Original Research

Molecular detection of type III secretory toxins in *Pseudomonas aeruginosa* isolates

Khanzad Khudhur Jarjees[*]

Department of Food Technology, College Agricultural Engineering Sciences, University of Salahaddin, Erbil, Iraq

Abstract: *Pseudomonas aeruginosa* has been known as a common unscrupulous pathogen that reasons cause nosocomial infections in patients with immuno-compromise. Infection with multi-drug resistant *Pseudomonas aeruginosa* infection in many patients is a public health problem. The bacterium causes urinary tract infections, respiratory tract infections, skin inflammation, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and various systemic infections, especially in patients with severe burns, cancer and AIDS, whose immune systems are suppressed. Among diverse virulence factors, the type III secretion system is known as a significant agent in virulence and development of antimicrobial resistance in *P. aeruginosa*. A total of 50 isolates of *P. aeruginosa* were gathered from burn wound and milk specimens. Documentation and antimicrobial susceptibility evidence were performed using the VITEK 2 system. Multiplex PCR was done to detect the secretion toxins-encoding genes. Out of 50 samples: 45/225 (20%) burn wound and 6/120 (5%) raw milk samples were found positive for *P. aeruginosa*. The multiplex PCR analysis of ExoT and ExoY genes showed that all *P. aeruginosa* 50 (100%) were positive. The occurrence of the ExoS and ExoU genes was 97.7% and 86.6% among clinical isolates while none of the raw milk isolates harbored the ExoU gene and 60% of them carried the ExoS gene. The results found 20 (40%) of isolates were multidrug resistance and the most effective antibiotics against clinical isolates were Ciprofloxacin and Meropenem. The aim of this study was to prevalence the exotoxin genes encoded type III secretion system and pattern of antimicrobial susceptibility of *P. aeruginosa* isolated from clinical and raw milk specimens.

Key words: Exotoxins, Clinical sample; Raw milk; Multiplex PCR; VITEK 2 system; Multidrug resistance.

Introduction

Pseudomonas aeruginosa is unique to patients with cystic fibrosis and is a common source of infection in the lungs. It is highly associated with cancer and burns patients and patients with suppressed immune systems. The mortality rate of people with bacteria is about 50%. The bacterium is basically a hospital pathogen, usually found in a humid hospital environment. The bacteria can be cloned in normal people; in this case, it can be used as a saprophyte. This bacterium can cause illness in people with immune system diseases (1-3).

The diagnosis of *P. aeruginosa* infection is carried out by isolation and laboratory diagnosis. This aerobic bacterium is mandatory and grows well in most laboratory culture media. The bacteria can be separated on blood agar and blue agar eosin methyl methionine (Eosin Methylene Blue Agar), and the former colonies are smooth and round. The detection of bacteria is based on warmth, no spores, flagella morphology, positive, exercise catalase, lactose intolerance (positive oxidase reaction), fruit odor (grape flavor) and ability to grow at 42 ° C. Ultraviolet fluorescence This function is also very effective in the immediate detection of *P. aeruginosa* colonies and helps to detect their presence in wounds (1, 2-4).

Due to the overproduction of alginate, an exopolysaccharide, cultures prepared from cystic fibrosis (CF) patient samples often form mucoid colonies. In CF patients, it seems that the exogenous polysaccharides produced to provide a platform for the survival of organisms in biofilms (2-4). However, the diagnosis based on morphological features is always flawed. The most accurate way to identify it is through molecular methods and genetic research (1-5).

Protein secretion plays a key role in regulating the interaction of bacteria with their environment. This is especially true when the bacteria are symbiotic bacteria (whether pathogenic, common or mobile) that interact with larger hosts. The bacteria are equipped with several types of secretion systems (1, 4, 5).

The *P. aeruginosa* virulence is related to the several cell-associated secretions and extracellular factors, such as exotoxin A, exoenzyme S, endotoxin (lipopolysaccharide), exoenzyme U, phospholipase C, elastase, neuraminidase, sialidase, and alkaline protease and exhibits antibiotic resistance (4, 5).

