Archives of Razi Institute, Vol. 78, No. 1 (2023) 397-403

DOI: 10.22092/ARI.2022.359035.2360

Original Article

Isolation of *Pseudomonas aeruginosa* from Persistent Bacterial Coinfection of a COVID-19 Patients with Molecular Detection of Antibiotics Resistance Genes

Miri Mizher Al Muhana, B[1 *], Ali, S. A[2], Sabri Jabbar, A[2]

1. Veterinary Microbiology Department, College of Veterinary Medicine, Al-Qadisiyah University, Al-Qadisiyah, Iraq
2. Clinical Laboratory Sciences Department, College of Pharmacology, Al-Qadisiyah University, Al-Qadisiyah, Iraq

Received 13 June 2022; Accepted 10 July 2022
Corresponding Author: balsam.mizher@qu.edu.iq

Abstract

Pseudomonas aeruginosa (P. aeruginosa) have a considerable risk to public health in the world, due to its high ability to develop resistance to different classes of antibiotics. It has been discovered as a prevalent coinfection pathogen that causes sickness exacerbation in COVID-19 patients. This study aimed to determine the prevalence of *P. aeruginosa* from COVID-19 patients in Al Diwaniyah province, Iraq and to identify its genetic resistance pattern. 70 clinical samples were obtained from severe cases of patients (RT-PCR positive for SARS-COV-2 on a nasopharyngeal swab) who attended Al Diwaniyah Academic Hospital. 50 *P. aeruginosa* bacterial isolates were detected via microscopic examination, routine cultured and biochemical testing, then validated by the VITEK-2 compact system. VITEK reported 30 positive results, which later confirmed through molecular detection using 16s RNA specific for detection and a phylogenetic tree.20 isolates had positive PCR findings and 5 isolates submitted to GenBank with accession numbers OL314557.1, OL314556.1, OL314555.1, OL314554.1, OL314553.1.For antibiotic resistance genes, the number of the isolates containing *bla*OXA-1 and *bla*CTX-M 18 (90 percent) and 16 (80 percent) respectively. To study its adaptation in a SARS-CoV-2 infected environment, genomic sequencing investigations were undertaken with phenotypic validation. In conclusion, we demonstrate that multidrug resistant *P. aeruginosa* play an important role in *in vivo* colonization in COVID-19 patients and could be one of the causes of death of these patients which indicates the great challenge to clinicians in the facing of this serious disease.

Keywords: COVID-19; *Pseudomonas aeruginosa*; *bla OXA-1*, *bla CTX-M*; antibiotic resistance; coinfection

1. Introduction

Coronavirus Disease 2019 (COVID-19) is a lethal lung infection caused by the novel coronavirus (SARS-CoV-2). The COVID-19 pandemic has resulted in millions of deaths worldwide (1-3). It damages the lungs, as well as other organs like as the heart, liver, and kidneys, severely (4, 5). COVID-19 patients with advanced age and underlying illnesses had a higher mortality rate (6). After a viral infection, a shift in the microbial ecology of the lungs often worsens the condition and makes the host more susceptible to subsequent bacterial coinfection (7). In the last century's influenza pandemics, bacterial coinfection was one of the leading causes of death (8-10). During the 2009 H1N1 influenza pandemic, bacterial coinfection was identified in about 30% of cases, despite antibiotic therapy (7, 11).Several retrospective studies based on cases from various geographical regions have also revealed bacterial coinfection with SARS-CoV-2 (12-15).

In a retrospective cohort study in Wuhan, China, Zhou found that bacterial infections were more

common in fatal COVID-19 cases, compared with recovered cases , 28/191 (15%) of patients had a culture-positive bacterial infection, and of these patients, all but one died (6).

Haemophilus influenzae, Staphylococcus aureus, Klebsiella pneumonia, Mycoplasma pneumoniae, Streptococcus pneumoniae, and *Pseudomonas aeruginosa* are some of the most prevalent bacterial co-infections (16). According to the results in Lansbury *et al* study, *P. aeruginosa* is the second most often found pathogen in COVID-19 patients (12). *P.aeruginosa* is a biofilm-forming opportunistic bacteria that causes life-threatening chronic infections in immunocompromised people who have burn wounds, urinary tract infections, or respiratory infections (17).

