Down Regulation of Biofilm and Quorum Sensing Genes of *Pseudomonas aeruginosa* and *Pasteurella multocida* Isolated from Broiler Chicken Pericarditis Lesions by the Action of Some Essential Oils

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ABSTRACT

Pseudomonas and *Pasteurella* species have variant of virulence factors as biofilm genetic elements that form a thick coat surface surround pathogen and prevent its phagocytosis; also, quorum sensing genes which responsible for cell-to-cell communication and its inhibition are considered a new approach of antimicrobial therapy. The present investigation successes to identify of *P. aeruginosa* (85.2%) and *P. multocida* (41.8%) from broiler chickens pericarditis lesions by using Vitek2 compact system. Disc diffusion antibiogram analysis showed that *P. aeruginosa* were complete resistant to amoxicillin, kanamycin and lincomycin, while *P. multocida* show complete resistance to amoxicillin, doxycycline, gentamycin, florfenicol and lincomycin 30% of the tested isolates for biofilm formation by Congo red assay showed positive results. Down regulation of biofilm genes *pslA*, *tadG* and quorum sensing genes *lasI*, *luxS* in *P. aeruginosa* and *P. multocida*, respectively after exposure to 0.01% concentration of cinnamon and clove oils were detected by using RT-PCR.

Key words: *P. aeruginosa*, *P. multocida*, Biofilm, Quorum sensing, Virulence, Antimicrobial resistance, RT-PCR, Essential oils.

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INTRODUCTION

Biofilm is a complex mixture of bacterial and host cells, exopolysaccharide, nucleic acids, trapped nutrients in water, and proteins at which the bacteria are protected from adverse conditions in the surrounding environment (Costerton et al. 1995). Pericarditis involved in many diseases that affect poultry including Gram-negative oxidase positive and oxidase negative bacteria. *Pseudomonas* infections of birds are of great importance due to its high morbidity and mortality rate in all ages poultry flocks (Borlaug and Reddy 2019). *P. aeruginosa* consider as the most predominant species causing mortality among chickens. *P. aeruginosa* is G-ve, aerobic, motile, non-capsulated and non-spore forming bacteria (Zhang et al. 2017). *P. aeruginosa* infections are difficult to treat due to presence of intrinsic and acquired antibiotic resistance mechanisms (Lister et al. 2009), also due to impermeability, multi-drug efflux, numbers of extracellular virulence factors and cellular components and a chromosomal AmpC β -lactamase (Odumosu et al. 2016).

However, *Pasteurella* is a part of upper respiratory tract normal flora of many animal species but is also a

potential veterinary pathogen (Ewers et al. 2006), with major economic importance due to its high mortality (Glisson et al. 2008), as it is the causative agent fowl cholera in birds (Hatfaludi et al. 2012). The critical virulence factor of *P. multocida* is polysaccharide capsule that allows the bacteria to evade host immune defense mechanisms and play an important role in *P. multocida* pathogenesis (Chung et al. 2001; Boyce and Adler 2000). *P. multocida* are grouped into 5 capsular serogroups (from serogroups A to F) and 16 somatic serotypes (serotypes 1 to 16) based on capsule and LPS, respectively (Carter 1952; Heddleston et al. 1972). The antibiotic resistant pathogen can be transmitted from poultry to humans through the food chain with serious problems on public health (Apata 2009; McDermott et al. 2002). This, therefore, necessitates a need for better antimicrobial drugs.

Plants have been studied as one of the sources that possess antimicrobial traits which are chiefly synthesized during metabolism as a secondary product (Kokoska, 2002; Rusenova, 2009). Plant antimicrobial compounds have potent therapeutic values as they can serve the functions without any side effects (El-Mahmood 2008; Gous 2010).

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Essential oils are extracted from plants by different methods as steam distillation, hydrodistillation, or solvent extraction which all can create a concentrate of aromatic and volatile therapeutic molecules (Raut and Karuppayil 2014). Essential oils of aromatic that have phenolic groups as the oils of cinnamon, thyme, and rosemary are the most effective against foodborne microorganisms (Rezaei et al. 2010).

