Indian Journal of Microbiology Research 2023;10(3):149–154

Content available at: https://www.ipinnovative.com/open-access-journals

Indian Journal of Microbiology Research

Journal homepage: https://www.ijmronline.org/

Original Research Article

A study on siderophore production and biofilm formation of *Klebsiella* isolated from urine samples and detection of antibiotic resistance mechanisms

Lakshmipriya I S[1,*], **Raksha Yoganand**[1], **Shoorashetty Manohar Rudresh**[1]

[1]*Dept. of Microbiology, ESIC Medical College & PGIMSR, Bangalore, Karnataka, India*

ARTICLE INFO

Article history:
Received 02-05-2023
Accepted 08-08-2023
Available online 10-10-2023

Keywords:
UTI
Klebsiella
ESBL
AmpC
CarbaNP
Biofilm
Siderophore

ABSTRACT

Background: One of the most encountered infections by the physician in the community is Urinary tract infections, and over the years most of the pathogens responsible for the etiology have become resistant to antimicrobials. In nosocomial infections and immunocompromised individuals the most common pathogen implicated in UTI and catheter associated UTI are *Klebsiella pneumoniae*. *Klebsiella* are notorious for their antibiotic resistance and also cause systemic dissemination.

Materials and Methods: A cross-sectional study that included 184 *Klebsiella* species isolated from urine samples collected from January 2020 – June 2021. Identification of isolates and speciation was done by biochemical reactions, antibiotic susceptibility pattern determined by Kirby Bauer disc diffusion method, virulence factors and antibiotic resistance mechanisms were detected by standard phenotypic methods.

Results: Among the patient's female: male ratio was 1.7:1 and maximum number of cases were seen in the 21-30 age group.

Maximum number of isolates belonged to *Klebsiella pneumoniae* (79.89%), followed by *Klebsiella oxytoca* (20%). *Klebsiella* species showed maximum sensitivity to Imipenem, Meropenem, gentamicin, and amikacin. Out of the 184 isolates 22.86% were ESBL producers, 17.93% were Amp C producers and 9.24% were Carbapenamase producers. Among all isolates 94.56% were found to be biofilm producers, and all biofilm poducers were strongly associated with ESBL and Amp C production. Also 61.41% of total isolates were Siderophore producers.

Conclusion: UTI is a predominant infection among younger age group females. High level of resistance to commonly used antibiotics were found, also the rising rate of antibiotic resistance mechanisms require further studies into the matter for ensuring better treatment success. Empirically amikacin and gentamicin could be used for treatment of UTI as they were found to be highly sensitive.

1. Introduction

Rise in antibiotic resistance has definitely had its implications in UTI, contributing to treatment failure. Worth mentioning are the plasmid mediated extended-spectrum β-lactamases ESBLs (resistance to third generation cephalosporins) mainly in *E.coli* and *K.pneumoniae*. Other plasmid mediated resistance mechanism is AmpC enzymes among other *Enterobacteriaceae* (resistant to 3^{rd} generation cephalosporins and beta lactamase inhibitors.[1]

Emergence of ESBL (extended spectrum beta lactamases) producing and Carbapenem resistant *Klebsiella* strains have limited the therapeutic options.

Only limited data is available on study of *Klebsiella* isolates from Urine samples. Hence, this study was designed to know the occurrence, detection of predominant *Klebsiella* species, resistance mechanisms and optimum antibiotic

* *Corresponding author*.
E-mail address: lakshmipriya31113@gmail.com (Lakshmipriya I S).

https://doi.org/10.18231/j.ijmr.2023.026

susceptibility pattern so that it would help the clinicians for empirical choice of antibiotics.

Several virulence factors mediate infectivity of *Klebsiella*, and studying them will help in further decoding the pathogenesis of *Klebsiella* at molecular level.[2]

Siderophores are non-redundant virulence factors used to enhance the pathogenesis of bacteria, they enhance competition among bacteria, modulate host cellular pathways, and determine the bacterial "*replicative niche*" during infection.[3] They mediate cellular iron homeostasis, promote dissemination of bacteria, and regulate the production of multiple bacterial virulence factors.[4]

Biofilm formation of gram-negative bacteria greatly influences the effectiveness of antibiotic therapy, because as widely studied, biofilm formation greatly deters the penetration of drugs also contributes to the resistance mechanisms of the bacteria. Hence, the need to study further to detect biofilm formation and find ways to prevent its growth. These steps will help in prevention of propagation of resistant strains and also prevent relapse after treatment.

