Virulence, Resistance Genes Detection and Sequencing of *gyrA* Gene of *Pseudomonas aeruginosa* Isolated from Chickens and Human in Egypt

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Abstract: In this study, a total of 200 samples (100 samples from broiler chickens at different ages and 100 samples of human origin) were screened bacteriologically for the occurrence 0f P. aeruginosa. A total of 47 isolates of P.aeruginosa (23.5%) confirmed and were further tested for antimicrobial susceptibility. Theisolates had the highest levels of resistance against cefotaxime (100%), ciprofloxacin (40.4 %), gentamicin (31.9%), ceftazidime (31.9%), amikacine (6.4%), colstinsulphate (2.1%), meropenem (2.1%) and imipenem (0%). 15 strains (4 strains of human origin and 11 strains of chicken origin) were multidrug resistant P.aeruginosa (31.9%). Polymerase chain reaction (PCR) was applied to detect virulence genestoxA (95%), lasB (90%), exoS (70%), exoU (35%) and resistance genes bla_{TEM} (75%) and aac (3)-Ia (100%) of P.aeruginosa isolates. gyrA gene sequencing was applied, Thr-83 → Ile substitution was found in examined ciprofloxacin resistant P. aeruginosa isolates when compared to the P. aeruginosa PAO1 (Accession: AAG06556.1).

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1. Introduction:

Pseudomonas aeruginosa is an opportunistic pathogen because of its innate resistance to many disinfectants and antibiotics. It is also the most common Gram negative bacterium found in nosocomial infections (Devanand and Saxena 2013). It causes a wide range of variety of systemic infections, especially in patients with severe burns, bed ulcers, and in immunosuppressed patients who are suffered from cancer or AIDS (Aloush et al., 2006 and Gad et al., 2007). P. aeruginosainfects birds of any age; young birds are most susceptible, also severely stressed or immunodeficient birds. Concurrent infections with viruses, other bacteria and mycoplasmas, enhance susceptibility to infection (Peterson1975, Randall et al., 1984 and Stipkovitset al., 1993). Virulence of P. aeruginosa controlled by many factors, it has been attributed to cell associated factors like flagellum, alginate (algD), lipopolysaccharide (LPS), pilus and non-pilus adhesions as well as with exoenzymes or secretory virulence factors like elastase B (lasB), pyocyanin, protease, phospholipase (plcH and plcN), exoenzyme U (exoU), exoenzyme S (exoS), exotoxin A, fimbrial biogenesis protein (pilB), hemolysins (rhamnolipids), siderophores and neuraminidase (nan1) (Ra'oof, 2011and RashnoTaeeet al.,2014). P. aeruginosa defined as Multidrug-resistant (MDR) when resist to one anti-microbial agent in three or more anti-pseudomonal anti-microbial classes (fluoroquinolones, carbapenems, aminoglycosides and penicillins /cephalosporins) (Magiorakoset al.,2012). The resistance of MDR P. aeruginosa developed by several mechanisms as multi-drug resistance efflux pumps, production of β- lactamases, biofilm formation and aminoglycoside modifying enzymes (Carmeliet al., 2002).

Therefore, the present study was planned for bacteriological characterization of P. aeruginosa isolated from chickens with different ages and human, also detection of virulence, resistance genes and examine the identity and mutations in gyrA gene codon 83 and 87 in ciprofloxacin resistant strains when compared with the P. aeruginosa PAO1 (Accession: AAG06556.1) in GenBank.

2. Materials and Methods:

2.1. Samples collection

A total of 200 samples were collected from different ages of broiler chickens and human. Among them 100 samples were collected from broiler chickens of different ages and 100 samples from human sources from Tanta cancer center and Tanta university hospitals in Egypt during December 2014 up to April 2017.

All samples were aseptically collected to prevent cross contamination and transferred immediately in ice box to the laboratory.

2.2. Bacteriological examination of P. aeruginosa

The samples were streaked onto selective media (pseudomonas cetrimide agar) and incubated

aerobically for 24 hours at 37°C. The suspected colonies were inoculated for 24 hours at 37°C onto nutrient agar, sheep blood agar and MacConky's agar for purification and pigment production, hemolysis properties and lactose fermentation detection respectively. The characteristic colonies of P. aeruginosa were selected and stained by Gram's stain. Each colony showing typical colonial appearance were subjected to biochemical identification and examined for oxidase reaction, catalase reaction, urea hydrolysis, indole test, MR test, and Simmon's Citrate agar utilization. Vitek2 was used as a confirmatory biochemical test.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed according to the Kirby- Bauer disk diffusion method (Finegold and Martin1982), using Muller Hinton broth, Muller Hinton agar and the following antibiotic discs: cefotaxime (30mg), imipenem (10mg), gentamicin (10mg), amikacine (30mg), colstinsulphate (10mg), meropenem (10mg), ceftazidime (30mg) and ciprofloxacin (5mg) (Oxoid). The results were interpreted according to NCCLS (2007).

