Original Article

DOI: 10.7860/NJLM/2024/69555.2872

Microbiology Section

Multidrug Resistance in Bacterial Isolates from Pus Samples: A Hospital-based Descriptive Cross-sectional Study

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ABSTRACT

Introduction: Antimicrobial Resistance (AMR) is a growing public health threat. Multidrug-resistant (MDR) bacteria, initially associated with healthcare-associated infections, have now spread and become a major cause of community-acquired infections, as well. Therefore, there is a need for monitoring MDR bacterial infections in healthcare settings to establish policies for antimicrobial therapy and effective infection prevention strategies. As pyogenic infections were a major burden of infectious diseases in our healthcare setup, the present study was conducted.

Aim: To estimate the prevalence of Multidrug Resistance (MDR) and Extensive Drug Resistance (XDR) in bacterial isolates from pus samples.

Materials and Methods: The present hospital-based descriptive cross-sectional study was conducted in the Microbiology Laboratory, SMBT Hospital, Nashik, Maharashtra, India, from June 2022 to May 2023. A total of 360 bacterial isolates from pus samples received from the General Surgery, Orthopaedics and Otorhinolaryngology Departments were tested for antimicrobial susceptibility according to the Clinical and Laboratory Standards Institute (CLSI) 2023 guidelines. Isolates were classified as MDR and XDR as per standard definitions. All *Escherichia coli (E. coli)* and *Klebsiella pneumoniae (K. pneumoniae)* isolates were tested for Ampicillinase C (AmpCs) production by disk approximation test and double disk synergy test. Microsoft Excel 2013 was used for data analysis.

Results: The prevalence of MDR and XDR in Gram-negative bacteria was 175 (68.8%) and 120 (47.2%), respectively. The prevalence of MDR and XDR *Staphylococcus aureus (S. aureus)* was 59 (67%) and 10 (11.3%), respectively. The prevalence of Methicillin Resistant S. aureus (MRSA) was 45.4% (n=40). Out of the total 111 isolates of *E. coli* and *K. pneumoniae*, five isolates were AmpC beta-lactamase producers (4.5%).

Conclusion: In the present study, a high prevalence of MDR was observed in both Gram-negative and Gram-positive bacteria isolated from different pus samples. This is an alarming situation, and further in-depth studies need to be conducted to assess the association of MDR bacterial infections with particular sites and types of infections to develop effective therapeutic and infection control policies.

Keywords: Ampicillinase C beta-lactamase, Antimicrobial resistance, Extensive drug resistance, Multidrug-resistant

INTRODUCTION

The AMR is a growing public health threat. The crude mortality rate in India due to infectious diseases is 417 per 100,000 persons. Therefore, the impact of AMR is expected to be higher in India. Many clinically important pathogens are exhibiting increasing rates of AMR. The emergence of resistance is not limited to older and more frequently used antimicrobial agents but also includes a rapid increase in resistance to newer and more expensive drugs, such as carbapenems. Therefore, periodic monitoring of AMR patterns is essential for formulating an effective empirical treatment plan and implementing containment interventions [1].

Pus is one of the major samples received in the Microbiology Laboratory for wound infections. Globally, the rate of septic wound infections ranges between 2.5-17.4%, with the highest rates observed in developing countries [2]. The overall incidence of wound sepsis in India ranges from 10-33% [3]. Chronic wound infections trigger inflammation, further tissue damage and a slow healing process, leading to longer hospital stays and the indiscriminate use of antibiotics, which in turn results in the development of AMR [4,5]. Bacteria acquire resistance through four mechanisms:

- Decreased entry of the antibiotic into the bacterial cell;
- Increased extrusion of the antibiotic by bacterial efflux systems;
- Mutational modification of the antibiotic's target;
- Production of antibiotic-inactivating enzymes.

Among the different mechanisms of AMR, the production of beta-lactamases is of major importance as these enzymes are commonly transferable and can inactivate multiple beta-lactam antibiotics [6]. Among the beta-lactamases, the most common are the production of Extended-spectrum Beta-lactamases (ESBLs) and AmpCs [7]. AmpC are cephalosporinases that confer resistance to Cephalothin, Cefazolin, cefoxitin, Penicillin, and β-lactamase inhibitor-β-lactam combinations [8].

