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RESEARCH ARTICLE OPEN ACCESS

Extended-spectrum β-Lactamase (ESBL) Producing Bacterial Pathogens Associated with Respiratory Tract Infections

Priyanka Lakshman[1], Shilpa Borehalli Mayegowda[2] and Manjula Nagalapur Gadilingappa[1]*

[1]Department of Microbiology, School of Basic and Applied Sciences, Dayananda Sagar University, Bengaluru, India.
[2]School of Psychological Sciences, CHRIST University, Kengeri Campus, Bengaluru, India.

Abstract

Respiratory tract infections (RTIs) have been critically associated with health care problems globally. Subsequently, increased antibiotic resistance rates have limited treatment options that are further exaggerated due to lack of newer novel drugs and therapies. Current study highlights, antibiotic resistance profiling along with extended-spectrum beta-lactamase (ESBL) producers of RTI pathogens from Bengaluru. During June 2020-May 2021, 1016 clinical samples collected, prevalence rate of 22.4% was exhibited, with highest in male (74.5%). Following age group, 30-35 years displayed highest (24.1%) though, lowest was in 45-50 years (1.3%). The standard microbiological characterization revealed *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli, Acinetobacter baumannii* as predominant bacterial pathogens associated with RTIs. While, Antibiotic susceptibility test (AST) exhibited highest resistance rates for different antibiotics in the following pathogens, as *K. pneumoniae* for ampicillin (74.8%), *P. aeruginosa* for doripenem (66.6%), *A baumannii* to piperacillin/tazobactam (76.9%), *E. coli* for penicillin and β-lactamase inhibitors ranging between 56-92%, *E. cloacae* to ticarcillin/clavulanic acid besides cefuroxime (100%). However, prevalence of Gram-positive strains were lowest and exhibited highest resistance to penicillin, and fluoroquinolone (83.3%). ESBL producers were predominantly *K. pneumoniae,* followed by *E. coli,* and *E. cloacae* with 21.9%, 6.5% and 1.3%, respectively. Notably, all the Gram-negative strains showed 100% sensitivity towards colistin with remarkable sensitivity was observed in oxazolidinone, glycopeptides by *S. aureus* and Coagulase-neagtive *Staphylococcus aureus* (CoNS). The study emphasizes increased antimicrobial resistance antimicrobial and ESBL resistance, suggesting AST as a systematic approach for apprising treatment guidelines in current scenario. The present study denotes polypeptide colistin as choice of drugs for treating RTI pathogens, however its not recommended in all cases.

Keywords: Antibiotic Resistance, Respiratory Tract Infections, Extended-spectrum beta-lactamase, Colistin

***Correspondence:** manjulang-sbas@dsu.edu.in

Abbreviations: RTI-Respiratory tract infections; AMR-Antimicrobial resistance; ESBL-Extended-spectrum beta-lactamase; MBL-Metallo-beta-lactamase; MDR-Multidrug-resistance; COVID-Coronavirus disease; ICU-Intensive care unit; OD-Optical density; CFU-Colony forming unit; WHO-World health organization; MHA-Mueller Hinton Agar; EMB-Eosine Methylene Blue; PPE-Personnel protective equipment; TS-Tracheal secretions; CLSI-The Clinical and Laboratory Standards Institute; DDST-Double Disc Synergy Test; EDTA-Ethylenediaminetetraacetic acid

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INTRODUCTION

The rise of novel antibiotic-resistant nosocomial pathogens associated with respiratory tract infections (RTIs) is becoming a threat to human development, food security, and global health. Hence, antibiotics must be used appropriately to save them for the future as there is a decline in the discovery of novel antibiotics.[1] The collective infections restricted to lower RTIs such as pneumonia, bronchitis, and upper respiratory tract infections namely tracheitis pharyngitis, sinusitis, and rhinitis are referred to as RTIs.[2] The diseases caused by RTI are quite common however, with being as life-threatening it contributes to millions of deaths across the globe. Moreover, being 3rd leading cause of morbidity and mortality, RTI accounts for 4.25 million deaths annually in children and adults.[3,4]

Major bacterial pathogens causing RTI include *K. pneumoniae, A. baumannii, P. aeruginosa, E. coil,* and *S. aureus.* A substantial increase in antimicrobial resistance (AMR) among these pathogens has made antibiotic therapy ineffective.[5] Some other pathogens causing RTI recently emerged as novel pathogens including coronavirus, influenza virus along with fungal pathogens such as *Aspergillus* spp., *Pneumocystis* spp., and bacterial pathogens like *Bacillus anthracis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *P. aeruginosa.*[6] The emergence of AMR in respiratory tract pathogens has led to life-threatening situations claiming approximately 2,603,913 deaths worldwide by 2019.[7] It has been estimated that, by 2050 around 10 million people may die annually due to AMR, with an account of about 3,90,000 in Europe, 47,30,000 in Asia, and 41,40,000 in Africa.[8] Eventually, multidrug-resistance (MDR) being detected with the drastic rise in respiratory pathogens was found to escalate management and treatment options in AMR. Hence, an immediate measure to control the spread of AMR in medically important respiratory pathogens is needed on an emergency basis.[9]

β-lactam antibiotics has been a key factor for treating bacterial infections globally with 65% of usage.[10] The classified groups include penicillin, cephalosporins, monobactam, cephamycin, carbapenems and β-lactamase inhibitors that block cell wall synthesis by preventing penicillin-binding protein (PBP), leading to cell death.[11] The mechanism for production of β-lactamases in Gram-positive and Gram-negative that acts as the most predominant source of resistance towards β-lactam antibiotics. The lactamases enzymes inactivate β-lactam antibiotics by binding covalently to their carbonyl group and hydrolyze the ring enabling them resistance.[12] Additionally, these β-lactamases can be inhibited by β-lactamases inhibitors like clavulanic acid, tazobactam and sulbactam.[13] ESBL producers have increased in frequent times and has become a worldwide threat that may also induce the resistance in non β-lactam drugs.[14,15]

The present investigation was carried out to understand the resistant pathogenic bacteria observed in patients admitted to the intensive care unit (ICU), in-patient (IP), and out-patients (OP) during the year 2020 COVID-19 pandemic. Following the signs and symptoms, samples were collected from RTI patients, and bacterial pathogens were isolated using specific microbiological techniques. Further, pure cultures of RTI bacterial pathogens thus obtained were identified by biochemical reactions, screened for antibiotic susceptibility test (AST) and VITEK® 2 system. Following with the results obtained from AST, the prevalence of antibiotic resistance was calculated and these isolates were additionally verified for their ESBL production.

