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Study of biofilm formation in *Klebsiella* species

Rakesh Prasad Sah[1], Rakesh Kumar Mukhia[2], A D Urhekar[3]
[1] Assistant Professor, Department of Microbiology, Saraswati Medical College, Unnao, Uttar Pradesh, India
[2] Assistant Professor, Department of Microbiology, Hind Institute of Medical Sciences, Ataria, Uttar Pradesh, India
[3] Professor and HOD Microbiology, Department of Microbiology, MGM Medical College, Navi Mumbai, Maharashtra, India

Abstract
Background: *Klebsiella* is a gram-negative, encapsulated, facultative anaerobic bacterium which causes hospital and community acquired infections. *Klebsiella* species has ability to grow *in vitro* as a biofilm which helps bacteria to survive within host leading to chronic infections as well as development of resistance to antibiotics and disinfectants. Due to development of resistance to antibiotics it is responsible for causing high morbidity & mortality.
Objectives: To study biofilm formation in *Klebsiella* species along with comparison of different phenotypic methods i.e. microscopic determination, Congo Red Agar (CRA) & Tissue culture plate (TCP). Further it was correlated the biofilm formation with antibiotic drug resistance.
Material and Methods: A prospective and experimental study was carried out over a period of three years (August 2013 to July 2016). Total 200 isolates of *Klebsiella* species were tested for the biofilm formation by three different methods i.e. Congo Red Agar, Tissue culture plate and Microscopic determination method. Antibiotic sensitivity was done by Kirby-Bauer Disc diffusion method and the final data was presented using frequency & percentage.
Results: Out of 200 isolates of *Klebsiella*, biofilm formation was detected by Microscopic determination 105 (52.5%), Congo red agar 108 (54%) & Tissue culture plate 119 (59.5%). In the 119 biofilm producing strains detected by TCP method, 94 (79%) strains were MDR (Multi-drug resistant). Highest biofilm producers *Klebsiella* strains were detected from Accessory devices (83.3%), followed by sputum (76.4%), Endotracheal aspirate (60%), Pus (59.4%), Urine (54.7%), vaginal swabs (50%), Throat swabs (40%), Blood (29.4%) and Stool (25%) by TCP method.
Conclusion: TCP is more sensitive & specific method for detection of biofilm and most of the biofilm producing strains were MDR (Multi-drug resistant).

Keywords: biofilm formation, *Klebsiella* species

Introduction
Klebsiella is a gram-negative, encapsulated, facultative anaerobic bacterium which causes hospital and community acquired infections such as pneumonia, urinary tract infection, septicaemia, soft tissue infections [1, 2, 3]. *Klebsiella* species have ability to grow *in vitro* as a biofilm since the end of the 1980s but clear evidence was provided in 1992 [4]. Biofilm formation means the aggregation of bacterial cells embedded within a self-produced matrix of extracellular polymeric substances adherent to each other or to an inert or living surface [5]. Biofilm forming bacteria are responsible for causing chronic infections within host which finally leads to development of resistance to antibiotics & disinfectants [6, 7]. Biofilm along with other virulence factors ultimately are responsible for high morbidity & mortality.

Materials and Methods
A prospective and experimental study was carried out over a period of three years (August 2013 to July 2016) in microbiology laboratory of MGM Medical College & Hospital Kamothe, Navi Mumbai, Maharashtra, India. The research topic was cleared by Ethical committee for research on Human subjects on 27th October 2014 via letter no. MGM/HIS/RS/2014-15. Inclusion criteria: Sample showing pus cells and bacteria were included in the study. Exclusion criteria: Sample not showing pus cells and bacteria were excluded from the study. A total of 200 isolates of *Klebsiella* species were isolated from different clinical specimens which were characterised into different species by standard protocols. Antibiotic sensitivity was done by Kirby-Bauer Disk diffusion method using following antibiotics (1st line: Augmentin, Tobramycin, Cefazolin, Gentamicin, Cefuroxime, Amikacin, Cefotaxime, Ciprofloxacin, Cefoperazone, Ofloxacin, Ceftazidime, Ceftizoxime, Tetracycline, Norfloxacin & Nitrofurantoin, 2nd line: Ceftazidime/ Tazobactam, Imipenem, Ceftriaxone/sulbactam, Meropenem, Cefoperazone/ sulbactam, Levofloxacin Cefepime, Prulifloxacin, Piperacillin/Tazobactam, Cefotaxime/clavulanic acid, Ticarcillin/ clavulanic acid & Cefixime/ clavulanic acid and 3rd line: Polymyxin B, Aztreonam, Ertapenem & Tigecycline) [8]. These isolates were tested for the biofilm formation by three different methods.