In recent years, there has been a rise in the prevalence of infections with MDR organisms producing AmpC Beta-lactamases (βLs), which have become a major therapeutic challenge. AmpC producers may initially appear susceptible to extended-spectrum Cephalosporin when tested, leading to the inappropriate selection of antimicrobial regimens and therapeutic failure [9].

AmpC beta-lactamase production can be chromosomal or plasmid-mediated. Chromosomal AmpC genes are expressed constitutively at low levels and are not easily transferable to other bacterial species. Inducible resistance, due to chromosomal AmpC genes, is present in *Enterobacter* species (spp.), *Citrobacter* spp. and *Serratia* spp. Exposure to antimicrobial agents like cefoxitin and Imipenem induces AmpC beta-lactamase production in these bacteria. Non-inducible chromosomal resistance is seen in *E. coli* and *Shigella* spp. Plasmid-mediated AmpC βLs have arisen through the transfer of chromosomal genes for AmpC βL onto plasmids. These genetic determinants can spread laterally to other bacteria through the transfer of plasmids. This phenomenon is observed in

*Klebsiella*pneumoniae, *E. coli*, and *Salmonella spp*. [10]. Therefore, in addition to monitoring MDR, timely detection of resistance mechanisms is crucial to prevent treatment failure and the spread of these resistant organisms in healthcare settings.

As a tertiary healthcare centre, many patients referred to the present study facility are already on certain antimicrobial agents and are more prone to developing MDR bacterial infections. Studying MDR bacteria is necessary to formulate hospital antimicrobial policies. Since the burden of MDR bacterial infections at the present study healthcare facility has not been studied before, the present study was conducted with the following aim to estimate the prevalence of MDR and XDR in bacterial isolates from pus samples. The primary objective of the study was to estimate the proportion of MDR Gram-negative bacteria susceptible to different antimicrobial agents and the secondary objective of the study was to estimate the proportion of *E. coli* and *K. pneumoniae* isolates producing AmpC Beta-lactamase.

MATERIALS AND METHODS

The present hospital-based descriptive cross-sectional study was conducted in the Microbiology Laboratory, SMBT Hospital, Nashik, Maharashtra, India, from June 2022 to May 2023. Permission was obtained from the Institutional Ethics Committee for the study, with approval letter No: IEC/22/46. Since the study focused on bacterial isolates from pus samples received in the Microbiology Laboratory, patient demographic details were not collected. Only information regarding the name of the Clinical Department from which the pus sample was sent for culture and sensitivity testing was collected during data collection.

A total of 360 aerobic bacterial isolates from various pus samples were included in the study. All pus samples were received from the General Surgery, Orthopaedics and Otorhinolaryngology Departments in the Microbiology Laboratory for culture and sensitivity testing.

Inclusion criteria: All known pathogenic bacteria isolated from pus samples weere included in the study.

Exclusion criteria: Commensal or contaminant bacteria isolated from pus samples were excluded from the study.

Sample size calculation: The sample size (n = 360) was calculated using the formula, $N=z^2pq/e^2$, where, z=1.96, p=63% and e=5%, with 95% confidence level and 6% acceptable error [11]. After calculating the sample size was derived to be as N=360.

Study Procedure

All isolates were identified using standard conventional microbiological methods [12]. Antimicrobial susceptibility testing was conducted using the Modified Kirby-Bauer disk diffusion method in accordance with the CLSI 2023 guidelines for antimicrobial classes as depicted in [Table/Fig-1] [13,14].

Gram-negative bacteria	Gram-positive bacteria
Beta-lactum combination agents	Aminoglycosides
Cephalosporins	Quinolones
Monobactums	Folate pathway antagonist
Carbapenems	Macrolides
Aminoglycosides	Lincosamides
Quinolones	Tetracycline
Glycylcyclines	Oxazolidinones

[Table/Fig-1]: List of antimicrobial classes used for antimicrobial susceptibility testing.

Quality control in antimicrobial susceptibility testing was conducted as per CLSI guidelines using *E. coli* ATCC 25922, *Pseudomonas aeruginosa (P. aeruginosa)* ATCC 27853 and *S. aureus* ATCC 25923 [13].

