The relationship between the site of infection and virulence of Pseudomonas aeruginosa experimental infection in mice

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Accepted on: 20/10/2013

Summary
The aims of this study were to investigate the virulence of Pseudomonas aeruginosa depending on infection site from otitis media in mice and compared with inter peritoneal infection. Suspension of bacteria was prepared in three doses (10^3, 10^6 and 10^8 ) CFU/ml live attenuated P. aeruginosa from otitis media and peritoneum infection of mice were tested in sixty healthy mice (White PALB/C), which randomly divided in two groups, first group isolated from otorrhea of otitis media, second group isolated from interperitonium infection. Both groups were subdivided in to 5 groups, each subgroup have 6 mice, were infected orally, subcutaneous, urinary tract and eye infection with 0.5 ml of bacterial suspension. While the 5th group was control negative. The present study found that the adhesion of P. aeruginosa isolated from otitis media isolates to mice epithelial cell was significantly increased compared with the isolates from peritoneal infection, the resistance to infection showed multiple resistances of otitis media isolates to antibiotics more than peritoneum isolates.

Keyword: Otorrhea of otitis media, Pseudomonas aeruginosa, site of infection.

Introduction
Pseudomonas aeruginosa is a ubiquitous pathogen capable of infecting virtually all tissues. A large variety of virulence factors contribute to its importance in burn wounds, lung, eye, urinary tract, joint, gastro-intestinal and a variety of systemic infections (1 and 2). Also classified as an obligate aerobes organisms, gram –ve, motile, rod-shaped and occurs as single bacteria, in pairs and occasionally in short chains (3). Pseud. aeruginos secretes a varies pigments including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent) and pyorubin (red-brown) (4).

P. aeruginosa can cause chronic infections, which are a serious problem for medical care, especially for abnormal host defenses. They often can’t be treated effectively with traditional antibiotics therapy (5). It has the capacity to carry multi-resistance plasmids, and this feature has led to the appearance of some Pseudomonas strains those are resistant to all reliable antibiotics (6).

The aim of this study is to evaluate the ability of P. aeruginosa pathogenicity according to the site of infection.

Materials and Methods
Depending on the nature of infection, an appropriate specimen are collected and a Gram stain is performed, which may show Gram –ve rod are often seen in smears are plated on blood agar and differential media commonly MacConkey agar colonies of P. aeruginosa characteristic grape-like odier on bacteriological media. In mixed cultures, it can be isolated as clear colonies on MacConkey agar (as it doesn’t ferment lactose) which will test positive for oxidase. Confirmatory tests include production of the blue-green pigment pyocyanin on cetrimide agar and other specific media like Pseudomonas agar and King media that growth at 42ºC, a T.S. I. slant is often used to distinguish non fermenting Pseudomonas Spp. (3 and 7).

For determination of the challenge dose suspension of Pseudomonas aeruginosa: prepared according to live account method (8). The mice were infected with 10^3, 10^6 and 10^8 C.F.U./ml/0.5 ml dose (9).

They sixty white Balb/C mice weight 25-30 gm. were used, obtained from the animal house of the Zoonotic Diseases Unit - Veterinary Medicine - Baghdad University, and were allowed to acclimatize in the research
laboratory for 4 days before the commencement of the study and were fed with standard pellets and allowed unrestricted access to clean drinking water.

Animals were divided randomly into two equal groups (1<sup>st</sup> group injected with isolate of <i>P. aeruginosa</i> from otitis media and 2<sup>nd</sup> group injected with isolate from interperitonium infection. Each experimental animal subdivided into 5 equal subgroups.

First group were challenged orally with 0.5 ml of bacterial suspension containing 1X10<sup>3</sup>, 10<sup>6</sup> and 10<sup>8</sup> CFU/ml of viable virulent <i>P. aeruginosa</i>, second group. Injected with 0.5 ml subcutaneously 10<sup>3</sup>, 10<sup>6</sup> and 10<sup>8</sup> CFU /ml interval, third group injected with 0.5 ml of10<sup>3</sup>, 10<sup>6</sup> and 10<sup>8</sup> CFU /ml ascending to urinary tract, fourth group eye dropping with 0.5 ml of10<sup>3</sup>, 10<sup>6</sup> and10<sup>8</sup> CFU /ml under light with ether anesthesia and fifth group kept as control negative.

