



Detection of Virulence factors of *Pseudomonas* species isolated from fresh water fish by PCR

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ABSTRACT

This study was conducted on 100 diseased Nile tilapia (*O. niloticus*) fish of various sizes collected from different fish markets in Kaliobia Governorate to estimate the prevalence of *Pseudomonas* infection and detection of some virulence genes in the isolated *P. aeruginosa* strains. The results of bacteriological examination revealed that the prevalence of *Pseudomonas* septicemia with *Pseudomonas* species isolation was 17.0% (17 \ 100 examined fish). These cases were attributed to *P. anguilliseptica*; *P. aeruginosa* and *P. fluorescens* (14/43.7%; 12/37.5% and 6/18.8%), respectively. In addition, 32 *Pseudomonas* species were isolated, 11 from liver samples (34.4%); 10 from kidney samples (31.2%); 6 from gill samples (18.8%) and 5 from skin samples (15.6%). Moreover, 14 *P. anguilliseptica* were isolated with an incidence of 35.7%, 28.6%, 21.4% and 14.3% followed by 12 *P. aeruginosa* 33.3%, 25%, 16.7% and 25% respectively; 6 *P. fluorescens* 33.3%; 50.0%, 16.7% and 0.0% from the liver, kidney, gill and skin samples respectively. The in-vitro antimicrobial sensitivity test showed that the isolated *Pseudomonas* strains were sensitive to gentamycin; enrofloxacin; norfloxacin; ciprofloxacin and florphenicol. Meanwhile; they were intermediate sensitive for doxycycline; sulfa-trimethoprim; oxytetracycline; nalidixic acid and streptomycin. In contrast, they were resistant for cefotaxime; erythromycin; amoxicillin; methicillin; oxacillin and ampicillin. Moreover, the PCR results revealed that, *opr L* and *exo S* virulence genes were detected in all six studied strains (100.0%). Meanwhile, *phz M* virulence gene was detected in 5 out of 6 studied strains (83.3%) and *tox A* virulence gene was detected in 4 out of 6 studied strains (66.7%) i.e., all studied strains were *Ps. aeruginosa* and all of them were virulent strains.

Key words: Fish, bacteriological evaluation, *Pseudomonas* species, PCR, *oprL*, *exoS* genes.

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1. INTRODUCTION

Bacterial Fish diseases were considered the main cause of high mortalities and economic losses among fish and fish farms (Austin and Austin, 2007). *Pseudomonas* are considered one of the most important fish pathogens which are responsible for ulcer type diseases including ulcerative syndrome, *Pseudomonas* septicemia (Wiklund and Bylund, 1990 and El-Nagar, 2010). The disease is characterized by petechial hemorrhage, darkness of the skin, detached scales, abdominal ascites and exophthalmia (Ilhan et al., 2006; and Hanna

et al., 2014). Moreover, *Pseudomonas* can cause a problem for human consumers too, generally caused by only one species (most frequently *Ps. aeruginosa*), cause health-care associated illnesses (Bagshaw et al., 2006 and Zilberberg and Shorr, 2009). Contamination with enterotoxigenic *Pseudomonas* has been reported from fish, food and drinking water resulting in diarrhoea and skin infections in immuno deficient individuals (Adlard et al., 1998 and Wong et al., 2000). The genus *pseudomonas* is the most heterogeneous and ecologically significant group of known

