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Research Article

EXTENDED SPECTRUM β-LACTAMASES PRODUCING AND BIOFILM FORMING MULTIDRUG RESISTANT *KLEBSIELLA PNEUMONIAE* FROM BACTERIAL COINFECTION AND SECONDARY INFECTION IN PATIENTS WITH CORONAVIRUS DISEASE AT A TERTIARY CARE HOSPITAL, KATHMANDU, NEPAL

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ABSTRACT

Bacterial co-infections and secondary infections in Coronavirus disease (COVID-19) patients can significantly worsen patient outcomes. Excessive use of antibiotics without prescription during viral infections might result in increase in antimicrobial resistance. This study aimed to determine the prevalence of these bacterial infections and assess multidrug resistance (MDR), Extended Spectrum β -Lactamases (ESBL) production, and biofilm formation among predominant isolates. A prospective study was conducted at Sukraraj Tropical and Infectious Diseases Hospital, Kathmandu, from July 2022 to June 2023. A total of 141 sputum samples from confirmed COVID-19 cases were processed using conventional culture methods. Antimicrobial susceptibility testing was done using the modified Kirby-Bauer Disc Diffusion method following Clinical Laboratory Standard Institute guidelines. Biofilm formation was detected by microtitre plate assay and ESBL production was confirmed phenotypically by the combined disc test. Deoxyribonucleic acid from ESBL-positive isolates was tested for the *blaCTX-M* gene using conventional polymerase chain reaction. Out of 141 patients, 9 (6.38%) showed bacterial co-infections and 11 (7.80%) showed secondary bacterial infections. Gram-negative bacteria were predominant, with *Klebsiella pneumoniae* 12 (60.00%) being the most common isolate. High resistance rates were observed in *K. pneumoniae* for Ceftriaxone 8 (66.70%) and Amikacin 7 (58.30%), while Imipenem remained effective for 11 (91.7%) isolates. Among the bacterial isolates 14/20 (70.00%) were MDR, 8/20 (40.00%) were biofilm producers and 8/19 (42.10%) were ESBL producers, with 6/8 (75.00%) harboring the *blaCTX-M* gene. To the best of our knowledge, this is one of the few studies to report ESBL producing MDR bacteria isolated from co-infection and secondary bacterial infection among COVID positive cases in Nepal. Continuous monitoring of bacterial infections and resistance patterns is crucial for improving infection control and managing the pandemic.

KEYWORDS: Bacterial coinfection, $bla_{CTX\text{-}M,}$ ESBL, *Klebsiella pneumoniae*, Multidrug resistant, Secondary bacterial infection

INTRODUCTION

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its associated illness, coronavirus disease 2019 (COVID-19), marked a turning point in global health since its discovery in Wuhan, China, in December 2019. While the primary focus has been on managing viral infections, evidences suggest a significant interplay between viral and bacterial pathogens, particularly in severe cases requiring intensive care unit (ICU) admission. Previous studies have highlighted the propensity for viral infections, such as influenza, to predispose patients to secondary bacterial pneumonia, a phenomenon commonly observed in critically ill patients (Liu *et al.*, 2021).

Among the bacterial pathogens complicating COVID-19, *Klebsiella pneumoniae* has been particularly concerning. This gram-negative bacterium is notorious for its ability to acquire and disseminate resistance genes, making infections difficult to treat. Of particular importance are strains that produce Extended Spectrum Beta-Lactamases (ESBLs), enzymes that confer resistance to a broad spectrum of beta-lactam antibiotics, including penicillins and

cephalosporins. Additionally, the ability of *K. pneumoniae* to form biofilms further complicates treatment, as biofilms protect the bacteria from both the host immune system and antibiotic therapy, leading to persistent and chronic infections. The excessive and often unregulated use of antibiotics during the COVID-19 pandemic has exacerbated the problem of antimicrobial resistance. Many patients have received antibiotics empirically to prevent or treat suspected bacterial infections, even in the absence of confirmed bacterial etiology. This practice has likely contributed to the selection and proliferation of multidrug-resistant (MDR) bacteria (Yang *et al.*, 2024).

