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Prototheca spp. co-infections and their virulence factors in human protothecosis in Hillah city, Iraq

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ABSTRACT

Background: The objective of the present study was to investigate coinfection of *Pseudomonas aeruginosa* and *Prototheca* species and other microorganisms in human protothecosis infections.
Methods: The current study included a collection of 275 clinical samples in Hillah, Iraq. Following bacterial identification, virulence factors genes were screened using polymerase chain reaction (PCR) technique.
Results: The results showed four types of *Prototheca* species *Prototheca zopfii genotype1*, *Prototheca zopfii genotype2*, *Prototheca stagnora and Prototheca blaschkeae* responsible for human protothecosis infections in burns, urinary tract infection (UTI), leukemia and dialysis. Furthermore, this study clarified coinfection of *Prototheca* spp. and *P. aeruginosa*. The predominant *Prototheca* spp. virulence genes included *lasR* (41.7%), *ecfXECF* (26.6%), *rhlR* (19.6%) and *toxAETA* (21.3%), with significant difference (P-0.047). furthermore, there was an association between *Prototheca* spp. and *P. aeruginosa* virulence genes such as *PA-GS 16sRNA*, *lasB ELA520*, *toxAETA397*, *phzM PYO330*, *ecfXECF164*, *lasR*, *rhlR*, *PlcH*.
Conclusion: This study revealed the existence of Prototheca spp. carrying various virulence genes among burns and UTI in Hillah city, Iraq. Bacterial co-infections exacerbate the severity of infections, particularly those potential nosocomial pathogens. Identification of *P. aeruginosa* virulence genes among Prototheca spp. highlighted their role in severe infections.

1. Introduction

The *Prototheca* spp. are large non-budding organisms observed in tissue (Pore, 1985). It is spheroid, ovoid, or elliptical microorganism with a prominent wall, and the round cell (theca) contains several thick-walled Autospores. The genus *Prototheca* includes unicellular yeast-like, colorless microalgae (phylogenetically related to *Chlorella*), and its reproduction is asexual, and during cell maturation, the cytoplasm undergoes a process of cleavage to form endospores. After release from the maternal cell, these spores increase in size and undergo an assimilatory stage, more or less a pair of twenty endospores develop, initially in irregular form and therefore the sporangia break underneath the pressure of the increasing spores (Sudman and Kaplan, 1973), what's more, their number and size change among the *Prototheca* species (Padhye et al., 1979). Spore discharge happens each five to six hours visible of adequate supplements ample enhancements (Joshi et al., 1975). Protothecosis is a zoonotic infection, as well as certain *Prototheca* species, e.g. *P. zopfii*, and *P. wicherhamii*, are human protothecosis etiological microalgae agents (Buzzini et al., 2004). Davies et al. (1964) firstly described the human infection of *Prototheca* species. *Pseudomonas aeruginosa* (*P. aeruginosa*) is the most important type of Gram negative bacteria that cause disease in human history because of various virulence factors such as a number of toxins, the production of extracellular enzymes as well as their resistance to phagocytosis (Laxmi and Sarita, 2014). Pathogenic *P. aeruginosa* is among substantial nosocomial agents in Iraq (Balaky et al., 2019; Ali et al., 2020; Al-Sa'ady et al., 2020; Hussen, 2020). Then forming the biofilm in the position of infection, after they are collected in large numbers, and the membrane protects them from the defenses of the body and increases its resistance to antibiotics (Zubair et al., 2011; Fazeli and Momtaz, 2014). The first time record and was detected genetically for *Prototheca* species such as *P. zopfii* genotype 1, *P. zopfii* genotype 2, and *P. blaschkeae* in Iraq

Abbreviations: PCR, polymerase chain reaction; UTI, urinary tract infection; EDTA, ethylenediaminetetraacetic acid; TBE, tris borate EDTA; SDA, Sabouraud's dextrose agar; PIM, *Prototheca* isolation medium.

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(Shanon et al., 2019). The current study was aimed to investigate coinfections of *P. aeruginosa* and *Prototheca* species in human protothecosis infections in Hillah city, Iraq.

