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PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF PSEUDOMONAS AERUGINOSA RECOVERED FROM KIDNEY LESIONS OF BROILER CHICKENS.

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ABSTRACT

This work was planned to investigate the prevalence of *P. aeruginosa* in broiler chickens kidneys suffered from naked eye pathological lesions. Out of 240 examined kidneys samples, 25 isolates (10.4%) of *P. aeruginosa* were recovered. Antimicrobial sensitivity pattern against 14 different antimicrobials comprising nine groups proved that isolates were resistant to most of the used antimicrobial agents. Phenotypic detection of extended spectrum beta-lactamases (ESBLs) producing isolates using modified double disk diffusion test revealed that, ten (40%) were ESBLs producers. Genotypically using PCR, three ESBLs genes (TEM, Ctx-M and Shv) and three virulence genes (lasI, Tox A and Plc H) were detected with different percentages. Cinnamon and carvacrol oils were found bactericidal for *P. aeruginosa* at concentrations of 4% and 0.4% respectively.

Key words: *P. aeruginosa*, inflamed kidneys, ESBLS.

INTRODUCTION

Bacterial lesions in kidneys usually developed when bacteria enter the kidney following systemic disease through the renal arteries or the renal portal system or as a result of ascending infection (Schmidt *et al.*, 2015). A wide range of bacteria has been reported to cause bacterial nephritis including Pseudomonas spp, (Al-Hiyali, 2005).

The environment of chickens house is ideal for growth of various microorganisms and thus serves as potential source of infection in chicken farms specially those organisms which are resistant to various antibiotic treatment. *Pseudomonas aeruginosa* considered as a good example of this environment associated infections (Kebede, 2010). *Pseudomonas aeruginosa* is a motile, Gram-negative, oxidase positive, non-spore forming rods with single arrangement or short chains (Elsayed *et al*., 2016). It producesa water soluble green pigment composed of fluorescentand pyocyanin with a specific fruity odor. *P.aeruginosa* considered being an opportunistic pathogen that can cause respiratory infections, septicemia and other forms when affecting birds (Fekadu, 2010). It affects newly hatched chickens drastically causing high mortality and mass death of embryos (Dinev *et al*., 2013) resistance of *P. aeruginosa* to various antimicrobial agents is contribute to impermeability, multi-drug efflux, and a chromosomal AmpC β-lactamase. Noticeable resistance found among Amino-penicillins, third generation Cephalosporins, Monobactams, Carbapenems, aminoglycosides, and Fluoroquinolones. (Odusanya, 2002) and (Ogbulie *et al*., 2008) Outer membrane lipoprotein (*Opr*L) implicated in efflux transport systems and affecting cell permeability (De Vos *et al*., 1997). Numerous virulence factors implicated in pathogenesis of *P. aeruginosa* as pyocyanin, pyoverdin, lipopolysaccharide, proteases, elastase, alkaline haemolysins, phospholipase C, rhamnolipids, biofilm, Pilli, and flagella (Gupta *et al*., 2011). The complex type III secretion system recognized virulence determinant of *P. aeruginosa* capable of injecting proteins and secreted toxins into the host cell (Hauser 2009). Extended spectrum β-lactamases and AmpC β-lactamases are enzymes encoded by the chromosomal and plasmid genes of many bacteria including *p. aeruginosa* and have the ability to hydrolyze beta-lactam antibiotics leading to multiple antibiotic resistances (Black *et al*., 2005; Jacoby, 2009). Some *p. aeruginosa* found to be Co-producers of AmpC together with ESBL making the organisms non-suceptible to β-lactam antibiotics including β-lactam and beta-lactam-inhibitor combinations (Feglo and Opoku 2014). Extended-spectrum β-lactamases (ESBLs) share the ability to hydrolyze third-generation cephalosporins and aztreonam yet are inhibited by clavulanic acid. Typically, they derive

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from genes for CTX-M, TEM, or SHV, OXA, PER-1, VEB-1, BES-1, and Other ESBLs by mutations that alter the amino acid configuration around the active site of these β-lactamases. This extends the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes (Paterson and Bonomo, 2005).

Aim of work:
Characterization of *P. aeruginosa* recovered from kidney lesions of broiler chickens with special reference to ESBLs producing isolates and determination of the antibacterial effect of two selected essential oils namely: carvacrol and cinnamon oil on multi drug resistant isolates.