In Nepal, where healthcare resources are limited, the burden of bacterial co-infections and the prevalence of antibiotic-resistant pathogens pose a significant challenge. Despite the critical importance of understanding the dynamics of bacterial infections in COVID-19 patients, there has been a paucity of data from this region. This study aims to fill this gap by investigating the prevalence and characteristics of ESBL-producing and biofilm-forming multidrug-resistant *K. pneumoniae* in COVID-19 patients at a tertiary care hospital in Kathmandu. By assessing the antibiotic resistance profiles, biofilm formation capabilities, and the presence of specific resistance genes such as *blaCTX-M*, this study seeks to provide valuable insights into the management of bacterial co-infections and secondary infections in COVID-19 patients. The findings are expected to inform local and regional infection control policies, guiding more effective antibiotic stewardship and helping to mitigate the impact of antimicrobial resistance during and beyond the COVID-19 pandemic.

MATERIALS AND METHODS

Study Design, Period and Setting

This was a cross-sectional prospective study conducted at Sukraraj Tropical and Infectious Disease hospital (STIDH), Teku, Kathmandu from July 2022 to June 2023. STIDH is the only infectious and tropical disease hospital in Kathmandu, Nepal. It is the national referral hospital with the capacity of 100 beds inpatient service and receives patients referred from all over the country.

Study Population

Patients visiting STIDH and confirmed as COVID positive by laboratory through polymerase chain reaction (PCR) were included in the study. Considering the estimated prevalence of bacterial infection in COVID cases (p) to be 7% with 95% confidence interval (z), 5% maximum tolerable error, the minimum sample size was calculated to be 100. Altogether, 141 COVID positive cases were included in the study. Bacterial coinfection was defined as the detection of bacteria in clinical sample collected within 48 hours of hospitalization in COVID positive cases. Secondary bacterial infection was defined as the detection of bacteria in clinical sample collected after 48 hours of hospitalization in COVID positive cases (Duan *et al.*, 2024).

Criteria for Sample Acceptance

Only clinical samples with appropriate volume aseptically in a clean, sterile, leak proof container with no visible signs of contamination and labeled and transported properly with demographic information of the patients were accepted for the study.

Criteria for Sample Rejection

Specimen with insufficient volume and improperly labeled demographic information were excluded. Saliva containing sample was excluded.

Sample Collection and Transportation

For the detection of bacterial co-infection and secondary bacterial infection, sputum sample was collected from the laboratory confirmed COVID case in a sterile leak proof container. Each sample was transported to the microbiology laboratory for further processing. Before accepting the sample, the sputum's quality was examined macroscopically for the presence of mucopurulent components.

Isolation and Identification of Bacterial Isolates

Each sample was processed for bacterial culture by following standard microbiological techniques. Using an inoculating loop, the mucopurulent portion of sputum sample was immediately inoculated on MacConkey Agar (MA) with aerobic condition and Blood Agar (BA), and Chocolate Agar medium in CO2 enriched condition and incubated at 37°C for 24 hours. Identification of bacterial isolate was done by observation of colony morphology, Gram's staining and biochemical tests (Tille, 2020).

Antibiotic Susceptibility Testing

Antibiotic susceptibility pattern of the identified bacterial isolates was done by modified Kirby-Bauer Disc Diffusion method following CLSI (Clinical and Laboratory Standards Institute) guideline. Bacterial isolates resistant to two or more classes of antibiotics were considered as multidrug resistant (MDR) organisms (CLSI, 2020).

Detection of Biofilm Production

Biofilm producing ability of the bacterial isolates was done using 96- well microtiter plate method by quantitative adherence assay as described previously (Nirwati *et al.*, 2019).

Phenotypic Detection of ESBL Producers

The screening test for the production of ESBL producing Gram negative bacterial isolates was performed by measuring the diameters of zone of inhibition around Ceftazidime (30µg) and Ceftriaxone (30 µg)/Cefotaxime (30µg) on MHA media by disc diffusion method following CLSI guidelines. The suspected ESBL producing isolates i.e. screen test positive isolates were subjected to phenotypic confirmation by combination disc method (CLSI, 2020).

