Pathogens and Disease, 74, 2016, ftw056

doi: 10.1093/femspd/ftw056
Advance Access Publication Date: 7 July 2016
Short Communication

SHORT COMMUNICATION

Penetration barrier contributes to bacterial biofilm-associated resistance against only select antibiotics, and exhibits genus-, strain- and antibiotic-specific differences

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One sentence summary: This study utilizes a disk diffusion assay to determine the role of penetration limitation in bacterial biofilm-associated drug resistance, with multiple genera, strain/isolate and antibiotic combinations.
Editor: Tom Coenye

ABSTRACT

Bacterial biofilms are implicated in a wide range of implant-based and chronic infections. These infections are often associated with adverse therapeutic outcomes, owing to the decreased antibiotic susceptibility of biofilms compared with their planktonic counterparts. This altered biofilm susceptibility has been attributed to multiple factors, including a reduced antibiotic penetration. Although several studies have addressed the role of penetration barrier in biofilm-associated drug resistance, it remains inconclusive. This study was done to elucidate antibiotic penetration through biofilms formed by *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli* and *Klebsiella pneumoniae*, using an agar disk diffusion assay. Penetration capacity of six antimicrobial drugs from different classes (β-lactams, aminoglycosides, tetracyclines, phenicols, fluoroquinolones and glycopeptides) through biofilms formed by standard strains and clinical isolates from catheter-related bloodstream infections (CRBSI) was elucidated by measuring their growth-inhibition zones in lawn cultures on Mueller–Hinton agar, following diffusion of an antibiotic from an overlying disk through their biofilm to the agar medium. Penetration of only select antimicrobials (vancomycin and chloramphenicol) was hindered through biofilms. There was considerable variation in biofilm-permeating capacity depending upon the genus, strain/CRBSI isolate and antibiotic tested. Furthermore, antibiotics failed to kill the biofilm cells independent of penetration, indicating that other factors contributed substantially to biofilm resistance.

Keywords: antibiotics; biofilms; disk diffusion; matrix; penetration

Received: 22 April 2016; **Accepted:** 2 June 2016

Biofilms are aggregates of microbial cells adhering to a surface or an interface or to each other, and encased in an extracellular-polymeric-substance matrix comprising of polysaccharides, proteins and DNA. These microbial communities have been implicated in a wide range of implant-based and chronic infections, which are often difficult to treat owing to the significantly decreased antibiotic susceptibility of biofilm cells compared with the planktonic cultures. The biofilm-associated antibiotic resistance has been attributed to multiple factors, including barrier effect of the matrix, slow growth, spatial heterogeneity, increased expression of antibiotic-degrading enzymes and efflux pumps, stress response, signaling pathways and the presence of drug-resistant or tolerant physiologies (Sun *et al.* 2013). Amongst these mechanisms, the role of reduced antibiotic penetration in conferring biofilm resistance has received considerable attention, but still remains inconclusive.

The decreased antibiotic permeation through biofilms may result from the biofilm architecture per se—the thick, multilayered organization, presence of biofilm matrix, antibiotic-degrading enzymes or antibiotic adsorption by the microbial cells. Many studies indicate that the biofilm matrix retards antibiotic penetration via diffusion limitations or ionic interactions, leading to a reduced drug exposure of biofilm cells and hence an apparent decrease in drug efficacy; others however suggest that it does not contribute to biofilm recalcitrance (Farber, Kaplan and Clogston 1990; Dunne, Mason and Kaplan 1993; Darouiche *et al.* 1994; Kumon *et al.* 1994; Stewart 1994; Anderl, Franklin and Stewart 2000; Stone *et al.* 2002; Zahller and Stewart, 2002; Jefferson, Goldmann and Pier 2005; Mathur *et al.* 2005; Rani, Pitts and Stewart 2005; Rodríguez-Martínez, Ballesta and Pascual 2007; Drăcea *et al.* 2009; Stewart, Davison and Steenbergen 2009; Singh *et al.* 2010). Furthermore, while the role of penetration limitation has been extensively studied in staphylococci, only limited literature is available on antibiotic penetration through biofilms formed by clinical isolates of *Escherichia coli* (Stone *et al.* 2002; Rodríguez-Martínez, Ballesta and Pascual 2007; Drăcea *et al.* 2009) and *Klebsiella pneumoniae* (Anderl, Franklin and Stewart 2000; Zahller and Stewart 2002), another two common bacteria implicated in such infections apart from *Staphylococcus aureus* and *S. epidermidis*.

