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Original Research Article

To study drug resistance & biofilm production in gram negative isolates from clinical samples

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ABSTRACT

Background: Biofilms are groups of microorganism encased in a matrix of extracellular polysaccharide (slime), called polysaccharide intercellular adhesion (PIA). Bacteria commonly involved include *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*. The present study was performed to identify antibiotic resistance pattern & their ability to form biofilm in gram negative clinical isolates.

Material and Methods: All clinical samples received in laboratory for microbial culture during study period of 12 months (2017 to 2018) were included in this study Antibiotic susceptibility testing, ESBL & MBL detection was done for clinical isolates. Biofilm productions were determined by Congo red agar method, Christenson's Test Tube method and Tissue culture plate method.

Result: 327 gram negative isolates were detected. Maximum were *Klebsiella pneumoniae* (32.72%) followed by *Escherichia coli* (28.44%), *Acinetobacter baumanii* (16.51%), *Pseudomonas aeruginosa* (16.51%), Citrobacter species (3.97%). Maximum isolates showed resistance to ampicillin (93.27%) followed by amoxiclave (87.46%), ceftazidime (74%). Out of 327 GNB isolates, biofilm produced by 64 (19.57%) isolates by Tissue culture plate (TCP) method, 38(11.62%) by Congo red agar (CRA) method and 23 (7.03%) by Tube method. Maximum biofilm were detected in *Klebsiella pneumonaie* (24.29%).

Conclusion: There is increase prevalence of multidrug resistant& biofilm forming bacteria. The routine monitoring of multidrug resistance pattern & biofilm detection can be recommended in clinical laboratories to guide proper antibiotic treatment.

1. Introduction

Biofilm are groups of microorganism encased in a matrix of extracellular polysaccharide (slime), called polysaccharide intercellular adhesion (PIA).[1] They have been associated with a variety of chronic persistent infections. Biofilm formation is an adaptive protected mode of growth enabling bacteria to survive in hostile environments as in the human host. This mode also enables them to disperses and colonize new niches as per their need which is mediated by their chemical cross-talk called quorum sensing. In most cases, chronic infections are accompanied by the formation of bio films.

Over the past decade it was observed that bacteria has acquired biofilm as to survive under stressful environment where mechanical stress, desiccation and biocides are common threats. Biofilm forming bacteria are the cause of many nosocomial infections.[2] According to some reports, over 65% of hospital-acquired infections occur by the infecting organisms that have the ability of producing

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biofilms.[3] Biofilms are associated with many medical conditions including indwelling medical devices, catheters, urinary tract infections, dental plaque, upper respiratory tract infections, peritonitis, and urogenital infections and may establish chronic and recalcitrant infections.[4–7] Both Gram's positive and Gram's negative bacteria have the capability to form biofilms. Bacteria commonly involved include *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa*.[4,8] Two important bacterial pathogens that have developed a complex network of evasion, counter-inhibition, and subjugation in their battle for space and nutrients are Pseudomonas aeruginosa and Staphylococcus aureus.[4,6,7]

Biofilm producing organisms have more antibiotic resistance compared to planktonic cells. It can increase upto 1000 folds. This is because of the failure of the antibiotic to penetrate the polysaccharide matrix.[9] Inside the host, the matrix protects biofilm bacteria from exposure to innate immune defenses (such as opsonization and phagocytosis) and antibiotic treatments. It is now well documented that biofilms are notoriously difficult to eradicate and are often resistant to systemic antibiotic therapy and removal of infected device becomes necessary.[3,9] The present study was performed to identify antibiotic resistance pattern & their ability to form biofilm in gram negative clinical isolates.

2. Materials and Methods

This is a hospital based prospective study which was approved by Institutional Ethical Committee & was conducted in Child and Maternity tertiary care hospital in Western Rajasthan. All clinical samples (Urine, pus, blood, sputum and swabs (wound, throat, vaginal, tracheal, endotracheal or any device) received in laboratory for microbial culture during study period of 12 month (2017 to 2018) were included in the study.