Detection of *bla*$_{CTX-M}$ Gene

DNA extraction from the bacterial isolates was done by boiling method. A loopful of fresh bacterial culture was suspended in 50 mM NaOH and heated at 100°C water bath for 20 minutes to extract the Deoxyribonucleic acid (DNA). Subsequently 20 µl Tris-HCl (pH 7.5) was then added followed by gently mixing by inverting several times and centrifuged at 13,000 rpm for 10 minutes. The upper aqueous phase was transferred into a sterile clean tube and stored at −20°C until used. DNA quantitation was done by using Nano drop. The required *bla*$_{CTX-M}$ gene was detected by conventional PCR method with 521 bp amplicon size using a set of following primers (Kot, 2019) :

CTX-M-Forward 5′–TTTGCGATGTGCAGTACCAGTAA–3′
and
CTX-M-Reverse 5′– CTCCGCCTGCCGGTTTTAT –3′

The PCR reaction mixture of volume was prepared consisting of 5 µL of 5X Qiagen master mixes, 0.5 µL of 10 picomole each of forward and reverse primers, 16 µL nuclease free water and 3 µL of extracted DNA template. The PCR amplification conditions were initial denaturation of 95°C for 15 minutes; followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 30 s, and a final extension at 72°C for 10 minutes. Positive and negative template controls were included during PCR.

Data Analysis

All the collected data were entered into Microsoft Office Excel and subsequently into Statistical Package for Social Sciences (SPSS) (version 26.0). Descriptive analysis was performed using Chi-square test. A p-value of <0.05 was considered to be significant.

RESULTS

Proportion of Co-infection and Secondary Bacterial Infection among COVID Positive Cases

Out of 141 COVID positive cases enrolled in the study, 9 (6.38%) showed co-infection and 11 (7.80%) showed secondary bacterial infection. Among the total cases, 32 (22.69%) were admitted to hospital whereas 109 (77.31%) were OPD patients (Table 1). Hospitalized patients had higher bacterial growth rate as compared to non-hospitalized patients.

Table 1. Demographic characteristics of patients.

Variable	Frequency, n (%)
Type of infection	
Co-infection	9 (6.38)
Secondary bacterial infection	11 (7.80%)
Patient type	
Hospitalized patients	32 (22.69%)
OPD patients	109 (77.31%)
Age category of patients in years	
Children (0-14)	1 (0.71%)
Youth (15-24)	28 (19.86%)
Adults (25-64)	94 (66.67%)
Seniors (65 and above)	18 (12.77%)
Gender	
Male	83 (58.86%)
Female	58 (41.13%)

Relationship between Age of COVID Patients with Hospital Stay

The median hospital stay was 3 days with a range from 1 day to 21 days. Older patients tend to have longer hospital stay but the lack of statistical significance indicates that, within this dataset, age is not a definitive factor in determining the length of hospital stay for COVID patients (Table 2).

Table 2. Relationship between age of COVID patients with hospital stay.

	No. of patients with hospital stay				Total	P-value
Age group in years	**≤3 days**	**4-7 days**	**8-14 days**	**15-21 days**	**no.**	
Children (0-14)	1	0	0	0	1	
Youth (15-24)	1	1	0	0	2	
Adults (25-64)	3	9	2	0	14	0.738
Seniors (65 and above)	6	6	2	1	15	
Total	11	16	4	1	32	

Distribution of Co-morbidities among Inpatients

Pneumonia was the most prevalent co-morbidity among the infections followed by diabetes and dengue. Conditions like hypertension, pulmonary TB and hypothyroidism showed moderate prevalence (Table 3).

Table 3. Distribution of co-morbidities among hospital admitted COVID cases with secondary bacterial infections.

Co-morbidities	Number of cases	Percent
Hypertension	2	10.50%
Diabetes	3	15.80%
HIV	1	5.30%
Pulmonary TB	2	10.50%
Dengue	3	15.80%
Pneumonia	5	26.30%
Hypothyroidism	2	10.50%
Thrombocytopenia	1	5.30%

Relation between Gender and Bacterial Growth Among COVID Positive Cases

There was no significant difference in gender with the occurrence of bacterial growth among both outpatients and inpatients (Table 4).

