Bacteriological Profile and Antibiotic Resistance Pattern of Pathogens Causing Pyogenic Infections At A Tertiary Care Hospital in Central India

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Abstract

Background: Pyogenic infection is a bacterial infection that leads to the formation of pus. Antibiotics are routinely prescribed to treat these pyogenic bacterial infections, but their toxicity poses a severe threat. It is important to know the antimicrobial resistance profile of such pathogens for proper management of the patients.

Objectives: To determine the bacteriological profile and antibiotic resistance pattern of pyogens.

Patients and Methods: In this cross sectional study, a total of 678 pus samples were received and processed for aerobic culture from various departments. Standard techniques were used to identify isolates from positive pus cultures, and CLSI standards were used to identify antimicrobial susceptibility patterns.

Results: Out of the 678 samples, 347 (51.18%) showed growth of pathogenic bacteria. Two hundred seventeen two (40.11%) Gram positive cocci and 75 (11.06%) Gram negative bacilli isolates were identified. *Staphylococcus aureus* was the most common isolate (57.5%). Most of the isolates were highly resistant to commonly prescribed antimicrobial drugs like Amoxycillin clavulanate. Most of the gram positive isolates were susceptible to vancomycin, linezolid, and teicoplanin. Most of the Gram-negative isolates were sensitive to imipenem.

Conclusion: Microbiological profile findings of pus culture isolates as well as their pattern of antimicrobial resistant may aid in the formulation of antibiotic policies for pyogenic infections.

Keywords: Wound; Pyogenes; Antibiogram; Multidrug Resistance; MRSA.

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Introduction

Simply, pyogenic infection refers to bacterial infection that leads to the production of pus. It's an invasion by and multiplication of pathogens in a tissue, which may produce subsequent injury and progress to overt disease through a variety of cellular or toxic mechanisms, generally caused by one of the pyogenic bacteria. Pus is yellow, white-yellow, or yellowbrown exudates made up of dead leucocytes, cellular debris, and necrotic tissues can form after infections of the skin and soft tissue caused by trauma, surgery, or burns (Dryden, 2010).

There is a long list of bacterial species known to be responsible for causing infections in humans. It most commonly include Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Escherichia coli, Streptococcus pneumoniae, Klebsiella pneumoniae, Salmonella Pseudomonas typhi, aeruginosa, Acinetobacter baumanii. Candida spp, Aspergillus spp, Neisseria *Mycobacterium* gonorrhoeae, tuberculosis, etc (Efstrtiou, 1989).

Antibiotics that treat these pyogenic bacterial infections are routinely prescribed, the toxicity of which is a serious threat and makes chemotherapy more difficult. Management of pyogenic infections consist of surgical drainage or fluid aspiration followed by appropriate antibiotics. Impetigo, osteomyelitis, sepsis, septic arthritis, spondylodiscitis, otitis media, spondylitis, cystitis, and meningitis are some common disease process caused by pyogenic infections. Pyogenic bacteria are known to cause inflammation and suppuration (Chong, 2009).

Pyogenic infections are still abundantly seen in developing countries and are a therapeutic challenge despite advances in microbiological techniques & therapeutic (**Singh et al., 2013**). To ensure an adequate and efficient therapy, it is necessary to identify and treat the focus of inflammation. Advances in a treatment facility are not enough to completely wipe out this problem because of the emergence of drug resistance (**Diwakar et al., 2019**).

Antibiotic resistance is seen as a potential danger around the world, and infection caused by such bacteria increases morbidity and mortality, resulting in significant economic loss due to the usage of more expensive antibiotics to treat infection (**Subedi et al., 2016**).

Different studies are being conducted across the globe to access the bacterial profile in pyogenic wound infection. The appropriate knowledge of the pathogens, their resistance, and their updated antimicrobial therapy plays a crucial role in therapeutic management as well as in infection control practices (Mohammed et al., 2017; Mehta and Diwakar, 2021)

Patients and methods

Design Overview: In this cross sectional study, a total of 678 pus samples were collected as per inclusion and exclusion criteria from various departments of Government Medical College Datia (MP) from April 2019 to September 2020 and processed for aerobic culture & antibiotic sensitivity, and were included in the study. All disinterested patients, those currently on antibiotics and patient whose duplicate sample showed mismatched isolate were excluded from the study.

