ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Drug Resistance, Biofilm Formation and the Occurrence of *mrk*ABCDF operon in Plasmid-Bearing Clinical Isolates of *E.coli* and *Klebsiella* spp. from Kerala, India.

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ABSTRACT

Biofilm comprises of a complex microbial community with a secreted polymeric matrix which imparts high tolerance to antimicrobials, resists host immune response and facilitates promiscuous horizontal gene exchange. The present study analyses the extent of biofilm formation, drug resistance and the prevalence of biofilm-associated *mrk*ABCDF operon in plasmid-bearing clinical isolates of *E.coli* and *Klebsiella* spp. *In vitro* microtiter plate-based biofilm assay and a PCR based screening for the presence of *mrk*B-C region of the operon was performed. The resistance of isolates, assessed by disc diffusion method, against 10 antibiotics belonging to 5 different classes, ranged from a minimum of 6 to all of the 10 antibiotics tested. Most of the isolates were resistant to more than 3 different classes of antibiotics. The association observed between antibiotic resistance and biofilm formation was more evident in *E.coli* than in *Klebsiella.* Although biofilm formation was found to be higher in *E.coli*, the prevalence of *mrk* operon in these isolates was lower compared to *Klebsiella* spp. This inverse relationship observed between biofilm formation and the occurrence of *mrk* operon suggests that the mere existence of this operon does not ensure biofilm forming ability in these isolates.

Keywords: Biofilm, type 3 fimbriae, plasmids, *mrk*ABCDF operon.

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ISSN: 0975-8585

INTRODUCTION

Biofilms constitute an organized heterogeneous microbial society, co-existing on biotic or abiotic surfaces, capable of combating hostile, stressful and selective conditions [1-4]. Apart from their presence in the human body surfaces like heart valve, lungs and teeth, biofilms assume clinical relevance in nosocomial infections as they pervade medical devices such as catheters, artificial heart valves and prosthetic joints. They can remain dormant on these surfaces, escape from host immune mechanisms such as phagocytosis and from the action of different antibiotics due to poor penetration [3, 5, 6, 7]. Biofilm community acts as a hot spot of horizontal gene exchange [2, 8-12]. Occurrence of wide host range plasmids in multi-species biofilms can act as a platform for interspecies gene exchange. The incidence of high antibiotic resistance reported in biofilm bacteria than in dispersed cells thus lends credence to the influence of the above mentioned aspects [3].

Several proteinaceous appendages that emanate from bacterial surfaces, in addition to cell surface adhesins, aid in adhesion and attachment during biofilm formation. These include fimbriae (type 1, 3 and curli), flagella and conjugative pili [2, 13, 14]. While type 1 fimbriae were reported earlier in most of the Enterobacteriaceae, type3 were reported only from *Klebsiella* spp. Athough, initially, type 3 fimbriae were not found to be associated with *E.coli*, later studies have reported plasmid-encoded expression of the same in these species [2, 13, 15, 16]. Type 1 and 3 of the chaperone-usher class fimbriae, are encoded by gene clusters *fim*ABCDEFGHIK and *mrk*ABCDF respectively [13, 14] whereas, curli - a proteinaceous extracellular fiber, is encoded by genes present on two operons *csg*BA(C) and *csg*DEFG [4].

The present study was carried out to understand the extent of biofilm formation, drug resistance and prevalence of *mrk*ABCDF operon in plasmid-bearing clinical isolates of *E.coli* and *Klebsiella*. The structural genes - *mrk*A-F - encode major subunit protein, chaperone, usher, adhesin and minor subunit proteins of type 3 fimbriae respectively [13, 14]. The *mrk*B-C region encoding the chaperon-usher region was employed for PCR detection as it has been reported from both of the genera under study.

MATERIALS AND METHODS

Bacterial isolates

Bacterial isolates were collected in pure line form from a tertiary care center, Kerala, which included 44 *E.coli* and 41 *Klebsiella* spp. Isolate identity was confirmed by biochemical assays.