All samples were collected as per standard protocol with proper aseptic precaution and were processed using standard procedure for isolation & identification of pathogens.[10] Antibiotic susceptibility testing were done by Kirby bauer disc diffusion method on Muller Hilton Agar as per CLSI guideline 2017. The isolation were categorized into MDR organisms based on resistance to various antibiotics & ESBL, MBL detection were done in clinical isolates.[10] Phenotypic ESBL detection by CLSI confirmatory method: ESBL production was initially tested with the CLSI confirmatory test (Clinical Standard Laboratory Institute, 2017) using Ceftazidime (30 ug) and Cefotaxime (30 μg) disks alone and in combination with Clavulanic acid (10 μg).[10] Interpretation: ≥5 mm increase in zone diameter for ceftazidime or cefotaxime when tested in combination with clavulanic acid than its zone when tested alone.[10]

MBL were detected by: A. Imipenem-EDTA combined disc B. Double disc synergy test using Imipenem and EDTA and others. These methods depend on the ability of metal chelators like EDTA and thiol-based mpounds to inhibit activity of MBL. C. EDTA disc potentiation using ceftazidime, cefipime. Phenotypic methods :- A. Imipenem-EDTA combined disc test (IMP EDTA CDT): In this method, the test organism is inoculated on MHA. Two disks are placed on MHA plate. One is 10 ug imipenem, another is imipenem-EDTA (10/750 ug) combined disk. The increase in more than 7mm in the inhibition zone of the imipenem-EDTA disk over than imipenem disk alone, is considered as MBL positive.[11]

Biofilm production were determined by using three methods Congo red agar method, Christenson's Test Tube method and Tissue culture plate method.

2.1. Congo red agar method

Colonies were inoculated on agar plate and incubated at 37 degree centigrade for 24 hours. Blackcolonies with dry metallic consistency were considered positive for slime production.[9]

2.2. Test tube method

Colonies were inoculated in Brain heart Infusion broth supplemented with 1% sucrose. After overnight incubation at 37 degree centigrade, tubes decanted and washed three times with phosphate buffered saline & stained with 0.1% crystal violet. A visible violet film on wall & bottom of the tube were considered positive test.[9]

2.3. Tissue culture plate method[12]

Colonies from fresh agar plates were inoculated in media(BHI broth with 2% sucrose) and incubated for 18 hours at 37°C and diluted 1in100 with fresh medium. 200 μl of diluted broth were added in to 96 well microtiter plate. The tissue culture plates will be incubated for 18 hours and 24 hours at 37°C. After incubation content of each well gently removed by tapping the plates and the wells will be washed four times with 0.2 mL of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate will be fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v) and plates were kept for drying. Optical density(OD) of stained adherent bacteria were determined with a micro ELISA auto reader (Thermo LabSystems) at wavelength of 450 nm (OD 450 nm). These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

Table 1: Showing demographic profile of samples

Clinical Specimen	Total samples		IPD/OPD		Locality		Gender	
	N	%	IPD	OPD	Urban	Rural	Male	Female
Blood	96	29.35	96	0	18	78	50	46
Pus	38	11.62	32	6	11	27	06	32
Urine	78	23.85	62	16	22	56	11	67
Devices	86	26.29	86	0	19	67	44	42
Others	29	8.86	21	8	11	18	9	20
Total	327	100	297	30	81	246	120	207

Table 2: Distribution of Gram negative bacterial isolates

Isolates	No. of isolates	Percentage (%)
Lactose Fermenter(LF)		
Escherichia coli	93	28.44
Klebsiella pneumoniae	107	32.72
Citrobacter species	13	3.97
Enterobacter species	4	1.22
Non Lactose Fermenter		
Acinetobacter baumanii	54	16.51
Pseudomonas aeruginosa	54	16.51
Salmonella species	01	0.30
Edwardsiella species	01	0.30
Total sample	327	100

Table 3: Distribution of various GNB isolates in clinical samples

Organism isolated	Type of specimens											
	Blood		Pus		Urine		Devices		Others		Total Samples	
	N	%	N	%	N	%	N	%	N	%	N	%
Klebsiella penumoniae	40	41.66	8	21.05	8	10.25	42	49.41	9	30	107	32.72
Citrobacter sp.	1	1.04	2	5.26	2	2.56	6	7.05	2	6.66	13	3.97
Enterobacter sp.	1	1.04	00	00	00	00	3	3.52	00	00	4	1.22
Escherichia coli	3	3.13	16	42.10	63	80.76	3	3.52	8	26.66	93	28.44
Acinetobacter baumannii	28	29.16	5	13.15	3	3.84	12	14.11	6	20	54	16.51
Pseudomonas aeruginosa	21	21.87	7	18.42	2	2.56	20	23.25	4	13.79	54	16.51
Salmonella sp.	1	1.04	00	00	00	00	00	00	00	00	1	0.30
Edwardsiella sp.	1	1.04	00	00	00	00	00	00	00	00	1	0.30
Total samples	96	29.35	38	11.62	78	23.85	86	26.29	29	8.86	327	100

3. Result

Total 4688 different clinical samples were received in the laboratory for aerobic bacterial culture and sensitivity testing during study time. Out of these 327 non repetitive gram negative bacteria were included in the study.