Pathological study by macroscopical examination of postmortem examination was done for all animals; the macroscopical appearance was recorded to detect any abnormal gross change in the internal organs including location, color, size, shape, consistency and appearance of cut section.

Histopathological examination and bacterial cultivication after postmortem by take samples of internal organs for bacterial isolation on <i>Pseudomonas</i> agar (7) and other samples for histopathological examination (10).

**Results and Discussion**

Brain- heart infusion broth was used as the culture medium for growth of organisms, then specific media (<i>Pseudomonas</i> agar- King Media) the result showed heavy growth and showed the pyocyanin pigment of <i>P. aeruginosa</i> and the biochemical test was be employed like Oxidase +ve, Gelatinase +ve, Urea +ve, Cimon citrate –ve, Indol –ve and made Gram stain. The result showed pure colonies of <i>P. aeruginosa</i> from liver, intestine organs and urine, fecal samples (Fig. 1).

<i>P. aeruginosa</i> isolate from otitis media were sensitive to Azithromycin, Amoxicillin, Clavulnic acid, Doxycyclin, Carbincillin, Trimethoprime, Tetracyclin, Bacitricin, Cefatoxime and Erythromycin compared with the other isolate from interperitonium which was sensitive to Azithromycin, Gentamycin and Erythomycin (Table, 1).

The results of 1<sup>st</sup> group, of eye infection was normal, with non-congested blood vessels and no edema. While oral infection showed sever acute clinical signs during 10-16 hr like depression, diarrhea, increase of respiratory rate and pulse rate, then death occur during 22-24 hr.

The gross examination of internal organs of control group that scarified during the first 24hr post challenge, showed enlargement of organs especially intestine, spleen and liver and gall bladder (Fig. 2 and 3).

The histopathological finding after 24 hr post challenge showed an increased in thickness on inter alveolar septa due to congestion of capillary B.V. and neutrophils infiltration with emphysema (Fig. 4). Also spleen showed depletion of white pulp with neutrophils infiltration in the congested red pulp (Fig. 5).

The result of heart showed congestion of B. V. between muscles fibers with neutrophils in their lumen (Fig. 6). While liver showed granuloma with congestion of central vein and sinusoids with neutrophils in lumen as well as necrosis of hepatocytes (Fig. 7). In other section necrosis of hepatocytes with neutrophils and congested B.V. (Fig. 8). In other animals showed purulent granuloma due to aggregation of neutrophils and macrophage in liver (Fig. 9).

The result of intestine showed infiltration of macrophages in sub epithelium layer. (Fig. 10).The result of skin appear acute clinical signs after 48hr of infection represented by cyanosis of animal, stiffness and died of animals at 72hr.

The results of skin after 72hr of challenge showed necrotic and desquamation of epidermal layers as well as infiltration of neutrophils in the dermal layer (Fig. 11). Other section showed infiltration of neutrophils and monocytes in subcutaneous tissues (Fig. 12).

The result of urinary system infection by <i>P. aeruginusa</i> showed depression of animal, loss of appetite, increase of urination and <i>P. aeruginosa</i> isolated from urine, inflammation of genital organs in animals. Result of kidney was showed aggregation of neutrophils and
lymphocytes around congestion blood vessels also between kidney tubules with sever cytoplasmic vaculation of endothelium cells (Fig. 13). Other sections explained congestion of blood vessels with filtration neutrophils between kidney tubules (Fig. 14). In lung results were showed increased thickness of intralveular septa due to congestion of capillary blood vessels and proliferation of parenchymal cell (Fig. 15). Other section explained RBCs. and neutrophils in interalveolar spaces, also emphysema and inflammatory cell in the subepithelial bronchi (Fig. 16). Results of liver appear congestion and explanation of sinusoids with neutrophils in lumen added to necrosis hepatocytes. (Fig. 17). Other section explained granulomatous from aggregation neutrophils around central vein (Fig. 18).

