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PCR-based co-detection of respiratory bacterial pathogens among hospitalized COVID-19 patients: Early-pandemic data from Golestan Province, Iran

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Abstract

Background

The presence of opportunistic bacterial pathogens may complicate patients with coronavirus disease-2019 (COVID-19), and characterizing their prevalence and spectrum during the early phase of the pandemic is epidemiologically relevant. This study estimated the frequency and distribution of respiratory bacterial pathogens detected by polymerase chain reaction (PCR) in hospitalized COVID-19 patients during the first period of the pandemic in Golestan Province, Iran.

Methods

This retrospective study was performed on patients with COVID-19 confirmed by real-time reverse transcription (RT-PCR) who were hospitalized between June and November 2020. Nasal and nasopharyngeal swabs were tested by PCR for eight respiratory bacterial pathogens. Demographic, clinical, laboratory, and outcome data were extracted from medical records. Because upper-airway PCR cannot detect colonization, non-viable DNA, and true lower-respiratory tract infection, all positive results were interpreted as bacterial detection, not clinically confirmed co-infection.

Results

Among 254 hospitalized patients with confirmed COVID-19, 154 patients (60.6%) had detectable DNA from at least one bacterial target. Monobacterial and polybacterial detection was found in 111 (43.7%) and 43 (16.9%) patients, respectively. *Staphylococcus aureus* was the most common (22.8%) detected organism, followed by *Streptococcus pneumoniae* (21.3%), *Stenotrophomonas maltophilia* (20.5%), and *Pseudomonas aeruginosa* (11.4%). No significant associations were found between overall bacterial detection and age group, gender, clinical severity, ICU admission, mortality, length of hospital stay, or major laboratory parameters. In the unadjusted organism-specific analyses, *P. aeruginosa* detection was significantly associated with age group (P = 0.006), COVID-19 severity (P = 0.032), fever (P = 0.028), and ICU admission (P = 0.04). After multivariable adjustment, severe or critical COVID-19 remained independently associated with *P. aeruginosa* compared with mild disease (adjusted odds ratio [AOR] = 6.88; 95% confidence interval [CI], 1.02–46.46; P = 0.048). Hypertension was identified as a suggestive marker for polybacterial detection (AOR = 3.08; 95% CI, 0.98–9.7; P = 0.054). Mild COVID-19 was associated with lower odds of polybacterial presence compared with severe or critical COVID-19 (AOR = 0.16; 95% CI, 0.03–0.95; P = 0.04).

Conclusions

Bacterial DNA of several respiratory organisms was frequently detected by PCR in upper-airway specimens from hospitalized COVID-19 patients during the early pandemic. However, these results

should be interpreted cautiously; the observational nature of the study precludes causal interpretation. Because respiratory sampling occurred at variable times during hospitalization and PCR positivity cannot distinguish colonization from infection, our findings suggest a prevalence of bacterial DNA detected during the hospital stay rather than active infection. Prospective studies using lower-respiratory specimens when clinically indicated, culture and susceptibility testing are needed.

Introduction

The acute respiratory syndrome known as coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), carried an unprecedented global burden of morbidity and mortality the early phases of the pandemic [1–3]. Clinical manifestations of disease range from asymptomatic infection to life-threatening pneumonia, acute respiratory distress syndrome, intensive care unit (ICU) admission, mechanical ventilation, and death [4, 5]. In critically ill patients, prolonged hospitalization, invasive procedures, immunosuppression, and exposure to broad-spectrum antibiotics may increase the probability of bacterial colonization and secondary bacterial pneumonia [6–8].

Distinguishing COVID-19 pneumonia from bacterial pneumonia can be difficult because both conditions share overlapping clinical, laboratory, and radiological features, including fever, cough, dyspnea, hypoxemia, elevated inflammatory markers, and pulmonary abnormalities [9–11]. This diagnostic uncertainty contributed to the use of empirical antibiotic therapy during the pandemic for a high percentage of hospitalized patients with suspected or definite COVID-19 [12, 13].

It is widely known that bacterial infections are the most common and potentially life-threatening complication of viral respiratory tract infection, including pneumonia cases caused by the influenza virus [14, 15] and, more recently, SARS-CoV-2 [6, 16]. The pathogenesis of bacterial invasion following severe viral infection remains to be elucidated. However, disruption of epithelial barrier function, dysregulation of immune function, and inflammatory response may promote this process [17–19]. The rate of bacterial co-pathogen detection in COVID-19 varies greatly, partly because studies have used non-equivalent definitions and methods. Factors like the severity of the disease (all COVID-19 patients vs. critically ill patients), the length of hospitalization, the patient's comorbidities, prior antibiotic exposure, the diagnostic criteria used to define bacterial infection, the study design, and regional epidemiology significantly contribute to this variability [7, 20, 21]. In addition, early and later phases of the COVID-19 pandemic differ markedly in terms of circulating variants, population vaccination coverage, and clinical management strategies [22–24]. Several studies conducted during both the early and later phases of the COVID-19 pandemic have reported identification of bacterial co-pathogens in all hospitalized and ICU-admitted patients with a diagnosis of COVID-19 infection [7, 20, 25–28]. Furthermore, recent studies confirm rates ranging from 2–7% overall to over 50% in critically ill cases, influenced by the severity of the patient's condition and other variables [29–31]. Early pandemic reports showed wide variability in prevalence, ranging from 2% to as high as 45%, due to inconsistent definitions of co-infection and diagnostic criteria. However, more recent multicenter cohort studies with standardized definitions have estimated the prevalence of bacterial co-infection in hospitalized patients with COVID-19 to be

approximately 2% to 10% and the rate in ICU populations to be between 14% and 17% [32, 33]. A recent systematic review in Iran found that the pooled prevalence of bacterial pathogens among the COVID-19 patients was 19.6%, predominantly involving Gram-negative bacteria, such as *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Escherichia coli*, and *Pseudomonas aeruginosa*, in hospitalized patients. ICU admission and mechanical ventilation were identified as the main risk factors for these secondary infections [27]. Similarly, patients with severe or critical COVID-19 who require ICU admission were shown to be at the highest risk of infection-related complications and death [8, 34]. Furthermore, culture-based studies, lower-respiratory molecular testing, bloodstream infection studies, and upper-airway PCR surveys demonstrate distinct clinical and epidemiological features.