2. Materials and Methods

All the *Klebsiella* isolated from urine samples received in the department of Microbiology laboratory at ESIC-MC and PGIMSR Bengaluru. From January 2020 to June 2021.

2.1. Sample size

Based on hospital data the proportion of *Klebsiella* species isolated from urine samples with significant bacteriuria calculated Sample size 184.

2.2. Inclusion criteria

Urine samples showing growth of *Klebsiella* pneumoniae with significant colony count ($\geq 10^5$ cfu/ml).

2.3. Exclusion criteria

1. *Klebsiella* species isolated from clinical specimens other than urine.
2. Urine samples showing mixed or insignificant growth.

2.4. Methods of detection of antibiotic resistance

2.4.1. Phenotypic confirmatory disc diffusion test (PCDDT) for ESBL detection

A Mueller Hinton agar plate is lawned with an overnight incubated broth of required organism to be tested. Two antibiotic discs are used a Ceftazidime disc and a combination of clavulanic acid (10 mcg) and ceftazidime (30 mcg). They are placed at a distance of 15mm. The plates are incubated for 24 hrs at 37 C. The zone diameters of both discs are measured and a difference in zone diameter of more than 5 mm for Ceftazidime- Clavulanic acid disc from Ceftazidime was considered as positive ESBL production.[5]

2.4.2. Amp C disk test

Lawn the surface of MHA plate with 0.5 McFarland bacterial suspension of *E. coli* ATCC 25922. On the surface of agar plate place a 30 μg cefoxitin disc, almost touching the disk place AmpC disk (rehydrated with 20 μl of saline) several colonies of each test organism to be applied on to the surface of Amp C disc where it touches the agar plate. Both discs should be kept almost touching each other. Invert the plate and incubate overnight at 35°C. After overnight incubation, check the plate for an indentation or a flattening of the zone of inhibition. If there is any indentation of zone of inhibition indicates that Cefoxitin was inactivated by the Amp C enzymes released by the test organism (positive result). If no indentation of zone of inhibition, indicates cefoxitin has not been inactivated, i.e. no Amp C production, (negative result).[6]

2.4.3. Modified Carba NP test

Inoculum was prepared by inoculating test strain in 200 μl peptone water for 2 hours (pH 7). To prepare solution A Clinical Laboratory Reagent Water phenol red (0.05%) and ZnSO4.7H2O (0.1 mmol/L) was added; pH was adjusted to 7.8 ± 0.1. Solution was stored in amber-colored bottles at 4°C for up to 15 days. The solution B was prepared by adding 12 mg/ml imipenem-cilastatin injectable form to solution A and should be stored at 4°C till it is used. Bacterial lysate (100 μl) was divided into two parts and added to two microcentrifuge tubes labeled a and b. Reagents A and B were added to tubes a and b, respectively. Both tubes incubate at 37°C and readings were taken at intervals of 10 min, 30 min, and 120 min by three different observers. Tube (a)was red and tube (b) was orange/yellow test was considered positive. Both tubes remained red test is considered negative. QC *Klebsiella* pneumoniae ATCC BAA 1705 (positive control), *K.pneumoniae* ATCC BAA 1706 (negative control), and plain A and B reagents with lysis buffer (reagent control).[7]

2.5. Biofilm detection

Fresh Luria-Bertani (LB) broth 100 μl is fill in each well of a 96-well polystyrene plate. Inoculate 1 μl of an overnight culture into each well. Incubate for 5 hours at 37°C. For staining of bacteria add 25 μl of 0.5% crystal violet for 20 min to each well. Discard the supernatant and wash the plates three times with deionized water to remove unbound cells. The biofilm-bound dye is then eluted with 95% ethanol, and the optical density at 550 nm (OD550) was determined. Each assay should be performed three times on at least three occasions.[8]

Characterization of isolates based on biofilm production.[9]

ODCUT = OD avg of negative control + 3(SD of ODs of negative control)

1. OD <=ODCUT = Non-Biofilm former (NBF)
2. ODCUT <OD<=2 x ODCUT = Weak biofilm former (WBF)
3. ODCUT <OD <4 x OD CUT = Moderate biofilm former (MBF)
4. OD>4x ODCUT = Strong biofilm former (SBF)

