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Phylogenic diversity some of the pseudomonas Isolate from wound infection in Babylon province

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***Abstract*---**The current study involved the collection of (75) swabs were collected from different animal wounds distributed Geographically in all parts of Babylon province, for the period from October 2021 to the end of February 2022. The results showed only 42 samples were identical to positively infected in microscopic examination, including 31 (%) samples contained Pseudomonas spp, while 44 samples were not infected with any type of bacterial infection. Bacteria were diagnosed based on a culturing on different agars according to the type of bacteria (Chrom agar), as well as by using VITEK 2 system. Pseudomonas spp. was the most dominant bacteria that isolated in the current study. So the antibiotic susceptibility of the Pseudomonas aeruginosa against 16 antibiotics belonging to 9 classes of antimicrobial agents was tested. The results showed that the resistance percentage of Pseudomonas aeruginosa was (100%) towards Ampicillin, Piperacillin, Amoxicillin, Cefepime, Cefotaxime, Ceftazidime, Aztreonam, trimethoprim and Chloramphenicol. followed by (80%) resistance to Amoxicillin clavulanate and Doxycycline. Also Nitrofurantoin showed (60%) resistance, Levofloxacin and Azithromycin showed (40%) resistance against isolated Pseudomonas . While Meropenem and amikacin have (20%) resistance against isolated Pseudomonas. The results of 16S rRNA revealed the following species Enterobacter, Erwinia.sp, Serratia marcescens, Escherichia coli, Pseudomonas aeruginosa and Pantoea dispersa. Some important virulence factors related genes with their Phylogenetic tree for Pseudomonas aeruginosa also studied , Alginate production, Elastase enzyme, Exoenzyme and Phospholipases of bacteria. The results showed that Pseudomonas aeruginosa isolates have 5(100%) algD gene (responsible for alginate

production), While 10(100%) for lasB gene encoding Elastase enzyme. For Exos gene which responsible for exoenzyme, the results of PCR also showed that all isolates 5(100%) possess this gene. Finally phospholipases encoded gene (plcH) revealed that 10(100%) possess this gene. In conclusions, Pseudomonas which characterized by higher levels of virulence factors compared to other local studies of other areas in Iraq. Meropenem and Amikacin antibiotics had remarkable activity against Pseudomonas.

***Keywords*---**wound, pseudomonas, babylon, PCR, gene.

Introduction

Wound healing is a public health concern (Doaa *et al.,* 2019). The immune system acts as a master key that orchestrates wound healing process via the collaborative efforts of numerous pro-angiogenic cells including fibroblasts, leukocytes, endothelial cells, and epidermal cells (Savari *et al.,* 2019). The progression of wound to an infected state is likely to involve a multitude of microbial or host factors including the type, site, and depth of wound, the extent of nonviable exogenous contamination, the general health and immune status of the host, the microbial load, and the combined virulence expressed by the types of microorganisms involved (Tottoli *et al.,* 2020).Antimicrobial resistance (AMR) has become a growing threat to human life globally, especially in developing countries (CDC, 2019;Sandar *et al.,* 2021). The increasing incidence of AMR has been attributed to patients' behavior of not completing a course of treatment or non-adherence to their treatment schedule; the challenges of supply chain management of antimicrobials in the population, leading to interruptions in treatment; unregulated use of antimicrobials, including their availability over-the-counter, and, most importantly, the irrational prescribing practices of healthcare professionals (Ayukekbong *et al.,* 2017).

In the United States, more than 2.8 million antibiotic-resistant infections occur each year, and more than 35,000 people die as a result (CDC, 2019).The burden of antibiotic-resistant infections across different wound types and care settings has been increasing in these settings (Woodmansey and Roberts, 2018). The predominant bacterial isolates from the infected wounds include *Staphylococcus aureus, Escherichia coli, Proteus* species, *Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumannii* (Janssen *et al.,* 2018). Wound infections with antibiotic-resistant bacteria may lead to higher mortality and prolonged debility of the patient, which may result in a longer hospital stay and increased healthcare costs. The following organisms are commonly associated with wound infection; *Streptococcus pyogenes, Staphylococcus aureus, Pseudomonas aeruginosa., Escherichia coli, Klebsiella species, Proteus* species, *Clostridium* species (Abrahamian and Goldstein, 2011). Molecular biology has proven that culture methodologies can be markedly less sensitive to bacterial detection than DNA detection methodologies (Han *et al.,* 2012). Also, the identification of bacteria is shifting away from the traditional metabolic biochemical testing of cultures and moving towards genetic or proteomic identification of the bacteria (Seng *et al.,* 2010). The advent of molecular microbiology has caused medicine to reexamine

Koch's postulates (Falkow, 2004).

