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Virulence Repertoire of *Pseudomonas aeruginosa* from some Poultry Farms with Detection of Resistance to Various Antimicrobials and Plant Extracts

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Abstract

Pseudomonas aeruginosa is a serious poultry pathogen and zoonotic bacterial agent causes nosocomial infections. The prevalence of virulence determinants among *P. aeruginosa* appears to be lacking in Egypt. Therefore, this study investigated occurrence, antimicrobial resistance and virulence gene profiling of *P. aeruginosa* in broiler chicken. Thirty eight cases (22.9%) were infected with *P. aeruginosa*, high isolation from dead-in-shell embryos 26 (52%) and significantly different ($p<0.0001$) when compared with that from diseased and freshly dead 12 (12%). Haemolysin and lipase gave highest rates 28 (73.68%) and 28 (73.68%). While, gelatinase, lecithinase and protease represented 24 (63.1%), 26 (68.42%) and 26 (68.42%) respectively. High distribution of Exotoxin A (*Exo* A) and Outer membrane protein (*opr* L) genes 100% with strong uphill correlation (r):1. Strong relationship between oprL and antibiotic resistance. High resistance 100% to Amoxicillin (AMX), E- Moxclav (AMC). While, resistance to Cotrimoxazole (CMX), Ceftriaxone (CRO), Ofloxacin (OFX) showed 90%, 80% and 30%. Besides, no resistance to Ciprofloxacin (CIP) and Gentamycin (CN). Significant difference between efficacy of Formalin, EDTA, Savlon and Thyme ($p<0.0001$). Although, great efficacy differences between the MIC of EDTA, Formalin, Savlon and Thyme with 1%, 3%, 6% and 8%, Formalin gave the highest efficacy with 10%. Uphill strong correlation between the efficacies and concentrations of Formalin, EDTA, Savlon and Thyme (r): 0.97, 0.91, 0.92 and 0.80.

In this study, we focused on elucidating virulence arsenal and resistance of *P. aeruginosa* to most antimicrobials. Providing evidential clues about efficacy of some antimicrobial compounds and plant extracts.

Keywords: Poultry; *Pseudomonas aeruginosa*; Virulence; PCR; Resistance

Introduction

P. aeuroginosa is a motile, gram negative, oxidase positive, rod shaped with single arrangement or short chains. The organism is a strict aerobe, ubiquitous and often associated with soil, water and humid environment. It affects newly hatched chickens drastically causing high mortality and mass death of embryos [1-8]. *P. aeruginosa* got a huge arsenal of virulence repertoire implicated in pathogenesis. Attributed to the numbers of extracellular virulence factors and cellular components as lipopolysaccharide, elastase, alkaline proteases, pyocyanin, pyoverdin, haemolysins, phospholipase C, rhamnolipids, biofilm, Pilli, and flagella. The complex type III secretion system recognized virulence determinant of *P. aeruginosa* capable of injecting proteins and secretion toxins into the host cell. Four secretion toxin identified; exoenzyme S, exoenzyme U, exoenzyme T and exoenzyme Y [9-15].

Outer membrane lipoprotein (*Opr*L) implicated in efflux transport systems and affecting cell permeability (4). The Exotoxin A is produced by most of *P. aeruginosa* strains with great similarity to diphtheria toxin. It can inhibit eukaryotic protein biosynthesis at the level of polypeptide chain elongation factor 2 [16].

P. aeruginosa is resistant to various antimicrobial agents due to impermeability, multi-drug efflux, and a chromosomal AmpC β-lactamase. Prominent resistance found among α-carboxy- and Amino-penicillins, third and fourth-generation Cephalosporins, Monobactams, Carbapenems, aminoglycosides, and Fluoroquinolones. Resistance to any of these classes could be due to various mutations that result in upregulation of efflux or down regulation of permeability. Besides, hyperproduction of the chromosomal AmpC β-lactamase in case of Aminopenicillins and Cephalosporins [17-23].