MATERIALS AND METHODS

Chemicals and microbiological Media: All the chemicals and reagents such as antibiotic discs, Glycerol, Peptone water, Nutrient broth, Mueller Hinton Agar (MHA), Blood agar, MacConkey agar, etc. were procured from Hi Media Laboratory Pvt. Ltd. (India).

Location and design of cross-sectional study

In the present investigation, prior to the sample collection, consent from the patients was taken and in case of critical patients informed consent was obtained from the patient's relatives. The study was conducted from June 2020 to May 2021 following appropriate personnel protective equipment in the Department of Microbiology. In the present study, following the signs and symptoms of RTI, the patients from different

sections such as the intensive care unit (ICU), in-patients (IP), and outpatients (OP) were selected and screened for antibiotic resistance pattern of bacterial isolates. The study included three different types of samples from the patients such as sputum, suction tip, and tracheal aspirate samples.

Clinical sample collection and isolation of RTI pathogens

The clinical respiratory tract samples were collected using sterile peptone water in an aseptic container along with the patient's details such as gender, age, IP, OP, etc. Following which the collected samples were transferred to the laboratory and processed immediately using standard microbiological techniques, if failed to process immediately, the samples were stored at 4 °C in a refrigerator. The clinical samples were sub-cultured on sterile nutrient agar along with specific media such as blood Agar, chocolate agar and MacConkey agar. The inoculated plates were incubated at 37 °C for 24-48 hours in a pre-set bacteriological incubator. Subsequently, the bacterial isolates were observed for their cultural characteristics on specific media and morphological characteristics was analysed by Gram staining, motility, and biochemical characteristics.

Identification of RTI pathogens by biochemical tests

Following preliminary identification of bacterial isolates based on their colony morphology, these isolates were further sub-cultured and subjected to biochemical characterization. Various biochemical reactions such as mannitol fermentation, citrate utilization, urease production, growth on Triple Sugar Iron (TSI) agar, and coagulase tests were performed using standard protocols.[16] The identified pure cultures of RTI causing pathogenic bacteria were preserved in glycerol stocks at -20 °C for further use.

Determination of antibiotic-susceptibility test (AST) of respiratory pathogens

The AST assay was performed as per Clinical and Laboratory Standards Institute guidelines.[17] The method adopted is Kirby Bauer's disc diffusion assay for analysing the antibiotic resistance in the pathogens. A 24 hour grown bacterial colony was inoculated in peptone water and inoculum size was adjusted to MacFarland's constant. Following the inoculum of 10^6 CFU/ml was used to spread uniformly on sterile MHA plates and allowed to set for 5 minutes. The antibiotic discs with difference of 6 mm diameter apart were dispensed on plates using sterile forceps, and incubated at 37 °C for 24 hours. The zone of inhibition formed around the antibiotic discs was recorded for each antibiotic and the results were interpreted as per CLSI guidelines.[18]

The AST for the isolated RTI pathogens was also analysed utilising a VITEK® 2 (BioMerieux) for identification using susceptibility cards as (ID-GN for Gram-negative, ID-GP for Gram-positive), the AST-628 (for *Staphylococci*), AST-658 (for *Enterococci* and *S. agalactiae*), AST-ST03 (for *Pneumococci*), AST-280 (for *Enterobacteriaceae*), and AST-281 (for Gram-negative non- fermenters). Following which the antimicrobial results as resistant, sensitive and intermediate were recorded as per EUCAST guidelines.[19] *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 was used as negative and positive control strain respectively for the effectiveness of the drugs.

The antibiotics used in the present study consisted of Ampicillin (10 mcg/disc), Ertapenem (10 mcg/disc), Imipenem (10 mcg/disc), Meropenem (10 mcg/disc), Doripenem (10 mcg/disc), Gentamicin (10 mcg/disc), Colistin (10 mcg/disc), Penicillin (10 units/disc), Amoxicillin/clavulanic acid (20/10 mcg/disc), Cefoperazone/Sulbactam (75/10 mcg/disc), Piperacillin/tazobactam (100/10 mcg/disc), Ticarcillin/clavulanic acid (75/10/disc), Cefuroxime (30 mcg/disc), Cefuroxime Axetil (30 mcg/disc), Ceftriaxone (30 mcg/disc), Ceftazidime (30 mcg/disc), Cefepime (30 mcg/disc), Amikacin (30 mcg/disc), Linezolid (30 mcg/disc), Teicoplanin (30 mcg/disc), Vancomycin (30 mcg/disc), Tetracycline (30 mcg/disc), Ciprofloxacin (5 mcg/disc), Levofloxacin (5 mcg/disc), Rifampicin (5 mcg/disc), Benzylpenicillin (2 units/disc), Daptomycin (5 mcg/disc), Erythromycin (15 mcg/disc), Trimethoprim/Sulfamethoxazole (1.25/23.75 mcg/disc), Clindamycin (2 mcg/disc), and Oxacillin (5 mcg/disc).

Phenotypic Confirmation of Extended-Spectrum β-lactamases (ESβL) production

The antibiotic-resistant clinical isolates of Gram-negative bacteria such as *K. pneumoniae*, *P. aeruginosa, A. baumannii, E. coli,* and *E. cloacae* were further tested for ESBL production by Double Disc Synergy Test (DDST) using CLSI guidelines.[20] A fresh bacterial inoculum equivalent to 0.5 McFarland standard was prepared from each 24-hour bacterial culture and inoculated on sterile MHA by spread plate technique and allowed to stand for 5 minutes. The antibiotic discs containing cefotaxime+clavulanic acid (30/10 mcg) along with cefotaxime (30 mcg) and ceftazidime+clavulanic acid (30/10 mcg) along with ceftazidime (30 mcg) were placed opposite to each other at an appropriate distance. The plates were incubated at 37 °C for 24 hours in an inverted position and the results were analysed. As recommended by CLSI guidelines, formation of ≥5 mm diameter of zone inhibition with combination versus cefotaxime and ceftazidime alone is indicated as positive for ESBL production.

