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RESEARCH ARTICLE

ISOLATION OF BIOFILM FORMING BACTERIA FROM UTI INFECTED PATIENTS AND ANTIBACTERIAL ACTIVITY OF ANTIBIOTICS AGAINST THE ISOLATES

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Abstract

In the present study the urine sample was collected from various hospitals in Thanjavur. The samples were stored on specific leek proof aseptic container. Bacterial species were isolated from the urine sample using Nutrient agar, MacConkey agar medium. The bacteria isolated were identified with the help of culture morphological and biochemical characteristics. The biofilm forming ability of the collected pathogens was determined by both tube assay and spectrophotometric analyses. The isolated human pathogenic bacteria *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Staphylococcus aureus* were observed positive results. In the present study exopolysaccharide and the protein content of EPS can be determined. Chemical analysis of EPS matrix was done and it was observed that content of protein was more as compared to carbohydrate. Antibacterial activity test was carried out following the modification of the method originally described by Bauer *et al.,* (1996). In bacteria the maximum zone of inhibition was observed in antibiotic ciprofloxacin. To conclude, there was significant bacteriuria in all the symptomatic catheterized patients and *Proteus vulgaris* was the most frequent isolate in the urinary tract infections in the catheterized patients. The microbial biofilms may pose a public health problem for the persons who require indwelling medical devices, as the microorganisms in the biofilms are difficult to treat with antimicrobial agents.

INTRODUCTION

A biofilm is any group of microorganisms in which cells stick to each other on a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilm EPS, which is also referred to as slime, is a polymeric conglomeration generally composed of extracellular DNA, proteins, and polysaccharides. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings (Hall-Stoodley *et al.,* 2004). Biofilms have been found to be involved in a wide variety of microbial infections in the body, by one estimate 80% of all infections (Schwermer *et al.,* 2008). Microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics (Karatan and Watnick, 2009). In the present study the bioflim bacteria were isolated from urine sample. The organisms were identified by Gram staining method, Biochemical method and analyzed biofilm activity by screening method (Tube assay, Congo red) and the inhibitory effect of organism was determined by antibacterial activity test.

MATERIALS AND METHODS

Collection of Urine Samples

In this study 100 infected urine samples were collected from different hospital in Thanjavur. The urine samples were collected in sterile, dry, wide necked, leak proof container. Clean catch method is used to collect mid-stream urine, first urine is not collected because it is contaminated with microbes from lower portion of the urethra. If immediate delivery to the laboratory in not possible, the urine should be refrigerated at 4˚C.

Isolation Identification of Bacteria

Bacterial species were isolated from the collected urine sample using Nutrient agar and Mac Conkey agar by streak plate method. After the streaking the bacterial plates were incubated at 37˚C for 24-48hrs. After incubation Cultural characteristics and colony morphology were observed. These colonies were sub cultured and stored in refrigerator for further study. Isolated colonies with visually distinguishable morphologies were randomly selected and isolated by directly streaking on Nutrient agar plates and incubated for another 12-18 hours.

Screening of Biofilm Bacteria

Primary biofilm screening was done using tube staining assay (Christensen *et al.*, 1982). LB broth (5mL) was inoculated with 100µl of overnight culture broth and incubated for 48 hours at 37°C. The tubes were decanted and washed with Phosphate Buffer Saline (PBS) (pH 7.3) and dried. Staining of dried tubes was done with 0.1% crystal violet. Excess stain was removed by washing the tubes with deionized water. Biofilm formation in tubes was then observed. The attached biofilm was stained with 10 times diluted Grams Crystal Violet for 15 minutes followed by destaining with 95% ethyl alcohol. The alcohol was rinsed and allowed to dry. The OD of the adherent biofilm was taken using a spectrophotometer reader at 570nm.

Screening of Biofilm Bacteria Plate Assay Method

The investigation of the biofilm production by the Congo red agar method was proposed by Freeman *et al.* (1989). The suspensions of the tested strains were inoculated into tubes which contained a specially prepared solid medium- Brain Heart Infusion broth (BHI) which was supplemented with 5% sucrose and Congo Red. The medium was composed of BHI (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and the Congo Red stain (0.8 gms/L). Congo red was prepared as a concentrated aqueous solution and it was autoclaved at 121°C for 15 minutes, separately from the other medium constituents and it was then added when the agar had cooled to 55°C. The plates were inoculated and incubated aerobically for 24-48 hours at 37°C. A positive result was indicated by black colonies with a dry crystalline consistency.

