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BIOLOGICAL CHARACTERIZATION OF PSEUDOMONAS AERUGINOSA ISOLATED FROM DISEASED CHICKENS

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SUMMARY

In the present study, *Ps. aeruginosa* was isolated from diseased chickens with different clinical manifestations. The incidence of *Ps. aeruginosa* infection in different tissues and organs was detected. The isolated *Ps. aeruginosa* strains were identified by traditional methods in addition to one available recent method. Moreover, the antibiogram of different chemotherapeutic agents against *Ps. aeruginosa* isolates was carried out. Morphological, biochemical and serological identification methods were able to specify different strains of *Ps. aeruginosa*. Pyocin production was also detected using the available indicator strains of *Ps. aeruginosa*. An approximate correlation was detected between Pyocin production and the serotyping. Some serotypes were more potent pathogens for chickens than others. Plasmid profiling was detected and proved to be a good tool to follow the epidemiological picture of *Ps. aeruginosa* outbreaks among our livestock as well as human populations. However, these was no clear evidence to refer certain plasmid profiles to the phenotypes of *Ps. aeruginosa* that were investigated in this study.

INTRODUCTION

Ps. aeruginosa is a Gram-negative, medium-sized bacteria which was first isolated by Gessard (1882). The organism is encountered in a variety of diseases of man, animals fish and birds. *Ps. aeruginosa* is capable of secreting many extracellular products such as fibrinolysin, collagenase, hemolysins and enterotoxins. These products have a major role in the virulence of pathogenic strains of *Ps. aeruginosa*. Infection of birds with *Ps. aeruginosa* is very crucial, as epidemics may spread rapidly within flocks causing severe economic losses due to high mortality, reduction in egg production, or at least loss of marketability (El-Ged et al., 1993). The clinical picture of *Ps. aeruginosa*

infection in a poultry farm includes septicemia, diarrhoea and respiratory symptoms (Awaad et al., 1981 and Abdalla, 1987). Within the veterinary diagnostic laboratories, resistance to antibacterial therapeutic agents is a common problem when dealing with *Ps. aeruginosa*.

Bacteriocins are antibiotic substances produced by *Ps. aeruginosa*, called pyocin. Recently, it was found that production and sensitivity patterns of pyocin vary considerably among different strains of *Pseudomonas*. This variation came out with a number of different methods for epidemiological identification of *Ps. aeruginosa* by pyocin typing (Gillies and Govan, 1966; Rose et al., 1971; Jones et al., 1972 and John and Lioyd, 1974). From the above mentioned background it could be concluded that, epidemiological identification and eradication of *Ps. aeruginosa* infection in birds is a difficult task and no single method for typing of such bacteria has been generally accepted.

To figure out to which limit *Ps. aeruginosa* exists as a bacterial pathogen for chickens, the present study was conducted. The overall objective was evaluation of different traditional laboratory methods to identify *Ps. aeruginosa* by serotyping, pyocin typing, antibiogram, and pathogenicity test in chickens. At the same time, a trial was carried out to use some recent methods based on DNA dissimilarity.

MATERIAL AND METHODS

1- Bacteriological examination

a) Sampling

A total of 700 samples were collected from diseased chickens. The birds were obtained from diseased flocks which were introduced to the Veterinary Research and Analysis Center, Faculty of Veterinary Medicine Cairo University, from different localities in Egypt. The clinical pictures were usually respiratory symptoms (343), diarrhoea (91), diarrhoea and respiratory manifestation (77) as well as joint affection (175). The samples were collected from the infra-orbital sinuses, trachea, lungs, heart blood, liver, intestine and joints under aseptic conditions. The samples were inoculated into nutrient broth and incubated at 37°C for 24 hours. At the end of incubation a loopfull from each tube was streaked onto the surface of pseudosel TM agar (BBL) and blood agar. The plates were incubated at 37°C for 24 hours.

b) Identification of isolates

The suspected isolates were purified and identified by using the general techniques described by Koneman et al. (1992) and Quinn et al. (1994). Serotyping of the isolates was performed according to the manufacturer's instructions by the slide agglutination test with *Ps. aeruginosa* specific antisera (Denka Seiken Co., Tokyo, Japan) polyvalent I (A, C, H, I, L-group), Polyvalent II (B, J, K, M. group) and Polyvalent III (D, E.F, G, N- group).