bacteria, and includes Gram-negative rods that are straight or slightly curved and are 0.5 – 1.0µm, strict aerobic, non-spore forming, motile, oxidative, catalase-positive and oxidase-positive. Their nutritional requirements are very simple and most will grow on MacConkey agar as lactose non-fermenters as well as converting nitrate to nitrite or nitrogen gas. Other characteristics that tend to be associated with *Pseudomonas* species (with some exceptions) include secretion of pyoverdine, a fluorescent yellow-green siderophore under iron-limiting conditions. Certain *Pseudomonas* species may also produce additional types of siderophore, such as pyocyanin by *P. aeruginosa* and thioquinolobactin by *P. fluorescens* (Markey et al. 2013 and Public Health England 2015). The most prevailing *Pseudomonas* species affecting fish are *P. fluorescens*, *P. anguilliseptica*, *P. putida*, *P. putrefaciens* and *P. aeruginosa* (Atwa, 2007; Sakr and Abd El-Rahman, 2008; Abd-Elghany et al. 2009 ; El-Hady and Samy, 2011 and Abdullahi et al. 2013). *P. aeruginosa* possesses cell-associated virulence factors such as pili; flagella; lipopolysaccharide and alginate/biofilm. It also produces a number of extracellular products such as protein exotoxin A; proteases; type III secretion system exoenzymes; rhamnolipid, haemolysin with lecithinase activity; elastase (Las B and Las A); siderophores (pyochelin, pyocyanin, and pyoverdine); phospholipase C and able to develop resistance to a wide variety of antimicrobial agents (Delden and Iglewski, 1998; Mavrodi et al. 2001 and Markey et al. 2013). As *pseudomonas* is considered one of the most important fish pathogens and can be a problem for human consumers too and *Tilapia* fish had attained a great economic importance in Egypt, so, the present study was conducted to estimate the prevalence of *Pseudomonas* infection in fresh water fish, Nile tilapia fish (*O. Niloticus*) at Kaliobia governorate. To clarify the phenotypic characterization of isolated *Pseudomonas*

strains and to carry out the antimicrobial sensitivity testing of them. In addition, detection of some virulence factors of *P. aeruginosa* by PCR technique.

2. MATERIAL AND METHODS

2.1. Samples collection:

A total of 100 diseased Nile tilapia (*O. niloticus*) fish of various sizes were collected from different fish markets in Kaliobia Governorate. Each examined sample was taken alone in strong sterile plastic bag, kept in icebox and transferred with minimum delay to the laboratory for clinical and bacteriological examination.

2.2. Clinical and postmortem examinations were performed using the method described by Schaperdaus et al. (1992).

2.3. Bacteriological examination

2.3.1. Sampling:

A total of 400 samples were taken from 100 diseased fishes (liver; kidney; gills and skin from each one) after clinical and postmortem examination. The surface of skin and organs were seared by hot spatula, then a sterilized loopful was introduced through seared portion. Loopfuls were taken from liver; kidney; gills and skin then inoculated in Tryptone soya broth then incubated aerobically at 25°C for 24 hours.

2.3.2. Isolation and identification of suspected *Pseudomonas* species:

A loopful from incubated Tryptone soya broth was streaked onto nutrient agar and incubated aerobically at 25°C for 24 hours. Suspected colonies were picked up and purified by further sub culturing on nutrient agar. Suspected purified colonies were picked up and streaked on the following media: MacConkey's agar; *Pseudomonas* agar; *Pseudomonas* Cetrimide agar; blood agar and Milk agar and incubated for another 24-48 hours at 25°C. Suspected colonies were picked up and kept in semi-solid nutrient agar. The purified isolates of *Pseudomonas* species were

morphologically identified by Gram stain and biochemical tests according to Quinn et al. (2002); Austin and Austin (2007) and Markey et al. (2013) and through using API 20 E test (BioMérieux, France)

2.3.3. *In-Vitro anti-microbial sensitivity test:*

In-Vitro sensitivity test was done for the isolated *Pseudomonas* species according to Koneman et al. (1997) and interpretation of results were carried out according to NCCLS (2002).

2.3.4. *Genotypic detection of virulence genes in P. aeruginosa using polymerase chain reaction (PCR)*

Four sets of primers were used for genotypic detection of four virulence genes that may play a role in virulence of *P. aeruginosa*. These genes were outer membrane lipoprotein L (*oprL*); exotoxin S gene (*exoS*); phenazine-modifying gene (*phzM*) and exotoxin A gene (*toxA*). It was applied on 6 random isolated *P. aeruginosa*, following QIAamp® DNA Mini Kit instructions (Catalogue no. 51304), Emerald Amp GT PCR mastermix (Takara) with Code No. RR310A and 1,5% agarose gel electrophoreses (Sambrook et al., 1989).

3. RESULTS

The clinical examination of studied fish showed irregular hemorrhages all over the fish body especially at the ventral part of abdomen, base of the fins. Some fish showed congestion in the fins, loss of fin membrane and sometimes loss of fin rays with grey patches at the tip of them (fins rot). Others showed eye cloudiness, detachment of scales and skin ulceration and abdominal distention. Internally these fishes showed abdominal dropsy with reddish ascetic exudates, liver paleness and enlargement in some fishes and congested with necrotic patches in other fishes; congested kidneys; congested and enlarged spleen and hemorrhagic enteritis.