P. aeruginosa ATCC9027strain was used as the quality control

2.4. Detection of virulence genes toxA, lasB, exoS, exoU and resistance genes bla$_{TEM}$ and aac (3)-Ia of P. aeruginosa isolates by PCR.

2.4.1. Extraction of DNA according to instructions

DNA was extracted from the isolated P.aeruginosa using QIAamp DNA mini kit. PCR Master Mix and cycling conditions of the primers during PCR was prepared according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit. Oligonucleotide primers used in PCR have specific sequence and amplify a specific product as shown in table (1). DNA samples were amplified in a total of 25 µl as follows: 12.5 µl of Emerald Amp GT PCR Master Mix**,** 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of template DNA. The reaction was performed in a Biometra thermal cycler. Temperature and time conditions of the primers during PCR were applied (table 2). Aliquots of amplified PCR products were electrophoresed in 1.5% agarose gel (ABgene) in 1x TBE buffer at room temperature. For gel analysis, 15 µl of PCR products were loaded in each gel slot. A 100 bp DNA Ladder (QIAGEN Inc, Valencia, CA, USA) was used to determine the fragment sizes. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

2.5. Gene Sequencing

PCR products were purified using QIA quick PCR Product extraction kit (QIAGEN Inc, Valencia, CA, and USA). Big dye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster City, CA) was used for the sequence reaction and then it was purified using Centrisep (spin column). DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan) and a comparative analysis of sequences was performed using CLUSTAL W. multiple sequence alignment program, version 1.83 of Meg Align module of Laser gene DNA Star software.

3. Results:

3.1. Identification of P. aeruginosa

P. aeruginosa on pseudomonas cetrimide agar medium appeared as small and smooth with different blue – green pigment colonies while it showed Beta hemolysis on blood agar medium. On MacConky's agar medium, the colonies were large, pale with greenish coloration. On nutrient broth, greenish yellow pigments were seen.

All isolates showed similar pattern of reaction despite of the source of isolation. oxidase, citrate utilization, urea hydrolysis and catalase tests showed positive results while MR, Indole and H_2S production showed negative results.

3.2. Prevalence of P. aeruginosa

P. aeruginosawas isolated totally in an incidence of 23.5%. Thirty four isolates of P. aeruginosa wereisolated from a total of 100 samples obtained from broiler chickens at different ages with a percentage of 34% and 13 strain from a total of 100 samples obtained from human samples with a percentage of 13%.

3.3. Antimicrobial resistance test for P.aeruginosa

All isolates were resistant to cefotaxime (100%), while ciprofloxacin (40.4 %), gentamicin (31.9%), ceftazidime (31.9%), amikacine (6.4%), colstinsulphate (2.1%), meropenem (2.1%) and imipenem (0%) as shown in Table (3). 15 strains (4 strains of human origin and 11 strains of chicken origin) were multidrug resistant P.aeruginosa in an incidence of 31.9%.

3.4. PCR results

The current results showed that 95% of tested isolates harbored toxA gene, other virulence factors studied in this research, were found as follow lasB (90%), exoS (70%) and exoU (35%) while aac (3)-Ia resistance gene was found in all tested isolates 100% but bla$_{TEM}$ was present in 75% of P. aeruginosa isolates as shown in Table (4).

Incidence of virulence geneslasB, toxA, exoS and exoU in multidrug resistant P.aeruginosawas 100%, 92.9%, 71.4% and 42.9% respectively, while resistance genes aac (3)-Ia was found in all tested isolates (100%) and bla$_{TEM}$ was (85.7%) as shown in Table (5).

3.5. Sequence analysis of gyrAgene of P.aeruginosa

Thr-83 → Ile substitution in gyrA was found in examined ciprofloxacin resistant P. aeruginosa isolates when compared to the P. aeruginosaPAO1 (Accession: AAG06556.1), but no amino acid change was detected in codon 87 (Aspartate) in the examined strains.