Antimicrobial penetration through biofilms has been tested in the literature by a variety of methods, including equilibrium dialysis experiments (Dunne, Mason and Kaplan 1993), diffusion-cell bioassays (Anderl, Franklin and Stewart 2000), sandwich-cup methods (Kumon *et al.* 1994), fluorescent tracers (Stone *et al.* 2002; Jefferson, Goldmann and Pier 2005; Rani, Pitts and Stewart 2005) or susceptibility testing in media containing slime (Farber, Kaplan and Clogston 1990; Mathur *et al.* 2005). In a previous publication, we reported the use of an agar disk-diffusion-based assay to elucidate the penetration of antibiotics through *S. aureus* (ATCC 29213) and *S. epidermidis* (ATCC 35984) biofilms. The assay is based upon Kirby–Bauer disk diffusion technique and measures the diameter of zones of growth inhibition (ZOI) in lawn cultures on Mueller-Hinton agar plates, following diffusion of an antibiotic from an overlying antibiotic disk through the biofilm to the agar medium versus the respective control assemblies (Singh *et al.* 2010). In the present study, this assay was further improved for better inference and employed to determine the penetrating capacity of different antibiotic classes through biofilms formed by standard strains and clinical isolates of four common bacteria involved in biofilm-associated infections, namely *S. aureus*, *S. epidermidis*, *E. coli* and *K. pneumoniae*.

The work was carried out on standard strains *S. aureus* ATCC 29213; *S. epidermidis* ATCC 35984; and *E. coli* ATCC 25922, and the clinical isolates of *S. aureus* ($n = 5$), *S. epidermidis* ($n = 3$), *E. coli* ($n = 5$) and *K. pneumoniae* ($n = 4$) from catheter-related bloodstream infections (CRBSI). The strains were preserved in brain–heart infusion broth with 15% glycerol at –20°C. Six routinely used representative antibiotics belonging to different classes were tested. These included imipenem (β-lactam; penem), cefotaxime (β-lactam; the third-generation cephalosporin), amikacin (aminoglycoside), tetracycline (tetracycline), chloramphenicol (phenicol) and ciprofloxacin (fluoroquinolone) for *E. coli* and *K. pneumoniae*, and vancomycin (glycopeptide), cefotaxime, amikacin, tetracycline, chloramphenicol and ciprofloxacin for *S. aureus* and *S. epidermidis*. The antibiotic contents chosen (Table 1) represent the concentrations recommended by Clinical and Laboratory Standards Institute (CLSI, 2008, 2012 and 2014).

The method of Singh *et al.* (2010) was followed to prepare colony biofilms. Briefly, overnight cultures grown in tryptic soy broth at 37°C were diluted in the same medium to an OD_{600} = 0.05 with normal saline, and a 10 μl drop (20 μl for *S. epidermidis*) was used to seed polycarbonate membranes (diameter, 13 mm; pore size, 0.4 μm; Whatman International Ltd.) placed on tryptic soy agar plates. The plates were incubated at 37°C for 48 h, with transfer of membrane-supported biofilms to fresh culture medium in between after 24 h. Inoculation volume, density and incubation time were chosen to obtain mature biofilms with mean diameter more than 6 mm, i.e. size of overlying antibiotic disk used during antibiotic penetration experiments, to prevent false negative results. Biofilm structure was confirmed by scanning electron microscopy.