RESULTS

Clinical samples collection and analysis of data

During the study period of 12 months interval, i.e. June 2020 - May 2021, a total of 1016 samples (n = 1016) were collected from patients attending with signs and symptoms for RTI. The detailed data of RTI samples collected from the patients belonging to different wards with their numbers and percentages are indicated below (Table 1 and Figure 1). The highest number of clinical samples of suction tips, i.e. 600 was collected from the ICU section contributing to 59.05% of the total clinical sample collected. In

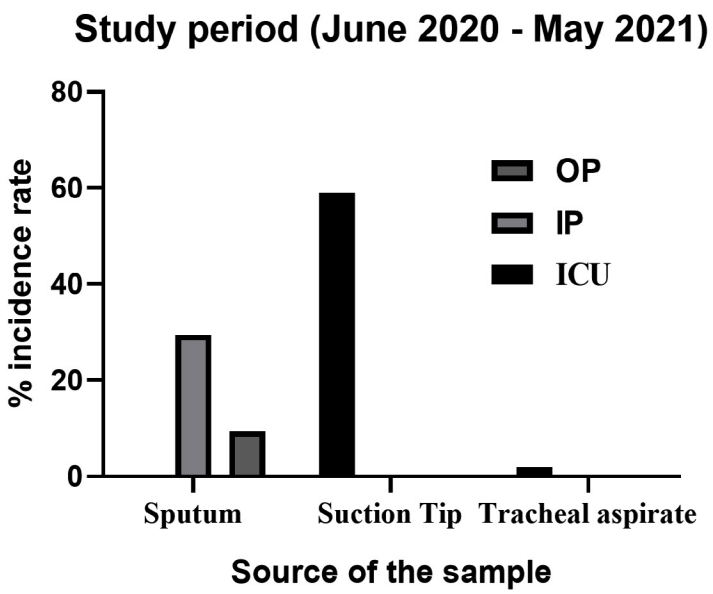

Figure 1. Total number of clinical samples of RTI collected from different hospital wards

Table 1. Total number of clinical samples of RTI collected from different hospital wards

RTI samples	Study period: June 2020 - May 2021					
	ICU	%	IP	%	OP	%
Sputum	00	00	300	29.52	96	9.44
Suction Tip	600	59.05	00	00	00	00
Tracheal aspirate	20	1.96	00	00	00	00
Total isolates			1016			

the total samples, sputum samples collected from IP and OP consisted 29.52% (300) and 9.44% (96) respectively. However, lowest of 1.96% consisted of the tracheal aspirate samples collected from the ICU section. Of the 1016 RTI samples collected, a total of 228 samples indicated the presence of respiratory tract pathogens corresponding to a prevalence rate of 22.4%.

The clinical samples collected from both the genders indicated highest prevalence in male RTI patients, i.e. n = 170 as compared to female patients (n = 58). The study indicated a 74% incidence with an overall carriage rate of 16.6% RTI pathogens in male patients in comparison with 25% in females with a carriage rate of 5.7%. The detailed number of clinical samples and percentage of incidences of RTI pathogens isolated from clinical samples of both genders are indicated in Table 2 and Figure 2.

The study also highlighted the age-wise assessment for the prevalence of RTIs, the analysis indicating highest percentage of prevalence in the age groups of 30-35 years (24.1%) followed by 25-35 years (19.7%). The younger patients with an age group of 20-25 years and elderly group of 60-65 years indicated an incidence of RTIs with 8.77%. The results shows that incidence of RTIs was found to be with increasing trend from age 0-5 years to 35 years of age. Subsequently, the older patients with above 50 years of age except 60-65 years of age group were less prone to RTIs as compared to those below 50 years of age (Figure 3).

Cultivation of clinical samples of RTI patients and preliminary identification

The clinical samples of RTI processed by standard microbiological culture methods revealed different colony morphologies on specific media as indicated in Figure 4. The clinical samples inoculated on MacConkey agar indicated dominant colonies with identical morphologies such as pink, cream, and colourless colonies. The lactose fermenters indicating pink coloured colonies belonging to *Enterobacteriaceae* family *viz., E. coli, Klebsiella, Enterobacter* sp*., Citrobacter* sp. while, colourless colonies were *Pseudomonas* sp. The colonies formed on Blood agar were analysed for haemolytic and non-haemolytic activity. Based on

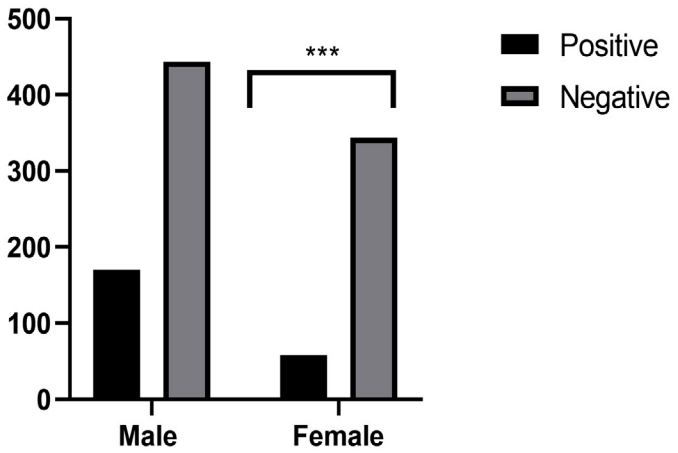

Figure 2. Strong association between the gender was observed for incidence rate of RTI in clinical samples of patients. ***p < 0.001, Male is distant pattern for male compared to female, Chi-square was using by GraphPad Prism

Table 2. Incidence of RTI observed in clinical samples of patients concerning gender

Gender	No. of samples (n = 1016)	No. of positive isolates (n = 228)	%incidence (n = 228)	%Carriage rate (n = 1016)
Male	614	170	74.5	16.6
Female	402	58	25.4	5.7

the colony characteristics, all the bacterial colonies were categorized into seven groups of isolates, i.e. *K. pneumoniae, P. aeruginosa, E. coli, A. baumannii E. cloacae* and *S. aureus.*

Identification of RTI pathogens by biochemical reactions and VITEK® 2 system

Following the cultural identification, all the positive clinical isolates of RTI (n = 228) were subjected to biochemical reactions, revealing majority of clinical isolates to be Gram-negative as compared to Gram-positive pathogens. The detailed results of biochemical reactions for 228 positive isolates are indicated in Table 3.