Table 1 showed demographic profile of samples. Maximum microorganism isolated were from blood culture (29.35%) followed by devices (26.29%) & urine (23.85%). Majority of samples were IPD (90.82%), rural (75.22%) and from female patients (63.30%). In age distribution maximum samples were in age group > 19 years (37.17%) followed by age <1 month (30.50%).

Out of total 327 GNB isolates, maximum were *Klebsiella pneumoniae* (32.72%) followed by *Escherichia coli* (28.44%), *Acinetobacter baumanii* (16.51%), *Pseudomonas aeruginosa* (16.51%), *Citrobacter species* (3.97%).

Table 3 shows distribution of total isolates in different clinical samples. *Klebsiella pneumoniae* were isolated maximum from blood (41.66%) and devices (49.41%). *Escherichia coli* was predominant in Urine (80.76%)sample. Nonfermenters like *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were detected maximum in Blood samples (29.16%, 21.87% respectively) and devices (14.11,23.25% respectively). In pus samples predominant organisms were *Escherichia coli* (42.10%)

followed by *Acinetobacter baumannii* (13.15%) and *Pseudomonas aeruginosa* (18.42%).

Table 4 showed Antibiotic Resistance patterns among GNB isolates. Maximum isolates showed resistance to ampicillin (93.27%) followed by amoxiclave (87.46%), ceftazidime (74%) and Ciprofloxacin (71.25%) and were sensitive to meropenem (70.34%), piperacillin-tazobactam (68.20%) and cefepime (55.05%). *Klebsiella pneumoniae*, *Acinetobacter baumannii* & *pseudomonas aeruginosa* were found to be multidrug resistant.

Out of 327 GNB isolates, 39(12.66%) isolates were ESBL & 26(8.44%) isolates were MBL producer. Amongst them maximum ESBL were produced by *Klebsiellap neumonia* (19.62%). Among nonfermenters, *Pseudomoas aeruginosa* showed maximum ESBL (14.81%) & MBL (14.81%) then *Acinetobacter baumannii* ESBL (7.40%) & MBL (7.40%).

Table 6 shows detection of biofilm production by three different methods. Out of 327 GNB isolates, biofilm produced by 64 (19.57%) isolates by Tissue culture plate (TCP) method, 38(11.62%) by Congo red agar (CRA) methods and 23 (7.03%) by Tube methods. Maximum biofilm were detected in *Klebsiella pneumoniae* (24.29%), *Citrobacter species* (23.07%), *Escherichia coli* (17.20%), *Acinetobacter baumannii* (22.22%) & *Pseudomonas aeruginosa* (12.96%). All species of Enterobacter, salmonella and Edwardsiella were non biofilm producer.

4. Discussion

In present study total 327 GNB isolates were detected with predominance of *Klebsiella pneumonia*e (107) & *Escherichia coli* (93). The study done by Dumaru et al[13] & Fatima et al[14] showed *E.coli* as predominant bacterial isolate. This could be because of different geographical area, different population types. In our study maximum positive isolates were seen in blood culture (29.35%) & similar observation was seen in the study done by Khanal et al, Roy et al.[15,16]

In present study *Klebsiella pneumoniae* were isolated maximum from blood (41.66%). Which is concordant with the study done by Negussie A et al, Jyoti et al, Vanitha RN et al & Nidhi Pal et al.[15–18]

Escherichia coli was predominant in Urine (80.76%)sample which was similar to finding with Alanazi MQ et al,[19] & Isaac Odongo et al.[20]

Nonfermenters like *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were detected maximum in Blood samples (29.16%, 21.87% respectively) and devices (14.11, 23.25% respectively). Which is similar to Usha Arora et al R & Rattanaumpawan et al & Juyal D et al.[21–23] In pus samples predominant organisms were *Escherichia coli* (42.10%) followed by *Acinetobacter baumannii* (13.15%) and Pseudomonas aeruginosa (18.42%) in this study almost all GNB isolates were highly resistant to

