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EXPERIMENTAL ARTICLES

Immunoassay Method to Check the Flagellin Mediated Binding of *Stenotrophomonas maltophilia* to Polystyrene[1]

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Abstract—Plate count and spectrophotometric methods have been used to asses the ability of an organism to attach to different surfaces and form biofilms. In the present study we report a highly sensitive, specific and quick method to check the role of flagellin in bacterial adhesion to polystyrene. Flagellin from *Stenotrophomonas maltophilia* showed high affinity for polystyrene ($P < 0.05$), which decreased on pretreatment of flagellin with anti-flagellin in a dilution dependent manner. In an enzyme immunoassay format a positive correlation was detected between the anti-flagellin dilutions and flagellin attachment to polystyrene (correlation coefficient +0.860155). These evidences conclusively prove the involvement of flagella in the adhesion of *S. maltophilia* to polystyrene surface and enzyme immunoassay, a quick and reliable method to check this phenomenon.

Keywords: adhesion, enzyme, immunoassay *Stenotrophomonas maltophilia*

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INTRODUCTION

Bacterial adhesion is an essential step in the pathogenesis of infections of prosthetic surfaces [1]. However the molecular and physical interactions that govern bacterial adhesion to biomaterials have not been understood in detail. Both specific and non-specific interactions may play an important role in the ability of the cells to adhere to (or to resist detachment from) the biomaterial surface [2]. *Stenotrophomonas maltophilia* strains of both clinical and environmental origin have been reported to adhere and form biofilm on abiotic surfaces such as glass, teflon, polystyrene, stainless steel [3, 4]. Adhesion of these bacteria to abiotic surfaces such as those of medical implants and catheters suggest the development of a biofilm that protects bacteria from natural immune defenses or from the action of antimicrobial compounds. Biofilms are made up of a community of bacteria immobilized and embedded in an organic polymer matrix composed of polysaccharides and proteins of bacterial origin [5].

Adhesion to abiotic surfaces is mediated by different appendages. In case of *S. maltophilia* it has been shown that binding to abiotic surfaces is mediated through flagellalike structures [6]. Flagella are composed of several thousand copies of flagellin subunits, the major component being flagellin C (FliC) [7]. To study this binding either plate count method or spectrophotometric method has been used. These methods tend to be complicated and time consuming.

In this study we report the utility of a new method to check the involvement of *S. maltophilia* flagella in attachment to abiotic surfaces. We evaluated the potential of an immunoassay method for checking the ability of flagellin to adhere to polystyrene. For this pure flagellin and anti-flagellin anti-sera were applied.

MATERIALS AND METHODS

Clinical isolate: A clinical isolate of *S. maltophilia* (Sm2) was used in this study. This isolate was procured from the Department of Medical Microbiology, Post Graduate Institute of Medical Education and Research, Chandigarh, India. Bacteria was preserved by lyophilization and also maintained at 37°C on Luria Bertani (LB) agar plates (Himedia), subcultures were made every week.

Flagellin and anti-flagellin preparation: Flagellin was isolated from *S. maltophilia* (Sm2) according to the procedure described earlier [8]. Briefly, *S. maltophilia* (Sm2) was grown in LB broth overnight and pelleted by centrifugation. Pellet was suspended in potassium phosphate buffer (0.01 M, pH 7.0) and sheared for 1 min in commercial blender. The sheared suspension was centrifuged for 30 min at 5000 × *g* and then centrifuged for 15 min at 16000 × *g*. The supernatant was centrifuged at 100000 × *g* for 3 h. The pellet was collected and kept at −80°C. Anti-flagellin antiserum was prepared by immunizing rabbit with flagellin. Complement was inactivated by incubating sera at 56°C for 30 min and presence of anti-flagellin was detected by immunoblotting.

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Determination of Sm-flagella affinity to polystyrene by enzyme immunoassay: The method of Cogan, et al. (2004) [9] was followed with modification, to check the ability of flagellin molecule to attach to polystyrene. Briefly, three rows of microtiter plate (Nunc, Denmark) were used, first and second were incubated for 1 h with 100 µl of 20 µg/ml Sm-flagellin at 37°C. Rows were washed four times with PBS (0.1 M pH 7.2) containing 0.05% Tween 20 (Himedia) and then all wells were blocked with 200 µl of PBS containing 3% dried milk for 1 h at 37°C. 100 µl of rabbit Sm anti-flagellin (1 : 320) suspended in PBS containing 0.1% dried milk was added to each well except second row (normal rabbit sera was used). Plate was incubated at 37°C for another 1 h. Goat anti-rabbit horseradish peroxidase conjugate antibody (Sigma) was added to the wells and incubated for 1 h, followed by addition of tetramethylbenzidine substrate (TMB) (Sigma). The reaction was stopped with 2 M sulphuric acid and absorbance read at 450 nm. In this experiment, first row acted as a test row (flagellin + anti-flagellin) where as 2*nd* row was taken as control (flagellin + normal rabbit serum) and 3*rd* row was control 2 with anti-flagellin only.

Following similar protocol, another experiment was conducted to check the adherence of flagellin to polystyrene, using pretreated flagellin with different dilutions of anti-flagellin.

Biofilm formation: Overnight cultures of *S. maltophilia* in 3 ml of Tryptose soy broth (TSB) (Himedia) were washed three times with fresh TSB, and bacterial count adjusted to1×10^7 cfu ml^{-1}. Aliquots (200 µl) of standardized inoculum were added to the wells of sterile flat-bottom polystyrene tissue culture plates, and incubated at 37°C for different time intervals (0.5 1, 2, 4, 6, 8, 24, 48 and 72 h) to study the kinetics of adhesion in a closed and humidified plastic container. The medium was then discarded, and non adherent cells were removed by washing three times with sterile PBS (0.1 M, pH 7.2). Quantitatiation of adherent *S. maltophilia* was performed by viable cell enumeration as well as by spectrophotometric method as described previously by Bonaventura et al., [1].

