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RESEARCH ARTICLE

Prevalence and Antibiotic Resistance Profile of Secondary Bacterial Pneumonia and Bloodstream Infection Among Hospitalized COVID-19 Patients and Their Relation to Procalcitonin

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ABSTRACT

Background and Objectives: in COVID-19 patients, secondary bacterial infections (SBIs) are a known complication of viral respiratory infections and are significantly associated with poorer outcomes.Our study aimed to determine the prevalence, clinical profile, antimicrobial resistance profile, and patient outcomes of secondary bacterial pneumonia and blood infections in COVID-19 hospitalized patients and their correlation with procalcitonin (PCT) levels.

Method: During 7 months' study, 260 clinical samples (blood and respiratory specimens) were collected from 130 hospitalized COVID-19 patients, of which 90 were from intensive care units (ICUs) and 40 from non-ICU departments, at six hospitals in Erbil city, Iraq. All samples were applied for bacterial identification via traditional method, Vitek-2 compact system, and molecular (PCR) detection. The antibiotic resistance profile was obtained via Vitek-2 compact system and the Standard International Kirby-Bauer disc diffusion method. Finally, the inflammatory biomarkers (PCT, C-reactive protein CRP, and WBC count) were evaluated.

Results: Among 130 patients, 64.16% were positive for SBIs, of which 86.9% from the ICU and 13.1% from the non-ICU department. The most prevalent isolates were gram-negative (77.7%) versus gram-positive (22.3%) bacteria. *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and coagulase-negative staphylococci were the predominant isolates in both blood and respiratory specimen. There was an obvious relation between mortality rate and SBIs in the studied patients, which reached 81%. Most of the isolated bacteria, especially ICU isolates, were multidrug resistant. PCT increased in 79 (89.8%) of the patients with SBIs. The highest PCT level was found in patients with bloodstream infection.

Conclusion: There is a high prevalence of bacterial superinfections in COVID-19 patients during hospitalization. Gram negative bacteria, especially *Klebsiella pneumoniae* and *Acinetobacter baumannii* were the main bacteria, and the antimicrobial resistance rates against the major isolated bacteria were generally high. The PCT level was positively associated with secondary bacterial infection and patient outcome.

Keywords: Covid-19, Secondary bacterial infections, Antibiotic resistance, Procalcitonin

INTRODUCTION

Coronavirus disease-2019 (COVID-19) is a highly contagious respiratory infection caused by sever acute respiratory syndrome coronavirus2 (SARS Cov2) which is a single stranded RNA virus (Wang et al., 2020, Hui et al., 2020). It was first reported in December 2019 in Wuhan, China (Huang et al., 2020), then spread rapidly to the majority of countries worldwide .This disease was declared a global pandemic by the World Health Organization (WHO) on March 11, 2020 (Organization, 2020).

Epidemiological data recorded more than 503.148 million COVID infection cases and over 6,223 million deaths by 14 April 2022 worldwide, including more than 2,322 million infections and 25,192 thousand deaths in Iraq (World Health Organization, 2022, Worldometer, 2022).

Pandemic COVID 2020 has a wide range of clinical manifestations, which are ranged between asymptomatic to severe pneumonia that might result in death (Guan et al., 2020). Fortunately, the majority of infections present mild to moderate symptoms including fever, dry cough, and fatigue (Wang et al., 2020, Hui et al., 2020) , whereas acute respiratory distress syndrome (ARDS) and respiratory failure are the major clinical challenges that may require intensive care unit (ICU) for prolonged hospitalization, mechanical ventilation (Lai et al., 2020).

Bacterial infection is associated to respiratory viral infection such as bacterial coinfection with influenza viruses (Morris et al., 2017, Morens et al., 2008) and SARS COVID-2 (Li et al., 2020, Falcone et al., 2021) it has been described that secondary bacterial infections (SBIs) could increase mortality by increasing the risk of death. Corona virus invasion inhibits immune system and initiates hyperinflammatory phase in multiple organs, especially lungs (Bakaletz, 2017, Mirzaei et al., 2020, Hanada et al., 2018) . Hence, it could result in alteration of lung microbiota which causes secondary bacterial infection. According to previous studies, 3.1-3.5% of COVID-19 patients had bacterial co-infections upon admission, while up to 15% of COVID-19 patients who were hospitalized had secondary bacterial infections (Garcia-Vidal et al., 2021, Langford et al., 2020, Zhou et al., 2020, Li et al., 2020). Zhou et al. (2020) showed that, in a cohort, 50% of non-survivors of COVID-19 had a secondary bacterial infection.

Several bacterial species are associated to ICU admission and mortality rate in hospitalized COVID-19 patients (Contou et al., 2020). The most common detected pathogenic bacterial species were Klebsiella pneumoniae, Acinetobacter sp. and Pseudomonas sp. (Sharifipour et al., 2020).

Various inflammatory biomarkers are related to severity COVID infection, including C reactive protein (CRP), ferritin, lymphocyte count and procalcitonin (PCT) (Lippi and Plebani, 2020, Malik et al., 2021). PCT is dramatically elevated in response to bacterial induced cytokines, such as TNF alpha, IL-1and IL-6 and (Christ-Crain et al., 2006). PCT is considered as helpful biomarker for detection of respiratory tract bacterial infection in COVID-19 patients (Guan et al., 2020, Feng et al., 2020). Furthermore, it can be used as biomarker for evaluation of antibiotic efficiency against invaded bacteria (Christ-Crain et al., 2006, Mewes et al., 2019).

Therefore, the main goal of our study is to identify the prevalence, clinical profile, and outcomes of secondary bacterial pneumonia and blood infections in patients hospitalized with COVID-19 in Erbil city- Iraq, evaluate the antimicrobial resistance profiles of these infections, as well as testing the correlation with PCT levels by comparing with other commonly used biomarkers in predicting microbiologically proven secondary bacterial infections.

SUBJECTS AND METHODS

Detection of secondary bacterial infections

Three clinical diagnostic criteria were used to predict bacterial superinfection in COVID-19 patients: A purulent sputum with a persistent fever (> 38.3°C), deteriorating white blood cell count than normal (leukocytosis or leukopenia), an increase in procalcitonin or C-reactive protein and positive radiologic signs (Cataño-Correa et al., 2021). When all three diagnostic criteria were met, lower respiratory tract and blood specimens were taken to determine the etiological agent of the possible secondary bacterial infection.

