Garlic blocks quorum sensing and attenuates the virulence of Pseudomonas aeruginosa

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Abstract
Pseudomonas aeruginosa is an opportunistic pathogen that colonizes urinary catheters, forms biofilms, and is responsible for causing persistent and recurrent nosocomial catheter-associated urinary tract infections (UTIs). These infections show increased morbidity and mortality in immunocompromised patients. Quorum sensing in P. aeruginosa plays a key role in biofilm formation, virulence factor production and antimicrobial resistance. Because of emerging antimicrobial resistance in P. aeruginosa, there is a need to find an alternate nonantibiotic agent for the control of infections caused by this organism. In the present study, garlic was evaluated as a prophylactic agent in vivo in a mouse UTI model. Oral treatment with garlic significantly lowered renal bacterial counts and protected mouse kidney from tissue destruction. In vitro data showed decreased elaboration of virulence factors and reduced production of quorum-sensing signals by P. aeruginosa in the presence of fresh garlic extract. The results suggest that decreased virulence of P. aeruginosa in garlic-fed mice can be attributed to the quorum-sensing inhibitory property of garlic. This might have contributed towards reduced production of virulence factors, as seen in vitro.

Introduction
Pseudomonas aeruginosa is responsible for about 11% of nosocomial infections and is one of the most common pathogens responsible for nosocomial urinary tract infections (UTIs) (Goldsworthy, 2008). It has a tendency to form biofilms, which lead to chronicity and recurrence of disease. Antipseudomonal treatment always comprises antibiotic therapy. However, therapeutic inefficiency of antibiotics at the highest deliverable doses is observed due to development of resistance in persistent biofilms formed by this organism, and is the major drawback of this approach to such infections.

Pseudomonas aeruginosa regulates biofilm formation and production of extracellular virulence factors such as alginate, elastase, rhamnolipids, exotoxin A, exoenzyme S, siderophores pyochelin and pyoverdin, proteases and haemolysins through cell-to-cell communication known as quorum sensing. This bacterium produces small diffusible chemical molecules in its environment known as acyl homoserine lactones (AHLs), which are used as quorum-sensing signal molecules (Smith et al., 2002). Quorum sensing has been shown to be important in the pathogenesis of various infections such as respiratory tract infections, burn wound infection and keratitis caused by P. aeruginosa (Rumbaugh et al., 1999; Pearson et al., 2000; Zhu et al., 2002).

Because of these reasons, researchers have become interested to use quorum sensing as an ideal target for controlling such infections. Although inhibition of quorum sensing has been shown by furanone compounds (Hentzer et al., 2002), use of these compounds may be associated with toxicity in vivo. Therefore, there is a need to search for herbal products that are able to block quorum sensing and inhibit the expression of virulence determinants of P. aeruginosa leading to attenuation of its virulence. There has been renewed interest in the development of newer compounds from medicinal plants with the aim of treating various diseases with negligible side-effects (Ahmad et al., 1998).

Of the large collection of herbal products, garlic (Allium sativum) has been used traditionally as a protective agent against a number of infections including bacterial, fungal and viral. Diallyl thiosulphate (allicin) and related derivatives have been identified as antimicrobial ingredients (Weber et al., 1992; Ankri & Mirelman, 1999). Besides its antimicrobial properties, garlic also has antioxidant,
anti-inflammatory and immunomodulatory properties due to which it has been used widely in a number of infectious conditions (Feldberg et al., 1988). Studies are available in which treatment with garlic has been shown to provoke a higher degree of inflammation and clearing of infecting bacteria in pulmonary infections of mice (Bjarnsholt et al., 2005). The present study investigated the potential of garlic to attenuate the virulence of P. aeruginosa in vitro and in vivo in an experimental UTI model of mice.

Materials and methods

Bacterial strains

Ten clinical isolates of P. aeruginosa obtained from urine samples of catheterized patients attending Government Medical College and Hospital, Chandigarh, India, were used. Pseudomonas aeruginosa was identified biochemically according to Bergey’s Manual of Determinative Bacteriology. Dr B.H. Iglewski (USA) kindly provided the reporter strain Escherichia coli MG4. The isolates were stored in 20% glycerol Luria–Bertani (LB) broth at −80°C.