The World Health Organization (WHO) has categorized *P. aeruginosa* as a critical priority pathogen, which needs urgent novel antibiotics intervention and was given a serious threat level due to multidrug resistance displayed to many antibiotics. Different mechanismsincluded in the resistance patterns of *P. aeruginosa* such as over expression of efflux pump, acquisition of Extended-Spectrum β-Lactamases (ESBLs) and Metallo-β-Lactamases (MBLs) (18). ESBLs are a cluster of β-lactamases that inactivates β-lactams, they are encoded on plasmids and can easily be conveyed from one organism to another. ESBL enzymes classified into A and D. The most prevalent enzymes in class A include *bla*TEM, *bla*CTX-M and *bla*SHV, and class D (OXA type) has been described in *P. aeruginosa* strains (19). The production of these enzymes is a going concern for infection controls supervision because it restricts therapeutic choices. Antibiotic surveillance studies are important for the design of control strategies for preventing bacterial resistance and establishing therapeutic guidelines. Despite the fact that *P. aeruginosa* coinfection has been observed, to the best of our knowledge, there are few reports on prevalence of antimicrobial resistant clinical isolates of *P. aeruginosa* from samples obtained from COVID-19 patients particularly in Iraq.

2. Materials and Methods

2.1. Patients

70 very critical cases of patients admitted to ICU wards in Al Diwaniyah Academic Hospital, Iraq, were enrolled in this study. Patients were administered antibiotics before being admitted to the ICUs. Inclusion criteria included being infected with COVID-19 (RT-PCR positive for SARS-COV-2 on a nasopharyngeal swab), being hospitalized, intubated, and mechanically ventilated in ICUs for more than 48 hours.

All of our patients had elevated C-reactive protein (CRP) values, high erythrocyte sedimentation rates, neutropenic, previously had cough, sore throat, or shortness of breath.

2.2. Samples Collection and Identification

Sputum samples were collected fromeach patient who remained in ICUs. All samples were routinely cultivated on nutrient, MacConkey and Blood agar plates. Suspected colonies were sub-cultured on Cetrimide agar for selective isolation and identified by Gram staining, colony characteristics, motility and pyocyanin production. Strains were identified to the species level with Vitek 2 (bioMérieux, Inc. USA) *P. aeruginosa* isolates were transferred to 1 percent nutritional agar slant and kept at 4 C° in the refrigerator.

2.3. Extraction of DNA

DNA was isolated from bacterial broth according to the Genomic DNA Mini Kit's manufacturer's instructions (Geneaid). The extracted DNA was electrophoresed on an agarose gel (1 percent agarose stained with 3L of ethidium bromide) to ensure that DNA was present in each sample, and then microcentrifuge tubes containing DNA were stored at -20°C in a deep freezer and used in PCR.

2.4. Primer Preparation and PCR Reaction

5µl of the template DNA, 12 PCR water Bioneer (South Korea). Amplification was carried out in thermocycler (Eppendorf mastercycler ®) (bioneer-south korea). Agarose gel electrophoresis (1.5%) of PCR products was carried out using mM Tris-Borate-EDTA(TBE) buffer at 70V for 1hour, and the DNA

bands were stained with ethidium bromide (sinaclon Iran) 100bp DNA ladder was used to confirm the size specific. To detect 16S rRNA as diagnosis of *Pseudomonas aeruginosa, bla- OXA and CTX* of , PCR reactions performed in a total volume of 25 μl containing 5 μl of the DNA sample, 2 μm of each primers, 2 μm Magnesium chloride ($MgCl_2$), 10 μl PCR buffer AMS, 200 μm dNTPs, and 1 unit of Taq DNA polymerase .The PCR assay was performed at 95C° for 5 minutes and then for 35 cycles of 94C° for 30 second , 60C° for 40 seconds, 72C° for 30 seconds, and a final extension at 72C° for 5 minutes, with a final hold at 4C° in a thermal cycler (Thermo cycler; Eppendorf, Germany) for housekeeping gene 16s RNA .while For *bla -OXA and CTX* amplifications, conditions for thermal cycling remained the same except for the annealing temperature (55C°).

The primer sequences for 16S rRNA as diagnosis of *Pseudomonas aeruginosa,CTX and OXA* are shown in table 1. Agarose gel 1.5% was used to run the amplified products and staining with ethidium bromide (3µl) in a darkness. The electrode buffer used wasTris-borate-EDTA (TBE), which consists of Tris-base 10.8 g 89 mM, boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 mL of 0.5 M EDTA (pH 8.0) (all components were combined in sufficient H_2O and stirred to dissolve). A 100-bp ladder molecular weight marker (Roche, New Jersey, USA) was used to measure the molecular weight of the amplified products.