The bacteriological examination of studied samples revealed that the prevalence of *Pseudomonas* septicemia with *Pseudomonas* species isolation was 17.0% (17 \ 100 examined fish) and 4.3% (17 \ 400 examined samples). A total of 32 *Pseudomonas* species were isolated, 14 *P. anguilliseptica* (43.7%); 12 *P. aeruginosa* (37.5%) and 6 *P. fluorescens* (18.8%) (Table, 1). These species were isolated from liver samples 11 (34.4%); kidney samples 10 (31.2%); gill samples 6 (18.8%) and skin samples 5 (15.6%). Moreover, 14 *P. anguilliseptica* were isolated with incidence of 35.7%, 28.6%, 21.4% and 14.3% respectively; followed by 12 *P. aeruginosa* 33.3%, 25%, 16.7% and 25% respectively; 6 *P. fluorescens* 33.3%; 50.0%, 16.7% and 0.0% respectively, from liver, kidney, gill and skin samples, respectively. The recovered isolates grow well and showed large, flat, spreading and irregular colonies with greenish-blue coloration on the culture with a characteristic fruity, grape-like odour of aminoacetophenone (*P. aeruginosa*); yellowish colonies (*P. fluorescens*); pale colonies (*P. anguilliseptica*) on nutrient agar. Large, pale colonies on MacConkey's agar (unable to utilize lactose) with greenish-blue pigment superimposed. Yellowish green colonies *P. fluorescens*, bluish green colonies *P. aeruginosa* and non-pigmented colonies *P. anguilliseptica*, on *Pseudomonas* agar. Small and smooth with blue – green pigmented colonies, *P. aeruginosa* on *Pseudomonas* Cetrimide agar. Irregular colonies surround by a clear zone of beta haemolysis, *P. aeruginosa* on blood agar. On milk agar protease production was shown by the formation of a clear zone caused by casein degradation. All the pseudomonads are medium-sized Gram-negative, straight or slightly curved rods, motile by polar flagella, non-sporulated, non-capsulated and non-sporulated.

The results of in vitro sensitivity test Table (2) showed that the isolated *Pseudomonas* strains were sensitive to gentamycin (93.8%); enrofloxacin (90.6%), norfloxacin

(84.4%); ciprofloxacin (75.0%) and florphenicol (71.9%). Meanwhile, they were intermediate sensitive for doxycycline (65.6%); sulfa-trimethoprim (65.6%); oxytetracycline (56.2%); nalidixic acid and streptomycin (53.1%) for each. In contrast, they were resistant for cefotaxime (75.0%); erythromycin (68.8%); amoxicillin; methicillin; oxacillin with (84.4%) for each and ampicillin (87.5%).

The PCR results for *P. aeruginosa* showed that, *opr L* and *exo S* virulence genes were detected in all six studied strains (100.0%). Meanwhile, *phz M* virulence gene was detected in 5 out of 6 studied strains

(83.3%) and *tox A* virulence gene was detected in 4 out of 6 studied strains (66.7%) as shown in Table (3). The *oprL* gene was amplified in all six studied strains (100.0%) giving product of 504 bp as shown in Fig. (1- a). The *exo S* gene was amplified in all six studied strains (100.0%) giving product of 118 bp as shown in Fig. (1- b). The *phzM* gene was amplified in 5 out of 6 studied strains (83.3%) giving product of 875 bp as shown in Fig. (2- a). The *tox A* gene was amplified in four out of 6 studied strains (66.7%) giving product of 396 bp as shown in Fig. (2- b).