Many disinfectants and antiseptics are now commercially available. Great progress made to understand mechanisms of their antimicrobial actions. They include alcohols, aldehydes, halogens, phenols, halogenated phenolic and substituted phenolic compounds. Additional preparations are biguanides, peroxygens, tar acid phenol, quaternary ammonium compounds and chlorohexidine gluconate. Many of them are used extensively for variety of animate and inanimate surface applications. In particular, their application constitutes an essential part of infection control practices and aid in infection prevention [24].

The antimicrobial efficacy of plant extracts and phytochemicals evaluated [25] with antibiotic susceptible and resistant microorganisms. *P. aeruginosa* showed interesting, results since it was inhibited by clove, jambolan, pomegranate and thyme extracts. This inhibition observed with single extract and when used with lower concentrations of ineffective antibiotics. Many regions of the world privileged with medicinal plants rich in active phytochemicals with potential medicinal values. Even so, many studied species considered promising and representing natural settlement of antibiotic resistance problem [26,27].

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Material and Methods

Sampling

A total of 150 broiler cases, 100 from 2-40 days old diseased and freshly dead (Nasal swabs, Throat swabs, Heart blood, Cloacal swabs and Liver tissue) were collected. And 50 from dead-in-shell embryos in hatcheries (Heart blood, Lung tissue, Peritoneal samples, Liver tissue, Intestinal samples and Yolk sac). Added to them 8 water and 8 litter samples.

Media and Biochemical Identification

Cetimide agar medium was used, colonial morphology, microscopic examination and biochemical identification according to [26], then confirmation using API 20 NE (Bio-Meriux) after the producer instructions.

Molecular Identification

DNA extraction

Bacterial genomic DNA was extracted from confirmed cultures by QIAamp DNA extraction Mini prep Kit after manufacturer's instructions. Extracted DNA stored at -80°C before PCR amplification. For each batch of extractions, a negative control containing reagents minus cultures and positive control of *P. aeruginosa* ATCC 27853.

PCR amplification

The used primers, PCR protocol and program according to [33]: Targeted primers, sequences, amplicon sizes and accession number listed in Table 1.

Antimicrobial susceptibility testing

Detection of bacterial count after 24 hrs growth according to [31] has identified *P. aeruginosa* cultivated onto Todd-Hewitt broth for 24 hrs. Then concentration of bacterial cells in 1 ml medium by centrifugation. Application of spectrophotometer at 660 nm to adjust the concentration to 1×10^6 colony forming unit (CFU) per 1 ml sterile TSB for dilution of the concentrated bacterial isolates. From the adjusted 106 CFU /l-1 1 ml taken and spread on the surface of Muller-Hinton agar plates and the excess decanted away. Plates left to dry at 40 0C for 20 minutes in the incubator. Plates adjusted to be enough for antibiotic discs Amoxicillin AMX 25 mcg, Ciprofloxacin CIP 5 mcg, Ceftriaxone CRO 30 mcg, Cefuroxime CXM 30 mcg, E- Moxclav AMC 20 mcg, Cotrimoxazole CMX (Trimethoprim/ Sulphamethoxazole 25 mcg), Gentamycin CN10 mcg and Ofloxacin OFX 5 mcg. Agar plates for chemicals and plant extracts (Formalin, EDTA, Ethyl alcohol Only 70%, Isopropyl alcohol, Chlorohexidine cetramid (Savlon), Iodine, Sodium citrate, Allicin, Basil, Lemon citrus oil, Pomegranate and Thyme [27-31].

Wells made by the wide end of blunted sterile Pasteur pipette. Inserting it and twisting to remove the plug of agar a pair of forceps sterilized by flaming in alcohol used to remove the agar. Wells numbered and filled with 120 µl of chemicals or plant extracts then incubated at 37°C for 24-48hrs. The inhibition zone measured by ruler and efficacy determined after [3].