Table 1. The primer sequences for 16S rRNA as diagnosis of *Pseudomonas aeruginosa* and antibiotic resistance gene *bla CTX*-M and *bla OXA*

Primer		Sequence	Amplification
16s RNA	F	TCAACCTGGGAACTGCATCC	668 bp
	R	CAGACTGCGATCCGGACTAC	
bla - OXA	F	ATATCTCACTGTTGCATCTCC	618bp
	R	AAACCCTTCAAACCATCC	
bla CTX-M	F	CGCTTTGCGATGTGCAG	550bp
	R	ACCGCGATATCGTTGGT	

Aliquots (10 µl) of PCR products were applied to the gel. A constant voltage of 80 V for 1 hour used for product separation. The images of ethidium bromide-stained DNA bands were digitized using an UVItec (UVItec, Paisley, UK).

3. Results

On nutrient agar, 50 (71.4%) of the sputum samples yield good findings, producing circular mucoid smooth colonies with a sweat grape odor (Figure 1-left). It was seen to produce -hemolysis on blood agar and grew on Cetrimide agar, producing fluorescein and pyocyanin pigments with blue colors, indicating that it was *P. aeruginosa* (Figure 1-right).

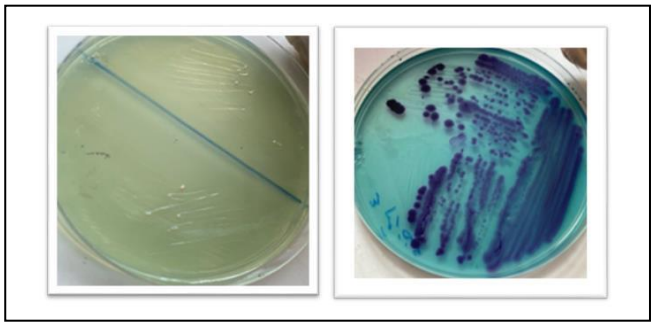

Figure 1. Left picture *P. aeruginosa* on nutrient agar.Right picture *P. aeruginosa* grow on Cetrimide agar

In Vitek2, only 30 (60 %) of positive cultivated bacteria confirmed as *P. aeruginosa*, and molecular detection by polymerase chain reaction is the endpoint diagnosis, giving 20 (66 %) positive results by employing 16s RNA for detection (Figure 2). Table 2 and chart 1 illustrate the results of each diagnostic method used for detection of *p.aeruginosa* in present study.

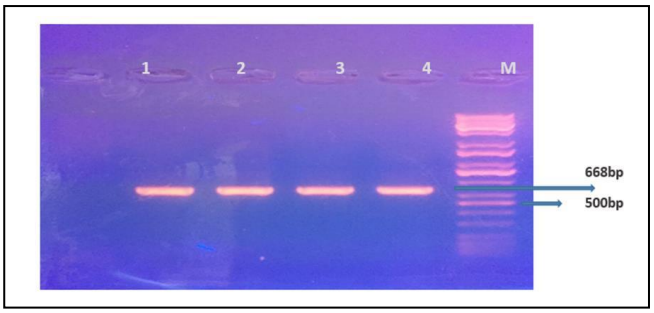

Figure 2. PCR for the detection of 16s RNA housekeeping gene (668bp) of *Pseudomonas aeruginosa*

Table 2. Numbers of positive and negative results from culture method, VITEK, and PCR

Samples	Culture	VITEK-2	PCR
Positive	50(71.4%)	30(60%)	20(66.6%)
Negative	20(29.6%)	20(40%)	10(34.4%)
Total	70(100%)	50(100%)	30(100%)

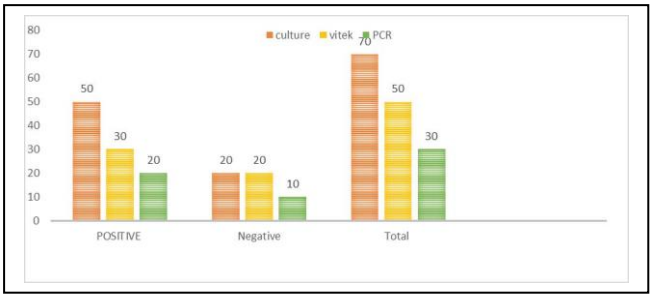

Chart 1. *pseudomonas aeruginosa* number and their isolation percentages of samples by cultured, vitek2, PCR methods

P. aeruginosa has the highest resistance rate to oxacillin and cefotaxime, and this resistance was driven by ESBL producing strains, $bla_{\text{OXA-1}}$ was found among 90% of positive results of *P. aeruginosa* (18 isolates),while 80% (16 isolates) were producing $bla_{\text{CTX-M}}$ (Figure 3).In addition, bla_{SHV} and bla_{TEM} were not detected in all tested strains.