Table (1): Oligonucleotide primers sequences (Metabion- Germany).

Target gene	Primers sequences	Amplified segment (bp)	Reference
bla_{TEM}	F: ATCAGCAATAAACCAGC R: CCCCGAAGAACGTTTTC	516	Colom et al., (2003)
ToxA	F:GACAACGCCCTCAGCATCACCAGC R:CGCTGGCCCATTCGCTCCAGCGCT	396	Matar et al., (2002)
ExoS	F: GCGAGGTCAGCAGAGTATCG R: TTCGGCGTCACTGTGGATGC	118	Winstanley et al., (2005)
ExoU	F: CCGTTGTGGTGCCGTTGAAG R: CCAGATGTTCACCGACTCGC	134	
lasB	F: ACAGGTAGAACGCACGGTTG R: GATCGACGTGTCCAAACTCC	1220	Finnan et al., (2004)
aac (3)-Ia	F: TTGATCTTTTCGGTCGTGAGT R: TAAGCCGCGAGAGCGCCAACA	150	Frana et al., (2001)
gyrA	F: AAATCTGCCCGTGTCGTTGGT R: GCCATACCTACTGCGATACC	344	Fàbrega et al., (2009)

Table (2): Cycling conditions of the different primers during PCR

Target gene	Primary den.	Sec. den.	Ann.	Ext.	No. of cycles	Final ext.
bla_{TEM}	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
ToxA	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.
ExoS	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
ExoU	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
lasB	94˚C 5 min.	94˚C 30 sec.	57˚C 50 sec.	72˚C 1 min.	35	72˚C 12 min.
aac (3)-Ia	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
gyrA	94˚C 5 min.	94˚C 30 sec.	58˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.

Table (3): Antimicrobial resistance of P. aeruginosa isolated from chickens and human.

Antibacterial agents		Disk contents (Ug/disk)	No. of resistant strains	%
Amikacin	**AK**	30mg	3	6.4
Gentamycin	**CN**	10mg	15	31.9
Cefotaxime	**CTX**	30mg	47	100
Ceftazidime	**CAZ**	30mg	15	31.9
Colstinsulphate	**CT**	10mg	1	2.1
Ciprofloxacin	**CIP**	5mg	19	40.4
Imipenem	**IPM**	10mg	-	0
Meropenem	**MEM**	10mg	1	2.1

% acc. to number of resistant strains.n= 47

Table (4): Incidence of virulence and resistance genes of P. aeruginosa in both chickens and human.

Type	Gene	Human source Examined n=10		Chicken source Examined n=10		Total n=20	
		No.	**%**	**No.**	**%**	**No.**	**%**
Virulence genes	toxA	10	100	9	90	19	95
	exoU	4	40	3	30	7	35
	exoS	5	50	9	90	14	70
	lasB	8	80	10	100	18	90
Resistance genes	bla_{TEM}	6	60	9	90	15	75
	aac (3)-Ia	10	100	10	100	20	100

Examined numbers were 20 strains (10 strains of chicken origin and 10 strains of human origin) from total 47 strains. % acc. to number of positive results. n=10

Table (5): Incidence of virulence and resistance genes in multidrug resistant P. aeruginosa.

Type	Gene	No. of +ve	%
Virulence genes	toxA	13	92.9
	exoU	6	42.9
	exoS	10	71.4
	lasB	14	100
Resistance genes	bla_{TEM}	12	85.7
	aac (3)-Ia	14	100

Examined numbers were 14 strains from 15 multidrug resistant strains.% acc. to number of positive results. n=14