Table 4. Relation between gender and bacterial growth among COVID positive cases.

Gender	Outpatients		Total	P-value	In patients		Total	P-value
	Growth	No growth			Growth	No growth		
Male	6 (8.9%)	61 (91%)	67	0.738	7 (43.8%)	9 (56.7%)	16	0.246
Female	3 (7.1%)	39 (92.9%)	42		4 (25.0%)	12 (75.0%)	16	
Total	9	100	109	-	11	21	32	

Relation between Age and Bacterial Growth among COVID Positive Cases

Bacterial infection varied across different age groups, with a notable increase in the proportion of individuals showing growth as age increases. However, for children specifically, the lack of statistical significance suggests that the result for this group may not be conclusive (Table 5).

Table 5. Relation between age and bacterial growth among COVID positive cases.

Age group in years	Growth status		Total n (%)	P-value
	Growth n (%)	No growth n (%)		
Children (0-14)	0 (0%)	1 (100%)	1 (0.71%)	0.1
Youth (15-24)	1 (3.6%)	27 (96.4%)	28 (19.86%)	
Adults (25-64)	14 (14.89%)	80 (85.10%)	94 (66.67%)	
Seniors (65 and above)	5 (27.8%)	13 (72.2%)	18 (12.77%)	
Total n (%)	20 (14.2%)	121(85.1%)	141	

Bacterial Isolates from COVID Positive Cases

There was a strong predominance of Gram-negative bacteria among COVID positive cases, with *K. pneumoniae* being the most frequently encountered pathogen (Table 6).

Table 6. Bacterial isolates from COVID positive cases.

Bacteria	Number	Percentage
Gram negative bacteria		
K. pneumoniae	12	60%
P. aeruginosa	2	10%
E. coli	1	5%
A. baumannii complex	2	10%
K. aerogenes	2	10%
Gram positive bacteria		
S.aureus	1	5%
Total isolates	20	100%

Antibiotic Resistance Pattern of Different Bacterial Isolates

Overall, aminoglycosides like Gentamicin and Amikacin appear to be the most consistently effective antibiotics across the tested bacterial strains, while other classes of antibiotics showed variable efficacy depending on the bacterial strain (Table 7).

Table 7. Antibiotic resistance pattern of different bacterial isolates.

Antibiotics class	Antibiotics	*K. pneumoniae* n=12	*E .coli* n=1	*P. aeruginosa* n=2	*A. baumannii complex* n=2	*K. aerogenes* n=2
Beta-lactams	Cefixime	6 (50%)	1 (100%)	2 (100%)	1 (50%)	1 (50%)
	Cefotaxime	5 (41.7%)	0 (0%)	1 (50%)	0 (0%)	1 (50%)
	Ceftazidime	5 (41.7%)	1 (100%)	1 (50%)	1 (50%)	1 (50%)
	Amoxicillin	5 (41.7%)	1 (100%)	0 (0%)	2 (100%)	1 (50%)
	Piperacillin/ Tazobactam	4 (33.3%)	1 (100%)	1 (50%)	0 (0%)	1 (50%)
	Imipenem	1 (8.3%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)
	Ceftriaxone	8 (66.7%)	0 (0%)	1 (50%)	1 (50%)	0 (0%)
Fluoroquinolo-nes	Ciprofloxacin	5 (41.7%)	1 (100%)	1 (50%)	0 (0%)	1 (50%)
Aminoglycosi-des	Gentamicin	6 (50%)	1 (100%)	1 (50%)	1 (50%)	1 (50%)
	Amikacin	7 (58.3%)	1 (100%)	1 (50%)	1 (50%)	1 (50%)
Sulfonamides	Co-trimoxazole	5 (41.7%)	1 (100%)	1 (50%)	2 (100%)	0 (0%)

*n denotes number of bacterial isolate

MDR Status of Bacterial Isolates

Out of 20 isolates from covid positive cases, 14 (70%) were MDR and 6 (30%) were non-MDR. Among all the isolates, the MDR prevalence rate was highest among *K. pneumoniae* from co-infection cases and secondary infected cases, followed by *A. baumannii* complex and *K. aerogene*s among secondary bacterial infected cases only (Table 8).

