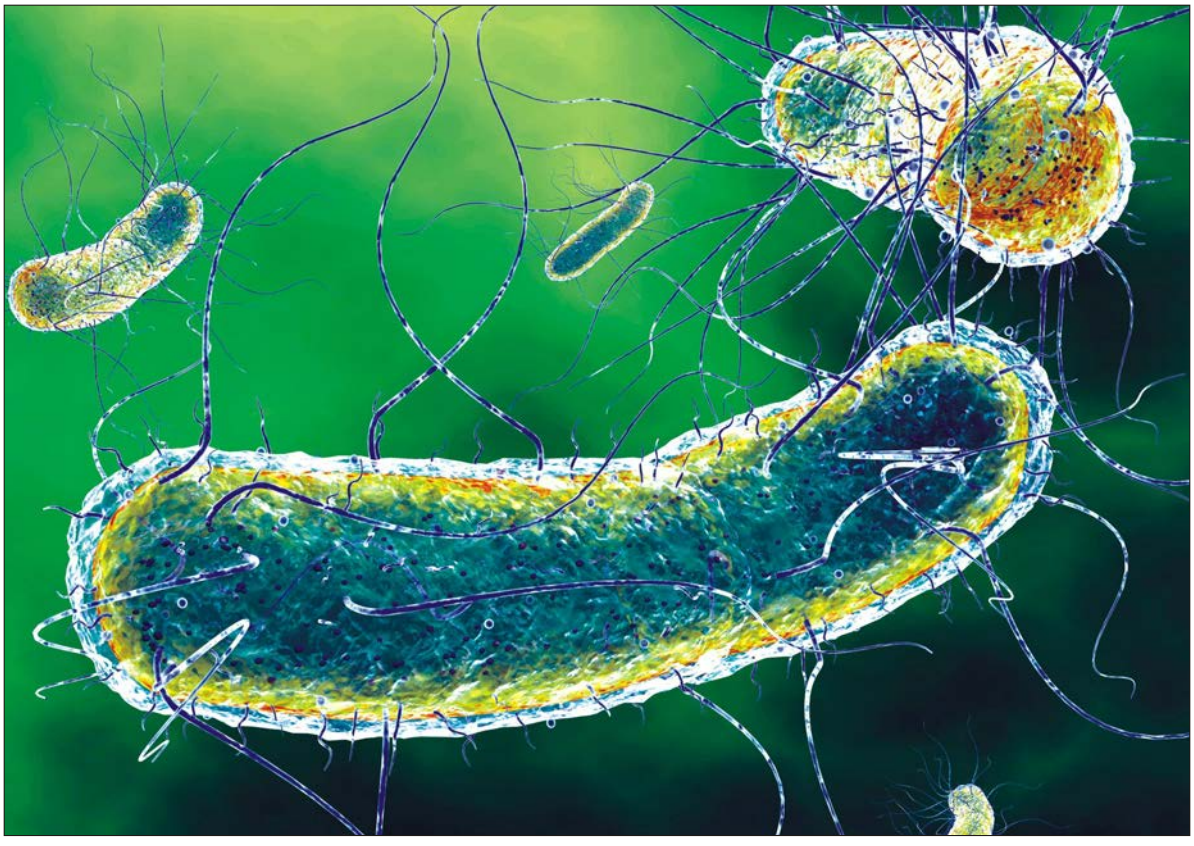

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Biofilm Formation by Methicillin Resistant *Staphylococcus aureus* and their Antibiotic Susceptibility Pattern: An *in vitro* Study

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ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) is associated with high morbidity and mortality rates because of the development of multi-drug resistance and their ability to form biofilms. The present study was conducted to detect the biofilm formation among MRSA isolates and to determine their antibiogram-resistogram profile. A total of 259 *S. aureus* isolates were collected from tertiary care hospitals in Coimbatore, South India. They were subjected to antibiotic susceptibility testing by Kirby Bauer technique and thereafter screened for biofilm formation by three methods viz., Congo Red Agar Method, Tube Method and Tissue Culture Plate method. Out of 259 *S. aureus* isolates, 209 (80.69%) were confirmed as MRSA and only 35 (16.76%) of the MRSA isolates were found to be strong biofilm producers by Tissue Culture Plate method on the basis of optical density values. The antibiotic resistance pattern of biofilm producing MRSA isolates were found to be highly variable with maximum resistance (100%) to ampicillin, ampicillin/sulfbactam, ofloxacin, tetracycline, ciprofloxacin, cotrimoxazole and 100% sensitivity observed towards vancomycin. The statistical analysis revealed that TCP method showed higher specificity when compared to other two methods. Thus, biofilm producers were found to be highly resistant to almost all the classes of antibiotics except vancomycin. According, to this study, vancomycin was the effective drug of choice for the treatment of biofilm producing MRSA infections and Tissue culture plate is the reliable method for detecting the biofilm formation.