Evaluation of quorum-sensing signals (AHLs)

Cross-feeding assay

LB agar plates covered with 40μL of X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, 20 mg mL⁻¹ in dimethyl formamide) were used for the assay. Detection of exogenous AHLs was done using E. coli MG4 (pKDT17) as reporter strain. Plasmid pKDT17 contains LasR under control of lac promoter and a LasB::LacZ translational fusion. Reporter strain and test culture were streaked about 1 cm apart on plates. AHLs produced within the culture diffused through the agar and resulted in activation of the tral–lacZ fusion in the reporter strain.

Quantitative measurement

Supernatant of the centrifuged culture was extracted twice with ethyl acetate containing 0.01% acetic acid (Zhu et al., 2002). For quantitative determination, a bioassay tube containing 2 mL of reporter strain and 0.5 mL of extracted supernatant was incubated at 30°C in a water bath for 5 h with rotation at 100 r.p.m. After centrifugation (3200 g for 15 min), each pellet was suspended in an equal volume of Z buffer (Na₂HPO₄·7H₂O, 0.06 M; NaH₂PO₄·H₂O, 0.04 M; KCl, 0.01 M; MgSO₄·7H₂O, 0.001 M; β-mercaptoethanol, 0.05 M; pH 7.0). To 1 mL of these cells 1 mL of Z buffer, 200 μL chloroform and 100 μL of 0.1% sodium dodecyl sulphate was added to lyse cells, and 0.4 mL of O-nitrophenol-β-D-galactopyranoside [4 mg mL⁻¹ in phosphate-buffered saline (PBS)] was also added. After the development of yellow colour, 1 mL of 1 M Na₂CO₃ was added to stop the reaction. OD of the reaction samples was taken at 420 and 550 nm. Units of β-galactosidase were calculated as 1000 × A₄₂₀ nm − (1.75 × A₅₅₀ nm)/time × volume × A₄₀₀ nm. Based on qualitative and quantitative assay for AHL production, one clinical isolate was selected for further study.

Biofilm generation

Biofilm was allowed to develop on sterile foley’s catheter (Rusch) pieces (1 cm) under static conditions for 7 days in the presence and absence of fresh garlic extract (FGE) (Mittal et al., 2006). Catheter pieces were transferred to fresh medium every 24 h. Catheter pieces were removed in duplicate daily and rinsed three times with PBS (pH 7.2). Cells were removed from the surface by scraping with a sterile scalpel blade, sonicated via a low-level sonication cycle and vortexed for 30 s. Dispersed sample was then centrifuged and biofilm cells were suspended in 1 mL PBS. Serial dilutions were made and counted on nutrient agar plates.

Preparation of FGE

Peeled garlic cloves were weighed, crushed and stock solution of 40 mg mL⁻¹ was made with normal saline. The solution was allowed to stand for 1 h followed by centrifugation at 3000 g for 10 min. Supernatant was filter sterilized and different concentrations ranging from 5 to 40 mg mL⁻¹ were prepared. A clinical isolate of P. aeruginosa was grown in the presence of different concentrations of FGE. The minimum concentration showing no growth inhibition was selected for further experiments.

Anti-infective potential of garlic in vivo

Induction of acute ascending pyelonephritis

The method of Hagberg et al. (1983) was used for the induction of UTI in mice. Prior approval of the Panjab University Animal Ethics Committee was obtained. Female LACA (Swiss Webster) mice, 6–8 weeks old, free of bacteriuria were used for the experiment. Animals were divided into two groups. Group 1 was given preinfection prophylactic treatment with 1 mL of selected concentration of FGE orally for 14 consecutive days and group 2 (control) was not given any prophylactic treatment. In the latter group, mice were further subgrouped as infected with no treatment, orally for 14 consecutive days and group 2 (control) was not given any prophylactic treatment. In the latter group, mice were further subgrouped as infected with no treatment, infected with saline treatment and only garlic fed without infection. Each group consisted of eight animals and experiments were performed in triplicate. Fifty microlitres of 10⁸ CFU mL⁻¹ of 4-day-old biofilm cells was introduced into the bladder of mice (Mittal et al., 2004) using a soft non-radio-opaque polyethylene tubing (outer diameter
Bacteriological examination

Animals were sacrificed on day 5 postinfection. Aseptically, kidneys were weighed and homogenized in 1 mL sterile saline solution. Quantitative bacterial counts were calculated per gram of kidney tissue as reported by Kakkar et al. (1986).

