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Characterization of *Pseudomonas aeruginosa* isolated from ration and its viability in the environment

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Summary
Out of 642 samples of different feedstuffs 33 samples were positive to *P. aeruginosa* with an incidence (5.1%). The isolated strains belonged to (8) serogroups (A-B–D-F-H–K-L and M). In general, the predominant serogroup was H (9 isolates), K (6 isolates), M (5 isolates) followed by A, B, L(3 isolates each) and finally F and D(2 isolates each). The antibiotic sensitivity test against *P.aeruginosa* revealed that ciprofloxacin was the most effective drug (93.1%) followed by norfloxacin (84.8%) and enrofloxacin (78.8%). *P.aeruginosa* was low in susceptibility to gentamicin (33.3%), tetracycline (18.2%) and streptomycin (12.1%) and finally erythromycin (3%).Regarding to pathogenicity in laboratory animals (I.P. inoculation in mice) serogroup D, H and K gave higher mortalities (100%) followed by M (70%) and A, L, B (60%) finally F yielded (50%), and selected 2 isolates (H and K) were studied for viability in feed, water and environmental samples. It was clear from plasmid profiles of 8 selected *P. aeruginosa* including (B – K – H – M – F – L – A - D) that they harboured one plasmid in each strain their molecular weight were 23000, 27542, 25098, 30090, 32000, 29870, 32895 and 27000, BP respectively, No clear pattern could be established relative to isolation of specific serogroups of *P. aeruginosa* in relation to plasmid profiles.

Key words: *P. aeruginosa*, *pathogenicity, laboratory animals*

Introduction

P. aeruginosa is an opportunistic pathogen of medical and veterinary importance due to its capability of infecting human, animals and poultry. *P. aeruginosa* has been implicated as food and water borne pathogens and now is considered as primary infectious agent **(Anonymous, 1991).**

Pseudomonas species can be found in water, soil, sewage and vegetation. It can also be found in the intestinal tract. *P.aeruginosa* is of public health importance as it produces infection of wound, eye giving rise a blue green pus, also cause meningitis and urinary tract infection.

P.aeruginosa is naturally found in rice paddy fields; the mud of river banks, and surface stagnant water, the organism can infect cattle, pigs, and other animals.

It also can invade fertile eggs causing death of embryos and virulent strains can cause diarrhea, dehydration, dyspnea, septicemia and death to newly hatched chick **(Walker *et al*., 2002).**

Feeds are considered as a favorable media for *P. aeruginosa* growth which may be further stimulated by environmental factors as temperature, deficient manufacturing practices and poor hygienic standards further attributed to contamination of animal feeds (**Durand *et al*., 1990**). In Egypt **(Ibrahim, 2002**) isolated *P. aeruginosa* from feed stuffs with an incidence of 4% .

The current study was designated to spot light on isolation and identification of *P.aeruginosa* from ration and concentrates. Biochemical and serological identification of the isolated *P. aeruginosa*. Sensitivity test to the different antibiotics. Pathogenicity in mice. Viability of *P. aeruginosa* in feedstuffs and environmental samples as well as, Plasmid DNA profiles of *P. aeruginosa* using the agarose gel electrophoresis and study

the correlation among the viability of *P. aeruginosa*, sensitivity and serogroups.

Material and Methods

Sampling.

A total of 642 samples from animals and poultry feedstuffs were collected from different areas of Egypt and from the Serology Unit in Animal Health Research Institute, Dokki, Giza. Feedstuffs included77 corn samples, 92Pelleted feed,245concentrate samples,50Poultry residue samples,178 Meals samples. Each sample was collected in sterile polyethylene bag and send to laboratory with minimal delay.

Isolation and identification of *P. aeruginosa* in animal feedstuffs by the conventional cultural method according to I.C.M.S.F. (1978).

25 grams of each sample were mixed with 225 ml buffered peptone water (pH 7.2) and incubated at 37°C for 24 hours then streaked onto *Pseudomonas* agar base with 0.1 % cetramide and inoculcated at 37°C for 24 to 48 hours. Pure culture was made from colonies suspected to be *P. aeruginosa* and was later identified Biochemically according to **(Koneman *et al.*, 1997 and Quinn *et al.*, 2002)** and through using API – 20E (Biomerieux).

Serological identification.

P. aeruginosa isolates were serogrouped according to **(Homma, 1982)** using antisera from Denka Seiken Co. Ltd, Tokyo, Japan.