Polymerase chain reaction (PCR)-based testing has high analytical sensitivity and can rapidly detect bacterial nucleic acids. The present retrospective study was conducted during the first period of the COVID-19 pandemic in Golestan Province, Iran. The primary aim was to describe the frequency and nature of several respiratory bacterial pathogens detected by PCR in upper-airway specimens from hospitalized COVID-19 patients. The secondary aim was to explore associations between bacterial detection and demographic, clinical, laboratory, and outcome data.

Materials and methods

Study design and setting, participants, and clinical data

This retrospective observational study was conducted in Golestan Province, Iran. The study included patients admitted to hospitals and medical centers between June and November 2020, during the early phase of the COVID-19 pandemic. Patients with COVID-19 whose infection with SARS-CoV-2 was confirmed by real-time reverse transcription (RT)-PCR were eligible for inclusion in the study. Nasal and nasopharyngeal swab specimens were collected at the time of SARS-CoV-2 confirmation by the SARS-CoV-2 Diagnostic Laboratory in Golestan Province. Confirmation occurred at variable times during the hospitalization and the precise interval between admission and bacterial sampling was not consistently documented. Demographic parameters, including age and gender, were recorded. Information on clinical presentations, comorbidities, laboratory parameters, oxygen saturation, ICU admission, length of hospital stay, COVID-19 severity, and mortality was extracted from medical records.

Classification of COVID-19 severity

Patients were classified according to the World Health Organization (WHO) criteria for COVID-19 clinical severity [1] into three categories: mild disease, defined as symptomatic illness in the absence of viral pneumonia or hypoxia; moderate disease, characterized by clinical signs of pneumonia, such as such as fever, cough, dyspnea, fast breathing but no features of severe pneumonia and an oxygen saturation (SpO_2) values ≥ 90%; and severe/critical disease, defined by clinical signs of pneumonia plus SpO_2 < 90%.

PCR detection of bacterial targets

Eight respiratory pathogenic bacteria, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, *Stenotrophomonas maltophilia*, *Bordetella pertussis*, and *Mycoplasma pneumoniae*, by conventional PCR using specific primers (Table 1) [35–42]. Extraction of bacterial nucleic acid was performed from clinical specimens using the SinaPure-DNA kit (SinaClon BioScience, Tehran, Iran), according to the manufacturer's instructions. The extracted DNA was stored in a freezer at − 80 ºC. PCR reactions were performed in a total volume of 25 µL containing 12.5 µL of Ready-to-Use PCR Master Mix 2x (SinaClon BioScience, Tehran, Iran), 0.4 µM of each primer, 1 µL (10 ng) DNA, and 10.7 µL of sterile distilled water. Amplification of each gene was carried out in a thermal cycler (Eppendorf, Master Cycler Gradient, Germany) under the following parameters: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56–64 ºC for 30 s depending on the target, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. PCR products were electrophoresed on a 1.5–2% agarose gel containing DNA Safe Satin (SinaClon BioScience, Tehran, Iran) at 100 V for 1 h and visualized by DNA Safe Stain under a UV transluminator. A positive PCR result was considered only as evidence of bacterial DNA detection and was not taken to represent a clinically confirmed bacterial infection.

Table 1
The specific primers used for the detection of bacterial respiratory pathogens

Targeted bacteria	Gene	Primer (5'→3')	Amplicon size (bp)	T_m (°C)	Reference
Streptococcus pneumoniae	*lytA*	F: ACGCAATCTAGCAGATGAAGCA R: TCGTGCGTTTTAATTCCAGCT	80	60	35
Staphylococcus aureus	*nuc*	F: GCGATTGATGGTGATACGGTT R: GCCAAGCCTTGACGAACTAAAGC	279	60	36
Klebsiella pneumoniae	*mrkD*	F: AAGCTATCGCTGTACTTCCGGCA R: GGCGTTGGCGCTCAGATAGG	340	64	37
Pseudomonas aeruginosa	*gyrA*	F: CGGTCAATATCGAAGACGAGC R: ACGGTTTCCTTTTCCAGGTC	621	60	38
Acinetobacter baumannii	*ITS*	F: CATTATCACGGTAATTAGTG R: AGAGCACTGTGCACTTAAG	208	56	39
Stenotrophomonas maltophilia	*23S rRNA*	F: GCTGGATTGGTTCTAGGAAAACGC R: ACGCAGTCACTCCTTGCG	278	60	40
Bordetella pertussis	*IS481*	F: GATTCAATAGGTTGTATGCATGGTT R: TTCAGGCACACAAACTTGATGGGCG	181	60	41
Mycoplasma pneumoniae	*P1*	F: CCAACCAAACAACAACGTTCA R: ACCTTGACTGGAGGCCGTTA	76	60	42

Statistical analysis

Statistical analysis were performed using Statistical Package for the Social Sciences (SPSS) Software version 26.0 for Windows (SPSS Inc., Chicago, IL, USA). Categorical variables are expressed as frequency and percentage and were compared using Pearson's chi-square or Fisher's exact test. Continuous variables are presented as median and interquartile range (IQR) and were compared using analysis of variance (ANOVA) or the Kruskal–Wallis test, as appropriate. Multinomial logistic regression analysis was used to compare monobacterial and polybacterial detection with the PCR-negative reference group. Multivariable binary logistic regression analyses were performed to examine selected organism-specific (single-species) outcomes. Adjusted odds ratios (AORs) with 95% confidence intervals (CIs) are reported. A two-sided P value < 0.05 was considered statistically significant. All organism-specific analyses were exploratory and hypothesis-generating.

Results

Characteristics of patients

Table 2 shows demographic features and clinical and laboratory characteristics of patients hospitalized for COVID-19. A total of 254 patients with laboratory-confirmed COVID-19 were included in this study. Of these, 113 (44.5%) were male and 141 (55.5%) were female. The distribution of patients by age was as follows: <15 years (n = 10, 3.9%), 15–44 years (n = 99, 39%), 45–64 years (n = 95, 37.4%), and ≥ 65 years (n = 50, 19.7%). The median age was 48 years (IQR, 35.75–63 years).

