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Risk of Free Living Birds (Pigeons, Iibises, Hoopoes and Crows) in Transmission of Multidrug Resistant *Pseudomonas aerouginosa* in Sohag Governorate

[1]Mona I.H. El-Enbaawy, [2]Nagwa A. Shalaby and [3]Gamal A.M. Omran

[1]Department of Microbiology,Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt
[2]Department of PoultryDiseases, Animal Health Research Institute, Provincial Laboratory, Tanta, Egypt
[3]Department of Microbiology, Animal Health Research Institute,Provincial Laboratory, Sohag, Egypt

Abstract: *Pseudomonas aeruginosa (P. aeruginosa*) is one of the common bacterial pathogens in free living birds. Multidrug resistance strains of *P.aeruginosa* are common throughout the world. A total of 17 isolates of *P.aeruginesa* were collected from free living birds (pigeons, ibises, hoopoes and crows) with an incidence of 3.63%. Isolates were tested for their virulent activities using Congo Red (CR) bindingassay and hemagglutination (HA) of erythrocytes from different species. Antimicrobial susceptibility of the isolates was determined against 10antimicrobial agents related to 5 antimicrobial classes (Beta lactams, Phenicols, Cephalosporins, aminoglycosides and Quinolones). All the isolates were found to be positive for Congo red binding activity, hemagglutination except *P.aeruginosa* of pigeon. Also, all *P. aeruginosa* isolates that showed hemagglutination were mannose resistant hemagglutination (MRHA). All the isolates showed multidrug resistance against more than 3 antimicrobial classes. In conclusion, the finding of this study show that the isolated *P. aeruginosa* from pigeons, ibises, hoopoes and crows produce virulence factors with multidrug resistance.

Key words: *Pseudomonas aeruginosa* · Free living birds · Virulence factors · Multidrug resistance

INTRODUCTION

Nowadays, infectious pathogens in wild life have become increasingly important throughout the world, as they have substantial impacts on human health and agricultural production [1]. Various species of free living birds, because of their propensity to nest and roost near human activity, may harbor and disseminate various species of bacterial microorganisms to domestic birds and animals [2]. Free-living bird populations can also be reservoirs of drug-resistant bacterial pathogens or resistant genetic element [3]. *P. aeruginosa*, *Escherichia coli* (*E. coli*), *Klebsiella* spp., *Salmonella* spp., *Pasteurella* spp*., Staphylococcus aureus (S. aureus*) and *Proteus*spp.were recovered from hoopoes, ibises, sparrows, dovesand quails with variable rates in Egypt [4]. Among these pathogens, the intrinsic resistance of *P. aeruginosa* to many antimicrobialagents and its ability to develop multidrug resistance (MDR) imposes aserious therapeutic problem [5] with the possibility for transfer of theresistance determinants [6,7]. since free living birds act as introductory hosts for several kinds of bacteria, viruses and parasites and MDR to human and animal population [8].

Recently, in Egypt, the number of crows and white ibises that was found in agricultural placeshave increased significantly in Cairo and other Governorates as Sohag. Crows and ibises become highly adaptable urban environment. Feralpigeons (*Columba liviadomestica*), white ibises (*Nipponianippon*), hoopoes (*Upupaepops*) and crows (*Corvuscornix*) are free living birds found everywhere in the our cities. They may carry causal agents of highly pathogenic with zoonotic infectivity. The fecal shedding may contaminate the environment and could play a role in the geographic spread of some of them.There are few reports dealing with the studies on various strains of *P. aeruginosa* isolated from free living birds.

Corresponding Author: Mona, I.H. El-Enbaawy, Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.

This study to investigate the *P. aeruginosa* inpigeons, ibises, hoopoes and crows in Sohag Governorate, Egypt and to detect the virulence activities (pigment production, Congo red binding ability, hemagglutination activity) and antimicrobial resistance of *P. aeruginosa* isolates to 10 major drugs.

MATERIALS AND METHODS

One hundred and fifty three free living birds (pigeons, ibises, hoopoes and crows) were captured or shot by hunters in different places in Sohag Governorate, UpperEgypt, over a period of 12 months fromDecember. 2005- December. 2006. Birds were transferred as soon as possible to the Bacteriology Department, Animal Health Research Institute, Sohage, Egypt. Samples (infra-orbital sinusesswabs, lungs and liver) from each bird were collected under sterile conditions to be examined bacteriologically for *Pseudomonas aeruginosa*.

