)	0 (0%)	2 (10%)	1 (5%)	3 (15%)	0 (0%)
	R	**N/ %**	16 (80%)	16 (80%)	15 (75%)	14 (70%)	18 (90%)	18 (90%)	17 (85%)	18 (90%)	16 (80%)	18 (90%)	12 (58%)	1 (5%)

Table 8. *Acinetobacter baumannii* isolates showing simultaneous detection of multiple resistance genes.

Detected genes patterns	**Number**	**Percentage**
bla$_{\text{OXA-51}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{ADC}}$, *bla*$_{\text{CIT}}$, *aph6*, *aphA1*	2	10%
bla$_{\text{OXA-51}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{ADC}}$, *bla*$_{\text{CIT}}$, *aph6*	1	5%
bla$_{\text{OXA-51}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{KPC}}$, *aacC1*, *aacA4*, *acc(6')*, *aadA1*	1	5%
bla$_{\text{OXA-51}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC}}$, *aacC1*, *aacA4*, *acc(6')*	1	5%
bla$_{\text{OXA-51}}$, *bla*$_{\text{OXA-48}}$, *aacC1*, *aacA4*, *acc(6')*	1	5%
bla$_{\text{OXA-51}}$, *aacC1*, *acc(6')*, *aadA1*	2	10%

Figure 2. Percentage of MDR organisms detected among group I patients.

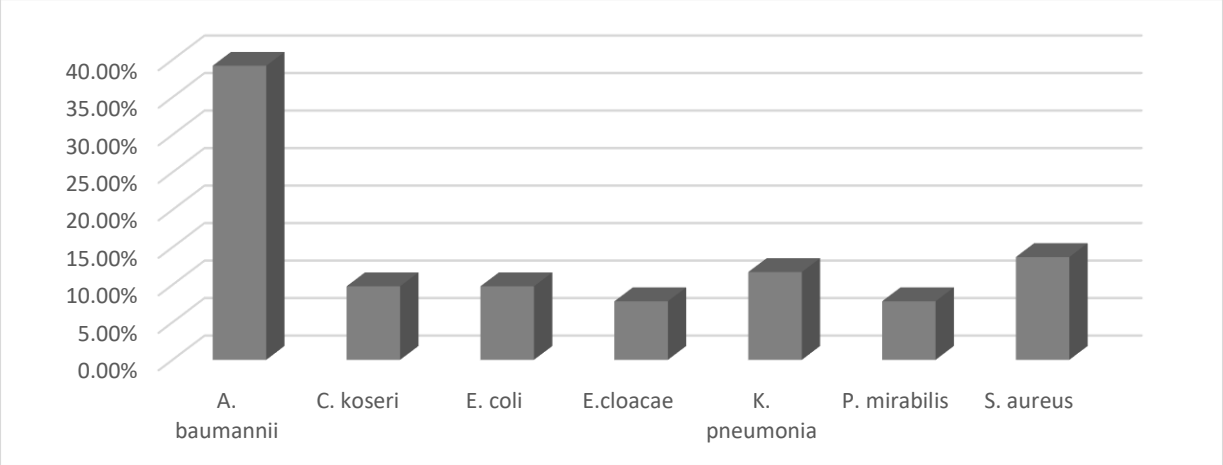


Figure 3 a & b. *bla*$_{\text{OXA-51}}$ gene (353 bp) in all of the 20 isolates of *A. baumannii*