Previous research has shown that multi-drug resistant strains have inappropriate chemotherapy, especially in burn patients. According to reports, the resistance rate of the isolated *P. aeruginosa* to most antibiotics is higher than 70%. An important measure of toxicity is the type III secretion system TTSS, which exists in several Gram-negative bacilli such as *Salmonella*, *Shigella*, and *Yersinia* (6).

The pathogenic system depends largely on Gram-negative bacteria: two layers of cell lining secrete cytoplasmic synthetic proteins. In Gram-negative pathogens,

these systems are usually associated with the secretion of toxic factors. They are involved. Some type III systems are protein complexes, which act like hypodermic needles and can directly inject proteins into the host's cytoplasm without exposing them to the extracellular environment (2-5).

The system does not depend on seconds. Type III secretion system is activated by the contact of bacteria with the host surface. By binding to secreted proteins, chaperone proteins can prevent reactive proteins from reacting with other proteins and protect their structure (3-5). Secretion-dependent TTSS is usually associated with unique host cells. The export mechanism of TTSS usually consists of 20 different proteins, including cytoplasmic soluble protein, outer membrane protein and inner membrane protein. TTSS has the ability of bacteria. Send various effects directly to the host's cytoplasm, manipulate the host's cellular processes and destroy them to benefit themselves. The genes encoding the type III secretion system are located on unstable genetic elements, plasmids and pathogenic islands, including hrp-PAI in *P. syringae* (2-6).

P. aeruginosa can directly produce and secrete virulence factors into the cytoplasm of host cells through TTSS mediated with cell contact. The system consists of three independent protein complexes; the secretion device itself, the translocation or targeting device, and the secreted enzyme (effector protein) and its cognate chaperons (7). Upon establishing intimate contact with host cells, effecter proteins are injected into host cells.

Exoenzyme S encoded by the exoS gene.   It has been proposed that ExoS is the main virulence factor required for colonization, invasion and bacterial transmission in burns and chronic lung infections. (8, 9). Similarly, this exoenzyme is a bifunctional effector protein with GTPase activating protein (GAP) and ADP-ribosyltransferase (ADPRT) activity, which can play a complex role, leading to pathogen escape and apoptosis from the host immune system. Exo T is a 53 kDa protein with high homology and enzyme activity to Exo S. Existing research shows that the GAP activity of Exo S and Exo T can prevent wound healing, which may be due to the destruction of the actin cytoskeleton, bacteria and inhibition. Phagocytosis and host cell rounding (10-12).

ExoS and ExoT have a 75% amino acid identity. ExoS and ExoT are bifunctional exotoxins with c-terminal ADP ribosylation activity, but ExoT's catalytic activity is very low, accounting for only 0.2% of ExoS ADP-ribosyltransferase activity (13, 14). Acute cytolytic factor (ExoU) has phospholipase A2-like activity, leading to extensive tissue destruction and regulation of host inflammation. In addition, it has been characterized as a major virulence factor in lung injury. This protein greatly promotes the pathogenesis of highly virulent strains and is mainly related to the severity of P. *aeruginosa* infection (13, 15, 16).

ExoU toxin can solubilize mammalian cells, including macrophages, neutrophils, epithelial cells and fibroblasts. ExoU and ExoS are different and important in the pathogenesis and almost isolates ExoT and ExoY encode and have little effect on virulence (17, 18). Previous studies have shown that the production of ExoU is associated with increased virulence. ExoU toxin can solubilize mammalian cells, including macrophages, neutrophils, epithelial cells and fibroblasts (19). Other studies have shown that invasive strains tend to express ExoS, while non-invasive cytotoxic strains express ExoU (20). Exo Y with natural adenylate cyclase activity causes an increase in intracellular signal messengers, leading to the destruction of the actin cytoskeleton and endothelial barriers. The result of these events is that *P. aeruginosa* spreads into the blood and increases the risk of septic shock (10). ExoY is an extracellular adenylate cyclase which has a certain homology with the extracellular adenylate cyclase of *B. pertussis* and *B. anthracis* (21). ExoY increases cytosolic cAMP, enhanced by a eukaryotic cofactor. This increased cytoplasmic cAMP leads to increased intracellular space formation in the lung microvessels and increased lung permeability (22).