Characterization of Biofilm Matrix (EPS)

The isolated biofilm bacteria were inoculated into Brain Heart Infusion broth separately. The inoculated broths were incubated at 37ºC for 48 hrs. After biofilm samples were placed on ice for thawing and centrifuged at 15000× g for 20 minutes. The biofilm pellets were resuspended in about 30 ml of cold sulfuric acid, i.e., 0.2 M sulfuric acid, pH 1.1 and the biofilm matrix was broken using a glass homogenizer tube and pestle. The cell suspension was kept at 4ºC for 3 hours with occasional stirring before centrifugation at 15000 × g for 20 minutes. The resulting supernatant containing total EPS was used for further analysis (Jiao *et al.*, 2010). The content of the EPS extracts is done by chemical analyses. The exopolysaccharide content of EPS can be determined by the phenol-sulphuric acid method described by Dubois *et al.* (1956), with glucose as the standard. The protein content of EPS can be determined by the Lowry's method (1951) with bovine serum albumin as the standard.

Antibacterial Activity

The antibiotic sensitivity of isolated biofilm bacterial species to the commercial antibiotic tests was analyzed by disc diffusion method. Antimicrobial activity test was carried out following the modification of the method originally described by Bauer *et al.*, (1996).

Statistical Analysis

The results obtained in the present investigation were subject to statistical analysis like Mean ($\bar{x}$) and Standard Deviation (SD) by Zar (1984).

RESULTS AND DISCUSSION

The present study was carried out on the isolation, identification, screening, characterization and various biochemical tests of bioflim bacteria from urine sample. The growth of 6 bacterial colonies was observed from the sample. The isolated colonies were named as KB1, KB2, KB3, KB4, KB5, and KB6. The colony characters of isolated organism was found to be gram positive for Bacillus subtis (KB1) and staphylococcus aureus (KB6) and it was a non-motile organism respectively. The isolated organism was found to be gram negative for E.coli (KB2), Klebsiella pneumonia (KB3), Pseudomonas auroginosa (KB4), Proteus vulgaris (KB5) and it was motile organism for E.coli, Pseudomonas auroginosa (KB4), Proteus vulgaris (KB5) and non-motile for Klebsiella pneumonia (KB3).

The organism was confirmed by different Bio-chemical test. *Bacillus subtilis* (KB1) showed negative for indole, MR, Urease, catalase and positive for VP, Citrate, Oxidase and Acid production for TSI, Dextrose and sucrose. *E.coli* (KB2) showed negative for VP, Citrate, Urease, oxidase and positive for indole, MR, Catalase. Acid production for TSI and sucrose. Acid and gas production for lactos and dextrose. *Klebsiella pneumoniae* showed negative for indole, MR. Urease, oxidase and positive for VP, citrate, catalase and acid production for TSI, Acid and gas production for lactose, dextrose and sucrose. *Pseudomonas auroginosa* showed negative for indole, MR, VP, urease, dextrose, sucrose. Alkaline slant and alkaline butt for TSI. *Proteus vulgaris* showed negative for indole, MR, VP, citrate, urease, oxidase, lactose and positive for catalase. Acid and gas production for dextrose and sucrose TSI. *Staphylococcus aureus* showed negative VP, citrate, oxidase and positive for indole, MR, urease, catalase and acid production for TSI, lactose, dextrose, sucrose (Table – 1).

The present study showed that among these 100 strains, *Proteus vulgaris* was the most frequently isolated pathogen 32 (32%), followed by *Escherichia coli* 20 (20%), *Pseudomonas aeruginosa* 20 (20%), *Klebsiella pneumoniae* 16 (16%), coagulase negative *Staphylococcus aureus* 8 (8%) and *Bacillus subtilis* 4 (4%). The investigated results were presented in Fig -1.

The bioflim bacteria were screened by tube assay method and congo red method for the organisms KB1, KB2, KB3, KB4, KB5, and KB6. Ring formation was observed and confirmed with the presence of attachment on the wall and bottom of the tube and black colonies with a dry crystalline consistency was observed in Congo red method (Table – 2 & 3). To characterize the bioflim matrix (EPS) from urine sample for chemical analysis of EPS matrix was done and it was observed that protein content was more as compared to carbohydrate (Table - 4). The different organism such as *Bacillus subtilis, E.coli, Klebsiella pneumonia, Pseudomonas auroginosa, Proteus vulgaris, S.aureus* were used for antibacterial activity by using various antibiotics (Fig – 2). The ciprofloxacin have maximum antibacterial activity against all bacterial isolates when compared to other antibiotics. At the same time minimum inhibitory activity observed against *Escherichia coli*. The piperacillin, ampicillin, and carbenicillin have resistant to all the bacterial isolates. *Pseudomonas aeruginosa* has maximum sensitivity was observed in ciprofloxacin whereas minimum range of sensitivity was showed by *Escherichia coli*.