Samples were collected under complete sterile conditions and inoculated into nutrient broth and incubated at 37°C for 24 hours. After incubation, nutrient broth was streaked on nutrient agar, pseudosel agar, sheep blood agar and MacConkey agar. These plates were incubated at 37°C for 24 hours. The suspected colonies were examined for pigment production, hemolytic activity on blood agar, microscopic morphology and motility, growth at 5°C and 42°C and biochemical characters according to Quinn *et al*. [9].

All *P. aeruginosa* isolates were tested for their Congo red (CR) binding ability [10] Hemagglutination (HA) of Guinea pig, sheep, chicken, horse and human (O blood group) erythrocytes in the presence and the absence of 2% D-mannose as described by Evans *et al.* [11].

Isolates of *P. aeruginosa*were submitted for *in vitro* susceptibility test against 10 antimicrobial agents by Kirby-Bauer disc diffusion method [12], using Muller-Hinton broth and agar (Oxoid) and antibiotics discs (Bioanalyse). The following antibiotic discs were used: penicillin (P, 10 IU), chloramphenicol (C, 30 µg), cephalexin (CL, 30 µg), cefixime (CFM, 5 µg), gentamicin (CN, 10 µg), amikacin (AK, 30 µg), streptomycin (S, 10 µg), ofloxacin (OFX, 5µg), norfloxacin (NOR, 10µg) and naldixic acid (NA, 30µg). The inhibition zones were measured in millimetres after 24 hours of growth and interpretation was according to NCCLS [13].

RESULTS AND DISCUSSION

The isolates were bacteriologically identified as *P.aeruginosa* depending on Gram stain, characteristic features and standard biochemical reactions included fruity grape-like colonies odor, pigment production, growth on MacConkcy agar,haemolysis on sheep blood agar, growth at 5°C and 42°C, motility, oxidation of lactose and glucose,oxidase, catalase, nitrate reduction and gelatin liquefaction test [8].

As shown in Table (1), from samples of infra-orbital sinuses swabs, lungs and liver tissues of 153 (live and freshly dead) pigeons, ibises, hoopoes and crows, 17 isolates were identified as *P. aeruginosa.* The most effective medium for isolation was Pseudosel (Cetrimid) agar, it is a selective medium for the isolation and identification of *P. aeruginosa*. it is a modification of Tech. agar developed by King *et al.* [14]. that improved pyocyaninand fluorescein production by *Pseudomonas* spp.

The isolation rates of *P. aeruginosa*were 3.63 % (17/459 samples). 1/18 (5.5%), 2/22 (9.0%), 5/43(11.6%) and 9/70 (12.8%) from pigeons, ibises, hoopoes and crows, respectively. Many investigators isolated *P. aeruginosa* from pigeons, ibises and they declared the role of *P. aeruginosa* as significant pathogen in domestic and free living birds; In Egypt, Nasif and Hassan [15]. and El-Sheshtway and Moursi [4]. isolated *P. aeruginosa* from pigeons and ibises. Hedawy [16] and Ibrahim [17] recovered *P. aeruginosa* isolates from diseased pigeons and squabs with different incidences. Hedawy and El-Shorbagy [2] isolated *P. aeruginosa* from ibises with an incidence of 20%. In contrary, Awad-Alla *et al.* [18] could not isolate *P. aeruginosa* from the internal organs of free living white ibis (*Nipponianippon*) in Egypt and they added that the world population of white ibis has increased significantly since 1983 and these birds are frequently observed in close contact people. This has led to concern that ibis may transmit pathogen that threatens not only the poultry industry, but also public health. Little is known about the incidence of *P. aeruginosa*in Egyptian hoopoesandcrows [4,2].

P. aeruginosa produce two types of soluble pigments, the fluorescent pigment pyoverdin and the blue pigment pyocyanin. The latter is produced by many, but not all strains of *P. aeruginosa.* Pseudosel agar is highly selective for the growth of *P. aeruginosa* and is also valuable for demonstrating pyocyanin pigment production [19].