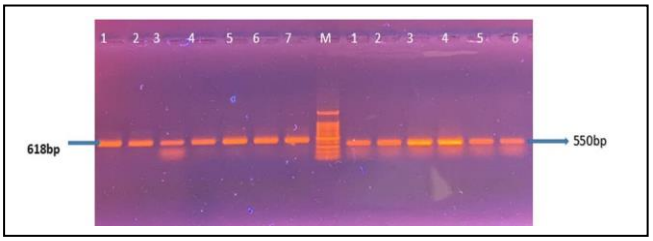

Figure 3. PCR for the detection of *bla*CTX-M (550 bp) and *bla*OXA gene (618bp) of *Pseudomonas aeruginosa*

Sequencing and phylogenetic tree construction of 16S rRNA gene

P.aeruginosa isolates with accession numbers OL314557.1, OL314556.1, OL314555.1, OL314554.1, OL314553.1 were linked with global reference strains for recording in the GenBank from 20 (66.6 %) PCR positive results.

The phylogenetic tree of *P. aeruginosa* revealed that local strains were 100 % identical to isolates from both human and near relatives of *P. aeruginosa* strain PAO1 (Figure 4).

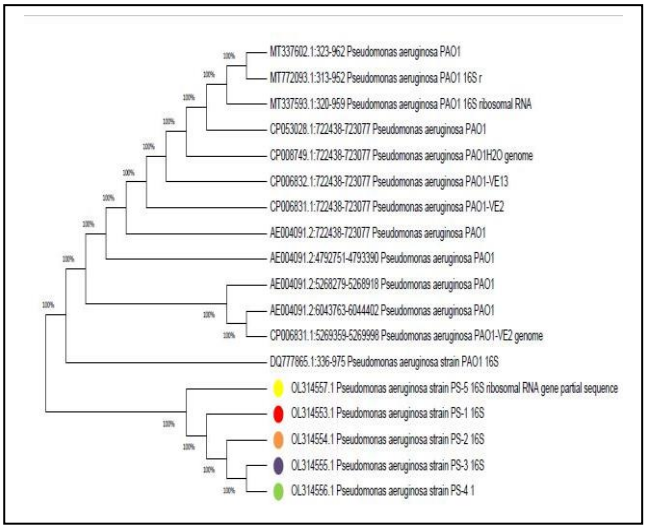

Figure 4. Phylogenetic tree of *Pseudomonas aeruginosa* with world strain

4. Discussion

The COVID-19 pandemic has affected many countries, including Iraq. From 24February 2020 until 1 January 2022, there have been over 2093891 confirmed cases and over 24163 deaths reported nationwide.

Coinfection with bacteria, fungi and other respiratory viruses, in COVID-19 patients has been reported to occur. Bacterial coinfection is a major causative agent of coinfection in particular is a worrisome issue as it complicates treatment in these patients and may increase the possibility of fatality (1, 20).

Our results demonstrated the high presence of *p.aeruginosa* among COVID-19 patients. The results are in line with previous studies reporting that Gram-negative pathogens were predominant secondary infection in COVID-19 patients, and the most common bacterial infections in patients with either influenza or COVID-19 was *P. aeruginosa* (21).