2. Methods

2.1. Samples collection

The present study was extended for the period between September 2018 to March 2019 and included 275 patients with varying degrees of burns (82 patients), Urinary tract infection (UTI) (94 patients), Leukemia (46 patients), Dialysis (53 patients) group from Hillah General Teaching Hospital, lobby burns, Margan hospital, Iraq.

2.2. Isolation of bacterial species

Prototheca species were isolated using Sabouraud's dextrose agar (SDA) and selective media called *Prototheca* isolation medium (PIM) was prepared and incubated for 5–7 days at 25 °C according to (Pore, 1973), from a purified agar base with the addition of selective agents that inhibit bacterial growth and cultivate *Prototheca* (Pore, 1998; DiPersio, 2001; Roesler et al., 2006).

The following culture media were used according to the requirements growth of organisms. Nutrient agar medium to isolate, cultivate, and to save bacterial strains, MacConkey agar medium to isolate Gram-negative bacilli and to differentiate lactose fermented from non-lactose fermented bacteria. The API-20E platform was also performed to confirm the identified species. Blood agar medium was used to cultivate bacterial strains with ability of blood haemolysis. Additionally, mannitol salt agar was used to detect the growth of *Staphylococcus* species that have the ability of mannitol fermentation.

2.3. Polymerase chain reaction

In this study, eight primers (Ligo/USA) were used to detect diagnostic genes as shown in the Table 1.

Isolation of genomic DNA was performed using Promega Genomic DNA Purification Ki. Bacteria species genes were amplified using conventional polymerase chain reaction (PCR). The conditions of PCR for *P. aeruginosa* detection were as following: initial denaturation at 95 °C for 4 min followed by Denaturation at 95 °C for 1 min, annealing at 61 °C for 55 s and extension at 72 °C for 55 s continued for 40 cycles and thereafter, the final extension was at 72 °C for 8 min. The PCR reaction mixture for gradient consisted of 5 μL template DNA, 5 μL master mix, 5 μL of each forward and reverse primer reaching totally 20 μL reaction volume.

Ethical Approval: A valid consists was achieved from hospitals administration and from each patient before their inclusion in the study. For every patient or their followers, the procedure had been informed before the samples were collected, making absolutely sure that they understood the procedure. The subjects were sentient that they had the right to reject to be included in the study without any detrimental effects.

3. Results

The study included the collection of 275 samples from different types of infections as follow: Burns (82 patients), UTI (94 patients), Leukemia (46 patients), Dialysis (53). The identification of *Prototheca* was done mainly based on the morphological characteristics of the organism on the affected loci or directly on the damaged tissue. We observed a typical appearance of the characteristic ovoid to globose sporangia with sporangiospores in several developmental stages (the so-called morula form). *Prototheca* species rate included 49 (32.2%) from burns, 53 (34.9%) from UTI, 22 (14.5%) from leukemia and 28 (18.4%) from dialysis. Accordingly, the highest ratio was detected in UTI (34.9%) followed by Burns (32.2%), Dialysis (18.4%) and Leukemia (14.5%) (Table 2).

The following results shows that *Prototheca* infections are always associated with *P. aeruginosa* infection, where note that the highest percentage of infection was *P. zopfii* genotype 1 (45%), followed by *Prototheca zopfii* genotype 2 (27.6%), and *P. blaschkeae* infection by (15.1%) and finally *P. stagnora* injuries by (11.8%), (Table. 3). Through the results can also note that the highest infection of *P. zopfii* genotype 1, *P. zopfii* genotype 2, *P. stagnora* and *P. blaschkeae* were with *P. aeruginosa* by 19.1%, 15.1%, 7.2%, 9.2%, respectively. According to the statistical analysis of the results there was no significant relation regarding coinfections (p-value > 0.05). It is possible to attribute why *Prototheca* spp. infection is associated with *P. aeruginosa* as opportunistic organisms that exploit the rapid growth of bacteria as well as sharing the virulence factors. Other coinfection with *Prototheca* spp. included *Escherichia coli* (*E. coli)* (n = 33, 21.7%), *S. aureus* (n = 33, 15.1%), *Streptococcus pyogenes* (*S. pyogenes*) (n = 11, 7.2%) and *Acinetobacter baumannii* (*A. baumannii*) (n = 8, 5.3%).

