Phenotypic Characterization of Extensively Drug Resistant *Pseudomonas aeruginosa* (XDR) from Broiler Chickens in Sharkia Province, Egypt.

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Abstract

Pseudomonas aeruginosa (P. aeruginosa) is considered the most predominant *pseudomonas species,* causing mortality in chickens at all ages. Two handered samples were collected from broiler chickens of ages ranging from 1 day to 35 days in Sharkia Province that suffered from respiratory distress, diarrhea, and omphalitis in chicks for isolation of *P. aeruginosa* and detection of its sensitivity to various antimicrobial agents. The prevalence of *P. aeruginosa* in broiler chickens was 10%. All the isolates showed beta hemolytic activity on sheep blood agar. The most effective antibiotics were amikacin (100%) and colistin sulphate (95%), followed by norfloxacin (70%) and ciprofloxacin (60%). The antimicrobial resistance pattern of the isolates revealed that all isolated *P. aeruginosa* were extensively drug-resistant. Subsequently, a farm is difficult to be cleared from *P. aeruginosa* because of its high resistance to numerous antibiotics so that strict hygienic measures should be applied inside poultry farms for controlling *P. aeruginosa*.

Key words: *P. aeruginosa,* chickens, Prevalence, Antimicrobial resistant

Introduction

Avian Pseudomoniasis (*Pseudomonas* infection) in all species is considered a secondary bacterial infection because of many stress factors that result in a reduction in the normal flora, immunosuppression, minor injuries of the mucosal membranes, general weakness, or systemic diseases ***(Samour, 2006)***.

According to ***(Shukla and Mishra, 2015),*** the main pathogenic forms of

P. aeruginosa infection are respiratory affections and septicemia. *P. aeruginosa* is a gram-negative, non-sporulating, non-fermenting, oxidase positive, catalase positive, motile (with one polar flagellum), strictly aerobic, straight or slightly curved rod belonging to the bacterial family *Pseudomonadaceae* ***(Sarma, 2002)***.

The cultural characters of *P. aeruginosa* are β hemolysis on blood agar, non-lactose fermentation on MacConkey's agar; while on pseudomonas agar medium, colonies have greenish coloration with a fruity smell. *P. aeruginosa* is characterized by light greenish yellow pigments when cultivated on Tryptic soya agar ***(Bakheet and Torra, 2020)***.

P. aeruginosa's susceptibility to a lot of types of antimicrobials is minor, making it a very hard pathogen to eliminate ***(Khattab et al., 2015)***. According to ***(CLSI, 2016),*** multidrug-resistant (MDR) *P. aeruginosa* has increased because of the misuse and overuse of antibiotics.

This work was done to investigate the antibiogram of *P. aeruginosa* isolated from broiler chickens.

Material and methods

Samples:

Two hundred (200) samples were collected from broiler chickens of ages ranging from 1 to 35 days in Sharkia Province that suffered from respiratory distress (pneumonia with deposition of cheesy material on the serous membranes of the peritoneal cavity and air sacs) and diarrhea with swelling and necrotic foci in the liver and spleen, also perihepatitis, pericarditis, congested internal organs, and omphalitis in chicks. Samples were collected aseptically in sterile separate plastic bags and transferred as soon as possible in an ice box to the reference laboratory of the veterinary quality control of poultry production in Sharkia Province for bacteriological examination.

Isolation and identification of *P. aeruginosa*

Non-selective pre-enrichment:

For enrichment, 1 gm of each sample was inoculated in buffered peptone water (9ml) and incubated for 24 hours at 37 ºC.

Selective plating.

MacConkey's agar ***(Oxoid, CM0007)*** and Pseudomonas agar ***(HIMEDIA, M085)*** were streaked with a loopful from the incubated broth culture of each sample and incubated at 37 ºC for 24 hours. Non-lactose fermenting colonies (pale colonies) on MacConkey's agar and blue-green or brown colonies on Pseudomonas agar were subcultured on Nutrient agar ***(Oxoid, CM0003)*** and incubated at 37 ºC for 24 hours for observation of pigmentation. The slant cultures are stored at 4ºC for investigations.

Identification of isolates:

Suspected *pseudomonas* colonies were identified phenotypically

according to ***(Konemann et al., 1997 and Quinn et al., 2011)*** by Gram′s staining, motility test, and biochemical tests.