Essential oils have potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* and a variety of other bacteria (Beg and Ahmad 2000; Aqil et al. 2005) such as *Escherichia coli*, *Klebsiella pneumoniae* (Djeussi et al. 2013), *Pseudomonas aeruginosa*, *Proteus mirabilis* (Panghal et al. 2011). Recently essential oils antimicrobial chemotherapy act as anti-QS compounds (Rasmussen and Givskov 2006; Roy et al. 2011) which inhibit a wide variety of prokaryotic phenotypes including biofilm, virulence expression, and disease occurrence (Antunes et al. 2010; Li and Tian 2012), so the present study aimed to control biofilm formation and quorum sensing ability of *Pseudomonas aeruginosa* and *Pasteurella* species isolated from cases of broiler chicken pericarditis by using some essential oils.

MATERIALS AND METHODS

Sample Collection

Samples were collected from 150 broiler chickens of different ages (3-5 weeks) and from different Egyptian farms. These chickens were suffering from high mortalities and respiratory manifestations, so the chickens were subjected to postmortem examination for detection of the affected tissues and pericarditis lesions. All samples were given a serial numbers and detailed information (Quinn et al. 2011).

Isolation and Identification of *Pseudomonas* and *Pasteurella* spp.

Inoculation the samples into tryptone soya broth and aerobically incubated at 37°C for 24 hrs. Then, a loopful of this culture was streaked onto tryptone soya agar and cetramide agar. Colonies grown on cetramide agar were picked up and considered presumptively *Pseudomonas*, while colonies grown on TSA were picked up and examined for oxidase enzyme test. Pure cultures of *Pseudomonas* and *Pasteurella* suspected isolates were identified biochemically using Vitek2 compact system following the procedures of kit manual (Quinn et al. 2011).

Antimicrobial Susceptibility and Biofilm Formation Assay

The tested isolates were sub-cultured into Muller – Hinton broth and incubated at 37°C for 18 hours. The standardized culture was swabbed thoroughly on completely dried Muller Hinton agar plates then dried for 5-10 minutes at 37°C then the antibiotic discs were placed aseptically; incubated over night at 37°C then examined by measuring zone of inhibition produced by diffusion of antibacterial agent from the discs into surrounding medium. The recovered isolates were examined for the antimicrobial sensitivity using disk diffusion according to (CLSI 2018). The Multi drug resistant isolates were selected for detection of biofilm formation using YESCA-CR medium according to (Zhou et al. 2013).

Antibacterial Activity of Some Essential Oils

The antibacterial activity of some essential oils as; cinnamon, clove, peppermint, black pepper, ginger, oregano and capsicum oil” against MDR selected isolates was tested. Briefly, the tested bacteria were propagated on tryptone soya agar at 37°C for 24hr, then cells were suspended in physiological saline, and the suspension was adjusted to 1.5×10^8 cfu/ml. Tryptone soya agar was prepared and autoclaved then the tested oils were mixed with TSA according to the concentration. The oil-agar medium was then poured into sterile petri dishes and was solidified. Bacterial suspensions were swabbed and speared onto the agar plates. The plates were then incubated at 37°C for 36 hrs (Jeff-Agboola et al. 2012).

Real-Time PCR Screening of Biofilm and Quorum Sensing Genes Expression after Exposure to Some Essential Oils

Enzymatic lysis procedure of QIA amp RNeasy Mini kit (Qiagen, Germany, GmbH) used for RNA extraction according to (Yuan et al. 2006). PCR reaction was applied in a Stratagene MX3005P real-time PCR machine where the specific primers (Table 1) for *P. aeruginosa*; *16s rDNA* (housekeeping), *pslA* (biofilm), *lasI* (Quorum sensing) according to (Spilker et al. 2004; Ghadaksaz et al. 2015; Bratu et al. 2006) respectively, while for *P. multocida* *kmt1* (housekeeping) and *tadG* (Biofilm) according to (OIE 2012; de Emery et al. 2017) while *luxS* (Quorum sensing) designed in this study, in brief preparation of PCR mix according to quantitect sybr green PCR kit as the following : 2x QuantiTect SYBR Green PCR Master Mix 12.5µl, Reverse transcriptase 0.25µl, each primers 0. 5µl, RNase Free Water 8.25µl, Template RNA 3µl.