Among clinical strains isolated from burn patients and raw milk isolates, the type III secretion system exonuclease-encoding gene is prevalent. Evidence that causative pathogenic bacteria belong to a specific subgroup of *Pseudomonas aeruginosa* will support the further development of strategies for better disease control. The purpose of this study was to determine the antibiotic sensitivity pattern, the multidrug resistance *P. aeruginosa*.

Materials and Methods

Collection and preparation of the samples
Wound burn specimens
   The clinical isolates of *P. aeruginosa* were supplied from hospitalized patients of the Teaching and West Emergency Hospitals from February to June 2018. The bacterial isolates of *P. aeruginosa* gathered from wound burn specimens.

Raw milk samples
   One 120 random samples of cow s raw milk were gathered aseptically from various milk markets in Erbil City. All milk samples were transported in an ice box to be tested bacteriologically for *P. aeruginosa* isolation. 25 ml of each sample was aseptically transferred to 225 ml of 0.1% sterile buffered peptone water and homogenized for 2 min by stomacher Lab-Blender (Bagmixer 400, Interscience, Paris, France). The homogenized sample was incubated at 37 ºC for 24 hours.

Isolation and identification of *P. aeruginosa*
   All isolates were identified as *P. aeruginosa* based on colonial morphology, Gram staining, oxidase test, catalase test, growth at 42 ºC, and the presence of characteristic pigments. Microbial identification was accomplished on the VITEK 2 automated system (bio Merieux). Isolates identification was confirmed by using 16SrDNA. All strains were incubated in tryptic soy broth containing 30% glycerol at -80 ºC.

Antimicrobial susceptibility test
Antimicrobial susceptibility testing was performed on the VITEK 2 automated system (bio Merieux) for the following antimicrobials: Ticarcillin, piperacillin, Ticarcillin/Clavulanic Acid, piperacillin/Tazobactam, Ceftazidime, meropenem, Cefepime, Amikacin, Imipenem, Gentamicin, Tobramycin, Ciprofloxacin.

Table 1. Primers used for detection of ExoS, ExoT, ExoU, and ExoY genes and length of the PCR products.

Target gene	Primer sequences (5 ' - 3 ')	Amplification size (bp)
exo S	FW(5 '-GCG AGG TCA GCA GAG TAT CG- 3 ')	118
	RV(5 '-TTC GGC GTC ACT GTG GAT GC- 3 ')	
exo T	FW(5 '-AAT CGC CGT CCA ACT GCA TGC G - 3 ')	152
	RV(5 '-TGT TCG CCG AGG TAC TGC TC - 3 ')	
exo U	FW(5 '-CCGTTG TGG TGCCGT TGA AG - 3 ')	134
	RV(5 '-CCA GAT GTT CAC CGA CTC GC - 3 ')	
exoY	FW(5 '-CGG ATT CTA TGG CAG GGA GG - 3 ')	289
	RV(5 '-GCC CTT GAT GCA CTC GAC CA - 3 ')	

Table 2. PCR program for amplification of ExoS, ExoT, ExoU, and ExoY genes.

Gene	Denaturation	Annealing	Extension	Cycles
exo S	Initial: 94 ºC 3 min	58 ºC 40 sec		1
	Cycle: 94 ºC 40 sec		Cycle: 68 ºC 1 min	36
			Final: 68 ºC 1 min	1

Molecular examination of *P. aeruginosa*
DNA purification

Bacterial DNA was purified from *P. aeruginosa* isolates sub-cultured on nutrient agar plates by a DNA extraction kit (generated, Korea**)** according to the manufacture's protocol. The extracted DNA was checked by measuring the optical density (OD) at 260 nm and 280 nm using a spectrophotometer. The DNA extracted samples were stored at -20 ºC until being used.