Table 8. Distribution of MDR isolates according to COVID status.

Bacterial species	Coinfection		Secondary bacterial infection	
	No of isolates	MDR n (%)	No of isolates	MDR n (%)
K. pneumoniae	7	4 (57.1%)	5	5 (100%)
E. coli	-	-	1	1(100%)
P. aeruginosa	1	-	1	1(100%)
A. baumannii complex	-	-	2	2 (100%)
K. aerogenes	-	-	2	2 (100%)
S. aureus	1	-	-	-
Total	9	4 (44.4%)	11	11 (100%)

*The symbol – indicates zero isolates

Detection of Biofilm Production

There was a slightly higher proportion of weak and moderate biofilm producers from secondary infections as compared to those with coinfections (Table 9).

Table 9. Biofilm producing isolates among coinfection and secondary bacterial infection.

Biofilm production	COVID status		Total	P-value
	Coinfection	Secondary Bacterial infection		
Biofilm non producer	5 (55.6%)	7 (63.6%)	12 (60%)	0.166
Weak biofilm producer	4 (44.4%)	2 (18.2%)	6 (30%)	
Moderate biofilm producer	0 (0%)	2 (18.2%)	2 (10%)	
Strong biofilm producers	0 (0%)	0 (0%)	0 (0%)	

Detection of Extended Spectrum *β*- lactamases Producing Isolates

Among the 19 isolates tested for ESBL production, 8 (42.10%) were found to be confirmed ESBL producers (Table 10).

Table 10. Detection of extended spectrum β- lactamases producing isolates.

Bacterial species	Total no. of isolates	No. of suspected ESBL producers	No. (%) of confirmed ESBL producers
K. pneumoniae	12	6	5 (41.67%)
E. coli	1	1	1 (100.00%)
P. aeruginosa	2	1	1 (50.00%)
K. aerogenes	2	1	1 (50.00%)
A. baumannii complex	2	0	0 (0%)
Total	19	9	8 (42.10%)

Detection of *blaCTX-M* Gene

Out of total 8 phenotypically confirmed ESBL producers, 6 (75%) harbored *blaCTX-M* gene in their plasmid. All phenotypically confirmed *E. coli* and *P. aeruginosa* contained *blaCTX-M* gene, while only 80% of ESBL producing *K. pneumoniae* isolates harbored *blaCTX-M* gene (Table 11).

Table 11. Prevalence of bla_{CTX-M} gene among ESBL phenotypes.

Bacterial species	No. of confirmed ESBL producers	No. (%) of isolates postive for bla_{CTX-M} gene
K. pneumoniae	5	4 (80%)
E. coli	1	1 (100%)
P. aeruginosa	1	1 (100%)
K. aerogenes	1	0 (0%)
Total no. (%)	8 (42.10%)	6 (75%)

DISCUSSION

The prevalence of secondary bacterial infection and co-infection in COVID positive patients was similar to that reported previously (Karataş *et al.*, 2021). In contrast, our results depicted lower frequency of secondary bacterial infection as compared to studies from India and China (Khurana *et al.*, 2021; Lansbury *et al.*, 2020). *This could be because the current study used a considerably smaller sample size than those studies, which were conducted in the early stages of the pandemic.* The higher prevalence of secondary bacterial infection compared to co-infection might be as a result of ICU patients using more catheters, such as endotracheal, arteriovenous, and urine tubes.

In this study, the majority of infected patients were male 83 (58.8%) and 58 (41.2%) were from female patients and these results agreed with those found by a study which reported in comparison to women, men have been found to have a higher risk of developing serious disease from COVID-19 (Alkhouli *et al.*, 2020). This may be due to females have better immune systems than males; females frequently have reduced susceptibility to viral infections. This may be because sex hormones are crucial for both innate and adaptive immunity.