Table 4: Antibiotic resistance profile of gram negative isolates(GNB)

Antibiotics	Organisms																	
	Escherichia coli		*Klebsiella pneumoniae*		*Citrobacter species*		*Enterobacter species*		*Salmonella species*		*Edwardsiella species*		*Acinetobacter baumannii*		*Pseudomonas aeruginosa*		Total	
	N= 93	%	N = 107	%	N=13	%	N = 4	%	n=1	%	N=1	%	(n=54)	%	(n= 54)	%	(N= 327)	%
AMP(10μg)	80	86.02	105	98.13	13	100	4	100	1	100	0	0	50	92.59	52	96.29	305	93.27
AMC (20/10μg)	67	72.04	99	92.52	12	92.30	3	75	0	0	0	0	52	96.29	53	98.14	286	87.46
GEN(10μg)	17	18.27	62	57.94	7	53.8	2	50	0	0	0	0	31	57.41	35	64.81	154	47.09
PIT (100/10μg)	12	12.9.	50	46.72	4	30.76	1	25	0	0	0	0	23	42.59	14	25.93	104	31.80
CTX(30μg)	30	32.25	76	71.02	6	46.15	1	25	0	0	0	0	42	77.78	48	88.89	203	62.07
CPM(30μg)	27	29.03	57	53.27	5	38.46	1	25	0	0	0	0	31	57.41	26	48.15	147	44.95
MRP(10μg)	13	13.97	27	25.23	5	38.46	0	0	0	0	0	0	28	51.85	24	44.44	97	29.66
AK(30μg)	28	30.10	71	66.35	8	61.53	3	75	0	0	0	0	27	50.00	36	66.67	173	52.90
CIP(5μg)	59	63.44	81	75.70	10	76.92	1	25	0	0	0	0	39	72.22	43	79.63	233	71.25
CAZ(30μg)	52	55.91	96	89.71	10	76.92	3	75	0	0	0	0	47	87.04	34	62.96	242	74.00
AT(30μg)	28	30.10	68	63.55	8	61.53	2	50	0	0	0	0	37	68.52	25	46.30	168	51.37

Abbreviations: AMP- Ampicilin, AMC- Amoxicillin Clavulanate, GEN- Gentamicin, PIT- Piperacillin-tazobactam, CTX- Cefotaxime, CPM- Cefepime, MRP- Meropenem, AK- amikacin, CIP-Ciprofloxacin, CAZ- Ceftazidime, AT- Aztreonam

Table 5: Distribution of ESBL & MBL among GNB isolates

		Blood	Pus	Urine	Devices	Others	Total N(%)
	Total	40	8	8	42	9	107
Klebsiella pneumoniae	ESBL	10	2	0	8	1	21 (19.62%)
	MBL	1	1	0	8	1	11 (10.28%)
	Total	3	16	63	3	8	93
E.coli	ESBL	1	1	3	0	1	6 (6.45%)
	MBL	0	1	1	1	0	3 (3.22%)
	Total	21	7	2	19	5	54
Pseudomonas aeruginosa	ESBL	5	2	0	1	0	8 (14.81%)
	MBL	2	0	0	6	0	8 (14.81%)
	Total	28	5	3	12	6	54
Acinetobacter baumannii	ESBL	2	0	1	0	1	4 (7.40%)
	MBL	1	0	0	2	1	4 (7.40%)

Table 6: Detection of biofilm production by three different methods & their distribution among GNB isolates.

Methods	***E.coli* (93)**		***K.p (107)***		***Citrobacter species* (13)**		***Pseudomonas aeruginosa* (54)**		***Acinetobacter baumannii* (54)**		***Enterobacter species* (4)**	***Salmonella species* (1)**	***Edwardsiella Species* (1)**	**Total (327)**	
	N	%	n	%	n	%	n	%	n	%				n	%
TCP	16	17.20	26	24.29	3	23.07	7	12.96	12	22.22	0	0	0	64	19.57
Tube	5	5.37	13	12.14	1	7.69	3	5.55	1	1.85	0	0	0	23	7.03
CRA	13	13.97	14	13.08	2	15.38	3	5.55	6	11.11	0	0	0	38	11.62

ceftazidime. This could be due to the widespread & misuse of third-generation cephalosporins without knowing the severity of infections.[23,24]

In present study maximum biofilm producers were seen in devices (25.77%) followed by blood (23.15%) while maximum non biofilm producers were seen in Urine (82.06%) & other sample (83.33%). Out of 327 GNB isolates, biofilm produced by 64 (19.57%) isolates by Tissue culture plate (TCP) method, 38(11.62%) by Congo red agar (CRA) methods and 23 (7.03%) by Tube methods.