Of different etiological agents isolated from different RTI samples, *K. pneumoniae* was predominant with an incidence rate of 62.71%, followed by *P. aeruginosa, E. coli,* and *A. baumannii* with prevalence rate of 13.15%, 10.96%, and 5.70%, respectively. However, the incidence of *E. cloacae* was lowest with an incidence rate of 2.63%. Meanwhile, the Gram-positive pathogens such as *S. aureus* and CoNS were found to be lowest as compared to Gram-negative bacteria with 2.19%. The total number of positive isolates of RTI along with their % incidence and overall % incidence of each clinical isolate is as shown in detail in Table 4 and Figure 5.

Antibiotic resistance profiles of clinical pathogens of RTI

The positive isolates of RTI evaluated for AST using the disc diffusion method indicated various antibiotic resistance profiles as shown in Table 5. The isolates of *K. pneumoniae* showed a complete resistance to ampicillin (100%), followed by piperacillin/tazobactam (80.4%), ceftriaxone and ciprofloxacin with 74.8%. While, cephalosporin group of antibiotics revealed resistant rate varying between 69.9%-65.7%, for cefuroxime, cefuroxime axetil, cefepime, and cefoperazone/sulbactam. In contrast, *P. aeruginosa* isolates revealed a moderate resistance against most of the antibiotics used in the study. The highest resistance rate was observed against doripenem (66.6%), followed by amikacin, imipenem, and levofloxacin with 46.6%, 43.3% and 40%, respectively. However, the resistance rate towards remaining isolates was less than 30% and none of the isolates showed 100% resistance towards any selected antibiotics.

A. baumannii exhibited highest resistance towards piperacillin/tazobactam (76.9%), followed by 3rd generation cephalosporins (Cefoperazone/sulbactam) and carbapenems (ertapenem, imipenem, and meropenem) with 69.2%. The following isolates also displayed 61.5% resistance against ciprofloxacin and trimethoprim/

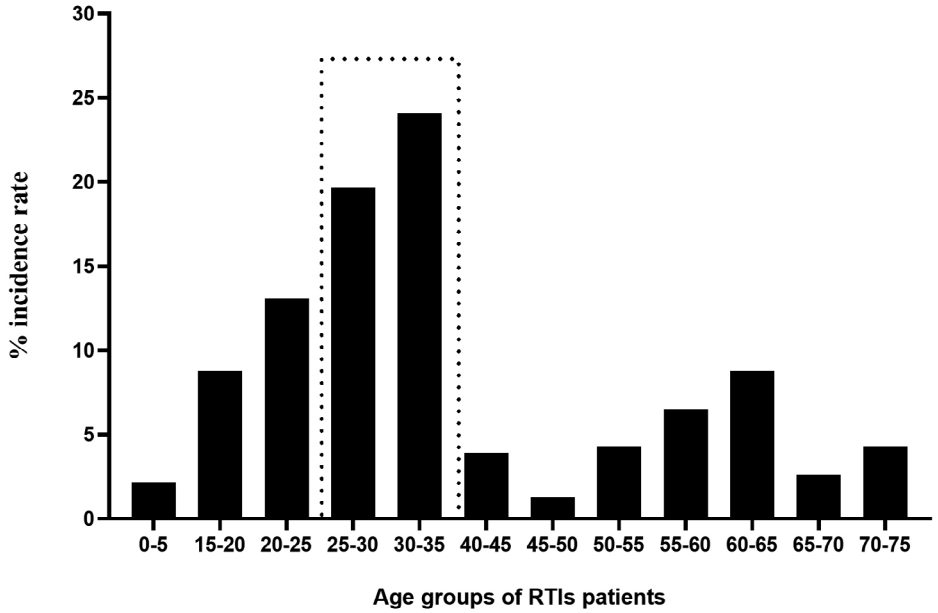

Figure 3. Incidence of RTI pathogens observed in clinical samples of patients concerning different age groups