The result of 2nd group of *P. aeruginosa* from intra pretonium infection, the skin infection in mice in 10^3, 10^6 and 10^8 CFU/ml of *P. aeruginosa* isolated from (I.P.) showed sever clinical signs at 3-14 days after infection represented by erythemia and purulent exudates with swelling around the infected area then reduce infection and scar tissue formation after 21 days (Fig. 19). The result of eye infection showed no clear clinical signs. Also urinary tract infection showed no clear clinical signs just increase pulse rate and respiratory rate and shedding of bacteria after 24 hr of infection in urine by *P. aeruginosa* as ascending infection, then mild or moderated these isolate from urine after 2-3 weeks after infection when isolated this bacteria on selective media.

Oral infection show sever clinical signs represented by diarrhea and depression of animal with rough of hair and loss of appetite after 7 days of infection and detection of *P. aeruginosa* in feces by isolation of bacteria in selective media. *P. aerogenous* produced several substances that are thought to enhance the colonization and infection of the host tissues, these substances, together with a variety of virulence factors including alginate, lipopolysaccharides (L.P.S.), flagellum, pills and non-pills adhesions as well as with exoenzymes or secretory virulence factors like protease, elastase, phospholipase, pyocyanin, exotoxin A, exoenzymes, haemolysin (rahnmolipids and siderophores) (2 and 7). This evidence agreed with those reported by (11) who have been shown an important role in pathogenesis of *P. aerogenosa* induced infection like tract infection, burn-wound infection and keratitis. This idea was supported by evidence which explained that *P. aerogenosa* pathogenicity (12). The present findings were probably indicated that highly virulent isolate from otitis media overcome the local defense mechanism of the host and rapidly disseminated from the site of inoculation to the internal organs inducing septic shock with multi organs dysfunction and sudden death of the challenged mice, so the result of culture of internal organs after death showed heavy growth of bacteria with production of green-blue color due to pyocyanin that agreed with (7), who showed the role of pigmentation in pathogenicity. This evidence was in agreement with (13), who showed the inter pretation of isolation from internal organs may be to the ability of *P. aerogenosa* to transmit through the blood (bacteremia) and spread to organs on distant location inside the body leading to invasive infection, so *P. aerogenosa* from otitis media may be toxigenic strain than the same strain from other organs in the same animals that lead to septic shock and death of all groups in multiple doses (14).

The result of culture of organs in the king media (Fig. 1), the isolate production of pyocyanin after 48hr of inoculation referred to role of pathogenicity (7), that agree with (15), who showed pyocyanin in virulence factor of *P. aeruginosa* and has been known to cause death in isolated in several study.

The result of eye infection show no clear clinical signs after eye infection by dropping of suspension in multiple dose, the fact that *P. aerogenosa* invasion of corneal epithelial cell has not been noted previously in mice after dropping may indicated that difference in the method used to infect eye affects *P. aerogenosa* invasion that agreed with (16), who mentioned that infected mice eye after abrasion injury, and explained that *P. aerogenosa* infection develops in one of three types of cases, those with traumatic cornial abrasions and with epithelial defect due to intrinsic disease e.g. dry eye, exposure keratitis, neurotrophic keratitis and post infection persistent epithelial defect.
Finally those who wear contact lenses especially extended-wear hydrophilic lens. These three cases defect in in the corinal epithelium to which bacteria must be adhere in order to initiate the infection. Further than the *P. aerogenosa* invades the eye ulcers or contaminated with treatment or foreign body entered the eye, this study was in agreement with the observation by (17) who explained that eye mucosal surface inhibit bacterial colonization in natural case for bacterial to infect a mucosal surface, they must adhere to that surface to resist the natural mechanisms. Other worker (18) show the presence of antibacterial substances in the tears including lysozyme, lactoferrin, betalysin and IgA antibodies bacteria do not adhere well to intact corinal epithelium to injure or disease epithelium at the edge of an epithelial defect. Also agreed with this study (19) who explained the adherence of *P. aerogenosa* to injured epithelium is known to be the initial step in the pathogenesis of corneal ulceration. The exact natural of interaction between bacterial and epithelial cell complex is filamentous cell wall appendages called fimbria are important for adhesion. In one study (20) showed that bacterial producing elastase and alkaline protease have been found to contribute to its virulence (were to be highly virulence) were as strain *P. aerogenosa* that were deficient in their production could not cause corneal infection in mice. The Balb/C strain mice had natural resistance to experimental corneal infection with *P. aerogenosa* when compared with their heterozygot (nu/t) or mice lack failure mature T. cell due to an embryological failure (21).