Minimum Inhibitory Concentration (MIC) of chemicals and plant extracts determined using the broth dilution method in Todd-Hewitt broth [28]. Each compound diluted 1 to 10% (v/v) only Ethyl alcohol was 70%. Transfer 1 ml of bacterial suspension (106CFU/ml) and 0.1 ml of each compound showing antibacterial efficacy added to 2.9 ml of Todd-Hewitt broth. After 24 hrs of incubation at 37°C under agitation in culture tubes, the MIC determined as the lowest concentration inhibit bacterial growth turbidity. To detect the MBC, 10 µL of bacterial inoculum removed from tubes with no turbidity and spread onto Todd-Hewitt agar. These plates incubated at 37°C for 48 hrs. The MBC considered as the lower concentration that shows no bacterial growth on Todd-Hewitt agar plates. Each MIC and MBC value obtained from three independent experiments and controls without test compounds used [32,33].

Statistical Analysis

Data analyzed by Statistical Analysis System software package SAS for Windows, version 8 (SAS Institute, Cary, NC). Independent t-test assesses the significance of the difference between numbers of isolates, efficacy of different chemicals and plant extracts. Statistical detection of the correlation coefficient of the presence of Exo A and opr L within isolates and efficacies and concentrations of antimicrobials was performed.

Results

Results of bacteriological examination: the total isolation result of *P. aeruginosa* was 38 /166 (22.9%). High isolation rate from dead-in-shell embryos yolk sac 26/50 (52%). This result showed significant difference ($p<0.0001$) when being compared with that of liver of 2-40 days old diseased and freshly dead 12/100 (12%). Distribution of phenotypic virulence factors, haemolysin and lipase gave highest rates 28/38 (73.68%) and 28/38 (73.68%). While, gelatinase, lecithinase and protease represented 24/38 (63.1%), 26/38 (68.42%) and 26/38 (68.42%) respectively. These results proved high virulence repertoire owned by the *P. aeruginosa* confirming pathogenicity.

Molecular detection of *Exo*A, *opr*L genes, and phenotypic susceptibility to antimicrobials. There located high distribution of both genes within 100% of the obtained isolates with strong uphill correlation (r):1. A strong resistance of *P. aeruginosa* to screened antimicrobials with strong relationship between the presence of *opr*L and phenotypic antibiotic resistance (Regression line equation (y): (62.5). All isolates showed complete resistance 100% to Amoxicillin (AMX), E- Moxclav (AMC). While, resistance to Cotrimoxazole (CMX), Ceftriaxone (CRO), Ofloxacin (OFX) showed 90%, 80% and 30% respectively. Besides, no resistance to Ciprofloxacin (CIP) and Gentamycin (CN).

Concerning the results of testing the efficacy of various chemical substances, disinfectants, and essential oil extracts on *P. aeruginosa*. There located significant difference between the efficacy of Formalin, EDTA, Savlon and Thyme ($p<0.0001$). Although, great differences between the MIC of EDTA, Formalin, Savlon and Thyme with concentrations of 1%, 3%, 6%, and 8%. Formalin gave the highest efficacy with 10% concentration. Uphill strong correlation expressed between the efficacies and increased concentrations of Formalin,

Target Gene locus	Primer	Amplicon (bp)	Accession number
*opr*L	f: 5'-ATG GAA ATG CTG AAA TTC GGC-3' r: 5'-CTT CTT CAG CTC GAC GCG ACG-3'	504	JQ228528.1
*Exo*A	f: 5'-GAC AAC GCC CTC AGC ATC ACC AGC-3' r: 5'-CGC TGG CCC ATT CGC TCC AGC GCT-3'	396	K01397.1

Table 1: Targeted primers, sequences, amplicon sizes and accession number.