Antibiotic sensitivity test of isolated *P. Aeruginosa*.

Antibiotic sensitivity test was performed by the disk diffusion technique according to **(Finegold and Martin 1982, Quinn, *et al.*, 2002)** with Mueller Hinton medium, using the following 10 Antibiotic discs (Oxoid) discs, ciprofloxacin (5 µg), enrofloxacin (5 µg), norfloxacin (5 µg), lomefloxacin (10 µg), tobramycin (10 µg), ceftrioxone (30 µg), gentamicin (10 µg), streptomycin (10 µg), tetracycline (30 µg) and erythromycin (15 µg). The results were interpretated according to the manual supplied by Oxoid Company.

Pathogenicity in laboratory animals (Mice).

A total 90 Albino white mice with average weight of about 18-20 g and aged 28-30days old were used to investigate the pathogenicity of *P. aeruginosa*. Five mice were used for examination of each isolate. All mice were examined bacteriologically to ensure theyare free from pathogens. The mice were inoculated I.P. with 0.1 ml of bacterial suspension of 18 hour old culture of the tested isolate in peptone water and matched with standard Mcferland tube No. 0.5 containing approximately $1.5x10^8$ C. F. U/mouse of the tested isolate (**Cruckshank, *et al.*, 1975**). All mice receiving the same serogroup were kept together. Last five mice were kept as control and injected only with saline. Mice were kept under observation for 7 days, the number of dead mice was recorded and re-isolation of the inoculated isolates was done.

Viability test.

1 ml of isolates *P. aeruginosa* count 9 x 10^8 CFU/ml. were injected in 100 ml of sterile water, 25 gm of concentrated feed and polluted environmental samples (Sand, Sawdust, Plastic). Viability of injected organism was tested periodically for 2 months **(Mezyed *et al.*, 2011*)*.**

Plasmid profiles: - was performed in Biotechnology Center Faculty of Veterinary Medicine Cairo University.

Extraction and characterization of Plasmid DNA (miniprep) (Ausubel *et al.*, (1987).

One colony of *P. aeruginosa* was picked and isolated in 10 ml LB broth containing 12 mg/ml tetracycline, 50 mg/ml ampicillin and 50 mg/ml chloromphenicol then incubated at 37°C overnight with shaking, centrifuged at 3000 rpm/ 5minutes at 4°C.The supernatant was discarded, and then 250 ml ice-cold tris EDTA buffer solution (TES) pH 7.8/ 1.5ml culture was added, and then shacked well Ice-cold solution I 100 ml/ 1.5ml bacterial pellet was added, shacked vigorously and put on ice for 10 minutes. Freshly prepared solution II 200

ml/ 1.5ml bacterial suspension was added, mix the content by inverting the tube rapidly.150 ml/ 1.5ml culture from ice-cold solution III was added and mixed gently by inverting the tube several times then put on ice for 10 minutes, Centrifuged at 14000 rpm for 10 minutes at 4°C. Proteins and genomic DNA was extracted from the supernatant with phenol: chloroform: iso amyl alcohol mixtures. Plasmid DNA precipitated by double volume of ethanol to the above separated aqueous layer and kept for 30 minutes ice than centrifuged at 14000 rpm for 30 minutes at 4°C.The pellet was washed with 5 ml 70% ethanol and centrifuged at 14000 rpm for 10 minutes at 4°C. After drying the pellet it was dissolved in 10 ml trisEDTA (pH8).

Characterization of extracted plasmid DNA using agarose gel electrophoresis (Davis *et al.*, 1986).

Ten ml of plasmid samples were electrophoresis through 0.9 agarose gel in tris borate EDTA buffer at 150 V, 60 mA for 5.5 hrs. the gel was stained for 2hrs. in 0.5 mg/ml of ethidium bromide. The experiment was evaluated under UV lamp.Visible bands being seized by DNA molecular weight marker.

Results

Table (1). The prevalence of *P. aeruginosa* isolated from animal feedstuffs.

Out of 642 samples of different feedstuffs 33 samples were positive to P. *aeruginosa* with an incidence (5.1%).

Samples	No. of examined samples	Bacteriological findings	
		No. of +ve samples	%*
Corn	77	4	5.2
Pellet feed	92	5	5.4
Concentrates:			
a-Broiler conc.	86	3	3.5
b-Layer conc.	93	3	3.2
c- Ration conc.	66	5	7.6
Poultry residue	50	4	8
Meals:			
a-Bone meal	67	2	2.9
b-Fish meal	50	4	8
c-Meat and bone meal	61	3	4.9
Total	642	33	5.1**

* The percent was calculated according to the number of each type of examined animal feedstuff samples.