Patient's specimens

A total of 260 clinical samples were included, including 130 lower respiratory samples and 130 blood samples from the same 130 patients. The 130 lower respiratory samples include [75 endotracheal aspirates, 5 bronchoalveolar lavage (BAL), and 50 sputum samples]. After instructing patients to rinse their mouths with water and expectorate with the help of a deep cough in the early morning, sputum samples were collected and placed directly into a labeled and sterile wide-mouth screw cap container. A pulmonologist aspirated bronchial wash samples in the bronchoscopy unit and collected them in a labeled, sterile screw-cap container (Mahon et al., 2018). Endotracheal aspirates of patients requiring invasive mechanical assistance (tracheal intubation, tracheotomy) were obtained (Green and Liddy, 2020). In addition, 130 blood samples were collected for blood culture in BACT/ALERT®

aerobic and anaerobic blood culture bottles from patients with a clinical suspicion of a secondary bacterial infection who were admitted to the COVID-19 ICU and Non-ICU departments from six different hospitals in Erbil City of Kurdistan Region, Iraq, for the period from August 2021 to March 2022.A questionnaire form was designed, including: age, gender, date of admission, presence of comorbidities (if any), length of hospital stays vaccinated or not, smoker or not, antibiotics administered, and patient outcome for all patients admitted to ICU and non-ICU departments from those hospitals that are specialized for COVID-19. The samples were submitted to the microbiology laboratory for these hospitals. All adult (≥18 years old) patients with laboratory-confirmed COVID-19 (RT-PCR positive on a nasopharyngeal or oropharyngeal swab) were included. Only samples that were collected from patients ≥ 48 h after admission to exclude potential community-acquired infection, those who had respiratory cultures positive for fungal and blood cultures positive in one or a single bottle within a set for skin normal flora (ie, coagulase-negative staphylococci [CoNS], micrococcus, gram-positive bacilli) were excluded.

Bacterial isolation and identification

All the sputum specimens or a pellet of centrifuged BAL specimens were handled according to good microbiological laboratory practice, and all the blood samples were incubated in a Bact/ALERT® 3D system (BioMerieux, France) based on guidelines from the Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2007). Later on, both respiratory samples and positive blood bottles were cultured on each of the Blood, MacConkey, and Chocolate agars (Oxoid, UK) and incubated under aerobic conditions for 18–24 hours at 37 °C. A positive anaerobic bottle was inoculated on blood and chocolate agar using a candle jar for anaerobic conditions. Isolated bacteria were identified using conventional methods according to microbiology standard techniques (Hall, 2013), including gram staining, cultural characteristics, biochemical tests, and sensitivity to some antibiotic disks. For more confirmation, the identification was performed by both the VITEK 2 Compact system from bioMérieux, France (Pincus, 2006) and the molecular approach of the PCR Max test.

Molecular Detection

DNA extraction

Genomic DNA for each isolated strain of some common bacteria was obtained. A single bacterial colony from an 18-24 hours subculture plate was inoculated into a tube containing 5 ml of Luria-Bertani (LB) broth and incubated cultured in Luria-Bertani (LB) broth in an orbital shaker (37°C, with 180 rpm, overnight). A commercial kit was used to extract the bacterial DNA in accordance with the instructions provided by the kit's manufacturer (Add bio, Korea).The extracted genomic DNA was kept in a freezer at -20 °C for further usage (Brewster and Paoli, 2013).

PCR Assay

PCR amplification was performed using a thermal cycler machine (Alpha PCR, UK) for further identification of the isolated bacteria through the detection of one target gene that related to some commonly isolated bacteria according to the following mixture: 0.5 µL of each primer,3 µL of DNA, 12.5 µLof a master mix (Amplicon, Denmark), then sterile distilled water was added to the final volume of 25 µL. All primers and programs were shown in (Tables 1and 2), which were purchased from (Macrogen /South Korea). Finally, the PCR products were separated on 1.5% agarose gel, contained safe stain in TBE buffer. The amplicons' sizes were confirmed by comparing them to a 100-bp DNA ladder (SMOBiO, Taiwan) and DNA bands were visualized with UV- transilluminator. All the reference strains ATCC of *Staphylococcus aureus (S. aureus,* 25923), *Klebsiella pneumonia (K. pneumonia,* 13883), *Acinetobacter baumannii (A. baumannii, 19606), Pseudomonas aeruginosa (P. aeruginosa,* 27853*), and Escherichia coli (E. coli*, 25922) were provided from Erbil's Media Diagnostic Center, and used as the positive control for the (16S rRNA and nuc),16SrRNA, bla OXA-51,16SrDNA, uspA respectively, and distilled water (DW).

Antibiotic Susceptibility Testing (AST)

Susceptibility testing to different antibiotics was performed by the VITEK 2 system for all isolated bacteria according to the manufacturer instructions, using specific cards AST-P580 for Gram-positive bacteria and AST (GN-204, GN-326) for Gram-negative bacteria. To detect MRSA strains, the VITEK2 cefoxitin screen test was used (Karataş et al., 2021). According to the updated CLSI 2019, the results of Vitek susceptibility testing were obtained as MIC values and classified as susceptible, intermediate, or resistant (Wayne, 2019), and the standard international Kirby-Bauer disc diffusion method was also applied for confirmation of MRSA, extend spectrum beta-lactamase (ESBL) test, and

some further antimicrobial susceptibility testing for the isolated bacteria. This method was performed on Mueller-Hinton agar (Oxoid, UK) plates and incubated for 18–24 h at 35°C, then the results were compared with guidelines from the Clinical and Laboratory Standards Institute (CLSI 2019). As quality control strains, *E. coli ATCC* 25922 *and S. aureus ATCC* 25923 were used.

Table 1. Primers used for the identification of some common bacterial species by PCR.

Gene name	Nucleotide sequences (5' to 3')	Bacterial Strains	Amplicon size(bp)	References
16S rRNA-F **16S rRNA-R**	GTTGACTGCCGGTGACAAAC GCTGTTACGACTTCACCCCA	*Staphylococcus Sp.*	372	(Hasan and Hoshyar, 2019)
nuc gene-F **nuc gene-R**	AGCCAAGCCTTGACGAACTAAAGC GCGATTGATGGTGATACGGTT	*S. aureus*	279	(Kalorey et al., 2007)
16S rRNA-F **16S rRNA-R**	GGT GAA TAC GTT CCC GG TAC GGC TAC CTT GTT ACG ACT T	*K.pneumonia*	144	(Shimpoh et al., 2017)
blaOXA-51- F **blaOXA-51-R**	TAA TGC TTT GAT CGG CCT TG TGG ATT GCA CTT CAT CTT GG	*A.baumannii*	353	(Turton et al., 2006)
16SrDNA -F **16SrDNA-R**	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	*P.aeruginosa*	956	(Megahed et al., 2015)
uspA-F **uspA-R**	CCG ATA CGC TGC CAA TCA GT ACG CAG ACC GTA GGC CAG AT	*E. coli*	884	(Chen and Griffiths, 1998)

Table 2. The PCR program used for amplifying genes for some common bacteria in this study.