The result of the skin infection showed death of animal after 72 hr of challenge by multiple dose of *P. aerogenosa* suspension of otitis media isolate with sever clinical and histological changes this agreed with further experimental, burned tissue produced a lethal toxin (22) this also suggested presence of an extracellular toxin, (22) these workers suggest that lethal process had already been initiated and was independent of contained microbial growth in the host. Other study suggested that production of protease at site of infection may aid in the systemic dissemination of toxin and organism (23) these studies implicated protease as well as exotoxin A as virulence-associated factors of *Pseudomonas* infection in wound mice that agree with (24 and 25) whom showed the ability of protease in wound and burn infection by *P. aerogenosa*.

The result of oral infection showed sever clinical signs (enlargement of intestine, spleen, liver and showed sever hemorrhage in all organs) this agreed with (7) who recorded necrotising enterocolitis, the result showed that all animal died after 24 hr post challenge, this disagree with study that showed some animals die after 4 weeks (25) whom is agreed with this study that said the oral administration of *P. aerogenosa* to rat at dose a $2 \times 10^{10}$ elicited a systemic and mucosal antibodies response in intestine and respiratory (26). All most oral administration infected lung and respiratory tract but moderated infection of intestine in mice (27) this disagreement with (1) *P. aerogenosa* more fatal septicemia from animal than recombined death from other G-ve bacteria that agree with (28) which showed the high virulence characteristic of these isolate from otitis media

The result of urinary infection showed sever clinical signs and pathological changes in kidney, liver and gall bladder then death of animals after 18-24 hr these signs may be due to change of mucosal layer of urinary tract which disrupts the natural barrier and allows bacterial colonization this agree with (29) who explained the ability of *P. aerogenosa* as second most common infection in the body.

High levels of elastase and phospholipase were produced by most isolates obtained from trachea, urinary tract and wound that agreed who showed significantly higher levels of toxin a was produced by wound isolates while significantly higher level of exoenzymes was produced by wound and urinary tract isolates, the persistent infection isolated from different site produce significantly higher level of exoenzymes Some workers investigated the relationship between production of virulence straits and site of infection (11).

The sever clinical signs showed in skin infection in 2nd groups due to characteristic of this bacteria and virulence factor like exotoxin A, protease, alkalinease and pyocyanin production (7 and 24) as they recorded *P.
*P. aeruginosa* is the most common cause of burn-wound infection in mice, that agreement with (30) who showed the pathogenicity of *P. aeruginosa* in skin and soft tissue. The result showed no clear clinical signs in eye infection this due to cellular immunity against *P. aeruginosa* like T-Cell which control of it (2). This idea was disagreement with (31) who recorded eye infection with injury and immune suppression. But it agreed with recent evidence that *P. aeruginosa* enter lung and corneal epithelial cell during infection (32). The result of urinary tract infection showed isolated of bacteria after 24hr of infection in this study may be due to suggested that moderated of virulence of *P.aerugenosa* isolated from 2nd group that agree with (33) who showed little is known about the pathogenicity of *P.aerugenosa* of (U.T.S.).

