Detection of virulence factors of *Pseudomonas aeruginosa* in different animals by using bacteriological and molecular methods

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Abstract

The aim of this study was to detect the presence of virulence factors of *Pseudomonas aeruginosa* in different animals. For this purpose 120 samples were collected and examined to detect fourteen virulence factors by using biochemical and molecular methods. The results showed that the highest isolation rate was recorded in doges (29.6%) among studied animals, and highest isolation rate was recorded in milk samples (26.8%) among the studied samples. The virulence factors were detected in different ratio, and highest of them were capsule detected in 50% from skin isolates, amylase enzyme detected in 28.5% from milk isolates, hemolysin enzyme detected in 75% from wound isolates, protease detected in 100% from skin isolates, phospholipase enzyme detected in 56.1% from milk isolates, urease enzyme detected in 50% from skin isolates, gelatin liquefaction detected in 100% from skin and ear isolates, β -lactamase production detected in 100% from skin and wound isolates, pigments production detected in 100% from skin and ear isolates, *oprI, oprL* and *exoT* detected in 100% from skin and wound isolates, exoS detected in 100% and 85.7% from skin and milk isolates respectively. We conclude from his study that the dogs are more sensitive in compare with studied animal, while the milk sample is more susceptible to contamination by *Pseudomonas aeruginosa.* Regarding the virulence factors we noticed that the appearance of it basis on infection state.

Keywords: *Pseudomonas aeruginosa*, virulence factors, molecular methods
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الكشف عن عوامل الضراوة للزوائف الزنجارية في الحيوانات المختلفة باستخدام الطرق الجرثومية والجزيئية

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الخلاصة

هدفت هذه الدراسة الى تحديد تواجد عوامل الضراوة للزوائف الزنجارية من انواع مختلفة من الحيوانات، ولهذا الغرض جمعت ١٢٠ عينة مختلفة وفحصت لتحدد ١٤ عامل ضراوة باستخدام طرق جرثومية وجزيئية. اظهرت النتائج ان اعلى نسبة عزل سجلت في الكلاب (٢٩,٦%)، كما ان اعلى نسبة عزل كانت من عينات الحليب (٢٦,٨%).كما اظهرت الدراسة اختلافا في ظهور عوامل الضراوة باختلاف مصدر العزل، حيث سجلت اعلى نسب لظهور عوامل الضراوة كالاتي: ظهرت المحفظة في ٥٠% في الجراثيم المعزولة من الجلد، كشف انزيم الامايليز بنسبة ٢٨,٥% في الجراثيم المعزولة من الحليب، كشف الانزيم الحال الدموي بنسبة ٧٥% في الجراثيم المعزولة من الجروح، كشف الانزيم المحلل للبروتين بنسبة ١٠٠% في الجراثيم المعزولة من الجلد، كشف الانزيم الحال للدهون الفوسفاتية بنسبة ٥٦,١% في الجراثيم المعزولة من الحليب، كشف الانزيم الحال للبول بنسبة ٥٠% في الجراثيم المعزولة من الجلد، كشف الانزيم المذيب للجيلاتين بنسبة ١٠٠% في الجراثيم المعزولة من الجلد والاذن، كشف انزيم البيتا لاكتيميز بنسبة ١٠٠% في الجراثيم المعزولة من الجلد والجروح، كشف افراز الصبغات بنسبة ١٠٠% في الجراثيم المعزولة من الجلد والجروح، ظهر الجين *oprI, oprL, exoT* في ١٠٠% من

الجراثيم المعزولة من الجلد والجروح، ظهر الجين *exoT* بنسبة ١٠٠% و ٨٥,٧% في الجراثيم المعزولة من الجلد والحليب على التوالي. نستنتج من هذه الدراسة ان الكلاب هي الحيوانات الاكثر حساسية من بين الحيوانات المدروسة، وعينات الحليب هي الاكثر عرضة للتلوث بالزوائف الزنجارية، وان ظهور عوامل الضراوة يعتمد على نوع الاصابة.

Introduction

Pseudomonas aeruginosa is a Gram negative bacteria, non spore forming, obligate aerobes, motile by one or more polar flagella, most isolates are oxidase and catalase positive (1,2). Pyocyanin, pyoverdin, fluorescence, pyorubin and pyomelanin are a pigments produced by *Pseudomonas* spp. (3).