Key words: Methicillin resistant *Staphylococcus aureus*, biofilm, tissue culture plate method, vancomycin, multi-drug resistant

INTRODUCTION

Staphylococcus aureus is a significant human pathogen in both hospitals and community and the first Methicillin resistant *Staphylococcus aureus* (MRSA) isolates were detected in the hospital settings in the early 1960 (Durand *et al*., 2006). The MRSA represents a major challenge to hospitals all over the world due to the emergence and spread of isolates with decreased susceptibilities to numerous antibiotics classes, in addition to methicillin and other members of the β-lactam family (Gomes *et al*., 2006). Moreover, there is a possibility of extensive epidemic with multiple drug resistant MRSA which would be difficult to control (Pai *et al*., 2010). As MRSA infection's therapeutic outcome is worse than those from methicillin-sensitive *S. aureus* (MSSA) (Cosgrove *et al*., 2003).

Microbial biofilms constitute a major reason for infections to occur as well as to persist at various sites in the human body. *Staphylococcus aureus* being one of the major gram positive pathogens

which causes an array of infections has the ability to colonise biofilms on damaged tissue and/or implanted biomaterials (Donlan, 2001). These organisms can persist in clinical settings and gain increased resistance to antimicrobial agents through biofilm formation which appears to be a bacterial survival strategy (Donlan and Costerton, 2002; Hall-Stoodley *et al.*, 2004). Therefore, MRSA biofilms becomes resistant to almost all available antimicrobial agents used for its treatments (Gotz, 2002). Production of biofilms can be a marker of virulence which can be detected phenotypically (Jain and Agarwal, 2009). The information on the capacity of a clinical isolate to produce biofilm would help a clinician to evaluate the measure of its virulence and devise an appropriate treatment plan for the patient. In the present study, the biofilm producing potential of MRSA isolates by Tissue Culture Plate method (TCP), Tube Method (TM) and Congo Red Agar (CRA) method were detected in order to assess the reliability of these methods to be used as a suitable screening technique in routine clinical laboratories, to determine their antibiotic profile and analyze their association which assumes great significance.

MATERIALS AND METHODS

Bacterial isolates: A total of 259 consecutive non-duplicate clinical isolates of *S. aureus* from various clinical specimens such as pus, urine and blood were obtained from tertiary care hospitals in Coimbatore, South India from August 2010 to November 2012. The organisms were identified on the basis of colonial morphology, gram-positive cluster-like appearance on staining, positive catalase and coagulase tests. The cultures were inoculated on trypticase soy broth with 16% glycerol (v/v) and kept at -20°C (Kloos and Bannerman, 1999). Methicillin resistance was confirmed using a cephoxitin (30 μg) disk on Mueller-Hinton agar (Hi-media, Mumbai, India) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011).

Antibiotic susceptibility testing: Antibiogram of MRSA was based on the susceptibility patterns for selected antibiotics representing various classes of antimicrobial agents (Table 1). The antibiotic susceptibility pattern was determined by Kirby Bauer disc diffusion method. The entire surface of the Mueller-Hinton Agar (MHA) plate with 2% NaCl was covered with inoculums of *S. aureus*, turbidity matching 0.5 McFarland standards, by a sterile cotton swab stick and the plate was air-dried before antibiotic discs were laid on the surface (CLSI, 2011).