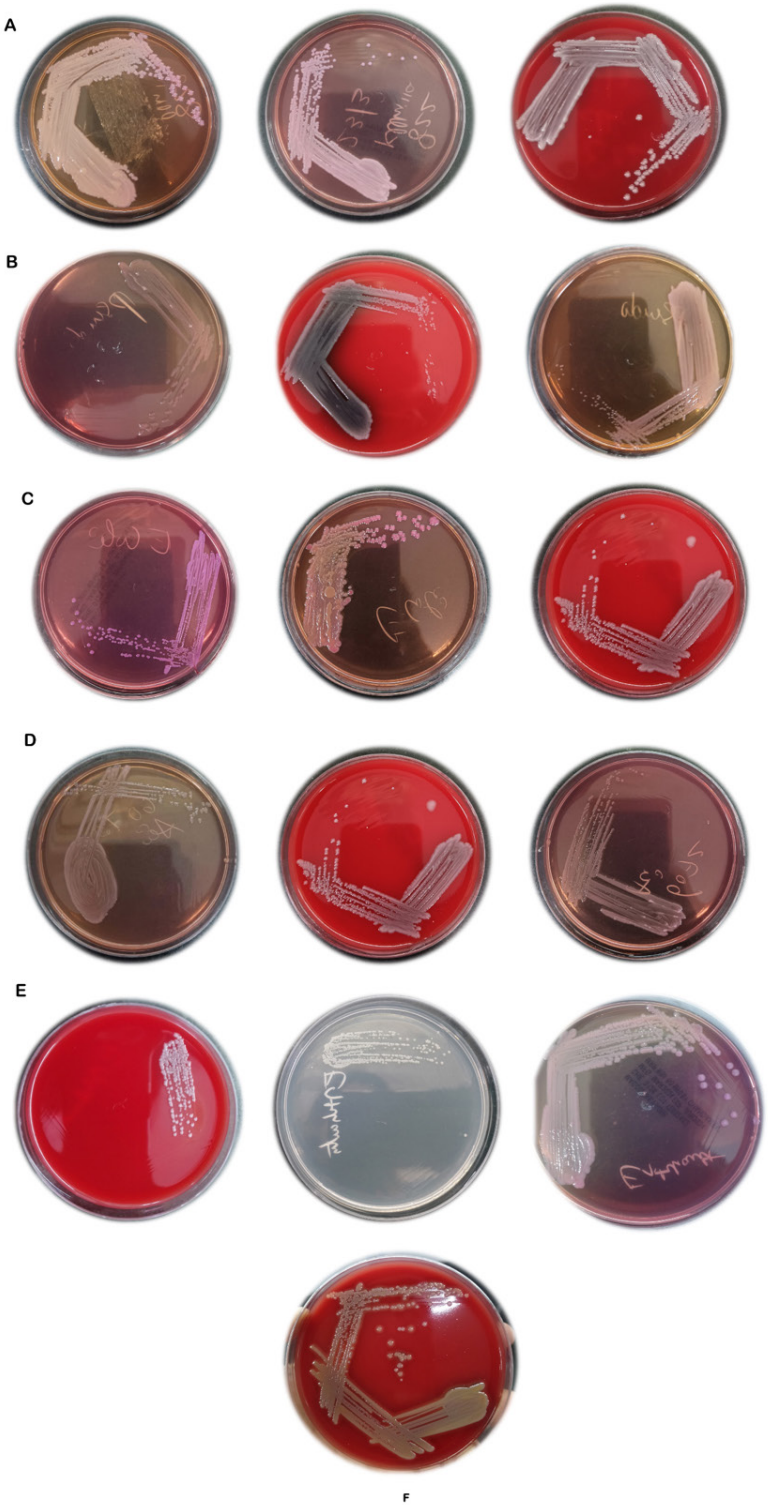

Figure 4. Colony morphologies of clinical isolates on differential media. (A). *K. pneumoniae,* (B). *P. aeruginosa,* (C). *E. coli,* (D). *A. baumannii,* (E). *E. cloacae* and (F). *S. aureus*

Table 3. Biochemical tests performed for positive clinical pathogens (n = 228) of RTI

Biochemical parameters	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7
Gram staining	Gram-negative bacilli	Gram-negative bacilli	Gram-negative bacilli	Gram-negative coccobacilli	Gram-negative bacilli	Gram-positive cocci	Gram-positive cocci
Motility	Non-motile	Motile	Motile	Motile	Motile	Non-Motile	Non-Motile
Colony colour on:	Pink	Colourless	Pink	Pale pink	Pink	No growth	No growth
MacConkey agar	Pink	Cream	colour	Pink	Pink	No growth	No growth
EMB	Haemolytic	Haemolytic	GMS	Non-haemolytic	Non-haemolytic	Haemolytic	Haemolytic
Blood agar			Non-haemolytic				
Mannitol fermentation	Negative	Negative	Negative	Negative	-	Positive	Positive
Citrate utilization	Positive	Positive	Negative	Positive	-	-	-
Urease production	Positive	Negative	Negative	Negative	-	-	-
TSI	Positive	Positive	Positive	Negative	-	-	-
Coagulase test	Negative	Negative	Negative	Negative	-	Positive	Negative
RTI pathogen identified	*K. pneumoniae*	*P. aeruginosa*	*E. coli*	*A. baumannii*	*E. cloacae*	*S. aureus*	CoNS

Note: GMS-Green metallic sheen, TSI-Triple Sugar Iron, CoNS-Coagulase-negative *Staphylococcus aureus,* RTI-Respiratory tract infection

sulfamethoxazole. The isolates *E. coli* analysed had substantial resistance against ampicillin with 92%, followed by 2nd generation cephalosporins (80%), cefepime (76%), and ciprofloxacin (76%). Meanwhile, *E. cloacae* exhibited highest resistance rate to ticarcillin/clavulanic acid and cefuroxime (100%), followed by ampicillin and selected 2nd, 3rd, and 4th generation cephalosporins, ciprofloxacin (83.3%). Interestingly, the resistance rate towards antibiotics like amikacin and gentamicin were represented at 50%. Remarkably, all the six isolates of *E. cloacae* were found to be 100% sensitive to carbapenems. The most striking results in the present work highlights that all aetiological pathogens isolated were 0% resistant to colistin making it a remarkable choice of drug for treating

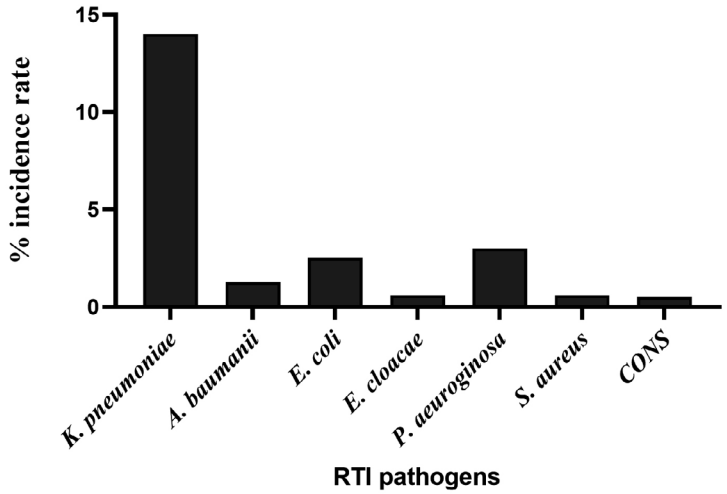

Figure 5. Percentage incidence of RTI pathogens collected from different clinical specimens (June 2020 - May 2021)

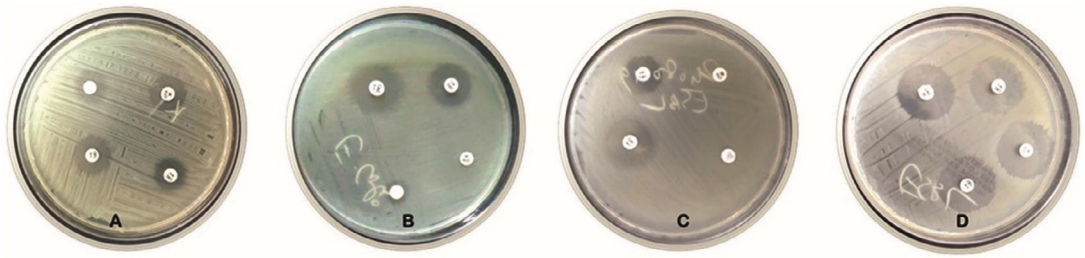

Figure 6. Phenotypic characterization of ESBL productions by clinical isolates of *K. pneumoniae* (A), *E. coli* (B), *E. cloacae* (C), and the negative control of ESBL (D)