Congo Red Agar method (CRA)
As per method given by Freeman DJ *et al* [9]. Briefly, Congo red was prepared as concentrated aqueous solution separately from other constituents of media (Brain Heart infusion broth,

5% sucrose, 1% Agar no. 1), and autoclaved at 121°C for 15 minutes, and then added to the agar which is cooled at 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. Positive result was indicated by black colonies.

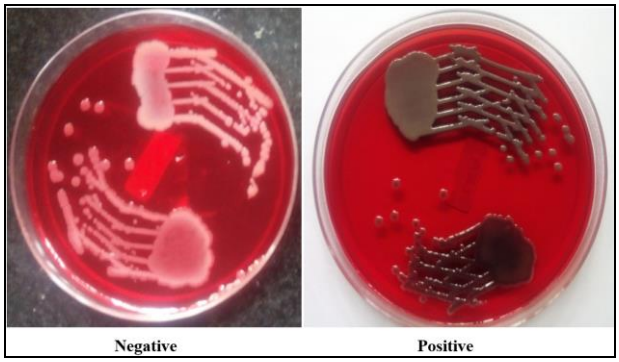


Fig 1: Congo Red Agar

Microscopic determination of biofilm formation (MD) [10]

As per method given by Magesh *et al* [10]. Briefly, The test strain was cultured in Brain-heart infusion (BHI) broth. A sterile glass slide was kept in a sterile petriplate and overlaid with 20µl of test strain inoculated in BHI. After 24 and 48h of incubation, the slide was taken out aseptically and washed with PBS (7.2) to remove free floating planktonic bacteria. The biofilm was fixed with 2% sodium acetate and stained with Safranin stain, washed and air dried and observed under Binocular microscope at 100x oil immersion. Photomicrographs of adhered bacterial biofilms were recorded. Clustered form bacteria within Extracellular polymeric substances observed in biofilm producers categorized in to three catagories i.e. weakly positive, moderately positive & strongly positive while scattered form of bacteria observed in non-biofilm producers.

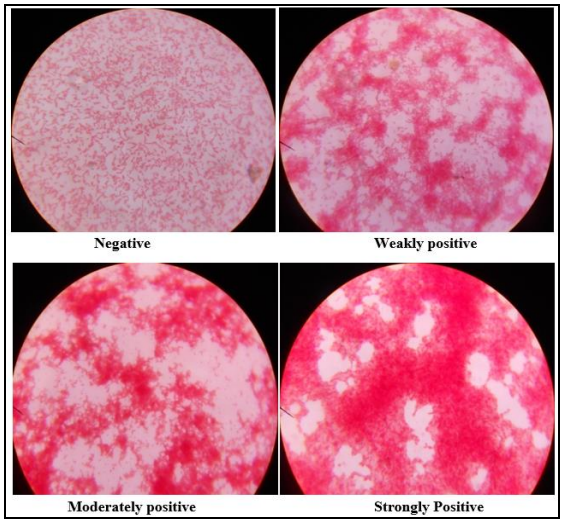


Fig 2: Microscopic determination

Tissue culture plate method (TCP)

As per method given by Eftekhar & Speert et.al. [11] and Interpretation of biofilm formation was classified given by Mathur et.al [12]. Briefly, an overnight culture of each isolate of *Klebsiella* in Trypton soya broth (TSB) with 1% glucose was adjusted to 0.5 McFarland standard. 200µl of diluted aliquots were inoculated to tissue culture plates and only broth was kept as control to check sterility and non-specific binding of media and known positive were used as positive control then incubated for 24-48hrs at 37^0C. After incubation, the planktonic cells were aspirated then wells were washed with PBS 7.2 to remove fee floating *Klebsiella*. Biofilms which adhere to the wells were fixed with 2% sodium acetate and stained with Safranin. Excess stain was washed with deionised water and plates were dried for further processing. The absorbance of stained adherent *Klebsiella* was determined by absorbance reader at 492nm. To compensate for background absorbance, O.D. values from sterile medium well were averaged and subtracted from all the test values. The experiment was repeated twice. Each isolates was analyzed in triplicate.