Table - 1 Biochemical Test for Bacterial Identification

S.No	Isolated organisms	Gram staining	Motility	Shape	Indole	Methyl red	VP	Citrate	Urease	TSI	Catalase	Oxidase	Carbohydrate fermentation		
													Lactose	Dextrose	Sucrose
1.	KB1	+	+	rod	-	-	+	+	-	A/A	-	+	+	A	A
2.	KB2	-	+	rod	+	+	-	_	-	A/A	+	-	+ (AG)	+ (AG)	+/-A
3.	KB3	-	-	rod	-	-	+	+	-	A/A	+	-	+ (AG)	+ (AG)	+ (AG)
4.	KB4	-	+	rod	-	-	-	+	-	K/K	+	+	-	-	-
5.	KB5	-	+	rod	-	-	-	-	-	A/K	+	-	-	AG	AG
6.	KB6	+	-	cocci	+	+	-	-	+	A/A	+	-	A	A	A

+ – Positive, – – Negative
± – Variation reaction

K/K – Alkaline slant Alkaline butt

K/A – Alkaline slant Acid

Fig – 2 Assay of Antibacterial Activity

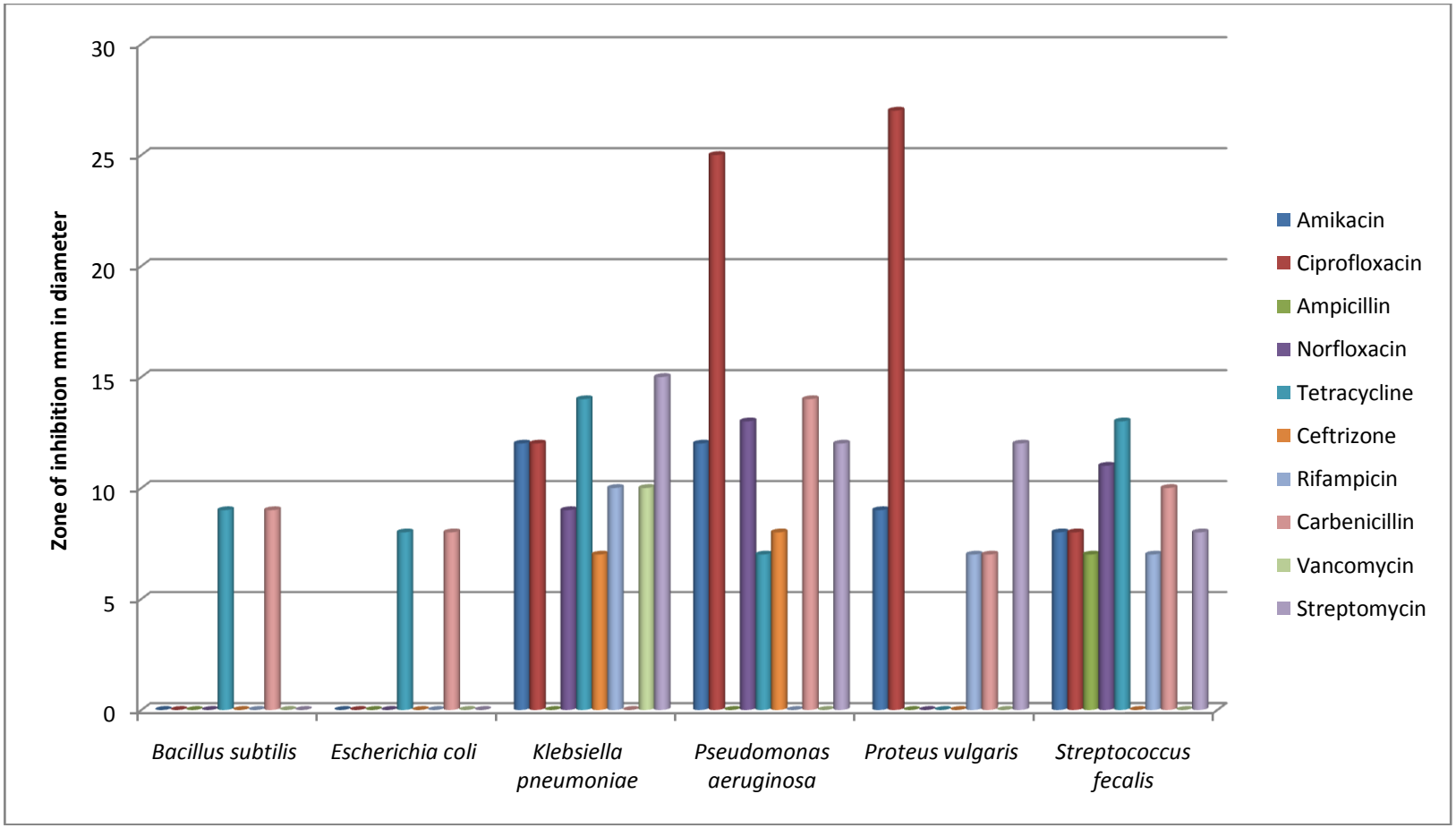
Zone of inhibition mm in diameter
30
25
20
15
10
5
0
Bacillus subtilis
Escherichia coli
Klebsiella pneumoniae
Pseudomonas aeruginosa
Proteus vulgaris
Streptococcus fecalis
Amikacin
Ciprofloxacin
Ampicillin
Norfloxacin
Tetracycline
Ceftrizone
Rifampicin
Carbenicillin
Vancomycin
Streptomycin