PCR amplification of type III secretion toxins-encoding genes

The type III secretion enzymes were amplified using multiplex PCR (p22) with specific primers (Table 1). The total of 25 µl PCR master mix reaction volume was performed containing 4µl of genomic DNA, 12.5 µl of 2X GoTaqGreen Master Mix (Promega, USA) and 1.5µl was added for each of the forward and reverse primer for all genes (Table 2).

PCR products were separated in 2% agarose gel stained with Safe dye (GenetBio/Korea). The gel was visualized under UV transilluminator (Synegene/ UK). Amplified genes were identified on the basis of fragment size as compared with the DNA marker (Norgen Biotech, Canada).

Results

Six samples positively contained *P. aeruginosa* this 6(5%) were found from the total of 120 caw's raw milk samples also out of 225 clinical samples, 45 (20%) were found to be positive with *P. aeruginosa*.

According to the morphology of the colony, the isolated colony is identified,

oxidase and catalase positivity, the presence of characteristic pigments and growth at 42 ºC (23), also *P. aeruginosa* not ferment lactose can easily be differentiated from lactose-fermenting bacteria on MacConkey agar Fig. 1.

All *P. aeruginosa* isolates were identified and screened for 12 antimicrobial agents by the VITEK 2 compact system.

In the present study, all isolates were found to be

Figure 1. *P. aeruginosa* on MacConkey agar**.**
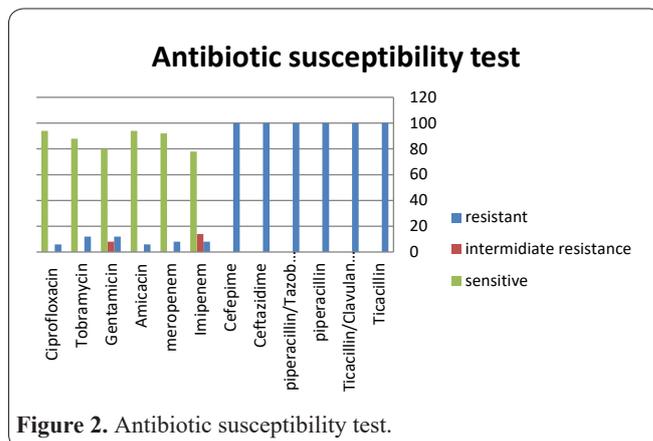

Figure 2. Antibiotic susceptibility test.

resistant to Ticarcillin, Ticarcillin/Clavulanic Acid, piperacillin/Tazobactam, piperacillin, Ceftazidime, and Cefepime. On the contrary, resistance to Imipenem, meropenem, Amikacin, Gentamicin, Tobramycin, and Ciprofloxacin were 8%, 8%, 6%, 12%, 12%, and 6% respectively. In addition, intermediate resistance to Imipenem and Gentamicin was 14% and 8%.

In the present research, a total of (20) 40% of the 50 *P. aeruginosa* isolates were multi-resistance to three or more antimicrobial agents.

The results showed that all bacterial isolates carried ExoT and ExoY gene, while 97.7% and 86.6% of clinical isolates harbored ExoS and ExoU genes, however, the incidence of ExoS gene was 60% of raw milk
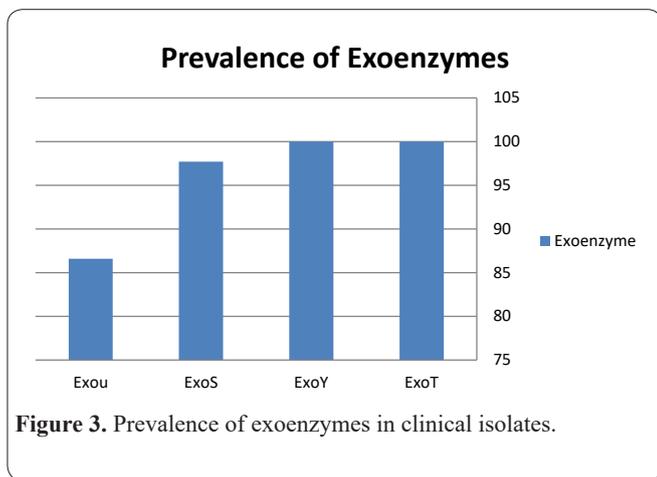
Prevalence of Exoenzymes