Plasmid isolation

Plasmid DNA was isolated by alkaline lysis method [17]. The DNA samples were electrophoresed at 100V for 1h and 30 min in 0.5x Tris-Borate-EDTA (TBE) on 0.8% agarose gel.

Antibiotic sensitivity assay

Antibiotic sensitivity was tested by Kirby-Bauer disc diffusion method [18] according to CLSI manual [19]. Five different classes of antibiotics were used which included β-lactams, aminoglycoside (gentamicin - GEN), macrolide (azithromycin - AZM), quinolone and chloramphenicol (C). Antibiotics of β-lactam group included ampicillin (AMP), 3rd generation cephalosporins (ceftazidime - CAZ and cefotaxime - CTX), meropenem (MRP) and a combination antibiotic – Piperacillin/Tazobactam (PIT). Among the quinolones, the 1st and 2nd generations -nalidixic acid (NA) and ciprofloxacin (CIP) respectively were included.

Crystal violet assay for biofilm formation

Biofilm growth was analyzed in microtiter plates using crystal violet as mentioned elsewhere [20]. Sterile Luria-Bertani media served as control. Optical density of tests and control has been measured at 570 nm. The isolates were grouped into three categories based on the O.D. values [21, 22, 23] as given below:

a. Strong biofilm producers – OD_T (Test) > 4 x OD_C (Control)
b. Moderate biofilm producers – 2 x OD_C < OD_T ≤ 4 x OD_C
c. Weak biofilm producers - OD_C < OD_T ≤ 2 x OD_T

Molecular identification of *mrk*B-C region of *mrk*ABCDF operon

The *mrk*B-C region of mrkABCDF operon was amplified with primers mrkC rev – 5'-ACCAGGTTGTCTTTGACG-3'and mrkB fw - 5'-GCAGTGAAAGTGGCCGTT-3' [13] using plasmid DNA as template. Genomic PCR was also carried out with the same primer set for isolates which were found to be negative with respect to *mrk* to ascertain its presence, if any, within the genome. The PCR reactions were performed in a MiniCycler™ (MJ Research, USA) in a volume of 25µl containing 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 2 U of Taq DNA polymerase, 0.5 µM of each primer and 100 ng template DNA. All reagents were purchased from Bangalore Genei Pvt. Ltd. HPLC purified primers were purchased from Sigma Aldrich Chemicals Pvt. Ltd. (Bangalore). Conditions for amplification were as follows: Initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min and a final extension step at 72°C for 10 min. The PCR products (10 µl each) along with the molecular weight markers were resolved on 1% (w/v) agarose gels as mentioned above, and the results were visualized and photographed using Alpha imager™ 2200 (Alpha Innotech corporation,USA).

The PCR products were sequenced at a commercial facility (Xcelris Labs Limited, Ahmedabad). The nucleotide and deduced protein sequences were analyzed with programs such as BLAST / FASTA and ORF finder of NCBI (www.ncbi.nlm.nih.gov).

Statistical analysis

Statistical analysis was carried out by Z-test using SPSS software version 20. Differences with $P<0.01$ were considered significant.

RESULTS

Plasmid analysis

Plasmids were obtained from 55% (n = 24) of *E.coli* and 56% (n = 23) of *Klebsiella* isolates and were observed as both single as well as multiple bands in the plasmid profiles.

Antibiotic sensitivity test

The bacterial isolates displayed high resistance against AMP, 3rd generation cephalosporins, and 1st and 2nd generation quinolones. Resistance was found to be low against MRP, AZM, GEN and C. Percentage resistances of *E.coli* and *Klebsiella* spp. against each antibiotic are shown in Table 1**.** The resistances displayed ranged from a minimum of 6 to 10 antibiotics belonging to 5 different classes. Table 2 shows percentage of multidrug-resistant isolates (resistant to ≥ 3 different classes of antibiotics) of *E.coli* and *Klebsiella* respectively. The degree of resistance was significantly higher (P<0.01) in *Klebsiella* spp. than in *E.coli* as evident from the number of resistant isolates resistant to > 3 different classes of antibiotics.