Materials and Methods

- Animal wound Swabbing: Swaps were taken from all animals wounds suspected, which were diagnosed based on clinical signs by veterinary specialist at the Teaching Veterinary Hospital in Babylon, and 11 Veterinary Clinic. Seventy five (75) according to table (3-8), were included and transferred directly to the laboratory.
- Chrom Agar orientation: Selective Chromogenic medium was used for the isolation and differentiation of Pseudomonas, according to (Dortet et al., 2014).
- Staining with Gram Stain: This set was equipped by manufacturing company and used to study morphology and arrangement of bacterial cells (Forbes et al., 2007).
- Swab Culture. In order to cultivate the swab samples, an equal amount of physiology saline solution was added to the swab and homogenized by mixing by a shaker for 30 seconds according to (Vandepitte et al., 2003).
- Diagnosis of Bacteria by VITEK 2 Systems.
  The VITEK 2 system Version: 07.01 device is used to diagnose bacteria with a high degree of accuracy, it includes 64 biochemical tests in each card used for the diagnosis of bacteria so that the accuracy of the device reach to 99%. The samples were prepared according to the company instructions (Biomerieux, French). The result of the diagnosis of bacteria appears after 24 h of incubation.
- Antimicrobial Susceptibility Test.
  The antimicrobial susceptibility test was applied using disc diffusion manner by Kirby–Bauer according to (Hudzicki, 2009).
- Detection of Pseudomonas Virulence Factors by PCR.
  Nucleic acid (DNA) extracted from high frequency bacterial cells was used as a template in specific PCR for the detection of virulence genes listed in Table (3-13)(3-14) A single reaction mixture containing 3□l of upstream primer, 3□l of downstream primer, 5□l of extracted DNA, 25□l of master mix and 14□l of nuclease free water was used. The resulting PCR products were run in 1.5% agarose gel (Abd El Tawab et al., 2020).

Results

Collection of the Swab Samples and Direct Examination

The results showed only 42 samples were identical to positively infected in microscopic examination, including 31 (%) samples contained Pseudomonas spp, while 44 samples were not infected with any type of bacterial infection. Bacteria were diagnosed based on a culturing on different agars according to the type of bacteria (Chrom agar), as well as by using VITEK 2 system.

Table 1
Geographically distribution of different infected wounds in all parts of Babylon province

Province	Positive swabs	Pseudomonas
Babylon Veterinary hospital	18	10
Abi-Gharak clinic	11	8
Al-Dabla clinic	8	6
Al-niel clinic	7	2
Al-Qasim clinic	4	13
Al-Sada clinic	1	5
Al-Imam clinic	0	2
Al-Shomali clinic	1	3
Private clinic in Hilla	4	12
Private clinic in Al-Qasim	0	0
Private clinic in Abi-Gharak	0	0
Private clinic in Al-Nel	0	0
Animal market in Hilla	1	4
Total	29 (31.1%)	42

Figure 1. The isolated bacterial colony on CHRO Magar

Characterization of Pseudomonas aeruginosa by using VITEK2 system

All positive isolates of Pseudomonas aeruginosa that isolated from animals wound were confirmed with VITEK 2 systems, which have high sensitivity and specificity .VITEK 2 systems are characterized by the ability to diagnose bacterial isolates faster and more efficiently. Using (64) biochemical tests for diagnosis with special kits and away from the contamination that may cause confusion in the detection of the pathogen .The number of P. aeruginosa isolates in the current study was 42 isolates, which were diagnosed in different animals wound.

Antibiotic sensitivity test of Pseudomonas aeruginosa

Antibiotic susceptibility of Pseudomonas aeruginosa against 16 antibiotics belonging to 9 classes of antimicrobial agents was tested, as shown in Table (4-3). Almost Pseudomonas aeruginosa isolates were a multidrug resistant, the highest resistance was found in isolate No.2,3,4,5 that was resistant to 13 classes of antimicrobial, followed by isolate No.1 showing resistant to 10 classes of antibiotics. The results showed that the resistance percentage of Pseudomonas aeruginosa was (100%) towards Ampicillin, Piperacillin, Amoxicillin, Cefepime, Cefotaxime, Ceftazidime, Aztreonam, trimethoprim and Chloramphenicol. followed by (80%) resistance to Amoxicillin clavulanate and Doxycycline. Also Nitrofurantoin showed (60%) resistance, Levofloxacin and Azithromycin showed (40%) resistance against isolated Pseudomonas .Finally, the results showed that Meropenem and, amikacin have (20%) resistance against isolated Pseudomonasas shown in the Table (4-3)