In conclusion: The result of this work showed that *P.aeruginosa* isolates from otitis media was more fatal and virulent than other isolates for another part of body infected by the same isolates. The fact that *P.aeruginosa* invasion of corneal epithelial cells has not been noted previously in white mice after abrasion injury may indicate that differences in the method used to infected eye affects *P.aeruginosa* invasions.

### Table 1: Inhibitory effect of some antibiotic disc against *P. aeruginosa* on bacteria.

<table>
<thead>
<tr>
<th>No</th>
<th>Antibiotic disc</th>
<th><em>P. aeruginosa</em> zone inhibition from otitis media</th>
<th><em>P. aeruginosa</em> zone inhibition from perito-neum</th>
<th>S.I.R.</th>
<th>Standard of S.I.R. according to (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Azithromycin (Azm) 15mcg</td>
<td>25mm</td>
<td>35mm</td>
<td>S</td>
<td>20 of sensitivity</td>
</tr>
<tr>
<td>2</td>
<td>Gentamycin(CN)10mcg</td>
<td>7mm</td>
<td>18mm</td>
<td>S</td>
<td>12 of resistance</td>
</tr>
<tr>
<td>3</td>
<td>Amoxicilin +Clavulanic acid (Amc) 30mg</td>
<td>Zero</td>
<td>Zero</td>
<td>R</td>
<td>13 of resistance</td>
</tr>
<tr>
<td>4</td>
<td>Doxyclyline(Do)30mcg</td>
<td>8mm</td>
<td>10mm</td>
<td>R</td>
<td>13 of resistance</td>
</tr>
<tr>
<td>5</td>
<td>Cephalexin(Cl)30mg</td>
<td>Zero</td>
<td>Zero</td>
<td>R</td>
<td>14 of resistance</td>
</tr>
<tr>
<td>6</td>
<td>Erythromycin(E)15mcg</td>
<td>Zero</td>
<td>18</td>
<td>S</td>
<td>13 of resistance</td>
</tr>
<tr>
<td>7</td>
<td>Carbenicilin(Py)100mcg</td>
<td>Zero</td>
<td>Zero</td>
<td>R</td>
<td>13 of resistance</td>
</tr>
<tr>
<td>8</td>
<td>Ampicilin(Am)10mg</td>
<td>Zero</td>
<td>Zero</td>
<td>R</td>
<td>13 of resistance</td>
</tr>
<tr>
<td>9</td>
<td>Trimethoprin (Tmp)</td>
<td>Zero</td>
<td>Zero</td>
<td>R</td>
<td>10 of resistance</td>
</tr>
</tbody>
</table>

S: Sensitive  I: Intermediate  R: Resistance

**Figure 1:** Plate of King media show the heavy growth of *P. aeruginosia* which isolated from organs after challenge note the pyocyanin product (green- blue color).

**Figure 2:** Show enlargement of organs especially intestine, spleen and liver.
Figure 3: Show thickness and enlargement of gall bladder when infected urinary system by *P. aeruginosa*.

Figure 4: Lung at 24hr post-challenge showed increased thickness on interalveolar septa congestion of capillary B.Vs. and neutrophils infiltration in oral infection H&E stain X 40.

Figure 5: Spleen at 24hr post-challenge showed depletion of white pulp with neutrophils infiltration in the congested red pulp (oral infection) H&E stain X40.

Figure 6: Heart at 24hr post-challenge showed congestion of B.Vs. between muscles fibers with neutrophils in their lumen H. and E. staineX40.

Figure 7: Liver at 24hr post-challenge showed granuloma with congestion of central vein and sinusoids with neutrophils in lumen with necrosis of hepatocyte (in oral infection) H& E stain

Figure 8: Liver explained necrosis of hepatocyte and replicated with neutrophils and red blood vessels H& E. stain X40.
Figure, 9: Liver explained purulent granuloma due to aggregation of neutrophils and macrophages (oral infection) H & E. stain X40.

Figure, 10: Intestine at 24hr post-challenge show infiltration of macrophage cells in sub epithelium layer (oral infection) H& E. stain X40.