Table 1: Types and number of birds, types of samples and the incidence of *P. aeruginosa* among the free living birds

Birds	Number of examined birds	Type of samples	*P.aeruginosa* Positive	Percent
Pigeons	18	infra-orbital sinuses	1	5.5
Ibises	22	liver	2	9.0
Hoopoe	43	infra-orbital sinuses	1	11.6
		Liver	4	
Crows	70	infra-orbital sinuses	3	
		liver	6	12.8
Total	153		17	11.0

Table 2: The incidence of *P.aeruginosa* pigment production, Congo red binding activity and hemagglutination properties among pigeons, ibises, hoopoesand crows

				HA					MRHA					MSHA				
				Types of RBCs					Types of RBCs					Types of RBCs				
Free living birds	*P. Aeruginosa number (17)*	Pi	CR	Gp	S	C	H	Hu	Gp	S	C	H	Hu	Gp	S	C	H	Hu
Pigeon	1	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total		1	1	0.0					0.0					0.0				
Ibis	2	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Ibis	3	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Total		1	2	2					2					0.0				
Hoopoe	4	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Hoopoe	5	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Hoopoe	6	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
Hoopoe	7	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
Hoopoe	8	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total		2	5	4					4					0.0				
Crow	9	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Crow	10	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Crow	11	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Crow	12	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Crow	13	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Crow	14	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-
Crow	15	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
Crow	16	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
Crow	17	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-
Total		2	9	9					9					0.0				

Total= Total Positive for *P.aeruginosa*

Pi= pigment production, CR= Congo red binding ability, HA= hemagglutination,

MRHA=Mannose resistance hemagglutination, MSHA=Mannosesensitivehemagglutination.

Gp=Guinea pig, S= Sheep, C = Chicken, Hu = Human.

As shown in Table(2), 1/1, 1 /2, 2/5 and 2/9 of *P. aeruginosa* of pigeons, ibises, hoopoes and crows origins, respectively were pigment (blue-green) producer. Pyocyanin pigment is a redox active virulence factor produced by *P. aeruginosa* [20]. In contrary, Lau *et al*. [21]. stated that it is difficult to define the contribution of pyocyanin among thenumerous virulence factors produced by *P. aeruginosa* during infection.

P. aeruginosa is able to form biofilm on different animate or inanimate surfaces; this property helps the survival and protects the bacteria from the immune system and antimicrobial agents inside the host [22]. Flagella and twitching motility have been shown to be involved in regulation of biofilm formation and they are important for initial attachment to surfaces [23].

Ghafoor *et al.* [24] used the Congo red binding assay to assess the ability of *P. aeruginosa* mutants to produce Pel polysaccharide (an exopolysaccharides that contribute to the formation of biofilms in this organism) this assay was adapted from Spiers *et al*. [25]. Although the specificity of this Congo red binding assay has not been established, it has been widely used as an indicator of Pel production [26].

Table 3: Antimicrobial susceptibility testing of *P.aeruginos* frompigeons, ibises, hoopoes and crows

		Antimicrobial classes										
		Beta Lactms	Phenicols	Cephalosporins		Aminoglycosides			Quinolones			MDR
No	Birds	P10 Iu	C30 µg	Cl30 µg	CF5 µg	CN10 µg	AK30 µg	S10 µg	OFX,5µg	NOR10µg	NA30µg	*P.aeruginos*
1	Pigeon	R	R	R	R	S	S	I	S	S	I	MDR+
2	Ibis	R	R	R	R	S	S	R	S	S	R	MDR+
3	Ibis	R	R	R	R	I	S	I	S	S	R	MDR+
4	Hoopoe	R	R	R	R	R	S	I	S	S	I	MDR+
5	Hoopoe	R	R	R	R	I	S	S	S	S	R	MDR+
6	Hoopoe	R	R	R	R	S	S	I	S	R	I	MDR+
7	Hoopoe	R	R	R	R	I	S	R	S	S	S	MDR+
8	Hoopoe	R	R	R	R	S	S	I	S	S	I	MDR+
9	Crow	R	R	R	R	R	S	I	S	S	R	MDR+
10	Crow	R	R	R	R	S	S	I	S	S	*R*	MDR+
11	Crow	R	R	R	R	S	S	I	S	S	*R*	MDR+
12	Crow	R	R	R	R	S	S	I	S	S	R	MDR+
13	Crow	R	R	R	R	S	S	I	S	S	R	MDR+
14	Crow	R	R	R	R	S	S	I	S	S	R	MDR+
15	Crow	R	R	R	R	S	S	S	S	S	I	MDR+
16	Crow	R	R	R	R	S	S	S	S	S	I	MDR+
17	Crow	R	R	R	R	S	S	S	S	S	I	MDR+
	Total of R	17	17	17	17	2	0	2	0	1	9	