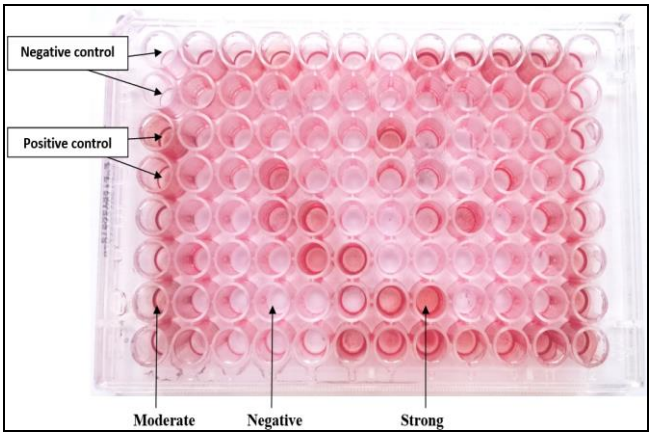


Fig 3: Biofilm detection by Tissue Culture plate method

Interpretation

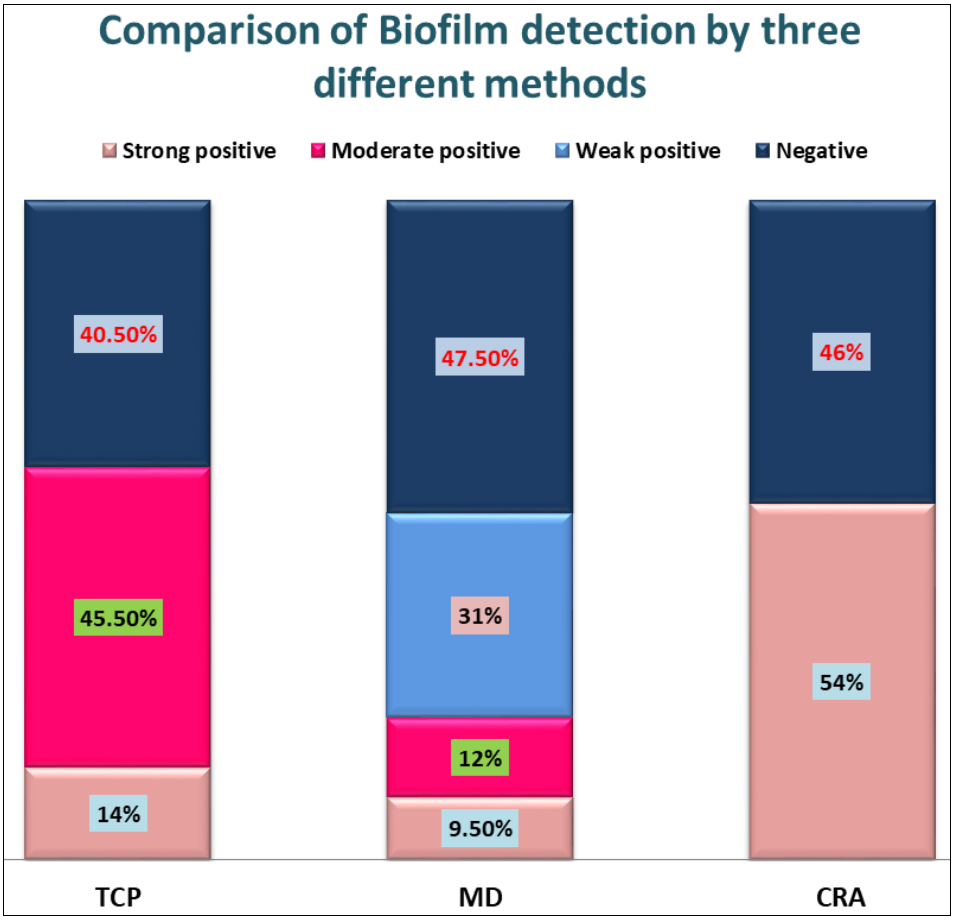
Table 1

O.D. Value	Biofilm Producer
< 0.120	Non Biofilm producer
0.120 – 0.240	Moderate Biofilm producer
> 0.240	Strong Biofilm producer

Results

Out of 200 isolates of *Klebsiella*, 105 (52.5%), 108 (54%) & 119 (59.5%) isolates were biofilm producers by Microscopic determination method, Congo red agar and Tissue culture plate method respectively. In the 119 biofilm producing strains detected by TCP method, 94 (79%) strains were MDR (Multi-drug resistant).