Histopathological examination

Kidneys were fixed in 10% buffered normal saline and dehydrated in 30–100% gradient ethanol. Paraffin wax blocks were prepared and 5-μm thin sections were stained with haematoxylin and eosin. Medulla, cortex, calyx and subcalyx of each kidney were evaluated. Histopathological examination of kidney was carried out to evaluate interstitial and perivascular infiltration of neutrophils, destruction of blood vessels, renal tubules and glomerular destruction. All histopathological investigations were performed under the supervision of an expert histopathologist.

Effect of garlic on virulence factors

Different virulence factors elaborated by *P. aeruginosa* were estimated in the culture supernatant of 4-day-old biofilm cells grown in the presence of selected concentration of FGE.

Alginate

Estimation was done according to the method of Mathee et al. (1999). Alginase present in culture supernatant was precipitated using an equal volume of 2% (w/v) cetylpyridium chloride followed by centrifugation at 8900 g for 20 min. Each pellet was suspended and precipitated with 5 mL of cold 2-propanol followed by centrifugation at 10 000 r.p.m. for 10 min. Each pellet was resuspended in 1 mL of normal saline. Alginase was determined using D-mannourate lactone used to calibrate a standard curve.

Pyochelin

Quantification of pyochelin was performed according to Arnow (1937). One millilitre of cell-free supernatant, 1 mL each of 0.5 N hydrochloric acid, nitrite molybdate reagent and 1 N NaOH were added and the final volume was made to 5 mL with distilled water. OD$_{510}$ nm was taken.

Pyoverdin

Quantification of pyoverdin was done by the method of Ankenbauer et al. (1985). One millilitre of cell-free culture supernatant was diluted with 50 mM Tris-HCl (pH 7.4) and fluorescence was measured at 460 nm while the samples were excited at 400 nm in a Gibson Spectro glo fluorometer.

Haemolysin

Both cell-free and cell-bound haemolysin were estimated following the method of Linkish & Vogt (1972).

Cell-bound haemolysin

Cell-bound haemolysin was determined by adding 1.5 mL of 2% washed human red blood cell (RBC) suspension to 1.5 mL of 4-day-old biofilm cells and incubating at 37 °C for 2 h. The assay mixture was centrifuged at 8000 g for 15 min. Supernatant was collected and $A_{545}$ nm was read. Haemolysin (mg mL$^{-1}$) was calculated using lyophilized haemoglobin as standard curve.

Cell-free haemolysin

For cell-free haemolysin, 1.5 mL of 2% washed human RBC suspension was mixed with 1.5 mL of cell-free supernatant and incubated at 37 °C for 2 h. Assay mixture was centrifuged at 5000 g for 5 min and OD$_{545}$ nm was taken.

Phospholipase C (PLC)

This was estimated by following the method of Habermann & Hardt (1972). Briefly, 50% (v/v) egg yolk suspension was prepared in saline and added to premelted agar to a final concentration of 10% with 0.1% sodium azide as preservative. Wells (8 mm) were cut into the agar plates and 100 μL of culture supernatant was added. Plates were incubated at 37 °C for 24–48 h. The diameter of hydrolysis of egg yolk was measured.

Phagocytic assay

Phagocytosis was performed according to the method of Allen et al. (1987). Peritoneal macrophages were isolated and washed twice with RPMI-1640.

Uptake

For determination of uptake, 0.4 mL of normal mouse serum, 0.5 mL of macrophage cell suspension (10$^6$ cells mL$^{-1}$) and 0.1 mL of bacterial suspension (10$^6$ CFU mL$^{-1}$) was mixed and incubated at 37 °C under 5% CO$_2$. Aliquots of 20 μL were transferred to 2 mL of cold RPMI-1640 after 0, 30, 60 and 90 min. Bacterial counts were determined by plating appropriate dilutions on MacConkey’s agar plates. Results were expressed as percentage bacteria taken up by the macrophages.