Crystal violet assay for biofilm formation

All the plasmid bearing isolates were found to produce biofilm either strongly, moderately or weakly. The number of strong biofilm producers was found to be higher among *E.coli* than in *Klebsiella* spp. (P<0.01). Percentage of isolates producing each type of biofilm is given in Fig. 1(a)**.** In *E.coli* isolates, a significantly high number of strong biofilm producers were found to be resistant to ≥ 3 different classes of antibiotics (P<0.01); isolates resistant to > 3 antibiotics were totally absent in weak biofilm producing *E.coli* [Fig. 1(b)]. In the case of *Klebsiella*, even though highly resistant isolates were included under weak biofilm producers, those with low resistance (resistant to < 3 different classes of antibiotics) were totally excluded from strong and medium biofilm producers (Fig. 1(c)).

PCR based detection of *mrk* gene

A 500bp amplicon was obtained after amplification of *mrk*B-C region (Fig. 2). The percentage detection of this sequence was found to be significantly higher on plasmids in *Klebsiella* isolates (65%, n = 15) than in *E.coli* isolates (25%, n = 6; P<0.01). Further, the amplicon was not detectable in genomic PCR with

ISSN: 0975-8585

E.coli DNA. Interestingly, in weak biofilm producing *Klebsiella* spp. the number of *mrk*-positive isolates was found to be significantly higher than *mrk* -negative ones (P<0.01) (Fig. 3). Sequence analysis of the 500bp amplicon showed 99% identity with type3 fimbriae gene cluster. An analysis of the ORF obtained within the amplicon showed 100% identity with outer membrane usher protein.

Table 1: Percentage of isolates resistant to each of the 10 different antibiotics

Isolates	AMP	CAZ	CTX	PIT	MRP	AZM	GEN	NA	CIP	C
E.coli	100	100	100	100	83	58	38	100	100	17
Klebsiella spp.	100	96	96	100	74	96	83	100	100	70

AMP – Ampicillin, CAZ – Ceftazidime, CTX – Cefotaxime, PIT – Piperacillin/Tazobactam, MRP – Meropenem, AZM – Azithromycin, GEN – Gentamicin, NA – Nalidixic acid, CIP – Ciprofloxacin, C – Chloramphenicol

Table 2: Percentage of Multidrug-resistant E.coli and Klebsiella spp.

Isolates	Percentage of isolates resistant to		
	< 3 different classes of antibiotics	3 different classes of antibiotics	>3 different classes of antibiotics
E.coli	33	38	29
Klebsiella spp.	4	22	74