RESULTS

Occurrence of *Pseudomonas* and *Pasteurella* in Broilers Pericarditis Lesions

The obtained results revealed that the recovery rate of oxidase positive bacteria from broiler chicken pericarditis lesions was 39.6% (n=55/150), while the biochemical Vitek2 compact system for these isolates showed that the occurrence rate of *Pseudomonas* and *Pasteurella* species was 58.2% (n=32/55) and 41.8%: (n=23/55) respectively. The most common isolated *pseudomonas* species were *P. aeruginosa* (n=29/32) followed by *P. putida* (n=3/32), in the other hand *Pasteurella* species were *P. multocida* (n=14), *P. trehalosi* (n=6) and *P. avium* (n=3).

Antimicrobial Susceptibility and Biofilm Formation of the Recovered *P. aeruginosa* and *P. multocida*

Antibiogram results in Table 2 recorded that all *P. aeruginosa* isolates were highly resistant to amoxicillin, kanamycin and lincomycin, while sensitivity to amoxicillin+clavulanic acid, doxycycline, trimethoprim-sulfamethoxazole and florfenicol was 93.1%. Sensitivity to gentamycin, cefotaxime and ciprofloxacin was 86.2%. On other hand all *P. multocida* isolates were highly resistant to amoxicillin, doxycycline, gentamycin, florfenicol and lincomycin. Four isolates were sensitive to cefotaxime 28.6%. Two isolates were sensitive to amoxicillin+clavulanic acid, kanamycin and ciprofloxacin 14.3%. One isolate was sensitive to trimethoprim- sulfamethoxazole

Table 1: Oligonucleotide's sequence used for screening of virulence genes in both *P. aeruginosa* and *P. multocida*

Target bacteria	Gene& Function	Primer sequence (5'-3')	References
<i>P. aeruginosa</i>	<i>16s rDNA</i> (Housekeeping)	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCCACCCG	Spilker et al. (2004)
	<i>pslA</i> (Biofilm)	TCCCTACCTCAGCAGCAAGC TGTTGTAGCCGTAGCGTTTCTG	
	<i>lasI</i> (Quorum sensing)	ATGATCGTACAAATTGGTCGGC GTCATGAAACCGCCAGTCG	Ghadaksaz et al. (2015)
	<i>kmt1</i> (Housekeeping)	ATC-CGC-TAT-TTA-CCC-AGT-GG GCT-GTA-AAC-GAA-CTC-GCC-AC	
<i>P. multocida</i>	<i>luxS</i> (Quorum sensing)	GCTTGGTTAGCCTCAATGCG ATTACACTCACACCACGCT	Bratu et al. (2006)
	<i>tadG</i> (Biofilm)	AACTTGCCCAATTGTTCTCG CCTTCTGGTTGGACTTCTGC	
			OIE (2012)
			Designed in this study
			de Emery et al. (2017)

Table 2: Antibigram of *P. aeruginosa* and *P. multocida* recovered from examined samples

Chemotherapeutic Discs	Conc. (µg)	<i>P. aeruginosa</i> (n=29)				<i>P. multocida</i> (n=14)			
		Resistant		Sensitive		Resistant		Sensitive	
		N	%	n	%	n	%	n	%
Amoxicillin	10	29	100	0	0	14	100	0	0
Amoxicillin+Clavulanic acid	30	27	93.1	2	6.9	12	85.7	2	14.3
Doxycycline	30	27	93.1	2	6.9	14	100	0	0
Kanamycin	30	29	100	0	0	12	85.7	2	14.3
Gentamycin	10	25	86.2	4	13.8	14	100	0	0
Cefotaxime	30	25	86.2	4	13.8	10	71.4	4	28.6
Ciprofloxacin	5	25	86.2	4	13.8	12	85.7	2	15.4
Trimethoprim- sulfamethoxazole	25	27	93.1	2	6.9	13	92.9	1	7.1
Florfenicol	30	27	93.1	2	6.9	14	100	0	0
Lincomycin	2	29	100	0	0	14	100	0	0

Table 3: Results of the prevalence of some virulence in *P. aeruginosa* using RT-PCR before and after exposure to 0.01% concentration of cinnamon and clove oils.