Hemolytic activity:

Tested and control organisms were plated on tryptose blood agar (Difco) with 5% defibrinated washed sheep blood and incubated at 37 °C for 24 hours. The plates were then examined for alpha or beta hemolytic activity (greening or clearing of the agar around the colonies, respectively) ***(Forbes et al., 1998)***.

Antibiogram of recovered *P. aeruginosa* isolates:

Antibiotic sensitivity pattern of the isolates was performed and interpreted according to ***(CLSI, 2016)*** by Kirby-Bauer disc diffusion test against 20 antibiotic discs ***(Oxoid)*** within 12 Antimicrobial classes where a loopful of each tested organism was inoculated in Muller-Hinton broth and adjusted with 0.5 Macferland standard tube then swabbed on the surface of Muller-Hinton agar plates. Antibiotic discs are placed on the surface of inoculated Muller-Hinton plates 2 cm apart, and then incubated at 37 °C for 24 hours.

The antimicrobial resistance pattern of recovered *P. aeruginosa* isolates and the multiple antibiotic resistant (MAR) index.

According to ***(Magiorakos et al., 2012),*** bacteria are identified as MDR when non-susceptible to at least one agent in three or more antimicrobial categories, while bacteria are considered as XDR when non-susceptible to at least one agent in all but two or fewer antimicrobial categories. Bacteria that show resistance to all examined antimicrobial agents are identified as Pan drug resistant (PDR). The MAR index is calculated with the formula (a/b), where ‘a’ represents the antibiotics that were resistant in examined isolates, while ‘b’ represents the total used antibiotics (***Rasmussen-Ivey et al., 2016)***.

Results

A total of 20 isolates showing the character of *P. aeruginosa* were recovered from 200 examined samples, as shown in **Table 1**.

Results of *Pseudomonas* isolation

Cultural characters of *P. aeruginosa:*

Pseudomonas cultures are characterized by non-lactose-fermenting colonies on MacConkey's agar and brown or blue-green colonies on pseudomonas agar after overnight incubation at 37 ºC.

Detection of hemolytic activities in the isolates:

A clear, colourless zone that appears around the colonies is produced on 5% sheep Tryptose Blood Agar (**Beta Hemolysis**).

Identification of *Pseudomonas* isolates

A) Gram′s stain

Gram′s stained fixed film of Pseudomonas spp. revealed gram-negative coccobacilli with the X100

magnification power of an ordinary microscope.

B) Motility Test

Isolates showed extension and diffusion of the growth towards the sides, and the bottom of the inoculated tubes were recorded as motile bacteria.

C) Biochemical tests

Pseudomonas aeruginosa gave positive reactions for oxidase, catalase tests, citrate utilization, urea hydrolysis, and nitrate reduction, while giving negative reactions for H2S production, Vogues-Proskauer, Methyl Red, and Indole tests.

Antimicrobial sensitivity test and MAR index results

As shown in **Table 2**, the isolates were sensitive to amikacin (100%), colistin sulphate (95%), norfloxacin (70%), ciprofloxacin (60%), florfenicol (15%), doxycycline, neomycin, and tetracycline (5%). There was intermediate sensitivity to apramycin (45%), ciprofloxacin and neomycin (25%), erythromycin, florfenicol, and norfloxacin (10%), and cefotaxime (5%). While all tested isolates showed complete resistance (100%) to amoxicillin, ampicillin, cephradin, fosfomycin, sulfamethoxazole trimethoprim, kanamycin, spiramycin, streptomycin followed by 95% resistant to cefotaxime, doxycycline, gentamycin and tetracycline, then 90 %, 75%, 70%, 55%, 20%, 15% and 5% resistant to erythromycin, florfenicol, neomycin, apramycin, norfloxacin, ciprofloxacin and colistin sulphate respectively. All tested *P. aeruginosa* isolates were extensively drug resistant (XDR), with the MAR index ranging between 0.5 and 0.9.