Pseudomonas aeruginosa: is a saprophytic bacteria in soil, water and plants, in addition to its apart of the normal flora in animals and human (4). In animals, it causes mastitis, metritis, pneumonia, dermatitis and enteritis (1).

Pseudomonas aeruginosa possess many virulence factors including: Protease: its two types: Elastase and Alkaline protease; that causes tissue necrosis, distraction of immunoglobulin and inhibition of tumor necrosis factors and gamma interferon (5). Ureases: its play a role in urea hydrolysis and ammonia releasing; these events which lead to increase of urine pH (6). Lipases and phospholipases: distraction of lipids surfactant and phospholipids of host cell membranes (7). Haemolysin: play role in pulmonary infection, its involved two types: Heat-labile (Phospholipase C) and Heat-stable (Glycolipid) (8). Motility : *Pseudomonas aeruginosa* motile by single polar flagellum, the bacterium can adhere to host epithelial cells through the binding of its flagellum to the asialyated glycolipid (9). Pigments: its causes host oxidative stress, damage of host catalase enzyme and mitochondrial electron transport (10). Also so it apple to cause inhibition of chemokine production and intra-phagocytic killing (11). Outer membrane proteins of *Pseudomonas aeruginosa* (*OprI* and *OprL*): play important roles in the interaction of the bacterium with the environment as well as the its inherent resistance to antibiotics, where the consequence of the presence of these specific outer membrane proteins that have been implicated in efflux transport systems that affect cell permeability (12). As these proteins are found only in this organism, they could be a reliable factor for rapid identification of *Pseudomonas aeruginosa* in clinical samples (13). Exoenzyme S: encoded by the *exoS* gene, is an ADP ribosyltransferase that is secreted by a type-III secretion system directly into the cytosol of epithelial cells (14).

Materials and methods

This study was designed in Salahaldeen province on animals with different age, sex and species in period extended from February to May 2017. The samples were collected and transported immediately for bacterial culturing then molecular technique done in Tikrit university, laboratory of Veterinary Medicine College.

Samples

Skin scraps, swabs from wound and ear, and samples from digestive system and milk were collected. The species of animals and number of samples as in table 1.

Bacterial isolation

it was performed by using (Pseudomonas agar base - Himedia), with nalidixic acid (500 mg dissolved in 100 ml of distilled water then add of 3 ml of suspension to each 1L of medium) and tetrazolium bromide (3g dissolved in 100 ml, then add of 10 ml of suspension to each 1 L of medium) (1).

Biochemical tests which include

Oxidase test, catalase test, Urease test, nitrate reduction test, H2 S production test, Lysine decarboxylase test, indole test and Oxidation –fermentation test (1).

Table 1: species of animals and number of samples

Animals species	Samples					
	Skin infection	Wound infection	Ear infection	Digestive system infection	Milk samples (mastitis milk)	Totals
Dogs	4	3	8	12	0	27
Sheep	2	15	2	5	4	28
Goat	3	11	3	1	7	25
Cow	1	3	6	4	8	22
Hours	3	3	8	4	0	18
Total	13	35	27	26	19	120`

Serotyping

By used monoclonal antibodies (Sanofi Diagnostics Pasteur- France) which are anti O- poly saccharide (1P-16p). appearance of agglutination between bacteria and monoclonal antibodies refer to its serotype.

Detection of virulence factors

Capsule detection: by using of Indian ink, Motility activity detected by used of Triple Sugar Iron sugar iron and according to (15). Production of amylase enzyme detected by using of Starch medium and according to (16). Production of hemolysin enzyme detected by using of blood agar. Production of protease enzyme detected by using of skim milk agar and according to (17). Production of phospholipase enzyme detected by using of nutrient agar, Nacl and egg yolk and according to (18). Production of Urease enzyme detected by using of urea media Production of DNAase detected by using of DNA media. Gelatin liquefaction test: by used of gelatin media and according to (15). Detection of β -lactamase production applied according to (19).