The experimental setup used to track the penetration of antibiotics through biofilms is based on the method reported earlier (Singh *et al.* 2010), with slight modifications. Forty-eight hour old membrane-supported biofilms were transferred to Mueller Hinton agar plate pre-inoculated with a bacterial suspension set to McFarland standard 0.5, so as to give a confluent lawn of growth after incubation. In a partial modification to the previous study (Singh *et al.* 2010), lawn inoculations were done with the same strain/isolate whose biofilm was being tested, instead of a common standard reference strain used earlier in the indicator lawns for all biofilms; this is likely to yield results with more relevance for the concerned strain/isolate. An antibiotic disk pre-moistened with 25 μl of sterile water was then placed above the biofilm. Control assemblies comprising of sterile membranes and antibiotic disks, without biofilm, were set up in parallel. Another set of controls with antibiotic disk alone was also tested to confirm that the presence of the polycarbonate membrane does not affect antibiotic permeation or ZOI diameters (and susceptibility profiles) of the lawn cultures. The plates were incubated for 24 h at 37°C, and ZOI diameters were measured up to the nearest whole millimeters. Lower limit of detection for this assay was 13 mm, i.e. the diameter of membranes used for biofilm culture. Two biological replicates and two technical replicates were performed for each experiment. The results are presented as an average of all these four replicate setups. Two tailed, unpaired t-test assuming unequal variance was used to statistically compare the ZOI in test versus the respective control assemblies for each strain/CRBSI isolate and drug combination, as a measure of antibiotic penetration through biofilms. Percentage reduction in antibiotic penetration was determined by calculating the area (πr^2) of ZOI in test versus control assemblies. The ZOIs were also interpreted for susceptibility according to the guidelines of CLSI (2014), except for cefotaxime and vancomycin against *Staphylococcus* spp., which were interpreted

Table 1. Penetration of antibiotics through bacterial biofilms.