Type and No. of Cases Haemolysin		Collected samples		Results of Biochemical tests and API 20NE	Results of phenotypic detection of virulence factors				
		Type	No.		Gelatinase	Haemolysin	Lipase	Lecithinase	Protease
Birds (2-40 days old) (diseased and freshly dead)	100	Nasal swabs	100	0	-	-	-	-	-
		Throat swabs	100	0	-	-	-	-	-
		Heart blood swabs	100	0	-	-	-	-	-
		Liver	100	12/100 (12%)	12	12	12	12	12
		Cloacal swabs	100	0	-	-	-	-	-
Dead -in- shell embryos from hatcheries	50	Heart	50	0	-	-	-		-
		Lung	50	0	-	-	-	-	-
		Peritoneal samples	50	0	-	-	-	-	-
		Liver	50	0	-	-	-	-	-
		Intestinal samples	50	0	-	-	-	-	-
		Yolk sac	50	26/50 (52%)	26	26	26	26	26
Environmental samples	16	Litter samples	8	0	-	-	-	-	-
		Water samples	8	0	-	-	-	-	-
Total	166			38 /166 (22.9%)	24/38 (63.1%)	28/38 (73.68%)	26/38 (68.42%)	28/38 (73.68%)	26/38 (68.42%)

Table 2: Genotypic detection of Exo A and opr L with relation to sensitivity to various antimicrobials.

EDTA, Savlon and Thyme with correlation coefficients (r): 0.97, 0.91, 0.92, and 0.80 respectively.

Discussion

The pathogenicity of *P. aeruginosa* in birds is related to keratitis, keratoconjunctivitis, septicemia, respiratory infections, sinusitis, and soared embryonic death rates in hatcheries [10]. The total isolation rate (Table 1) was 38/166 (22.9%) of them 12/100 (12%) and 26 (52%) from liver of freshly dead and yolk sac of dead -in-shell embryos respectively, these results similar to [14]. While from the dead- in-shell embryos was higher than 2004 records from Egypt [19] which could be interpreted by the increased virulence and antimicrobial resistance which lead to existence of serious types of *P. aeruginosa.* The high mortalities reflected in high isolation results were due to the colonization of *P. aeruginosa* in eggs and degradation of yolk proteins making the environment conductive to the proliferation and installation of other pathogens. Localized or septicemic forms of *P. aeruginosa* infections dependent on its path of entrance, age and resistance of the host. Concerning the results of phenotypic virulence factors (Table 1), gelatinase and Lecithinase activities were like results reported from Romania in 2013 [11]. In addition to that, haemolysin production was similar to data from India in 2006 [18]. *P. aeruginosa* lipase is an important virulence factor induces harmful effects with other bacterial enzymes, in particular, Phospholipase C. Production of lipase activity by *P. aeruginosa* isolates contribute to pathogenicity which may suppress the immune response [8]. The result of protease production comes consistent with data from Iraq 2013 [20]. The relative contribution of each of these exoproducts implicated in virulence of *P. aeruginosa* may vary depending on site and type of infection [32].

The results of virulence factors clarified that most of the isolates expressed high virulence degrees. The interaction between virulence factors and host immune response determines severity and type of infections.

Molecular detection of (*opr*L) and (*Exo*A) gene loci confirmed distribution pattern in 38/38 (100%) of all *P. aeruginosa* with strong uphill correlation (r):1 (Table 2). There located significant resistance of *P. aeruginosa* to screened antimicrobials ($p<0.0001$). There found strong relationship between the presence of oprL and phenotypic antibiotic resistance (Regression line equation (y): 62.5. The highest resistance 100% showed to Amoxicillin (AMX), E- Moxclav (AMC). While, resistance to Cotrimoxazole (CMX), Ceftriaxone (CRO), and Ofloxacin (OFX) were 90%, 80%, and 30% respectively. Besides, no resistance to Ciprofloxacin (CIP) and Gentamycin (CN). Furthermore, the high results of *Exo*A coincides with published data from National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas in 1994 [16]. The results of genetic detection of outer membrane protein L agree with published results from Belgium in 1997 [4]. Regarding the antimicrobial sensitivity testing results, high sensitivity to gentamicin [25], Also the high *P. aeruginosa* sensitivity to Ciprofloxacin concurred with reported results from Iraq in 2013 [20] while, ceftriaxone like stated data from Lagos Nigeria in 2002 [23]. Resistance to Amoxicillin, E- Moxclav similar to data published on isolated *P. aeruginosa* from cattle in Bangladesh in 2013 [13]. Sensitivity to Cotrimoxazole agree with [30] while, resistance to Cefuroxime like published results from Tamale teaching hospital at the north of Ghana in 2013 (Table 3) [1].