Intracellular killing

For measurements of intracellular killing, 4.5 mL of bacterial suspension (10$^8$ CFU mL$^{-1}$) was mixed with 0.5 mL of
normal mouse serum and kept for 30 min at room temperature. Bacterial suspension was washed with half volume of normal saline and added to 0.5 mL of macrophages in RPMI-1640 supplemented with 5% foetal calf serum (FCS). Cells were incubated for 20 min at 37°C. Free bacteria were removed by centrifugation at 1800 g for 5 min. Each pellet was resuspended in 2 mL of RPMI-1640 containing 5% FCS with 50 μL of gentamycin per millilitre and incubated at 37°C. Cells were lysed at 0, 1, 2, and 3 h postincubation with normal saline containing 0.5% sodium deoxycholate. CFUs were calculated after plating the dilutions on MacConkey agar plates.

**Statistical analysis**

All the experiments were repeated three times to validate reproducibility. SIGMASTAT software was used to analyse the results and to calculate P-values statistically by Student’s t-test.

**Results**

**Production and estimation of autoinducers (AHL)**

Production of quorum-sensing signal molecules was detected qualitatively based on development of blue colour by the reporter strain *E. coli* MG4 in all clinical isolates. Quantitative induction of β-galactosidase was also estimated in all the strains. Based on the highest level of quorum-sensing signal production in miller units, one clinical isolate was chosen for further study.

The selected clinical isolate was grown in the presence of different concentrations of FGE. The minimum concentration of FGE (35 mg mL⁻¹) showing no growth inhibition as compared with control was chosen for further *in vitro* and *in vivo* experiments (data not shown).

**Biofilm formation**

Biofilm of the clinical isolate was generated on catheter pieces and log CFU was calculated. *Pseudomonas aeruginosa* showed an increase in biofilm mass from day 1 to day 4 followed by a decline in the biofilm mass until day 7. Biofilm generated in the presence of FGE was reduced significantly as compared with control (Fig. 1). As maximum biofilm formation was observed on day 4, 4-day-old biofilm cells were used for all further *in vivo* and *in vitro* experiments.

**Effect of garlic on outcome of ascending pyelonephritis**

The efficacy of garlic on establishment of ascending UTI and renal bacterial colonization was evaluated in an experimental model of UTI in mice. Mice were divided into two groups. One group was given a prophylactic treatment with 1 mL of the selected concentration of FGE (35 mg mL⁻¹) orally for 14 days. The control group was split into three. The test group was given saline treatment orally for 14 days, a second group was not given any treatment while the third group of mice was only given garlic treatment without any infection. On day 15, all mice in the control group (saline and untreated) and treatment group (FGE) were challenged with 4-day-old biofilm cells of *P. aeruginosa*. Mice were sacrificed on day 5 postinfection. Kidneys were collected aseptically and were subjected to bacteriological and histopathological investigations.

In the untreated group and saline-treated group, mice infected with biofilm cells of *P. aeruginosa* showed comparable renal log CFU of 7.3 and 7.1, respectively. By contrast, the FGE-treated group without infection did not show any renal colonization. Infected mice which had undergone prophylactic treatment with FGE showed statistically reduced renal colonization with log CFU of 4.5 (*P* < 0.001) (Table 1). On histopathological examination, renal tissue of untreated but infected mice and saline-treated mice showed severe inflammation with infiltration of lymphocytes and plasma cells in glomeruli and subepithelium of renal pelvis. By contrast, infected mice given prophylactic treatment with FGE showed mild inflammation with normal glomeruli and tubules in medulla and cortex region of renal tissue of mice. FGE treatment in mice (without infection) did not show any histopathological changes in renal tissue, suggesting no effect of FGE on renal tissue (Figs 2–4).

**Effect of garlic on attenuation of *P. aeruginosa* virulence**

**Estimation of AHL**

AHL production (176 MU) was maximum in 4-day-old biofilm cells of the selected clinical isolate grown in the
absence of FGE. AHL production was reduced significantly (81 MU) when biofilm cells were generated in the presence of FGE \((P \leq 0.001)\) (Table 1).