Table 2
The Antibiotic Susceptibility of *Pseudomonas aeruginosa* isolates against 16 antibiotics

No	Antibiotic	Percentage%	Sample no.				
			w1	w2	w3	w4	w5
1	Ampicillin	100%	R	R	R	R	R
2	Piperacillin	100%	R	R	R	R	R
3	Amoxicillin	100%	R	R	R	R	R
4	Amoxicillin/clavulanate	80%	S	R	R	R	R
5	Cefepime	100%	R	R	R	R	R
6	Cefotaxime	100%	R	R	R	R	R
7	Ceftazidime	100%	R	R	R	R	R
8	Aztreonam	100%	R	R	R	R	R
9	Meropenem	20%	S	R	S	S	S
10	Amikacin	20%	S	S	R	S	S
11	Azithromycin	40%	S	S	S	R	R
12	Doxycycline	80%	S	R	R	R	R
13	Levofloxacin	40%	S	S	S	R	R
14	Trimethoprim	100%	R	R	R	R	R
15	Chloramphenicol	100%	R	R	R	R	R
16	Nitrofurantoin	60%	R	R	R	S	S

Genotype Study of Some Virulence Factors genes for pseudomonas spp. Isolates

The genes presence of algD gene, LasB gene, Exos and plcH gene in all studied isolates were confirmed by PCR i.e. amplification of 1310,300,504 and 307 bps respectively table (4-5).

Table 3
Sequence of Some Virulence Factors genes for pseudomonas spp

Gene	Sequence (5'-3')	product Size (bp)	Reference
LasB	GGA ATG AAC GAG GCG TTC TC	300	(Strateva *et al*, 2008)
	GGT CCA GTA GTA GCG GTT GG		
Exos	CTT GAA GGG ACT CGA CAA GG	504	(Strateva *et al*, 2008)
	TTC AGG TCC GCG TAG TGA AT		
algD	ATG CGA ATC AGC ATC TTT GGT	1310	(Strateva *et al*, 2008)
	CTA CCA GCA GAT GCC CTC GGC		
plcH	GAA GCC ATG GGC TAC TTC AA	307	(Strateva *et al*, 2008)
	AGA GTG ACG AGG AGC GGTAG		

Alginate production gene (algD)

The results of PCR for algD gene (the gene responsible for alginate production) showed that all isolates 4(100%) possess this gene, since gel electrophoresis results showed DNA bands with molecular size 1310 bp in comparison with DNA marker for all tested isolates (as shown in figure 2).

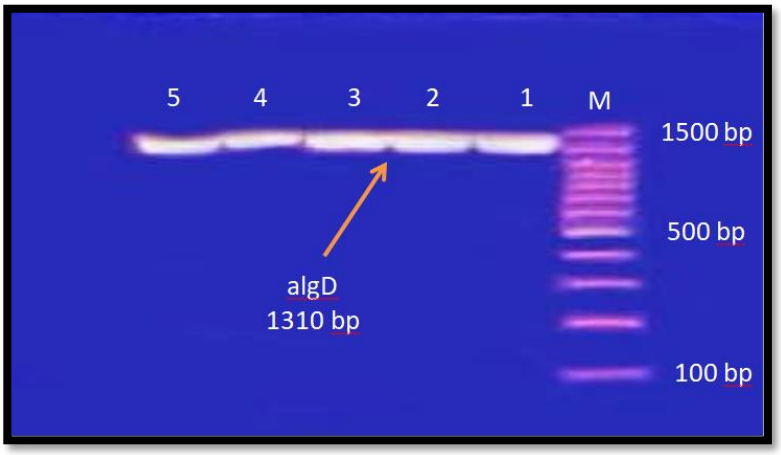

Figure 2. Agarose gel electrophoresis (1.5%) showing the PCR products of *algD* gene after partial amplification using gene specific primers. M:100 -1500 bp DNA ladder. Lanes (1-5): PCR products of *algD* gene partial amplification (1310 bp) from five *pseudomonas aeruginosa* clinical strains namely AAQ1-5

Elastase enzyme responsible gene(lasB gene)

For *lasB* gene, the results of PCR also showed that all isolates 4(100%) possess this gene, since gel electrophoresis results showed DNA bands with molecular size 300 bp in comparison with DNA marker for all tested isolates as shown in figure(3).