Highest biofilm producers *Klebsiella* strains were detected from Accessory devices (83.3%), followed by sputum (76.4%), Endotracheal aspirate (60%), Pus (59.4%), Urine (54.7%), vaginal swabs (50%), Throat swabs (40%), Blood (29.4%) and Stool (25%) by TCP method.



P-value = 0.333, no significant difference in the three methods

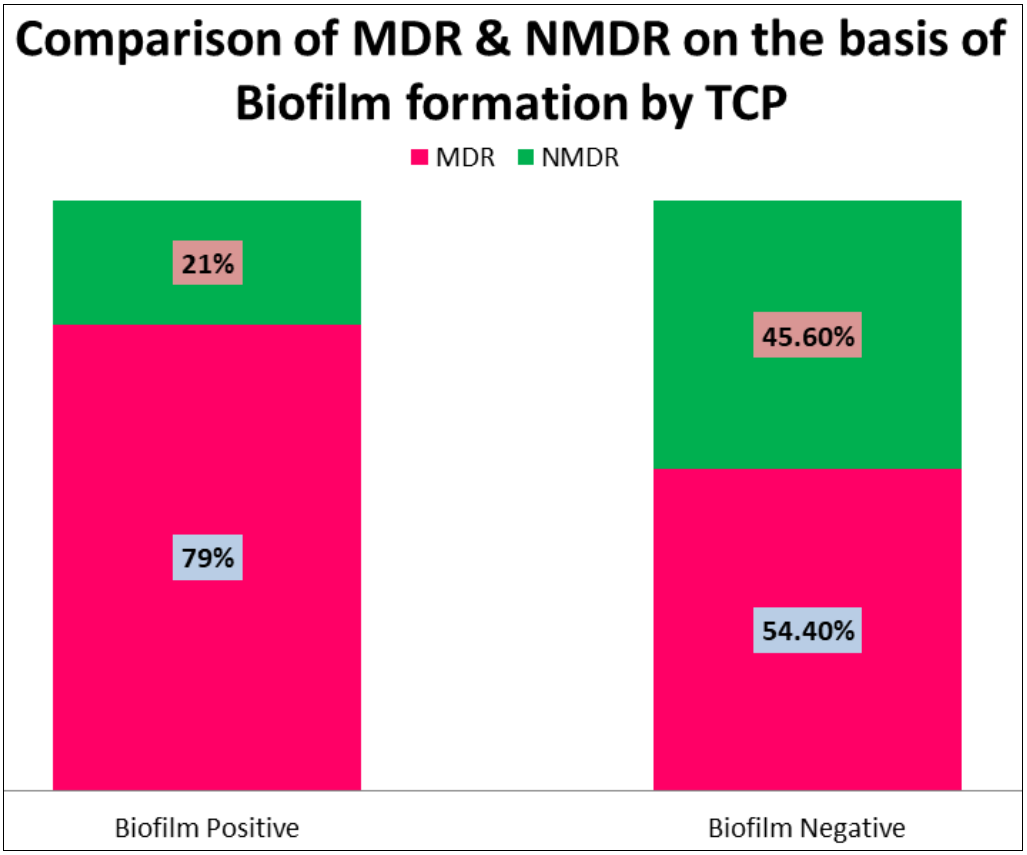
Fig 4: Bar diagram showing comparison of methods for the detection of Biofilm

Table 2: Sample wise distribution of Biofilm production by *Klebsiella species*

S. No.	Sample	Total Isolates	Congo Red Agar		Microscopic determination		Tissue Culture Plate	
			Positive	Negative	Positive	Negative	Positive	Negative
1	Accessory devices	6	4 (66.6%)	2	4 (66.6%)	2	5 (83.3%)	1
2	Sputum	51	40 (78.4%)	11	38 (74.5%)	13	39 (76.4%)	12
3	Endotracheal aspirate	25	12 (48%)	13	11 (44%)	14	15 (60%)	10
4	Pus	37	16 (43.24%)	21	22 (59.4%)	15	22 (59.4%)	15
5	Urine	53	25 (47%)	28	25 (47%)	28	29 (54.7%)	24
6	Throat swabs	5	1 (20%)	4	1 (20%)	4	2 (40%)	3
7	Blood	17	7 (41%)	10	2 (11.7%)	15	5 (29.4%)	12
8	Stool	4	2 (50%)	2	1 (25%)	3	1 (25%)	3
9	Vaginal swabs	2	1 (50%)	1	1 (50%)	1	1 (50%)	1
Total		200	108 (54%)	92	105 (52.5%)	95	119 (59.5%)	81

