Characterization of *Pseudomonas aeruginosa* exotoxin recovered from camels

By

Ansam M Salman

Immunology Dept., Animal Health Research Institute, Dokki, Giza

**SUMMARY**

Eight *Pseudomonas aeruginosa* isolates were recovered from a total of 53 pneumonic lung samples of camels in a percentage of 15.1%. Application of the salt aggregation test showed that 75% of the isolates were hydrophobic. Results of bacterial survival test in camel serum showed that 87.5% of the recovered isolates survived in camel serum. β-galactosidase activity of the isolates ranged from 401-840 Miller unit. Results of intradermal injections of *P. aeruginosa* crude exotoxins in guinea pigs induced increase in skin thickness at the site of injection that varied from moderate to severe skin reactions. *P. aeruginosa* prepared exotoxin induced significant morphological changes in Vero cells. Sera collected from mice immunized with *P. aeruginosa* crude exotoxin demonstrated increase in antibody level (measured with ELISA) as compared to the non immunized mice. Administration of gentamicin with the vaccine was associated with reduction of antibody production, whereas norfloxacin have no effect on antibody formation.

**INTRODUCTION**

*Pseudomonas aeruginosa* is highly pathogenic to respiratory system of camels (Dapgh, 2000 and Saddek, 2002). It causes severe infections among immunocompromised hosts (Cripps et al., 1997) and considered as one of the main causes of bacteremia (Montanaro et al., 2006). *Pseudo- monas aeruginosa* cause exuberant chronic host inflammatory response, which together with the bacterial products damage host tissues leading to clinical deterioration of lung function (Fawthrop et al., 1991). It infects the bronchial trees of animals and once colonization is established, the bacteria is rarely eliminated (Fick, 1989).
P. aeruginosa exotoxins are cytotoxic to numerous mammalian cells and inhibit protein synthesis in vitro and in vivo (Melstrom et al., 2007). Exotoxin has been implicated as a major pathogenic factor in P. aeruginosa infections. It is highly immunogenic and produces highly specific antibodies (Cross, 1980).

Immunosuppression still represent the bad adverse reaction that may be induced by antibiotics, which suppress the immune system even at therapeutic level (Nagwa et al., 2005). Immunosuppressive properties of antibiotics are among the main causes of inhibition of humoral immune responses against vaccines (Shalaby, 1989). Several antibiotics suppress the immune response in man and animals by their ability to interfere with immunoglobulin synthesis (Panigrahy et al., 1978).

The purposes of this study were to isolate P. aeruginosa from diseased camels, to study some of its virulence factors and extraction of crude exotoxin and studying its biological effects using cytotoxicity assays and delayed skin hypersensitivity reaction. It was also planned to elucidate the immunological characteristics of the crude exotoxins and the effect of some antibiotics on the antibody produced against it.

MATERIALS AND METHODS

Bacteriological examination:
A total of 53 lung specimens from pneumonic lungs were collected from slaughtered camels at Kerdasa abattoir. These specimens were subjected to bacteriological examination. Cultivation and identification were done according to Quinn et al. (1994). Serotyping of P. aeruginosa were performed by slide agglutination test using P. aeruginosa specific antisera (Difco, Detroit, Michigan, USA).

Virulence factors of P. aeruginosa:
Survival in camel serum:
Using a straight wire, saline bacterial suspension of P. aeruginosa isolates was inoculated into a well of hemagglutination tray containing 150 µl of camel serum. After incubation at 37°C for 1, 3, 6 hours 20 µl were plated into tryptose agar and the number of colonies were counted (Barrow and Hill, 1989).

Hydrophobicity examination (Erganis et al., 1989):
All isolates were grown on colonization factor agar "CFA" (Evans et al., 1979) for 18 hour at 37°C. Growing colonies were washed twice and resuspended in PBS. Equal volume of P. aeruginosa bacterial suspension (10^8 CFU/ml) and ammonium sulphate of differ-
ent molarities (0.01-3 M) were mixed on glass slides at room temperature for 4 minutes. Isolates with clumbing activity at ≤ 1.5 M were hydrophobic

**Measurement of β-galactosidase activity:**

It was carried out according to the standard procedure described by Miller (1972). About 100 μl of cells with known optical density at 600 nm, were resuspended in 0.9 ml of PBS lysed with 10 μl of the 0.1%SDS, 20 μl of chloroform and 200 μl of ONPG 4mg/ml was added each min until the mixture turned yellow. The OD 420 and OD 550 of the reaction mixture, the reaction duration in min and the OD 600 of bacterial culture were used to calculate the corrected β-galactosidase activity. Each assay was repeated at least two times.