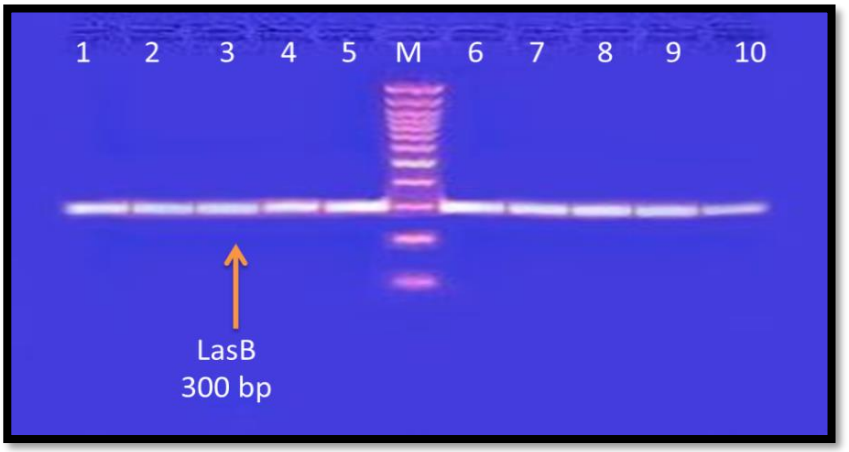

Figure 3. Agarose gel electrophoresis (1.5%) showing the PCR products of LasB gene after partial amplification using gene specific primers. M: 100-1500 bp DNA ladder. Lanes (1-5): PCR products of LasB gene partial amplification (300 bp) from five *pseudomonas aeruginosa* clinical strains namely AAQ1-5.

Exoenzyme encoded gene (Exos gene)

For Exos gene, the results of PCR also showed that all isolates 4(100%) possess this gene, since gel electrophoresis results showed DNA bands with molecular size 504bp in comparison with DNA marker for all tested isolates as shown in figure (4).

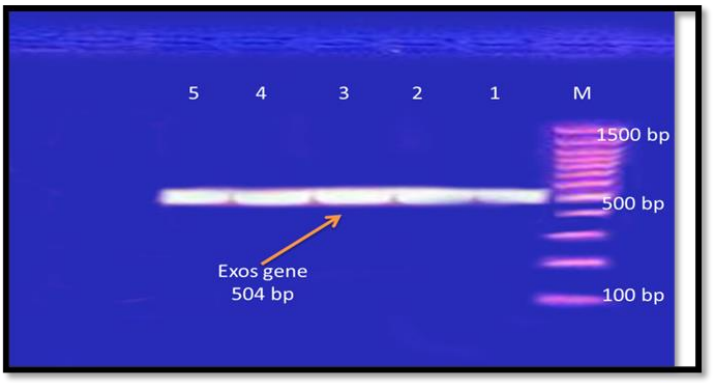

Figure 4. Agarose gel electrophoresis (1.5%) showing the PCR products of Exos gene after partial amplification using gene specific primers. M: 100 bp DNA ladder. Lanes (1-5): PCR products of Exos gene partial amplification (504 bp) from *pseudomonas aeruginosa* clinical strains namely AAQ1-5

Phospholipases encoded gene(plcH)

For plcH gene, the results of PCR also showed that all isolates 4(100%) possess this gene, since gel electrophoresis results showed DNA bands with molecular size 307bp in comparison with DNA marker for all tested isolates as shown in figure (5).

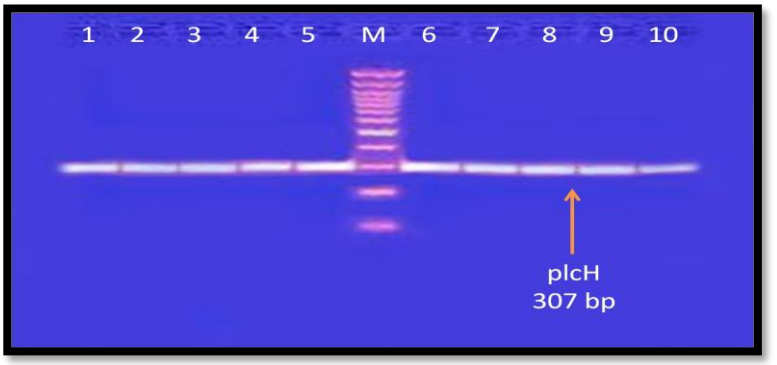

Figure 5. Agarose gel electrophoresis (1.5%) showing the PCR products of *plcH* gene after partial amplification using gene specific primers. M: 100 bp DNA ladder. Lanes (1-5): PCR products of *plcH* gene partial amplification (307 bp) from five *pseudomonas aeruginosa* clinical strains namely AAQ1-5.a