The median length of hospital stay in our study was similar to that reported by other researchers (Zhou *et al.*, 2020). The most common bacterial species found in the inpatient and outpatient COVID-19 patients was *K. pneumoniae*. This bacterium produces a variety of virulence factors and has significant levels of antibiotic resistance, which results in a high mortality rate. Among gram positive isolates only *S. aureus* (1/20, 5%) was found. This finding was found to be similar with the study in China, where most prevalent pathogens identified from respiratory tract samples were Gram negative (26, 65%) followed by Gram positive (14, 34.99%) pathogens. The presence of bacterial co-infection in patients with coronavirus infection appears to be significantly influenced by the patient's location, the presence of an antimicrobial policy, appropriate stewardship interventions, robust surveillance for healthcare-associated infections, and antimicrobial resistance. The implementation of strong infection control measures is particularly crucial because the majority of the pathogens found are significant sources of nosocomial infections in the hospital setting.

Additionally, an increase in AMR during the pandemic compared to the year before COVID-19 has also been reported, emphasizing that antibiotic misuse creates an abundance of opportunities for bacterial pathogens will increasingly develop defenses against antibiotics and other drugs (Clancy & Nguyen, 2020). Gram negative isolates from COVID-19 patients revealed considerably higher drug resistance to fluoroquinolones, cephalosporins and carbapenems than isolates from non-COVID-19 patients. In our study, from COVID and COVID negative patients, *K. pneumoniae* was the most frequently isolated bacterial species. This bacterium produces a variety of virulence factors and has significant levels of antibiotic resistance, which results in a high mortality rate. High percentage of *K. pneumoniae* were resistant towards ceftriaxone 8 (66.7%), amikacin 7 (58.3%) while susceptible to imipenem 11 (91.7%). This finding is similar to that of the research by Khurana *et al.* (2021) which revealed higher resistance among *Klebsiella* species, making treatment options more challenging for COVID-19 affected patients hospitalized to an intensive care unit (Khurana *et al.*, 2021). In contrary of the study conducted by Sharifipour *et al.* (2020) indicated that *Acinetobacter* species were a major contributor to a widespread resistance (Sharifipour *et al.*, 2020). The discrepancy may result from local cleanliness precautions taken for COVID-19 patients or from the different drug resistance profiles between countries.

The isolated gram-negative bacteria were shown to be primarily multidrug resistant based on the antibiotic susceptibility testing. The COVID-19 patients' recovery and treatment processes may be delayed as a result, and the mortality rate may also rise. As a result, selecting an antibiotic program that can treat infections caused by gram-negative bacteria that are resistant to

many drugs may be more effective (Acharya *et al.*, 2017; Devkota *et al.*, 2018; Dhungana *et al.*, 2019; Shakya *et al.*, n.d.; Shrestha *et al.*, 2010). In our study, analysis of COVID-19 positive individuals who had bacterial co-infection at the time of admission revealed the presence of multidrug resistance among gram negative bacteria. This study suggested the prevalence of MDR among COVID positive patient was 70%. Out of 14 MDR gram negative isolates, prevalence of MDR isolates in co-infection and secondary bacterial infection was 15% and 55% respectively. Based on this finding, MDR in Gram-negative isolates is on the rise and implies that an immediate control mechanism is needed to reduce them. Mutua *et al.* (2022) suggested that the majority of bacterial isolate (64.3%) were MDR, particularly in gram negative bacteria (69.6%) with all isolates of *K. pneumonia**e*** (100%), *E. coli* (100%) and *A. calcoaceticus* (100%) presenting as MDR organisms (Mutua *et al.*, 2022). From another study, Saeed *et al.* (2021) suggested that individuals with SARS-CoV-2 infection had a 65.8% MDR in GNB rate in the Kingdom of Bahrain (Saeed *et al.*, 2021). MDR bacterial infections increase the length of stay in critically ill COVID-19 patients, and their frequencies range from 32 to 50%. The rising rate of MDR discovered in this study may be related to antibiotic abuse in Nepal, including non-empirical use, taking medicines improperly doses and the easy availability to antibiotics without a doctor's prescription.