Statistical analysis: All the experiments were carried out in triplicate and all values have been taken as mean value and standard deviation calculated. The differences between test and control were analyzed by using Student's *t* test and correlation coefficient calculated by employing Origin 6.0 version Software. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

The results in Fig. 1 show the high affinity of Sm flagellin for polystyrene. The flagellin on pretreatment with different dilutions of anti-flagellin showed significant reduction in attachment compared to positive control (CP). The observed decrease was dose dependent and a positive correlation was detected between the anti-flagellin dilutions and adhesion to polystyrene.Though both classical plate count method and spectrophotometric method have been employed by different investigators [1, 6, 10], but these methods are time consuming with low sensitivity and specificity. However, the enzyme immunoassay to study the adhesion of flagellin to polystyrene surface, as reported in this study, is highly sensitive and specific as this method is based on the principle of antigen-antibody interaction. It is a rapid test as the results were available in lesser period of time.

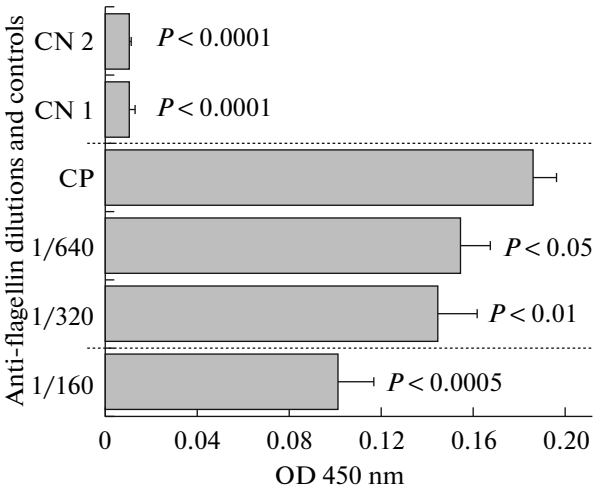

Fig. 1. Ability of flagellin to attach to polystyrene as checked by enzyme immunoassay. Positive relation was detected between adhesion of flagellin to polystyrene and anti-flagellin dilution(correlation coefficient: +0.860155). CP: Control positive (flagellin + anti-flagellin), CN1: control negative 1 (flagellin + normal rabbit serum), CN2: Control negative 2 (only anti-flagellin).

The results in Fig. 2 show the ability of this strain of *S. maltophilia* to form biofilm on polystyrene plate. Flagella are present on the bacterium (*S. maltophilia*) as single or multi polar flagella [6]. The flagella probably contribute to biofilm formation as well by intercalating the different bacteria which finally get enclosed in a polysaccharide matrix [11] secreted by them. In this study adherence to polystyrene through flagella resulted in biofilm formation as well. This is important as it will not only help them in their attachment to abiotic surface but will also allow them to survive over a longer period of time. Biofilm mode of growth not only leads to morphological and physiological changes in bacteria but also makes them recalcitrant to a number of antibiotics [12]. This property may be of significance in their disease causing ability as it has been reported that once organisms like *S. maltophilia* colonize and form biofilm on the prosthetic surfaces, it gives rise to serious infections especially in situations where host's immune status is lowered [13, 14]. Since *S. maltophilia* is an organism known to cause serious infection in such patients' hence utmost care should be

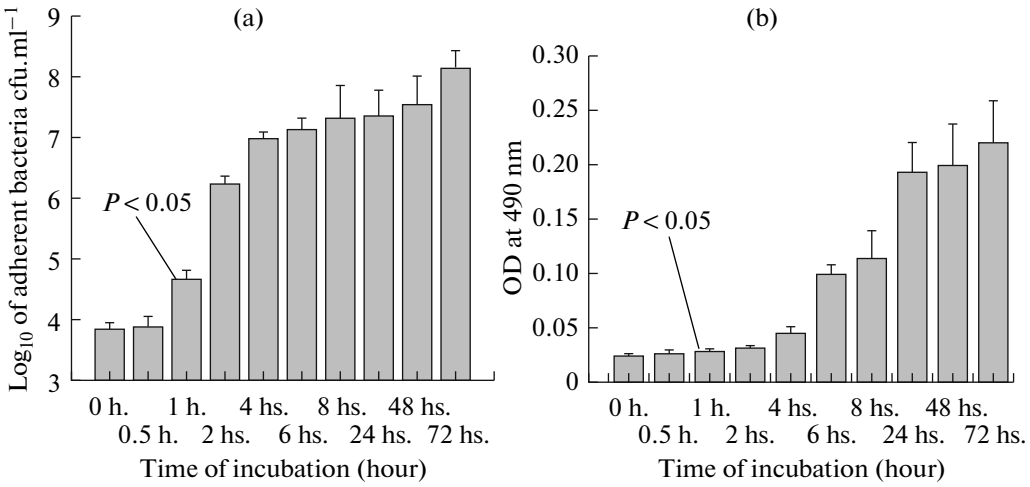

Fig. 2. Kinetics of biofilm formation on polystyrene microtiter plate. (a) By plate count method, (b) By spectrophotometric method. Significant adhesion of *S. maltophilia* Sm2 was detected after 1 h of incubation by both the methods.

taken while handling and implanting such materials in these patients.

This study provides evidence to show that flagella of *S. maltophilia* mediated adhesion of bacteria to polystyrene surface that was easily detected by applying a sensitive and specific technique like immunoassay.

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