The rate of virulence factors among *Prototheca* spp. has been represented in Table 4. Accordingly, *lasR*, *toxAETA*, *rhlR*, *ecfXECF* and *plcH* genes were predominant virulence genes in *Prototheca* spp. Other virulence factors included *PA-GS*, 16s*RNA*, *lasB ELA*, and *phzM PYO* genes.

Among *Prototheca* spp., *P. zopfii* genotype 1, *P. zopfii* genotype 2, *P. stagnora* and *P. blaschkeae*, respectively mostly carried *lasR* (41.7%), *ecfXECF164* (26.6%), *rhlR* (19.6%), *toxAETA* (21.3%) with significant difference (p = 0.047) (Table 4 and Fig. 1).

LasR gene and *rhlR* were identified by trademark band designs on 2% Agarose. The PCR items were 450 bp for lasR and 900 bp for rhlR as appeared in the accompanying (Fig. 2).

The PCR gel electrophoresis of *ToxAETA397*, phzM PYO330 and

Table 2
Number and percentage of positive cases for *Prototheca* spp. growing on PIM and SDA with all cases including in this study.

Cases	No.	No. of positive	%
Burns	82	49	32.2
UTI	94	53	34.9
Leukemia	46	22	14.5
Dialysis	53	28	18.4
Total	275	152.0	100.0

Table 1
Sequence of primers used in this study.

Gene	Primers		Size (bp)	Reference
	Forward	Reverse		
lasB ELA520	ACATCGCCCAACTGGTCTAC	ACCAGCGGATAGAACATGGT	520	
*toxA*ETA397	ATGGTGTAGATCGGCGACAT	AAGCCTTCGACCTCTGGAAC	397	(Shi et al., 2012)
phzM PYO330	CGGCGAAGACTTCTACAGCT	AGGTAGATATCGCCGTTGGA	330	
*ecfX*ECF164	ATGCCTATCAGGCGTTCCAT	GGCGATCTGGAAAAGAAATG	164	
lasR	ATCTTGTGGGCTGACTGGAC	CTGCGGCAGTCGTTTCGAGAAT	450	(Köhler et al., 2010)
rhlR	ACGGTGCTGGCATAACAGATAGG	CCTCTCAGTCGGAGGACATACCA	900	
PlcH	GAAGCCATGGGCTACTTCAA	AGAGTGACGAGGAGCGGTAG	307	(Ullah et al., 2017)
PA-GS 16sRNA	GACGGGTGAGTAATGCCTA	CACTGGTGTTCCTTCCTATA	599	(Ardura et al., 2013)

Table 3
The coinfection of *Prototheca* spp. with other nosocomial pathogens.

Prototheca spp.	*P. aeruginosa*		*E. coli*		*S. aureus*		*S. pyogenes*		*A. baumannii*		Total		p-Value
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
P. zopfii genotype 1	29	19.1	15	9.9	13	8.6	7	4.6	5	3.3	69	45.4	>0.05
P. zopfii genotype 2	23	15.1	11	7.2	6	3.9	2	1.3	0	0.0	42	27.6	
P. stagnora	11	7.2	3	2.0	1	o.7	2	1.3	1	0.7	18	11.8	
P. blaschkeae	14	9.2	4	2.6	3	2.0	0	0.0	2	1.3	23	15.1	
Total	77	50.7	33	21.7	23	15.1	11	7.2	8	5.3	152.0	100.0	

Table 4
Carriage of *P. aeruginosa* virulence genes by *Prototheca* spp. isolated from clinical samples.

