Enterotoxicity and related virulence factors of diarrhoeagenic local isolates of *Pseudomonas aeruginosa*

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**Abstract**

A total of 23 isolates of *Pseudomonas aeruginosa* were isolated from watery and soft frequent stool samples. Results for enterotoxicity, invasiveness, serum resistance and phagocytosis inhibition showed that 4 of 6 isolates isolated from watery stool samples were heat-labile enterotoxin producers, whereas 2 of 17 of soft stool were heat-labile enterotoxin producers. The 6 *P. aeruginosa* watery stool samples were invasive and phagocytosis inhibitors, whereas not all soft stool isolates of *P. aeruginosa* were invasive or phagocytosis inhibitors. All *P. aeruginosa* isolates were serum resistant. *P. aeruginosa* isolates of watery stool samples were most virulent than soft stool samples as diarrhoeagenic isolates, giving a strong evidence for their potential role as an intestinal pathogens.

**Introduction**

*P. aeruginosa* is an environmental organism (water, soil and on plants), commonly present in moist environment in hospitals (7). It can colonize normal humans, in whom it is a saprophyte and cause disease in humans with abnormal host defenses (7).

*P. aeruginosa* infects body sites devoid of normal immune defenses as wounds and burns, and also responsible for device-associated infections as urinary tract infection, when introduced by catheters and instruments, and respiratory tract infections, especially from contaminated respirators, results in necrotizing pneumonia (17).

*P. aeruginosa* can cause serious infections in immunocompromised and critically ill patients, as it causes an invasive (malignant) otitis media in diabetic patients (7). In infants or debilitated person, *P. aeruginosa* may invade the bloodstream and result in fatal sepsis; this occurs commonly in patients with leukaemia or lymphoma who have received antineoplastic drugs or therapy and in patients with severe burns (17).
Enterotoxicity and related virulence factors of diarrhoeagenic local isolates of *Pseudomonas aeruginosa* Sadik, Tahreer

The association of *P. aeruginosa* with diarrhoea was noticed since fourteenths of the last century, especially with severe watery diarrhoeal cases (2, 16), but they didn't reveal the mechanism of diarrhoea induced by this bacterium. This research was aimed to reveal the mechanism of *Pseudomonas*-induced diarrhoea and other related virulence factors as invasiveness, serum resistance, and phagocytosis inhibition, as a preliminary study to establish *P. aeruginosa* as an intestinal pathogen.

**MATERIALS AND METHODS**

**Specimens.** A total of 244 stool samples were collected in stool examination & culture unit of medical laboratories in Al-Yarmouk general hospital in Baghdad from 18/5/2005 to 20/7/2005. All samples were cultured on Blood agar and McConkey agar (Himedia, India). Watery diarrhoea samples were cultured on TCBS agar (Himedia, India), whereas bloody stool samples were cultured on SS agar (Himedia, India). Plates were incubated on 37°C for 18 hr. Bacterial growth were purified to isolated colonies for each sample, and isolated colonies were diagnosed by using colony morphology, slide morphology, Biochemical tests, and API 20E and API Staph and API Strep systems (Bio-Merieux, France).

**Assay for Vascular Permeability factor activity** (26)

This assay was done according to Sandefur & Peterson (26). Adult albino rabbits were shaved, and the remaining hair was removed with a depilatory lotion (Kasanova, Paris) prior to skin testing. Culture filtrate were prepared by cultivation of the isolate to be tested for Vascular permeability factor on Mueller-Hinton agar (Himedia, India) supplied with 0.6% yeast extract (BBL, USA) in 37°C for 18 h with shaking. The 18 h. Culture were centrifuged at 10,000 RPM. Culture supernatant was filtrate through 0.45 μm filter (Oxoid, England).

One-tenth-milliter injections of supernatant filtrate were given intradermally using 26-gauge insulin syringe. Each animal were gagged separately to avoid friction with each other. Sterilized Muller-Hinton agar supplied with 0.6% yeast extract were used as a negative control, whereas *Vibrio cholerae* culture filtrate used as a positive control.

Result were interpreted as a positive for rapid vascular permeability factor in the case of development of erythema at the site of injection directly after the injection (an indication for heat stable (ST) enterotoxin), and interpreted as a positive for delayed vascular permeability factor in the case of development of an induration in the
site of injection represented by an odema and vascular hemorrhage 18 h after the injection (an indication of heat-labile (LT) enterotoxin).