Figure 3. Prevalence of exoenzymes in clinical isolates.
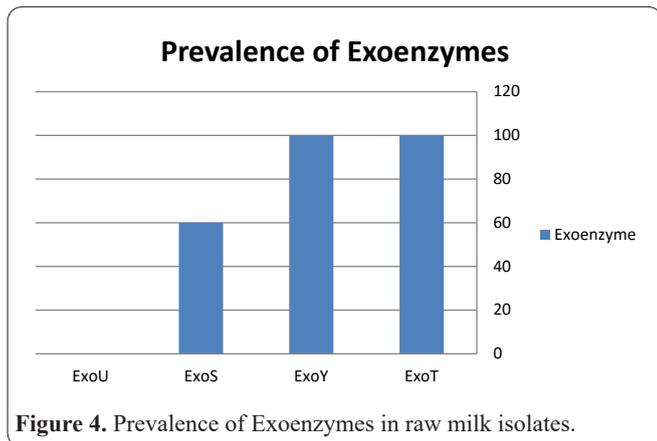
Prevalence of Exoenzymes

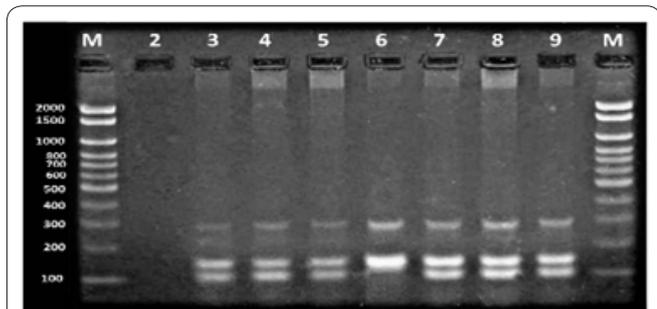**Figure 4.** Prevalence of Exoenzymes in raw milk isolates.

Figure 5. Multiplex PCR specific for type III Secretion Toxins. Lane: 1-10 ladder of 1kb, lanes 2: Negative control, Lane 3-5: represent exoU (137 bp), ExoT (152 bp) and ExoY (289 bp) genes, Lane 6: ExoT (152 bp) and ExoY (289 bp) genes, Lane 7-9: show ExoS (118 bp), ExoT (152 bp) and exoY (289 bp), genes.

isolates and none of them were positive for ExoU gene (Fig. 2,3,4,5).

Discussion

Pseudomonas aeruginosa is an opportunistic pathogen with different virulence factors that can help bacteria colonize different niche in their host and is the main cause of acquired infections in hospitals and communities around the world (24, 25). It is expected that the presence of ExoS (invasive) and ExoU (acute) will be consistent with the invasive and acute cytotoxic phenotype (16, 26-29). Most *P. aeruginosa* strains carry ExoT and ExoY genes. However, the existence of ExoS and ExoU is significantly different between the isolates and appear to be mutually exclusive (30).

They suggested that the production of ExoS may provide *P. aeruginosa* isolates with the advantages of

colonization or persistence in CF lung (28).

The variation observed in the prevalence of Exo genes may be the consequence of clinical specimens used for collecting isolates (31).

In this research, the prevalence of the ExoT and ExoY gene was 100%. This result was in agreement with findings of other previous studies, that have shown that ExoT and ExoY are existing in about all clinical isolates (16, 32).