Table 3: Species wise distribution of Biofilm

Species	Total no.	Methods					
		TCP		CRA		MD	
		Positive	Negative	Positive	Negative	Positive	Negative
K. pneumoniae pneumoniae	169	109 (64.5%)	60	97 (57.4%)	72	99 (58.6%)	70
K. pneumoniae. ozaenae	7	3 (42.9%)	4	2 (28.6%)	5	2 (28.6%)	5
K. oxytoca	24	7 (29.2%)	17	9 (24.1%)	15	4 (16.7%)	20
Total	200	119 (59.5%)	81	108 (54%)	92	105 (52.5%)	95



P-value = <0.001** Finding that MDR strains produce biofilm is statistically significant.
Note: "**" = Highly significant and MDR (Multi drug resistance): MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories [13].

Fig 5: Bar diagram showing comparison of MDR & NMDR on the basis of Biofilm

Discussion

Studies on Biofilm formation detection by a) Congo red agar method b) Tissue culture plate method c) Microscopic determination method was carried out by some researchers in India & other countries. Test procedures were all standard methods. However, different values were reported. Some results were similar to ours; others reported higher or lower values. These differences could be because of variations in the genetic makeup of *Klebsiella* strains at various places as regards expression of biofilm formation.

For *Klebsiella species* by TCP (Tissue culture plate method)

Biofilm formation by TCP in our study was 59.5% of *Klebsiella* strains, higher values were shown by Harith J.F. Al-Mathkhury et.al. (100%) from Iraq [14] and Zubair et.al. (70%) from Karnataka, India [15]. Lower value was shown by Devesh et.al. (32%) from Pondicherry, India [16].

For *Klebsiella pneumoniae* by TCP (Tissue culture plate method)

Our study (64.5%) is similar to Maqbool et.al. (66.6%) from Maharashtra, India [17]. Higher values were shown by Seifi et.al. (93.6%) from Teharn, Iran [18] Deotale et.al. (90.91%) from Wardha, India [19], FM Patel et.al. (90.90%) from Ahmedabad, India [20], Dr. chndana devaraj et.al. (80%) from Karnataka, India [21], Zubair et.al. (77.7%) from Aligarh, India [15] and. Lower values were shown by H. Magesh et.al. (57.14%) from Chennai, India [22], Devinder Kaur et.al. (46.67%) from Ambala, India [23], Ruchi Tayal et.al. (18.75%) from Mumbai, India [24], and Tayal et.al. (16.7%) from Mumbai, India [25].

For *Klebsiella oxytoca* by TCP (Tissue culture plate method)

Present study results (29.2%), higher value was shown by Zubair et.al. (63.4%) from Karnataka, India [15].

For *Klebsiella species* by Congo red agar method

Present study results (54%), higher value was shown by S. Niveditha et.al. (63%) from Pondicherry, India [26], while lower values were shown by Sabina Fatima et.al. (45%) from Telangana, India [27], and Abdagire et.al. (36.11%) from Maharashtra, India [28].

For *Klebsiella pneumoniae* by Congo red agar method

Our study result (57.4%) is similar to Devinder kaur et.al. (60%) from Ambala, India [29]. Higher values were shown by Subramanian et.al. (63%) from Pondicherry, India [30] and Kabir et.al. (100%) from Bangladesh [31]. Lower value was shown by Chandana devaraj et.al. (30%) from Karnataka, India [32].

Conclusion

To conclude this study, our study suggest that TCP is the best

method for the detection of biofilm in Klebsiella species as it showed more sensitive and specific method than others. Most of the positive isolates of biofilm are isolated from accessory devices in which it is very difficult to treat them with available antimicrobial agents so there is need to establish standard guidelines to care these accessory devices in order to prevent such infections.

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