MATERIALS AND METHODS

1. Samples
Two hundred and forty kidney Samples of broiler chickens [Hubbard and Ross] 3-5 weeks from El-fayoum and Beni-suef governorates. These chickens were suffered from high mortalities respiratory manifestation with or without ruffled feathers and white or greenish white diarrhea was noticed in most of chickens. At P/M examination there was pericarditis in some chickens, air sacculitis in other, most of collected samples were from chickens have congested, pale and enlarged kidneys, also liver was enlarged and congested in many chickens. All samples were collected under complete aseptic conditions and cultivated on laboratory media according to (Quinn *et al*., 2002).

2. Isolation and identification of P. aeruginosa
2.1. Growth Media (Oxoid):
Trypton soya broth and trypton soya agar, MacConkys agar and nutrient agar were used for recovery of *P. aeruginosa* from kidney tissues.

2.2. Biochemical and biological testes:
Pigment production, Oxidase test, Urease test, Indoltest, Citrate utilization test, Methyl red test, Voges–Proskauer test, Esculin hydrolysis, Gelatin liquefaction, Hemolysis on blood agar (β), Growth at 4°C and 42°C and Fermentation tests all were done according to (Quinn *et al*., 2002).

2.3. API 20 NE (bioMérieux, france):
Furthermore, all *P. aeruginosa* isolates were then confirmed using API 20 NE (Bio-Meriux) according to producer instructions.

3. Antimicrobial susceptibility test:
The disk diffusion method was used to determine the susceptibility of *P. aeruginosa* isolates to several antibiotics of veterinary significance according to the standards and interpretative criteria described by (CLSI, 2013). Fourteen different antibiotic disks (Oxoid) with the following concentrations were used: ampicillin (10 µg), amoxicillin (10 µg), amoxicillin–clavulanic acid (20/10 µg) cefotaxime (30 µg) ceftriaxone (30µg), ceftazidime (30µg), aztreonam (30 µg), Doxycycline (30µg), Lincomycin (10 µg), Clindamycin (2µg), Fosfomycin (200 µg), Florfenicol (30 µg), colistinsulphate (10µg), and Apramycin (30 µg), the results were recorded based on CLSI guidelines (CLSI, 213).

4. Detection of ESBL in P. aeruginosa using modified double disk diffusion (Kaur *et al*., 2013):
Bacterial isolates of *P. aeruginosa* showed diameter of less than 27mm for cefotaxime and less than 25mm for ceftriaxone were tested to confirm the ESBLs production using the Modified Double Disc Synergy Test (MDDST).

Culture of the organisms was made on a Mueller-Hinton agar plate, as was recommended by (CLSI, 2013) then a disc of amoxicillin-clavulanate (20/10 µg) was placed in the centre of the plate. Disks of cefotaxime, ceftazidime, aztreonam and ceftriaxone were placed with 20 mm apart center to center around amoxicillin-clavulanic disk and incubated at 37°C for 18 h. ESBLs confirmed when any distortion or increase in the zone towards the disc of amoxicillin-clavulanate was occurred.

5. Molecular Identification
5. 1.Extraction of DNA from P.aeruginosa isolates
The bacteria were grown on *TS-YE* agar plates at 37°C and up to 100 colonies from the plates were combined. The DNA was obtained by suspending colonies of bacteria in 500 µl of PBS, pH 7.2, then washing 3 times in PBS. The cell suspension was centrifuged for 10 min at 14,000 xg the bacterial pellets were resuspended in 400 µ l Tris-EDTA buffer (pH 8.0) and DNA extracted by heat block method at 98 °C for 10 min.

5.2. Multiplex PCR for the detection of ESBL genes in *P.aeruoginosa* isolates
The amplified reactions were performed in 50 ul volumes in micro-amplification tubes (PCR tubes). The reaction mixture consisted of 10 µl (200 ng) of extracted DNA template from bacterial cultures, 5 µl 10X PCR buffer, 0.375 µl $MgCl_2$ (1.5 mM), 1.25 µl dNTPs (250 µM), 0.25 µl (1.25 Unit) Taq DNA polymerase, 0.25 µl (0.5 µM) from each primer pairs and the volume of the reaction mixture was completed to 50 µl using DDW.

PCR amplifications were performed with a thermal cycler adjusted as follows:

Table 1: Cycling conditions of the different primers during PCR.

	Initial Denaturation	Secondary Denaturation	Annealing	Extension	No. of amplification cycles	Final extension
Temperature	95°C	95°C	55°C	72°C	30	72°C
Time	2 min	40 sec	40 sec	2 min		10 min

The PCR products were stored in the thermal cycler at 4°C until used.