Detection of biofilm formation: All the 209 MRSA isolates were subjected for biofilm formation. The methods used in this study include TCP (Christensen *et al.*, 1985), TM (Christensen *et al.*, 1982) and CRA method (Freeman *et al.*, 1989) without any modifications. Black colour colonies were observed in CRA method which was considered as positive and pink colour colonies suggested negative biofilm phenotype. In TM, adherence was observed as ring formation on the inside walls of the test tube when stained with crystal violet. Optical Densities (OD) of stained adherent bacteria in TCP method were determined with a micro ELISA auto reader at wavelength of 570 nm (OD 570 nm). These OD values were considered for an index of bacteria adhering to surface and forming biofilms. OD<0.120 were considered for weak biofilm producers. Optical Densities (OD) values between 0.120-0.240 were considered for moderate biofilm producers and OD>0.240 were considered strong biofilm producers.

Statistical analysis: Statistical Package for the Social Sciences (SPSS) software (SPSS Inc no.16) was used for data analysis. Chi-square test was used for analysis of categorical data. Statistical analysis was performed for all the three biofilm screening methods by using 2×2 table

Table 1: Antibiotic used in the study (classification and mechanism)

Classes of antibiotics	Antibiotics	Dose (µg)
Inhibitors of cell-wall synthesis		
Cephamycin (new C2G)	Cephoxitin	30
Glyco peptides	Vancomycin	30
Lipoglyco peptides	Teicoplanin	30
Beta-lactams		
Aminopenicillins	Ampicillin	10
Penicillin stable penicillins	Oxacillin	1
β-lactam/β-lactamase inhibitor combinations	Ampicillin/sulfbactam	10/10
	Amoxyclav	30
Inhibitors of protein synthesis		
Tetracyclines	Tetracycline	30)
Aminoglycosides	Gentamicin	10
Lincosamides	Clindamycin	2
Phenocols	Chloramphenicol	30
Oxazolidinones	Linezolid	30
Ansamycins	Rifampicin	5
Fusi danes	Fusidic acid	30
Monocarboxylic acid	Mupirocin	5
Tetracycline family group	Minocycline	30
Inhibitors of nucleic acid synthesis		
Fluoroquinolones	Ciprofloxacin	30
	Ofloxacin	5
	Levofloxacin	5
Antimetabolites	Co-trimoxazole	25

described by Greenhalgh (1997) and Gardner and Greiner (2006). A $p<0.0001$ was considered statistically significant. Parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy were also calculated. True positives are biofilm producers by TCP, TM and CRA method. False positives are biofilm producers by TM and CRA method and not by TCP method. True negatives are those which were non biofilms producers by all the three methods.

RESULTS

Antibiogram-resistogram profile of MRSA: Out of 259 *S. aureus* isolates, 209 (80.69%) isolates were found to be methicillin resistant and exhibited diverse resistant profile against 18 commonly used antibiotics and antibiotic modifications (Fig. 1). All the isolates showed 100% resistance to cotrimoxazole, tetracycline whereas, 100% sensitivity to vancomycin. The percentage of resistance to antibiotics such as amoxyclav, ciprofloxacin, ampicillin, gentamicin, ofloxacin, ampicillin/sulfbactam, mupirocin, levofloxacin, rifampicin, chloramphenicol, fusidic acid, teicoplanin, linezolid, clindamycin and minocycline was found to be in the order of 90.91, 87.57, 81.82, 65.56, 65.55, 62.20, 58.37, 55.68, 53.59, 51.67, 44.01, 35.89, 32.54, 24.40 and 31.58%, respectively.

Detection of biofilm formation: By the standard TCP assay, 35 (16.74%) isolates displayed strong biofilm formation. Optical Densities (OD) value of the stained adherent biofilm was