Methicillin-resistant Staphylococcus aureus (MRSA) detection by cefoxitin disc diffusion test: All strains of *S. aureus* isolated from infected samples were screened for mecA-mediated methicillin resistance using a 30 µg cefoxitin disc (HiMedia) by the Modified Kirby-Bauer disc diffusion method, and the results were interpreted using CLSI 2023 guidelines [13].

Vancomycin resistance detection: Screening for vancomycin resistance in MRSA isolates was conducted using Vancomycin screen agar. Spot inoculation (10 µL) of a 0.5 McFarland suspension of the MRSA isolate was performed on Mueller Hinton Agar (MHA) plates containing 6 µg/mL of Vancomycin. The agar plates were then incubated for 24 hours at 35°C following the criteria outlined by CLSI. Resistance was indicated by the presence of >1 colony or a light film of growth [13].

All bacterial isolates were classified as MDR and XDR based on standard definitions [15].

MDR: An isolate that is not susceptible to at least one agent in three or more antimicrobial classes tested.

XDR: An MDR isolate that is not susceptible to at least one agent in all but two or fewer antimicrobial classes.

All *E. coli* and *K. pneumoniae* isolates were screened for AmpC production using the cefoxitin (30µg) disk diffusion method on Mueller-Hinton agar. All isolates that were resistant (Zone diameter ≤18mm) to cefoxitin were confirmed for AmpC production using the Disk approximation test and cefoxitin-cloxacillin double disk synergy test [16-18].

Disk approximation test: In this test, Mueller-Hinton agar was inoculated with the test isolate with 0.5 McFarland turbidity. An Imipenem disk (10 µg) was placed at the center of the plate as an inducer disk, and cefotaxime (30 µg), aztreonam (30 µg) and piperacillin-tazobactam (100/10 µg) disks were positioned around the inducer disk at a distance of 20 mm between each disk. The plate was then aerobically incubated at 35-37°C. The test was considered positive, if there was blunting of the inhibitory zone of any of the substrate disks towards the inducer disk.

Cefoxitin-coxacillin double disk synergy test: This test is based on the inhibition of AmpC beta-lactamase by cloxacillin. Initially, Mueller-Hinton agar was inoculated with the test isolate at a 0.5 McFarland turbidity. Two disks were placed on the Mueller-Hinton agar: one cefoxitin disk (30 µg) and the second cefoxitin disk impregnated with Cloxacillin (HiMedia CXX 30 µg + 200 µg). The plate was then aerobically incubated at 35-37°C. The test was considered positive if there was an increase in the zone diameter by ≥5 mm around the cefoxitin+cloxacillin disk.

STATISTICAL ANALYSIS

Microsoft Excel 2013 was used for data analysis and data was presented as descriptive statistics.

RESULTS

[Table/Fig-2] shows the distribution of bacterial isolates (N=360) from pus samples received from the General Surgery, Orthopaedics and Otorhinology Departments. The majority of them were Gram-negative bacteria, 254 (70.5%). *P. aeruginosa*, 80 (22.2%), *E. coli*, n=69 (19.1%) and *K. pneumoniae*, n=42 (11.6%) were predominant Gram-negative bacteria. Among Gram-positive bacteria *S. aureus*, 88 (24.4%) was the most common. The predominant bacteria in the general surgery, orthopaedics, and otorhinology departments were *E. coli*, 51 (33.5%), *S. aureus*, 39 (39.7%) and *P. aeruginosa,* 48 (43.6%), respectively. Out of the total Gram-negative bacteria (n=254), 175 bacteria were MDR (68.8%) and 120 bacteria were XDR (47.2%).

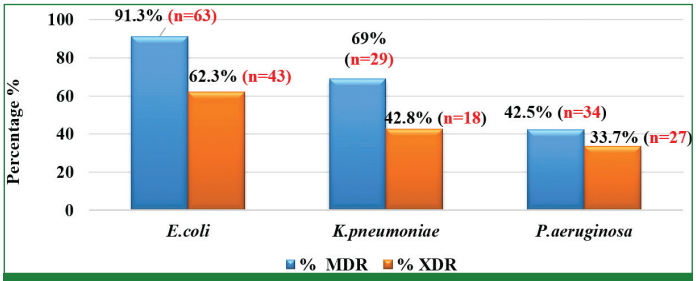
The percentage of MDR and XDR in *E. coli*, *K. pneumoniae* and *P. aeruginosa* is seen in [Table/Fig-3]. The highest percentage was seen in *E. coli* (MDR=91.3%, XDR=62.3%).