Table 2
Demographic features, clinical symptoms, and laboratory findings of COVID-19 patients with or without bacterial co-detection

Characteristics	All patients (n = 254)	Patients without bacterial detection (n = 100)	Patients with monobacterial detection (n = 111)	Patients with polybacterial detection (n = 43)	P value
Demographic features					
Age (Years; Median, IQR)	48 (35.75-63)	48 (35–60)	51 (38–64)	50 (35.5–63.5)	0.53
Age group (Years)					
< 15	10 (3.9)	5 (5)	4 (3.6)	1 (2.3)	0.69
15 – 44	99 (39)	38 (38)	42 (37.9)	19 (44.2)	
45 – 64	95 (37.4)	42 (42)	39 (35.1)	14 (32.6)	
≥ 65	50 (19.7)	15 (15)	26 (23.4)	9 (20.9)	
Gender					
Male, n (%)	113 (44.5)	42 (42)	48 (43.2)	23 (53.5)	0.42
Female, n (%)	141 (55.5)	58 (58)	63 (56.8)	20 (46.5)	
Comorbidities					
Diabetes, n (%)	54 (21.3)	21 (21)	23 (20.7)	10 (23.3)	0.94
Hypertension, n (%)	52 (20.5)	24 (24)	23 (20.7)	5 (11.6)	0.24
Clinical presentations					
Fever, n (%)	49 (19.3)	17 (17)	20 (18)	12 (27.9)	0.29
Cough, n (%)	54 (21.3)	18 (18)	25 (22.5)	11 (25.6)	0.54
Dyspnea, n (%)	88 (34.6)	35 (35)	40 (36)	13 (30.2)	0.79
Malaise and fatigue, n (%)	71 (28)	28 (28)	28 (25.2)	15 (34.9)	0.49
Laboratory parameters					

Cr, Creatinine; CRP, C-reactive protein; IQR, Interquartile range; LDH, Lactic dehydrogenase; WBC, White blood cell; SpO_2, Oxygen saturation.

Characteristics	All patients (n = 254)	Patients without bacterial detection (n = 100)	Patients with monobacterial detection (n = 111)	Patients with polybacterial detection (n = 43)	P value
WBC (10^3/μL; Median, IQR)	6.4 (4.67–8.2)	6.1 (4.65–7.6)	6.5 (4.6–8.4)	7.1 (5-9.15)	0.28
Lymphocyte (%; Median, IQR)	20 (13–26)	20 (13–26)	18 (13–26)	18 (12.5–24)	0.41
LDH (U/L; Median, IQR)	548 (460-647.5)	542 (465–637)	551.5 (460–650)	530 (468–676)	0.85
Cr (mg/dL; Median, IQR)	1 (0.9–1.2)	1 (0.9–1.2)	1 (0.9–1.2)	1.1 (0.9–1.3)	0.49
Urea (mg/dL; Median, IQR)	22 (14.75-32)	21.5 (15–31)	21 (14-31.5)	26 (16–36)	0.40
CRP, n (%)					
Negative	57 (22.5)	28 (28)	21 (18.9)	8 (18.6)	0.42
Positive (+)	73 (28.7)	29 (29)	35 (31.6)	9 (20.9)	
Positive (++)	72 (28.3)	27 (27)	30 (27)	15 (34.9)	
Positive (+++)	52 (20.5)	16 (16)	25 (22.5)	11 (25.6)	
SpO_2 (%; Median, IQR)	93 (90–96)	93.5 (91–96)	93 (91–96)	93 (89–96)	0.69
Length of hospital stay (Days; Median, IQR)	5 (4–8)	6 (4–9)	5 (4–7)	5 (4-7.5)	0.58
ICU admission, n (%)	18 (7.1)	5 (5)	9 (8.1)	4 (9.3)	0.56
Mortality, n (%)	25 (9.8)	10 (10)	11 (9.9)	4 (9.3)	0.99
COVID-19 severity, n (%)					
Mild	103 (40.6)	46 (46)	43 (38.7)	14 (32.6)	0.49
Moderate	94 (37)	34 (34)	44 (39.6)	16 (37.2)	

Cr, Creatinine; CRP, C-reactive protein; IQR, Interquartile range; LDH, Lactic dehydrogenase; WBC, White blood cell; SpO_2, Oxygen saturation.

Characteristics	All patients (n = 254)	Patients without bacterial detection (n = 100)	Patients with monobacterial detection (n = 111)	Patients with polybacterial detection (n = 43)	P value
Severe/Critical	57 (22.4)	20 (20)	24 (21.6)	13 (30.2)	

Cr, Creatinine; CRP, C-reactive protein; IQR, Interquartile range; LDH, Lactic dehydrogenase; WBC, White blood cell; SpO_2, Oxygen saturation.

Regarding COVID-19 severity, 103 (40.6%) were mild cases, 94 (37%) were moderate cases, and 57 (22.4%) were severe/critical cases. The relationship between the COVID-19 severity and demographic characteristics of age and gender was described in Table 3. More women than men were observed in mild (n = 60, 58.3% vs. n = 43, 41.7%) and moderate (n = 55, 58.5% vs. n = 39, 41.5%) categories, whereas more patients in the severe/critical group were men (n = 31, 54.4%). Furthermore, the median age of patients with severe/critical COVID-19 was significantly higher than that of patients with mild or moderate disease (60 years vs. 43 and 47.5 years, respectively; P < 0.001).

Table 3
The relationship between the COVID-19 severity and demographic characteristics of age and gender in the patients studied

Characteristics	All patients (n = 254)	Mild (n = 103)	Moderate (n = 94)	Severe/Critical (n = 57)	P value
Gender					
Male, n (%)	113 (44.5)	43 (41.7)	39 (41.5)	31 (54.4)	0.23
Female, n (%)	141 (55.5)	60 (58.3)	55 (58.5)	26 (45.6)	
Age (Years; Median, IQR)	48 (35.75-63)	43 (33–56)	47.5 (36–62)	60 (48–71)	**< 0.001**
Age group (Year)					
< 15	10 (3.9)	6 (5.8)	3 (3.2)	1 (1.8)	**0.001**
15 – 44	99 (39)	48 (46.6)	39 (41.5)	12 (21)	
45 – 64	95 (37.4)	36 (35)	37 (39.4)	22 (38.6)	
≥ 65	50 (19.7)	13 (12.6)	15 (15.9)	22 (38.6)	

The median length of hospital stay was 5 days (IQR, 4–8 days) in the overall cohort. ICU admission occurred in 18 patients (7.1%), and 25 patients (9.8%) died.