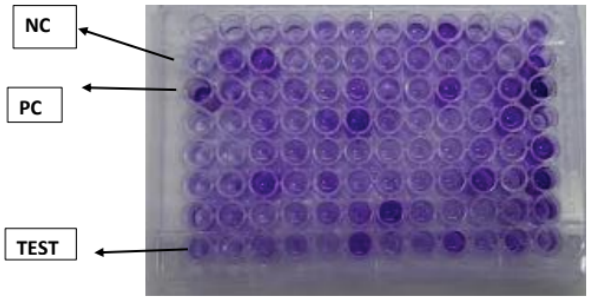

Fig. 1: Biofilm production by microtiter plate method

2.6. *Siderophore production*

2.6.1. *Qualitative method*

This assay was performed according to modified method given by Hu and Xu (2011). 100 ml CAS reagent is mixed in 900 ml sterilized LB agar medium to prepare CAS agar plates. Bacterial strains were inoculated by spot inoculation on each plate. After inoculation, plates were incubated for 5–7 days at 28 °C and look for the formation of orange zone around the bacterial colonies. Orange zone confirms the siderophore production by isolates.[10]

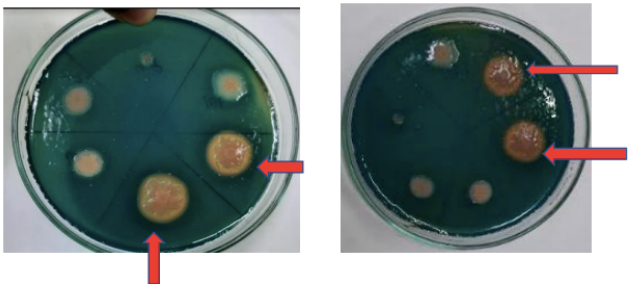

Fig. 2: Inoculated CAS agar plate showing orange clear zone around growth showing siderophore production

2.7. *Statistical analysis*

Statistical analysis was done using Epi InfoTM 7.1.4 software program. Simple frequencies were tabulated. Chi square test was done to determine the statistical significance.

2.8. *Ethical consideration*

Institutional ethical committee clearance was taken.

3. Results

Among the study population there was high preponderance of females 63.04%. Maximum number of cases were seen in the age group 21-30 age group. From study group maximum number of cases belonged to Wards (55.98%) followed by OPD (36.96%) and least number belonged to ICU(7.07%). Speciation done using usual biochemical reactions. Maximum number of isolates belonged to *Klebsiella pneumoniae subsp pneumoniae* (79.89%), followed by *Klebsiella oxytoca* (20%). Diabetes mellitus was found to be a major predisposing factor in the UTI patients, followed by presence of calculi, pregnancy in female patients also presence of invasive devices like catheter. *Klebsiella* species showed 80% sensitivity to Imipenem, 78% sensitivity to Meropenem, 76% sensitive to gentamicin, 74% sensitive to amikacin. Highest resistance was shown to Cefazolin, Cefotaxime & Cotrimoxazole. Out of the 184 isolates 22.8% were ESBL producers.

Out of 184 isolates, 83 were Cefoxitin resistant and these were screened by AmpC disc and 33 among 83 (39.76%) were found to be Amp C positive and, 50 were Amp C negative.

Out of the 184 isolates, 57 were found to be Carbapenem resistant isolates and they were subjected to Modified CarbaNP test. Out of the 57 only 17 (29.82%) gave positive CarbaNP test.

Out of the 184 isolates 83.15% were found to be strong biofilm producers, 5.43% were medium biofilm producers and 5.98% were weak biofilm producers. In total 174 out of 184 isolates were biofilm producers.

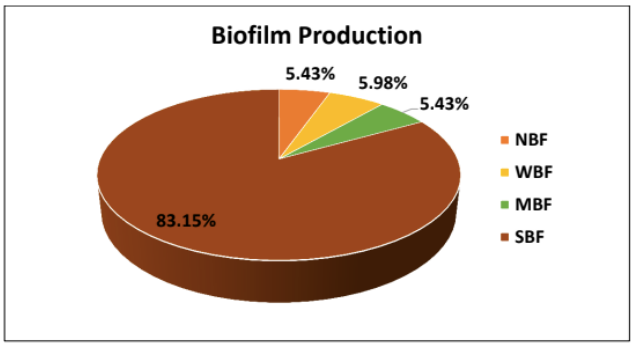

Fig. 3: Distribution of biofilm producers among all isolates NBF- Non-biofilm former, WBF- Weak biofilm former, MBF – Medium biofilm producer, SBF- Strong biofilm producer

Out of the 184, 113 (61.41%) were Siderophore producers, whereas 71(38.59%) isolates were siderophore non producers.