Table (1): Prevalence of *Pseudomonas* species isolated from different organs of studied fishes

Pseudomonas species	Positive Samples									
	Liver		Kidneys		Gills		Skin		Total	
	No.	% ¹	No.	% ¹	No.	% ¹	No.	% ¹	No.	% ²
<i>P. anguilliseptica</i>	5	35.7	4	28.6	3	21.4	2	14.3	14	43.7
<i>P. aeruginosa</i>	4	33.3	3	25.0	2	16.7	3	25.0	12	37.5
<i>P. fluorescens</i>	2	33.3	3	50.0	1	16.7	0	0.0	6	18.8
Total	11	34.4	10	31.2	6	18.8	5	15.6	32	100.0

%¹ Percentage in relation to no. of each isolated *Pseudomonas* species in each row. %² Percentage in relation to no. of isolated *Pseudomonas* species (32)

Table (2): In-Vitro anti-microbial Sensitivity test for isolated *Pseudomonas* strains

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		AA
		No.	%	No.	%	No.	%	
Amoxicillin	25µg	4	12.5	1	3.1	27	84.4	R
Ampicillin	20 µg	3	9.4	1	3.1	28	87.5	R
Cefotaxime	30 µg	5	15.6	3	9.4	24	75.0	R
Ciprofloxacin	5 µg	24	75.0	2	6.2	6	18.8	S
Doxycycline	30 µg	5	15.6	21	65.6	6	18.8	IS
Enrofloxacin	5 µg	29	90.6	0	0.0	3	9.4	S
Erythromycin	15 µg	5	15.6	5	15.6	22	68.8	R
Florphenicol	30 µg	23	71.9	4	12.5	5	15.6	S
Gentamicin	10 µg	30	93.8	1	3.1	1	3.1	S
Methicillin	5 µg	3	9.4	2	6.2	27	84.4	R
Nalidixic acid	30 µg	4	12.5	17	53.1	11	34.4	IS
Norfloxacin	10 µg	27	84.4	2	6.2	3	9.4	S
Oxacillin	1 µg	4	12.5	1	3.1	27	84.4	R
Oxytetracycline	30 µg	4	12.5	18	56.2	10	31.2	IS
Streptomycin	10 µg	3	9.4	17	53.1	12	37.5	IS
Sulfa-trimethoprim	1.25 µg	3	9.4	21	65.6	8	25.0	IS

No.: Number of isolates. %: Percentage in relation to total number of isolates (32). AA: Antibiogram activity.

Table (3): The results of PCR amplifications of different virulence genes of *P. aeruginosa*

Serial	Virulence genes			
	<i>oprL</i>	<i>exoS</i>	<i>toxA</i>	<i>phzM</i>
1	+	+	-	+
2	+	+	+	-
3	+	+	-	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
Total NO.	6	6	4	5
%	100	100	66.7	83.3

- *oprL* (outer membrane lipoprotein L) gene. • *exoS* (exotoxin S) gene.
- *toxA* (exotoxin A) gene. • *phzM* (phenazine-modifying) gene.

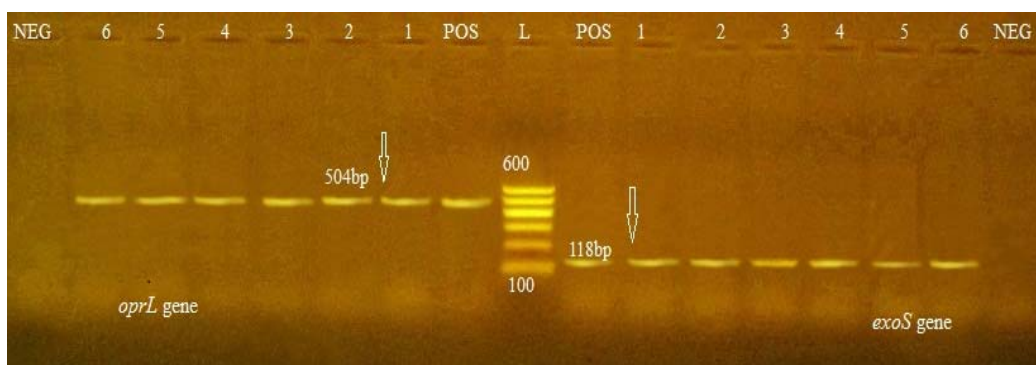


Fig. (1) Agarose gel electrophoresis of PCR amplification of *Ps.aeruginosa* extracted DNA. (a) outer membrane lipoprotein L (*oprL*) gene. (b) exotoxin S (*exoS*) gene. Lane L: 100-600 bp DNA Ladder. Lane L: 100-600 bp DNA. Neg.: Negative control. Neg.: Negative control. Pos.: Positive control (at 504 bp). Pos.: Positive control (at 118 bp). Lane 1- 6: *P. aeruginosa* (Positive). Lane1- 6: *P. aeruginosa* (Positive).