** The percent was calculated according to the total number of animal feedstuff samples (642).

Table (2). Serogrouping of *P. aeruginosa* isolated from examined animal feedstuffs.
It was evident from Table (2) that the isolated strains belonged to (8) serogroups (A-B–D-F-H–K-L and M)

Types of samples	No. of isolated strains	Serogroups	No.
Corn	4	B L K	1 2 1
Pellet feed	5	F K H	1 2 2
Concentrates: Broiler conc.	3	A B H	1 1 1
Layer conc.	3	D F M	1 1 1
Ration conc.	5	A H K	1 2 2
Poultry residue	4	H M	3 1
Meals: Bone meal	2	K L	1 1
Fish meal	4	H M	1 3
Meat and Bone meal	3	A B D	1 1 1

* The percent was calculated according to the number of isolated strains of each type of examined animal feedstuff samples.

Table (3). The antibiotic sensitivity test of *P. aeruginosa* isolated from animal feedstuffs.
The most strains were resistant to 6 antimicrobial drugs as ceftriaxone, erythromycin, gentamicin, streptomycin, tetracycline, and tobramycin.

Antibiotic disc	Conc. in µg	Serogroups								Total	
		3A	3B	2D	2F	9H	6K	3L	5M	No.	%
Ceftriaxone	30 µg	0	0	0	1	3	2	1	2	9	27.3
Ciprofloxacin	5 µg	3	3	2	2	8	6	3	4	31	93.1
Enrofloxacin	5 µg	2	1	2	2	8	5	3	3	26	78.8
Erythromycin	15 µg	0	0	0	0	1	0	0	0	1	3.0
Gentamicin	10 µg	0	0	0	1	4	2	2	2	11	33.3
Lomefloxacin	10 µg	2	2	0	2	8	5	0	0	19	57.6
Norfloxacin	5 µg	2	2	2	2	8	5	3	4	28	84.8
Streptomycin	10 µg	0	0	0	1	1	0	1	1	4	12.1
Tetracyclin	30 µg	0	0	0	1	2	1	1	1	6	18.2
Tobramycin	10 µg	0	0	0	1	5	2	1	1	10	30.3

No. = no. of sensitive strain to antibiotic.
The percent was calculated according to the number of each examined serogroup.

Table (4). Pathogenicity test of *P.aeruginosa* isolated from animal feedstuffs in mice.
P. aeruginosa isolates from animal feedstuffs show variations in pathogenicity in mice.

Serogroups	No. of tested serogroups	No. of dead mice/day							Dead	
		1	2	3	4	5	6	7	total	%*
A	2	0	4	4	1	1	0	0	10	100
B	2	0	2	1	1	1	0	1	6	60
D	2	1	2	2	2	2	1	0	10	100
F	2	1	2	1	1	1	0	0	6	60
H	2	2	2	2	2	2	0	0	10	100
K	2	2	2	2	2	2	0	0	10	100
L	2	0	0	0	2	2	1	1	6	60
M	2	0	0	2	2	2	1	0	7	70

*The percent was calculated according to the total numbers of mice (5).

Table (5). Viability test of *P. aeruginosa* isolated from animal feedstuffs.
Viability test of *P. aeruginosa* experimentally studied revealed still viable for 8 weeks in all samples except in ration viable only for 3 weeks.

Samples/ time	H Serotype						K Serotype					
	Water room temp.	Water cold	Ra-tion	San d	Saw dust	Plast ic	Water room temp.	Water cold	Ra-tion	Sand	Saw-dust	Plas tic
1st day	+	+	+	+	+	+	+	+	+	+	+	+
2nd day	+	+	+	+	+	+	+	+	+	+	+	+
3rd day	+	+	+	+	+	+	+	+	+	+	+	+
1st week	+	+	+	+	+	+	+	+	+	+	+	+
2nd week	+	+	+	+	+	+	+	+	+	+	+	+
3rd week	+	+	+	+	+	+	+	+	+	+	+	+
4th week	+	+	-	+	+	+	+	+	-	+	+	+
5th week	+	+	-	+	+	+	+	+	-	+	+	+
6th week	+	+	-	+	+	+	+	+	-	+	+	+
7th week	+	+	-	+	+	+	+	+	-	+	+	+
8th week	+	+	-	+	+	+	+	+	-	+	+	+