DNA extraction

For genetic methods performed by reactivation of *Pseudomonas aeruginosa* by culturing in trypton soya agar at 37c for 24 hours. and bacterial DNA extracted according to methods describe by (20). And by using of Genomic DNA Mini Kit (blood/cultured cell) (Geneaid). (C#:GB100/300) primers used in current study: these primers were chosen depending on (21) as table 2.

Compounds used in preparation of reaction mixture

The component that used in genetic included: Taq PCR Master Mix KIT (20µl), Forward primer (1.4µl), Reverse primer (1.4µl), DNA Template (2µl) and DNA free water (15.2µl).

Thermocycler programs

Thermocycler were set in three steps: First Denaturation step with 95°C for 5mints, Denaturation step with 95°C for 30 second, DNA extension step with 72°C for 30seconds and Primer-annealing step with 55°C, 58°C, 60°C and 55°C for 30seconds for*, oprI, oprL* and *exoT* respectively.

Results

The current study showed difference in isolation rate of *Pseudomonas aeruginosa* according type of samples, the highest isolation rate was recorded in milk and ear samples which are 26.8% and 25.9% respectively and lowest isolation rate in wound infection (11.4%).

The isolation rate differs according to species of animals the higher infection rate recorded in doges was 29.6%. and lowest rate recorded in horses (16.6%). Table 3 describe the infection rate in different samples for each animals spp.

Table 2: primers used in study

Gene name	Gene text	product size	Company
oprI	F *5'CGGCTGGGAGATTGCTGTTA3'* R *5'-CCTT* GCGATAGGCTTCGTCA*3'*	202 pb	(BioRad, USA)
oprL	F *5' GGA ATG AAC GAA GCG TTC TC 3'* R *5' GGT CCA GTA GTA GCG GTT GG 3'*	300 bp	(BioRad, USA)
exoT	F *5'*- AATCGCCGTCCAACTGCATGCG-*3'* R *5'*-TGTTCGCCAGAGGTACTGCTC-*3'*	150 bp	Alpha DNA, Canada
exoS	F *5' CTT GAA GGG ACT CGA CAA GG 3'* R *5' TTC AGG TCC GCG TAG TGA AT 3'*	504 bp	(BioRad, USA)

Table 3: *Pseudomonas aeruginosa* infection ratio in different animals and simples

Animals	Samples											
	Skin infection		Wound infection		Ear infection		Digestive system infection		Milk samples (mastitis milk)		Totals	
	No.	Infection No. and %	No.	Infection No. and %	No.	Infection No. and %	No.	Infectio No. and %	No.	Infection No. and %	No.	Infection No. and %
Doges	4	1 (25.0%)	3	0 (0.0%)	8	5 (62.5%)	12	2 (16.66)	0	0 (0.0%)	27	8 (29.6)
Sheep	2	0 (0.0%)	15	3 (20%)	2	0 (0.0%)	5	1 (20.0%)	4	1 (25.0%)	28	5 (17.8%)
Goats	3	1 (33.3%)	11	1 (9.0%)	3	1 (33.3%)	1	0 (0.0%)	7	3 (42.8%)	25	6 (24%)
Cows	1	0 (0.0%)	3	0 (0.0%)	6	0 (0.0%)	4	1 (25.0%)	8	3 (37.5%)	22	4 (18.1%)
Horse	3	0 (0.0%)	3	0 (0.0%)	8	1 (12.5%)	4	2 (50.0%)	0	0 (0.0%)	18	3 (16.6%)
Total	13	2 (15.38)	35	4 (11.4%)	27	7 (25.9%)	26	6 (23.0%)	19	7 (26.8%)	120	26 (12.6%)

Serotyping of *Pseudomonas aeruginosa*:

Table 4 shows serotypes of *Pseudomonas aeruginosa* that isolated in current study.

Table 4: Serotype of *P. aeruginosa* isolated from different animal

Serotypes	No. of samples	Rate
O3	3	11.5%
O6	5	19.2%
O9	17	65.38%
O11	1	3.8%
Total	26	100%

Virulence factors

The ratio of virulence factors were capsule: 19.2%, motility 100%, amylase enzyme: 100%, hemolysin enzyme: 61.5%, protase:30%, phospholipase enzyme: 34.6%, urease enzyme:19.2%, DNAase: 0%, gelatin l liquefaction test: 92.3%, B-lactamase production: 61.5%, Pigments:88.4%. Table 5 showed virulence factors of *p. aeruginosa* isolated from different animal samples.