2- Pyocin typing

a) Bacterial strains

(i) *Ps. aeruginosa strain*

A reference strain (ATCC, 29336) was compared to the *Ps. aeruginosa* isolates.

(ii) **Indicator strains**

A set of 8 indicator strains were necessary for pyocin typing of the bacterial isolates. All of the bacterial strains were submitted to Animal Health Research Institute, Giza, Egypt from Dr. R.R. Gillies. Department of Bacteriology, Medical School Edinburgh University, U.K. All strains were cultivated on pseudosel TM agar (BBL) and Blood agar plates.

b) **Typing of pyocin produced by *Ps. aeruginosa* isolates**

The typing method was performed as described by Gillies and Govar (1966). Each isolate was streaked diametrically across trypticase soya blood agar plates (Difco) and incubated for 14 hours at 32°C. The macroscopic growth was removed using glass slides and killed by inverting the plate over its lid containing 5 ml of chloroform, for 15 minutes then the chloroform was decanted and the plates were exposed to the air to get rid of any chloroform residues. The chloroform treated plates were streaked out by the eight indicator strains which were grown in nutrient broth for 4 to 6 hours at 37°C. The streaking of indicator strains was approximately in perpendicular lines with the original streaking lines of the test isolates of *Ps. aeruginosa*. The plates were incubated for 8 to 18 hours at 37°c and the pyocin types were indicated by the number of the indicator strains which were inhibited due to the pyocin produced by the test strains and diffused to the culture medium.

3- **Antibiogram assay**

The disc diffusion method was done to *Ps. aeruginosa* isolates on Mueller-Hinton agar (BBL) according to the method described by Finegold & Martin (1982). A total of 9 antibacterial agents were used in the present investigation (amikacin, cephalothin, chloramphenicol, gentamicin, neomycin, ofloxacin, streptomycin, tobramycin and trimethoprim-sulfamethoxazol). The results were interpreted according to Koneman et al. (1983) and Quinn et al. (1994).

4- Pathogenicity Test

Testing of the pathogenicity of 7 isolated *Ps. aeruginosa* strains of different serotype and pyocin types to chickens was carried out according to the method described by Markaryan (1975) and Andrew et al. (1982). In this experiment, 80 ten day old chicks (Al-Ahram Poultry Company, Giza, Egypt) were divided into 7 groups and each group was subdivided into 2 subgroups, five birds in each, in addition to one group which was left uoninoculated control group

Each two subgroups in the same group were inoculated, subcutaneously, with 0.5 ml, and 1 ml of the same strain of 9×10^8 bacterial cells/ml nutrient broth respectivly. Birds of the control group were injected, subcutaneously, with sterile nutrient broth. All chicks were kept under observation for 30 days. The clinical symptoms, P.M. exam. and trials for reisolation from infected chicks were done.

Plasmid Fingerprinting of *Ps. aeruginosa* Strains of Chicken Origin:

Plasmid DNA was isolated from 9 bacterial isolates representing different serogroups, accord-

ing to the methods described by Sambrook et al., (1989), Hashad (1995). In this method, a bacterial colony of each strain was picked up using a sterile tooth pick and transferred into 4 mls of Luria-Bertani (LB) broth (Sambrook et al. 1989). The inoculated tubes were incubated at 37ºC with shaking at 230 rpm for overnight. The cultures were transferred into 1.5 ml microfuge tubes and the bacteria were pellected down at 10,000 rpm centrifugation. The plasmid DNA was isolated by the minipreparation method

Finally, plasmid DNA was precipitated by absolute ethanol at room temperature followed by high speed centrifugation. After rinsing the DNA pellet with 70 % ethanol, the pellet was dried for 5-15 minutes and dissolved in 1 X Tris EDTA buffer with pH 8. Fingerprinting of plasmids isolated from different *Ps. aeruginosa* strains was achieved by electrophoresis of the DNA on 1 % agarose containing ethidium bromide (1 µg/ml gel). After running the gel at a voltage of 2V/cm gel using 1 X tris acetate EDTA buffer the bands were visualized using an ultraviolet transillumінator. The sizes of the DNA bands were approximately measured against molecular size DNA marker.

RESULTS AND DISCUSSION

Pseudomonas infection in poultry has been evidenced as a considerable cause for outbreaks and economical losses (Gross, 1984). It was only very recently when Egyptian researchers started to consider *Pseudomonas* as a bacterial pathogen which has a major role in chicken farms outbreaks (Awaad et al., 1981, Kheir Eldin et al., 1986 and Abdalla, 1987).