Meditating in results of antimicrobial effect of chemical substances (Table 4). Formalin gave notable efficacy with a concentration of 3% concurred with stated data from Al-Hilla teaching hospital Iraq in 2012 [9]. *P. aeruginosa* was sensitive to 1% of EDTA with a proportional relationship between concentrations and efficacies [2]. Resistance to Ethanol and Isopropyl alcohols disagree with [12]. While, susceptibility to Savlon with concentration of 6% agrees with their results. Essential oils produced no efficacy except Thyme at a concentration of 8% concurred with [21]. The presence of outer membrane proteins increased resistance to various tested antibiotics, chemicals, and plant extracts. Outer membrane proteins implicated in interaction with an environment, efflux transport systems, and cell permeability [22].

Results of Phenotypic antimicrobial susceptibility testing.	**Results of antimicrobial efficacy**			**Molecular Detection of Exo A And oprL genes**	
Types of used antibiotics	Sensitive (S)	Intermediate (I)	Resistant (R)	*Exo* A	*opr*L
Amoxicillin (AMX)	-	-	100%	38/38 (100%)	38/38 (100%)
Ceftriaxone (CRO)	20%	-	80%		
Cefuroxime (CXM)	-	-	100%		
Cotrimoxazole (CMX)	10%	-	90%		
Ciprofloxacin (CIP)	60%	40%	-		
E- Moxclav (AMC)	-	-	100%		
Gentamycin (CN)	100%	-	-		
Ofloxacin (OFX)	70%	-	30%		

Table 3: Susceptibility testing of *P. aeruginosa* with chemical substances, disinfectants and essential oil extracts from some medicinal herbs.

Concentrations / Type	**1%**	**2%**	**3%**	**4%**	**5%**	**6%**	**7%**	**8%**	**9%**	**10%**
Formalin	R*	R	21.25 ± 0.25	21.75 ± 0.45	26.5 ± 0.204	26.85 ± 0.312	35.58 ± 0.217	45.88 ± 0.426	53.67 ± 0.311	61.62 ± 0.625
EDTA	17.57 ± 0.217	25.45 ± 0.210	25.62 ± 0.239	26.37 ± 0.239	27.4 ± 0.244	28.32 ± 0.235	29.55 ± 0.239	30.57 ± 0.209	31.6 ± 0.212	32.5 ± 0.279
Ethyl alcohol Only 70%	R									
Isopropyl alcohol	R	R	R	R	R	R	R	R	R	R
Chlorohexidine cetramid (Savlon)	R	R	R	R	R	16 ± 0.248	17.85 ± 0.064	20.92 ± 0.047	23.82 ± 0.143	25.47 ± 0.213
Iodine	R	R	R	R	R	R	R	R	R	R
Sodium citrate	R	R	R	R	R	R	R	R	R	R
Allicin	R	R	R	R	R	R	R	R	R	R
Basil	R	R	R	R	R	R	R	R	R	R
Lemon citrus oil	R	R	R	R	R	R	R	R	R	R
Pomegranate	R	R	R	R	R	R	R	R	R	R
Thyme	R	R	R	R	R	R	R	17.5 ± 0.221	20.6 ± 0.216	21.67 ± 0.228

R*: Resistant

Table 4: Antimicrobial effect of chemical substances.

Conclusion

In conclusion, the gained results proved high virulence repertoire owned by the *P. aeruginosa* confirming pathogenicity. Outer membrane protein is responsible for most of resistance expressed by *P. aeruginosa.* Expressed sensitivity to EDTA, Formalin, Savlon and Thyme with a proportional relationship between concentrations and efficacies. Further investigations are required for new antibacterial components and vaccine formulation.

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