Genetic methods used in detection of virulence factors

The *OprI* and *exoT* genes were detected in about 88% of pseudomonas isolates, *exoS* gene detected in 76.9% while *OprL* detected in 69.2% from total number of pseudomonas isolates, as in table 6 and figure 1-4 shows result of PCR test.

Table 5: Virulence factors of *P. aeruginosa* isolated from different animal samples

Virulence factors	Samples types											
	Skin infection (2)		Wound infection (4)		Ear infection (7)		Digestive system infection (6)		Milk samples (mastitis milk) (7)		Totals (26)	
	No	%	No	%	No	%	No	%	No	%	No	%
Capsule	1	50%	0	0%	2	28.5%	1	16.6%	2	28.5%	5	19.2%
Motility	2	100%	4	100%	7	100%	4	%	7	100%	26	100%
amylase enzyme	0	0%	0	0%	0	0%	0	0%	1	14.2%	1	3.8%
hemolysin enzyme	1	50%	3	75%	4	57.1%	4	66.6%	4	57.1%	16	61.5%
Protase	2	100%	1	25%	2	28.5%	1	16.6%	2	28.5%	8	30%
phospholipase enzyme	1	50	1	25%	2	28.5%	1	16.6%	4	56.1%	9	34.6%
Urease enzyme	1	50%	0	0%	1	14.2%	2	33.3%	1	14.2%	5	19.2%
DNAase	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Gelatin l liquefaction test	2	100%	3	75%	7	100%	5	83.3%	7	100%	24	92.3%
β -lactamase production	2	100%	4	100%	5	71.4%	4	66.6%	3	42.8%	16	61.5%
Pigments	2	100%	3	75%	7	100%	5	83.3%	6	85.7	23	88.4%

Table 6: virulence factors of *Pseudomonas aeruginosa* isolated from different animal samples

Virulence factors	Samples											
	Skin infection (2)		Wound infection (4)		Ear infection (7)		Digestive system infection (6)		Milk samples (mastitis milk) (7)		Totals (26)	
	No	%	No	%	No	%	No	%	No	%	No	%
oprI	2	100%	4	100%	7	100	4	66.6%	5	71.4%	23	88.4%
oprL	2	100%	4	100%	6	85.7%	4	66.6%	2	28.5%	18	69.2%
ExoT	2	100%	4	100%	4	57.1%	5	83.3%	6	85.7%	23	88.46%
exoS	2	100%	3	75%	6	85.7%	3	50%	6	85.7%	20	76.9%

Discussion

In this study, *P. aeruginosa* isolated from all animal samples with different levels. This result was in agreement with the study of AL-hadithi (22) who isolated *P. aeruginosa* from different animal samples in Baghdad. The highest isolation rate was recorded in samples of dogs ear and this was in agreement with Doge *et al.* (23).

P. aeruginosa can causes otitis externa in canines it cause inflammation and ulceration within the external ear canal (13).

Figure 1: Agarose gel electrophoresis of PCR products. M: 100 bp DNA ladder, lines (1-8) positive result at 202 bp for *oprI,*of *P. aeruginosa.*

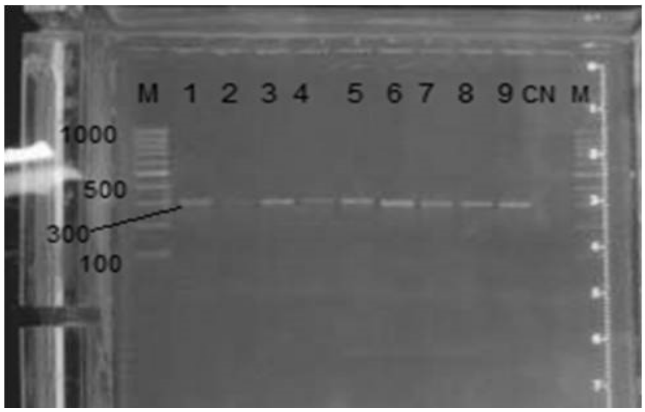

Figure 2: Agarose gel electrophoresis of PCR products. M: 100 bp DNA ladder, lines (1-8) positive result at 300 bp for *oprL,*of *P. aeruginosa.*