5.3. Screening of PCR products by agarose gel electrophoresis:-

Two grams agarose was added to 100 ml Tris Acetate EDTA (TAE) buffer. The agarose was autoclaved for 10 minutes and 0.5 µg/ml ethidium bromide was added and then left to cool to room temperature. The gel tray was tapped and the warm agarose was poured in. The comb was inserted in the agarose which was left to polymerize. After hardening, the tray was untapped, the comb was removed and the gel was applied to the electrophoresis cell. The cell was filled with TAE buffer. 10 µl of each of the PCR product samples were applied to the gel along with 5 µl molecular weight marker after mixing each with 1µl loading buffer on a piece of parafilm. Each mixture was applied to a slot using 10µl micropipette. The electrophoresis cell was covered and the power supply was adjusted at 10 Volt/cm. The gel was taken out from the cell and examined under short wave UV trans illuminator. The gel was photographed in order to obtain a permanent record using digital camera (Acer CR-5130, China).

Table 2: Oligonucleotide primers sequences and size of the PCR-targeted products PCR for *P.aeruoginosa* isolates (ESBL Genes and virulence Genes).

Primer		Sequence	Amplification product	Reference
TEM	F	5- TCCGCTCATGAGACAATAACC-3	296 bp	**(Sturenburg *et al*., 2004)**
	R	5- ATAATACCGCACCACATAGCAG -3		
Ctx-M	F	5- TCTTCCAGAATAAGGAATCCC-3	909 bp	
	R	5- CCGTTTCCGCTATTACAAAC-3		
Shv	F	5- TACCATGAGCGATAACAGCG -3	450 bp	
	R	5-GATTTGCTGATTTCGCTCGG -3		
lasI	F	5-CGTGCTCAAGTGTTCAAGG -3	295bp	**(Sabharwal *et al*., 2014)**
	R	5-TACAGTCGGAAAAGCCCAG -3		
Tox A	F	5-GGAGCGCAACTATCCCACT -3	150 bp	
	R	5- TGGTAGCCGACGAACACATA -3		
Plc H	F	5-GAAGCCATGGGCTACTTCAA -3	307 bp	
	R	5-AGAGTGACGAGGAGCGGTAG -3		

6. Detection of anti-bacterial effect of essential oils (cinnamon and carvacrol) on bacterial isolates using agar dilution method (Koneman *et al*., 1992):

All tested oils were sterilized by filtration with 0.45 µm pore diameter cellulose filter membrane and Tween 20 was added (0.5% v/v) to facilitate the emulsification of the oil.

The oils then mixed with TSA medium at concentrations (0.5, 1, 2, 4 and 5% for cinnamon oil) and (0.01, 0.02, 0.1 and 0.4 for carvacrol oil) and poured in sterile petri dishes.

The standardized bacterial suspensions (adjusted to McFarland standard tube No. 0.5) were swabbed thoroughly on agar plates containing different concentrations of examined oils and incubated at 37º and examined daily for bacterial growth up to three days.

RESULTS

1. Recovery rate of *P.aeruginosa* in bacteriologically examined kidney lesions:

Out of 240 bacteriologically screened pathologically affected kidneys, 25 isolates (10.4%) of *p.aeruginosa* were recovered.

2. Results of Antimicrobial sensitivity of examined isolates:

A strong resistance of *P. aeruginosa* to examined antimicrobials have been noticed, all isolates showed complete resistance (100%) to Amoxicillin (AMX), Ampicillin (AMP), Clindamycin (DA) followed by amoxicillin–clavulanic acid (AMC) (96%). While, resistance to Apramycin and Colistinsulphate (CT) were 92% for each and 84% for Doxycycline (DO), 80% for both Florfenicol (FFC) and Lincomycin (MY), 72% for Fosfomycin (F+T). cephalosporins and Monobactam antibiotics Including Ceftriaxone (CRO) Cefotaxime (CTX) Ceftazidime (CAZ) and aztreonam (ATM) showed resistance of 64%, 60%, 56%, 56% respectively.

3. Results of phenotypic detection of ESBL sprodusing isolates:

All 25 *P. aeruginosa* isolates under study showed either resistance or intermediate susceptibility pattern to one or more of the extended spectrum beta-lactamases were subjected to modified double disk diffusion test to confirm ESBLs production. ESBLs were confirmed phenotypically in 10 (40%) out of 25 isolates of *P. aeruginosa* recovered from inflamed kidneys.