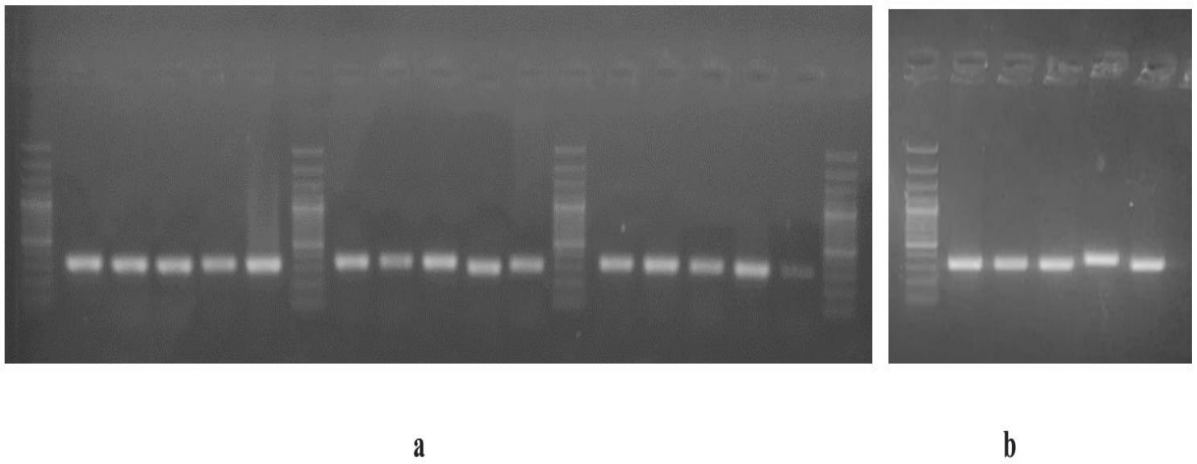


Figure 4. A. OXA-type genes M-PCR positive for *bla*$_{\text{OXA-24}}$ gene (246 bp). b, c, & d S-PCR positive for *bla*$_{\text{OXA-48}}$ gene (389 bp).

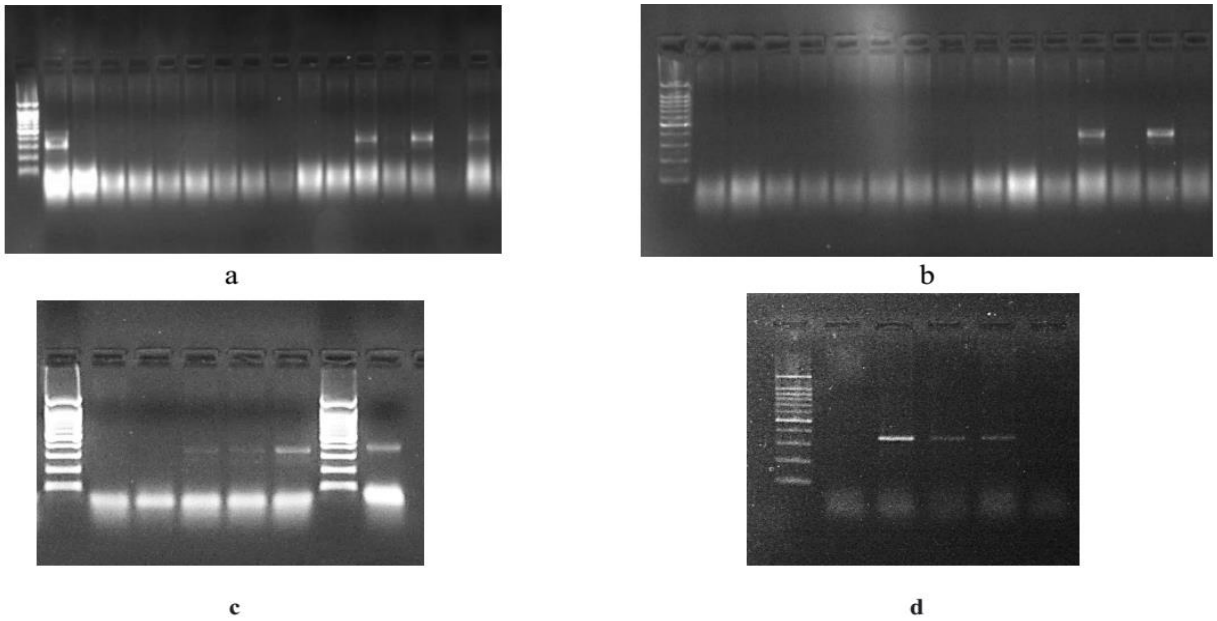


Figure 5. A. AmpC genes M-PCR positive for bla_{ADC} gene (663 bp) and bla_{CIT} gene (462 bp). b. S-PCR positive for bla_{KPC} gene (538 bp).