Type of *Prototheca*	*P. aeruginosa* virulence genes																p-Value
	PA-GS 16sRNA		*lasB ELA*		*toxAETA*		*phzM PYO*		*ecfXECF*		*lasR*		*rhlR*		*PlcH*		
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
P. zopfii genotype 1	29	24.8	21	31.8	24	32.0	22	37.9	20	31.3	20	41.7	18	39.1	16	38.1	0.047
P. zopfii genotype 2	23	19.7	14	21.2	16	21.3	11	19.0	17	26.6	12	25.0	9	19.6	11	26.2	
P. stagnora	11	9.4	8	12.1	11	14.7	6	10.3	7	10.9	5	10.4	9	19.6	3	7.1	
P. blaschkeae	14	12.0	12	18.2	16	21.3	10	17.2	7	10.9	6	12.5	8	17.4	4	9.5	
Non- *Prototheca spp.*	40	34.2	11	16.7	8	10.7	9	15.5	13	20.3	5	10.4	2	4.3	8	19.0	
Total	117	100	66	100	75	100	58	100	64	100	48	100	46	100	42	100	

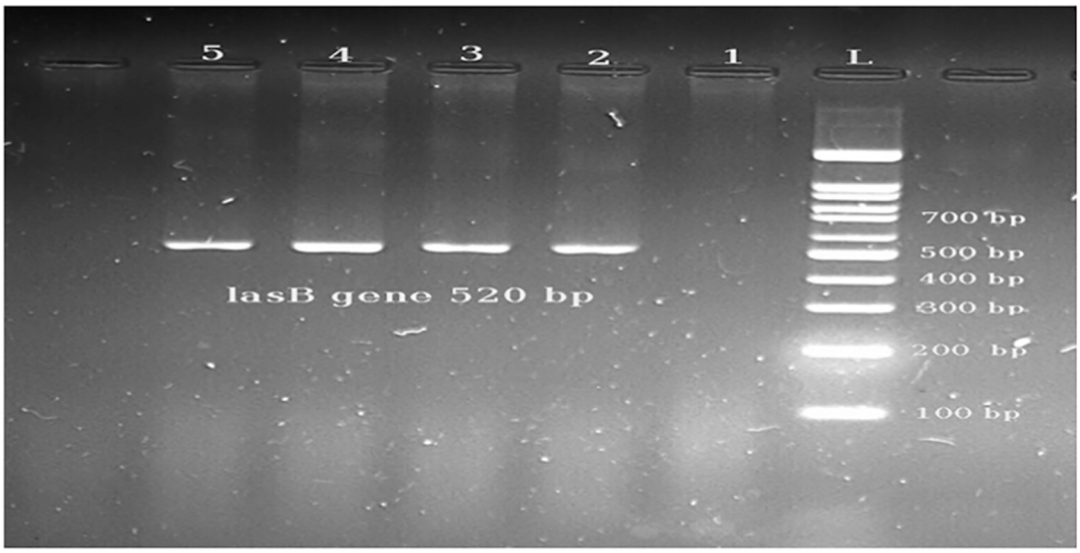

Fig. 1. The electrophoresis of *lasB* gene in 5% NuSieve® 3:1 agarose gel in 1× TBE (Tris borate ethylene di-amide tetra acetic acid) buffer containing 5 μL red safe; L lane exhibits 100 bp DNA Ladder, well 1: negative control, well 2: positive control and wells 3–5: positive samples with 520 bp size.

ecfXECF164 genes has been depicted in Fig. 3.

Moreover, the electrophoresis of the *PA-GS* 16 s RNA gene has been depicted in Fig. 4.

4. Discussion

In this study, the *Prototheca* spp. were mostly isolated from UTI (n = 82, 49%) and burns (n = 94, 53%). Prototheca spp. have been isolated from infections. The rate of infections caused by *Prototheca* spp. has been different in various studies possibly due to sources of isolation, geographical or epidemiological differences, number of samples, the degree of hygiene and the type of sterilizers and disinfectants in the hospitals, as well as body neutrophils which play an important role in host defense against different species of *Prototheca*. Prototheca spp. are opportunistic pathogens which carry various virulence factors (Sari et al., 2018).

Our results revealed that *Prototheca* infections were associated with *P. aeruginosa* infection, where the highest percentage of infection was regarding *P. zopfii* genotype 1 (45%), followed by *P. zopfii* genotype 2 (27.6%), and *P. blaschkeae* infection by (15.1%) and *P. stagnora* (11.8%). The coinfection of *P. zopfii* genotype 1, *P. zopfii* genotype 2, *P. stagnora* and *P. blaschkeae* were with *P. aeruginosa* included 19.1%, 15.1%, 7.2%, 9.2%, respectively. According to the statistical analysis of the results there was no significant relation regarding co-infections (p-value > 0.05). Other coinfections with *Prototheca* spp. included *E. coli* (n = 33, 21.7%), *S. aureus* (n = 33, 15.1%), *S. pyogenes* (n = 11, 7.2%) and *A. baumannii* (n = 8, 5.3%).