The details of bacterial detection

At least one bacterial target was detected in 154 of 254 COVID-19 patients (60.6%), a finding that may reflect both true co-pathogens and colonizing organisms. Of the overall cohort, 111 patients (43.7%; 72.1% of PCR-positive patients) had monobacterial detections, while 43 patients (16.9%; 27.9% of PCR-positive patients) showed polybacterial detections. As shown in Fig. 1, S. *aureus* (n = 58, 22.8%), *S. pneumoniae* (n = 54, 21.3%), and *S. maltophilia* (n = 52, 20.5%) were the most common co-pathogens, followed by *P. aeruginosa* (n = 29, 11.4%), *A. baumannii* (n = 17, 6.7%), *M. pneumoniae* (n = 6, 2.4%), *B. pertussis* (n = 4, 1.6%), and *K. pneumoniae* (n = 1, 0.4%).

Table 2 shows demographic characteristics, clinical symptoms, and laboratory findings of COVID-19 patients with or without bacterial detection. Seventy-one (46.1%) and 83 (53.9%) of male and female COVID-19 patients were positive for bacteria in PCR assay, respectively (P > 0.05). Forty-eight (43.2%) of males showed monobacterial detection compared to 63 (56.8%) of females. The opposite was true for polybacterial presence (n_{male} = 23, 53.5% and n_{female} = 20, 46.5%) (P > 0.05). The highest rate of co-detection was observed in patients between 15 and 44 years (n = 61, 39.6%), while the lowest rate was found in patients below 15 years of age (n = 5, 3.2%). However, the overall rate of bacterial detection did not differ significantly among age groups. The rate of monobacterial detection was higher than polybacterial detection in all age groups of COVID-19 patients except those aged 15 to 44 (n = 42, 37.9% vs. n = 19, 44.2%). As shown in Fig. 2, all eight bacteria studied were detected in the 45 to 64 years age group, followed by the ≥ 65 years, 15–44 years, and < 15 years age groups, with 7, 6, and 3 bacteria detected, respectively. Three bacterial species, *S. aureus*, *S. pneumoniae*, and *S. maltophilia*, were identified across all age groups.

Data from the organism-specific analyses showed that *P. aeruginosa* detection differed significantly by age groups (P = 0.006), with a higher proportion of positivity among patients aged ≥ 65 years (n = 11, 37.9%) (Supplementary file 1, Table S4). Furthermore, patients with a positive PCR result for *B. pertussis* were older than those with a negative PCR (median age 71.5 years, IQR 58–78 vs. median 48 years, IQR 35–62; P = 0.035) (Supplementary file 2, Table S7). Regarding gender, a higher percentage of male patients were positive for *M. pneumoniae* compared to females, although this was not statistically significant (P = 0.09) (Supplementary file 2, Table S6).

The highest rate of co-detection was found in the moderate category of COVID-19 patients (n = 60, 38.9%) and the lowest among the severe/critical cases (n = 37, 24%) (Table 2). As shown in Fig. 3, most bacterial targets were detected in all severity groups. All studied bacteria, except *B. pertussis* and *K. pneumoniae*, were present in all COVID-19 severity groups, with no statistically significant differences (P > 0.05). The highest rates of positivity for both polybacterial and monobacterial presences were found among the moderate cases of COVID-19 patients (n = 44, 39.6% and n = 16, 37.2%, respectively) (Table 2).

When the distribution of each bacterial co-detection was analyzed separately by the clinical type of COVID-19 patients (Supplementary file 2, Table S1-S8), a significant association was found between the rate of *P. aeruginosa* positivity and disease severity (P = 0.032). Of the 29 patients with *P. aeruginosa* detection, 6 (20.7%) had mild disease, 12 (41.4%) had moderate disease, and 11 (37.9%) were

severe/critical cases. In contrast, among *P. aeruginosa*-negative patients, 97 (43.2%) had mild disease, 2 (36.4%) had moderate disease, and 46 (20.4%) had severe/critical disease (Supplementary file 2, Table S4).

Generally, there was no significant difference between patients with or without bacterial detection in clinical presentations, presence of underlying comorbidities, COVID-19 severity, and mortality. Moreover, no significant difference was found in the proportion of hospital stay, SpO_2, and ICU admission. Similarly, laboratory findings were not significantly different between the two groups. In unadjusted analyses, several individual associations were exploratory observed; these are reported below and should be interpreted with caution (Supplementary file 2, Table S1-S8). Compared with patients with only COVID-19, a significantly higher percentage of patients with *P. aeruginosa* developed fever (34.5% vs. 17.3%, P = 0.028) and were admitted to the ICU (17.2% vs. 5.8%, P = 0.04) (Supplementary file 2, Table S4). Patients with a positive *B. pertussis* result had lower oxygen saturation than those without detection of this organism (median SpO_2 87%, IQR 65.5–91% vs. median SpO_2 93%, IQR 91–96; P = 0.033) (Supplementary file 2, Table S7). A higher frequency of fever and higher creatinine values were observed among patients with *S. aureus* detection than negative group, although these findings were not statistically significant (P = 0.07 for both comparisons) (Supplementary file 2, Table S2). Similarly, patients who were positive for *S. pneumoniae* showed numerically higher rates of diabetes, lower SpO_2, and higher lactate dehydrogenase levels, but these associations did not reach statistical significance (Supplementary file 2, Table S1).

Multinomial showed that mild COVID-19 was significantly associated with a lower risk of polybacterial detection compared with severe/critical disease (AOR = 0.16; 95% CI = 0.03–0.95; P = 0.04). Hypertension was associated with an increased likelihood of polybacterial detection, although this association was borderline or suggestive rather than statistically significant (AOR = 3.08; 95% CI = 0.98–9.7; P = 0.054) (Table 4). When the impact of these factors on the likelihood of species-specific co-detections was analyzed with the multivariable approach (Supplementary file 3, Table S1-S5), severe or critical COVID-19 remained significantly associated with the presence of *P. aeruginosa* compared with mild cases (AOR = 6.88; 95% CI = 1.02–46.46; P = 0.048), consistent with the unadjusted analysis. However, ICU admission was no longer associated with *P. aeruginosa* detection (AOR = 2.46; 95% CI = 0.67–9.00; P = 0.17), so it is reported as an unadjusted association only and is not interpreted as an independent risk factor (Supplementary file 3, Table S4). Moreover, malaise and fatigue (AOR = 1.88; 95% CI = 0.95–3.74; P = 0.07) and severe/critical COVID-19 (AOR = 3.99; 95% CI = 0.92–17.23; P = 0.06) were found to be suggestive risk factors for *S. pneumoniae* and *S. aureus* co-detections, respectively (Supplementary file 3, Tables S1 and S2).