As shown in Table (1), the incidence of *Ps. aeruginosa* infection among the examined chickens was 9.43 %. This percentage should not be ignored because it becomes higher if compared with the percentages detected over the past few years (2-6 %) as mentioned by Markaryan (1975), Koncicki and Szubsvaska (1988) and Younes et al. (1990). This increase could be attributed to the emergence of many antibiotic resistant *Ps. aeruginosa* strains due to the improper use of antibacterial agents. Actually, antibiotic multi-resistant bacteria is considered a dangerous hazard for human as well as animal populations (Cruickshank et al., 1975). The highest incidence was found in the sample obtained from chickens with respiratory symptoms simultaneously with joint affection (35.7 %). Lin et al. (1993) isolated *Ps. aeruginosa* from the respiratory tract of birds with a long history of respiratory illness. The obtained data in Table (1) revealed also that, *Ps. aeruginosa* was isolated in pure culture from the sinuses with the highest recovery rate (4.43 %) followed by the liver (1.57 %), heart blood (1.29 %), lungs (0.86 %), joints (0.71 %), intestine (0.43 %) and trachea (0.14 %). In this concern Bapat et al. (1985); Abdall (1987) and Utomo and Pornome (1990).

As a confirmatory test to identify *Ps. aeruginosa*, serological examination was done using the available specific agglutinating sera. The most common serotypes found among the diseased chickens were poly III groups D&G (27.27 % and 18.18 respectively) as shown in Table (2).

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It looks like that these strains should be considered when preparing diagnostic or immunizing kits because this finding was previously mentioned by many investigators (Wokatsch, 1964; Srinivasan, 1977 and Schildger et al., 1989). The importance of these two serotypes was confirmed once more with the pathogenicity test where these strains in addition to the serotype Poly III E resulted in mortalities for 10 days-old chicks ranging from 40 to 100 %. This adds one more important *Ps. aeruginosa* serotype which is Poly III E.

Results of pyocin typing are clearly shown in Table (3). Out of 66 *Ps. aeruginosa* isolates, 27 were positive for pyocin production with a percentage of 40.9%. This incidence was nearly similar to that reported in a study carried out in India (38 %) by Sarma and Srinivas (1980). The most common types were P_4 (Poly III, G). While serotypes poly II groups K & M produced pyocin of type P5. Six strains belonging to the serotype poly III D were positive for pyocin production of two types of pyocin P_2, 4 (4 strains), P_1, 5 (1) and P_2,3 (1).

Regarding the antibiogram assay as shown in Table (4) reveals a serious problem which is the multiple resistance of almost all *Ps. aeruginosa* strains recovered in the present study.

The percentage of resistance to gentamicin, neo-

Table (1): The incidence of *Ps. aeruginosa* isolates recovered from diseased chickens

Clinical signs	No. of samples	Site of isolation														Total number of isolates	
		Sinus		Trechea		Lung		Blood		Intestine		Liver		Joint			
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Respiratory manifestation	343	17	4.96	-	0	5	1.46	5	1.46	-	0	8	2.33	-	0	35	10.2
Respiratory manifestation with joint affection	14	2	14.29	1	7.14	-	0	-	0	-	0	-	0	2	14.29	5	35.7
Respiratory manifestation with diarrhoea	77	5	6.49	-	0	1	1.3	1	1.3	1	1.3	-	0	-	0	8	10.39
Respiratory manifestation with diarrhoea and joint affection	175	7	4	-	0	-	0	3	1.71	2	1.14	3	1.71	3	1.71	18	10.29
Diarrhoea	91	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Total	700	31	4.43	1	0.14	6	0.86	9	1.29	3	0.43	11	1.57	5	0.71	66	9.43

% was calculated to the number of samples

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Table (2): Serotyping of *Ps. aeruginosa* strains isolated from chickens

Serotype	Number of isolates	Percentage **
* Poly. II, B.	2	3.03 %
Poly. II, K.	1	1.51 %
Poly. II, M.	1	1.51 %
Poly. III, D.	18	27.27 %
Poly. III, E.	4	6.06 %
Poly. III, G.	12	18.18 %
Untypable strains	28	42.42 %
Total	66	100 %

* Specific antisera were of two types: polyvalent (I, II, III) and monovalent which is indicated by uppercase alphabets.