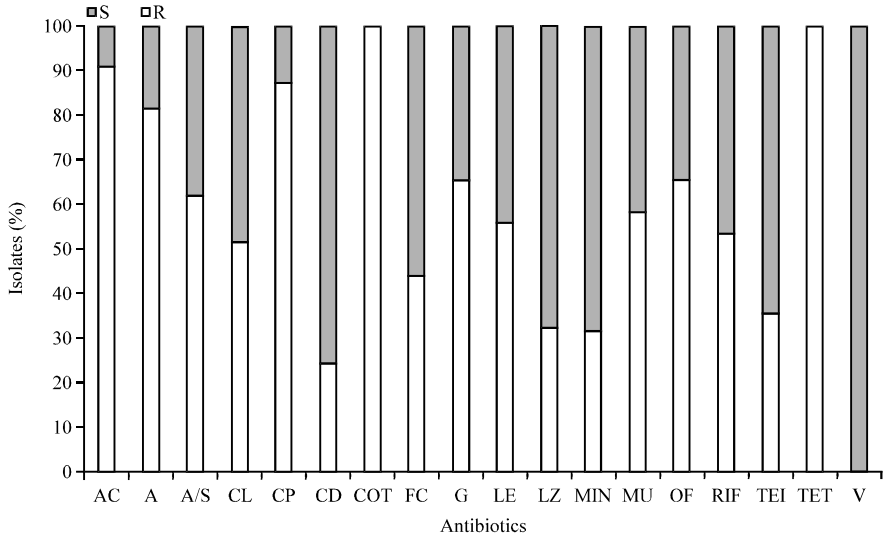

Fig. 1: Antibiotic susceptibility profile of methicillin resistant *Staphylococcus aureus* (n = 209). S: Sensitive, R: Resistant, AC: Amoxyclav, A: Ampicillin, A/S: Ampicillin/Sulfbactam, CL: Chloramphenicol, CP: Ciprofloxacin, CD: Clindamycin, COT: Co-trimoxazole, FC: Fusidic acid, G: Gentamicin, LE: Levofloxacin, LZ: Linezolid, MIN: Minocycline, MU: Mupirocin, OF: Ofloxacin, RIF: Rifampicin, TEI: Teicoplanin, TET: Tetracycline and V: Vancomycin

Table 2: Screening of MRSA isolates (n = 209) for biofilm production by three different methods

	TCP		TM		CRA	
Biofilm formation	No.	%	No.	%	No.	%
Strong	35	16.76	33	15.78	9	4.30
Moderate	71	33.97	65	31.10	20	9.56
Weak	103	49.28	111	53.11	180	86.12

Table 3: Statistical evaluation of TCP, TM and CRA methods for detection of biofilm formation in MRSA (n = 209)

Screening method	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
	(%)				
TCP	81.30	93.02	94.34	77.76	86.12
TM	75.22	86.46	86.73	74.77	80.38
CRA	14.10	86.79	75.86	25.56	32.54

read in a micro ELISA reader at wavelength 570 nm. By tube method, only 33 (15.78%) isolates were positive for biofilm formation and by Congo Red Agar method only 9 (4.30%) isolates showed positive biofilm phenotype with black colour colonies (Table 2). The TCP method was considered as the gold-standard for this study when compared with the data obtained from TM and CRA methods. Sensitivity and specificity of TCP, TM and CRA was found to be 81.30 and 93.02%; 75.22 and 86.46% and 14.10 and 86.79%, respectively (Table 3).

Table 4: Antibiotic resistance pattern in biofilm producing and non producing methicillin resistant *Staphylococcus aureus*

Antibiotics	Biofilm producers (n = 35)	Non biofilm producers (n = 174)
Amoxyclav	100.00	89.20
Ampicillin	100.00	78.41
Ampicillin/Sulfbactam	93.94	56.25
Chloramphenicol	100.00	42.61
Ciprofloxacin	100.00	85.23
Clindamycin	63.64	17.05
Cotrimoxazole	100.00	100.00
Fusidic acid	81.82	36.93
Gentamicin	87.88	61.36
Levofloxacin	75.76	52.27
Linezolid	48.48	29.55
Minocycline	54.55	27.27
Mupirocin	72.72	55.68
Ofloxacin	100.00	100.00
Rifampicin	66.67	51.14
Teicoplanin	36.36	35.79
Tetracycline	100.00	100.00
Vancomycin	0.00	0.00

Antibiotic resistance in biofilm and non-producing MRSA: Biofilm producing MRSA isolates were selected on the basis of TCP method screening as it was highly sensitive and reliable. The antibiotic resistance pattern of the biofilm producing (35) and biofilm non producing (174) was observed towards amoxyclav (100% vs. 89.20%), ampicillin (100% vs. 78.41%), ampicillin/sulfbactam (93.94% vs. 56.25%), chloramphenicol (100% vs. 42.61%), ciprofloxacin (100% vs. 85.23%), fusidic acid (81.82% vs. 36.93%), mupirocin (72.22% vs. 55.68%) and rifampicin (66.67% vs. 51.14%), levofloxacin (75.76% vs. 52.27%) (Table 4). There was no significant difference observed in susceptibility to each antibiotic between biofilm producing and non producing MRSA isolates. A $p<0.0001$ was considered significantly associated.