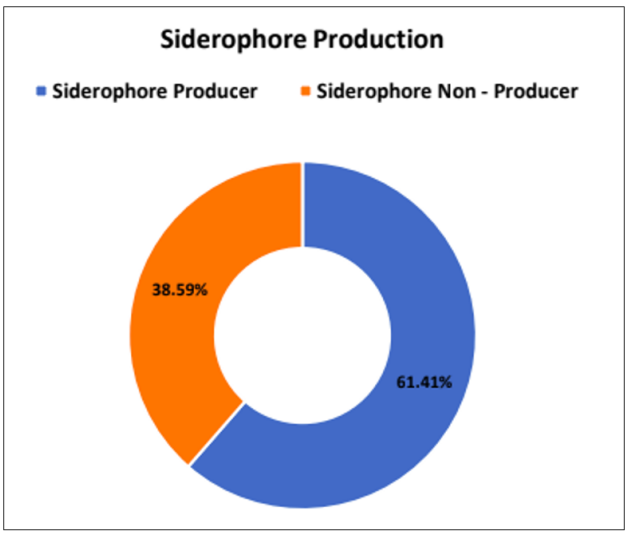

Fig. 4: Percentage of Siderophore producers and non-producer

3.1. *Species wise virulence factor production*

Among the 147 *Klebsiella* pneumoniae isolates 11 produced Siderophore alone, 58 produced biofilms alone and 78 produced both biofilm and siderophore.

Table 1: Species wise virulence factor production of *Klebsiella* pneumoniae

	Siderophore producers	Biofilm producers	Producing both
Klebsiella pneumoniae	11	58	78
Percentage (%)	7.48	39.4	53.06

Among the 37 *Klebsiella oxytoca* isolates 3 produced Siderophore alone, 7 produced biofilms alone and 27 produced both biofilm and siderophore

Table 2: Species wise virulence factor production of *Klebsiella* oxytoca

	Siderophore producers	Biofilm producers	Producing both
Klebsiella oxytoca	3	7	27
Percentage (%)	8.1	18.91	72.97

It was shown that among the Amp C producers 75.75% were biofilm producers.

Among all ESBL producers 88% were associated with biofilm production.

4. Discussion

Increased incidence of UTI in women when compared to men are due to anatomic differences. Relatively short

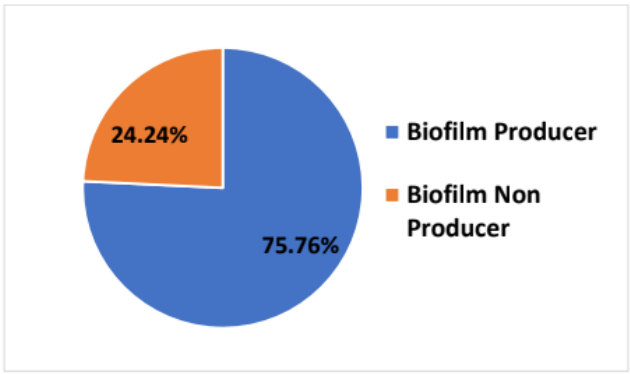

Fig. 5: Pie chart showing Association of AmpC with Biofilm producers

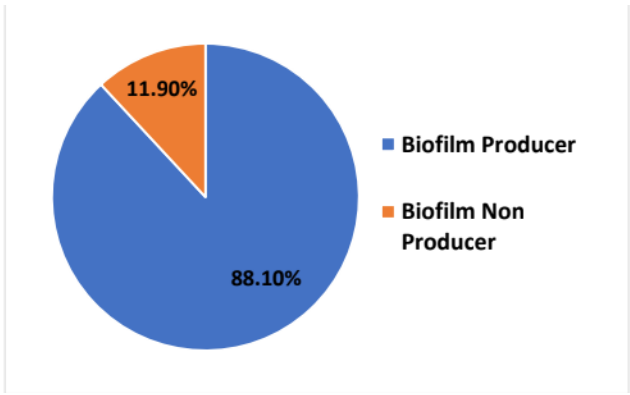

Fig. 6: Pie chart showing Association of ESBL with Biofilm production

Table 3: Relationship between *Klebsiella* with biofilm and without biofilm among Upper and lower UTI

	Upper UTI	Lower UTI	
Klebsiella with biofilm	19	90	109
Klebsiella without biofilm	3	4	7
Total	22	94	116

P value < 0.05, there is significant association between *Klebsiellae* isolates producing biofilm and Upper and Lower UTI.