<i>P. aeruginosa</i> Sample No.	<i>16S rDNA</i>		<i>pslA</i>		<i>lasI</i>	
	CT	CT	Fold change	CT	Fold change	
P	19.28	20.06	-	19.75		
P1	19.46	22.80	0.1696	23.02	0.1174	
P2	20.11	22.07	0.4414	22.90	0.2003	
PS	19.33	20.35	-	20.25	-	
PS1	19.52	23.14	0.1649	22.18	0.2994	
PS2	19.97	22.59	0.3299	22.10	0.4323	

P: *P. aeruginosa* isolate No. 1 (control); Ps: *P. aeruginosa* isolate No. 2 (control); 1: After exposure to 0.01% concentration of cinnamon oil; 2: After exposure to 0.01% concentration of clove oil.

Table 4: Results of the prevalence of some virulence in *P. multocida* using RT-PCR before and after exposure to 0.01% concentration of cinnamon and clove oil

<i>P. multocida</i> Sample No.	<i>Kmt1</i>		<i>luxS</i>		<i>tadG</i>	
	CT	CT	Fold change	CT	Fold change	
A	21.17	22.08	-	21.64	-	
A1	20.53	22.97	0.3463	23.12	0.2300	
A2	20.70	22.60	0.5035	22.47	0.4061	

A: *P. multocida*(control); 1: After exposure to 0.01% concentration of cinnamon oil; 2: After exposure to 0.01% concentration of clove oil.

7%. MDR isolates screened by CR assay for detection of biofilm formation at which CR positive isolates represent 30%.

Antibacterial Activity of Some Essential Oils against MDR Recovered Isolates

MDR isolates were selected to detect effect of some essential oils as; cinnamon, clove, peppermint, black pepper, ginger, oregano and capsicum oil, the results showed that all examined isolates grown when exposed to black pepper, peppermint, capsaicin, and ginger oils in both concentrations 0.1% and 0.01%. On other hand oregano, cinnamon, and clove oils inhibited growth of all isolates at concentration of 0.1%.

Down Regulation of Biofilm and Quorum Sensing Genes of *P. aeruginosa* and *P. multocida*

The recovered *P. aeruginosa* and *P. multocida* isolates were proved to be pathogenic as RT-PCR was able to detect specific virulence genes related to different virulence determinants; biofilm (*P. aeruginosa pslA* gene, *P. multocida tadG*) and quorum sensing (*P. aeruginosa lasI* gene, *P. multocida luxS* gene). Table 3 and Fig. 1-3 discussed the down regulation in fold change for *pslA* gene of *P. aeruginosa* was 0.1696 after exposure to 0.01% concentration of cinnamon oil, while were 0.4414 when exposure to 0.01% concentration of clove oil. On other hand for *P. multocida tadG* down regulation as showed in Table 4 and Fig. 4-6 was 0.2300 and 0.4061 with 0.01% cinnamon and clove oil treatment, respectively.

Results of some virulence genes in *P. aeruginosa* isolate using RT-PCR before and after exposure to 0.01% concentration of cinnamon and clove oils are shown in Fig. 1 to Fig. 3.

Fig. 1: Down regulation in 16s rRNA.

Fig. 2: Down regulation in *pslA*.

Fig. 3: Down regulation in *lasI*.

Results of some virulence genes in *P. multocida* isolate using RT-PCR before and after exposure to 0.01% concentration of cinnamon and clove oils are shown in Fig. 4 to Fig. 6.

Fig. 4: Down regulation in *Kmt1*.

Fig. 5: Down regulation in *luxS*.

Fig. 6: Down regulation in *tadG*.

DISCUSSION

Pasteurella species exhibit usually a widespread host specificity covering mammals and birds. The species with the broadest host spectrum is *P. multocida*. Fowl cholera, an acute to chronic disease of wild birds but especially of domestic fowl leads to high economic loss in breeding farms for turkeys and chicken (Hinz and Lüders 1991). *P. multocida* virulence arms include fimbriae, adherence, colonization factors, iron-regulated and acquisition proteins, extracellular enzymes such as neuraminidase, hyaluronidase and a variety of outer-membrane proteins (Tang et al. 2009).