DISCUSSION

Staphylococcus aureus infections are very common and MRSA continues to be a serious and dreadful challenge as their prevalence is reported to be increasing exponentially (Sanders *et al*., 2011). The present study reports a high prevalence (80.69%) of MRSA in Coimbatore, South India and considerable increase in the prevalence of MRSA has been observed globally (Tiemersma *et al*., 2004; Boucher and Corey, 2008). Many authors from various regions of India have reported varying and distinctive figures in the prevalence of MRSA (Verma *et al*., 2000; Rajaduraipandi *et al*., 2006; Tsering *et al*., 2011; Kumar *et al*., 2012). This could be due to several factors like efficacy of infection control practices, healthcare facilities and antibiotic usage that vary from hospital to hospital (Tsering *et al*., 2011). We obtained 259 *Staphylococcus aureus* isolates, among which 209 (80.69%) isolates were identified as methicillin resistant. In a study conducted in Kerala, the authors reported all the isolates to be methicillin resistant (Jeshina and Surekha, 2009). Various researchers worldwide have reported the prevalence rate of MRSA to be 68.6 (Sharif *et al*., 2013), 68.25 (Khatoon *et al*., 2010), 57 (Alizargar *et al*., 2013), 48.3 (Al-Baidani *et al*., 2013), 40.14 (Gayathri *et al*., 2013) and 32% (Sadek *et al*., 2013), respectively.

Studies by various researchers have showed 100% susceptibility towards linezolid and teicoplanin (Rajaduraipandi *et al.*, 2006; Tsering *et al.*, 2011; Kumar *et al.*, 2012; Jeshina and Surekha, 2009; Shanthi and Sekar, 2009) and 90% towards clindamycin (Perwaiz *et al.*, 2007) when tested against MRSA isolates. The noteworthy and clinically relevant observation of this study is the maximum resistance shown by MRSA to most of the conventional antibiotics. Although, ciprofloxacin was proposed to be an alternate therapy for MRSA infection (Pai *et al.*, 2010), there is a rapid developing resistance against antibiotic which has been reported worldwide (Blumberg *et al.*, 1991). Even in this study, 100% resistance was observed against ciprofloxacin indicating that this antibiotic has become completely irrelevant to cure MRSA infections. It was also observed that percentage of resistance to clindamycin was 24.40% which was similar to the results reported by Debdas and Joshi (2011) but low when compared to other studies (Yilmaz *et al.*, 2007; Gadepalli *et al.*, 2006).

The results of this study depicted 100% susceptibility of MRSA towards vancomycin which assures this drug to be an efficient choice for treatment. Several authors have also reported similar results (Tsering *et al.*, 2011; Kumar *et al.*, 2012; Murugan *et al.*, 2008; Kalyani *et al.*, 2012). But at the same instance, vancomycin resistant *S. aureus* has also risen in India (Thati *et al.*, 2011). The towering resistance of the isolates in the present study to fusidic acid, cotrimoxazole, ciprofloxacin, rifampicin, mupirocin, chloramphenicol and tetracycline can be attributed to the fact that these broad spectrum antibiotics are frequently used in treatment of common infections which concludes that monotherapy is associated with increased resistance as compared to combination therapy (Deresinski, 2009). The present study revealed a high average rate (58.37%) of mupirocin resistance when compared to other studies which was reported to be 24-30% (Gadepalli *et al.*, 2006; Nicholson *et al.*, 2010). Similar prevalence rate was also reported by Vasquez *et al.* (2000) and Orrett (2008).