Ethics: Ethical approval was obtained from the Institutional ethics committee before conducting the study (Approval Letter No-005/MIC/IECHP/DMC). Prior consent was obtained from all participants.

Sample collection: All specimens like wound exudates, abscess drainage, ear swab, wound aspirates were collected in duplicate from each wound at a point in time following standard microbiological techniques with aseptic precautions. Difference in bacterial isolates among duplicate swab samples is indicative of contamination. To reduce the chances of contamination by commensal flora, swabs were taken from wound after cleansing and debridement with sterile gauze & sterile normal saline. This not only eliminates the superficial normal resident flora but also increase the bacterial pathogens yield (Godebo et al., 2013; UK SMI, 2018).

Isolation, identification, and characterization of bacterial isolates: As per the standard microbiological protocol, all samples were processed for Gram staining and aerobic culture. They were inoculated on blood agar and Mac Conkey agar followed by overnight aerobic incubation at 37°C. Identification of the organism was done by standard microbiological techniques (Collee et al., 1996).

Antibiotics susceptibility testing: Depending on the isolates, a set of antibiotic discs was applied on a preseeded Muller-Hinton agar plate with 0.5 McFarland standard inoculums by modified Kirby Bauer's disc diffusion method and was interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines 2019 and 2020 (CLSI, 2019; CLSI, 2020). Antibiotics tested in the study were Amoxicillin/clavulanate (20/10mcg), Amikacin (30mcg),), Aztreonam (30mcg), Azithromycin (15mcg), Cefoxitin(30mcg), Clindamycin (2mcg), Cotrimoxazole (1.25/23.75mcg),

Ciprofloxacin (5mcg), Ceftriaxone (30mcg)., Cefotaxime (30mcg), Cefotaxime/clavulanic acid (30/10mcg),Ceftazidime (30mcg), Ceftazidime/clavulanic acid (30/10mcg), Cefepime (30mcg), Doxycycline(30mcg), Erythromycin (15mcg), Gentamycin (10mcg), Gentamicin High-Level (10mcg), (120mcg), Imipenem Levofloxacin (5mcg), Linezolid (30mcg), (10mcg), Piperacillin/ Meropenem tazobactam (100/10mcg),Tetracycline (30mcg), Tobramycin (10mcg), and Vancomycin (30mcg).

Staphylococcus aureus ATCC 43300, 25923 & 29213, Pseudomonas aeruginosa ATCC 27853, Klebsiella pneumoniae ATCC 700603, Escherichia coli ATCC 25922 and Enterococcus faecalis ATCC 29212 were used as quality control strains for antimicrobial susceptibility.

Methicillin Resistant (MRSA) was detected by taking cefoxitin as a surrogate marker. MRSA was detected by the cefoxitin disc diffusion test, using a 30 μ g disc (an inhibition zone diameter of \leq 21 mm was reported as methicillin resistant and a zone diameter of \geq 22 mm was considered as methicillin sensitive) and by growth on oxacillin screen agar, incorporating 4% NaCl and 6 μ g/ml of oxacillin (HiMedia, Mumbai), as per CLSI guidelines.

ATCC 43300 and ATCC 25923/ATCC 29213 were used as positive and negative controls respectively for methicillin resistance (**Diwakar et al., 2018; CLSI, 2019**)

High Level Aminoglycoside resistance (HLAR) was reported in *Enterococcus spp.* isolates using High content Gentamycin disk (120µgm) diffusion method and interpreted as per CLSI guideline as zone of inhibition <6mm suggestive of High level resistance; >10mm indicated absence of HLAR and zone size of 7-9 mm being inconclusive (CLSI, 2019; CLSI, 2020).

Extended spectrum **B** lactamase (ESBL) producers were identified by using Cefotaxime & Ceftazidime disc, alone and in combination with clavulanic acid by using disc diffusion method and interpreted as per CLSI guideline as increased difference in zone of inhibition > 5mm between Cefotaxime/Ceftazidime and clavulanic acid combination suggestive of ESBL producers. Klebsiella pneumoniae ATCC 700603 and Escherichia coli ATCC 25922 were used as control for ESBL production. **Statistical Analysis**

All data maintained in Microsoft Office Excel and analysis done using Microsoft Excel with appropriate statistical tools applied wherever required. The Chi-square test was applied to determine whether the resistance pattern of different organisms is statistically significant or not. A P-value of less than 0.05 was considered statistically significant.