Globally, the prevalence of ESBL-producing bacteria is steadily rising in hospital sector, primarily as nosocomial infections, and it is changing quickly over time and with a wide range of strains. Due to their ability to hydrolyze third- and fourth-generation of cephalosporins and monobactams, plasmid-mediated ESBL producers are currently a cause for concern. The current study shows that out of 9 positive isolates of *K. pneumoniae, E. coli, P. aeruginosa* and *K. aerogenes*, 8 were phenotypically confirmed ESBL producer. In the confirmed ESBL producer, the prevalence of *K. pneumoniae* was found to be 41.4% followed by *P. aeruginosa* and *K. aerogenes* (100%) whereas *E. coli* (50%). Similar to our study, a study in Nepal reported the cases of ESBL to be 7% for *E. coli* and 33.7% for *Klebsiella* spp. (Nepal *et al.*, 2018). Our finding is in contrast with one study where 13.33% of *Klebsiella* spp. and 36.36% of *E. coli* cases had ESBL producer respectively (Maharjan *et al.*, 2022). The significance of ESBL-producing strains comes from the fact that they are challenging to treat due to the presence of plasmids that confer resistance to the majority of commonly used antibiotics.

Lately, the emergence and spread of *CTX-M* type ESBL has been reported from different parts of the world. In our study, the molecular characterization by PCR detected the presence of $bla_{CTX\text{-}M}$ gene in 30% (6/20) of the total ESBL phenotype. Some isolates that gave positive ESBL test phenotypically did not contain *CTX-M* gene. It might be caused by the presence of ESBL genes other than the ones that are targeted (Parajuli *et al.*, 2016). A low level of ESBL gene expression could be another cause. Higher prevalence and dominance of $bla_{CTX\text{-}M}$ over other genotypes have been reported from the other parts of the world (Abrar *et al.*, 2019). These findings advocate the fact that the *CTX-M* type of ESBL is the mostly distributed and globally dominant ESBL genotype.

In Nepal, minimal research on the prevalence of co-infection and secondary infection has been conducted. Despite the fact that the issue is considered a worldwide one, Nepalese individuals are poorly aware about resistant gene. This could be as a result of a lack of information and experience in this field of study. Hence, this is one of the few studies conducted to investigate the molecular characterization of isolates from the patients with co-infection, secondary infection and COVID condition. Furthermore, this may contribute in providing knowledge about resistant genes and the degree of severity caused in public. This study could also be helpful for researchers and policymakers in determining the true status of anti-microbial resistance in Nepal. Similarly, the study's findings could also help in making public aware about the harmful outcomes of irrational use of antibiotics.

This study also has some limitations. It was a hospital-based study conducted in a limited time period. Samples were collected completely from one hospital setting and sample size was relatively low, thus result might not relate to larger population. Moreover, only $bla_{CTX\text{-}M}$ gene was detected, although ESBLs production is also related to other genes such as bla_{TEM} and bla_{SHV}. This work would have been more reliable if other ESBL genotypes were also characterized.

CONCLUSIONS

This study highlights the occurrence of bacterial co-infections and secondary infections among COVID-19 patients, emphasizing the higher prevalence of bacterial growth in hospitalized cases compared to outpatients. *K. pneumoniae* emerged as the most commonly isolated pathogen, particularly associated with multi-drug resistance. This study warranted for the continuous monitoring of bacterial co-infection, secondary infection and resistance patterns, as well as improving infection control measures are important to control the pandemic at a local and global level.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest.

ETHICAL STATEMENT

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Institutional Review Committee (IRC) of the Institute of Science and Technology (IOST), Tribhuvan University, Nepal (Reg. No. IRC/IOST 52/079/080). Written informed consent was obtained from all individual participants included in the study.

AUTHOR CONTRIBUTIONS

Conceptualization: SS, PP, KG SA, JT, PG; Investigation: SR, BSC, JT, SA; Data curation: SS; Data analysis: KRR; Writing- draft: SS; Writing-Review and editing: SS, SR, BSC, SA, KG, SA, JT, KRR, PP, PG.

DATA AVAILABILITY STATEMENT

All the data generated or analyzed during the study are included in the manuscript.

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