Bacterial strain/isolate		Diameter of the zone of growth inhibition (mm; Mean ± SD)						
		Imipenem (10 μg)	Cefotaxime (30 μg)	Amikacin (30 μg)	Tetracycline (30 μg)	Chloramphenicol (30 μg)	Ciprofloxacin (5 μg)	Vancomycin (30 μg)
E. coli								ND
ATCC 25922								
	T	29 ± 1 (S)	30 ± 3 (S)	27 ± 3 (S)	16 ± 2 (S)	≤13 ± 0 (I/R)*	32 ± 2 (S)	
	C	30 ± 1 (S)	33 ± 3 (S)	29 ± 1 (S)	20 ± 1 (S)	22 ± 1 (S)	37 ± 5 (S)	
CRBSI 1								
	T	25 ± 3 (S)	NA	18 ± 1(S)	NA	14 ± 3 (I)*	NA	
	C	30 ± 1 (S)	≤13 ± 0	20 ± 1 (S)	≤13 ± 0	20 ± 3 (S)	≤13 ± 0	
CRBSI 2								
	T	30 ± 0 (S)	34 ± 2 (S)	21 ± 4 (S)	NA	≤13 ± 0 (I/R)*	24 ± 1 (S)	
	C	31 ± 1 (S)	34 ± 3 (S)	26 ± 2 (S)	≤13 ± 0	22 ± 2 (S)	31 ± 4 (S)	
CRBSI 3								
	T	32 ± 4 (S)	37 ± 3 (S)	25 ± 2 (S)	29 ± 1 (S)	16 ± 4 (I)*	31 ± 8 (S)	
	C	37 ± 4 (S)	41 ± 4 (S)	30 ± 1 (S)	28 ± 0 (S)	28 ± 5 (S)	37 ± 6 (S)	
CRBSI 4								
	T	27 ± 1 (S)	NA	22 ± 1 (S)	NA	≤13 ± 0 (I/R)*	≤13 ± 1 (R)	
	C	31 ± 1 (S)	≤13 ± 0	25 ± 0 (S)	≤13 ± 0	25 ± 1 (S)	15 ± 0 (R)	
K. pneumoniae								ND
CRBSI 1								
	T	31 ± 4 (S)	NA	NA	NA	18 ± 0 (S)	NA	
	C	32 ± 3 (S)	≤13 ± 0	≤13 ± 0	≤13 ± 0	18 ± 0 (S)	≤13 ± 0	
CRBSI 2								
	T	25 ± 1 (S)	34 ± 2 (S)	19 ± 3 (S)	20 ± 2 (S)	14 ± 1 (I)*	31 ± 2 (S)	
	C	27 ± 1 (S)	35 ± 4 (S)	23 ± 4 (S)	23 ± 1 (S)	25 ± 2 (S)	36 ± 2 (S)	
CRBSI 3								
	T	25 ± 2 (S)	NA	16 ± 2 (I)	NA	NA	NA	
	C	26 ± 2 (S)	≤13 ± 0	18 ± 1 (S)	≤13 ± 0	≤13 ± 0	≤13 ± 0	
CRBSI 4								
	T	26 ± 1 (S)	NA	16 ± 1 (I)*	NA	14 ± 1 (I)*	≤13 ± 0 (R)	
	C	27 ± 1 (S)	≤13 ± 0	20 ± 1 (S)	≤13 ± 0	23 ± 1 (S)	16 ± 2 (I)	
S. epidermidis		ND						
ATCC 35984								
	T		21 ± 1 (I)	18 ± 2 (S)	35 ± 1 (S)	14 ± 1 (I)*	34 ± 3 (S)	14 ± 1 (R)*
	C		20 ± 1 (I)	23 ± 1 (S)	35 ± 1 (S)	22 ± 1 (S)	36 ± 3 (S)	21 ± 1 (S)
CRBSI 1								
	T		35 ± 1 (S)	26 ± 2 (S)	35 ± 1 (S)	20 ± 2 (S)	23 ± 1 (S)	≤13 ± 0 (R)*
	C		34 ± 1 (S)	28 ± 3 (S)	34 ± 1 (S)	20 ± 1(S)	24 ± 0 (S)	20 ± 1 (S)
CRBSI 2								
	T		17 ± 1 (I)	24 ± 1 (S)	36 ± 2 (S)	27 ± 2 (S)	20 ± 4 (I)	≤13 ± 0 (R)*
	C		20 ± 2 (I)	26 ± 1 (S)	36 ± 1 (S)	27 ± 1 (S)	23 ± 4 (S)	21 ± 1 (S)
CRBSI 3								
	T		38 ± 3 (S)	29 ± 1 (S)	31 ± 1 (S)	23 ± 4 (S)	36 ± 8 (S)	17 ± 4 (S)*
	C		38 ± 3 (S)	31 ± 0 (S)	30 ± 0 (S)	26 ± 1 (S)	36 ± 8 (S)	24 ± 0 (S)
S. aureus		ND						
ATCC 29213								
	T		17 ± 1 (I)*	23 ± 1 (S)	29 ± 2 (S)	24 ± 2 (S)	27 ± 4 (S)	≤13 ± 0 (R)*
	C		26 ± 1 (S)	25 ± 1 (S)	29 ± 1 (S)	26 ± 2 (S)	28 ± 4 (S)	20 ± 0 (S)
CRBSI 1								
	T		31 ± 1 (S)	20 ± 0 (S)	21 ± 0 (S)	27 ± 0 (S)	17 ± 1 (I)	≤13 ± 0 (R)*
	C		32 ± 3 (S)	21 ± 1 (S)	22 ± 1 (S)	27 ± 0 (S)	19 ± 2 (I)	23 ± 0 (S)

Table 1. *(Continued)*.