Table (1): Prevalence of *pseudomonas* isolated from examined samples

Sample Type	Samples NO.	Isolates NO.	Isolates (%)
Broiler Chickens	200	20	10%

NO. = Number

Table (2): *Results of Antimicrobial Sensitivity of 20 isolated Pseudomonas aeruginosa*

Antimicrobial agent Classes	Antimicrobial agents	Disc Conc	Antimicrobial sensitivity					
			Resistant		Intermediate		Sensitive	
			NO.	%	NO.	%	NO.	%
Aminocyclitol	Apramycin (APRA)	15 µg	11	55	9	45	0	0
Aminoglycosides	Amikacin (AK)	30 µg	0	0	0	0	20	100
	Gentamycin (CN)	10 µg	19	95	1	5	0	0
	Kanamycin (K)	30 µg	20	100	0	0	0	0
	Neomycin (N)	30 µg	14	70	5	25	1	5
	Streptomycin (S)	10 µg	20	100	0	0	0	0
Cephalosporin 1st generation 3rd generation	Cephradin (CE)	30 µg	20	100	0	0	0	0
	Cefotaxime (CTX)	30 µg	19	95	1	5	0	0
Fluoroquinolones 2nd **generation**	Ciprofloxacin (CIP)	5 µg	3	15	5	25	12	60
	Norfloxacin (NOR)	10 µg	4	20	2	10	14	70
Macrolides	Erythromycin (E)	15 µg	18	90	2	10	0	0
	Spiramycin (SP)	100 µg	20	100	0	0	0	0
Penicillins Aminopenicillin	Ampicillin (AM)	10 µg	20	100	0	0	0	0
	Amoxicillin (AX)	25 µg	20	100	0	0	0	0
Phenicols	Florfenicol (FFC)	30 µg	15	75	2	10	3	15
Polymyxin B	Colistin sulphat (CT)	10 µg	1	5	0	0	19	95
Sulfonatmides/ Trimethoprin	Sulfamethaxazole trimethoprin (SXT)	25 µg	20	100	0	0	0	0
Tetracyclines	Doxycycline (DO)	30 µg	19	95	0	0	1	5
	Tetracycline (TE)	30 µg	19	95	0	0	1	5
Others	Fosfomycin (FF)	50 µg	20	100	0	0	0	0

Conc. = concentration NO. =Number

Discussion

P. aeruginesa is a significant pathogen of poultry and a zoonotic pathogen causing nosocomial infections in immunized people ***(Elsayed et al., 2016)***.

In this study, the prevalence of *P. aeruginousa* isolation in broiler chickens was 10% **(Table 1).** This results is relatively higher than that of (***Mohamed, 2004)***, ***(Al-Adl, 2014)*** and ***(Khelfa and Morsy, 2015)*** who isolated *P. aeruginosa* from broiler chickens with a prevalence of 3.3%, 4.57% and 4.8% respectively, and lower than that obtained by ***(Hassan, 2013)***, ***(Shukla and Mishra, 2015), (Elghazaly et al., 2017), (El-Demerdash et al., 2020) and (Hassan et al., 2020)*** where *P. aeruginosa* isolated with a percentage of 25.3%, 30%, 26.9%, 20% and 18% respectively from broiler chickens.

All recovered isolates showed the typical colony characteristic of *pseudomonas*, where the isolates are G -ve rods, and appeared as pale colonies on MacConkey's agar, blue-green colonies on Pseudomonas agar. Similar results were noted by ***(Adams and Moss, 2008)*** *and* ***(Shahat et al., 2019).***

All isolates showed the typical biochemical reactions ***(Konemann et al., 1997 and Quinn et al., 2011),*** and beta hemolytic activities on sheep blood agar, which are similar to the results of ***(Farghaly et al., 2017).***

Antimicrobial sensitivity tests against 20 antimicrobial agents (**Table 2)** revealed that the most effective antibiotic was amikacin (100%), followed by colistin sulphate (95%), norfloxacin (70%), and ciprofloxacin (60%). These results are nearly identical to those obtained by ***(Al-Adl, 2014)*** which found that isolates of *P. aeruginosa* were highly susceptible to colistin sulphate (76.5%) and norfloxacin (52.9%), while gentamicin and ciprofloxacin gave 23.5% and 17.6%, respectively. Also, the results are nearly identical ***(Farghaly et al., 2017)*** found that *P. aeruginosa* isolates were highly sensitive to the quinolone group (norfloxacin, ciprofloxacin, and levofloxacin) with the percentages of (80.9%), (76.2%), and (73.8%), respectively. Also, colistin sulphate and gentamicin were shown (76.2%), and streptomycin (66.7%). ***(Tawakol et al., 2018)*** reported that *P. aeruginosa* isolates were highly sensitive
to colistin sulphate and amikacin (90%) for each, and ***(Hassan et al., 2020)*** recorded that the most effective antibiotics were imipenem and colistin (100% for each), and amikacin (92%). Colistin is considered a significant option for treatment of MDR-resistant *P. aeruginosa* infections due to its bactericidal effect ***(Gill et al., 2013)***.