P. aeruginosa and *P. multocida* are of major economic importance due to their high mortality rate and high economic loss (Glisson et al. 2008; Zhang et al. 2017). *P. aeruginosa* has numbers of extracellular virulence factors and cellular components which make its infections are difficult to treat due to its ability to resist many structurally unrelated antibiotics (Lister et al. 2009; Habeeb et al. 2012; Kebede 2010). The present survey recorded that the most common isolated *pseudomonas* species were *P. aeruginosa* 90% followed by *P. putida* 9%, in the other hand *Pasteurella* species were *P. multocida* 61%, *P. trehalosi* 26% and *P. avium* 13%.

Antibiogram results of the present study recorded that there were high resistant to resistant to amoxicillin, kanamycin and lincomycin among *P. aeruginosa* isolates, while sensitivity to amoxicillin clavulante, doxycycline, trimethoprim- sulfamethoxazole and florfenicol was 93.1%, this results were in accordance with those of recent studies (Elsayed et al. 2016) which record the antibiogram for *P. aeruginosa* isolates at which complete resistance (100%) to amoxicillin, While, resistance to cotrimoxazole, ceftriaxone, ofloxacin showed 90, 80 and 30%, respectively. Besides, no resistance to ciprofloxacin and gentamycin, another study showed that the majority of *P. aeruginosa* isolates were completely resistant to ceftazidime. The susceptibilities of the isolates to cefepime, ciprofloxacin and amikacin were 98, 96 and 88%, respectively (Odumosu et al. 2016). While Li et al. (2018) revealed that *P. multocida* isolates showed resistance rates to amoxicillin, sulfamethazine, tetracycline, doxycycline, florfenicol and streptomycin were 82.2, 71.1, 53.3, 48.9, 46.7 and 44.4%, respectively.

In the present study MDR isolates screened by CR assay for detection of biofilm formation at which CR positive isolates represent 30%, the biofilm process acquire the organisms a thick coat of extracellular polysaccharide which resistant to antibiotics as this coat protects the

pathogen from phagocytosis (Davies 2003), so in the present investigation MDR isolates were selected to detect effect of some essential oils as; cinnamon, clove, peppermint, black pepper, ginger, oregano and capsicum oil, the results in Table 5 showed that all examined isolates grown when exposed to black pepper, peppermint, capsaicin, and ginger oils in both concentrations 0.1% and 0.01%. On other hand oregano, cinnamon, and clove oils inhibited growth of all isolates at concentration of 0.1%, in contrast Aqil et al. (2005) found that plant extracts such as garlic, cinnamon, tulsi, ginger, turmeric, lemon, neem, yucca, thyme and rosemary have antibacterial activity against *S. aureus* and a variety of other bacteria. The antibacterial role of cinnamon and clove oil on different bacteria was previously studied by many authors who proved its powerful effect on *E. coli*, *S. aureus* and *P. aeruginosa* (Nuñez and Aquino 2012) and other respiratory bacterial agents (Erfan and Marouf 2019).

Quorum sensing inhibition is considered as a novel methods of antimicrobial therapy as anti-QS compounds target genes that are essential for basic metabolism in vitro and the microorganisms itself (Roy et al. 2011; Rasmussen and Givskov 2006). In the current study down regulation of *lasI* gene for *P. aeruginosa* was 0.1174 when exposure to 0.01% concentration of cinnamon oil, while was 0.2003 after treatment with 0.01 clove oil. However, *P. multocida* down regulation of *luxS* was 0.3463 when exposure to 0.01% concentration of cinnamon oil and was 0.5035 after treatment with 0.01% clove oil.

Conclusion

The obtained results have focus on the possible use of essential oils as cinnamon oil and other essential oils for the control of antibiotic-resistant bacterial pathogens isolated from pericarditis lesions in Egyptian broiler chicken's farms as it has a positive effect in controlling and down regulation of its virulence factors as biofilm and quorum sensing mechanism.

Author's Contribution

All authors contributed equally to study design methodology, interpretation of results, and writing of the manuscript.

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