** % was calculated to the total number of isolates.

- Serotyping was carried out by the slide agglutination method.

Table (3): Pyocin production by *Ps. aeruginosa* strains in relation to the serotypes

Serotype	Number of isolates	Number of Pyocin producing strains	Pyocin Type (P)
* Poly. II, B.	2	2	P3, 6
Poly. II, K.	1	1	P5
Poly. II, M.	1	1	P5
Poly. III, D.	18	6	P2, 4 (4); P1, 5 (1) & P2, 3 (1)*
Poly. III, E.	4	-	-
Poly. III, G.	12	6	P4
Untypable strain	28	11	P1, 5 (1); P3, 5 (1); P3 (2); P4 (1) & P3, 6 (6)

* These numbers = number of strains that produce the corresponding pyocin.

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Table (4): In vitro sensitivity of *Ps. aeruginosa* strains to different antibacterial agents by using the disc diffusion method

Antibacterial Agent	Degree of Sensitivity					
	Resistant		Intermediate		Sensitive	
	Number	% *	Number	%	Number	% *
Amikacin	64	96.96	-	0	2	3.03
Cephalexin	60	90.91	-	0	6	0.09
Chloramphenicol	61	92.42	-	0	5	7.58
Gentamicin	66	100	-	0	-	0
Neomycin	66	100	-	0	-	0
Ofloxacin	13	19.7	-	0	53	80.3
Streptomycin	66	100	-	0	-	0
Tobramycin	66	100	-	0	-	0
Trimethoprim & Sulphamethoxazole	48	72.73	18	27.27	-	0

* % was calculated to the total number of *Ps. aeruginosa* strains (66).

Table (5): The pathogenicity of different *Ps. aeruginosa* serotypes to chickens after subcutaneous inoculation

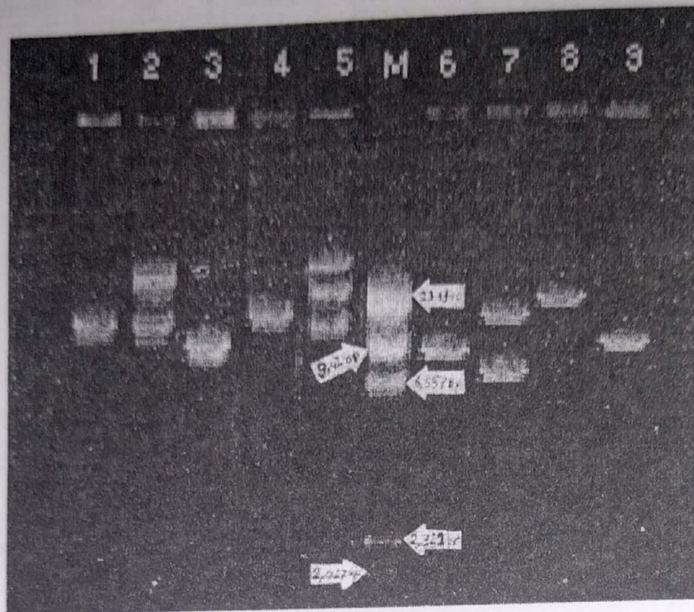
Chicken group	*Ps. aeruginosa* serotype	* Dose in ml	Number of dead birds	Mortality percentage ¶
1	Poly III (E) #	0.5	1	20 %
2	Po	1.0	2	40 %
3	Poly III (E) @	0.5	5	100 %
4	Po	1.0	5	100 %
5	Poly III (G) @	0.5	3	60 %
6	P4	1.0	4	80 %
7	Poly III (K) #	0.5	0	0 %
8	P5	1.0	0	0 %
9	Poly III (D) #	0.5	0	0 %
10	P2, 4	1.0	1	20 %
11	Poly II (B) #	0.5	0	0 %
12	P 3,6	1.0	0	0 %
13	Poly II (M) #	0.5	1	20 %
14	P5	1.0	2	40 %
15	Control	-	0	0 %