There are reports that patients with immunocompromised have neutrophils unable to kill *Prototheca* spp. (Phair et al., 1981; Sari et al., 2018).

Our results outlined that the *lasR*, *rhlR* and *plcH* genes were predominant virulence genes in *Prototheca* spp. Other virulence factors included *PA-GS*, 16s*RNA*, *lasB*, *ELA520*, *toxAETA*, *phzM PYO* and *ecfXECF* genes. There is no previous data regarding Prototheca spp. rate

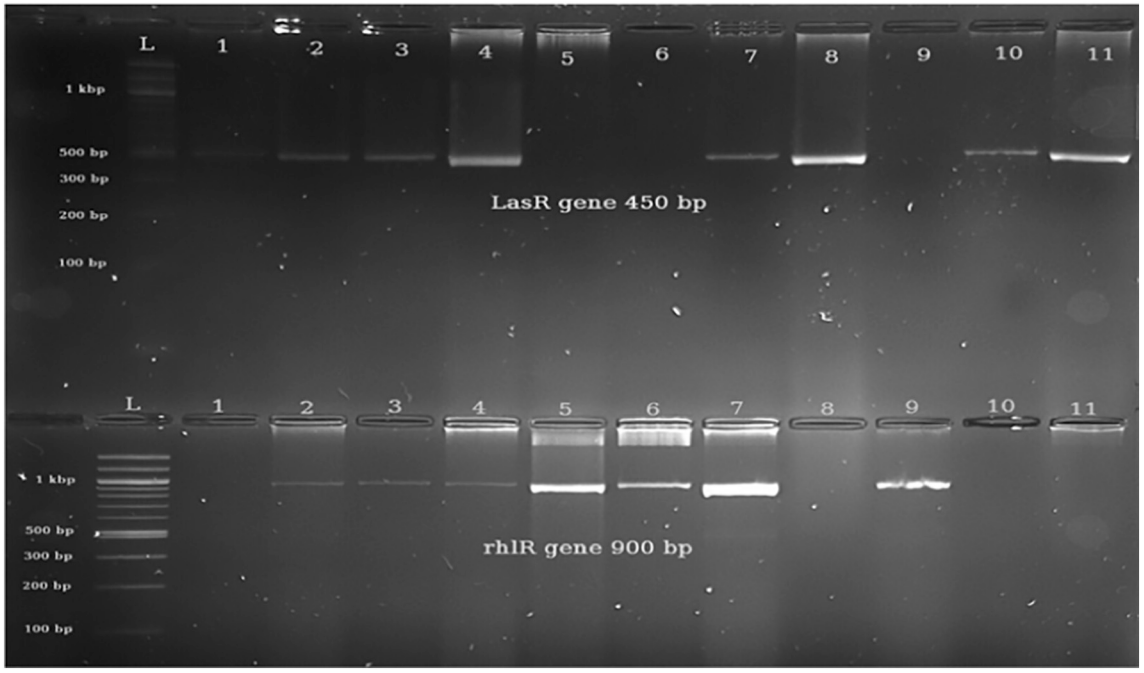

Fig. 2. The electrophoresis of *lasR* gene and *rhlR* products; well L: the 100 bp DNA Ladder, upper well 1: positive control, wells 2–4, 7, 8, 10, 11: *lasR* positive samples with 450 bp well 5: negative control, low wells 1 and 2: negative and positive control, respectively, 3–9: *rhlR* positive samples with 900 bp size.