Table 4. Etiology of RTI pathogens collected from different clinical specimens from June 2020 - May 2021

Organisms	No. of positive isolates (n = 228)	% incidence rate (n = 228)	Overall % incidence (n = 1016)
K. pneumoniae	143	62.7%	14%
A. baumanii	13	5.7%	1.28%
E. coli	25	10.9%	2.5%
E. cloacae	06	2.6%	0.6%
P. aeuroginosa	30	13.1%	3%
S. aureus	06	2.6%	0.59%
CoNS	05	2.1%	0.5%

Table 5. Antibiotic sensitivity assay of bacterial pathogens of RTI collected from June 2020 - May 2021

Antimicrobials			*K. pneumoniae* (n = 143)		*P. aeruginosa* (n = 30)		*A. baumannii* (n = 13)		*E. coli* (n = 25)		*E. cloacae* (n = 6)	
Class of antibiotics	Name	Conc. (mcg/disc)	No. of isolate; % Resistance	No. of isolate; % Sensitive	No. of isolate; % Resistance	No. of isolate; % Sensitive	No. of isolate; % Resistance	No. of isolate; % Sensitive	No. of isolate; % Resistance	No. of isolate; % Sensitive	No. of isolate; % Resistance	No. of isolate % Sensitive
Penicillin & β-lactamase inhibitors	Ampicillin	10	100	00; 00	NA	NA	NA	NA	23; 92	02; 08	05; 83.3	01; 16.6
	Piperacillin/ tazobactam	100/10	115; 80.4	28; 19.6	05; 16.6	25; 83.3	10; 76.9	03; 16.6	14; 56	11; 46	03; 50	03; 50
	Ticarcillin/ clavulanic acid	75/10	NA	NA	12; 40	18; 60	NA	NA	NA	NA	06; 100	00; 00
2nd Gen. Cephalosporins	Cefuroxime	30	100; 69.9	43; 30.06	NA	NA	NA	NA	20; 80	05; 20	06; 100	00; 00
	Cefuroxime Axetil	30	100; 69.9	43; 30.06	NA	NA	NA	NA	20; 80	05; 20	05; 83.3	01; 16.6
3rd Gen. Cephalosporins	Ceftriaxone	30	107; 74.8	36; 25.1	NA	NA	NA	NA	20; 80	05; 20	05; 83.3	01; 16.6
	Ceftazidime	30	NA	NA	09; 30	21; 70	NA	NA	NA	NA	05; 83.3	01; 16.6
Sulbactam	Cefoperazone/	75/10	94; 65.7	49; 34.3	06; 20	24; 80	09; 69.2	04; 30.8	06; 24	19; 76	05; 83.3	01; 16.6
4th Gen. Cephalosporins	Cefepime	30	100; 69.9	43; 30.06	08; 26.6	22; 73.4	NA	NA	19; 76	06; 24	5: 83.3	01; 16.6
Carbapenems	Ertapenem	10	71; 49.6	72; 50.4	NA	NA	09; 69.2	04; 30.8	07; 28	18; 72	00; 00	06; 100
	Imipenem	10	71; 49.6	72; 50.4	13; 43.3	17; 56.7	09; 69.2	04; 30.8	07; 28	18; 72	00; 00	06; 100
	Meropenem	10	71; 49.6	72; 50.4	14; 46.6	16; 53.4	09; 69.2	04; 30.8	07; 28	18; 72	00; 00	06; 100
	Doripenem	10	NA	NA	20; 66.6	10; 35.4	NA	NA	NA	NA	00; 00	06; 100
Aminoglycoside	Amikacin	30	85; 59.4	58; 40.6	13; 43.3	17; 56.7	NA	NA	08; 32	17; 68	03; 50	03; 50
	Gentamicin	10	85; 59.4	58; 40.6	05; 16.6	25; 83.4	NA	NA	07; 28	18; 72	03; 50	03; 50
Fluoroquinolone	Ciprofloxacin	05	107; 74.8	36; 25.2	05; 16.6	25; 83.4	08; 61.5	05; 38.5	19; 76	06; 24	05; 83.3	01; 16.6
	Levofloxacin	05	NA	NA	13; 43.3	17; 56.7	NA	NA	NA	NA	NA	NA
Polypeptide	Colistin	10	00; 00	143; 100	00; 00	30; 100	00; 00	13; 100	00; 00	25; 100	00; 00	06; 100
Sulfonamides	Trimethoprim/ Sulfamethoxazole	1.25/ 23.75	86; 60.1	57; 39.9	NA	NA	08; 61.5	05; 38.5	05; 20	20; 80	05; 83.3	01; 16.6

Note: R: Resistant, S: Sensitive, NA: Not Applicable

T**able 6.** Antibiotic sensitivity assay of *S. aureus* and CoNS causing RTI during June 2020 - May 2021

Class of antibiotics	Antibiotics Name	Conc. (mcg/disc)	*S. aureus* (n = 6)		CoNS (n = 5)	
			No. of isolate; % Resistant	No. of isolate; % Sensitive	No. of isolate; % Resistant	No. of isolate; % Sensitive
Oxazolidinone	Linezolid	30	00; 00	06; 100	00; 00	05; 100
Glycopeptides	Teicoplanin	30	00; 00	06; 100	00; 00	05; 100
	Vancomycin	30	00; 00	06; 100	00; 00	05; 100
Lincosamides	Clindamycin	10	03; 50	03; 50	02; 40	03; 60
Sulphonamides	Trimethoprim/ Sulfamethoxazole	1.25/ 23.75	01; 16.6	05; 83.3	03; 60	02; 40
Macrolactams	Rifampicin	05	01; 16.6	05; 83.3	00; 00	05; 100
β-lactam antibiotics	Penicillin	10 units	05; 83.3	01;16.6	03; 60	02; 40
	Oxacillin	05	NA	NA	02; 40	03; 60
	Benzyl penicillin	2 units	NA	NA	02;40	03;60
Fluoroquinolone	Levofloxacin	05	05; 83.3	01; 16.6	02; 40	03; 60
	Ciprofloxacin	05	05; 83.3	01; 16.6	02; 40	03; 60
Aminoglycoside	Gentamicin	10	01; 16.6	05; 83.3	01; 20	04; 80
Macrolides	Erythromycin	15	00; 00	06; 100	03; 60	02; 40
Lipopeptide	Daptomycin	05	00; 00	06; 100	03; 60	02; 40
Tetracyclines	Tetracycline	30	00; 00	06; 100	00; 00	05; 100

Note: R: Resistant, S: Sensitive, CoNS: Coagulase-negative *S. aureus*

these MDR strains associated with respiratory tract infections.