4. Results of molecular characterization of *P.aeruginosa* under study:

Concerning the molecular investigations of ESBLs gens namely (TEM, Ctx-M and Shv) two isolates which phenotypically proved to be ESBLS producer were found negative to the three intended gens. Meanwhile Ctx-M gene was the most prevalent gene with a percentage of 50% and five isolates harbored at least two different ESBLs gens. Regarding virulence gens eight out of ten tested isolates harbored one (50%) or two (50%) of the three intended gens. The most prevalent virulent gene was Plc H (60%).

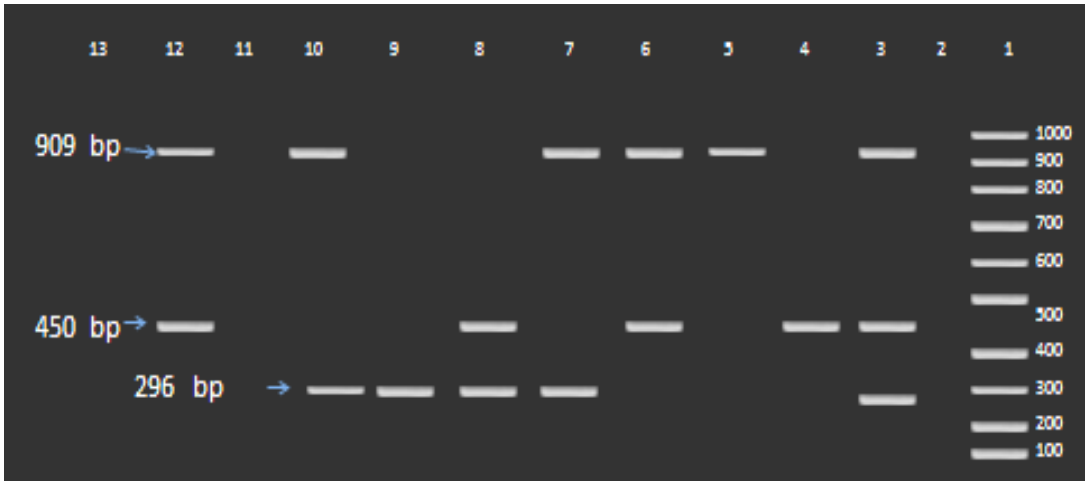

Fig. (1): Gel electrophoresis show positive amplification of *P.aeruginosa isolates* at 296, 909 and 450 bp of *TEM, CTX-M and SHV* respectively by using the specific primers.

Fig. (2): Gel electrophoresis show positive amplification of *P. aeruginosa isolates* at 150, 295 and 307 bp of *Tox A, lasI and Plc H* respectively by using the specific primers.

Table 3: Results of molecular characterization of tested genes.

	Genes	Positive		Negative	
		No.	%	No.	%
ESBL	***TEM***	4	40	6	60
	Ctx-M	5	50	5	50
	Shv	4	40	6	60
virulence	***lasI***	3	30	7	70
	Tox A	3	30	7	70
	Plc H	6	60	4	40

No.: Number of isolates.
%: was calculated according to the total number of the examined cases.

5. Results of antibacterial effect of carvacrol and cinnamon oils in *p.aeruginosa* isolates:
Two essential oils (cinnamon and carvacrol) were tested for their antimicrobial activity against the 25 *p.aeruginosa* isolates. Cinnamon oil completely inhibited the growth of all tested isolates at concentration of 4% while at concentration of 3% all *p.aeruginosa* isolates completely loss their ability to produce pigment and grown as white colonies. carvacrol oil exerted strong bactericidal effect against *p.aeruginosa* at concentration of 0.4% while at 0.2% concentration the isolates lost the ability for pigment production.