+ viable - no growth

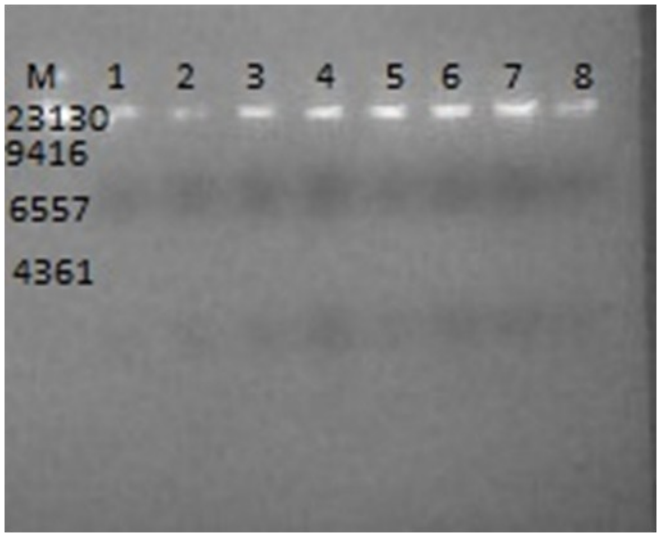

Photo (1). Plasmid DNA profiles of 8 selected *P. aeruginosa* isolated from feedstuffs.
Lane M: Hind III digested stander molecular weight marker.
Lane 1, serogroup B 23000BP.
Lane 2, serogroup K 27542 BP.
Lane 3, serogroup H 25098.
Lane 4, serogroup M 30090BP.
Lane 5, serogroup F 32000BP.
Lane 6, serogroup L 29870.
Lane 7, serogroupA 32895BP.
Lane 8, serogroup D 27000BP.

Discussion

P. aeruginosa has long been recognized **as** pathogen in hosts with impaired immune function and special precautions may be required to limit the exposure of these susceptible population so environment play great role in persistence and transport of the *P. aeruginosa* to animal and human. Out of 642 samples of different feedstuffs 33 samples were positive to *P. aeruginosa* with an incidence (5.1%). The prevalence differed according to kind of examined feedstuffs as 4 samples were positive from pellet feed with an incidence (5.4%), 11 samples were positive from concentrates with an incidence (4.5%), poultry residue produce 4 positive samples with an incidence (7.6%), meals produce 9 positive samples with an incidence(5.1%) as shown in Table (1). Isolation of *P. aeruginosa* from feedstuffs was recorded by many investigators such as **(Mashoor *et al.*, 1987**, **Heringand and Zimmermann, 1988)**. The present results were in accordance with **(Ibrahim, 2000)** and **(Ibrahim, 2002)** who found that the percent of *P. aeruginosa* in feedstuffs was 5% and disagreement with **(Barbour *et al.*, 1986)** who recorded that the rate of recovery of *P. aeruginosa* was high reaching 25.4% in feedstuffs and (**Alexander Boyer** *et al.,* **2011**) who isolated *P. aeruginosa* from environmental samples with an incidence (31%).

It was evident from Table (2) that the isolated strains belonged to (8) serogroups (A-B–D-F-H–K-L and M). In general, the predominant serogroup was H (9 isolates) then K (6 isolates) then M (5 isolates) followed by A, B, L (3 isolates) and finally F and D (2 isolates). Serogrouping of *P. aeruginosa* may be helpful in predicting the source of infection especially from environmental sample isolates, **(El-Gohary, 2004)** recorded that the more prevalent serogroups were H and B from water and

ration.

The obtained data in Table (2) showed that the most predominant serogroups were H and K. There were variations between different kinds of feedstuffs in incidence of *P.* aeruginosa and no present relation for the isolation of specific serogroups of *P. aeruginosa* from specific type of feedstuffs during the period of investigation could be established. These results were in agreement with **(Ibrahim, 2002).**

The obtained data in Table (3) showed that antimicrobial susceptibility of *P. aeruginosa*, the most strains were resistant to 6 antimicrobial drugs as ceftriaxone, erythromycin, gentamicin, streptomycin, tetracycline, and to bramycin. While the percentage of sensitivity were 93.1%, 78.8%, 84.8% and 45.6% respectively to ciprofloxacin, enrofloxacin, norfloxacin and lomefloxacin. Those results were in accordance greatly to **(Ali and Ibrahim, 2004)** who stated that the isolated *P. aeruginosa* were sensitive to enrofloxacin and **(Yah** *et al.*, **2006)** who found that *P. aeruginosa* were resistant to gentamicin, tetracycline, erythromycin and streptomycin. While high sensitivity to ciprofloxacin agreed with **(El-Bialy *et al.*, 2006).** The indiscriminate use of antibiotics is potentially capable of giving rise to a higher incidence of infection with resistant bacteria such as *P. aeruginosa* which may be transmitted from animals or poultry to human complicating the theory of human disease **(Ojeniy, 1989)**.