Results

A total 678 specimens were processed at the clinical microbiology laboratory; 347 (51.18%) of them showed significant bacterial growth confirming the pyogenic wound. Demographic profiles of pyogenic infections are shown in (**Table. 1**). The age group 21-30 years was prevalent for pyogenic infections and prone to Gram negative bacilli (P = 0.0001).

	Cases $(n = 347)$				
Age (years)	Gram positive cocci GPC		Gram negative bacilli GNB		
	Ν	%	Ν	%	
• 0-10	35	12.87	5	6.67	
• 11-20	34	12.50	8	10.67	
• 21-30	79	29.04	31	41.33	
• 31-40	43	15.81	9	12.00	
• 41-50	23	8.46	11	14.67	
• 51-60	29	10.66	7	9.33	
• 61-70	23	8.46	3	4.00	
• 71-80	6	2.21	1	1.33	
Gender					
• Male	145	53.3	41	54.67	
Female	127	46.7	34	45.33	
Total	272		75		

 Table 1. Demographic profile of pyogenic infections

The organism-wise percentage distribution and isolation rates have been depicted in (**Table 2**). *Staphylococcus aureus* (214, 61.67%) was the leading

bacterial pathogen followed by enteric coliforms (42, 12.1%), Non-fermenters (33, 9.49%) and *CoNS* (31, 9%). None of the cultures yielded polymicrobial growth.

Table 2. Bacterial isolates associated with pyogenic wound infections					
Bacterial isolates Frequency n=347 % of total isolates Culture positivity (%					
Gram-positive isolates	272	78.39	40.11		
CoNS	31	8.93	4.57		
MRCoNS	10	2.88	1.47		

Staphylococcus aureus	214	61.67	31.56
MRSA	112	32.28	16.52
Enterococcus spp.	25	7.2	3.69
Streptococcus spp.	2	0.58	0.29
Gram-negative isolates	75	21.61	11.06
P.aeruginosa	23	6.63	3.39
Citrobacter spp.	17	4.9	2.50
E.coli	15	4.3	2.21
Klebsiella pneumonia	7	2	1.03
Acinetobacter spp.	6	1.7	0.88
Enterobacter spp.	2	0.6	0.29
Morganella morganii	1	0.3	0.15
Serratia spp.	1	0.3	0.15
Proteus spp.	3	0.86	0.44
Total	347	100	51.18

CONS- Coagulase Negative Staphylococcus species, MRCONS- Methicillin Resistant Coagulase Negative Staphylococcus species, MRSA- Methicillin Resistant Staphylococcus aureus

The antibiotic resistance pattern of test isolates is depicted in (**Tables 3, 4, 5 & 6**). When compared to CONS, we found that MRCoNS showed statistically significant resistance to the following drugs: Ciprofloxacin (P <0.001), Levofloxacin (P <0.001), Gentamycin (P

<0.001), Linezolid (P 0.045), Teicoplanin (P 0.045), Vancomycin (P 0.045), Azithromycin (P 0.003), and Erythromycin (P 0.013), but not to Clindamycin (p=0.45), Co-trimoxazole (P=0.47), Doxycyclin (p= 0.29) & Tetracycline (p=0.182).

Antibiotic Resistance pattern					
CoNS (31) S.aureus (214) Enterococcus spp. (25					
Antibiotics	(N, %)	(N, %)	(N , %)		
Amoxycillin/clavulanate	18 (58.06)	156 (72.90)	20 (80.00)		
Azithromycin	19 (61.29)	142 (66.36)	25 (100)		
Erythromycin	21 (67.74)	148 (69.16)	16 (64.00)		
Clindamycin	11 (35.48)	128 (59.8)	-		
Cotrimoxazole	14 (45.16)	112 (52.34)	-		
Ciprofloxacin	14 (45.16)	147 (68.69)	-		
Levofloxacin	11 (35.48)	141 (65.89)	15 (60.00)		
Cefoxitin	10 (32.26)	112 (52.34)	-		
Doxycycline	2 (6.45)	15 (7.01)	2 (8.00)		
Tetracycline	4 (12.90)	40 (18.69)	3 (12.00)		
Linezolid	1 (3.23)	8 (3.74)	1 (4.00)		
Vancomycin	0 (0.00)	3 (1.40)	0 (0.00)		
Gentamicin (10)	5 (16.13)	68 (31.78)	-		
Gentamicin (High level)			9 (36)		