Table 4: Relationship between *Klebsiella* with siderophore and without siderophore among Upper and lower UTI

	Upper UTI	Lower UTI	
Klebsiella with Siderophore production	16	64	80
Klebsiella without siderophore production	6	30	36
Total	22	94	116

P value > 0.05. There is no significant association between Upper and Lower UTI caused by *Klebsiella* producing siderophore and *Klebsiella* not producing siderophore.

urethra of female, as compared to the male counterpart, the proximity of the urethra to the anus, and the colonization of the periurethral mucosa with bowel flora are the several the reasons. Antibiotic sensitivity pattern of organisms changes rapidly over a period of time. In the present study *Klebsiella* species showed 80% sensitivity to Imipenem, 78% sensitivity to Meropenem, 76% sensitive to gentamicin, 74% sensitive to amikacin. Highest resistance was shown to Cefazolin & Cefotaxime.

4.1. Antibiotic resistance mechanisms

In the present study out of the 184 cases 22.8% were ESBL positive. Extended-spectrum β-lactamases (ESBLs) are a group of plasmid-mediated, diverse, complex and rapidly evolving enzymes the ability to hydrolyze third-generation cephalosporins and monobactam (aztreonam) and yet are inhibited by clavulanic acid. Carbapenems are the treatment of choice for infections due to ESBL-producing organisms. ESBL- producing organisms exhibit co-resistance to many other classes of antibiotics also, resulting in limited therapeutic options.[11]

4.2. Amp C beta lactamases

Out of 184 isolates, 83 were Cefoxitin resistant and these were screened by AmpC disc and 33 among 83 (39.76%) were found to be Amp C positive and, 50 were Amp C negative. AmpC β-lactamases are clinically significant, and these confer resistance to cephalosporins in the oxyimino group (cefotaxime, ceftazidime, ceftriaxone), 7-α methoxy cephalosporins (cefoxitin or cefotetan) and are not affected by available β-lactamase inhibitors (clavulanate, sulbactam, tazobactam).

4.3. Carbapenamase producer

Out of the 184, 57 were found to be Carbapenem resistant isolates and they were subjected to modified CarbaNP test. Out of the 57 only 17(9.24%) gave positive CarbaNP test. Globally, common carbapenemases in Enterobacterales include the *Klebsiella* pneumoniae carbapenemases (KPC), oxacillinase (OXA)-48-like β-lactamases, and metallo-β-lactamases, such as New-Delhi-metallo-β-lactamases (NDM), the Imipenemase(IPM), and Verona integron-encoded metallo-β-lactamases (VIM).[12]

4.4. Virulence factors

4.4.1. Biofilm production

Out of the 184 isolates 83.15% were strong biofilm producers, 5.43% were medium, 5.98% were weak biofilm producers. 5.43% were non biofilm producers. It is to be noted that in the present study Strong biofilm producers were strongly associated with Upper UTI, i.e 58.38% of upper UTI were associated with SBF. Also, patients with history of indwelling catheter in the present study were 100% associated with SBF. Bacteria attaches firmly to the uroepithelium and forms biofilm. Such bacteria are capable of invading the renal tissue causing pyelonephritis.

Biofilms can develop on urethral stents and catheters causing and propagating infection and blockage. Thus, catheter-associated UTI (CAUTI) is one of the most well know infections in community and associated with health care around the world[13] One of the most important point of Biofilm formation induces antimicrobial resistance by several mechanisms, like.

1. Limiting the diffusion of antibiotic through the matrix.
2. Transmission of resistance genes within the community.

4.4.2. Siderophore production

Out of the 184, 113 (61.41%) were Siderophore producers, whereas 71(38.59%) isolates were siderophore non producers. There is significant connection between resistance to fluoroquinolones and siderophores production. Ciprofloxacin- and norfloxacin-resistant enterococcal strains produced siderophores in large quantity.[14] Siderophore synthesis lead to Fluoroquinolone resistance resulting in the increased virulence that will increase the severity of the infection and the effectiveness of treatment.[15]

In the present study out of the 36.9% isolates that were Ciprofloxacin resistant, 41.5% were Siderophore producers. Finally, among the total isolates 105 were found to be co-producers of biofilm and Siderophore ie 57%.

5. Conclusion

As found in the present study high level of prevalence of Amp C, ESBL and Carbapenamases is a matter of concern. Determination of their prevalence is essential to formulate an effective antibiotic policy and to prevent treatment failure. For any person with a urinary tract infection Amikacin and Gentamicin could be used as empirical therapy provided their renal parameters are within normal limits. Virulence factors that were studied showed biofilm and siderophore production to be significantly associated with antibiotic resistance. These antivirulence therapies would allow us to effectively neutralize, or disarm the virulence of bacteria, and decrease the capacity of UTI pathogens to cause disease. Antivirulence therapeutics target processes that are critical for UTI pathogenesis and will help combat this infection.

