Paraffin baiting system for demonstration of growth and biofilm production in *Pseudomonas aeruginosa*

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Received 14 June 2006; revised 16 October 2006

*Pseudomonas aeruginosa* is one of the commonest pathogens among the pseudomonads. This organism can grow in minimal nutritional requirements. Because of the ability of pseudomonads to grow on paraffin is not commonly found among other human pathogens and the primary human pathogen being *P. aeruginosa*, we studied the adaptation of this organism to paraffin baiting system for growth and biofilm formation. Strains were tested for the capacity to use paraffin as the sole source of carbon using Czapek’s minimal salt medium. Of the 53 clinical isolates of *P. aeruginosa*, 20 strains exhibited growth by 24 hrs and 42 strains by 48 hrs. The remaining strains did not show any growth in the paraffin baiting system. The oxidase test with the paraffin baiting system was also performed. This simple and inexpensive method can be used to isolate and demonstrate the biochemical and biofilm forming capacity of the organism.

**Keywords:** Paraffin baiting, *Pseudomonas aeruginosa*

Paraffin baiting was developed for isolating organisms like Nocardia and Mycobacteria from soil because of their ability to use paraffin wax as a sole source of carbon. The baiting was accomplished by growing the organisms in a medium lacking any carbon source into which were dipped glass rods coated with paraffin wax. *Pseudomonas aeruginosa* is the primary pathogen among pseudomonads and is a common cause of Nosocomial infection, especially in the Immunocompromised patients. These organisms are often multidrug resistant because they undergo mutational resistance to all relevant treatments.

These organisms are also known for Biofilm and microcolony formation. This is said to enhance their resistance to various antibiotics, against other microorganisms and protection against human immune system. Biofilm organism behave differently from planktonic form with respect to growth rates and ability to resist antimicrobial treatment. *Pseudomonas aeruginosa* is also commonly isolated from catheters on which these organisms form biofilms. The biofilm formation of the central venous catheters was universal, short term duration had biofilm formation on external surface of the catheter whereas long term duration the biofilm formation on the catheter inner lumen was found to be greater. It has also been found that the adherence to the catheter materials was dependant on the hydrophobicity of both the organism and their surfaces.

Pseudomonads can grow in minimal nutritional requirements and are able to oxidize n-alkanes including paraffin wax because of certain enzymes present in this organism. In the present study the ability of *Pseudomonas aeruginosa* to grow in the paraffin baiting system and also its capability to produce biofilms has been analysed.

**Materials and Methods**

Fifty three known isolates of *Pseudomonas aeruginosa*, obtained from various clinical specimens like sputum, urine, pus and burns wound were included in the study. Standard strains used for the study were *P aeruginosa* ATCC 27853, PAO1, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923. Bacterial strains were grown in peptone water at 37°C for 4 h and standardized using Mc farland tube No. 0.5 and used in the paraffin...
baiting test.

Paraffin baiting test method — Strains were tested for paraffinophilic capacity by growth in Czapek’s minimal salt medium. Tubes containing Paraffin coated slides and 4 ml of Czapek’s broth were inoculated with 100 µl of standardized inoculum of each strain. The growth was observed on the paraffin slide and broth from 6hrs after inoculation for 24 h and upto 48 h at 25° and 37°C. Positive growth was indicated by the presence of biofilm on the paraffin slide. The growth in the broth was demonstrated by preparing a smear and staining by simple carbol fuschin and observing under the microscope.

Oxidase test was performed from the paraffin baiting system as per the methodology described by Massengale et al.

Results

Paraffin coated slide inoculated with reference strains and Pseudomonas aeruginosa isolated from various clinical specimens were incubated at 37°C and 25°C. PA O1 and P. aeruginosa ATCC 27853 were able to use paraffin as a sole source of carbon by 24 and 48 hrs respectively. Where as Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923 were not able to grow in the paraffin baiting system. The strains isolated from clinical specimens were tested for the use of the paraffin as a sole source of carbon. Of the 53 clinical isolates 20 strains exhibited growth at 24hrs and 42 strains exhibited growth at 48 hrs (Table 1). The remaining 11 strains did not grow in the paraffin baiting system even after 2 days of incubation. Some of the Pseudomonas aeruginosa strains (8) exhibited formation of bubbles at the slide surface indicating carbon utilization from paraffin at 37°C. All the 42 strains of P. aeruginosa were positive for oxidase test after 48 h of incubation. There were no growth difference seen at 25 oC and 37°C. The statistical analysis of the growth and biofilm formation by P. aeruginosa using the paraffin baiting system was analysed using McNemar’s test and it was found to be statistically significant \( P < .001 \).