4. Discussion:

In this study, 34 isolates of P.aeruginosa (34%) was recovered from 100 samples of chickens. This results were more than that obtained by Elsayed, et al., (2016) who reported a total isolation result of P. aeruginosa was 38 /150 (25.3%), Mohamed, et al., (2002), Sidhom (2011), and Swatantra, et al., (2011) who reported an incidence of 26.69 %, 24.93% and 23.33% respectively. Also, clinical isolates were collected from Tanta University Hospitals Egypt and Tanta Cancer Center in this study; they were 13 strains of P. aeruginosa isolated from 100 hospitalized patients clinical samples in an incidence of 13%, this results were similar to that obtained by El-Ghannam (2014) who examined a total of 89 clinical samples including; urine, wound pus, stool, sputum, and blood, From these samples 11 P. aeruginosa isolates were obtained in an incidence of 12.4% and Yousef (2014) who found that the incidence rate was 15.9% (49 P.aeruginosafrom 307 hospitalized). Mostly used anti-pseududomonal agents in Egypt were tested against P. aeruginosa isolates to screen their resistant pattern; all isolates from different clinical sources were resistant to cefotaxime. A comparable level of resistance to cefotaxime was found by Sedighi, et al., (2015) and Norouzi, et al., (2010), while Jabalameli, et al., (2012) mentioned that more than 90% of the isolates were resistant to cefotaxime, also Ameen (2014), Corona-Nakamura, et al., (2001) found the resistance level for cefotaxime was 80%, third generation antibiotic ciprofloxacin resistance rate was 40.4%, this results were more than Elhariri, et al., (2017) results in Egypt who stated that 33.3% of strains were resistant, while Rajat and his co-worker found that (49%) of isolates from clinical sample were resistant to ciprofloxacin in India in 2012, also a study from India reported ciprofloxacin resistance rate was 63.1% by Mohanasoundaram, (2011), but Tam, et al., (2010) in their study reported 100% resistance against ciprofloxacin.31.9% of examined isolates were resistant to gentamycin antibiotic, while Elhariri, et al., (2017) found that gentamycin resistance rate was 28.5% in Egypt, an elevated rate of resistance was found in Bangladesh 93.7% Nasreen, et al., (2015), Two Indian study Rajat, et al., (2012) and Angadi, et al., (2012) showed 63% of P.aeruginosa isolates isolated from clinical samples were resistant to gentamycin. A study performed by Rashid et al., (2007) showed 77.3% resistance to gentamycin in P.aeruginosa isolates. A paper accomplished by Mahmoud, et al., (2013) found 43.9% gentamycin resistant isolates in clinical sample in Egypt. But the resistance rate in our study was higher than that recoded by Hassuna, et al., (2015), who found gentamicin resistance rate 20%. Ceftazidime is the prescribed anti-pseudomonal third generation cephalosporins, ceftazidime resistance rate was 31.9%, these results were less than that obtained by Ameen (2014) who found 42% of examined strains were resistant to ceftazidime and Angadi, et al., (2012) who showed (74.4%) resistance rateto ceftazidime. But the resistance to ceftazidime, in Ansari, et al., (2015) and Tavajjohi and Moniri (2011) study, was 22.03% and 25% respectively. Amikacin showed 6.4% resistance against P. aeruginosa while 9.32% was the resistance rateto amikacin in Ansari, et al., (2015) study but Meenakumari et al. (2011) in their study showed a higher resistance 56.86% to amikacin. No isolate, in Ansari, et al., (2015) study was found resistant to meropenem but in our results the resistance rate was 2.1% in isolated strains. Also Elhariri, et al., (2017) found 14.2% of examined strains were meropenem resistant in Egypt. 2.1% resistance rate to colistinsulphate was found in our study, this similar to that reported by Azimi, et al., (2016) who reported that 2.5% of the isolates were resistant to colistin, where an Indian study of Angadi, et al., (2012) found 15.2% resistance, also 12.5% resistance to colistin was found by Nasreen, et al., (2015). All P. aeruginosa isolates in our study were sensitive to imipenem, which is consistent with other studies carried in Egypt, as lowest resistance rate was recorded for imipenem (0%) by Hassuna, et al., (2015) and Osman, et al., (2012) while Badr et al., (2008) found resistance rate forimipenem was 4.3%. On the other hand, two other studies carried by Zafer et al., (2014) in Cairo, and Diab et al., (2013) found a higher frequency of imipenem resistance; 39.34% and 72% respectively. imipenem seems to be a promising therapy for pseudomonal infection. Multidrug resistant P.aeruginosa in this study was 15 strains (4strains of human origin and 11 strains of chicken origin) 31.9%.