Table 4
Multinomial logistic regression analysis for bacterial co- detection in COVID-19 patients

Risk factors*	Monobacterial detection		Polybacterial detection	
	AOR (95% CI)	P value	AOR (95% CI)	P value
Age	1.01 (0.99–1.03)	0.24	1.01 (0.98–1.03)	0.62
Length of hospital stay	0.97 (0.9–1.04)	0.38	0.96 (0.86–1.06)	0.4
Diabetes	1.14 (0.51–2.55)	0.74	1.21 (0.42–3.51)	0.72
Hypertension	1.39 (0.68–2.84)	0.37	3.08 (0.98–9.7)	0.054
Fever	1.13 (0.45–2.81)	0.79	0.83 (0.26–2.62)	0.75
Cough	0.98 (0.36–2.64)	0.97	1.23 (0.33–4.51)	0.76
Dyspnea	1.27 (0.47–3.4)	0.64	2.47 (0.68–8.94)	0.17
Malaise and fatigue	1.21 (0.63–2.29)	0.57	0.66 (0.29–1.52)	0.33
ICU admission	0.61 (0.18–2.05)	0.42	0.62 (0.13–2.85)	0.54
Severe/Critical COVID-19 cases (Referent)				
Mild	0.59 (0.14–2.37)	0.45	0.16 (0.03–0.95)	**0.04**
Moderate	0.97 (0.41–2.29)	0.94	0.44 (0.15–1.33)	0.15

*Risk factor analysis for bacterial co-pathogens was compared with the reference group with no detection.

AOR, Adjusted odds ratios.

Discussion

This retrospective study describes the frequency of eight respiratory bacterial targets in nasal and nasopharyngeal specimens from hospitalized COVID-19 patients during the early pandemic period, along with key patient characteristics. The presence of bacterial pathogens among COVID-19 patients was reported to be variable in different studies. Co-detection rate in our study was as high as 60.6%, which is comparable to the values of 62.9%, 51%, and 74% reported in three separate retrospective studies by

Louka et al. [43], Zong et al. [29], and Zuh et al. [44], respectively, lower than that reported by Zuh et al. (91.8%) [25], and higher than that found in other studies [45–47]. Furthermore, a meta-analysis study estimated the overall pooled prevalence of bacterial co-infection to be 20.97% [95% CI: 15.95–26.46], meaning that overall, 16–26 out of every 100 patients with COVID-19 will have the presence of bacteria detected simultaneously [48].

Several points are crucial to be considered to ensure accurate interpretation of the results. First, the discrepancy in results can be attributed to several factors. Patient characteristics (type, clinical stage, and the severity of COVID-19 infection), diagnostic criteria for bacterial infection, the number and type of clinical specimens, timing of sample collection, and the diagnostic method used (e.g., culture, PCR), geographical location, and healthcare practices related to antimicrobial use, surveillance, and infection control seem to have a significant impact on findings regarding the prevalence of bacterial co-pathogen in patients with coronavirus infection, so comparisons with published estimates require attention to these factors [49, 50]. Overall, reported figures derive from at least five non-equivalent designs: (i) upper-airway PCR or colonization surveys; (ii) lower-respiratory PCR studies; (iii) culture-confirmed community-acquired co-infection at presentation; (iv) hospital-acquired secondary infection; and (v) bloodstream infection. The current study evaluated the presence of bacterial DNA in upper-airway swabs collected at variable and undocumented times during hospitalization. A possible reason for the observed higher detection rate may be due to this sampling strategy, which likely includes a mixture of community-onset and hospital-onset cases [30, 51–53]. Exposure to antibiotics as well as the timing of sampling are of particular importance in that previous antibiotics can suppress bacterial detection, while late sampling may reflect colonization over acute co-infection [54]. Second, since the bacteria studied are common pathogens in patients with nosocomial pneumonia, it was difficult to distinguish colonization from active infection [55]. In particular, because nasal and nasopharyngeal carriage of *S. aureus* and *S. pneumoniae* is common in the absence of disease, detection rates based on molecular methods may substantially exceed culture-confirmed co-infection estimates and should not be interpreted as evidence of a higher burden of active infection in this cohort. Moreover, colonization may be more frequent in patients with greater disease severity or longer hospital stays, potentially resulting in differential misclassification and contributing to the observed associations between bacterial detection and disease severity [56]. Nonetheless, these bacteria may act as opportunistic pathogens and exacerbate disease severity, leading to increased morbidity and mortality, particularly in immunocompromised patients during viral pandemics [57, 58]. Finally, while risk factors for bacterial co-infection are clinically relevant in principle, the observational design of our study permits only associative observations rather than findings that can directly guide identification or management. Our risk analysis revealed that severe or critical COVID-19 and certain comorbidities, notably hypertension, were statistically associated with mixed bacterial detections, whereas mild disease, in contrast to severe disease, was associated with a lower likelihood of such detections. Although these associations require further validation and do not establish causation, our results are supported by previous studies reporting that bacterial co-pathogens predominantly affect critically ill patients, particularly those admitted to ICUs [30, 32, 59, 60].

Diagnostic tools like PCR, which are used for rapid identification of bacteria, are significant in the management of critically ill patients [61]. This is because broad-spectrum antibiotics are often administered before microbiological data are collected, making methods that provide a fast detection of infections essential to avoid false-negative results [62]. On the other hand, the high sensitivity of PCR-based tests compared with traditional culture techniques complicates the clinical interpretation of results and remains a significant obstacle to the widespread adoption of PCR assays for detecting causative microorganisms from clinical specimens [63]. Because PCR can detect nucleic acid material from non-viable and degraded pathogens, a positive PCR result does not necessarily reflect a true infection [63, 64]. Likewise, in SARS-CoV-2-infected patients whose bacterial culture from respiratory specimens is negative but PCR detection is positive, a clear differentiation between real infection and colonization cannot be made without further investigation into the clinical validity of the quantitative PCR test [43].