P. aeruginosa is a respiratory opportunistic pathogen, it is also known as the most common Gram-negative bacterial species associated with severe hospital-acquired infections (HAIs) in some hospitals.It is well known that most severe or moderate hospitalized COVID-19 patients are prescribed steroidsand sometimes have a prolonged hospital stay, rendering

them at risk to HAIs. Predominance of *P. aeruginosa* could be due to the invasive device-associated infections during hospitalization due to mechanical ventilation and central venous catheter implantation in these patients. Another interpretation for prevalence of these bacteria is the destruction of respiratory tract tissues by viral infection and modulation of immune cells/cytokines, causing microbiome dysbiosis and bacterial colonization (22). Virus infections have been shown to aid bacterial colonization and enhance biofilm development in previous research. After influenza virus infection, pathogenic factors were secreted by coinfecting bacteria such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, allowing patients to move from non-invasive colonization to secondary bacterial infection (18, 23). Damaged mucus limits *P. aeruginosa* biofilm dispersion and boosts the expression of virulence pathways through modulating motility, quorum sensing systems, and the generation of siderophore and toxins, according to a recent study (24). The *SARS-CoV-2* virus produces tissue destruction, such as diffuse alveolar injury and alveolar epithelial cell shedding. After viral infection, such abnormalities in tissues and decreased host immunity provide *P. aeruginosa* a chance to increase its virulence (25). These findings imply that *P. aeruginosa* pathogenicity is influenced by pathological changes in host tissues as well as variations in niche environmental factors.

The most recent clinical management interim guideline for COVID-19 from the WHO discourages the use of antibiotics in mild COVID-19 cases to prevent exacerbation of antibiotic resistance (26). However, antibiotics included in Iraqi COVID-19 treatment protocol. The inappropriate prescribing of antibiotics when not needed can amplify the increasing antibiotic resistance problem, especially as the prescribed antibiotics tend to be broad-spectrum (13).

In our study, we have highlighted the dissemination of *bla CTX-M* and *bla OXA-1*-producing *P.* aeruginosa in the ICU admitted COVID-19 patients. Several causes are attributed to the high prevalence of drug resistance pathogens. Limitation in implementing stewardship programs and strict infection control measures in the hospitals as well as over prescription of antibiotics, being available over the counter in outpatient settings. High ESBL rates among isolated pathogens in our study could be the consequence of excessive use of these antibiotics.Furthermore, ESBL-producing bacteria is mainly transmitted from patient to patient directly by the medical staff's hands, or indirectly via the environment (27).

We used molecular detection and a phylogenetic tree to characterize the *P. aeruginosa* isolates from COVID-19 patient sputum samples. The phylogenetic tree results similarity and identity with100% with *pseudomonas aeruginosa* strain PAO1,these strain of *Pseudomonas aeruginosa* PAO1 produces three polysaccharides, alginate, Psl, and Pel that play distinct roles in attachment and biofilm formation

P. aeruginosa, as a top coinfecting pathogen, was found to be capable of inducing bacterial coinfection throughout the crucial stage of COVID-19 pneumonia. Understanding these *P. aeruginosa* genetic modifications may substantially aid in illness prediction and treatment scheme selection for secondary infections caused by *P. aeruginosa* in COVID-19 patients.

Our report is one of the first to demonstrate the presence of *P. aeruginosa* co-infections in the respiratory tract of patients with COVID-19.Bacterial coinfection in COVID-19 patients has the potential to complicate treatments and accelerate the development of antibiotic resistance in the clinic due to the widespread use of broad-spectrum antibiotics. Consequently, it is important to pay attention to bacterial co-infections in critical patients positive for COVID-19. Overall, it is important to limit the risk of infection and the spread of these resistant strains through controlling nosocomial infections accurately and bringing secondary infections caused by resistant bacteria that can increase the mortality rate in COVID19 critical patients into attention.

Authors' Contribution

Study concept and design: S. A. A.
Acquisition of data: B. M. M. A. M.
Analysis and interpretation of data: A. J.
Drafting of the manuscript: A. S. J.
Critical revision of the manuscript for important intellectual content: B. M. M. A. M.
Statistical analysis: B. M. M. A. M.
Administrative, technical, and material support: B. M. M. A. M.