CONS- Coagulase Negative Staphylococcus species

Antibiotic Resistance pattern						
AntibioticsMRCoNS (N, %)MRSA (N, %)P value						
Amoxycillin/clavulanate	10 (100.00)	112 (100.00)	-			
Azithromycin	8 (80.00)	84 (75.00)	0.39			
Erythromycin	9 (90.00)	85 (75.89)	0.008*			
Clindamycin	3 (30.00)	13 (11.61)	0.002*			
Cotrimoxazole	4 (40.00)	76 (67.86)	<0.001*			
Ciprofloxacin	7 (70.00)	102 (91.07)	<0.001*			
Levofloxacin	6 (60.00)	11 (9.46)	<0.001*			
Cefoxitin	10 (100.00)	112 (100.00)	-			
Doxycycline	1 (10.00)	10 (8.93)	0.81			
Tetracycline	2 (20.00)	25 (22.32)	0.73			
Linezolid	1 (10.00)	5 (4.46)	0.096			
Vancomycin	1 (0.00)	2 (1.79)	0.155			
Gentamycin	4 (40.00)	42 (37.50)	0.77			

Table 4. Antibiotic resistance pattern of Methicillin-Resistant Gram Positive isolates

*P-value of less than 0.05 was considered statistically significant; MRCONS- Methicillin Resistant Coagulase Negative Staphylococcus species, MRSA- Methicillin Resistant Staphylococcus aureus

Table 5. Antibiotic resistance	e pattern of gram-r	negative isolates	(Enterobacteriacae)
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Antibiotic Resistance pattern (N, %)					
K. pneumoniae E. coli Citrobacter spp.					
Antibiotics	(N , %)	(N, %)	(N , %)		
Amoxicillin/clavulanate	5 (71.43)	9 (60.00)	-		
Amikacin	3 (42.86)	5 (33.33)	8 (47.06		
Gentamycin	3 (42.86)	4 (26.67)	5 (29.41		
Tobramycin	2 (28.57)	5 (33.33)	-		
Ciprofloxacin	4 (57.14)	7 (46.67)	8 (47.06		
Levofloxacin	2 (28.57)	5 (33.33)	5 (29.41		
Cotrimoxazole	5 (71.43)	11 (73.33)	-		
Ceftriaxone	4 (57.14)	9 (60.00)	13 (76.47		
Cefotaxime	5 (71.43)	11 (73.33)	12 (70.6		
Cefotaxime/clavulanic acid	3 (42.86)	8 (53.33)	10 (58.82		
Ceftazidime	5 (71.43)	10 (66.67)	11 (64.7		
Ceftazidime/ clavulanic acid	3 (42.86)	5 (33.33)	9 (53.0		
Piperacillin/tazobactum	5 (71.43)	7 (46.67)	11 (64.71		
Doxycycline	4 (57.14)	6 (40.00)	5 (29.41		
Tetracycline	3 (42.86)	7 (46.67)	6 (35.29)		
Imipenem	1 (14.29)	1 (6.66)	0 (0.00)		

 Table 6. Antibiotic resistance pattern of gram-negative isolates (Non-fermenters)

Antibiotic Resistance pattern					
Antibiotics	P.aeruginosa (N, %)	Acinetobacter spp. (N, %)	P value		
Amoxicillin/clavulanate	12 (52.17)	4 (66.67)	0.031*		
Amikacin	5 (21.74)	2 (33.33)	0.081		
Gentamycin	5 (21.74)	2 (33.33)	0.081		
Tobramycin	4 (17.39)	3 (50.00)	P<0.001*		



C iprofloxacin	8 (34.78)	4 (66.67)	P<0.001*
Levofloxacin	3 (13.04)	3 (50.00)	P<0.001*
Ceftriaxone	18 (78.26)	5 (83.33)	0.37
Ceftazidime	14 (60.87)	4 (66.67)	0.38
Piperacillin/tazobactum	11 (47.83)	4 (66.67)	0.007*
Cefepime	9 (39.13)	1 (16.67)	P<0.001*
Aztreonam	19 (82.61)	-	1.00
Tetracycline	19 (82.61)	2 (33.33)	P<0.001*
Imipenem	7 (30.43)	2 (33.33)	0.65
Meropenem	6 (26.08)	2 (33.33)	0.28