DISCUSSION

Pseudomonas aeruginosa is the one which can be cited as a good example of environment associated infection and may cause a serious problem in poultry farms. Generally, it is considered to be an opportunistic organism that produces septicemia and other disease forms when introduced into tissues of susceptible birds, *P. aeruginosa* can be highly virulent causing 50 -100% mortality in experimentally infected 4-week-old chickens. (kebede, 2010). in this work, a total of 25 *p.aeruginosa* isolates out of 240 bacteriologically examined 240 inflamed kidneys were recovered with a percentage of (10.4%). In chickens, *P. aeruginosa* produces dyspenia and cheesy deposits on the serous surfaces lining the air sacs and peritoneal cavity also congestion of internal organs, perihepatitis and pericarditis (Kheir El din *et al.,* 1985). (Shukla and Mishra 2015) induced experimental infection in 7 day old chicks with *p.aeruginosa* intra muscularly and found that the liver, lung and kidneys were highly congested with necrotic foci on the liver and visceral organs. Results of Antimicrobial sensitivity of examined isolates revealed that all isolates of *P. aeruginosa* were highly resistant to Amoxicillin, Ampicillin, Clindamycin and amoxicillin–clavulanic acid followed by Apramycin and Colistinsulphate. The recorded resistance to Cephalosporins and Monobactam antibiotics Including (Ceftriaxone, Cefotaxime, Ceftazidime, and aztreonam) were (64%, 60%, 56% and 56%) respectively. (Nikaido 1994 and Odusanya 2002), concluded that P.*aeruginosa* was resistant to various antimicrobial agents as α-carboxy- and Amino-penicillins, third and fourth-generation Cephalosporins, Monobactams, Fluoroquinolones, Carbapenems, and aminoglycosides might be due to up-regulation of efflux or down regulation of permeability also may due to hyper production of the chromosomal AmpC β-lactamase.

The cephalosporins had been developed in response to the increased prevalence of β-lactamases in certain organisms as *E.coli* and *K.pneumoniae* and the spread of these β-lactamases into new hosts.Using modified double disk diffusion test to confirm ESBLs production, ten (40%) of the tested isolates were confirmed phenotypically to be ESBLs producers. The molecularly examined 10 phenotypically ESBLs producing isolates for (TEM, Ctx-M and Shv), 8 isolates only were found to harbored one or more of the investigated gens Ctx-M gene was the most prevalent gene with a percentage of 50% and five isolates harbored at least two different ESBLs gens. ESBLs derive from genes for TEM-1, TEM-2, or SHV-1 and others by mutations that alter the amino acid configuration around the active site of these β-lactamases.

This extends the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes (Paterson and Bonomo 2005). The ESBLs are frequently plasmid encoded. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes. The virulence associated genes were lasI, Tox A and Plc H with the prevalence of 30%, 30% and 60% respectively. The Exotoxin A is produced by most of *P. aeruginosa* strains It can inhibit biosynthesis of eukaryotic protein at the level of polypeptide chain elongation (Khanand Cerniglia, 1994).

Cinnamon oil completely inhibited the growth of *p.aeruginosa* isolates at concentration of 4% and inhibited the exo-pigments production at 3% concentration. On the other hand carvacrol oil exhibited bactericidal effect at concentration of 0.4% and inhibited the exo-pigment production at 0.2% concentration. Essential oils (EOs) and other extracts of plants have evoked interest owing to other potential uses as alternative remedies for the treatment of many infectious diseases. Some of these Eos show inhibitory effect against multidrug resistant bacteria. Essential oils with its anti-quorum sensing activity that might be important to reduce virulence and pathogenicity of drug resistant bacteria (olveira and cunha, 2008). Cinnamon and carvacrol are phenolic compounds with both hydrophobic and hydrophilic properties, interacts with the lipid bilayer of the bacterial cytoplasmic membrane causing loss of its integrity (zhu *et al.*, 2016).

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التوصيف الشكلي والجيني للسودوموناس ايروجينوزا المعزولة من الكُلى المصابة في بدارى التسمين

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يهدف هذا العمل الى معرفة مدى تواجد بكتريا السودوموناس ايروجينوزا في كُلى بدارى التسمين التي تعاني من اعراض باثولوجية بالعين المجردة. تم عزل البكتريا من عدد ٢٥ عينة كُلى من اصل ٢٤٠ عينة فُحصت بكتريولوجياً بنسبة عزل ١٠.٤ % . بأجراء اختبار الحساسية للمضادات الميكروبية بأستخدام ١٤ نوع مختلف من المضادات الميكروبية والتي تُمثل ٩ مجموعات مختلفة ، ثبُت أن البكتريا مقاومة لمعظم المضادات الميكروبية المُستخدمة. بالفحص الشكلي لمعرفة مدى احتواء المعزولات البكتيريا على انزيمات البيتالاكتاميز طويل المدى باستخدام اختبار الحساسية على الاجار اعطت ١٠ عترات من البكتريا نتائج ايجابية وبالفحص الجزيئي ثبُت وجود ٣ جينات بيتالاكتاميز طويل المدى وثلاث جينات ضراوة بنسب مختلفة في المعزولات التي تم فحصها. زيت القرفه بتركيز ٤% وزيت الكارفاكرول بتركيز ٠.٤% ادى الى تثبيط كامل لنمو البكتريا بأستخدام المثتنت البكتيري.