Target gene	Amplified segment (bp)	Primary denaturation (°C/min)	Second denaturation (°C/sec)	Annealing (°C/sec)	Extension (°C/sec)	Final extension (°C/min)	Cycles
16S rRNA (*Staph.Sp.)*	372	94/5	95/30	59/45	72/40	72/5	35
nuc gene	279	94/5	94/180	58/30	72/45	72/10	30
16S rRNA *K.pneumonia*	144	95 /7	95/30	57/30	72/30	72 /7	35
blaOXA-51	353	94/3	94/45	57/45	72 /60	72/5	35
16SrDNA *P.aeruginosa*	956	94/10	94 /60	50/60	72 /60	72/12	35
uspA	884	95/ 5	95/30	57/45	72/60	72/10	40

Biological markers

In this study, the biological marker tests conducted for suspected bacterial infection included procalcitonin by using an automated rapid sensitive assay (Elecsys Brahms PCT on Roche Cobas E411, Germany, measuring range 0.02-100 ng/ml and human PCT serum levels are normally less than 0.1 ng/ml, but levels greater than 0.25 ng/ml may indicate a bacterial infection. CRP in serum were measured using the Cobas C111 (Roche Diagnostics) equipment, and normal values for adults being less than 5.0 mg/L, and the WBC count was performed by an automated CBC analyzer (Sysmex XP-300, Japan) with a normal range (3 - 11 × 10^9 /L) according to (Covington et al., 2018, Taha et al., 2022, Ali et al., 2022, Ahmed et al., 2021) respectively.

Statistical analysis

Data analysis was performed via using GraphPad prism version 9.0.0. The demographic characteristics and some other data were expressed as percent values. An unpaired t-test was applied for comparison between SBIs and non-SBIs patient samples. A one-way analysis of variance with Tukey's post-hoc tests was used for comparison between groups of patients according to their origin and gram of bacteria. The comparison data were expressed as mean and standard error.

RESULTS

Patients' clinical and demographics characteristics

Out of 130 patients, 46 patients had no secondary bacterial infection, while 84 patients have developed secondary bacterial infections (Table 3). There were 11 patients (13.1%) in non-ICU departments and 73 patients (86.9%) in ICU. Of the 73 ICU patients, 36 (85.7%) were males, and 37(88.1%) were female. Of the 11 non-ICU patients, 6 (14.3%) were male and 5 (11.9%) were female. (2/84) of the patient vaccinated admitted to ICU(Table4).

The most common comorbidities for Non-ICUs and ICU patients were; hypertension 5 (16.7%), 25 (83.3%), and diabetes mellitus 4 (23.5%), 13 (76.5%), respectively. Among the 84 positive cultures, 57 (67.9%) patients had only a respiratory tract infection, 14 (16.7%) patients had only a circulatory tract infection, and 13 (15.5%) patients had both a respiratory tract and a circulatory tract infection with the same or different organisms. The distribution ratios of respiratory and circulatory tract infections in ICUs and non-ICUs are shown in Table 4.

The patients with SBIs had a median age of 62 years, while for patients without SBIs, it was 55 years (Table 3). The median age for non-ICU and ICU patients were 58 years (with range between 37-80) and 61 years (with range 22-85) respectively (Table4).

For SBIs patients, the average length of stay in the hospital was 18.3 days, while for patients without SBIs was 10 days (Table 3). In addition, in non-ICU patients, it was 20 days, ranging from 8 to 42 days. In contrast, 18.1 days ranged from 3 to 75 days in ICU patients (Table 4). The total number of samples positive in ICU between 3 and 7 days of stay was 12 with the rate death 10.4%, and after 7 days it was 72 with the rate death 89.5%.

Among 84 patients with SBIs in the study, 94 % of the patients received at least one antibiotic therapy within 24 hr. of admission during their COVID-19 hospitalization before first positive culture. Antibiotics were given to ICU patients more frequently than to non-ICU patients (98.6% versus 63.6%). Patients on non-ICU ,40% were given one antibiotic, most commonly meropenem followed by ceftriaxone, while 60% were given two antibiotics, meropenem and levofloxacin. Whereas 45 % of ICU patients received two antibiotics and 55% received three or more, the most commonly used antibiotic combination was piperacillin and tazobactam with levofloxacin or vancomycin, or linezolid followed by colistin with tigecycline or meropenem, or linezolid with tigecycline, or a combination of more than three of these antibiotics.

In addition, out of 84 patients, 32 (38%) were on remdesivir as antiviral therapy three. Furthermore, out of 84 patients, 829 (7.6%) were on corticosteroid therapy, 100% of them were admitted to the ICU and 81.1% were in Non-ICU with a mean of 12 days. The most common was dexamethasone, followed by pulmicort (Table 4).The death rate was 68 (81%) among the 84 COVID-19 patients whose samples were positive for SBIs, compared to 34.8 % in 46 patients who were not infected with bacteria (Table 3). The most of the patients who died from COVID-19, 91.8% (67/68), were admitted to the ICU, while patient mortality in non-ICU settings was lower, at 9% (1/68) (Table 4).

Table 3. Comparison among patients hospitalized with COVID-19s (n= 130) based on SBIs.

	Patients with bacterial infections (n=84) (64.6%)	Patients without bacterial infections (n=46) (35.4%)
Death	68 (81%)	16 (34.8%)
Discharged	16 (19%)	30 (65.2%)
ICU admission	73 (86.9%)	17 (37.1%)
Age, years (median)	62 (22 ~ 85)	55 (31~ 84)
Comorbidity	53 (63%)	22 (47.8%)
Length of study, days(mean)	18.3	10.0
PCT >0.25 ng/ml	79 (89.8%)	9 (10.2%)
CRP >5.0 mg/L	83 (99%)	45 (98%)
WBC >11× 10^9 /L	71 (84.5%)	38 (83%)

Table 4. Data on the demographics, clinical characteristics, and outcomes of hospitalized COVID-19 patients with SBIs