**Suckling mouse assay**

Suckling mouse assay was by method of Gianella (12) as below:

**Animals.** Newborn albino suckling mice (1 to 3 days old) were separated from their mothers immediately before use and randomly divided into groups of three.

Each mouse was inoculated (Intragastrically) with 0.1 ml of crude culture filtrate containing 2 drops of 2% Evans blue dye (Fluka, Switzerland).

Mice were incubated at 25ºC for 3 h. The mice were killed by cervical dislocation, the abdomen was opened, and the entire intestine (not including the stomach), was removed with forceps. The intestines from each group of three mice were pooled and weighed, and the ration of gut weight to remaining carcass weight was calculated. Animals with no dye in the intestine or with dye within the peritoneal cavity at autopsy were discarded.

Animal with gut weight/carcass ratio ≥0.085 was interpreted a positive result, whereas gut weight/carcass ratio <0.085 interpreted as a negative result.

**Serény test**

Serény test were made on mice by method of Murayama et al. (20), as below:

Fresh bacteria grown overnight at 37ºC on Muller-Hinton broth (Himedia, India) were harvested and suspended in Muller-Hinton broth to give approximately 5x10^10 viable cells per ml. Each eye was infected with 1x10^8-5x10^8 viable cell per eye (10 µl). The right eye received the bacterial inoculum, and the left eye received sterile saline as a control for serény test.

Each mouse was gagged separately and monitored for development of macroscopic changes characteristic of conjunctivitis, which are redness and swelling of the palpera.

**Serum resistance**

Bacterial isolates were tested for their serum resistance according to method of Taylor & Hughes (25) as below:

Normal human serum was obtained from healthy volunteers and used immediately.

Bacterial isolate to be tested for their serum resistance was grown on Trypticas soy broth (Himedia, India). An early log phase of this Trypticas soy broth culture was washed in 0.06 M NaCl (BDH, England) and suspended in 0.05 M tris (hydroxymethyl) aminomethane (Tris)-hydrochloride (BDH, England) buffer (pH 8.4) to a concentration of 10^6 organisms per ml.
A sample (0.5 ml) of this suspension was added to 1.5 ml of serum, and viable counts were obtained at the beginning of the test and after 1,2, and 3 h of incubation at 37°C.

**Phagocytosis resistance**

Bacterial isolates were tested for their phagocytosis resistance according to Bastos *et al.* (4) as below:

An overnight Trypticase broth culture of tested bacteria were washed with 20 mM PBS, pH 7.4 and suspended with the same buffer to a concentration of $10^8$ organisms per ml.

The peritoneal cavities of 10-week-old abino female mice were stimulated by injecting a 10% peptone (Oxoid, England) solution. Three days later, mice were injected intraperitoneally (ip) with 0.2 ml of tested bacteria ($10^8$ cell/ml in 20 mM PBS, pH 7.4).

Thirty minutes after the ip injections, the mice were sacrified by cervical dislocation, and their peritoneal cavities were washed with 3 ml of 20 mM PBS containing 5 IU heparin/ml (Sigma-Aldrich, USA). The macrophages collected were resuspended to $2 \times 10^6$ in 20 mM PBS, pH 7.4.

This suspension was cytocentrifuged and the cells were cytcentrifuged and the cells were stained with Giemsa (Fluka, Switzerland).

The percentage of phagocytes was determined by counting phagocytic and non-phagocytic cells in a total of 100 cells. The numbers of macrophages were counted by light microscopy at a magnification of 100X.

**RESULTS AND DISCUSSION**

**Isolation of *P. aeruginosa* from diarrhoeal cases.** A total of 23 isolates of *P. aeruginosa* was isolated from 23 samples of watery and soft stool samples.

*P. aeruginosa* was isolated in 6 samples of 78 samples of watery diarrhoea (7.7%), and in 17 samples of 134 samples of semisolid stool (12.7%), whereas *P. aeruginosa* not isolated from 32 bloody diarrhoea samples.