6. Source of Funding

None.

7. Conflict of Interest

None.

References

1. Liu L, Li F, Xu L, Wang J, Li M, Yuan J, et al. Cyclic AMP-CRP Modulates the Cell Morphology of Klebsiella pneumoniae in High-Glucose Environment. *Front Microbiol*. 2020;10. doi:10.3389/fmicb.2019.02984.
2. Lenchenko E, Blumenkrants D, Sachivkina N, Shadrova N, Ibragimova A. Morphological and adhesive properties of Klebsiella pneumoniae biofilms. *Vet World*. 2020;13(1):197–200.
3. Namikawa H, Niki M, Niki M, Oinuma KI, Yamada K, Nakaie K, et al. Siderophore production as a biomarker for Klebsiella pneumoniae strains that cause sepsis: A pilot study. *J Formos Med Assoc*. 2022;121(4):848–55.
4. Faoagali JL. Klebsiella urinary tract infection. *N Z Med J*. 1975;81(536):286–8.
5. Iqbal R, Ikram N, Shoaib M, Shoaib M, Javaid M, Mehmood RT, et al. Phenotypic cofirmatory disc diffusion test (PCDDT), double disc synergy test (DDST), E-test OS diagnostic tool for detection of extended spectrum beta lactamase (ESB L) producing Uropathogens. *J Appl Biotechnol Bioeng*. 2017;3(3):344–9.
6. Gupta G, Tak V, Mathur P. Detection of AmpC β Lactamases in Gram-negative Bacteria. *J Lab Physicians*. 2014;6(1):1–6.
7. Rudresh SM, Ravi GS, Sunitha L, Hajira SN, Kalaiarasan E, Harish BN, et al. Simple, rapid, and cost-effective modified Carba NP test for carbapenemase detection among Gram-negative bacteria. *J Lab Physicians*. 2017;9(4):303–7.
8. Kirmusaoglu S. The Methods for Detection of Biofilm and Screening Antibiofilm Activity of Agents [Internet]. In: Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods. IntechOpen; 2019. doi:10.5772/intechopen.84411.
9. Kuineki S, Acharya J, Dhungel B, Adhikari S, Adhikari N, Shrestha UT, et al. Biofilm Formation and Phenotypic Detection of ESBL, MBL, KPC and AmpC Enzymes and Their Coexistence in Klebsiella spp. Isolated at the National Reference Laboratory, Kathmandu, Nepal. *Microbiol Res* . 2021;12(3):683–97.
10. Karimi K, Zarei O. Investigation of Antibiotic Resistance and Biofilm Formation in Clinical Isolates of Klebsiella pneumoniae. *Int J Microbiol*. 2021;2021:5573388. doi:10.1155/2021/5573388.
11. Rawat D, Nair D. Extended-spectrum β-lactamases in Gram Negative Bacteria. *J Glob Infect Dis*. 2010;2(3):263–74.
12. Podschun R, Fischer A, Ullmann U. Siderophore production of Klebsiella species isolated from different sources. *Zentralbl Bakteriol*. 1992;276(4):481–6.
13. Soto SM. Importance of Biofilms in Urinary Tract Infections: New Therapeutic Approaches. *Adva Biol*. 2014;2014. doi:10.1155/2014/543974.
14. Jasim SA, Abdulrazzaq SA, Hashoosh SI, Saleh RO. Virulence Factors of Klebsiella pneumoniae Isolates from Iraqi Patients. *Sys Rev Pharm*. 2020;11(6):916–21.
15. Lisiecki P. Antibiotic resistance and siderophore production in enterococci. *Med Dosw Mikrobiol*. 2014;66(1):1–10.

Author biography

Lakshmipriya I S, PG Student https://orcid.org/0009-0000-3793-7954

Raksha Yoganand, Professor and HOD https://orcid.org/0000-0003-2254-3893

Shoorashetty Manohar Rudresh, Assistant Professor https://orcid.org/0000-0003-0555-6748

Cite this article: Lakshmipriya I S, Yoganand R, Rudresh SM. A study on siderophore production and biofilm formation of *Klebsiella* isolated from urine samples and detection of antibiotic resistance mechanisms. *Indian J Microbiol Res* 2023;10(3):149-154.