Figure, 11: Skin after 72hr of challenge explained necrotic and desquamation of epidermal layer with infiltration of neutrophils in dermal layer (skin infection) H& E. stain X40.

Figure, 12: Skin after 72hr of challenge explained infiltration of neutrophils and monocytes in subcutaneous tissue (in skin infection) H. and E. stain X40.

Figure, 13: Kidney at 24hr post-challenge explain aggregation of neutrophils and lymphocytes around congestion B.Vs. also between kidney tubules which suffering from cytoplasmic vaculation for endothelium cell (urinary tract infection) H& E stain X40.

Figure, 14: Kidney at 24hr post-challenge explains congestion B.Vs. and filtration neutrophils between kidney tubules (urinary tract infection) H& E. stain X40.
Figure, 15: Lung at 24hr post-challenge showed increased thickness on intralevular septa due to congestion of capillary B.Vs. and proliferation of paranchemal cells (urinary infection) H& E. staineX40.

Figure, 16: Lung at 24hr post-challenge explain inflammatory cells in bronchioles H& E. stain X40.

Figure, 17: Liver at 24hr post-challenge revealed congestion and explantation sinusoid with neutrophils in lumen added to necrosis of hepatocyte (urinary tract infection) H& E. stain X40.

Figure, 18: Liver at 24hr post-challenge explain granulomatus from aggregation neutrophils around central vein H& E. staineX40.

Figure, 19: Show erythemia and swallowing of infected area of P. aeruginosa after 3 days of infection

Figure, 20: Show scar tissue after 21 days after infection with P. aeruginosa.

References


العلاقة بين مكان الأصابة والضراوة للاصابة التجريبية للزوائف الزنجارية في الفئران

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صممت هذه الدراسة لمعرفة دور ضراعة جرثومة الزوائف الزنجارية (المشخصة في وحدة الأمراض المشتركة) معتادة على مكان الأصابة، حيث استعملت الجرثومة المعزولة من فح الأذن الوسطي المصاب بالالتهاب في الفئران وقُورنت مع نفس الجرثومة المعزولة من التجويف البطني في نفس الحيوان. ومععرفة دور مكان الأصابة وعلاقته بعامل الضراوة فقد اعتمدت التجربة على 60 فأر، قسمت بتساوي إلى مجموعتين: عزلت المجموعة الأولى من تهاب الأذن الوسطي وحدها، حيث عزلت الجرثومة المعزولة من التهاب الأذن الوسطي في حين عزلت عزلة الأذن البطني في نفس الحيوان. وعُلِخلت المجموعة الثانية على مجموعتين ككل، كل مجموعة على 5 مجاميع: 6 جراثيم (كل مجموعات تحوي 6 فئران)، عزلت كل المجمعة بالعلاق البكتيري المعركة بطريقية العد الحي للجرثومة الزوائف الزنجارية (واقع ثلاث جرعات مختلفة في مجموعات) وحدة تكريس مستعمرة لكل مجمعة من العلاق البكتيري. حيث أعطيت المجموعة الأولى تجربة فورا، أما المجموعة الثانية فقد فرطت تحت الجلد، وروت المجموعة الثالثة بالجهاز البطني، أما المجموعة الرابعة فقد أعطيت ضراعة جرثومة الزوائف الزنجارية بوعكة مفتاحية، وأخذت الأذن الوسطي في الفئران في كل المجموعات لقياس الضداكات الحياتية المقارنة بعزلة الأذن. وتثبت هذه الدراسة ضراعة جرثومة الزوائف الزنجارية في مكان الأصابة من تهاب الأذن الوسطي وللمجتمعات مصابات بالهرمونات الحياتية بعزلة الأذن، ومع ذلك، يمكن أن تكون هذه الدراسة مفيدة للحصول على المعلومات لمعرفة مكان الأصابة في حالة تهاب الأذن الوسطي، مما يمكن أن يكون له تأثيراً على كفاءة العلاج.

كلمات المفتاحية: فح الأذن الوسطي، الزوائف الزنجارية، مكان الأصابة.