In comparison, the isolates showed low susceptibility to florfenicol (15%), doxycycline, neomycin, and tetracycline (5%). This is in accordance with ***(Badr et al., 2020)*** who recorded that the least sensitivity of *P. aeruginosa* isolates was recorded, for tetracyclines (doxycycline) and phenicoles (florfenicol), (9.375%) for each.

For the resistance, all recovered isolates were completely resistant (100%) to amoxicillin, ampicillin, cephradin, , sulfamethoxazole-tri-methoprim, kanamycin, spiramycin, streptomycin and fosfomycin, followed by (95%) resistant to cefotaxime, doxycycline, gentamycin and tetracycline, then (90 %), (75%), (70%), (55%), (20%), (15%) and (5%) resistant to erythromycin, florfenicol, neomycin, apramycin, norfloxacin, ciprofloxacin and colistin sulphate respectively, which all posess a significant warning to the public health. The antimicrobial resistance

pattern of the examined isolates revealed that all isolates were XDR with a MAR index ranging between 0.5 and 0.9. These findings go hand to hand with, ***(Badr et al., 2020)*** who mentioned that the MAR index for most isolates of *P. aeruginosa* from poultry farms was > 0.6, indicating discrimination and abuse of antimicrobials in poultry farms.

Antimicrobial resistance is one of the most important problems confronting the world, and it is escalating in developing countries. Therefore, it's important to detect *P. aeruginosa* precisely and quickly and identify its susceptibility pattern; this may avoid useless antibiotic treatment, which presents antibiotic-resistant pathogens ***(Hamisi et al., 2012)***.

Conclusion:

In this study, all isolates showed complete sensitivity to amikacin and only 95% to colistin, so these antibiotics can be recommended in the first line for the treatment of infections due to *P. aeruginosa.* All the isolates were extensively drug resistant, with a MAR index >0.5, which makes *P. aeruginosa* difficult to eliminate from poultry farms. So the strict hygienic measures should be applied inside poultry farms for controlling *P. aeruginosa*.

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التوصيف الظاهري لميكروب السيدوموناس ارجينوزا المقاوم لمضادات الميكروبات على نطاق واسع المعزول من دجاج التسمين من محافظة الشرقية ـ مصر

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الملخص العربي

تسبب بكيتريا السيدوموناس إريجينوزا مشاكل خطيرة في مزارع الدواجن و تعد إحدي مسببات العدوي الإنتهازية للإنسان. تم تجميع 200 عينة من دجاج التسمين و التي تتراوح أعمارها بين (1إلي35 يوم) في محافظة الشرقية والتي تعاني من أعراض تنفسية و إسهالات. تم التجميع لعزل السيدوموناس إرجينوزا والكشف عن حساسيتها لمختلف الأدوية المضادة للميكروبات. وقد تبين بالتحليل البكتيرى تواجد السيدوموناس إريجينوزا بنسبة (10 ٪) من العينات التي تم جمعها. أظهر إختبار الحساسية لمعزولات السيدوموناس إريجينوزا أن أكثر مضادات الميكروبات حساسية هي أميكاسين (100٪)، كبريتات الكوليستين (95٪)، نورفلوكساسين (70٪)، سيبروفلوكساسين (60٪)، فلورفينيكول (15٪)، دوكسي سيكلين، النيومايسين والتتراسيكلين (5٪). من ناحية أخرى كانت جميع المعزولات المختبرة مقاومة (100٪) لأموكسيسيلين، أمبيسيلين، سيفرادين، فوسفومايسين، كاناميسين، سبيرامايسين، سلفاميثوكسازول- تري ميثوبريم، تليها مقاومة 95٪ لسيفوتاكسيم، دوكسي سيكلين، جنتامايسين وتتراسيكلين، ثم 90٪، 75٪، 70٪، 55٪، 20٪، 15٪ و 5٪ مقاومة للإيرثروميسين، الفلورفينيكول، النيومايسين، الأبراميسين، النورفلوكساسين، سيبروفلوكساسين وكبريتات الكوليستين على التوالي.