Fig – 1 Percentage of Isolated Bacteria

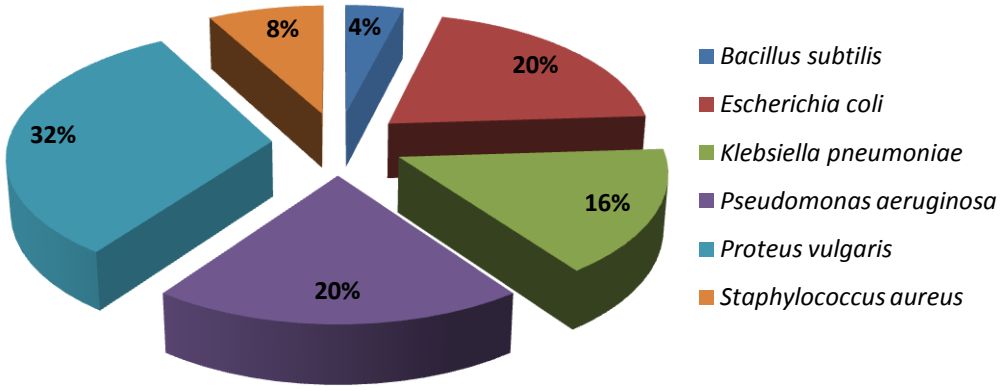

Table – 2 Screening of Biofilm Bacteria by Tube Assay Method

S. No.	Isolated Biofilm Bacteria	Tube Assay
1	*Bacillus subtilis*	+ve
2	*Escherichia coli*	+ve
3	*Klebsiella pneumoniae*	+ve
4	*Psedomonas aeruginosa*	+ve
5	*Proteus vulgaris*	+ve
6	*Staphylococcus aureus*	+ve

+ve Indicative positive
-ve Indicative negative

Table – 3 Screening of Biofilm Bacteria by Spectrophotometer

S. No.	Isolated Biofilm Bacteria	OD values (570 nm)	
		Control	Biofilm Bacteria
1	*Bacillus subtilis*	0.041	0.600
2	*Escherichia coli*	0.041	0.640
3	*Klebsiella pneumoniae*	0.040	0.700
4	*Psedomonas aeruginosa*	0.039	0.620
5	*Proteus vulgaris*	0.040	0.680
6	*Staphylococcus aureus*	0.040	0.720

Table – 4 Characterization of Biofilm Matrix (Eps)

S. No.	Isolated Biofilm Bacteria	Carbohydrate content in µg/ml	Protein content in µg/ml
1	*Bacillus subtilis*	147.5±3.00	1460±11.00
2	*Escherichia coli*	115.9±9.00	1244±09.00
3	*Klebsiella pneumoniae*	155.8±7.00	1293±13.00
4	*Pseudomonas aeruginosa*	105.3±3.00	1367±17.00
5	*Proteus vulgaris*	149.7±2.00	1692±10.00
6	*Staphylococcus aureus*	116.3±5.00	1128±08.00

Values are expressed in Mean± Standard Deviation, n=3.

CONCLUSION

To concluded, there was significant bacteriuria in all the symptomatic catheterized patients and *Proteus vulgaris* was the most frequent isolate in the urinary tract infections in the catheterized patients. The Diabetes and urogenital instrumentation were the major risk factors for the UTIs. The microbial biofilms may pose a public health problem for the persons who require indwelling medical devices, as the microorganisms in the biofilms are difficult to treat with antimicrobial agents. Also, there is a need to establish standard guidelines on the care of catheters for all the units in the hospital, with a view to prevent the nosocomial infections which are associated with the devices in the patients.

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