Bacterial strain/isolate		Diameter of the zone of growth inhibition (mm; Mean ± SD)						
		Imipenem (10 μg)	Cefotaxime (30 μg)	Amikacin (30 μg)	Tetracycline (30 μg)	Chloramphenicol (30 μg)	Ciprofloxacin (5 μg)	Vancomycin (30 μg)
CRBSI 2								
	T		33 ± 0 (S)	22 ± 2 (S)	16 ± 0 (I)	22 ± 2 (S)	15 ± 1(R)	≤13 ± 0 (R)*
	C		33 ± 1 (S)	25 ± 0 (S)	17 ± 1 (I)	23 ± 1 (S)	16 ± 0 (I)	22 ± 0 (S)
CRBSI 3								
	T		32 ± 1 (S)	26 ± 2 (S)	31 ± 1 (S)	29 ± 3 (S)	32 ± 2 (S)	14 ± 1 (R)*
	C		33 ± 1 (S)	32 ± 2 (S)	33 ± 2 (S)	30 ± 3 (S)	34 ± 1 (S)	21 ± 1 (S)
CRBSI 4								
	T		32 ± 3 (S)	25 ± 1 (S)	28 ± 5 (S)	26 ± 5 (S)	15 ± 3 (R)	15 ± 3 (R)
	C		34 ± 1(S)	27 ± 3 (S)	30 ± 2 (S)	28 ± 4 (S)	15 ± 3 (R)	20 ± 1 (S)

The results are represented as diameters of the ZOI, obtained on Mueller Hinton agar plates in test (biofilm) and control (membrane plus disk) assemblies. The data has been rounded up to the nearest whole mm and interpreted for susceptibility, according to the guidelines of Clinical Laboratory and Standards Institute (CLSI 2014), except for cefotaxime and vancomycin against *Staphylococcus* spp., which were interpreted according to CLSI (2012) and CLSI (2008) guidelines, respectively, as these antibiotics are presently not tested routinely by disk diffusion. The limit of detection for this assay was 13 mm i.e. the diameter of the polycarbonate membranes used for biofilm culture. CRBSI, isolates from catheter-related bloodstream infections; *, $P < 0.05$ in ZOI of test versus control assembly; S, susceptible; I, Intermediate; R, Resistant; NA, not applicable (for test set-ups with control assemblies showing ZOI ≤ 13); ND, not done (vancomycin for *E. coli* and *K. pneumoniae*; imipenem for *S. epidermidis* and *S. aureus*).

according to CLSI (2012) and CLSI (2008) guidelines, respectively, as these antibiotics are presently not tested routinely by disk diffusion. These susceptibility profiles in test and controls assemblies were noted with an aim to correlate whether a significant decrease in biofilm penetration, where observed, was substantial enough to limit the antibiotic level within biofilms to an extent that would alter the observable susceptibility profile of the indicator lawn beneath them—the same effect can then be inferred for the biofilm cells, since the indicator lawn inoculum comprised of the same strain/isolate whose biofilm was being tested. The association between a significant reduction in biofilm penetration and the corresponding change in susceptibility profiles was analyzed statistically by Fisher's exact test.

Mean CFUs in biofilms after antibiotic penetration were determined by drop plating of two representative test assemblies in each bacterium: one whose ZOI was similar in test and control (indicative of efficient antibiotic penetration) and the second whose ZOI differed significantly ($P < 0.05$) between test and control (indicative of a reduced antibiotic penetration). The CFU counts of pre-treatment and untreated biofilms were performed simultaneously.