	All patients (*n* = 84) (100%)	NON-ICU patient (n=11) (13.1%)	ICU patient ((*n*= 73) (86.9%)
Characteristics			
Age, years (median)	62 (22 ~ 85)	58 (37- 80)	61 (22 ~ 85)
Gender			
Male	42	6 (14.3%)	36 (85.7%)
Female	42	5 (11.9%)	37 (88.1%)
Vaccination	2	0 %	2 (100%)
Comorbidities			
Diabetic mellitus	17	4 (23.5%)	13 (76.5%)
Hypertension	30	5 (16.7%)	25 (83.3%)
Heart disease	10	1 (10%)	9 (90%)
Chronic renal disease	6	1 (16.7%)	5 (83.3%)
Thyroiditis	3	3 (100%)	0%
Cancer	2	1(50%)	1 (50%)
Smoking	7	0%	7(100%)
(†) SBIs			
Blood	14	1 (9%)	13 (17.8%)
Respiratory specimen	57	10 (90.9%)	47 (64.3%)
Both	13	0%	13 (17.8%)
(†) Medications Received within 24hr of admission			
Antibiotic therapy *	79	7 (63.6%)	72(98.6%)
Corticosteroid therapy**	82	9 (81.8%)	73 (100%)
Antiviral therapy	32	7 (63.6%)	25 (34.2%)
(†) Outcomes			
Length of study, days (mean)	18.3 (3-75)	20 (8-42)	18.1 (3-75)
Discharge	16	10 (90.9%)	6 (8.2%)
Death	68	1 (9%)	67 (91.8%)

Indicates meropenem, levofloxacin, ceftriaxone, Piperacillin/Tazobactam, linezolid, colistin, tigecycline

** Indicates dexamethasone, Pulmicort

(†) The percent ratio obtained according to the columns.

The etiology of secondary bacterial infection

A total of 260 samples were obtained for microbiological culture, which included 130 for each of the respiratory and blood specimens, 70/130 were positive for the respiratory cultures and 27/130 for the blood. A total of 103 bacteria were isolated from the 97 specimens that were positive for bacteria in culture. The most common 76 isolates received were from respiratory samples (50 endotracheal aspirates, 5 bronchoalveolar lavages (BAL), and 21 sputum samples), followed by 27 isolates from blood cultures. All isolates were identified based on colony morphology on culture media, some biochemical tests, and they were confirmed by VITEK 2. For further confirmation, most frequently, isolated genera were identified by conventional monoplex PCR assays using specific genes. All isolates showed positive results for the target gene (Figures 1 and 2). Gram-negative bacteria were the most prevalent among the isolated bacteria, making up 77.7% of them, while gram-positive bacteria made up 22.3%. *K. pneumonia* was the most prevalent bacteria cultivated in respiratory tract culture, accounting for 30.3% of all bacteria, followed by *A. baumannii*, 22.4%, and *Stenotrophomonas maltophilia (S. maltophilia*, 11.8%).100% of the isolates *K.pneumonia* were admitted to the ICU. The mortality rates among patients with *K. pneumoniae and A. baumannii* infections were 100% and 95%, respectively.

Coagulation-negative staphylococci were predominant in blood cultures (37%), followed by *K. pneumonia* 18.5% (Table 5). Six (7.1%) of the 84 culture-positive patients had secondary bacterial pneumonia with mixed bacteria, mostly *K. pneumoniae* mixed with *P. aeruginosa* and two other combinations. Moreover, 13 (15.5%) patients had a bloodstream infection along with a pneumonia, among them 7 (8.3%) with the same causative agent and 5 (6%) with different causative agents. Out of the patients who died with SBIs, (48/68) 70.6% had gram-negative infections, (9/68) 13.2% had gram-positive infections, and (11/68) 16.2 % had both gram-negative and gram-positive infections mixed together.

Table 5: Etiology distribution by site of SBIs in COVID-19 hospitalized patients.

Bacteria isolated	Respiratory specimen N (%)	Blood N (%)	Total N (%)
Gram-negative	68 (89.5)	12 (44.4)	80 (77.7)
K. pneumoniae	23 (30.3)	5 (18.5)	28 (27.2)
A. baumannii	17 (22.4)	3 (11.1)	20 (19.4)
Stenotrophomonas maltophilia	9 (11.8)	2 (7.4)	11 (10.7)
Pseudomonas aeruginosa	8 (10.5)	1 (3.7)	9 (8.7)
Enterobacter cloacae	4 (5.3)	1 (3.7)	5 (4.9)
Escherichia coli	3 (3.9)	0 (0)	3 (2.9)
Others *	4 (5.3)	0 (0)	4 (3.9)
Gram-positive	8 (10.5)	15 (55.6)	23 (22.3)
S. aureus	2 (2.6)	1 (3.7)	3 (2.9)
*Coagulase- negative Staph*ylococci (CoNS)			
Staphylococcus epidermidis	0 (0)	1 (3.7)	1 (1.0)
Staphylococcus hominis	0 (0)	3 (11.1)	3 (2.9)
Staphylococcus haemolyticus	6 (7.9)	4 (14.8)	10 (9.7)
Enterococcus faecium	0 (0)	3 (11.1)	3 (2.9)
Others**	0 (0)	3 (11.1)	3 (2.9)
Total N (%)	76 (100)	27 (100)	103 (100)

* Indicates two *Proteus mirabilis*, one *Morganella morganii*, one *Sphingomonas paucimobilis.*

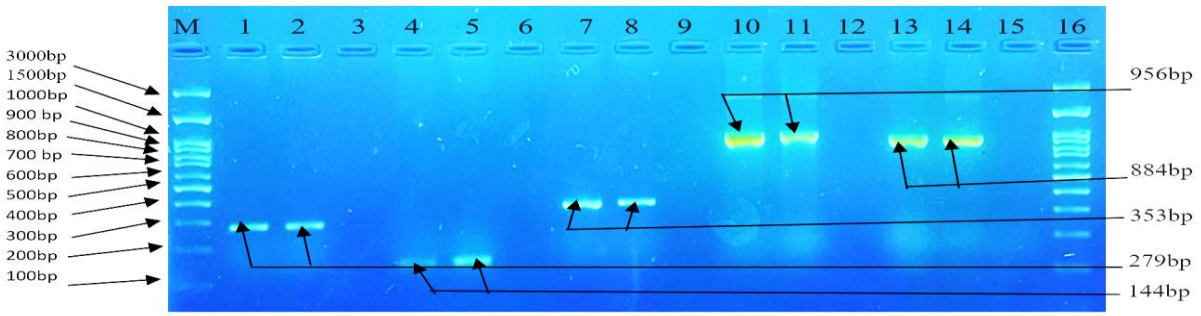

Figure 2: Amplification of PCR products

** Indicates one *Staphylococcus pseudintermidius*, one *Staphylococcus lugdunensis, one Enterococcus gallina*

Figure 2: Amplification of PCR products on an agarose gel. M: 100-3,000 bp DNA marker, **Lane 1** is a positive control for the nuc gene (*S.aureus* ATCC 25923), **Lane 2** is an isolate of *S.aureus* ,**Lane 3** is a negative control , **Lane 4** is a positive control of16SrRNAgene (*K. pneumoniae* ATCC 13883), **Lane 5** is *K. pneumoniae* isolate , **Lane 6** is a negative control , **Lane 7** is a positive control of bla OXA-51gene *(A.baumannii* ATCC 19606) ,**Lane 8** is *A.baumannii* isolate, **Lane 9** is a negative control, **Lane 10** is a positive control of 16SrDNA(*P.aeruginosa* ATCC 27853) ,**Lane 11** is *P.aeruginosa* isolate, **Lane 12** is a negative control, **Lane 13** is a positive control of the uspA gene *(E. coli* 25922), **Lane 14** is E. coli isolate, and **Lane 15** is a negative control ,Lane 16 is a marker.