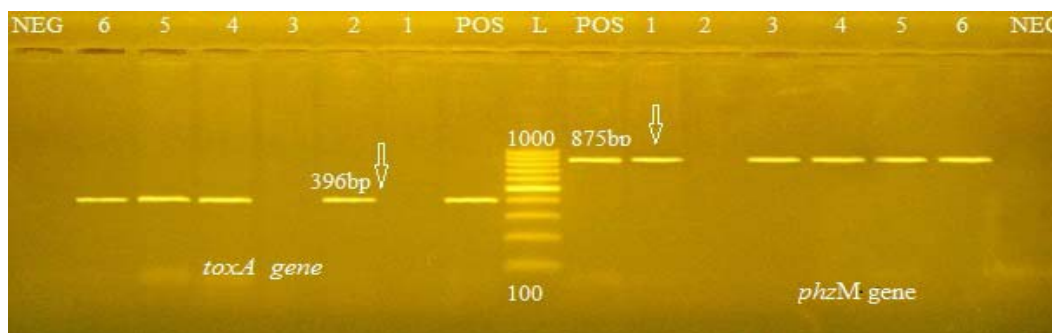


Fig. (2) Agarose gel electrophoresis of PCR amplification of *Ps. aeruginosa* extracted DNA (a) exotoxin A (*toxA*) gene. (b) phenazine-modifying (*phzM*)gene. Lane L: 100-1000bpDNA Ladder. Lane L: 100-1000bpDNA. Neg.: Negative control. Neg.: Negative control. Pos.: Positive control (at 396 bp). Pos.: Positive control (at 875 bp). Lane 1; & 3: *P. aeruginosa* (Negative). Lane 2: *P. aeruginosa* (Negative). Lane 2;4;5& 6: *P. aeruginosa* (Positive). Lane1;3;4;5& 6: *P. aeruginosa* (Positive).

4. DISCUSSION

Pseudomonas species is widely distributed in natural sources of water and considered as one of the primary cause of septicemia in both freshwater and marine fish throughout the world and causes severe economic losses and decreases fish farm efficiencies (Olsson et al., 1998 and Roberts, 2001). The results of clinical and postmortem examinations of studied fish were similar to that reported by Atwa (2007); Sakr and Abd El-Rhman(2008); Khalil et al.,(2010) and Hanna et al.,(2014). The prevalence of *Pseudomonas* septicemia with *Pseudomonas* species isolation revealed that, 17 out of 100 examined fish (17.0%) and 17 out of 400 examined samples (4.3%) were positive for *Pseudomonas* species isolation. These results came in accordance with those obtained by Atwa (2007) and Saad El-Deen (2014). Meanwhile, such results disagreed with others which recorded at higher incidence, {(El-Hady and Samy, 2011(55.3%); Ragab, 2011(47.3%) and Hanna et al., 2014 (34.4%)}. The results of bacteriological examination (Table, 1) revealed that *Pseudomonas* septicemia among examined fish was attributed to *P. anguilliseptica*; *P. aeruginosa* and *P. fluorescens* (14/43.7%; 12/37.5% and 6/18.8%) respectively. These results agree with those of Atwa (2007); Sakr and Abd El-Rhman (2008); Khalil et al. (2010); Mesalhy (2013) and Hanna et al. (2014). Also, 32 *Pseudomonas* species were isolated, 11 from liver samples (34.4%); 10 from kidney samples (31.2%); 6 from gill samples (18.8%) and 5 from skin samples (15.6%). Moreover, the table demonstrated that, 14 *P. anguilliseptica* were isolated with incidence of 35.7%, 28.6%, 21.4% and 14.3%; followed by 12 *P. aeruginosa* 33.3%, 25%, 16.7% and 25%. 6 *P. fluorescens* 33.3%; 50.0%, 16.7% and 0.0%, from liver, kidney, gill and skin samples respectively. Nearly similar results were recorded by Atwa (2007); Sakr and Abd El-Rhman (2008); Eissa et al. (2010); Khalil et

al. (2010); El-Hady and Samy (2011) and Hanna et al. (2014). The morphological characteristics of the culture, Gram staining and the biochemical profile of *Pseudomonas* species isolated such as the fermentation of certain sugars or enzymatic reaction as protease; lipase; extracellular pigmentation production and was similar to those previously reported (Buller, 2004; Austin and Austin, 2007; Jvo, 010; Markey et al. 2013; Panda et al. 2013; Hanna et al. 2014 and Darak and Barde, 2015). Moreover, the results of API 20E test for isolated strains showed characteristic identical biochemical reaction to be *Pseudomonas* species.