*P-value of less than 0.05 was considered statistically significant

Antimicrobial resistance in MRSA was found to be statistically significant for Co-trimoxazole (P = 0.021), Ciprofloxacin (P = 0.001), and Levofloxacin (P = 0.039), but non-significant for Azithromycin (p = 0.163), Erythromycin (p = 0.27), Clindamycin (p = 0.67), Doxycyclin (p = 0.60), Tetracycline (p = 0.60), Gentamycin (P=0.37), Teicoplanin (p= 0.41) and Vancomycin (p= 0.52) when compared with S. aureus.

In this study, 52.34% (112) of *S.aureus* isolates (214) and 32.26% (10) of *CoNS* isolates (31) were found to be Methicillin-Resistant.

Among pyogenic isolates, 14.3% of *K.pneumoniae* and 13.3% of *E.coli* isolates were found to be extended spectrum β lactamase producers (ESBL).

Discussion

Every year, millions of individuals in developing countries such as India suffer from pyogenic wound infections as a result of trauma, accidents, or burns, as well as the consequences that come with pathogenic microorganisms (Acharya et al., 2008; Muluye et al., 2014; Rai et al., 2017).

The ongoing rise in antibiotic resistance among pathogenic organisms has posed a therapeutic challenge in the treatment of pyogenic wound infections (**Belbase et al., 2017**). As a result, current knowledge of the etiology and antibiogram is particularly beneficial in reducing morbidity and consequences.

Overall, 347 (51.18%) of research participants had pyogenic wound infections based on substantial bacterial growth in clinical specimens. Authors from Nepal reported similar growth rates of 50.7% by Acharya et al., (2008) and 50.0% by Shrestha & Basnet (2009) in pyogenic clinical specimens.

Rai et al., (2017) (59%) and Trojan et al., (2016) (60.1%) from India and Bessa et al., (2015) (69.5%) from Italy reported much higher rates of growth. In addition, Mohammed et al., (2017) (83.9%) and Mama et al., (2014) (87.4%) from Ethiopia reported extraordinarily high rates of growth among pyogenic clinical specimens. These differences in pyogenic wound specimen growth rates could be due to the quality of the specimens treated, contamination with external microbiota, and routine wound care methods in the healthcare and bacterial cultivation facilities in the area (Bessa et al., 2015).

Poor wound care, increased microbial survival and inadequate antimicrobial treatment has been related to polymicrobial pyogenic wound infections (Mama et al., 2140). In this study Gram-positive cocci revealed as the primary source of pyogenic wound infections (272; 40.11%). This finding is in concordance with several previous studies (Acharya et al., 2008; Rai et al., 2017; Yakha et al., 2015).

The Gram-negative bacilli dominance in pyogenic wound infections has been reported by Trojan et al., (2016) from India, Bessa et al., (2015) from Italy, and Mama et al., (2014) from Ethiopia. In our investigation, however, Staphylococcus aureus (214; 31.56%) was the most common isolate responsible for pyogenic wound infections, which is closely similar to prior research (Acharya et al., 2008; Muluve et al., 2014; Rai et al., 2017).

In this study 186 males (53.6%) and 161 females (46.4%) had pyogenic infections. The infectivity among both sex did not showed any significant difference. The most prevalent age group for pyogenic infection was 21-30 years but, statistically non significant (P=0.103). This finding is in concordance with previous studies (Chakraborty et. al., 2021; Kalita et. al., 2021; Sujatha et. al., 2016). We found that 21-30 years age group was statistically prone (P = 0.0001) for Gram negative pyogenic infections in contrast to gram positive pyogens. While, many other researcher found other age group as prevalent for progenic infection (Kumari Pilli et. al., 2018; Rijal et. al., 2017; Sudhaharan et. al., 2018).

S.aureus and Gram-negative bacterial pathogens are well known for producing highly powerful virulence factors, which are responsible for maintaining the infection and delaying wound healing (**Bessa et al., 2015**).