The susceptibility pattern of MDR *E. coli*, *K. pneumoniae* and *P. aeruginosa* is seen in [Table/Fig-4]. *E. coli* and *K. pneumoniae* showed the highest susceptibility to tigecycline (84.1% and

79.3%, respectively) followed by meropenem (61.9% and 58.6%, respectively). *Pseudomonas aeruginosa* had the highest susceptibility to Meropenem (58.8%).

Name of bacteria	Name of the department			Total no. of bacterial isolates, n (%)
	General surgery, n (%)	Orthopaedics, n (%)	Otorhinology, n (%)	
Gram-negative bacteria	121 (79.6)	56 (57.1)	77 (70)	254 (70.5)
Escherichia coli	51 (33.5)	15 (15.3)	3 (2.7)	69 (19.1)
Klebsiella pneumoniae	19 (12.5)	13 (13.2)	10 (9.0)	42 (11.6)
Enterobacter spp.	9 (5.9)	10 (10.2)	4 (3.6)	23 (6.3)
Citrobacter spp.	8 (5.2)	8 (8.1)	5 (4.5)	21 (5.8)
Proteus spp.	5 (3.2)	2 (2.0)	7 (6.3)	14 (3.8)
Pseudomonas aeruginosa	24 (15.7)	8 (8.1)	48 (43.6)	80 (22.2)
Acinetobacter spp.	5 (3.2)	-	-	5 (1.3)
Gram-positive bacteria	31 (20.3)	42 (42.8)	33 (30)	106 (29.4)
Staphylococcus aureus	22 (14.4)	39 (39.7)	27 (24.5)	88 (24.4)
Enterococcus spp.	9 (5.9)	3 (3.0)	4 (3.6)	16 (4.4)
Streptococcus spp.	-	-	2 (1.8)	2 (0.5)
Total no. of bacterial isolates	152 (42.2%)	98 (27.2%)	110 (30.5%)	360

[Table/Fig-2]: Distribution of bacteria isolates from pus samples received from different Clinical Departments.



[Table/Fig-3]: Percentage of MDR and XDR *E. coli*, *K. pneumoniae* and *P. aeruginosa*.

Name of antimicrobial agent	MDR *E. coli* (n=63) susceptibility, n (%)	MDR *K. pneumoniae* (n=29) susceptibility, n (%)	MDR *P. aeruginosa* (n=34) susceptibility, n (%)
Amikacin	26 (41.2)	10 (34.4)	9 (26.4)
Gentamycin	29 (46)	12 (41.3)	10 (29.4)
Ciprofloxacin	4 (6.3)	2 (6.8)	10 (29.4)
Piperacillin tazobactum	20 (31.7)	7 (24.1)	11 (32.3)
Ampicillin-sulbactum	3 (4.7)	3 (10.3)	-
Cefoxitin	17 (26.9)	6 (20.6)	-
Cefepime	17 (26.9)	14 (48.2)	7 (20.5)
Cefotaxime	5 (7.9)	4 (13.7)	-
Ceftazidime	-	-	2 (5.8)
Aztreonam	11 (17.4)	9 (31)	9 (26.4)
Imipenam	34 (53.9)	11 (37.9)	9 (26.4)
Meropenam	39 (61.9)	17 (58.6)	20 (58.8)
Tigecycline	53 (84.1)	23 (79.3)	-

[Table/Fig-4]: Susceptibility patterns of MDR Gram-negative bacteria.
MDR: Multidrug resistant

Among all *S. aureus* isolates (n=88), 40 (45.4%) isolates were MRSA and the percentage of MDR and XDR in *S.aureus* was 67% (n=59) and 11.3% (n=10), respectively.