The reported bacterial species responsible for co-infections in COVID-19 patients show significant variability across studies. Since all samples in our study were collected between June and November 2020, the observed rate of bacterial positivity reflects early-pandemic COVID-19 epidemiology, a period that differed markedly from subsequent waves in terms of the circulating viral lineages, population vaccination coverage, and clinical therapeutic strategies [22, 28]. Accordingly, our analysis is framed at the level of this pandemic phase and does not distinguish community-acquired from hospital-acquired detection, nor does it claim bacterial co-detection present at COVID-19 presentation. The four most common identified bacteria in our study were *S. aureus* (22.8%), *S. pneumoniae* (21.3%), *S. maltophilia* (20.5%), and *P. aeruginosa* (11.4%). This is consistent with several studies evaluating bacterial co-pathogens in COVID-19, which found *S. aureus* and *S. pneumoniae* among the most common early-pandemic co-pathogens, as well as significant roles played by Gram-negative bacteria, like *S. maltophilia*, *P. aeruginosa*, and *A. baumannii*, particularly in hospital-acquired and ventilator-associated infections [55, 65, 66]. The data from Musuuza et al.'s systematic review and meta-analysis highlight that *K. pneumoniae* (9.9%), *S. pneumoniae* (8.2%), and *S. aureus* (7.7%) were the most commonly identified bacteria associated with co-infections at the time of SARS-CoV-2 diagnosis. The study also found that *Acinetobacter* spp. (22%), *Pseudomonas* spp. (10.8%), and *Escherichia coli* (6.9%) were the leading causes of superinfections during care for SARS-CoV-2 infection [67]. According to recent studies, these patterns of co-pathogens have shifted in subsequent pandemic waves due to evolving SARS-CoV-2 variants, changes in hospital infection controls, and advanced antimicrobial stewardship practices [22, 68–70]. Early pandemic phases had lower prevalence rates with a predominance of *S. aureus and S. pneumoniae*, followed by a higher rate of early pulmonary infections during later phases and waves, often dominated by Gram-negative bacilli during peaks, then varying post-vaccination due to variants, immunity, and management changes [22–24, 33, 71, 72].

As expected, we found that *S. aureus* was present in a sizeable number of cases. Co-/secondary infections caused by *S. aureus* are known to be a complication of other viral pandemics, such as the Spanish flu and the H1N1 influenza pandemic [73, 74]. In studies of culture-confirmed infection, *S. aureus* has been reported to act synergistically with respiratory viruses and to be associated with increased

mortality and severity [75]; our PCR-based data cannot establish that such synergism occurred in the present cohort. As we found in the present study, patients with severe or critical COVID-19 showed almost four times the odds of *S. aureus* co-detection compared to those with mild illness (P = 0.06). This borderline association, observed in a small subgroup, suggests a potential link between COVID-19 severity and the presence of *S. aureus*, warranting further investigation. Westblade et al. reported *S. pneumoniae* among the bacterial pathogen most frequently identified on admission in patients with COVID-19, suggesting the potential benefit of empirical antibacterial coverage against this organism in hospitalized patients with clinical indications for such treatment [76]. However, caution is warranted because our design does not allow any inference about empirical therapy: PCR detection in an upper-airway specimen, in the absence of culture and susceptibility data, cannot confirm or refute a treatment decision. We also found that patients who were positive for *S. pneumoniae* were more likely to have diabetes (P = 0.09) and exhibited lower SpO_2 and higher LDH levels (P = 0.09 and P = 0.056, respectively). The level of serum LDH in COVID-19 patients can be used as a warning indicator for the severity of the disease [77]. In addition, malaise and fatigue were also identified as suggestive risk factors for *S. pneumoniae* co-detection, although more research is needed to confirm this relationship [78, 79].

Gram-negative organisms, such as *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *S. maltophilia* have been reported in hospitalized patients with COVID-19, especially in ICU-acquired infections [45, 55, 67, 80–84]. A study showed that the prevalence of NF-GNB infections rose from 42% in the pre-COVID-19 period (2018–2019) to 58% during the pandemic (2020–2021) [85]. This increase is associated with factors, such as prolonged hospitalization in the ICU, use of high-risk invasive devices, including mechanical ventilation, vascular catheters, and urinary catheters in COVID-19 patients, which elevate the risk of acquiring HAIs [80, 81, 86]. In the present study, *P. aeruginosa* was detected in 11.4% of COVID-19 patients, consistent with the findings reported by Mahmoudi et al. [87]. In agreement with Yang et al. [55], an important finding was the association between severe/critical COVID-19 and *P. aeruginosa* detection after adjustment for potential confounders. The odds of *P. aeruginosa* detection were approximately sevenfold higher in severe/critical cases than in mild cases (P = 0.048, OR = 6.88 vs. mild). Severe illness may increase the risk of colonization due to hospitalization, invasive procedures, antibiotic exposure, frequent contact with healthcare facilities, and impaired host defenses [59, 88]. Conversely, bacterial detection may also be associated with greater severity of illness. As this study was retrospective and the time of sampling was not recorded, its directionality cannot be determined. In addition, the unadjusted associations of *P. aeruginosa* detection with older age, fever, and ICU admission are clinically plausible. Older adults and critically ill patients may have more comorbidities, prolonged exposure to hospital environments, higher use of invasive devices, and increased susceptibility to opportunistic organisms [59, 89–91]. However, ICU admission was no longer statistically significant after multivariable adjustment and was therefore not considered an independent risk factor.

Regarding the *B. pertussis*, we found PCR-positive patients older than negative individuals for this organism (P = 0.035). Duan et al. reported a significant difference between COVID-19 without bacterial infection and with bacterial secondary infection (P = 0.002) [47], suggesting elderly patients with COVID-19 are more susceptible to bacterial co-pathogen. In addition, detection of this bacterium among COVID-

19 patients was significantly associated with decreased SpO_2 levels (P = 0.033), consistent with the findings of a previous study [92].

This study has several strengths, including a relatively large early-pandemic cohort, systematic testing for multiple bacterial targets, and the inclusion of demographic, clinical, laboratory, and outcome data. Furthermore, it provides early-pandemic data from northern Iran, a region where molecular epidemiological data remain limited. However, there are several limitations. First, the retrospective nature of study limited the completeness of clinical, laboratory, and radiological findings, which in many cases precluded acceptable statistical analysis. Second, the interval between admission and respiratory sampling was not consistently recorded, and specimens were obtained at variable points during hospitalization; therefore, community-onset and hospital-onset detections could not be distinguished. Third, PCR positivity cannot differentiate viable organisms from nonviable DNA or distinguish colonization from infection. Fourth, no bacterial culture, antimicrobial susceptibility testing or detailed antibiotic-exposure data were available. Finally, subgroup sizes for several organisms were small, and organism-specific analyses should be considered exploratory.