**Table 1. Production of different virulence factors by the biofilm cells of *Pseudomonas aeruginosa* in the presence and absence of FGE**

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Parameter</th>
<th>In the absence of FGE</th>
<th>In the presence of FGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Renal colonization (log CFU)</td>
<td>7.3 ± 0.28</td>
<td>4.5 ± 0.29 ( (P \leq 0.001) )</td>
</tr>
<tr>
<td>2</td>
<td>Renal colonization saline group (log CFU)</td>
<td>7.15 ± 0.39</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>AHL production (MU)</td>
<td>176 ± 21</td>
<td>81 ± 11 ( (P \leq 0.001) )</td>
</tr>
<tr>
<td>4</td>
<td>Alginate (μg mL(^{-1}))</td>
<td>610 ± 11.7</td>
<td>507 ± 9.4 ( (P \leq 0.001) )</td>
</tr>
<tr>
<td>5</td>
<td>Pyochelin (OD(_{510\text{nm}}))</td>
<td>0.17 ± 0.001</td>
<td>0.16 ± 0.001</td>
</tr>
<tr>
<td>6</td>
<td>Pyoverdin (relative OD)</td>
<td>775 ± 13.4</td>
<td>410 ± 11.0 ( (P \leq 0.001) )</td>
</tr>
<tr>
<td>7</td>
<td>Cell-bound haemolysin (mg mL(^{-1}))</td>
<td>2.2 ± 0.2</td>
<td>1.9 ± 0.1 ( (P \leq 0.001) )</td>
</tr>
<tr>
<td>8</td>
<td>Cell-free haemolysin (mg mL(^{-1}))</td>
<td>3.25 ± 0.12</td>
<td>1.42 ± 0.1 ( (P \leq 0.001) )</td>
</tr>
<tr>
<td>9</td>
<td>Phospholipase C (zone of clearing, mm)</td>
<td>12.5 ± 0.3</td>
<td>7.5 ± 0.2 ( (P \leq 0.001) )</td>
</tr>
</tbody>
</table>

NA, not applicable.

**Fig. 2.** Photomicrograph showing severe inflammation between the tubules along with interstitial infiltration of lymphocytes, tubule destruction and formation of protein casting in tubules along with the renal pelvis of kidney tissue of control unfed mice infected with biofilm cells of *Pseudomonas aeruginosa* (haematoxylin and eosin, × 25).

**Fig. 3.** Photomicrograph showing mild inflammation with markedly reduced infiltration of lymphocytes in the interstitium, with normal tubules of the medulla region along the renal pelvis of kidney tissue of FGE-fed mice followed by infection with biofilm cells of *Pseudomonas aeruginosa* (haematoxylin and eosin, × 25).

**Fig. 4.** Photomicrograph showing normal glomeruli and tubules in renal tissue of FGE-fed mice followed by infection with biofilm cells of *Pseudomonas aeruginosa* (haematoxylin and eosin, × 100).

**Discussion**

*Pseudomonas aeruginosa*, a common nosocomial UTI pathogen, develops resistance to antibiotic therapy mainly because of its ability to form biofilms. This poses a great challenge for the treating clinicians as such infections often lead to
prophylactic treatment in an experimental UTI model. Mice were challenged with biofilm cells of P. aeruginosa and were sacrificed and establishment of pyelonephritis was evaluated on the basis of bacteriological and histopathological examinations. Prophylactic treatment with garlic was able to significantly lower renal bacterial counts in test group animals (P ≤ 0.001). Histopathological results were also in accordance with the bacteriological counts, showing significant differences between test and control groups. Renal tissue lesions were of less severity in the garlic-treated group, suggesting attenuation of virulence of P. aeruginosa in the UTI model. A protective role of garlic was also demonstrated by Bjarnsholt et al. (2005) in an experimental lung infection model. These workers observed that subcutaneous administration of garlic extract decreased the virulence of P. aeruginosa significantly and protected the mice from lethal lung infection. The pathogen neither formed biofilms nor expressed quorum-sensing-associated virulence factors. Based on the results of this study, these workers highlighted the importance of quorum-sensing inhibitory components in garlic. Although pulmonary infections are also an example of mucosal infections, the pathogenesis of these infections may differ from that of UTIs. Moreover, in comparison with the subcutaneous route followed by Bjarnsholt et al. (2005), the oral route of garlic administration, adopted in the present study, is more meaningful as garlic is generally consumed orally. Other routes may have deleterious effects on the host. To the best of our knowledge, no study regarding the role of garlic in a UTI model is available. The results of our study are also supported by observations made in another study (Rasmussen et al., 2005), which used a gene chip-based transcriptome analysis, and showed that garlic had specificity for quorum-sensing-controlled virulence genes in vitro in P. aeruginosa.