The susceptibility pattern of MDR *S. aureus* is seen in [Table/Fig-5]. The highest susceptibility was shown to linezolid (100%) followed by tetracycline (83%) and gentamycin (50.8%). All MRSA isolates were susceptible to vancomycin by Vancomycin screen agar method.

Name of antimicrobial agent	MDR S. aureus (n=59) susceptibility, n (%)
Cefoxitin (as a surrogate marker for Methicillin)	22 (37.2)
Gentamycin	50.8% (n=30)
Erythromycin	18.6% (n=11)
Clindamycin	35.5% (n=21)
Cotrimoxazole	42.3% (n=25)
Tetracycline	83% (n=49)
Ciprofloxacin	6.7% (n=4)
Linezolid	100% (n=59)

[Table/Fig-5]: Susceptibility pattern of MDR Staphylococcus aureus.
MDR: Multidrug resistant

Among 69 isolates of *E. coli,* 48 (69.5%) were AmpC screening test positive and 4 (5.7%) isolates were confirmed AmpC producers by the double disk synergy test [Table/Fig-6]. Additionally, out of 42 isolates of *K. pneumoniae*, 30 (71.4%) isolates were AmpC screening test positive and only one isolate was confirmed as an AmpC producer by the double disk synergy test. Therefore, the overall prevalence of AmpC-producing *E. coli* and *K. pneumoniae* was 4.5%.

Micoorganism	No. of isolates positive for AmpC screening test, n (%)	No. of isolates positive for AMPc confirmatory test, n (%)	
		Disk approximation test	Double disk synergy
E. coli (n=69)	48 (69.5)	0	4 (5.7)
K. pneumoniae (n=42)	30 (71.4)	0	1 (2.3)
Total (111)	78 (70.2)	0	5 (4.5)

[Table/Fig-6]: Number (%) of *E. coli* and *K. pneumonia* producing AmpC.

DISCUSSION

In the present study's laboratory, the majority of pus samples were received from the General Surgery, Otorhinology and Orthopaedics Departments during the study period. Out of the 360 bacterial isolates from pus samples included in the study, the predominant isolates were from the General Surgery Department, 152 (42.2%). Mukherjee S et al., also reported in their study that major positive pus samples (32%) were from the General Surgery Department [19].

Out of the 360 bacterial isolates from pus samples, 254 (70.5%) were Gram-negative bacteria. Similar results were reported by Wadekar MD et al., however Rai S et al., reported Gram-Positive, 162 (61%) as the predominant isolates, followed by Gram-negative, 102 (39%) in their study [20,21].

A study by Deboral A et al., reported *P. aeruginosa* as the predominant organism causing wound infections in their study [22]. Similarly, in the present study, *P. aeruginosa* {n=80 (22.2%)} was the major Gram-negative bacteria isolated from different pus samples.

The predominant bacteria isolated in pus samples received from the General Surgery Department were *E. coli*, while in the Orthopaedic and Otorhinology Departments it was *S. aureus* and *Pseudomonas aeruginosa*. This information will help the clinician in predicting the possible causative organism and choosing appropriate antimicrobial agents as empirical therapy for wound infections in the respective Departments.

The prevalence of MDR and XDR in Gram-negative bacteria was 68.8% and 47.2%, respectively in the present study. A study from Nepal and Bangladesh by Paudel P et al., and Alam MM et al., reported 62.96% and 67.1% MDR bacteria in pus samples [11,23]. However, a study by Lal A et al., reported the prevalence of MDR and XDR as 37% and 25%, respectively, in Enterobacterales isolated from various clinical samples [24]. In a four-year study by Odsbu I et al., on MDR in *Acinetobacter* spp. isolated from various clinical

samples, they reported the proportion of MDR isolates ranging from 89.4% to 95.9% in Western India [25].

The high prevalence in the present study may be due to the fact that, being a tertiary referral centre, the majority of patients with wound infections visiting the present study hospital have already been started on antimicrobial agents.