The main concern of this study includes high rates of antibiotic resistance

pathogenic microorganisms among with pyogenic infections. associated Antimicrobial resistance incidence and pattern among pyogenic bacterial isolates vary widely depending on geographic areas, climatic circumstances, and the endemicity of resistant pathogens in the area. (Rijal et.al., 2017; Sudhaharan et. al., 2018). Among Gram-positive bacteria, Staphylococcus aureus was found to be the resilient organism to most develop resistance in this investigation.

Tested Gram-positive cocci were found to have extensive resistance against Amoxicillin/clavulanic acid, erythromycin, ciprofloxacin, and azithromycin. This finding is in support of prior similar studies (Acharya et al., 2008; Rai et al., 2017; Yakha et al., 2015). Streptococcus pyogenes isolates, like those in earlier research, were promisingly sensitive to Amoxicillin/clavulanic acid. cotrimoxazole. erythromycin and (Acharya et al., 2008; Shrestha and Basnet, 2009). In this study, Grampositive bacteria showed remarkable susceptibility to Vancomycin, Teicoplanin, and Linezolid which may be a promising choice for pyogenic wound infections.

Furthermore, nearly half of the Gram-positive bacteria in our investigation were MDR, which is significantly higher than in earlier studies from Nepal (Acharya et al., 2008; Yakha et al., 2015). Several other investigations also showed a higher prevalence of MDR strains (Godebo et al., 2013; Mama et al., 2014; Bhattacharya et al., 2016; Dessie et al., 2016).

About 52.3 % of the Staphylococcus aureus isolates were methicillin-resistant and also resistant to other antimicrobial treatments. When compared to prior reports by **Acharya et**

al., (2008) (22.5%) and Rai et al., (2017) (19%), the MRSA rate is higher, but it is lower when compared to those by Belbase et al., (2017) (47.4%) and Khanal et al., (2010) (68%).

The discrepancy in medication susceptibilities could be due to differences in the study group, which includes hospitalized inpatients, who are more likely to have MDR strains. In addition, our findings show that E. coli, Klebsiella spp., and Citrobacter spp. are all extremely resistant to 3GC and β lactam- β lactamase inhibitors (BLBLI). Our Gram-negative isolates' susceptibility pattern matches with other prior reports from this location (Acharva et al., 2008; Rai et al., 2017; Shrestha and Basnet, 2009). Gramnegative resistance to routinely used antimicrobials in wound infections has become a growing problem in recent years (Simonsen et al., 2013; Trojan et al., 2016).

Gram-negative bacteria with significant resistance rates have previously been identified as lactamase makers (**Parajuli et al., 2016**). Non-lactam antibiotics, such as fluoroquinolones and aminoglycosides, might be better treatment regimens in our settings for pyogenic wound infections in this scenario. Our data suggest that large levels of medicationresistant bacteria are present in pyogenic wound infections.

The widespread use of β -lactam antibiotics in hospitals, as well as ineffective infection control practices, may cause increased rates of resistance among these bacteria. Furthermore, longer preventive antimicrobial exposure during surgical operations may lead to the development of resistance in organisms.

Medical practitioners in developing countries prescribe antibiotics most

frequently, inappropriately, and inadequately and thus became one of the highly abused agents. Moreover, the emergence of multidrug-resistant organisms limits the choice of appropriate therapy. The present situation needs an active interaction between a clinician and microbiologist to minimize the spread of multidrug-resistant strains in the hospitals as well as in the community and to ensure authentic treatment to the patients.

Conclusion

Pyogenic infections have been a major problem in the field of surgery for a long time. Advances in the control of infection are not enough to resolve this problem completely because of the emergence of multidrug resistance that increases complications and costs associated with treatment. Knowing the causative agent and their characterization among patients with pyogenic infections will be beneficial for the effective management of the antibiogram-oriented disease. So. an institutional antibiotic policy should be implemented in each institution for empirical therapy. The clinician should appropriate samples send for microbiological culture & sensitivity before starting antibiotic therapy and encourage the patient to take a complete regimen and follow up. People should also avoid self-medication. This will definitely control & decrease the emergence of resistance among pathogens.

Limitation of the study

The associated risk factors with the patient's antibiogram, duration, and outcome of the antimicrobial therapy were not analyzed. This was a cross-sectional study and the patients were not followed up.

Conflict of Interest: there is no conflict of interest.

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