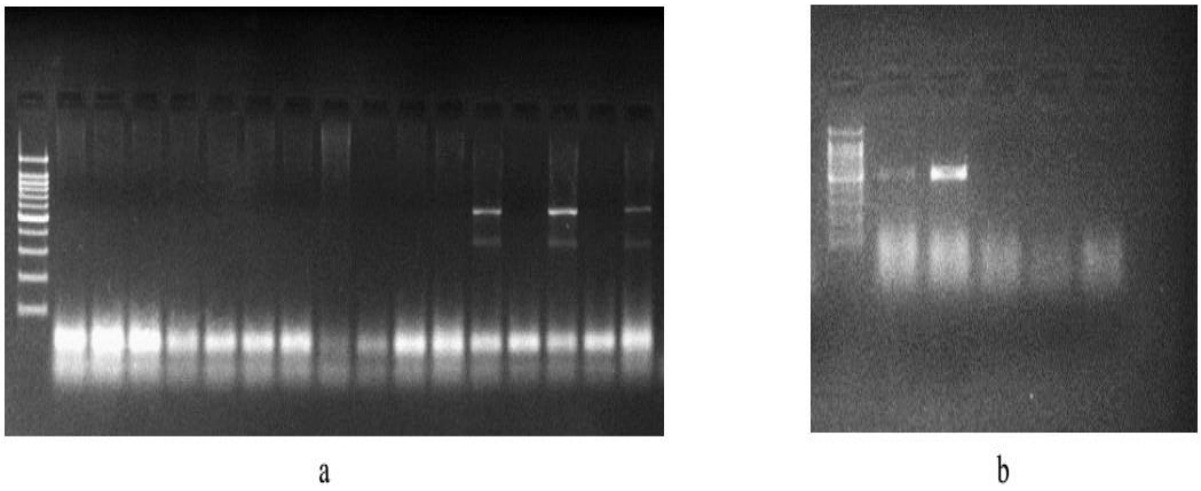
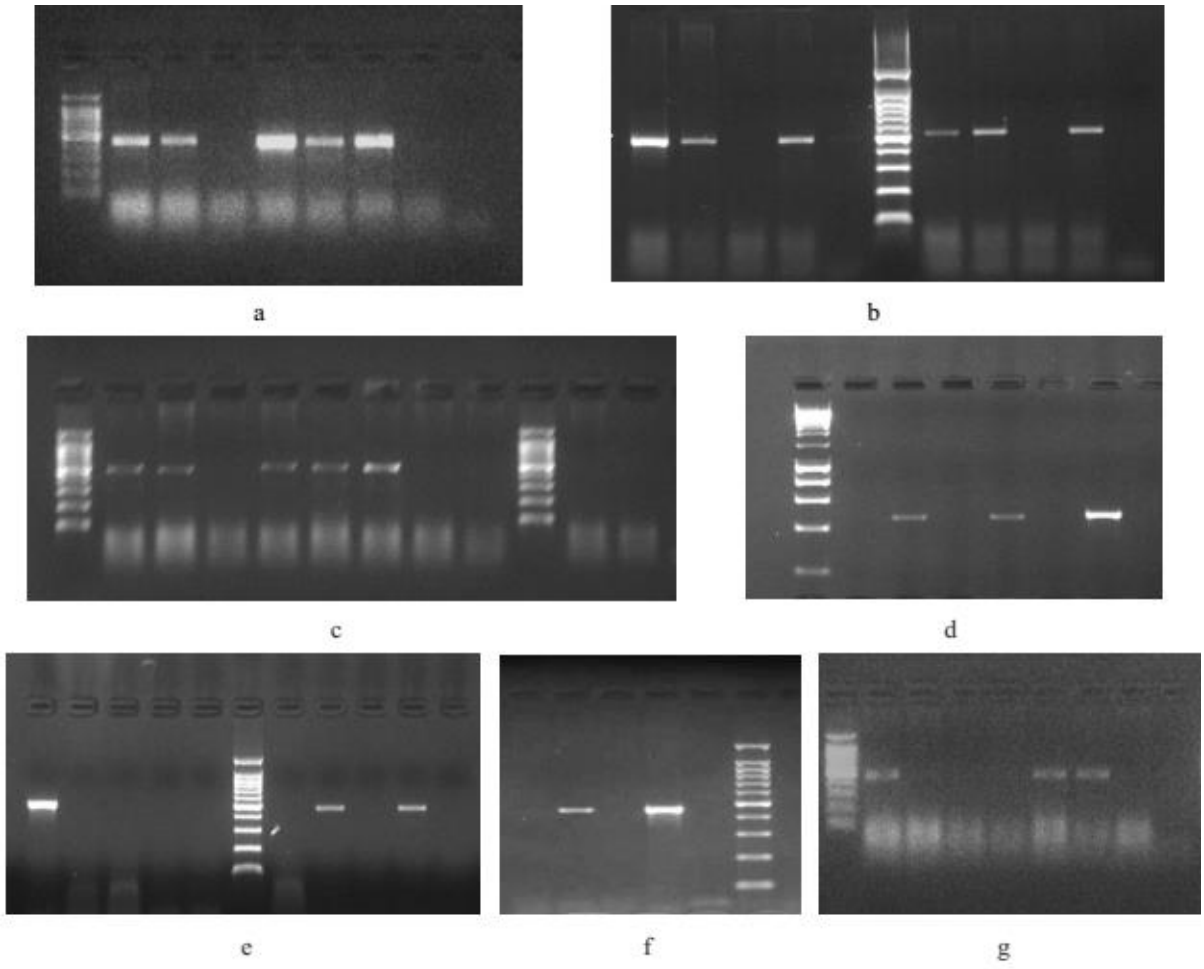