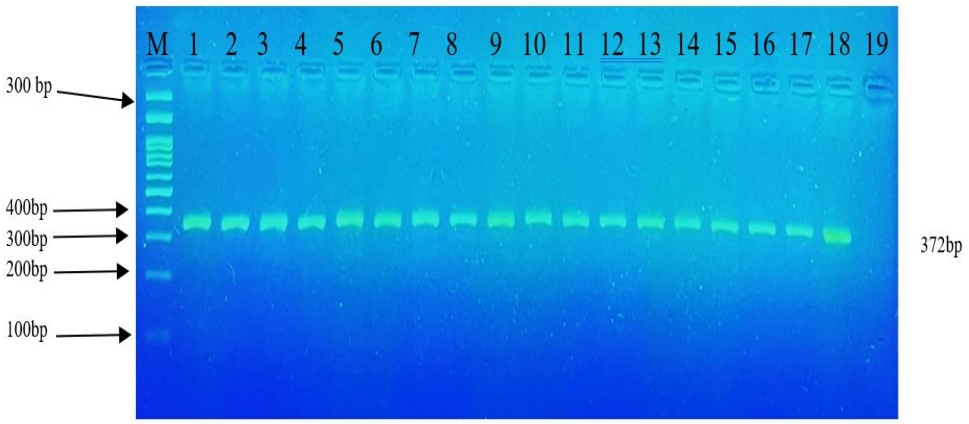

Figure 1: Agarose gel showing amplification of the PCR products

Figure 1: Agarose gel showing amplification of the PCR products. **M:** 100-3000 bp DNA Marker, **Lane 1** is a positive control (*S.aureus* ATCC 25923), **Lane 2-18** are *Staphylococcus sp.* isolates. **Lane 19** is a negative control (distilled water).

Antibiotic resistance (AR) profiles of the isolated bacteria

The results of antimicrobial resistance to different antibiotics for the most common Gram-negative and Gram-positive bacteria are shown in (Tables 6 and 7). In general, observed that the isolated bacteria from patients with SBIs had a high rate of antibiotic resistance.

Carbapenem-resistance (Imipenem and Meropenem) was higher in *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* than in *Enterobacter cloacae* and *E. coli*. The isolation rates

of ESBL producing *E. coli, and K.pneumoniae*, were 100%, and 78.6 % , respectively. All *K. pneumoniae, A. baumannii, E. coli*, and *Enterobacter cloacae (E. cloacae)* strains were only (100%) sensitive to colistin, While in *P. aeruginosa* stains showed the colistin-resistance (33.3%). *E. coli, K.pneumoniae*, and *Enterobacter cloacae* stains were only sensitive to tigecycline 100%. Whereas *A. baumannii* stains were 10% resistant to tigecycline (Table 6).

Methicillin resistance was found in 66.7% of *S. aureus*, whereas in coagulase-negative staphylococci strains, it was present in 100%. They were completely resistant to Clindamycin and Erythromycin. Only linezolid and tigecycline were completely effective against all gram-positive bacteria. In addition, 75% of CoNS isolates, 66.7 % of *S. aureus* and 33.3% of *Enterococcus faecium* were resistant to vancomycin (Table 7).

Table 5. The rate of major Gram-negative bacterial resistance against various antibiotics among hospitalized *COVID-19 patients.*

Antibiotics	*K. pneumoniae* (n=28)	*A. baumannii* (n=20)	*S. maltophilia* (n=11)	*P. aeruginosa* (n=9)	*E. cloacae* (n = 5)	*E. coli* (n= 3)
Amoxicillin-Clavulanic acid	22(78.6)	-	-	-	5 (100)	3 (100)
Ampicillin	28 (100)	-	-	-	5 (100)	3 (100)
Aztreonam	22 (78.6)	-	-	-	3 (60)	3 (100)
Cefepime	22 (78.6)	20 (100)	-	7(77.8)	2 (40)	3 (100)
Ceftriaxone	22 (78.6)	20 (100)	-	-	3(60)	3 (100)
Ciprofloxacin	21 (75)	20 (100)	-	4 (44.4)	2 (40)	2 (66.7)
Levofloxacin	21 (75)	20 (100)	6 (54.5)	4 (44.4)	3 (60)	2 (66.7)
Imipenem	18 (64.2)	19 (95)	-	4 (44.4)	1 (20)	1 (33.3)
Gentamicin	14 (50)	13 (65)	-	6 (66.6)	2 (40)	0 (0)
Meropenem	18 (64.2)	20 (100)	-	6 (66.6)	2 (40)	1 (33.3)
Trimethoprim/ sulfamethoxazole	24 (85.7)	20 (100)	4 (36.4)	-	2 (40)	1 (33.3)
Tetracycline	26 (92.9)	20 (100)	-	-	3 (60)	2 (66.7)
Tobramycin	12 (100)	13 (65)	-	-	3 (60)	0 (0)
Tigecycline	0 (0)	2 (10)	-	-	0 (0)	0 (0)
Ceftazidime	22 (78.6)	20 (100)	-	7 (77.8)	3 (60)	3 (100)
Piperacillin	28 (100)	20 (100)	-	4 (44.4)	4 (80)	3 (100)
Piperacillin/ Tazobactam	19(67.9)	20 (100)	-	4 (44.4)	2 (40)	1(33.3)
Amikacin	14 (50)	20 (100)	-	7 (77.8)	3 (60)	1(33.3)
Netilmicin	20 (71.4)	11 (55)	-	-	3 (60)	0 (0)
Colistin	0 (0)	0 (0)	-	3 (33.3)	0 (0)	0 (0)
ESBL positive*	22(78.6)	-	-	-	-	3 (100)

- indicates as (Not detected)
Values between ( ) indicates percent values
***ESBL= Extended Spectrum beta lactamase**

Table 6. The rate of major Gram-positive bacterial resistance against various antibiotics among hospitalized COVID-19 patients.