The highest prevalence of MDR in the present study was observed in *E. coli* (91.3%), followed by K. pneumoniae (69%). A study by Kalita JM et al., reported *Klebsiella* spp. (74.79%) and *Acinetobacter* spp. (74.32%) as the most common MDR isolates in pus samples [26]; however, a study by Alam MM et al., reported the highest percentage of MDR in *Proteus spp.* (75.9%) and *P. aeruginosa* (72.5%) [23]. These variations in resistance patterns could be due to differences in the type of pyogenic infections, previous antibiotic exposures, and hospital infection control practices. Therefore, knowledge of the resistance pattern at a particular healthcare setting is extremely important for the formulation of an effective antibiotic policy.

Among MDR *E. coli* and *K.pneumoniae* isolates, the highest percentage of susceptibility was observed for Tigecycline (84.1% and 79.3%, respectively), followed by Meropenem (61.9% and 58.6%, respectively). Meropenem also showed the highest susceptibility (58.8%) in MDR *Pseudomonas aeruginosa* isolates. This suggests that these antimicrobial agents must be reserved for MDR Gram-negative bacterial infections.

In the present study, 45.4% of *S.aureus* isolates were Methicillin-resistant. A study by Mukherjee S et al., and Wadekar MD et al., reported the prevalence of MRSA as 11% and 48.1%, respectively [19,20]. The prevalence of MDR and XDR *S.aureus* in the current study was 67% (n=59) and 11.3% (n=10) respectively. Gurung RR et al., reported 71.8% of MRSA as MDR in their study on different clinical samples in pediatric patients [27]. All MDR *S.aureus* isolates in the present study were predominantly sensitive to Linezolid (100%) and Tetracycline (83%). All MRSA isolates were also susceptible to Vancomycin by Vancomycin screen agar method.

Among the Gram-negative bacterial isolates, *E. coli* and K. pneumoniae were predominantly MDR in the present study, so the authors screened and then confirmed AmpC production in both of these isolates. Although standard methods for phenotypic detection of AmpC beta-lactamase in bacteria do not exist in the CLSI guidelines, some studies have evaluated various methods like the double-disk synergy test using inhibitors like boronic acid and Cloxacillin, disk approximation test, Modified three-dimensional test and AmpC disk test [17,18,28].

The prevalence of AmpC production in *E. coli* and K. pneumoniae isolates in the current study was 5.7% and 2.3%, respectively. A study by Maraskolhe DL et al., reported AmpC-producing *E. coli* at 4.44% and *Klebsiella* spp. at 5.19% [18]. Another study by Grover N et al., reported an overall rate of 14.8%. All AmpC-producing isolates in the present study were confirmed by the double-disk synergy test [29]. AmpC beta-lactamase production may be constitutive or inducible. The disk approximation test is used for the detection of inducible AmpC beta-lactamase. In the present study, all *E. coli* and *K. pneumoniae* isolates showed negative results with the Disk approximation test, suggesting that they must have genes coding for constitutively expressed AmpC Beta-lactamases.

Limitation(s)

Analysis of MDR bacteria as per community acquired and hospital acquired infections and patient locations like Ward, Intensive care unit and Outpatient Department was not done in our study due to lack of sufficient information in test requisition forms. Also, as antimicrobial susceptibility was performed by Modified Kirby –Bauer disk diffusion method, some of the antimicrobials for which breakpoints for disk diffusion testing not available in standard guidelines are not tested in our study. Another limitation of our study is that we have not studied for anaerobic bacterial infections due to lack of necessary set up for testing in our laboratory.

CONCLUSION(S)

The current study highlighted a high prevalence of MDR bacteria isolated from various pus samples received in the Microbiology Laboratory. Since this type of study has not been conducted previously at the author's healthcare setup, the present study will aid in formulating policies regarding empirical antimicrobial treatment for patients visiting the hospital with wound infections. Although the prevalence of AmpC-producing *E. coli* and *K. pneumoniae* was low in the present study, further investigation is needed with more bacterial isolates. Additionally, other mechanisms of AMR need to be studied.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jan 13, 2024
- Manual Googling: May 03, 2024
- iThenticate Software: Jun 17, 2024 (13%)

ETYMOLOGY: Author Origin

EMENDATIONS: 7

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? No
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jan 12, 2024**
Date of Peer Review: **Mar 14, 2024**
Date of Acceptance: **Jun 18, 2024**
Date of Publishing: **Jul 01, 2024**