The results of in-vitro sensitivity test for the isolated *Pseudomonas* strains Table (2) revealed that, the isolated *Pseudomonas* strains were sensitive to gentamycin; enrofloxacin; norfloxacin; ciprofloxacin and florphenicol. Meanwhile, they were intermediate sensitive for doxycycline; sulfa-trimethoprim; oxytetracycline; nalidixic acid and streptomycin. In contrast, they were resistant for cefotaxime; erythromycin; amoxicillin; methicillin; oxacillin and ampicillin. Nearly similar results were recorded by Atwa (2007); Saleh et al. (2008); Eissa et al. (2010); El-Hady and Samy (2011); Hanna et al. (2014) and Roy et al. (2014) .

The PCR results for *P. aeruginosa* virulent genes showed that, *oprL* and *exoS* virulence genes were detected in all six studied strains (100.0%). Meanwhile, *phzM* virulence gene was detected in 5 out of 6 studied strains (83.3%) and *toxA* virulence gene was detected in 4 out of 6 studied strains (66.7%) as shown in Table (3). These results were decided by (Somerville et al., 1999; Todar, 2011; Nafee ,2012; Nowroozi et al., 2012 and Markey et al. (2013)). They reported that all of these virulence factors participate in specific ways in the infection process, and in addition, each may affect host cell signal transduction in ways that enhance the spread of infection. Regarding to the occurrence of outer membrane

lipoprotein L (*oprL*) gene in *P. aeruginosa* isolates.

The obtained result revealed that it was amplified in all six studied strains (100.0%) giving product of 504 bp as shown in Fig. (1- a). These results came in accordance with those recorded by Abdullahi et al. (2013). The results of PCR for amplification of exotoxin S (*exoS*) gene in *P. aeruginosa* isolates (Fig., 1- b) showed that, the *exoS* gene was amplified in all six studied strains (100.0%) giving product of 118 bp. Similar findings were recorded by Finnan et al. (2004) and Todar, (2011).

Regarding to the occurrence of phenazine-modifying gene (*phzM*) gene in *P. aeruginosa* isolates. the obtained result revealed that the *phzM* gene was amplified in 5 out of 6 studied strains (83.3%) giving product of 875 bp as shown in Fig. (2- b). One strain showed absence of *phzM* gene. These results were agreed with those obtained by Nowroozi et al. (2012) who failed to detect *phzM* gene in 2 strains of *P. aeruginosa* and Igbinsosa et al. (2014) who failed to detect *phzM* gene in all studied *P. aeruginosa* and decided the fact of Mavrodi et al. (2001) and Nowroozi et al. (2012) who reported that some strains of *P. aeruginosa* do not produce phenazine pigment due to absence of the possible protein band of the *phzM* gene or did not have phenazine modifying gene bands. The results of PCR for amplification of exotoxin A (*toxA*) gene of *P. aeruginosa* (Fig., 2-a) revealed that the *toxA* gene was amplified in four out of 6 studied strains (66.7%) giving product of 396 bp. These results were agreed with those obtained by Somerville et al. (1999) and Nafee (2012).

Finally, one could be concluded that *Pseudomonas* species specially, *P. aeruginosa* are important pathogens causes *Pseudomonas* septicemia in fish. The isolated *Pseudomonas* species were sensitive to gentamycin; enrofloxacin; norfloxacin; ciprofloxacin and florphenicol, can be used for treatment of these cases and were resistant to cefotaxime; erythromycin;

amoxicillin; methicillin; oxacillin and ampicillin. In addition, PCR indicated that *oprL* and *exoS* virulence genes were detected in all six studied *Ps. aeruginosa* strains (100.0%). Meanwhile, *phzM* virulence gene was detected in 5 out of 6 studied strains (83.3%) and *toxA* virulence gene was detected in 4 out of 6 studied strains (66.7%).

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