In the present study, the frequency ExoU and ExoS genes of clinical isolates were 97.7% and 86.6%, were higher than that of the study conducted by Khodayary *et al*, who reported 59% and 41% in *P. aeruginosa* isolates (28).

The prevalence of ExoS genes of milk isolates was 60%, where it was higher than the studies conducted in India 36.8%(33). In the present research, in agreement with the previous study who reported that none of the isolates of raw milk harbored the ExoU gene (34).

According to the above study and comparison with the results of this study, the observed differences in the prevalence of ExoS and ExoU genes may be due to the types of samples, strain types, and the diversity of the geographic regions investigated.

Results showed a lower frequency of exoenzymes encoding genes in raw milk samples in comparison with clinical samples, this may be due to the presence of high mutation of virulence and antibiotic resistance genes in isolates collected from hospitalized patients, also the transfer of these genes among nosocomial isolates in hospitals.

ExoU[+] isolates may be more resistant and more toxic, so they may have evolutionary advantages. This is supported by a study which shows that ExoU has a higher prevalence among isolates collected from the hospital environment (35). However, because ExoS[+] strains can cause mammalian cells to take up antibiotics, they can be protected from antibiotics (so-called invasive strains). Their residence in mammalian cells can provide protection against antibiotics, thereby reducing selection pressure, which translates into antibiotic resistance. The ExoU[+] strain has more mutations in the resistance-determining genes, which is probably the reason for the higher antibiotic resistance in the ExoU[+] strain. The results showed all Ciprofloxacin resistant isolates were harbored ExoU gene, this agreement with the study conducted by Pena *et al*, 2015. (36). ExoU[+] isolates are more resistant than ExoS[+] isolates. Therefore, it appears that ExoU and increased resistance to Levofloxacin may be jointly selected traits (37, 38).

In surveys directed by Maatallah et al (39) and Pena et al (36), the occurrence of the ExoU gene was significantly associated with multidrug resistance and ciprofloxacin resistance. Alternatively, Preister et al. have reported that the gyr A mutation that confers fluoroquinolone resistance also affects supercoiling DNA, which has shown to affect virulence gene expression (40). The wide-ranging usage of antibiotics such as fluoroquinolones can be effective in generating the multi-drug resistant isolates. In a study by Angella et al., they were found that the presence of the ExoU gene was significantly correlated with fluoroquinolones-resistance-type III secretion system toxins-encoding genes were not found in all *P. aeruginosa* isolates. Most *P. aerugunosa*

isolates had at least two genes of these toxins (41).

The evolutionary history most likely involved transposition of the ExoU determinant onto a transmissible plasmid, then transferring the plasmid to different *P. aeruginosa* strains, t and then integrating the plasmid into tRNA (Lys) in each recipient chromosome, where sequences are inserted and deleted and rearranged (42). Given this, it points to the higher plasmid transmission among our isolates. The information obtained from the latest research on genes and genome editing helps researchers to study the paper in detail (43-44). Recently, *in silico* analysis and molecular modeling of *P. aeruginosa proteins* has helped to suggest vaccine candidates (45). Anti-adhesion-based vaccination showed promising results to overcome *P. aeruginosa* infections by inducing of specific humoral immune response in mice (46).

However, these observations require confirmation of a large sample size, which should include studies of isolates from various sources and related epidemiological data.

The results of this research showed that the rate of resistance to most antibiotic classes is high. The high resistance rate of *Pseudomonas aeruginosa* and its ability to produce biofilms alert public health. Genes of type III secretion system toxins were found in the isolated *P. aeruginosa*, in which ExoT and ExoY genes were detected in all isolates, ExoS genes were detected in most isolates, and ExoU gene was detected in some clinical isolates, while ExoU gene was not detected in the animal samples (raw milk). In addition, among *P. aeruginosa* isolates, ExoS and ExoU are variable traits with different prevalence rates. To avoid the spread of more virulent strains in health care institutions, molecular evaluation and antimicrobial susceptibility testing are recommended.

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