In conclusion, this retrospective study detected at least one of eight respiratory bacterial pathogens in upper-airway specimens from a large proportion of hospitalized patients with COVID-19 during the early pandemic period. Because PCR positivity may represent colonization rather than infection, our findings remain associative. In other settings, these organisms have been reported to opportunistically worsen severity and mortality, but our data cannot confirm such effects here. Severe COVID-19 remained independently associated with the presence of *P. aeruginosa*. In contrast, older age, and fever/ICU admission were associated with *P. aeruginosa* detection in the unadjusted analysis; we interpret these as associations in elderly or critically ill patients rather than evidence of a causal mechanism. Some trends, including *B. pertussis* in older patients and *S. aureus* with fever/creatinine level aligned with COVID-19 severity markers, but require prospective validation to rule out collinearity with COVID-19 progression. In addition, hypertension linked to polybacterial detection likely reflects comorbidities rather than direct causation. Future prospective studies using standardized sampling at admission and during hospitalization, alongside culture and susceptibility testing and detailed information on antibiotic exposure and invasive procedures are warranted to clarify these dynamics.

Abbreviations

AOR
Adjusted odds ratios
CI
Confidence interval
COVID-19
Coronavirus disease-2019
ICUs
intensive care units

IQR
Interquartile range
LDH
Lactate dehydrogenase
PCR
Polymerase chain reaction
RT-PCR
Real-time reverse transcription–polymerase chain reaction
SARS-CoV-2
Severe acute respiratory syndrome coronavirus 2
SPSS
Statistical package for the social sciences
WHO
World Health Organization

Declarations

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Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

Ethics approval and consent to participate

This study protocol was reviewed and approved by the Ethics Committee of Golestan University of Medical Sciences (approval no. IR.GOUMS.REC.1399.422). The study was conducted in line with the principles of the Declaration of Helsinki. The need for written informed consent was waived by the Ethics Commission for emerging infectious diseases.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author Contribution

AA, AV, and HRN conceptualized and designed the study. AV, HJ, AI, and MH performed the experiments. AT, AM, AH, and SAA contributed to the preparation of materials and the setting up of laboratory experiments. AA, AV, and HJ interpreted the results and collected and analyzed the data. AA drafted and critically revised the manuscript. HJ, MR, and MY contributed to the drafting of the manuscript. All authors read and approved the final version.

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

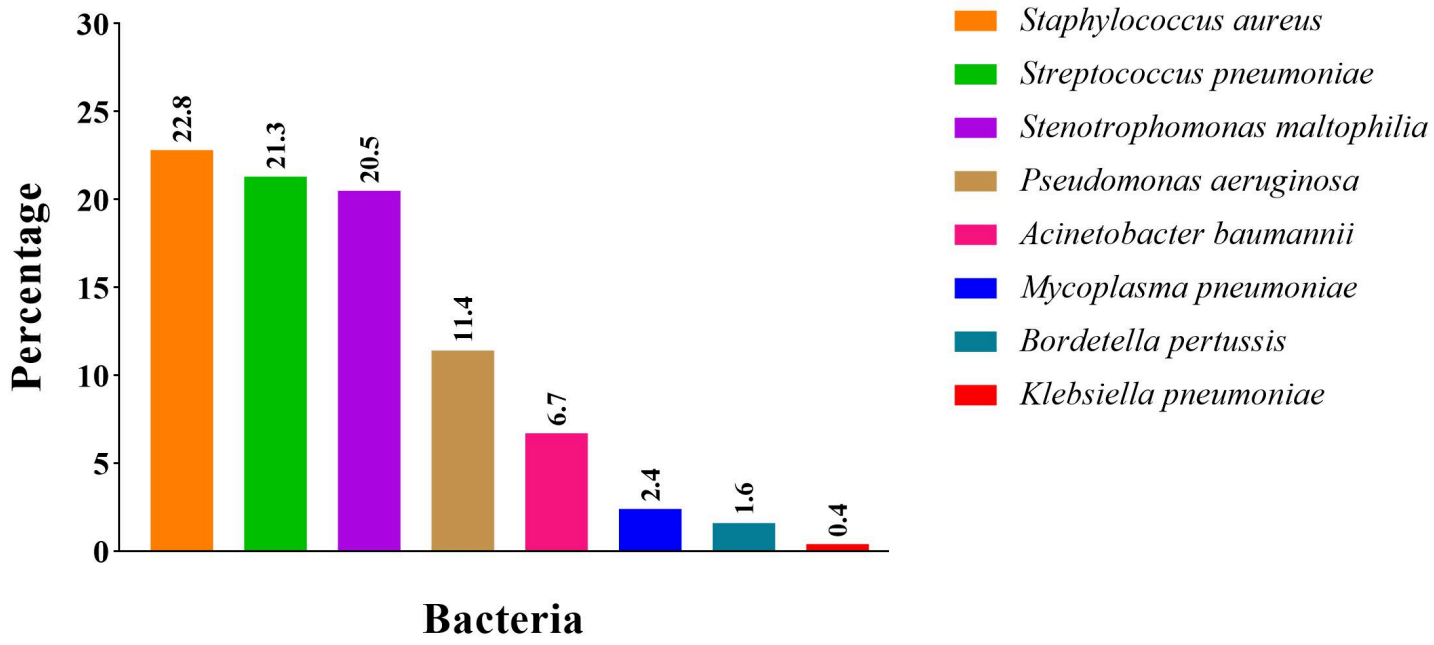


Figure 1

Distribution of respiratory bacterial co-pathogens in patients with SARS-CoV-2. Bars represent PCR positivity rates, not clinically confirmed infections. The X-axis shows bacterial species, while the Y-axis represents the percentage of each co-pathogen among 254 COVID-19 patients. The percentage value is displayed above each bar. *S. aureus*, *S. pneumoniae*, *S. maltophilia*, and *P. aeruginosa* were the four most common co-pathogens with frequencies of 22.8% (58/254), 21.3% (54/254), 20.5% (52/254), and 11.4% (29/254), respectively.