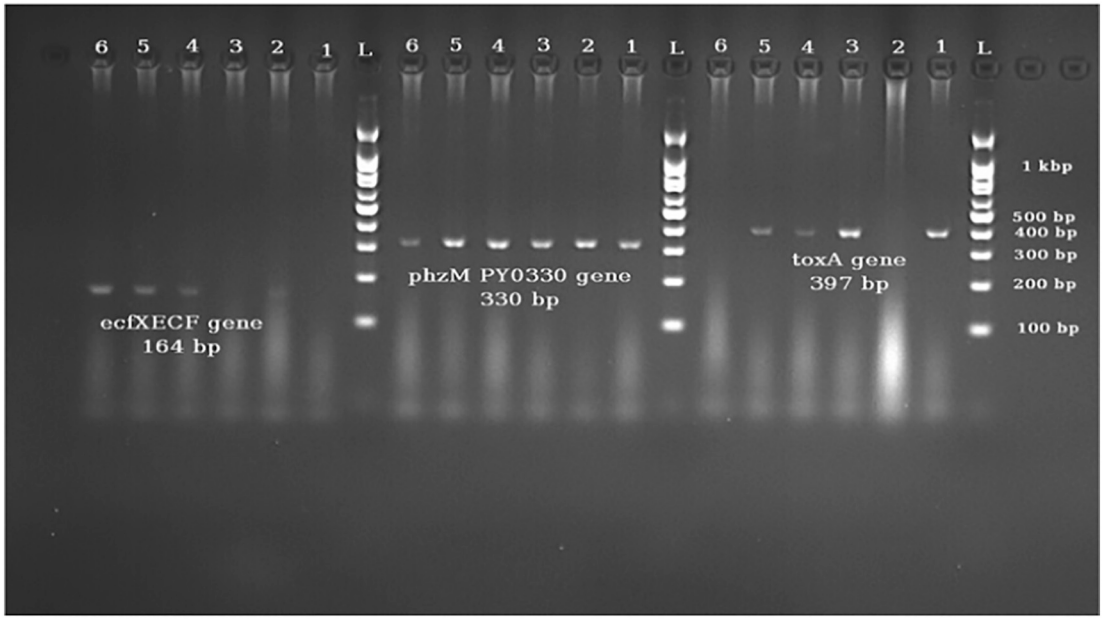

Fig. 3. The electrophoresis of *toxA*, *phzM* and *ecfXECF* gene products; from right hand: L well the 100 bp DNA Ladder, wells 1 and 2: positive and negative control, respectively, wells 3–5: *tox A* gene positive samples with 397 bp size; Center: well 1 exhibits positive control and wells 2–6: positive samples with 330 bp size; Left: wells 1 and 2: negative and positive control respectively and wells 4–6: positive samples with 164 bp size.

and virulence factors in Iraq. Among *Prototheca* spp., *P. zopfii* genotype 1, *P. zopfii* genotype 2, *P. stagnora* and *P. blaschkeae*, respectively mostly carried *lasR* (41.7%), *ecfXECF164* (26.6%), *rhlR* (19.6%), *toxAETA* (21.3%) with significant difference ($p = 0.047$). We suppose that *Prototheca* spp. infections are always associated with other pathogens, especially *P. aeruginosa* due to lethal agents, paving the way for the proliferation of *Prototheca* spp.

The *lasB* gene encodes one of substantial extracellular virulence factors, elastase classified as one of the proteolytic enzymes. *LasB* gene plays an essential function in the pathogenicity of *P. aeruginosa* through host infection (Caiazza et al., 2005). The reason of increases its ferocity attributed to interaction with Proteins of the immune defense system, especially the multi-nuclei white blood cells, natural killer cells and IgA IgG immunoglobulins. Thus, strains of *P. aeruginosa*, which produce this enzyme, regulate the immune, making the body more susceptible to infection with other microorganisms such as *Prototheca* (Cotar et al., 2010). The *LasR* protein is a pivotal transcriptional activator of *P. aeruginosa* quorum sensing (QS) and plays a fundamental function in the activation of many virulence genes. Repeatedly LasR mutated, which helps to continuation bacterial infection and thus have severe clinical