Antibiotics	*S.aureus (n = 3)*	*Coagulase- negative staphylococci (n = 16)*	*Enterococcus faecium (n = 3)*
Ampicillin	-	-	3 (100)
Ciprofloxacin	2 (66.7)	14 (87.5)	3 (100)
Clindamycin	3 (100)	16 (100)	-
Erythromycin	3 (100)	16 (100)	-
Gentamicin	2 (66.7)	10 (62.5)	-
Fusidic acid	1 (33.3)	16 (100)	-
Moxifloxacin	1 (33.3)	9 (56.25)	-
Levofloxacin	2 (66.7)	11 (68.8)	2 (66.7)
Teicoplanin	2 (66.7)	14 (87.5)	1 (33.3)
Oxacillin	2 (66.7)	16 (100)	-
Benzylpenicillin	3 (100)	16 (100)	3 (100)
Trimethoprim/sulfamethoxazole	0 (0)	7 (43.8)	-
Rifampicin	2 (66.7)	13 (81.2)	-
Tetracycline	3 (100)	13 (81.2)	3 (100)
Vancomycin	2 (66.7)	12 (75)	1 (33.3)
Tigecycline	0 (0)	0 (0)	0 (0)
Linezolid	0 (0)	0 (0)	0 (0)
Cefoxitin screen positive*	2 (66.7)	16 (100)	-
High level gentamicin resistance positive	-	-	3(100)

- indicates as (Not detected)
Values between ( ) indicates percent values
*Cefoxitin Screen = to detect MRSA

Inflammatory biomarkers

In this study, we included all three biomarkers: PCT, CRP, and WBC. We found elevated PCT, which was greater than 0.25 ng/ml in 89.8% of patients with secondary bacterial infections and 10.2% in those without secondary bacterial infections (Table 3). The PCT rise in secondary bacterial pneumonia was statistically significant ($p< 0.05$) when compared to no SBIs in respiratory. In blood infection (BSI), the PCT rise was also statistically significant, however, when compared to no BSI (Figure 3), it increased to ($p<0.001$).

We observed that CRP was higher than 5.0 mg/L in (99%) of the patients with SBIs, while the CRP in (98%) of the patients without SBIs were also elevated (Table 3). A statistically significant association was found between the rise of CRP in secondary bacterial pneumonia compared with no secondary bacterial pneumonia ($p <0.05$). While the rise of CRP in BSI compared with no BSI was statistically significant, it increased four times ($p<0.0001$) (Figure 3).

WBC count higher than 11×10^9 /L was present in (84.5 %) of the patients with SBIs, while WBC in (83%) of the patients without SBIs was also elevated (Table3). However, no statistically significant difference was found between secondary bacterial pneumonia or blood infections and no SBIs in the increase of WBC count (Figure 4).

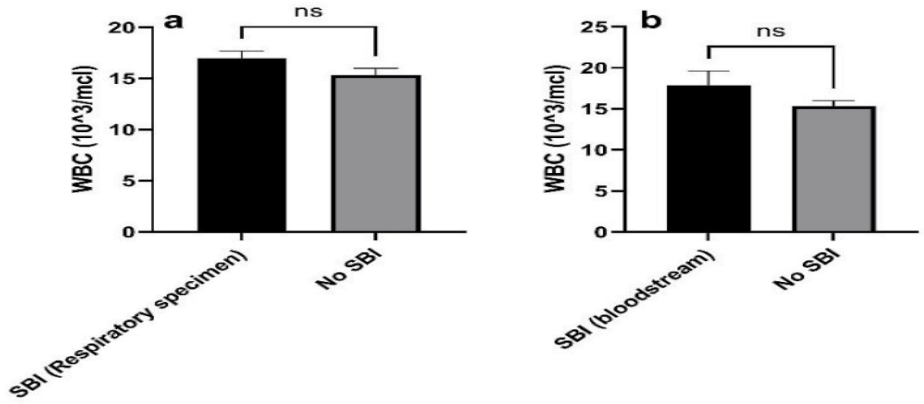

Figure 4: Association of WBC count with secondary bacterial infection among COVID-19

Figure 4: Association of WBC count with secondary bacterial infection among COVID-19 hospitalized patients. **a)** WBC count in respiratory SBIs specimens and no SBIs patients, **b)** WBC count in blood SBIs no SBIs patients Unpaired t-test applied for comparison. **ns** regarded as non-significant.

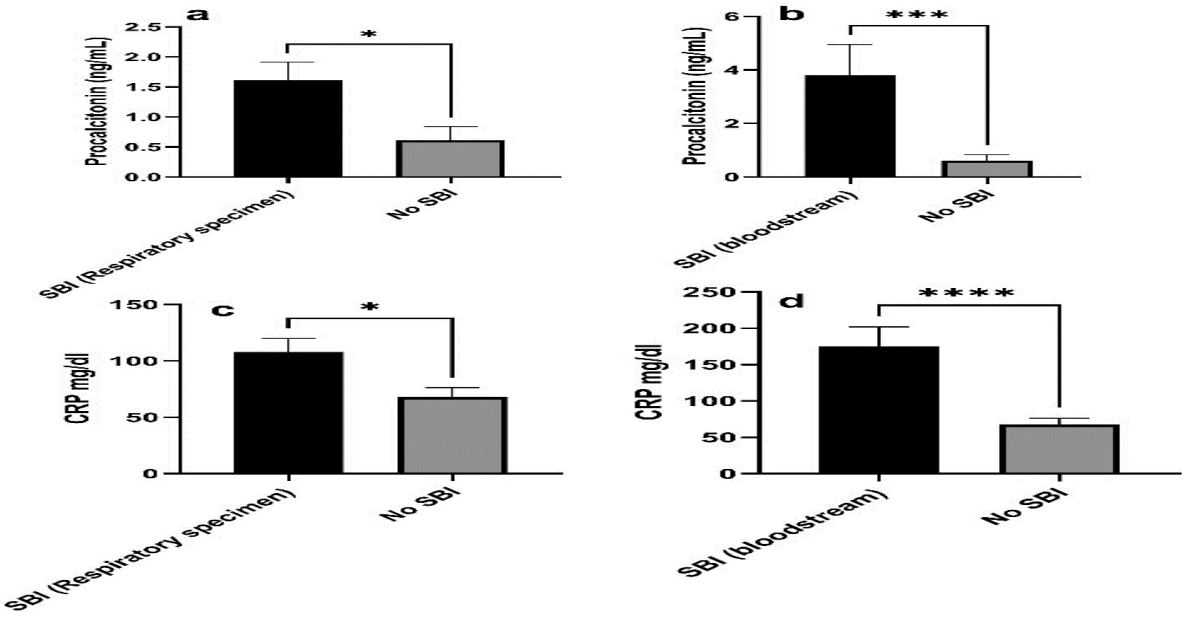

Figure 3: Association of serum concentration of inflammatory biomarkers with secondary bacterial infection (SBIs)

Figure 3: Association of serum concentration of inflammatory biomarkers with secondary bacterial infection (SBIs) among hospitalized COVID-19 patients. **a)** Procalcitonin (PCT) level (ng/mL) in respiratory SBIs specimens and no SBIs patients, **b)** Procalcitonin (PCT) level (ng/mL) in blood SBIs no SBIs patients, **c)** C-reactive protein (CRP) level (mg/L) in respiratory SBIs specimens and no SBIs patients, **d)** C-reactive protein (CRP) level (mg/L) in blood SBIs no SBIs patients. Unpaired t-test applied for comparison. $p<0.05$ regarded as significant. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$, ns regarded as non-significant.