The results are described in Table 1. Biofilm penetration was found to vary amongst different antibiotic classes used, bacterial genus tested, and for some antibiotics, even between multiple strain/isolates of the same species. The average percentage reduction in penetration was 14%, 11%, 22%, 9%, 34%, 18% and 57% for imipenem, cefotaxime, amikacin, tetracycline, chloramphenicol, ciprofloxacin and vancomycin, respectively. In *S. aureus* and *S. epidermidis*, the glycopeptide vancomycin was significantly hindered (average reduction, 57%; $P < 0.05$) through biofilms formed by standard strains and all CRBSI isolates, possibly owing to its high molecular weight (=1450) and thereby entrapment in the matrix (Souli and Giamarellou 1998). Chloramphenicol was impeded by all *E. coli* biofilms (average reduction, 64%; $P < 0.05$) but exhibited strain/isolate specific variation in biofilms of staphylococci and *K. pneumoniae*. Amikacin (aminoglycoside), cefotaxime and imipenem (β-lactams), ciprofloxacin (fluoroquinolone) and tetracycline penetrated without significant impediment through nearly all biofilms, except one *K. pneumoniae* CRBSI isolate for amikacin and the strain *S. aureus* ATCC 29213 for cefotaxime. Such variation observed amongst genera and/or isolates may be due to differences in the matrix composition and concentration, mesh size and viscosity, biofilm architecture or biofilm-forming capacity (Simitsopoulou *et al.* 2013).

The present results corroborate with some of the previous studies regarding vancomycin impediment in staphylococcal biofilms (Farber, Kaplan and Clogston 1990; Souli and Giamarellou 1998; Mathur *et al.* 2005; Kostenko, Ceri and Martinuzzi 2007; Doroshenko *et al.* 2014; Siala *et al.* 2014), except a few (Dunne, Mason and Kaplan 1993; Darouiche *et al.* 1994). These results also support the unrestricted penetration demonstrated for ciprofloxacin and tetracycline in an uropathogenic *E. coli* isolate (Stone *et al.* 2002; Rodríguez-Martínez, Ballesta and Pascual 2007), and that for beta-lactams (ampicillin) and ciprofloxacin shown in an environmental *K. pneumoniae* isolate (Anderl, Franklin and Stewart 2000; Zahller and Stewart 2002). In contrast to the present data, however, certain studies report that penetration of select beta-lactams is hindered by *E. coli* biofilms (Drăcea *et al.* 2009), and that of beta-lactams, aminoglycosides, fluoroquinolones and doxycycline is hindered by staphylococcal biofilms (Souli and Giamarellou 1998). Further, the difference observed in the results of cefotaxime penetration for *S. epidermidis* ATCC 35984 biofilms in our previous study (significant reduction in ZOI) versus the present work (insignificant reduction) is perhaps attributed to the difference in the indicator lawn used, which was a common standard strain (*S. aureus* ATCC 29213) in the previous study (Singh *et al.* 2010) compared with the same biofilm parent strain (*S. epidermidis* ATCC 35984) in the present study, as *S. epidermidis* ATCC 35984 is methicillin resistant whereas *S. aureus* ATCC 29213 is methicillin sensitive. Usage of the same biofilm isolate/strain in its indicator lawn is thus likely to yield results more relevant to the concerned isolate/strain. To our knowledge, not much data is available in the prior literature on the biofilm penetrating capacity of chloramphenicol.

A significant reduction in antibiotic permeation through biofilms, where observed, also shifted the susceptibility profile in the test assembly set-up to 'intermediate' or 'resistant'

compared with 'susceptible' in the control assembly set-up ($P < 0.001$) (Table 1). As the lawn inoculum comprised of the same strain/isolate whose biofilm was being tested, these results reflect the therapeutic consequence resulting from penetration limitation of the concerned antibiotic(s) through biofilms. No such shift in susceptibility profiles of test-assembly set-ups was noted for those antibiotics that permeated well (Table 1). These antibiotics nevertheless failed to efficiently kill all the biofilm cells, alike ($P > 0.05$) the antibiotics that were significantly hindered, thus, indicating the multifactorial nature of biofilm resistance and involvement of mechanisms other than the lack of drug penetration (Singh *et al.* 2009) in such recalcitrance. The mean $\log_{10}$ decrease in CFU counts for test setups with and without significant reduction in antibiotic penetration were 0.50 and 1.05 respectively for *E. coli*, 1.30 and 0.88 respectively for *K. pneumoniae*, 0.61 and 0.14 respectively for *S. epidermidis*, and 0.63 and 0.36 respectively for *S. aureus* compared with the pre-treatment biofilms. Similar results were obtained when the test setups were compared with the untreated biofilm controls, as the 48-h-old-biofilms used in the experiments did not grow further over the 24-h treatment period. The mean $\log_{10}$ CFU counts/membrane for pre-treatment and untreated biofilms were 9.86 ± 0.55 and 9.54 ± 0.57 respectively for *E. coli*, 9.93 ± 0.13 and 9.80 ± 0.76 respectively for *K. pneumoniae*, 9.63 ± 0.35 and 9.69 ± 0.32 respectively for *S. epidermidis*, and 10.09 ± 0.12 and 9.72 ± 0.63 respectively for *S. aureus*.