Microbial biofilms is considered a virulence factor that contributes to infections associated with various medical devices and cause nosocomial infection (Arciola *et al.*, 2001). Biofilm formation is a multistep process, starting with transient adherence to a surface. Subsequently, specific bacterial adhesins referred to as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMS) promote the actual attachment. The exact procedure by which biofilm producing organisms cause disease is poorly understood, however, the recommended mechanisms for biofilm formation are: Detachment of cells from medical device, biofilm causing bloodstream or urinary tract infection, endotoxin formation, resistance to host immune system and generation of resistance through plasmid exchange (Donlan and Costerton, 2002). In TCP method, biofilm formation was observed in 16.74% of isolates which was similar to observations reported by other researchers (Bose *et al.*, 2009; Mathur *et al.*, 2006) and contradictory to the observation made by Hassan *et al.* (2011). In tube method, 15.78% isolates were found to be biofilm producers which varied from other studies, which reported 19 and 13.36% (Hassan *et al.*, 2011; Taj *et al.*, 2012). Also, positivity rate of CRA method in this study was similar to the results (4.47%) obtained by Bose *et al.* (2009) and greater (1.31%) than that reported by Mathur *et al.* (2006). The necessity to perform the three tests was to compare their efficiency in detecting the biofilm formation. The sensitivity and specificity of TCP method was more than TM and CRA, so it is considered to be best in detecting biofilm formation. Also, false positive biofilm producers were found to be less through TCP method. The tube method correlates well with the TCP method for strongly biofilm producing isolates but it was difficult to discriminate between weak and negative isolates due to variability in the observed results by different observers. By TCP method, 6 false positive and 23 false negative isolates were

obtained; whereas, for TM (13 false positive and 28 false negative) and for CRA (7 false positive and 28 false negative) isolates were observed. CRA method showed very least correlation when compared with other two methods rendering it totally unsuitable for detection of biofilm formation. Hassan *et al.* (2011) also observed in their study that CRA method showed parameters of sensitivity (11%), specificity (92%) and accuracy (41%) through they concluded that CRA method did not correlate well with other three methods. Therefore, high variability was observed and classification in biofilm positive and negative was difficult by tube method. In accordance with the previous reports (Mathur *et al.*, 2006; Hassan *et al.*, 2011; Taj *et al.*, 2012), tube method cannot be recommended as general screening test to identify biofilm producing isolates. In CRA method, 9 (4.31%) positive isolates displayed black colonies with no dry crystalline morphology. In remaining isolates, no correlation was observed with TCP and TM. Based on the findings of this study, CRA and TM method cannot be recommended for detection of biofilm formation.

Moreover, in the present study, 16.76% of MRSA isolates by TCP method have shown the potential to produce biofilm which highlights the high prevalence of resistant microorganism in this region. Also, biofilm producing MRSA isolates showed high resistance to almost all the classes of antibiotics tested when compared to the biofilm non producers and this observation was also supported by other studies (Donlan, 2001; Bose *et al.*, 2009; Mathur *et al.*, 2006; Khan *et al.*, 2011). The main reason for antibiotic resistance is due to the decreased diffusion of antibiotics through the biofilm matrix and decreased metabolic activity of bacteria. Researchers have studied the strategies employed by microorganisms to produce biofilms and to understand the pathogenesis and they have concluded that biofilm producing bacteria secrete certain chemicals that protect them from disinfectants, antimicrobials and phagocytic host immune systems (Saitou *et al.*, 2009). Exploration to understand the detection and pathogenesis of MRSA biofilm infections has been focused upon the adherence of microorganisms to surfaces (Donlan *et al.*, 2001). The methods used for detecting biofilms focus on the capacity of each isolate to adhere to the surfaces and intake of nutrients so they are categorized into strong, moderate and weak. It is also hypothesized that the regulatory pathways controlling biofilm adhesins vary between isolates (Donlan *et al.*, 2001).

CONCLUSION

This study reports the higher prevalence of MRSA in the region, therefore judicious use of existing newer antibiotics and regular monitoring of isolates circulating in a particular hospital or community at regular time intervals is essential to tackle the spread of multidrug resistant pathogens like MRSA. As biofilm formation among the pathogens is increasing impeccably, screening through TCP method should be followed on regular basis as it is easy to perform and highly reliable. Biofilm formation can cause a multitude of problems in the medical field, particularly with prosthetic devices such as indwelling catheters and endo-tracheal tubes. Obtaining clinical samples from such devices for laboratory testing to identify biofilm formation can help prevent potentially fatal and persistent infections. The study strongly proposes that frequent monitoring of these pathogens in the hospital and community settings should be made mandatory and unambiguous antibiotic policy should also be formulated to decrease the spread of such pathogens.

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