**Extraction of crude exotoxin (Lynn and Callahan, 1976):**

One hundred 32- once (approximately 0.95-liter) prescription bottles containing tryptic soy broth dialysate with 1% glycerol and 1% agar were inoculated each with tryptic soy broth culture of *P. aeruginosa* isolate and incubated at 37°C for 24 h. after incubation, the cultures were frozen and thawed and then centrifuged at 16000 x g for 30 min. The supernatant fluid was passed through 0.45 μm membrane filter and stored at -20 ºC until used. This material is referred to as a crude exotoxin preparation.

**Determination of protein content:**

It was done to measure the protein content of exotoxin according to Lowry *et al.* (1951).

**Biological assays on the prepared *P. aeruginosa* crude exotoxins:**

**Estimation of delayed skin reaction (Liu, 1974):**

Guinea pigs serotype were shaved and 0.1 ml of crude exotoxin of each *P. aeruginosa* was injected intradermally and the reaction was recorded after 48 h. A zone of firm induration (oedema) without redness was recorded.

**Cytotoxicity assays (Speires *et al.*, 1977):**

Crude exotoxins of *P. aeruginosa* isolates were examined for their ability to induce cytopathic effect (CPE) on Vero cells. Plates of vero cell cultures containing different concentrations of the crude exotoxin were incubated for 24 and 48 h in CO₂ atmospheric tension and examined microscopically for cytopathic effect. Toxicity more than 50% was considered positive.

**Immunization and antibiotic treatment:**

Four groups of mice were used (each 8 mice), group 1 was
immunized subcutaneously with 50 µg of crude exotoxins twice at 15 day intervals without antibiotic. The second group was injected, in addition to the vaccine, with gentamicin (2.2 mg) given I/P diluted in saline with the vaccination process. The third group received norfloxacin (2.2 mg I/P) with vaccine process. The fourth group was considered as control negative. Blood samples from each group were used for serum collection. This immunization protocol was repeated separately for each four exotoxin preparations prepared from each P. aeruginosa serogroup namely, A, K, F and G (Udhayakumar and Muthukkaruppan, 1987).

Evaluation of antitoxin by ELISA:
Antitoxin level of immunized non-treated mice, immunized antibiotic treated groups and control non immunized groups were determined using ELISA (Hariharan et al., 1986).

RESULTS
The results recorded in Table (1) showed that eight P. aeruginosa isolates were obtained from pneumonic lungs of camels, poly I was belonging to serogroup A (1 isolate), poly II was K (two isolates) and poly III were F (2 isolates) and G (3 isolates). Results of hydrophopic activity are presented in Table (1). Out of the eight isolates isolated from diseased camels, 6 were hydrophobic (75%), which can aggregate in ammonium sulphate at concentration ≤ 1.5 M. Results of survival in serum (Table 1) indicated that 87.5% of isolates recovered from diseased camels were able to survive in camel serum.

The standard Miller procedure was used for quantitative measurement of β- galactosidase of the isolates (Table 1) showed wide variation between the mean value among the different serogroups where β-galactocidase activity ranged from 401 to 840 miller unit.

Results of intradermal injections of crude exotoxin recovered from P. aeruginosa in G. pigs (Table, 2 and Photo 1) showed that there was increase in the skin thickness and induration at the site of injection that varied from moderate to severe reaction (24-48 h) post-injections. Table (2) and photo (3) revealed that all P. aeruginosa recovered from diseased camels produce crude exotoxins with high cytotoxic activity to Vero cell monolayer as compared to untreated monolayer Vero cell culture (Photo, 2).

Table (3) showed the results of mean optical density (OD) of mice vaccinated with crude exotoxin. Significant rises of
ELISA antibodies level were detected till 4th week post-immunization (group 2) and then there was slight decline in mean OD as compared to non vaccinated mice in Group 1. Whereas animals vaccinated and simultaneously treated with gentamicin showed significant reduction in antibody production (Group 3). Animals, which were treated with norfloxacin (Group 4) showed no great differences in antibody optical density in comparison to vaccinated non treated group.

Table (1): Virulence attributes of *P. aeruginosa* isolates recovered from camels with respiratory problems.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Sero-groups</th>
<th>Hydrophobicity activity</th>
<th>Survival serum</th>
<th>β-galactocidase activity (Miller unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Poly I A</td>
<td>1.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Poly II K</td>
<td>0.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>K</td>
<td>0.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Poly III F</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>G</td>
<td>2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>G</td>
<td>0.05</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>G</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Total positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The percentage was calculated according to the total number of the isolates (8)
Table (2): Biological assays of crude exotoxins extracted from \textit{P. aeruginosa} isolates.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Sero-groups</th>
<th>Delayed type hypersensitivity</th>
<th>Vero cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>1</td>
<td>Poly I</td>
<td>A 1+</td>
<td>60 %</td>
</tr>
<tr>
<td>2</td>
<td>Poly II</td>
<td>K 1+</td>
<td>69 %</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>K 2+</td>
<td>77 %</td>
</tr>
<tr>
<td>4</td>
<td>Poly III</td>
<td>F 1+</td>
<td>85 %</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>F 2+</td>
<td>80 %</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>G 3+</td>
<td>87 %</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>G 1+</td>
<td>85 %</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>G 2+</td>
<td>71 %</td>
</tr>
</tbody>
</table>