Figure 6. AMEs genes S-PCR. a. *aacC1* gene (456 bp). b. *aacA4* gene (518 bp). c. *acc(6')* gene (482 bp). d. *aph6* gene (248 bp). e &f. *aphA1* gene (461 bp). g. *aadA1* gene (527 bp).



Discussion

Several variables, including the infection severity, steroid usage, lymphopenia, and the administration of antibiotics on empirical basis, contribute to the high rate of bacterial co-infection and superinfection among COVID-19 patients. The bacterial superinfection rate is higher in critically ill patients in ICU with lymphopenia [29].

Among COVID-19 patients, persistent fever and worsening of shortness of breath are alarming indicators of a secondary bacterial pneumonia. Signs of bacterial superinfection include the sudden onset of fever, altered sputum characteristics, oxygen requirements escalation, and the appearance of new abnormalities on serial CXRs or CTS. *Pseudomonas*, *K. pneumonia*, *E. coli*, *Enterobacter species*, and *A. baumannii* are the main cause of late superinfections [18]. With the rise in MDR and XDR *A. baumannii* strains, this pathogen has become a threatening hazard for ICU patients [30].

The present study was conducted on 50 critically ill patients with acute severe COVID-19 with evidence of secondary MDR bacterial pneumonia, 50 critically ill patients with acute severe COVID-19 without evidence of secondary bacterial pneumonia, and 30 clinically stable patients with acute moderate COVID-19 infections admitted to COVID-19 ICU and isolation ward, King Abdulaziz Hospital, Jeddah from August to October 2021. As of July 2021, four SARS-CoV-2 variants of concern (VOCs) had been announced by the WHO including alpha, beta, gamma, and delta. According to WHO, as of July 2021, the alpha variant had been reported in 178 countries, beta in 123 countries, gamma in 75 countries, and delta in 111 countries [31]. The study of **Alhamlan et al.** reported the prevalence of Delta variant (40.9%), Beta variant (15.9%), and alpha variant (11.6%) among SARS-CoV-2 strains sequenced in Saudi Arabia by the end of 2021 [32].

Among the studied groups, male gender represented the majority (≥80%) of critically ill COVID-19 patients, with and without secondary bacterial pneumonia, in groups I & II with mean age of 58.7 & 56.9 respectively. Similar observation was reported by **Alqahtani et al.** who reported that among critically ill COVID-19 patients, the average age of patients was 64 years, and 79.5% of patients were males [6].