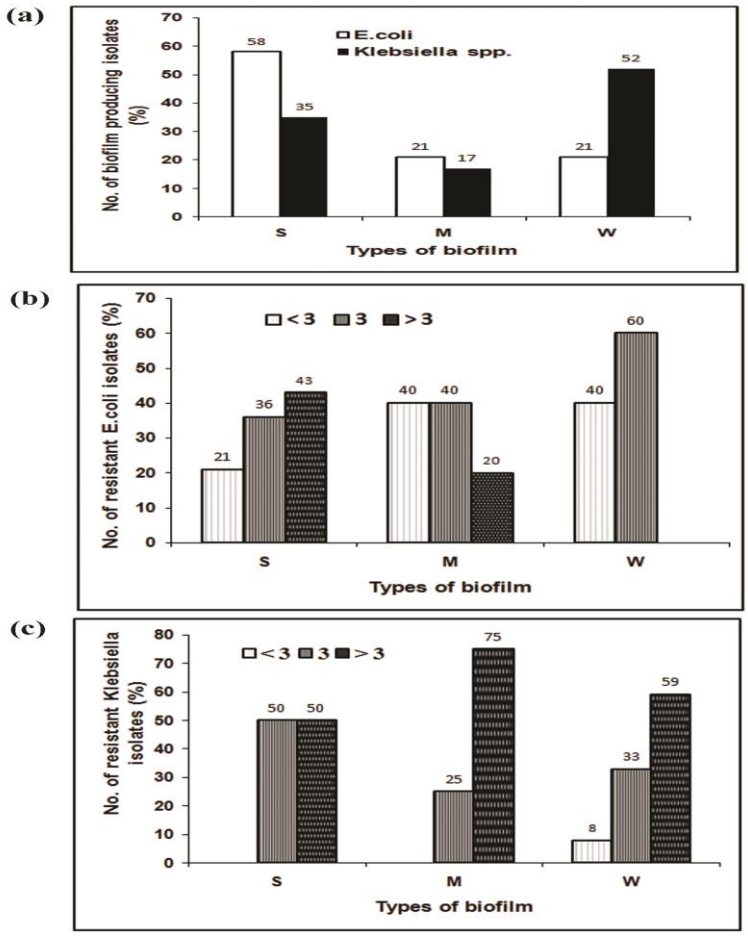

Figure 1: Biofilm formation and drug-resistance

(a) Percentage of *E.coli* and *Klebsiella* isolates producing strong (S), medium (M) and weak (W) biofilms. (b) and (c)Represents percentage of strong, medium and weak biofilm producing *E.coli* and *Klebsiella* spp. respectively, resistant to less than, equal to and greater than three different classes of antibiotics

ISSN: 0975-8585

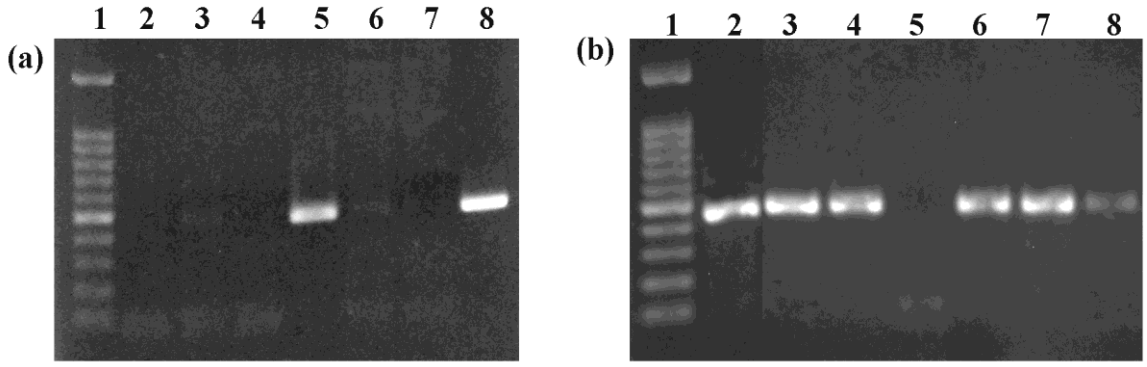

Figure 2: PCR amplification of *mrk*B-C region of *mrk*ABCDF operon

Lane 1 represents 100bp DNA ladder, Lanes 2 – 8 represents *mrk*$^{+}$ and *mrk*$^{-}$ isolates of (a) *E.coli* and (b) *Klebsiella* spp.