P, 10 Iu =Penicillin, C, 30 µg = chloramphenicol, CL, 30 µg = cephalexin, CFM, 5 µg =cefixime, CN, 10 µg=gentamicin, AK, 30 µg =amikacin, S, 10 µg= streptomycin, OFX, 5µg= ofloxacin NOR, 10µg= norfloxacin, NA, 30µg =naldixic acid, R = resistance, I = intermediate,
S = sensitive, MDR = Multidrug resistance.

In the present study, all isolates of *P. aeruginosa* from (pigeons, ibises, hoopoes and crows) were found to be positive for Congo red binding activity (colonies gave red color). Ezz El-Deen [27] from chickens, human as well as water and Ibrahim [28] and Aly [29] from animal and human origin examined *P. aeruginosa* isolates for Congo red binding affinity. The isolates were positive for Congo red binding affinity with different percentages. They concluded that Congored binding affinity could give an idea about pathogenic *P. aeruginosa.*

Hemagglutination properties of *P. aeruginosa* were tested using erythrocytes of different species as Guinea pig, sheep, chicken, horseand human.As shown in Table (3) *P. aeruginosa* of pigeon showed no hemagglutination. While, *P. aeruginosa* of ibises showed HA+ with Guinea pig RBCs only and*P. aeruginosa* from hoopoes showedHA+ with Guinea pig, sheep and chicken RBCs. On the other hand, *P. aeruginosa* isolated from crows showed HA+ with Guinea pig, chicken and horse RBCs. All *P. aeruginosa* strains that showed (HA+) were mannose resistant hemagglutination (MRHA) but no one of them was mannose sensitive hemagglutination (MSHA). In the present study hemagglutination was screened in microliter plates as described by Evans *et al*. [10] and supported by Stahlhut *et al.* [30]. *P. aeruginosa*pili facilitate attachment to epithelial cells and the value of hemagglutination in thepresence of D-mannose is an indication of the presence of adhesion antigens in *P. aeruginosa* strains [31]. Recently, Stahlhut *et al.* [30] mentioned that specific detection of fimbrial expression is an important task in virulence characterization and epidemiological studiesand they added thatthe use of Guinea pig RBCs to detect type 1 fimbriae in bacterial species able to express type 3 fimbriae is essential.

Although, the most active agents in this study were of loxacin, amikacin and norfloxacin, resistance of *P.aeruginos* atopenicillin, chloramphenicol, *cephalexin* and cefiximewas highly followed bynalidixic acid (Table, 3). In the present study the 10 used antibiotics were belonging to 5 antimicrobial classes: 1-Beta Lactams include penicillin (P, 10 Iu); 2-Phenicols include chloramphenicol (C,30 µg); 3-Cephalosporins (first generation) include cephalexin (CL, 30 µg); and (third generation) include cefixime (CFM, 5 µg);

4-Aminoglycosides include gentamicin (CN, 10 µg), amikacin (AK, 30 µg) and streptomycin (S, 10 µg); 5-Quinolones includeofloxacin (OFX, 5µg), norfloxacin

(NOR, 10µg) and naldixic acid (NA, 30µg). All isolates of *P. aeruginos* that isolated from pigeons, ibises, hoopoes and crows were MDR+, since isolates showing resistance to 3 or more antibacterial agents from different classes of antimicrobial agents were selected as MDR+. Multidrug resistance in *P. aeruginosa* is inincrease [7]. MDRis a major cause of treatment failure for infectious disease [32].

In conclusion,pigeons, ibises, hoopoes and crows are considered to be virulent multidrug resistant of *P. aeruginosa*. This MDR of *P. aeruginosa* may be a kind of the virulence factor. Screen out of free living birds is very important, since free living birds act as introductory hosts for several kinds of bacteria, viruses and parasites and MDR to human and animal population.

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