The AST was also analysed for Gram-positive pathogens, the antibacterial assay against *S. aureus* and CoNS are as recorded in Table 6. Following the total six *S. aureus* isolates, 5 isolates indicated highest resistance against penicillin, levofloxacin and ciprofloxacin (83.3%) followed by clindamycin (50%). Comparatively, low antibiotic resistance was observed in trimethoprim/sulfamethoxazole, rifampicin and gentamicin (16.6%). However, *S. aureus* was totally sensitive towards linezolid, teicoplanin, vancomycin, erythromycin, daptomycin, and tetracycline (100%). Among CoNS, three isolates exhibited higher resistance towards trimethoprim/ sulfamethoxazole, penicillin, erythromycin, daptomycin (60%). While, two isolates designated resistance towards clindamycin, oxacillin, benzylpenicillin, levofloxacin, ciprofloxacin with 40%, and a single isolate exposed to gentamicin. Remarkably, all the isolates were found to be 100% sensitive to linezolid, teicoplanin, vancomycin rifampicin, and tetracycline used in the study.

Production of extended spectrum β-lactamases

All antibiotic-resistant positive clinical isolates causing RTI were further assessed for phenotypic detection of ESBL production as indicated by Double Diffusion Synergy Test (DDST). After incubation of MHA plates inoculated with each positive isolate and placed with pairs of antibiotics, results revealed a significant formation of a zone of inhibition around cefotaxime/ clavulanic acid and ceftazidime/clavulanic acid. While, no zone of inhibition was formed around cefotaxime and ceftazidime antibiotic discs indicating negative ESBL production. Of all the positive isolates analysed for the prevalence of ESBL producers, 29.8% were found to be ESBL producers with a carriage rate attributing with 6.6% (Table 7). The highest ESBL producer were *K. pneumoniae* contributing about 21.9% of the total 228 positive isolates. While, *E. coli* indicated ESBL

production attributing to 6.5% and *E. cloacae* were found to be the least with 1.3% (with 3 isolates). None of *A. baumannii* and *P. aeruginosa* isolates were found to be ESBL producers. The details of clinical isolates of RTI-producing ESBL about total positive isolates and total clinical samples are shown in Figure 6 and Figure 7.

Table 7. Incidence of ESBL-producing RTI pathogens isolated from June 2020 - May 2021

RTI pathogen	No. of isolates	% of ESBL producers (n = 228)	% of ESBL Carriage rate (n = 1016)
E. coli	15	6.5%	1.4%
K. pneumoniae	50	21.9%	4.9%
A. baumannii	00	00%	00%
P. aeruginosa	00	00%	00%
E. cloacae	03	1.3%	0.29%
Total No. of ESBL isolates	68	29.8%	6.6%

DISCUSSION

One of the breakthrough pandemics of this era is COVID-19 which by the end of Jan-2021 claimed more than 2 million deaths all over the world.[21] However, the specific cause associated with the mortality of COVID-19 patients is still not clear yet to be well established. A study conducted to establish the cause of death in COVID-19 and non-COVID patients that included 17,456,515 subjects revealed 17063 patients died due to COVID-19 while the remaining patients, i.e. 134,316 died due to other causes.[22] Hence, deaths that occurred during the COVID pandemic were also associated with non-COVID causes and the present work is witnessing a similar instance of deaths due to non-COVID reasons.

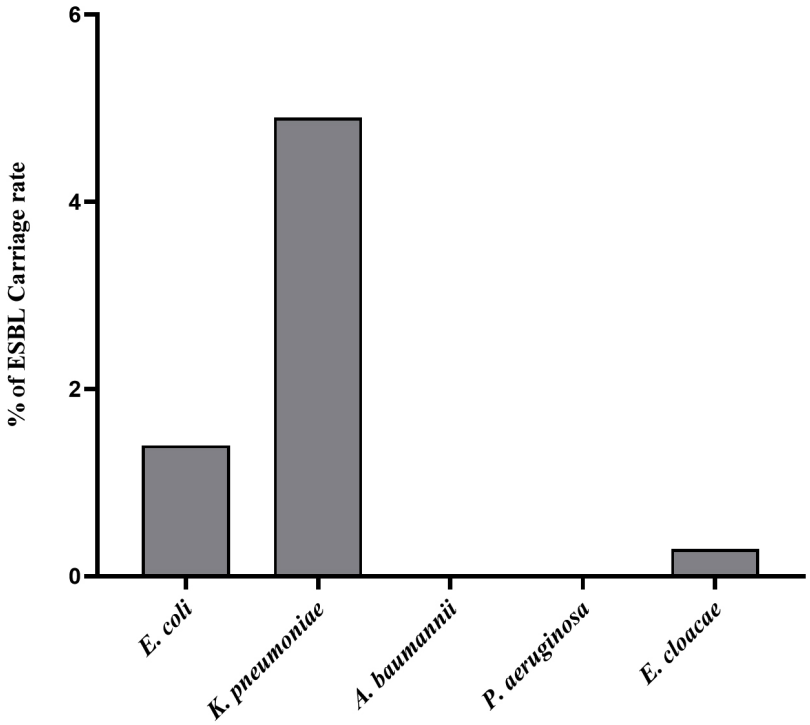

Figure 7. Incidence of ESBL-producing RTI pathogens isolated (June 2020 - May 2021)

Worldwide there is an increased prevalence to infectious diseases, in which RTIs stands in the fourth place globally. This statistically is a prominent cause of death worldwide which

claimed to be 2,603,913 deaths in 2019 all over the world. During the COVID-19 pandemic, about 567 million confirmed respiratory tract diseases along with 6.3 million deaths have been recorded across the globe.[23] The increased emergence of AMR in pathogens causing RTIs namely *K. pneumoniae, A. baumannii*, *P. aeruginosa, E. coli, S. aureus,* etc., has become more life-threatening and making it difficult to treat patients with respiratory tract diseases and challenging for need of significant antibiotic therapy.[24-27] Hence, in the current investigation, clinical samples of patients with RTIs were collected, bacterial pathogens were identified and their antibiotic resistance profile along with ESBL production was studied.