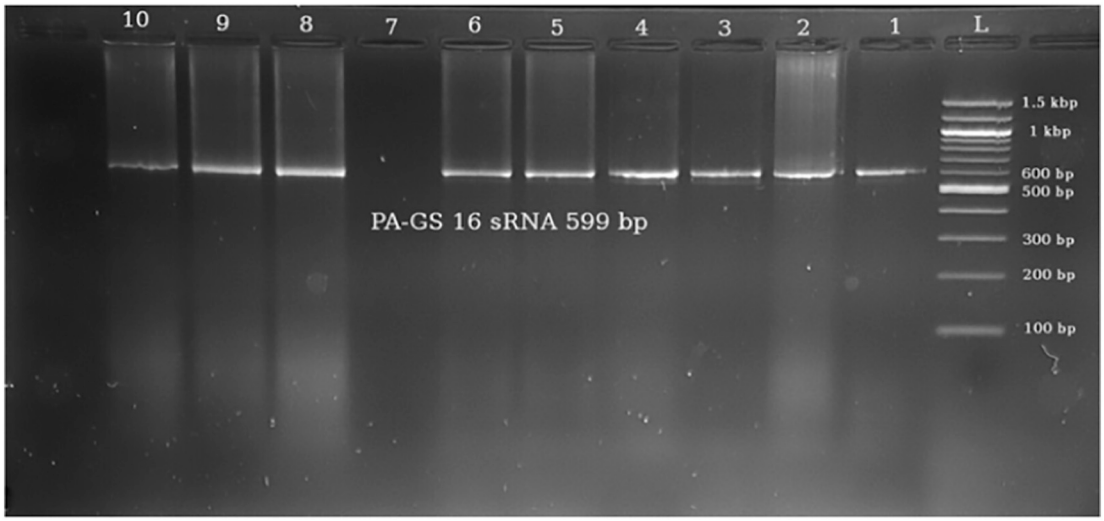

Fig. 4. The electrophoresis of products; well L: 100 bp DNA marker, well 1: positive control, wells 2–6 and 8–10: positive samples with 307 bp.

effects (Smith et al., 2006). While, *rhlR* is a virulence factor playing an important role in the pathogenicity of *P. aeruginosa* (Jimenez et al., 2012).

ToxAETA397 gene, phzM PYO330 gene and ecfXECF164: It can be described as the most deadly virulence factor and toxicity of the product by *P. aeruginosa*, which is considered a serious and often fatal inflammatory agent for the vast number of patients, the main function of this gene repression of protein synthesis, when it enters cytoplasm of eukaryotic cells through the protein part the molecular weight of 37 k Dalton, which works on the substance represented by of the elongation factor 2 which is In turn prevents elongation of the protein chain on the ribosome and eventual cell death (El-Zaim et al., 1998), It also has a practical role to grunt tissues and reduce phagocytosis in people with severe burns (Pillar and Hobden, 2002). As for the *phzM* gene can be seen as an important virulence factor in *P. aeruginosa*, which is involved in the bio cyanine biosynthesis process, which plays an important role in inhibiting the growth of other microorganisms, as well as its negative impact on the phagocytic cells in patients (Wang et al., 2012). While *ecfX* gene Considered is a kind of accurate diagnostic genes to *P. aeruginosa*, have extra cytoplasmic function encodes sigma factor which is restricted to *P. aeruginosa* as well might play major role in haem-uptake and the spread of bacteria in patients (Lavenir et al., 2007). The *plcH* gene is very important for *P. aeruginosa* playing a master role in hemolysis, interaction with host via protein secreted by type II secretion system, lipid catabolic process, pathogenesis and protein transport by the Tat complex (Hogan and Kolter, 2002). Identification of *P. aeruginosa* virulence genes among Prototheca spp. highlighted their role in severe infections.

5. Conclusion

This study revealed the existence of *Prototheca* spp. carrying various virulence genes mostly among burns and UTI in Hillah city, Iraq. Bacterial co-infections exacerbate the severity of infections, particularly those potential nosocomial pathogens. Identification of *P. aeruginosa* virulence genes such as *PA-GS 16sRNA*, *lasB ELA520*, *toxAETA397*, *phzM PYO330*, *ecfXECF164*, *lasR*, *rhlR*, *PlcH* among *Prototheca* spp. highlighted their role in severe infections.

Declaration of competing interest

This research has not been submitted for publication nor has it been published in whole or in part elsewhere. We attest to the fact that all authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission.

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