Regarding pathogenicity in laboratory animals in mice I.P. inoculation gave higher mortalities 100% in D, H and K serotypes, these results were in accordance greatly to**(Ibrahim, 2009).**

As recorded in Table (4) *P. aeruginosa* isolates from animal feedstuffs showed variations in pathogenicity in mice as serogroup D, H and K gave higher mortalities (100%) followed by M (70%) and A, L, B (60%) finally F gave (50%) and this was in accordance with **(Younes *et al.*, 1990).** The pathogenesis of *Pseudomonas* infections is multifactorial as suggested by the number and wide array of virulence determinates possessed by the bacterium.

Viability test cleared that *P. aeruginosa* isolated from animal feedstuffs experimentally studied was still viable for 8 weeks in all samples except in ration viable only for 3 weeks, this may attributed to heat treatment of ration. There was no variation between viability of *P. aeruginosa* in water kept in cold or in room temperature as illustrated in Table (5).

It was clear from plasmid profiles of 8 selected *P. aeruginosa* including (B, K, H, M, F, L, A, D) that they harbored one plasmid in each strain their molecular weight were 23 000, 27 542, 25 098, 30 090, 32 000, 29 870, 32 895 and 27 000 BP, respectively, as demonstrated in photo (1).

No clear pattern could be established relative to isolation of specific serogroups of *P. aeruginosa* in relation to plasmid profiles (plasmid molecular weight). In this concern**, (Negrete *et al.*, 2003)** reported that all the plasmids extracted from the *Pseudomonas* strains presented ranges in common, between 3000 and 16363 BP.**(Ibrahim, 2009)** reported that 5 strains of *P. Aeruginosa* harbored one plasmid in each and their molecular weights were 33 882 – 33 493 – 28 482 - 24 695 and 28 482 BP and **(Enany *et al.*, 2010)** stated that the molecular weights of*P. aeruginosa* from 20 213 to 32 498 BP. and finally **(El-Hadi and Samy, 2011)** reported that *P. aeruginosa* molecular weights of plasmid were 7126 to 6108 BP.

(Nordmann and Poirel, 2002) reported successful transformation experiments and carbapenem resistance *P. aeruginosa* is of particular importance because it is plasmid mediated and is transferable to Gram negative rods.

Our conclusion, device sanitary control measures must be adapted to improve the feed quality and prevent post-industrial contamination. This could be achieved by continuous examination of feedstuffs.

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خصائص السودوموناس ايرجينوزا المعزوله من الأعلاف وحيويته فى البيئه

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الملخص العربى

نظرا لتواجد عدوى السودوموناس اريجينوزا فى الحيوان والطيور و الآنسان بنسبه عاليه فى البيئه تم تجميع عينات من مصادر مختلفه لجميع العلائق من 642عينه علف منها 33 عينه أيجابيه للميكروب بنسبه (5.1%) . العترات المعزوله تشمل 8 أنواع سيرولوجيه وهى (A,B,D,F,H,K,L,M) ووجدأن العتره الأكثر عزلا هى H وعددها 9 و العتره Kو عددها 6 ثم Mو عددها 5 و يتبعها A,B,Lو عددهم 3 و أخيرا D و Fوعددهم 2.

بالنسبه لحساسيه الميكروب وجد أنه السبروفلوكساسين كان أكثر المضادات الحيويه تأثيرا يليه النورفلوكساسين و الأنروفلوكساسين.

بالنسبه لأختبار الضراوه فى حيوانات التجارب وجد أن العتره D,H,K أكثر العترات ضراوه فى نسبه الوفيات فى الفئران.

وقد تم أجراء أختبار الحيويه فى الأعلاف و المياه و بيئه المزرعه وقد تم أيضا عمل توصيف البلازميدات لمعرفه الوزن الجزيئى لعدد 8 عترات مختلفه.

وتم دراسه خصائص البلازميدات لعدد 8 من العترات للوزن الجزيئى .