Ethics

The human study were approved by the ethics committee of the Al-Qadisiyah University, Al-Qadisiyah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020;395(10223):507-13.
2. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. N Engl J Med. 2020;382(13):1199-207.
3. Shen C, Wang Z, Zhao F, Yang Y, Li J, Yuan J, et al. Treatment of 5 Critically Ill Patients With COVID-19 With Convalescent Plasma. JAMA. 2020;323(16):1582-9.
4. Liu K, Chen Y, Lin R, Han K. Clinical features of COVID-19 in elderly patients: A comparison with young and middle-aged patients. J Infect. 2020;80(6):e14-e8.
5. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. JAMA. 2020;323(11):1061-9.
6. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020;395(10229):1054-62.
7. Hanada S, Pirzadeh M, Carver KY, Deng JC. Respiratory Viral Infection-Induced Microbiome Alterations and Secondary Bacterial Pneumonia. Front Immunol. 2018;9:2640.
8. Bisno AL, Griffin JP, Van Epps KA, Niell HB, Rytel MW. Pneumonia and Hong Kong influenza: a prospective study of the 1968-1969 epidemic. Am J Med Sci. 1971;261(5):251-63.
9. Cilloniz C, Ewig S, Menendez R, Ferrer M, Polverino E, Reyes S, et al. Bacterial co-infection with H1N1 infection in patients admitted with community acquired pneumonia. J Infect. 2012;65(3):223-30.
10. Hers JF, Masurel N, Mulder J. Bacteriology and histopathology of the respiratory tract and lungs in fatal Asian influenza. Lancet. 1958;2(7057):1141-3.
11. Gill JR, Sheng ZM, Ely SF, Guinee DG, Beasley MB, Suh J, et al. Pulmonary pathologic findings of fatal 2009 pandemic influenza A/H1N1 viral infections. Arch Pathol Lab Med. 2010;134(2):235-43.
12. Lansbury L, Lim B, Baskaran V, Lim WS. Co-infections in people with COVID-19: a systematic review and meta-analysis. J Infect. 2020;81(2):266-75.
13. Rawson TM, Moore LSP, Zhu N, Ranganathan N, Skolimowska K, Gilchrist M, et al. Bacterial and Fungal Coinfection in Individuals With Coronavirus: A Rapid Review To Support COVID-19 Antimicrobial Prescribing. Clin Infect Dis. 2020;71(9):2459-68.
14. Zayet S, Kadiane-Oussou NJ, Lepiller Q, Zahra H, Royer PY, Toko L, et al. Clinical features of COVID-19 and influenza: a comparative study on Nord Franche-Comte cluster. Microbes Infect. 2020;22(9):481-8.
15. Zhu X, Ge Y, Wu T, Zhao K, Chen Y, Wu B, et al. Co-infection with respiratory pathogens among COVID-2019 cases. Virus Res. 2020;285:198005.
16. Faddin JFM. Biochemical Tests for Identification of Medical Bacteria: Lippincott Williams & Wilkins; 2000.
17. Gellatly SL, Hancock RE. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. Pathog Dis. 2013;67(3):159-73.
18. Pettigrew MM, Marks LR, Kong Y, Gent JF, Roche-Hakansson H, Hakansson AP. Dynamic changes in the Streptococcus pneumoniae transcriptome during transition from biofilm formation to invasive disease upon influenza A virus infection. Infect Immun. 2014;82(11):4607-19.
19. Zhao WH, Hu ZQ. Beta-lactamases identified in clinical isolates of Pseudomonas aeruginosa. Crit Rev Microbiol. 2010;36(3):245-58.

20. Chen X, Liao B, Cheng L, Peng X, Xu X, Li Y, et al. The microbial coinfection in COVID-19. Appl Microbiol Biotechnol. 2020;104(18):7777-85.
21. Shafran N, Shafran I, Ben-Zvi H, Sofer S, Sheena L, Krause I, et al. Secondary bacterial infection in COVID-19 patients is a stronger predictor for death compared to influenza patients. Sci Rep. 2021;11(1):12703.
22. Bakaletz LO. Viral-bacterial co-infections in the respiratory tract. Curr Opin Microbiol. 2017;35:30-5.
23. Reddinger RM, Luke-Marshall NR, Hakansson AP, Campagnari AA. Host Physiologic Changes Induced by Influenza A Virus Lead to Staphylococcus aureus Biofilm Dispersion and Transition from Asymptomatic Colonization to Invasive Disease. mBio. 2016;7(4).
24. Wheeler KM, Carcamo-Oyarce G, Turner BS, Dellos-Nolan S, Co JY, Lehoux S, et al. Mucin glycans attenuate the virulence of Pseudomonas aeruginosa in infection. Nat Microbiol. 2019;4(12):2146-54.
25. Martines RB, Ritter JM, Matkovic E, Gary J, Bollweg BC, Bullock H, et al. Pathology and Pathogenesis of SARS-CoV-2 Associated with Fatal Coronavirus Disease, United States. Emerg Infect Dis. 2020;26(9):2005-15.
26. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18(3):318-27.
27. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev. 2011;35(5):736-55.