* Concentration of bacterial suspension = 9×10^8 bacterial cells/ml

Ofloxacin sensitive strains

@ Ofloxacin resistant strains

¶ Percentage was calculated to the number of infected chickens in each group (5)



photograph (1). Indicates the plasmid pattern of 9 serotypes of *Ps. aeruginosa* (1 = Poly II group K *** (P5), 2 = Poly III group G *** (P4), 3 = Poly III group D *** (P2,4); 4 = Poly II group M *** (P5); 5 = Poly II group B *** (P3, 6); M = marker Hind III; 6 = Poly III group D *** (Po); 8 = Poly III group E *** (Po) and 9 = Poly III group E *** (Po), *** sensitive to ofloxacin, **** Resistant to ofloxacin.

mycin, streptomycin and tobramycin was 100 %. The resistance percentage for amikacin, chloramphenicol, cephalexin was higher than 90 % while the percentage of resistance was lower for trimethoprim-sulphamethoxazole (72.73 %). These findings should be underlined for many reasons, first; the negative role of gentamicin which was considered for a long time a drug of choice to treat *Pseudomonas* infection; second, the urgent need for an antibiogram screening to choose a suitable drug in case of isolating of multiresistant bacteria such as *P. aeruginosa* from diseased chickens. However, a promising result was obtained with ofloxacin which showed an inhibitory effect to 80 % of the tested *Ps. aeruginosa* strains. This may be attributed to the mode of action of this drug which inhibit the bacterial DNA topisomerase II, an important enzyme for DNA replication. The multiresistance property of *Pseudomonas* was reported by many authors and could be attributed to the physicochemical properties of the cell wall rather than antibiotic inhibiting enzymes (Koncicki and Szubstraska, 1988).

Ps. aeruginosa is a dangerous multiresistant bacteria (Randall et al., 1974). Seven bacterial strains, well-identified serologically and typed for pyocin production, were selected for experimental infection of chickens. Table (5) clearly indicates that some serotypes showed a mortality rate between 60 % and 100 %. These strains were found resistant to ofloxacin such as strain poly IIIE., poly IIIG. On the contrary, ofloxacin such as strains showed a mortality rate from 0 % to 40 % such as serotypes poly IIIK and poly IIID.

There was severe congestion of livers with focal necrosis, sever enteritis, congestion of the spleen, heart and lung. The post mortem lesions recorded are in accordance with the findings made by Narula & Kuppuswamy (1969) and Bapat et al. (1985).

Since *Ps. aeruginosa* is a particular troublesome pathogen due to the inherent resistance to all but a few drugs, makes the plasmid profile in the different isolates of this study, a way to reveal any possible segregation between plasmid profiles and the behaviour of *Ps. aeruginosa* toward antibacterial agents and pyocin production in vitro. Recently, fingerprinting of plasmid profiles isolated from *Ps. aeruginosa* strains were investigated. Three samples (1, 4 and 8) represent one pattern indicated by one band with a size of 20,000 bp. A second pattern consists of one band of a lower size (12,000 bp) is represented by samples 3, 6 and 9. Sample number 2 shows a third pattern which is indicated by 2 bands (20,000 and 30,000 bp). Another different pattern of 2 bands with sizes of 8000 and 20,000 bp is obtained from samples number 7. This is in addition to a pattern consisting of 3 bands of approximate molecular sizes of 20,000, 30,000 and 40,000 bp as shown in photograph (1).

Actually, no clear relationship was detected among the three criteria and this finding was not surprising. This can be explained according to the physiochemical nature of the bacterial cell wall being the main factor which determines the multiresistant nature of *Ps. aeruginosa* to different agents (Koncicki and Szubstraska, 1988). Moreover, the pyocin production was found to be

controlled by chromosomal genes and is in no way to be correlated with the plasmid profiles. However, this does not minimize the importance of plasmid fingerprinting as a recent tool to identify and follow up the epidemiology *Ps. aeruginosa* in animal and human diseases. We should mention that the number of strains used to detect the plasmid profiles in *Pseudomonas* was too little to announce important recommendations and nevertheless many different profiles came out of these few strains. Finally, we can say that to better identify *Ps. aeruginosa* isolated from diseased chickens one should put in consideration the biochemical characters, serodiagnosis, pyocin typing and plasmid profiling. A special request is passed to clinicians, laboratory diagnosticians in human and animal fields, in addition to producers of animals and animal products, not to use or prescribe antimicrobial drugs improperly. Otherwise, all of us are very close to a big catastrophe in which no drug will be effective to treat some infections caused by such bacteria as *Psudomonas* and *Mycobacterium tuberculosis*.

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