*P. aeruginosa* was isolated alone in 2 samples of watery diarrhoea, whereas isolated in concomitant with other intestinal pathogens in 4 samples of watery diarrhoea. These concomitant pathogens were *Escherichia coli*, *Proteus mirabilis*, and *Bacillus cereus*.

*P. aeruginosa* was isolated in concomitant with other intestinal pathogens in all semisolid stool samples. But it was the most prevalent bacteria of these samples.

**Assay for Vascular Permeability factor activity.** Results of this assay showed that 4 of *P. aeruginosa* isolates of watery diarrhoea were found to be positive for delayed vascular permeability factor (Heat-labile
enterotoxin producers), and negative for rapid vascular permeability factor (Non heat-stable enterotoxin producers), whereas the other 2 *P. aeruginosa* isolates of watery diarrhoea were negative for both rapid and delayed vascular permeability factor. Two isolates of *P. aeruginosa* isolates of 17 of soft stool isolates were found to be positive for delayed vascular permeability factor, and negative for rapid vascular permeability factor (figure1).

![Figure 1: Induration lesions on rabbit skin after 18 hr of subcutaneous injection of bacterial culture filtrate grown for 18 hr on brain-heart infusion broth supplemented with 0.6 yeast extract. A. Negative result, B. Positive result.](image_url)

The enterotoxigenic isolates of *P. aeruginosa* were isolated from different ages of both sexes, as table 1 showed.

<table>
<thead>
<tr>
<th><em>P. aeruginosa</em> isolate</th>
<th>Patient's sex (yr)</th>
<th>Patient's age</th>
<th>Stool consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pae56</td>
<td>4</td>
<td>male</td>
<td>Watery</td>
</tr>
<tr>
<td>Pae83</td>
<td>55</td>
<td>Female</td>
<td>Watery</td>
</tr>
<tr>
<td>Pae88</td>
<td>12</td>
<td>Female</td>
<td>Watery</td>
</tr>
<tr>
<td>Pae224</td>
<td>7</td>
<td>male</td>
<td>Watery</td>
</tr>
<tr>
<td>Pae18</td>
<td>28</td>
<td>male</td>
<td>Soft</td>
</tr>
<tr>
<td>Pa165</td>
<td>56</td>
<td>female</td>
<td>Soft</td>
</tr>
</tbody>
</table>
Enterotoxicity and related virulence factors of diarrhoeagenic local isolates of *Pseudomonas aeruginosa* Sadik, Tahreer

**Suckling mouse assay.** All *P. aeruginosa* isolates were negative for suckling mouse assay.

**Serény test.** The 6 *P. aeruginosa* isolates of watery diarrhoea were found to be positive for this test, whereas 8 of 17 *P. aeruginosa* isolates of semisolid stool samples were positive for this test.

**Serum resistance.** Most isolates of *P. aeruginosa* were serum resistant. The 6 *P. aeruginosa* isolates of watery diarrhoea were serum resistant, whereas 15 of 17 *P. aeruginosa* isolates of semisolid stool samples were serum resistant (figure 2).

**Phagocytosis inhibitor.** The 6 *P. aeruginosa* isolates of watery diarrhoea were phagocytosis inhibitors, whereas 8 of 17 *P. aeruginosa* isolates of soft stool samples were phagocytosis inhibitors (figure 3).

**Figure -2:** Bactericidal effect of human serum on isolates Pae 56, Pae 83, Pae88, and Pae224 of Enterotoxigenic *Pseudomonas aeruginosa*.

**Figure -3:** Percentage of phagocytosis of *Pseudomonas aeruginosa* isolates by mouse peritoneal macrophages after 30 min. of bacterial intraperitoneal injection.
Isolation of *P. aeruginosa* from watery and semisolid stool samples.

Culture results for watery and semisolid stool samples showed a strong association of intestinal colonization of *P. aeruginosa* with gastrointestinal disturbances. Isolation of *P. aeruginosa* alone from two watery stool samples is highly indicative for their diarrhoeagenicity in these samples, whereas their isolation in concomitant with other intestinal pathogens for the remaining 4 watery stool, and soft stool samples is indicative for their contribution as major intestinal pathogen, or an intestinal co-pathogen for these samples.

The association of *P. aeruginosa* with human diarrhoeal cases, especially watery diarrhoea cases is well-documented since fourteenth of the last century (6,11,15, 24).