DISCUSSION

Secondary bacterial infection has appeared to be one of the main complications causing the high death rates in patients hospitalized with a diagnosis of COVID-19 (Lansbury et al., 2020). ICU patients are more likely to develop secondary bacterial infections as a result of their prolonged ICU stay and increased mortality risk (Elabbadi et al., 2021). Therefore, this study found that the prevalence of SBIs and related antibiotic resistance profiles of COVID-19 patients admitted to the ICU were compared to those of non-ICU patients at different hospitals in Erbil, Iraq.

In this study, SBIs occurred in 84 (64.6%) of 130 COVID-19 patients who were examined ≥ 48 h after admission, indicating a high prevalence of nosocomial bacterial infection among hospitalized COVID-19 patients. While the death rate in COVID-19 hospitalized patients without secondary bacterial infection was 34.8%, it increased to 81% in patients with SBIs. According to Li et al. (2020), the mortality rate of SBIs in COVID-19 patients was increased up to 49.0%. Additionally, Sharifipour et al. (2020) reported a 95.0% mortality rate in the ICU patients in their study. We discovered that ICU patients were more likely than non-ICU patients to have SBIs (73/84 [86.9%] vs. 11/84 [13.1%]), with a higher fatality rate (Table 4).

SBIs in the lungs were the most common, which may be caused by invasive operations such as tracheal intubation and ventilator use during hospitalization, but patients with severe COVID-19 are also prone to blood infections (Qin et al., 2020, Cheng et al., 2020). In this study, the most common secondary bacterial infection identified was secondary bacterial pneumonia (83.3%), followed by a blood infection of unknown origin (32.1%). This sample tendency was similarly seen in the studies done by (Nori et al., 2021, Zhang et al., 2020).

In 27 patients, blood infections were present, with 13 of them also having infections in the lungs. When we examined the bacteria in mixed infections, we discovered that in 7/13 patients, the same bacteria were in both their circulation and lungs, including *K. pneumonia* (42.9%, 3/7), and *A. baumannii* (28.6%, 2/7). In these seven patients, blood infections came after lung infections, and the antimicrobial susceptibility testing results for these bacteria isolated from sputum and blood specimens were similar. Our results showed that the blood infections in these patients were caused by *K. pneumoniae* or *A. baumannii* migrating from the lungs and may be related to improper antibiotic treatment for these infections of the lungs (Lai et al., 2020).

According to our data, a total of 103 isolated bacteria were mostly caused by gram-negative bacteria (77.7%), with a death rate of 70.6%, followed by gram-positive bacteria (22.3%), with a death rate of 13.2%. The majority of secondary bacterial pneumonia was caused by gram-negative bacteria, including *Klebsiella pneumonia* (30.3%) and *Acinetobacter baumannii* (22.4%), followed by *Stenotrophomonas maltophilia* (11.8%) and *Pseudomonas aeruginosa* (10.5%). *Klebsiella pneumonia* and *Acinetobacter baumannii* were also found to be the most common pathogens in the respiratory specimens in the studies conducted by (Khurana et al., 2021, Pourajam et al., 2022). Among gram-positive bacteria, coagulase-negative Staphylococcus (6%), followed by *Staphylococcus aureus* (2%), were the most frequent causes of pneumonia. These rates for two bacteria were also seen in the study conducted by (Boorgula et al., 2022).

The antimicrobial susceptibility tests for gram-negative bacteria revealed that the majority of *A. baumannii* and *K. pneumoniae* were multi-drug-resistant bacteria with generally high rates of antibiotic resistance. The rates of carbapenem resistance (imipenem and meropenem) among these two bacteria were very high (64.2% and 95% to 100%, respectively). The mortality rates were 100% and 95%, respectively, for patients with *K. pneumoniae* and *A. baumannii* infections. These findings were consistent with previously reported high death rates associated with carbapenem-resistant *A. baumannii* and *K. pneumoniae* (Balkhair et al., 2019). The prevalence of these isolates in the present study might be attributed to invasive device-associated infections throughout the ICU stay as a result of

mechanical ventilation and empirical antibiotic treatment. An environmental source was also indicated, which pointed to inadequate hand hygiene and infection control methods. To prevent hospital infections, monitoring bacterial coinfection in COVID-19 patients is crucial, particularly with bacteria that are multidrug-resistant. This might not only delay COVID-19 patients' treatment and recovery but also raise their mortality rate (Alqahtani et al., 2022).

When considering blood infections, Ripa et al. (2021) indicate that coagulase-negative staphylococci are the most prevalent secondary bacterial infection agent. In this study, CoNS was the predominant bacteria, followed by K. *pneumoniae* from blood culture samples. This is consistent with the fact that CONS, a common skin commensal, are usually responsible for infections and catheter-related infections after central venous catheter placement. As a result, we believe that venous catheter care for critically ill patients should be improved in order to reduce blood infections. Coagulase negative staphylococcus can infect the lungs and cause pneumonia, especially in critically ill patients requiring prolonged mechanical ventilation via endotracheal tube or tracheostomy, due to extracellular proteins and DNA having greater functional relevance for biofilm accumulation, especially in *Staphylococcus haemolyticus* (Shi et al., 2019). These bacteria is widespread in hospitals and among medical staff, resulting in emerging microbe causing nosocomial infections. *S. haemolyticus*, especially strains that cause nosocomial infections, are more resistant to antibiotics than other coagulase-negative Staphylococci (Eltwisy et al., 2022). Because the majority of secondary infections in our study were nosocomial in origin and caused by highly drug-resistant bacteria, it revealed inadequate infection control methods and illogical antibiotic prescription (Vijay et al., 2021).