Discussion

In the present study, positives samples of bacteria were more represented (62%) in respect to negative samples (38%), confirming the results reported in other studies (Guan *et al.,* 2021). The majority of the wounds were colonized with a single bacterial species. These data are confirmed by Mohammed et al., that found single bacterial growth in the 81.7% of the swab cultures (Mohammed *et al.,* 2017), while Yeong *et al.* showed a higher number of polymicrobial resistant species in wound bacterial cultures within the first 72 hr (Yeong *et al.,* 2020). The high affinity of P. aeruginosa virulence by wound fascia. Fascia consists primarily of collagen fibers that enclose muscles and confer stability. Injury of fascia leads to the accumulation of macrophages and fibroblasts that are involved in the healing process (Lau and Pomahac, 2014). On the other hand, Rind and Khan(2000) surveyed wound samples located on the body surface of domestic animals and recorded 93.3, 90, 100 and 100% in buffaloes, cattle, sheep, and goats respectively (Rind and Khan(2000).

This result was similar with Hossain et al. (2013) when recorded that *Pseudomonas aeruginosa* showed highest sensitivity to Meropenem. The spread of multidrug-resistant P. aeruginosa is particularly concerning. Over recent years, the worldwide spread of so-called “high-risk clones” of multidrug-resistant or extensively drug-resistant (MDR/XDR) P. aeruginosa has become a public health threat that needs to be urgently and decisively studied and managed (Spagnolo *et al.,* 2021). The result of our study was closed to Al-Muhannak (2020) who found that Levofloxacin resistance 70% and similar to the result of Mohammed (2012) which was 69.3%. In 2002, Lambert mentioned that Levofloxacin, belong to

fluoroquinolone, inhibits bacterial growth by binding to A subunit of DNA gyrase (Lambert, 2002). In this study, P. aeruginosa isolates has revealed high resistance for group of beta lactam drugs Ceftazidime, Cefotaxime, Cefepime, Ampicillin, Piperacillin and Amoxicillin. Antibiotic resistance genes can be carried on plasmids, transposons, integrons and prophages, and bacteria can acquire these genes via horizontal gene transfer from the same or different bacterial species (Breidenstein *et al.,* 2011). Integrons are genetic elements that insert mobile gene cassettes into a specific genetic site via site-specific recombination and they have been shown to play a critical role in dissemination of antibiotic resistance among P. aeruginosa strains (Khosravi *et al.,* 2017).

The prevalence of *algD* is correlated with mucoid strains that over product alginate increasing the pathogenesis and resistance of the bacterium by protect it in a major exopolysacchrides in a biofilm matrix (Whitney *et al.,* 2015), almost, there is a high correlation between the chronic infection and *lasB* and *algD* genes (Mitov *et al.,* 2010).The current study results were in agreement with Al-Shimmary (2020), when she recorded that Positive results with algD gene is 110(63.6%) isolates involving 43(93.5%) with burns, 31(81.5%) with wounds, 6(25%) with keratitis, 4(14.3%) with otitis and 26(70.2%). Elastase (encoded by *lasB*) is a powerful T2SS secreted proteolytic enzyme. Bradbury *et al.*(2010), founded that vast majority of (184) tested P. aeruginosa strains shown to possess all virulence factor genes examined: apr (an alkaline protease gene), lasB (an elastase gene) . Our results were in agreement with the results reported by Mitov *et al.*(2010) whom found a widespread dissemination (100%) of *algD, lasB*, genes in (202) Pseudomonas aeruginosa isolated from CF , (92.5%) of *algD* and (100%) of lasB in isolates from urine, LRTIs, URTIs, blood and wound infections(4) and the results of Lanotte et al., who reported (100%) spread of these genes in (162) Pseudomonas aeruginosa isolated from CF patient (Mitov *et al.,* 2010). *ExoT* shows a lower ADP-ribosyltransferase activity than exoS (Azimi *et al.,* 2016). It has been shown that exoS and exoU were the major cytotoxins in both in vitro and in vivo assays (Hall-Stoodley and Stoodley, 2009). The majority of P. aeruginosa strains carry exoT and exoY genes; however, the presence of exoS and exoU differ noticeably between the isolates and appear to be mutually exclusive (Lee *et al.,* 2005). Different frequencies of cytotoxin encoding genes, however, have been reported in different studies (Azimi *et al.,* 2016).

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