Alard *et al.* (2) showed that *P. aeruginosa* is a cause of infectious diarrhoea, as isolated from 23 unrelated, hospital outpatients of a mean age of 60 years (3-90 years). All were reported recent, prolonged episodes of diarrhoea; several were had a history of recurrent diarrhoeal episodes (months to years).

Kim *et al.* (16) showed that *P. aeruginosa* is a cause of antibiotic-associated diarrhoea (AAD) of a seven patients, four were males and three were females. The median age of patients was 58 (41-71) yr. All patients had been receiving antibiotics and median duration of antibiotics before diarrhoeal onset was 6 (3-21) days.

**Assay for Vascular Permeability factor activity.** Production of heat-labile enterotoxin by 6 isolates of *P. aeruginosa* makes these isolates the main intestinal pathogens for cases of diarrhoea (Watery diarrhoea and soft frequent stool), as the only mechanism for production of watery diarrhoea is the production of heat-labile enterotoxin (13).

The results of this assay are consistent with all previous studies showed that isolation of *P. aeruginosa* were always correlated with cases of watery diarrhoea (2, 6, 11, 15, 16).

Table 1 showed that enterotoxigenic *P. aeruginosa* were isolated from different ages (4-56) yr with a mean age of 27 yr. This means that enterotoxigenic *P. aeruginosa* is virulent against different age groups and not confined into certain age group, as Enterotoxigenic *E. coli* and *Vibrio cholerae* (22), whereas some other enterotixigenic bacteria, as *Aeromonas* spp. and *Campylobacter jejuni* were confined to children (8,1).

**Serény test.** Majority of *P. aeruginosa* isolates (61%) were found invasive, including enterotoxigenic & non-enterotoxigenic *P. aeruginosa* isolates.

Invasiveness is common in clinical isolates of *P. aeruginosa*. Fleischig *et al.* (10) showed that *P. aeruginosa* isolates can be broadly
Enterotoxicity and related virulence factors of diarrhoeagenic local isolates of *Pseudomonas aeruginosa*

Sadik, Tahreer

differentiated into two groups, expressing either a cytotoxic, or an invasive phenotype.
The invasiveness of enterotoxigenic *P. aeruginosa* isolates indicates that the mechanism of diarrhoea produced by these isolates is similar to enterotoxigenic-invasive intestinal pathogens, as *Campylobacter jejuni* (23) and *Salmonella typhimurium* (3).

In this mechanism, after mucosal adherence and enterotoxin production, the enterotoxigenic bacteria translocate from enterocyte to lamina propria, when it drained into the mesenteric lymph node, where it reach the blood stream (18).

**Serum resistance.** Most *P. aeruginosa* isolates (91%) were serum resistant. Serum resistance of *P. aeruginosa* isolates is a representative for their pathogenicity and invasiveness, as non-pathogenic non-invasive bacteria is usually serum sensitive, whereas invasive pathogens is highly serum resistant, as serum resistance enables invasive pathogens to survive and spread inside infected host, resulting in new secondary infections in another sites of host's body (5, 21).

Studies on experimental animals showed that serum resistance of invasive bacteria is an important aspect for their pathogenicity (9).

**Phagocytosis resistance.** Phagocytosis resistance of *P. aeruginosa* isolates plays an important role in pathogenicity of these isolates, as antiphagocytic properties is a main indicator for pathogenicity in many bacterial pathogens, as enteropathogenic *E. coli* (14), *Yersinia enterocolitica* (19), *Neisseria gonorrhoeae* (27).

The possession of *P. aeruginosa* isolates for enterotoxicity, invasiveness, serum resistance, and antiphagocytic properties makes it potent intestinal pathogens, which are able to cause severe watery diarrhoea with other symptoms associated with translocation of bacteria to blood stream.

Further studies on animal model for *P. aeruginosa* - induced diarrhoea, and genetic studies on plasmid content, plasmid gene structure, and detection and expression of genes involved in enterotoxicity and other virulence factors is recommended to establish *P. aeruginosa* as an intestinal pathogen.

**REFERENCES**


Enterotoxicity and related virulence factors of diarrhoeagenic local isolates of \textit{Pseudomonas aeruginosa} \\
Sadik, Tahreer