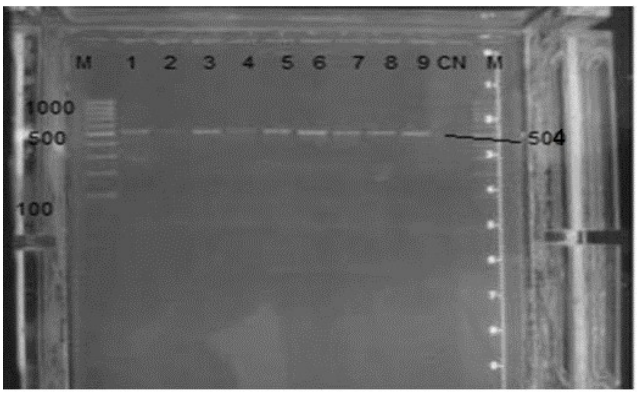

Figure 3: Agarose gel electrophoresis of PCR products. M: 100 bp DNA ladder, lines (1-8) positive result at 505 bp for *exoS,*of *P. aeruginosa.*

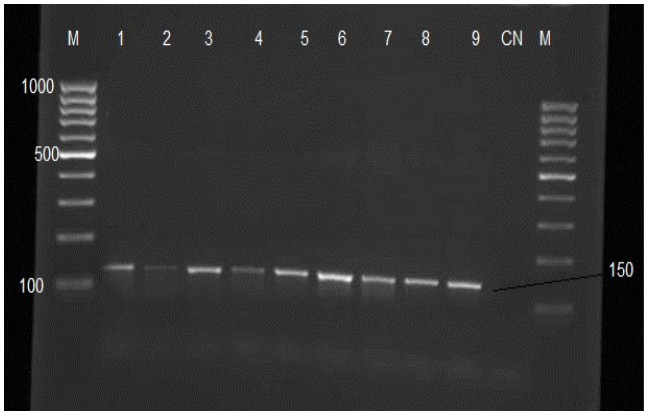

Figure 4: Agarose gel electrophoresis of PCR products. M: 100 bp DNA ladder, lines (1-8) positive result at 150bp for *ExoT,*of *P. aeruginosa.*

The isolation of *P. aeruginosa* from milk in the current study, may be referred to either ability of *P. aeruginosa* to live in wide range of temperature or its one the causes of mastitis also might be due to contamination after milking.

The present study also showed difference in the serotyping of the isolation. The dominance of one serotype in compare with others may be due to geographic distribution, resistance to host immunity and environmental prevalence, and antibiotics resistances (23,24).

Some isolates appeared to have capsule. This result differ from result recorded by Al-Mashhadani (25) were 0%, and result recorded by AL-Salihi and Hasan (26) were 42.8%, that's may be due to different in type of infection. Capsule play important role in prevention of phagocytosis so that its protect bacteria from anther types of immunological invasion (27).

Hemolysin enzyme detection in 61.5%, that agreement with (25). Hemolysin associated with necrotoxicity and cytotoxicity of the cells. It can form pores in the erythrocytes plasma membrane (28). All isolates were motile, DNAase negative, this agreement with (25). Most isolate were positive to pigments production and gelatin liquefaction. and that's was agreement with (25). Pigments play important role in pathogenicity of *P. aeruginosa* and reduce of host immunity (10,11).

Ureases enzyme detected in 19.2% of total isolates, this enzyme able to distract urea into CO_2 and NH_3 and increase pH which enhance bacterial growth (29).

Further more the rsults study showed many bacteria gave positive results in protease detection test. Protease enzyme enhance infection by damage of host cells and break down of immune defense mechanism like skin and mucous membranes (29,30).

In current study extracellular toxin (*exoS* and *exoT*) were detected in most isolates. The extracellular toxin can causes necrosis, edema and hemorrhage (14).

The outer membrane proteins OprI and OprL were detected in different rate according to isolate source. The outer membrane proteins effect in cellular permeability and increase antibiotic resistance (14).

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