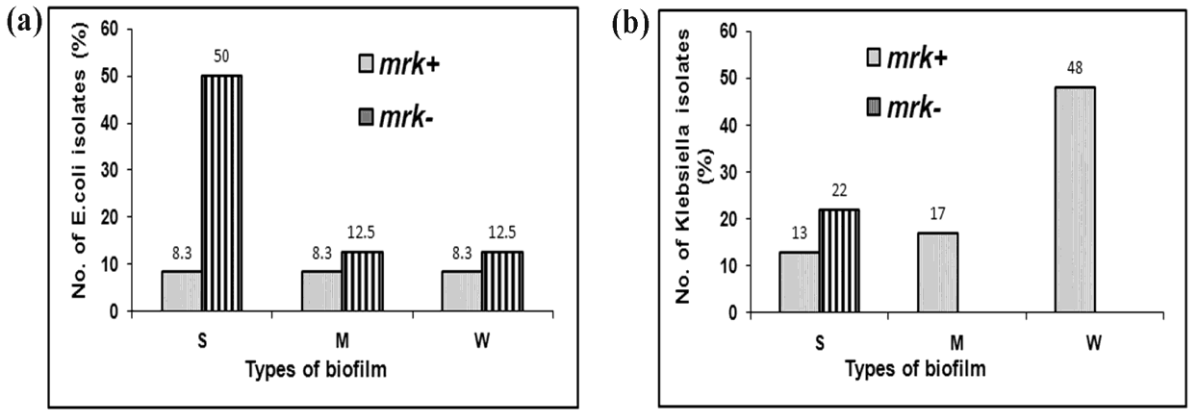

Figure 3: Biofilm formation and presence of *mrk*B-C region

(a) and (b) Represents percentage of strong (S), medium (M) and weak (W)) biofilm producing *E.coli* and *Klebsiella* spp. respectively, with (*mrk+*) and without (*mrk-*) *mrk* operon.

DISCUSSION

Biofilm formation on biotic and abiotic surfaces assume critical importance in healthcare settings enabling bacterial survival under tough conditions such as antibiotic selection, threat of destruction through the host immune system and nutrient deprivation. Bacteria harbouring conjugative plasmids can initiate biofilm formation, facilitating horizontal transfer of biofilm-associated genes along with resistance genes leading to rapid spread of drug resistance among the bacterial community [2, 12, 14]. This study was conducted on plasmid-bearing clinical bacterial isolates to investigate the relationship of the extent of biofilm formation with the degree of antibiotic resistance and the presence of a marker region of *mrk* operon strongly associated with biofilm formation. An association between biofilm production and antibiotic resistance was observed among *E.coli* isolates, in which most of the isolates resistant to ≥ 3 different classes of antibiotics were found to be strong or moderate biofilm producers. However, such an association could not be noticed in *Klebsiella* spp., in which biofilm production was found to be weak in spite of high antibiotic resistance. It has been shown previously that β lactasmases belonging to class A and D have an 'antibiofilm effect' as they interfered with peptidoglycan remodeling associated with attachment to surfaces prior to biofilm formation [24-26]. In fact, high resistance against β-lactams was observed in *Klebsiella* spp. tested in our study, which, in turn, explains the weak biofilm formation observed in these isolates. This observation is further supported by a recent report by Naparstek et al, 2014 [27] on reduced biofilm forming ability in KPC-producing *K.pneumoniae*.

The prevalence of the *mrk* operon encoding type3 fimbriae, strongly associated with biofilm formation, has been reported to be more common in *Klebsiella* spp. than in *E.*coli [14]. We have observed a

higher prevalence of the operon-associated *mrk* B-C region in plasmid DNA of *Klebsiella* in comparison to that found in *E.coli* despite the impaired potential for biofilm formation in the former. Moreover, in *Klebsiella,* overexpression of *mrk*J gene, known to be present adjacent to the *mrk*ABCDF gene cluster, encoding a phosphodiesterase has been reported to result in decreased fimbrial production [28]. Thus, the presence of *mrk* operon *per se* does not ensure biofilm formation. This view, indeed, is further reinforced by our results with *E.coli* isolates, wherein, the prevalence of *mrk*B-C was found to be low despite the capability for strong biofilm formation.

In conclusion, significant association between antibiotic resistance and biofilm formation was better evident in *E.coli* isolates than in *Klebsiella* spp. Also, an inverse relationship observed, in both of these isolates, between biofilm formation and prevalence of *mrk* operon allows us to logically conclude that the mere presence of an *mrk* operon does not confer biofilm forming ability.

ACKNOWLEDGEMENTS

This work was supported by a grant from Indian Council of Medical Research to N.N.

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