According to antibiotic susceptibility tests for gram-positive bacteria, there were also high rates of antibiotic resistance; methicillin resistance was found in 100% and 66.7% of coagulase-negative staphylococci and *Staphylococcus aureus*, respectively. No tigecycline or linezolid resistance was found; the resistance rate was 0%. As a result, it is suggested that tigecycline and linezolid may be used as the empirical option for gram-positive bacteria if secondary bacterial infections occur. Since gram-negative bacteria were responsible for 77.7% of secondary bacterial infections in our study and 75% of CONS isolates, 66.7% of S. aureus, and 33.3% of *Enterococcus faecium* were vancomycin resistant. As a result, vancomycin may not be required as an empirical cover for resistant gram-positive infections (Vijay et al., 2021).

The current study documented that among 84 patients, 79 (94%) received antibiotic therapy within 24 hrs. of admission. This is in agreement with Mondal et al. (2022), who demonstrated that before the samples were sent for microbiological testing, the majority of patients had already taken empirical antibiotics. In this study, antibiotic prescribing patterns ranged from 98.6% to 63.6% depending on the patient's severity of illness (ICU and non-ICU), rather than based on bacterial confirmation by culture and antibiotic susceptibility test results.

The high antibiotic use for treating COVID-19 patients observed in our study was inconsistent with the WHO's proposed guidelines, they do not recommend regular antibiotic treatment in COVID-19 patients with mild or moderate symptoms unless a bacterial infection is detected (Organization, 2020). According to the current information and our findings, several COVID-19 patients received unnecessary antibiotic treatment (Manohar et al., 2020, Microbiology, 2020). Antibiotic overuse may place stress on bacterial pathogens, leading to antibiotic resistance (Usman et al., 2020, Mirzaei et al., 2020). This is why significant attempts to combat antimicrobial resistance focus on minimizing incorrect or excessive antibiotic usage.

In this study, the observed risk factors associated with the acquisition of a secondary bacterial infection were first advanced age, with the median age of patients with secondary bacterial infections being 62, indicating that older patients have more complicated conditions that make their hospital stays longer. This makes them more susceptible to secondary bacterial or nosocomial infections, which are associated with a greater risk of death. As demonstrated by previous studies (De Smet et al., 2020, Santoso et al., 2022). This could be attributed to age-related B and T cell function defects, as well as excess production of cytokines type 2, which could cause prolonged proinflammatory responses as well as defects in viral replication control, resulting in a poor outcome (Zhou et al., 2020, Opal et al., 2005).

In this study, comorbid diseases, particularly hypertension and diabetes, are suspected to be the second risk factor. The majority of the patients (80.9%) with these comorbidities were admitted to the ICU, while the comorbidity with no SBIs was 47.8%. Other studies findings were consistent with these findings: that these comorbidities were found to be a risk factor for secondary bacterial infections in ICU patients (Chen et al., 2020). There is a proportionate decline in immunological response (a decrease in T cell proliferation and interleukin (IL)-12 production and an increase in IL-10 production in response to phytohemagglutinin (PHA) stimulation) with increased comorbidity in older adults (Castle et al., 2005).

The length of ICU stays due to mechanical ventilation, which was another major risk factor in this study, was

apparently related to an increase in the prevalence of SBIs and the mortality rate. Our finding was supported by the study of He et al. (2021) that indicated secondary bacterial infections increased ICU stays, which increased the risk of death.

De Bruyn et al. (2022) concluded that a higher corticosteroid dose was related to a higher risk of secondary bacterial infection. We observed that 100% of ICU patients, with 86.9% having SBIs, were on corticosteroid therapy for a long period of time, with a mean of 12 days, this indicates that the total cumulative dose of corticosteroids given in the ICU was also associated with an increased risk of secondary bacterial infection.

In this study, our data showed that 89.8% of the patients with secondary bacterial infections had serum PCT values higher than 0.25 ng/mL, and 81% of them died. This suggests that PCT levels were related to bacterial superinfection and worsening outcomes in hospitalized SARS-CoV-2 patients, which means that patients with PCT levels lower than 0.25 ng/mL were unlikely to be positive for blood or sputum cultures, which may aid in reducing or eliminating unnecessary antibiotic use. An increase in PCT indicates the presence of a secondary bacterial infection; there is a positive correlation with it, as well as a more severe clinical outcome for SARS-CoV-2 infection in hospitalized SARS-CoV-2 patients. Our findings agree with those of Atallah et al. (2022).

Furthermore, in this study, we discovered that PCT rise is statistically significant in the early detection of lower respiratory tract infections with a value of $p < 0.05$ and blood infections with a value of $p<0.001$, although its specificity in identifying respiratory infections is not as good as it is in diagnosing blood infections, and this corresponded with the findings of Zhu et al. (2015).

In COVID-19 hospitalized patients, PCT was a useful biomarker for distinguishing secondary infections from no SBIs. In contrast, neither CRP nor WBC were shown to have any significant discriminating correlations (Richards et al., 2021). In this study, we observed increases in CRP and WBC levels in hospitalized COVID-19 patients with both secondary bacterial infections and no SBIs, Our results indicated that there is no significant correlation to an increase in total WBC between secondary bacterial infection in blood or respiratory specimen compared to patients with no SBIs, it means that WBC is not considered a useful biomarker, whereas CRP has been shown to be a better biomarker for detection of secondary bacterial infections in conjunction with an elevated PCT level compared to the total WBC test, because the CRP level was elevated significantly ($p <0.05$) in patients infected with secondary bacterial pneumonia to near 100 mg/L, whereas the CRP level was elevated significantly ($p<0.0001$) in blood infected patients to >150 mg/L when compared to patients with no SBIs, our results are supported by the study of Seaton et al. (2020). In this study, it is clear that when there is a clinical suspicion of bacterial co-infection in COVID-19 patients, a combination of CRP and PCT may provide further results to guide treatment decisions. Recent findings demonstrate that combining PCT and CRP levels is more accurate in predicting bacterial co-infection in children with influenza H1N1 (Li et al., 2019, Carbonell et al., 2022).

CONCLUSION

Overall, secondary bacterial infections were higher in ICU patients with COVID-19 who required hospitalization compared with non-ICU patients, mainly in those with older age, hypertension and diabetes comorbidities, mechanical ventilation, prolonged stays, and corticosteroid treatment. They were more commonly associated with gram-negative than gram-positive bacteria, with coagulase-negative staphylococci being the most common in blood infections and *K. pneumonia* in respiratory tract infections. The mortality rate in patients with secondary pneumonia and blood infections was higher than that of those who had no secondary bacterial infections. The antimicrobial resistance rates are generally high against the main identified bacteria, indicating that precise antibacterial agent administration is required for SBIs in hospitalized patients. The PCT level was positively associated with secondary bacterial infections and patient outcomes.

RECOMMENDATION

Antimicrobial stewardship programs and teams, in collaboration with infection prevention programs, must be available at every hospital to reduce unnecessary antibiotic use. Empirical antibiotic medication shouldn't be initiated in ICU patients with low PCT levels.

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