1+ means 5 to 10 mm increase in skin thickness (induration)  
2+ means 10 to 15 mm increase in skin thickness (induration)  
3+ means 15 to 20 mm increase in skin thickness (induration)  

Photo (1): Skin reaction of guinea pigs 48 hrs following interdermal injection of \textit{P. aeruginosa} crude exotoxin.
Photo (2): Vero cell monolayer showing no alteration (Negative control).

Photo (3): Vero cell cytotoxicity in the presence of *P. aeruginosa* crude
Table (3): Evaluation of humoral immune response using ELISA in mice immunized with *P. aeruginosa* crude exotoxins in the presence or absence of antibiotic therapy.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Groups</th>
<th>Pre vaccination</th>
<th>1(^{st}) week</th>
<th>2(^{nd}) week</th>
<th>3(^{rd}) week</th>
<th>4(^{th}) week</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Group 1</td>
<td>0.120 ± 0.011</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>0.288 ± 0.011</td>
<td>0.589 ± 0.004</td>
<td>0.711 ± 0.001</td>
<td>0.890 ± 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 3</td>
<td>0.121 ± 0.001</td>
<td>0.188 ± 0.001</td>
<td>0.201 ± 0.001</td>
<td>0.299 ± 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 4</td>
<td>0.283 ± 0.002</td>
<td>0.611 ± 0.001</td>
<td>0.702 ± 0.002</td>
<td>0.840 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>Group 1</td>
<td>0.108 ± 0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>0.290 ± 0.001</td>
<td>0.499 ± 0.001</td>
<td>0.811 ± 0.001</td>
<td>0.881 ± 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 3</td>
<td>0.119 ± 0.001</td>
<td>0.109 ± 0.001</td>
<td>0.211 ± 0.001</td>
<td>0.119 ± 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 4</td>
<td>0.288 ± 0.001</td>
<td>0.489 ± 0.002</td>
<td>0.801 ± 0.001</td>
<td>0.850 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Group 1</td>
<td>0.121 ± 0.004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>0.281 ± 0.001</td>
<td>0.581 ± 0.001</td>
<td>0.699 ± 0.001</td>
<td>0.790 ± 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 3</td>
<td>0.120 ± 0.001</td>
<td>0.188 ± 0.001</td>
<td>0.220 ± 0.002</td>
<td>0.121 ± 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 4</td>
<td>0.287 ± 0.001</td>
<td>0.591 ± 0.001</td>
<td>0.680 ± 0.001</td>
<td>0.781 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Group 1</td>
<td>0.123 ± 0.006</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>0.292 ± 0.001</td>
<td>0.581 ± 0.001</td>
<td>0.680 ± 0.001</td>
<td>0.840 ± 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 3</td>
<td>0.121 ± 0.003</td>
<td>0.111 ± 0.002</td>
<td>0.230 ± 0.001</td>
<td>0.199 ± 0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 4</td>
<td>0.299 ± 0.002</td>
<td>0.579 ± 0.001</td>
<td>0.669 ± 0.00</td>
<td>0.810 ± 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Group 1: non immunized mice  
Group 2: mice immunized with crude exotoxin alone  
Group 3: immunized and gentamicin treated mice  
Group 4: immunized and norfloxacin treated mice
DISCUSSION

*Pseudomonas aeruginosa* is highly pathogenic for respiratory tract of camels (Saddek, 2002 and Ibrahim, 2003). From Table (1) it was interested to note that out of 53 lung samples from pneumatic camels *P. aeruginosa* was recovered from 8 cases with an incidence of 15.1 %. Studying the salt aggregation test for measuring cell surface hydrophobicity of the isolates showed that 75% were hydrophobic. There are close relationship between hydrophobic properties and the presence of fimbrial antigens (Osek, 1991). Pili and fimbriae are known to mediate the first stage of adherence (Ramphal et al., 1984). Regarding the results of survival in camel serum, 87.5% of the isolates survived. Virulence of pathogenic microorganism appears to be correlated with ability to survive in serum and to complement resistance and the interaction of this resistance with haemagglutination properties (Wooley et al., 1992). In the present study, β-galactocidase activity ranged from 401 up to 840 Miller unit in the examined *P. aeruginosa* isolates. Krause et al. (1992) reported that β-galactocidase activity reflects the degree of transcriptional activity by specific genes in the bacterium associated with virulence. Klose and Mekalanos (1997) found that the enzyme activity play the major role in the metabolic and nutritional activities required during infection.