Among critically ill patients in the present study, significantly higher percentage of mechanically ventilated patients (56%) was observed among critically ill patients with evidence of secondary MDR bacterial pneumonia in group I which was also associated with a higher mortality rate (90%) compared to group II patients (18%). This was in accordance with the results of **Sadigov et al.** that showed that the rate of bacterial pneumonia is high in critically ill patients with COVID19 and that this risk was maximized with increasing COVID-19 severity and patients' intubation [22]. Also, the results of **Alqahtani et al.** showed that individuals with COVID-19 who presented with bacterial coinfections were at higher risk for a longer ICU stay and higher mortality rate of 50% compared to only 18.7% among patients with COVID-19 infection alone [6].

Comparing laboratory data between groups of the present study showed a significant difference between group I patients compared to groups II and III as regards all data except ALT serum level.

Critically ill COVID-19 patients with secondary bacterial pneumonia showed the lowest lymphocyte and eosinophile counts which relates to the severity of COVID-19 among those patients' group. Also, among this group, higher hepatic and renal function tests were detected which was in agreement with the results of **Dudoignon et al.** who reported that critically ill COVID-19 patients with VAP had more respiratory distress, more renal insult, mechanically ventilated longer, and had a longer ICU stay [33].

In the present study, a significantly lower mean Ct value was detected among patients of group I compared to patients of group II and group III. Also, a significant difference was observed between survivors and non survivors as regards mean Ct value among patients of group I (24.2 & 21.02 respectively) and group II (30.2 & 20.3 respectively). which agreed with **Kurzeder et al.** who reported that Ct value ≤26 was a significant predictor for death among COVID-19 patients [34].

In the present study, among critically ill COVID-19 patients with secondary MDR bacterial pneumonia in group I, 28 patients were identified as VAP. Among whom, 4 patients had early onset VAP caused by *S. aureus* (n=3) and *K. pneumonia* (n=1), and 24 patients had late onset VAP caused by *A. baumannii* (n=18), *K. pneumonia* (n=3), *E.coli* (n=2), and *S. aureus* (n=1). Similarly, the results of

Dudoignon et al. showed that Gram-positive bacteria were the main pathogens in early-onset VAP, and that gram-negative bacteria including 8 nonfermenting bacilli and 4 *Enterobacterales* were the major causative pathogens in late-onset VAP [33].

The most prevalent pathogen, among group I patients, was MDR/XDR *A. baumannii* which was isolated from 40% (20 patients) of critically ill COVID-19 patients with secondary MDR bacterial pneumonia, with the highest cause specific mortality rate (38%). This was in line with the results reported by **Ceparano et al.** showing a cumulative incidence of *A. baumannii* acquisition of 36.8%. among COVID-19 patients admitted to the ICU with higher mortality and length of ICU stay [30].

The highest antimicrobial resistance rates, among the 20 *A. baumannii* isolates in the present study, were observed with carbapenems (90%), amikacin (90%) and gentamicin 85%, while 95% of the isolates were sensitive to colistin. Among the 20 *A. baumannii* isolates, 3 isolates (15%) were MDR and 17 isolates (85%) were extensively drug resistant (XDR). Similar susceptibility pattern was observed by **Camargo et al.** who reported that *A. baumannii* causing bacterial infections among COVID-19 patients showed high resistance rates against all the antimicrobial agents tested including carbapenems, amikacin and gentamicin (99.3%, 97%, 94.9% respectively), but not against polymyxin B (100% susceptibility) [35]. Also, the results of **Ceparano et al.** and **Shinohara et al.** showed that, among COVID-19 patients investigated, all *A. baumannii* isolates were resistant to gentamicin, meropenem, imipenem, and ciprofloxacin, while they were all susceptible to colistin [30, 36]. High carbapenem resistance (92.6%) was observed by **Syed et al.** and it was associated with high mortality rate of 98.2%, while all the isolates were sensitive to colistin [37].

Acinetobacter baumannii has the ability to gain antimicrobial resistance to cephalosporin, carbapenem, aminoglycoside, and fluoroquinolone. In *Acinetobacter* species, carbapenem resistance is usually caused by acquired carbapenemase synthesis. The most prevalent Class D β-lactamases are OXA-23-like, OXA-24/40-like, OXA-58-like, OXA-143-like, and OXA-235-like groups, besides the intrinsic chromosomal OXA-51-like group [38].