The majority of clinical samples collected in the present study were from the suction tips of the ICU section and sputum samples of IP and OP wards in which the highest disease prevalence was observed in male patients. The high prevalence of RTI in ICU may be associated with healthcare-associated infections (HAIs) and it has been reported that about 30% of HAIs acquired by patients admitted to the ICU section. The High risk of HAIs in ICU may also be due to the high prevalence of devices, intensive procedures, the microflora of critically ill patients, nurses (source of cross-contamination), microbiological sampling, cross contamination, etc. along with the emergence of novel SARS-CoV-2 (COVID-19) pathogen has further intensified the ICU acquired infections.[28]

The percentage incidences of respiratory tract diseases were increased between the age of 5 to 35 years and was found declined in older age patients. In 2018, the naive out-patients with RTI in referral hospitals of Meru, County, Kenya also showed a high prevalence of respiratory tract diseases in male patients (71.4%) as compared to female patients (28.6%) with a ratio of 5:2. Further, the disease was increasing with increased age of the patients' ages from 5 to 34 years which declined in the older patients of above 34 years.[29] Following the microbiological culture and biochemical-based identification of respiratory bacterial pathogens in clinical samples of RTI indicated the predominant Gram-negative species such as *K. pneumoniae, P. aeuroginosa, E. coli, A. baumanii* and *E. cloacae* compared to Gram-positive *S. aureus* and CoNS pathogens. These etiological agents except *E. cloacae* and CoNS were also reported by other studies as commonly detected respiratory pathogens. However, percentage of antibiotic resistance against the selected antibiotics differs from in the following studies as compared to the others documented.[30,31]

The AST performed for *K. pneumoniae* in the present study showed highest resistance towards ampicillin (100%), followed by cephalosporin group (65.7-74.8%), fluoroquinolone (74.8%) aminoglycoside (59.4%), and carbapenems (49.6%) as compared to the reports of resistance rate with penicillin class of antibiotics of 57.6 to 80.4%, cephalosporins with 44.9 to 82%, carbapenems resistance 45.8 to 72.5%, aminoglycosides and tetracycline resistance with 44.1-64.4%, and fluoroquinolone resistance of 53.3 to 82% as reported five years in previous studies (2015-2019). Another study also reported 2.3-42.5% resistance to colistin, a polypeptide antibiotic, noteworthy that our study showed 100% sensitivity towards the same antibiotic.[32] Among Gram-negative respiratory pathogens, *K. pneumoniae* is the 3rd most commonly detected pathogenic bacteria from patients with RTI (mean% of 10.9). The drug resistance rate worldwide for *K. pneumoniae* has been estimated to be more than 70% with rate of infection causing fatality rate of approximately 40-70%.[33]

P. aeruginosa strains from RTI patients in the present study showed the highest resistance to carbapenems (66.6%) compared to fluoroquinolone and aminoglycoside (43.3%), while, penicillin showed (16.6-40%). However, *P. aeruginosa* was reported as a major resistant pathogen as MDR, exclusively drug resistance (EDR), and difficult-to-treat.[34,35] The global antibiotics resistance level of *A. baumannii* in respiratory tract diseases has been reported to be 45%, being MDR strains they have been reported with carbapenem resistant and colistin sensitive.[36] However, high-rate resistance, i.e. about 70% of MDR strains was noticed in low-income nations.[37,38] The present study reports high resistance to antibiotics among *E. coli* isolates from RTIs as compared to earlier reports by Promite and Saha.[39] Their study highlighted the resistance range of 72%, 56%, 54%, 48%, 32%, 28%, and 24% resistance to amoxicillin, cefixime, sulfamethoxazole/

trimethoprim, ciprofloxacin, nalidixic acid, amikacin, and gentamycin, respectively in MDR *E. coli* of RTI as compared to varying antibiotics resistance of 50-92% in the present study.[39]

Majority of respiratory tract samples of *E. cloacae* isolated from 53 Chinese hospitals showed less than 8% resistance to meropenem, imipenem, polymyxin B, amikacin, and 10% resistance to carbapenem compared to high-level resistance to penicillin (100%), cephalosporin (83.3%), fluoroquinolone (83.3%), and sulphonamides (83.3%) antibiotics in our study indicating significant resistance level in the pathogens.[40] However, in our study *E. cloacae* was found to be 100% sensitive to carbapenems and colistin although colistin-resistant *E. cloacae* accounted for high morbidity and mortality rate of ICU patients.[41] Though, several studies have reported colistin resistance, our study significantly showed the inhibition of the growth of all respiratory pathogens indicating it as the choice of drug for the treatment of respiratory tract diseases. Among Gram-positive bacteria, the highest resistance was observed against β-lactam antibiotics, fluoroquinolone, and lincosamides in both *S. aureus* and CoNS and equivalent resistant against fluoroquinolone class of antibiotics, i.e. levofloxacin (80.4%) and ciprofloxacin (92.5%).[42] The antibiotic resistance pattern of each isolate in the present study is supportive in the treatment of RTI patients and one can anticipate the possible future course of resistance in these pathogens. Hence, the use of antibiotics against which resistance is anticipated can be limited.

CONCLUSION

A high prevalence of antibiotic resistance was observed in Gram-negative pathogens as *K. pneumoniae*, *P. aeruginosa*, and *E. coli,* along with few species of *A. baumannii* and *E. cloacae.* These pathogens revealed resistance to more or least one class of antibiotics and were found to be carbapenem-resistant and ESBL producers. Following MDR strains, there is a need for continuous search towards novel antibiotics and alternative treatment options. The present study encased the levels of antibiotic resistance patterns that has been high values. The possibilities of resistance in pathogens are varied reasons such as inappropriate and incorrect administration of antimicrobial agents, lack of apt infection control strategies that are alarming situation with increased MDR strains. The problem can be addressed by performing the antibiotic susceptibility testing before empirical treatment. Surveillance of such infections and monitoring of antimicrobial resistant patterns needs to be carried out in both hospital and community-acquired infections. Further, studies need to be designed for molecular characterization and validated with accuracy for guiding empirical treatment options in RTI using antibiotics.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

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

ETHICS STATEMENT

The study was approved by the Ethics Committee, Santosh Hospitals and Diagnostics, vide approval number SHIEC/ECAL/DSU/APR2020.

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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