A recent study, based on our earlier model, has also shown that efficient penetration may not correlate with killing efficacy in *Bacillus cereus* and *Pseudomonas fluorescens* biofilms (Araújo *et al.* 2014). These findings substantiate the hypothesis in our previous study (Singh *et al.* 2010) that the tested method is relevant for efficacy testing of only those antimicrobials where penetration limitation contributes to biofilm-associated drug resistance. Under such conditions, the ZOI diameters are significantly reduced in test versus control assemblies, and the susceptibility profile of the indicator lawn shifts from susceptible to intermediate or resistant. In contrast, when other factors govern the resistance of bacterial biofilms, the ZOI diameters obtained following drug penetration do not differ significantly between test and control assemblies, and are not an indicator of drug efficacy. The model can nevertheless be useful to determine biofilm-penetrating capacity of antibiotics and its clinical significance in multiple genera, isolate and drug combinations.

It should also be noted that the penetration profiles of the antibiotics might vary depending upon their concentrations tested. Given a high enough concentration, any molecule may freely penetrate, as long as the saturation point of the adsorbing material is surpassed. Similarly, given a low enough concentration, the diffusion of any molecule can be impeded. The present work was carried out at a fixed concentration for each antibiotic, and the results obtained are an indicator of the antibiotic's penetration at that specific concentration only. Nonetheless, the concentrations selected in this study represent the concentrations recommended by the CLSI guidelines for antibiotic susceptibility testing in patient care and diagnostics, based on the available *in vitro*, pharmacokinetics-pharmacodynamics and clinical studies (CLSI 2014). The results obtained with these concentrations are thus likely to be physiologically relevant. Further, the present assay is a modified, simpler version of the method reported by Anderl, Franklin and Stewart (2000). In that system, the antibiotic was incorporated into an agar plate, followed by placement of the biofilm-containing membrane, and then an empty disk over the biofilm. Penetration was noted in terms of the amount of antibiotic that diffused from the medium through the biofilm into the empty disk, and was measured as ZOI resulting from this disk using a separate bioassay plate containing *E. coli*. In the present method, a reverse setup has been used, wherein a pre-loaded antibiotic disk is overlaid on the biofilm, and the penetration from the disk through the biofilm to the agar medium is determined with an indicator lawn on the same plate and comprising of the same isolate/strain whose biofilm is being tested.

In conclusion, the present findings imply that penetration limitation contributes to biofilm-associated drug resistance for only select antimicrobials (such as vancomycin and chloramphenicol), and there is considerable variation in biofilm-permeating capacity depending on bacterial genus, interstrain/CRBSI isolate and antibiotic tested. Even antibiotics that penetrated well through the biofilms failed to kill all the biofilm cells, indicating that factors other than penetration limitation contribute substantially to biofilm resistance.

FUNDING

The study was partly supported by Research Associateship award no. 09/141(0178)/2011-EMR I and INSPIRE Faculty Scheme award no. IFA13-LSBM-88 granted to RS by Council of Scientific and Industrial Research (CSIR) and Department of Science and Technology (DST), Govt. of India respectively.

Conflict of interest. None declared.

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