Multiple AMEs, including aminoglycoside acetyltransferases (AAC), aminoglycoside phosphotransferases (APH), and aminoglycoside nucleotidyltransferases (AAD), are involved in *A. baumannii* resistance mechanisms. This class of enzymes is widely spread due to the presence of its genes on mobile genetic elements as transposons and plasmids. The synthesis of *AAC(3)-I*, *APH(3')-VI*, and *ANT(3")-I* is predominate, but there are significant regional differences in their genotypes [28].

Among the 20 *A. baumannii* isolates in the present study, identification was confirmed with the detection of *bla*OXA-51 gene, intrinsic in *A. baumannii* species, in 100% of the isolates. The detected OXA-type class D carbapenemases included the *bla*OXA-48 gene, detected in 8 isolates (40%), and the *bla*OXA-24 gene, detected in 4 isolates (20%). As regards other β-lactamases classes, AmpC genes including *bla*ADC gene and *bla*CIT gene were simultaneously detected in 3 isolates (15%). Also, the *bla*KPC gene was detected in 2 (10%) of the *A. baumannii* isolates. The AMEs genes detected among *A. baumannii* isolates were *aacA4* gene, *aacC1* gene, *acc(6')* gene, *aphA1* gene, *aph6* gene, and *aadA1* gene which were detected in 30%, 25%, 25%, 25%, 15%, and 15% of the isolates respectively. In line with the results of the present study, **Camargo et al.** investigated the clonal spread of MDR *A. baumannii* strains among COVID-19 patients in Brazil and reported that all the isolates were highly resistant (>95%) to aminoglycosides, fluoroquinolones and beta-lactams. However, they detected 2 OXA-type class D carbapenemases genes different than those detected in the present study including *bla*OXA-23 (85.4%) and *bla*OXA-72 (11.7%). They also detected several aminoglycosides resistance genes including *armA*; *ant(2")-Ia*; *aadA1*; *aph(3')-VIa*; *aph(6)-Id*; and *aph(3")-Ib*35. Among which, only the *aadA1* gene was detected in the present study. This may be due to different geographical distribution of the isolates investigated in the present study being epidemiologically originating in the middle east region.

In the present study, 8 MDR/XDR *A. baumannii* isolates (40%) co-harbored multiple resistance genes and 2 isolates (10%) were positive only for the intrinsic *bla*OXA-51 gene. None of the tested isolates were positive for *bla*OXA-23, *bla*OXA-58, *bla*OXA-143, *bla*OXA-235, *bla*MOX, *bla*DHA, *bla*ACC, *bla*EBC, *bla*FOX, *bla*NDM, and *aph(3')-IIb* genes. Similarly, the study of **Palavecino et al.** evaluated the prevalence of different β-lactamases genes among carbapenem

resistant *A. baumannii*. All the isolates met the definition of MDR phenotype. They reported the detection of *bla*OXA-23 (67%), and *bla*OXA-24/40 (14.9%). None of their isolates were positive for *bla*OXA-58, *bla*NDM, *bla*KPC, *bla*IMP, *bla*VIM, or *bla*OXA-48 genes and 18.1% of the isolates were negative for all carbapenemases genes tested in the study [39]. They concluded that, since those isolates also showed an MDR phenotype, this might be explained by other possible resistance mechanisms such as the loss or modification of the carbapenem-associated outer membrane protein or modification of penicillin-binding protein [40].

Conclusion

Secondary MDR bacterial pneumonia had a significant impact on critically ill COVID-19 patients' outcomes with multiple systems organ affection and was associated with significantly higher mortality rate. MDR-GN constituted the major pathogens detected with MDR/XDR *A. baumannii* counted as the most prevalent pathogen. Multiple genetic determinants of antibiotic resistance were detected among MDR/XDR *A. baumannii* isolates. However, further molecular studies on larger scale would help clarify the clonal relatedness between the prevalent MDR/XDR strains spreading in COVID-19 ICUs. Thus, preventing secondary MDR bacterial pneumonia through infection prevention measures, including standard precautions, infection prevention care bundles and antimicrobial stewardship programs, should be strictly implemented to protect critically ill COVID-19 patients and help avoid its detrimental effect on patients' outcomes.

Conflict of interest

The authors report no conflicts of interest.

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