Results of in vivo experiments encouraged us to explore further and check if the reduction in the severity of infection was due to attenuation of virulence factors in the presence of garlic. To evaluate this, quorum-sensing signals produced by P. aeruginosa were estimated in the presence and absence of FGE. These signals are presently being considered as probable virulence factors. Interestingly, garlic blocked production of these signals in vitro, qualitatively and quantitatively. As these signals are also known to be produced in vivo in sputum samples (Erickson et al., 2002) and lungs (Wu et al., 1999), the blocking of quorum-sensing signals in vivo by garlic may also be an important mechanism for decreased establishment of organisms in the urinary tract. The in vitro results of this study corroborate this hypothesis. Levels of alginate, considered as one of the virulence factor of P. aeruginosa, were estimated in biofilm cells. This mucoid exopolysaccharide helps in tissue colonization and formation of biofilms. Pseudomonas aeruginosa showed significantly less production of alginate when biofilm was formed in the presence of FGE. But biofilms were more sticky and thick when grown in the absence of FGE. Elastase and protease are also important virulence factors of P. aeruginosa and play a role in disseminating infections (Woods et al., 1986).
The contribution of these factors in UTIs has yet to be demonstrated. Estimation of these virulence factors was therefore not carried out in the present study.

A role of siderophores has been pointed out in the pathogenesis of UTI by many workers (Johnson, 1991; Visca et al., 1992), and therefore siderophores and haemolysins were estimated in the present study. *Pseudomonas aeruginosa* produces two types of siderophores, pyochelin and pyoverdin, to trap iron from the host tissues. Production of a low level of siderophores by biofilm cells grown in the presence of FGE suggested that garlic also inhibited iron acquisition mechanisms. Production of haemolysins led to the lysis of FGE suggested that garlic also inhibited iron acquisition mechanisms. Production of haemolysins led to the lysis of host cells and organelles and also helps the organism to acquire iron. It is considered as an important virulence factor in UTIs (Johnson & Allen, 1978; Gabisoniia et al., 1992). In the present study, growth of *P. aeruginosa* in the presence of FGE also showed reduced production of haemolysins, indicating decreased lysis of host cells in the presence of garlic. In addition, biofilms grown in the presence of FGE showed reduced production of PLC. The innate immune defence provided by PMNs forms the first line of defence in the body against invading organisms. Growth in the presence of FGE made *P. aeruginosa* more susceptible to the action of PMNs, resulting in increased uptake and killing of *Pseudomonas* by PMNs as compared with that of control. In *Pseudomonas*, the biofilm mode of growth protected the bacteria from phagocytosis (Elkins et al., 1999; Jesaitis et al., 2003). Increased phagocytic uptake and killing by macrophages along with reduced production of exoenzymes in the presence of FGE may have contributed towards the reduced virulence of *P. aeruginosa*.

Garlic is believed to be useful in prevention of diseases but conclusive evidence is lacking. Most research is directed towards the antibacterial effect of garlic as it has been reported to exhibit a broad antibiotic spectrum against a number of gram-positive and gram-negative bacteria, including *P. aeruginosa* (Ankri & Mirelman, 1999; O’Gara et al., 2000; Tsao & Yin, 2001). The present study highlights that garlic significantly blocks production of quorum-sensing signal molecules as well as extracellular virulence factors. Garlic also helps to attenuate the virulence of *P. aeruginosa* in *vivo*, as observed in this study, which resulted in reduced pathogenicity of this organism in an experimental UTI model. These observations suggest that garlic has the prophylactic potential to prevent nosocomial UTIs caused by biofilm cells of *P. aeruginosa* in catheterized patients.

**References**