While Ansari, et al., (2015) study showed a 32.6% frequency of MDR P. aeruginosa, also Gill et al. (2011) reported a 22.7% incidence in Islamabad. Another study conducted in Iran by Tavajjohi and Moniri (2011) reported 27.6% prevalence of MDR P. aeruginosa, also Hassuna et al., (2015) revealed that 56% of the isolates were multi-drug resistant, Yousef (2014) reported Twenty eight P. aerauginosa isolates were found to be MDR representing%57.1of the isolated strains and Abd El Hameid (2014) who found that 75.8% of P. aeruginosa isolates were multi-drug resistant.

exoS, exoT, exoU, and exoY are four effect or proteins secreted via the type III secretion system in P. aeruginosa Roy-Burman, et al., (2001). PCR results clearly indicated the presence of each virulence gene among studied strains. exoS was present in 70% of examined strains, while exoU 35% and lasB90%, this is nearly similar with other studies done by Habibi and Honarmand (2015) where the prevalence of exoS, exoU and lasB virulence factors in the P. aeruginosastrains of investigation were 75%, 37.5%, and 87.5% respectively, also Dadmanesh, et al., (2014) who reported that the most commonly detected virulence factors were exoS (73.91%). also Previous study which was conducted in Bulgaria by Mitov, et al., (2010) showed that a total prevalence of lasB, exoS and exoU factors in the clinical isolates of P. aeruginosa were 100%, 62.4%, and 30.2% respectively. 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 Khan and Cerniglia (1994). In this study 95% of tested isolates carry toxA gene, this is similar to Mitchell, et al., (2017) and Elsayed, et al., (2016) studies in which all tested isolates (100%) were positive for toxA, but it decreased to (69.56%) and (35.29%) in Dadmanesh, et al., (2014) and Fazeli and Momtaz (2014) respectively. The β-lactams antibiotics resistance is mainly associated with the production of enzymes called Temoniera (TEM) that are from chromosomal DNA, among 12 isolates of P. aeruginosa 6 strains showed positive results for the presence of bla_{TEM} gene 50%, Merina (2013), also Fazeli and Momtaz (2014) found the bla_{TEM} incidence was (94.11%), While frequency of bla_{TEM} among the isolates in our study was 75%, but Shahcheraghi, et al., (2009), Dadmanesh, et al., (2014), Chen, et al., (2015), Hassuna, et al., (2015), Pragasam, et al., (2016) and Farzali, et al., (2017) reported the incidence of gene encoding resistance against β-lactams antibiotic bla_{TEM} was 9%, 47.82%, 20.5%, 12.5%, 23% and 34.2% respectively. The AAC (3)-I family, of which three variants (IaTenover, et al., (1989), IbSchwocho, et al., (1995) and IcRiccio, et al., (2003)) have been described in P. aeruginosa, is a common determinant of gentamicin resistance in this organism. Frequency of AAC (3)-Ia among all isolates in our study either multidrug resistant or not multidrug resistant P. aeruginosa was 100%.

Fluoroquinolones such as ciprofloxacin and levofloxacin are an important class of antibiotics for the treatment of P. aeruginosa infections; the principle mechanism of fluoroquinolones resistance in P.aeruginosa involves mutations in the genes of DNA gyrase and topoisomerase IVLee, et al., (2005), also they found that a single alteration in gyrA (Thr-83-->Ile) was the most frequently detected. This agree with our results where the two examined strains showed mutation from threonine to isoleucine in codon 83 (Thr—>Ile) of gyrA gene when compared with the corresponding sequences of P. aeruginosa PAO1 (Accession: AAG06556.1) in genbank, while codon 87 had no mutation. This was consistent with the results of other studies as Gorgani, et al., (2009) who revealed that mutations related to antibiotic resistance were detected in codons 83 and 87 of gyrA, Wydmuch, et al., (2005) who reported that among 73 clinical isolates of P. aeruginosa 48 strains were ciprofloxacin susceptible and 25 ciprofloxacin resistant. The most frequently detected mutation in the gyrA gene (16 out of 25 mutants) was the Thr-83 --> Ile substitution. Also Salma, et al., (2013) stated that among 38 isolates, 11 were susceptible, 22 were resistant, and 5 were intermediate-resistant to ciprofloxacin. They found that 19 (50 %) of these strains had a mutation in the gyrA gene (Thr 83 Ile), the ciprofloxacin-sensitive strains had no mutations in the sequence area examined. Also they found that 81.8 % of the isolates that were resistant to ciprofloxacin had a mutation in the gyrA gene. So Thr-83 → Ile was shown to be the chief mechanism of fluoroquinolones resistance. This observation confirmed that the DNA gyrase was the primary target for fluoroquinolone resistance in the clinical isolates of P. aeruginosa (Nouri, et al., 2016).

In conclusion, P.aeruginosa had a high resistance to various antibiotics, antibiotic policies should be formulated to resist and overcome the antibiotic resistance problem. Every effort should be made to prevent the spread of multi-drug resistant strains.

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