Discussion

The paraffin baiting system can be used for both, the growth of P. aeruginosa using paraffin as a sole source of carbon and also to study the bacterial biofilm formation on the paraffin slide and also to isolate these organisms in pure form from specimens like sputum containing other organisms. The ability of P. aeruginosa to use paraffin as sole source of carbon was tested for 53 strains isolated from various clinical specimens out of which 20 strains grew within 24 h including the reference strains and the 42 strains grew after 48 h of incubation.

Pseudomonads are able to use wide variety of alkanes as a carbon and energy source by the presence of alkane hydroxylase system encoded by alkBFGHKL operon on the n–octane utilisation plasmid. Alkane hydroxylase, rubredoxin and rubredoxin reductase enzyme are required for alkane oxidation. The latter are soluble proteins that transfer electrons from NADH to membrane bound hydroxylase which in turn oxidizes the alkane to create a primary alcohol and water soluble molecule.

Most of the P. aeruginosa strains tested in the present study exhibited light growth on paraffin in the form of biofilm and 8 strains tested produced bubbles on the slide surface. In the paraffin baiting system, gas production at the slide surface is indicative of paraffin oxidation. However without the presence of bubbles on the surface of paraffin it is difficult to determine whether the P. aeruginosa is oxidizing the paraffin or simply producing a biofilm at paraffin surface. The biofilm and the growth of the bacteria on the glass slide coated with paraffin could be established by performing simple carbol fuschin staining of the slides. One more finding of this study was that the thin film like growth of P. aeruginosa produced iridescence after 48 hours, with a metallic sheen.

Pseudomonas commonly form adherent biofilms at the site of infection like urinary tract, airways and corneal infections. First the bacteria adhere to the surface with the help of pili, lipopolysacchride or alginate. Then they form a matrix of hydrated polysacchrides. Bacteria inside biofilm are resistant to the attack of immune system, surfactants and antibiotics. This is due to the glycocalyx and reduced growth rate of cells in the biofilm. This type of complicated process requires the need of further

<table>
<thead>
<tr>
<th>Source</th>
<th>Growth at 24 hr</th>
<th>Growth after 48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum (15)</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Urine (20)</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Pus (11)</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Burns wound (7)</td>
<td>3</td>
<td>5</td>
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research to prevent the protective nature of biofilm in the pathogenicity of *P. aeruginosa*.

Since *P. aeruginosa* grows as a biofilm on the paraffin, this paraffin baiting system can be used to study the growth pattern and antibiotic activity of the biofilms, this factor which is mostly responsible for long term persistence of *P. aeruginosa* in certain physiological environment like cystis fibrosis of the lung.<sup>7</sup><sup>17</sup>. Therefore, it is believed that paraffin baiting system can be used as a convenient *in vitro* method for the production of biofilms similar to that found in the physiological conditions and can be used to study the effects of antibiotics on these biofilms. Van Beilen et al.<sup>23</sup> have identified several hydrocarbons that can increase the expression of paraffin hydroxylase enzyme in *Pseudomonas aeruginosa*.

Future experiments will focus on detection of paraffin hydroxylase enzyme which is responsible for paraffin oxidation. Future studies can also focus on the addition of hydrocarbons which can improve the growth of the organism on the paraffin slide. Assay for detection of paraffin hydroxylase would further enhance the system for the identification of Pseudomonas. To the best of our knowledge this type of study has not been reported from this part of the country.

In conclusion, paraffin baiting system is a simplified and inexpensive method for selective isolation, identification and to study the biofilms produced by *P. aeruginosa* in the clinical and the research laboratories.

References