Our studies indicated that *P. aeruginosa* secrete exotoxins. Wilson and Mills (1983) stated that exotoxin of *P. aeruginosa* is the most toxic substance and exerts its lethal effect by inhibiting protein synthesis. Moreover, Stead (1992) detects the presence of exotoxin gene in 95% of *P. aeruginosa* strains. Brooks et al. (1995) concluded that many strains of *P. aeruginosa* produce exotoxin which cause tissue necrosis and is lethal for animals.

Results of interadermal injections of exotoxins in guinea pigs (Table 2 and Photo 1) showed that there was increase in skin thickness and induration at the site of injection that varied from moderate to severe reaction. Peterson and Sandefur, (1979) showed that exotoxin of *P. aeruginosa* was responsible for delayed skin reactions. Ornalowski et al. (2007) stated that exotoxin cause local immunosppression, cell death and fibrosis.

All *P. aeruginosa* recovered serovars from diseased camels produce exotoxins with high cytotoxic activity to Vero cells (Table, 2 & Photo, 3). Jiwa (1981) and Kioi et al. (2008) investigated morphological changes in Vero cells after
challenge with exotoxins. *P. aeruginosa* combats host and initiated oxidant stress through expression of the transactiviting factor which regulates cytotoxicity through exotoxin production (Melstrom et al., 2007).

Examination of sera collected from immunized mice (group 2) as demonstrated by ELISA showed significant increase in antibody optical density against *P. aeruginosa* exotoxins (Table, 3). This result indicated that the exotoxins is valuable immunogen and stimulate humoral immunity. This assumption was confirmed with Cross (1980). Lydick et al. (1985) studied the response of exotoxin vaccine and immunogenicity of this vaccine, which can enhance survival from *P. aeruginosa* infections through active and passive immunization.

Administration of gentamicin with the vaccine (group 3) resulted in suppression in antibody production (Table, 3). Similar results were reported by Panigrahy et al. (1978) and Naqi et al. (1984).

It is noted that administration of norfloxacin with vaccination (Group 4) had no effect on antibody formation (Table, 3). The present finding was consistent with that obtained by Shalit (1991) who mentioned that most quinolones have no direct effect on immunoglobulin production.

It is concluded that *P. aeruginosa* is highly virulent and immunization trials with exotoxins is highly effective. Treatment of animals may be obligatory during vaccination therefore, the effective antibiotic which is less harmful to the immune status and vaccination process should be used.

**REFERENCES**


Dapgh, A.W. (2000): "A contribution towards the bacterial har-


Lowry, O.H.; Rosenbrough, W.J.; Farr, A.L. and Randall, R.J. (1951): "Protein measurement..."


توضيح السم الخارجي لميكروب السوادوموناس أربوجينوزا المعزول من الجمال

أ.د. نسام محمد سلمان
قسم المناعة - معهد بحوث صحة الحيوان

الملخص العربي

تم عزل ميكروب السوادوموناس أربوجينوزا من حالات الالتهاب الرئوي في الجمال بنسبة 10.1%. وتم دراسة عوامل الضراعة للميكروب وذلك باختبار التجمع في ملح سلفات الأمونيوم وكانت خاصية التجمع 75% بالنسبة للعشرات المعزولة من الرئة المصابة. ووجد الميكروب حيا في سير المجلة بنسبة 87.5% ونسبة الأختبار البيتاغالاكتوسيداز وجد أن النشاط التراوح بين 84.1 - 88.6 وحدة ميلر في الميكروبات المعزولة.

وقد تم اختيار السم الخارجي المعزول من أربعة عشرات سودوموناس أربوجينوزا بالنسبة للحقن تحت الجلد ووجد أن هناك زيادة في سمك الجلد. أيضاً تم اختيار السم الخارجي للميكروب بالنسبة لأعداده تسمم في خلايا الفيرو ووجد أن جميع عشرات السوادوموناس أربوجينوزا أعطت سم خارجي قادر على أن أحدث تغيرات واضحة في خلايا الفيرو. تم تجميع عينات السيرم من الفئران المحصنة بالسم الخارجي ووجد أنه قادر على أعطاء سيرم عالى الأجسام المضادة باختبار الألزابا بالمقارنة بالسيرم الغير محصن. وجد أن أعطاء جناتينيسين مع التحصين بالسم الخارجي لميكروب السوادوموناس أربوجينوزا أعطى تربت في تكوين الأجسام المضادة. بينما أعطاء النورفوكسيسين لم يؤثر على تكوين الأجسام المضادة المصاحبة للتحصين بالسم الخارجي.

المحكون:

أ.د. جامع علاء الدين الجابي
استاذ الميكروبيولوجيا - كلية الطب البيطرى - جامعة القاهرة
أ.د. رفيق توفيق سليمان
استاذ الميكروبيولوجيا - كلية الطب البيطرى - جامعة القاهرة