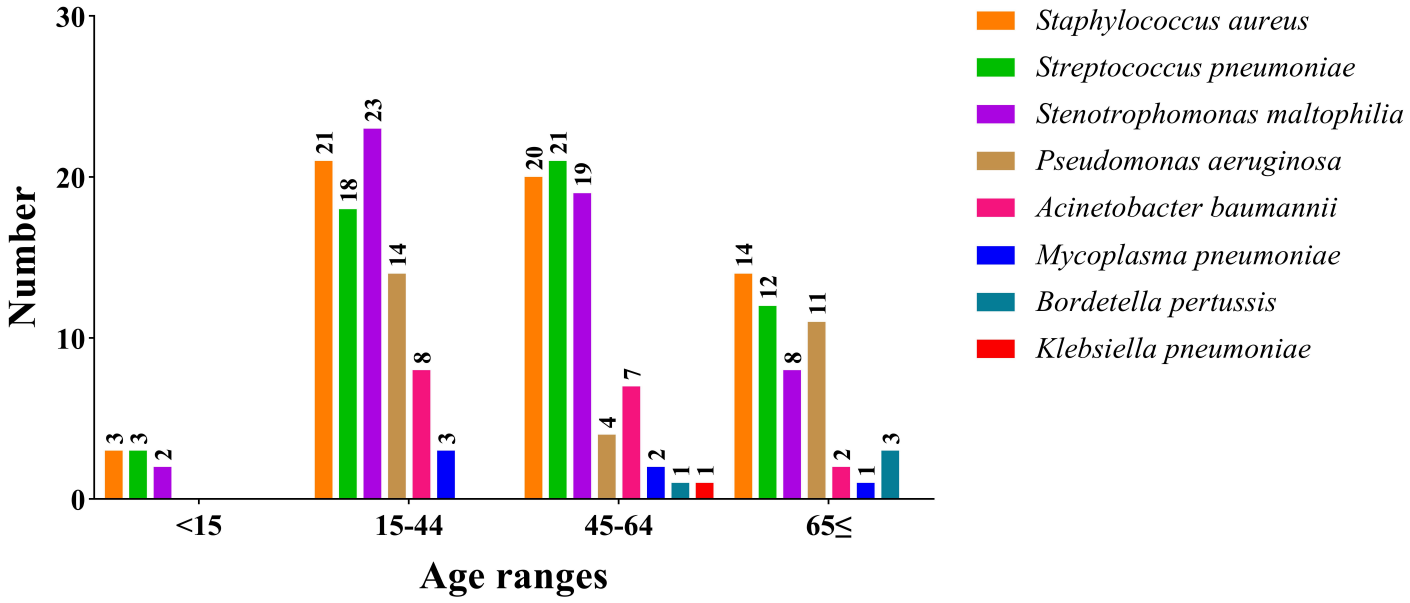


Figure 2

Distribution of respiratory bacterial co-pathogens in different age groups of COVID-19 patients. Bars represent PCR positivity rates, not clinically confirmed infections. The X-axis shows patients' age ranges (years), and the Y-axis indicates the number (count) of each bacterial co-pathogen, as is displayed above each bar. In the age group of 45 to 64 years, all eight bacterial species were detected, followed by ≥ 65 years, 15-44 years, and < 15 years groups, with 7, 6, and 3 bacteria discovered, respectively.

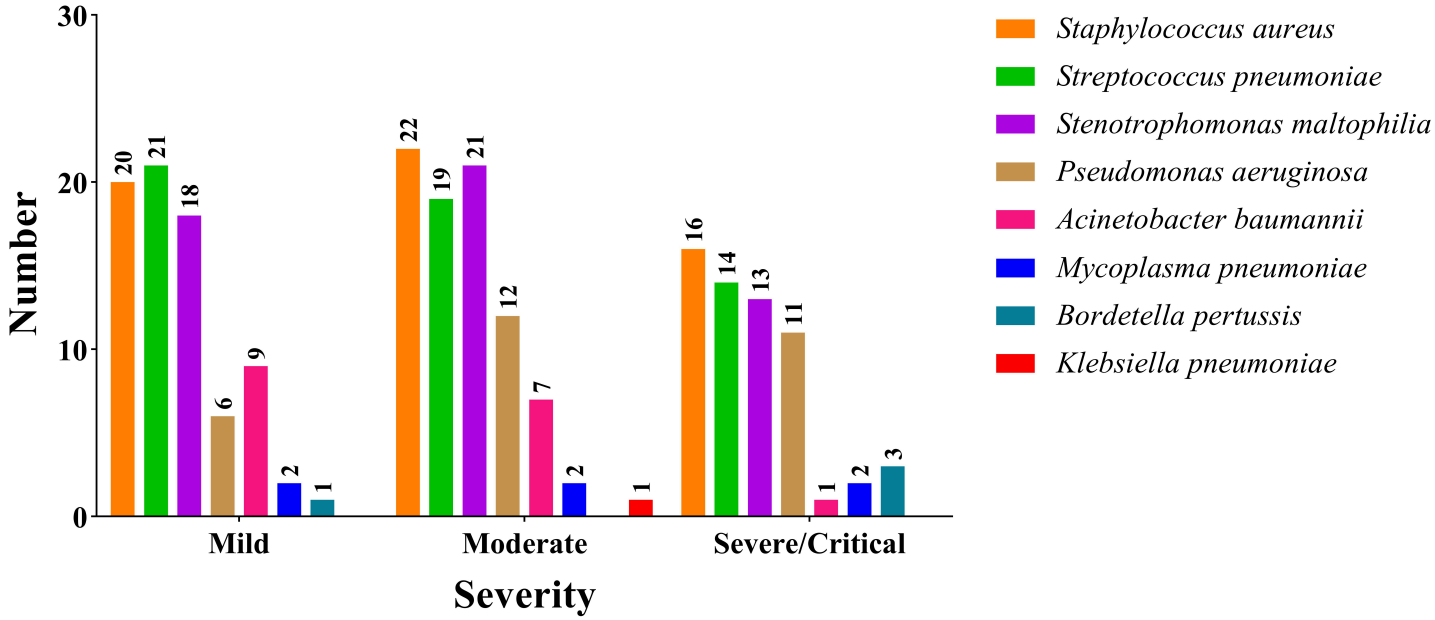


Figure 3

Distribution of respiratory bacterial co-pathogens in different severity groups of COVID-19 patients. Bars represent PCR positivity rates, not clinically confirmed infections. The X-axis shows COVID-19 severity, and the Y-axis indicates the number (count) of each bacterial co-pathogen, as is displayed above each bar. Except for *B. pertussis* and *K. pneumoniae*, other bacterial species were commonly found in all severity groups.

Supplementary Files

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