This is concordant with the study done by Pragyan et al,[25] they showed out of 300 isolates,137 isolates (45.6%) produced biofilm by the TCP method, Tube adherence method detected biofilm in 118 isolates (39.3%) & Congo red agar method detected biofilm in 33(11%) isolates. The TCP was found to be most sensitive followed by the TM & CRA.

In other study done Hassan et al[26] on 110 isolates showed that the TCP method detected biofilm in 70 isolates (63.6%), TM in 54 (49%) and CRA in 11 (10%) isolates. In Mathur et al[1] study done on 152 isolates for biofilm detection reported 47.3% biofilm by TCP, 41.4% by TM and 5.2% isolates were biofilm producers by CRA method. Baqai et al[27] showed biofilm production in 75% of the isolates as by TM while with CRA detected in 10% isolates only.

In this study maximum Biofilm production were shown by *Klebsiella pneumonaie* (24.29%), *Citrobacter species* (23.07%), *Escherichia coli* (17.20%), *Acinetobacter baumannii* (22.22%) & *Pseudomonas aeruginos*a(12.96%) while study done by Malvika et al[28] showed maximum biofilm production in *E.coli* (78.5%) followed by *Pseudomonas aeruginosa* (82%), *Acinetobacter species(*90%).

Many authors have reported that tissue culture plate method is considered to be better for the biofilm detection because it provides only true positive result thereby omitting the false negative & false positive results given by other two methods. In TM method there can be subjective observer's error while assessing biofilm result as compared to TCP method. It was observed that biofilm producers isolates were more resistant to antibiotics & were multidrug resistant similar observation was made by Carlos J Sanchez et al[29] who studied biofilm forming phenotype in large number of clinical isolates with MDR phenotype.

In present study maximum ESBL isolates were *Klebsiella pneumoniae* (19.62%) which is similar to study done by Sanghamithra Datta et al[30] who showed 50%, 35.33% ESBL isolates in *Klebsiella pneumoniae* & *E.coli* respectively.

In our study total 27 MBL isolates were detected. Maximum MBL were produced by *Pseudomoas aeruginosa* 8 isolates followed by *Klebsiella pneumoniae* 11 isolates & *Acinetobacter baumannii* 4 isolates & *Escherichia coli* 3 isolates. The study done by Sundaram et al[31] showed 53.83% & 41.7% MBL isolates in *Acinetobacter species* & *Pseudomonas aeruginosa* respectively variable results have been observed by different studies done across India & abroad. Tripathi et al.[31] demonstrated ESBL production in 29.9% isolates & MBL production in 86.9% isolates whereas Yong et al[32] observed 54.63% isolates were ESBL & 50% isolates were MBL producer. Which is comparable to studies conducted by Oberoi et al, Kaur et al and Rao et al.[33–35]

Maximum of them were isolated from Blood & devices sample. *Pseudomonas aeruginosa* is an notorious hospital acquired drug resistant bug, well-known for chronic infection due to potential to form biofilms.[36] *Acinetobacter baumannii* has emerged as one of the most troublesome pathogen responsible for various infections especially in the ICUs. In recent year *Acinetobacter baumannii* has emerged as hospital superbug as a result of their profundity in developing multidrug (MDR) & pandrug resistance(PDR). They have remarkable ability to acquire resistant determinant gene encoding for enzymes like ESBL & MBL which makes them resistant to all higher antibiotics.

5. Conclusion

The increase prevalence of multidrug resistant & biofilm forming organisms provides a glimpse of emerging threat of hospital superbugs in new era of world. The routine monitoring of multidrug resistance pattern & biofilm detection can be recommended in clinical laboratories along with strict adherence to Institutional antibiotic policy, proper implementation &monitoring of hospital infection control & prevention activities.

6. Source of Funding

None.

7. Conflict of Interest

None.

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