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Investigation of Phospholipase C Activity of Pseudomonas Species Isolated from Water and Soil Samples by Different Methods

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Abstract

Pseudomonas bacteria, which are widespread in nature and the bacteria can be isolated from soil and water. Among the members of the genus Pseudomonas, the species most commonly isolated from natural samples is *P. aeruginosa.* These bacteria have minimal nutritional needs and have many virulence factors. Phospholipase C (PLC), which is one of the important virulence factors and a heat-sensitive protein, may damage lipids and lecithin and cause necrosis in tissue. PLC-H (hemolytic) and PLC-N (nonhemolytic) break phosphatidyl choline into diacyl glycerol and choline. In our study, the presence of PLC was investigated in a total of 43 strains Pseudomonas isolated from soil and water samples. Two methods were used for the investigation of phospholipase C. In the first method, p-nitrophenylphosphorylcholine (NPPC) was used as substrate. In the second method, tryptic soy agar medium containing 8% egg was used. In the study, PLC activity was positive in 28 (65.11 %) of 43 natural samples according to first method. In addition, According to second method, PLC activity was found in 26 (60.46 %) of 43 natural isolates positive. Both methods are a fast and convenient option to determine the PLC properties of Pseudomonas strains in clinical laboratories.

Keywords: Nature, *Pseudomonas*, Phospholipase C

1. Introduction

Pseudomonas bacteria are the bacteria that are categorized in Pseudomonadaceae family. The genus that is commonly observed in soil and water covers many *Pseudomonas* species with gram-negative, nonfermentative and aerobic. Also, *Pseudomonas* bacteria can be found as a disease agent in humans, animals and plants (Palleroni, 2005).

Phospholipase C (PLC) is a substance that contains 1 rhamnose and 2 betahydroxidecanoic acids ad that weighs 78000 mol. *Pseudomonas* bacteria produce two different substances named PLC, which is a temperature sensitive protein with haemolysis feature and rhamnolipid, which is a temperature resistant glycolipids. These substances act together and damage lipid and lecithin and they may result in necrosis by increasing the tissue invasion in infections. Haemolytic phospholipase C (PLC-H), hydrolysis phosphatidylcholine and sphingomyelin while nonhemolytic phospholipase C (PLC-N), hydrolysis phosphatidylcholine and phosphatidylserine (Berka et al., 1981). It is reported to have a local effect in the diseases of patients with haemolytic characteristics, while it may colonize in respiratory tract of patients with cystic fibrosis. For this reason, in the pneumonias originating from *Pseudomonas*, it may result in the

surfactant deficiency, swelling, hemorrhagic necrosis, and tissue damages in lungs. Moreover, it brings about diseases by fractionating phospholipids, which are the membrane components (Pollack, 1995). *P. aeruginosa* creates rhamnolipid according to ambient conditions. Its hydrophilic part contains one or two rhamnose molecules and hydrophobic part creates biosurfactant in the structure of fatty acid. Rhamnolipid, as biosurfactants, are amphiphilic compositions lowering the surface and internal surface tension between solids, liquids, and gases. For this reason, they are used in eliminating the hydrocarbons with lower solubility from the ambient in the areas of industry and environment (Maier and Soberon-Chavez, 2000; Delden and Iglewski, 1998). *P.aeruginosa* is the important infection factor for the people with weak immune system and hospitalized patients. It is a significant opportunistic pathogen that causes respiratory tract, urinary, gastrointestinal and neural system infections, skin, eye, and bone infections such as bacteremia and septicemia (Gilligan, 1995). *Pseudomonas* bacteria cause infections in the patients with cystic fibrosis, weak defence mechanism, and those receiving corticosteroid, antibiotics, and chemotherapy. As *Pseudomonas* bacteria are the factors of high mortality and morbidity for the patients having hospital infections, they are listed among the important pathogens (Todar, 2004). Pseudomonas bacteria infect humans through the virulence factors they produce. Although the treatment of diseases varies depending on the diseases and infection regions, the virulence factors of Pseudomonas species have been reported to play a role in the treatment (Pollack, 1995). In addition to *P.aeruginosa* bacteria being a hospital infection factor, as it is hard to treat them yet they can survive at every ambient and resists antibiotics and disinfectants (Koneman et al., 1997). Pathogenesis of *P.aeruginosa* bacteria is dependent on many cellular products such as hemolysins (rhamnolipid, phospholipase C) and they are of a multifactorial character (Erdem, 1999). Besides called 'non-pathogenic' for P. aeruginosa from environmental samples (Ebadi et al., 2017) and studies in non-clinical settings continues to attract attention [11]. (Deredjian et al., 2014). *Pseudomonas* bacteria can survive between -7ºC and 43ºC while these bacteria can reproduce well at optimum pH 7. *P.aeruginosa* can adapt to ambient conditions and survive for a long period with sufficient moist and a small amount of nutrient. Further, they can also survive in such environments with NaCl at a minimum level (Palleroni, 1984; Gilligan, 1995). These bacteria have lower molecular weight. They do not require organic reproduction factors and they use more than thirty organic substances for their reproduction. Although *Pseudomonas* bacteria are aerobe, they can also reproduce as facultative anaerobe when nitrate and arginine exist (Koneman et al.,1997; Palleroni, 1984). Because of their ability to live in different environments, *P. aeruginosa* can live in a wide variety of ecological areas (Cabot et al., 2016) and *P. aeruginosa* isolates were obtained from plants, soil, water and clinical specimens. But it was found less frequently in arid or semi-arid regions (Schroth et al., 2018; Deredjian et al., 2014). Thanks to its metabolism, *P. fluorescence* can degrade chemicals that result in environmental pollution. They have also a toxic effect with the antimicrobial substances that they produce against plant pathogens. *P. putida* can be used for obtaining oil from microorganisms or in bioremediation. Further, they are effective in pesticide fractionation as biocontrol (Palleroni, 2005; Wang and Mulligan, 2009). In *Pseudomonas* strains isolated from nature samples, it was aimed to investigate the formation of phospholipase C activity, which is considered among the factors related to virulence.

2. Materials and Methods

2.1. Collection and identification of samples

The presence of PLC, one of the important virulence factors, was compared with different methods in Pseudomonas species isolated from soil and water samples. In this study, nature samples were collected from different parts of the southern Marmara region of Turkey. Pseudomonas isolates were researched in soil samples taken from Bilecik villages and water samples from the Hamsu river Iznik lake and Karasu in this region. In the study, total 43 *Pseudomonas* isolates were obtained from the soil and water samples taken from the areas. During the research, total 24 *Pseudomonas* isolates were found in the soil samples taken in the region. Further, total 19 *Pseudomonas* isolates were obtained from the water samples taken from the brooks and lakes in the region. The soil samples were taken from a 0-20 cm depth from the surface with a spatula and put in sterile containers of 100 ml. A sample weighing 10 grams was homogenized with 90 ml normal saline and their dilutions were prepared. In the study, the water samples were taken from brooks and the lake and put in sterile containers of 100 ml. A sample of 10 ml was homogenized with 90 ml normal saline and their dilutions were prepared. Both mixtures were maintained in refrigerator until the study is made. The

soil and water samples were diluted 10^{-3} to 10^{-5} with normal saline after inoculation to McConkey ve EMB (eozin metilen blue) medium from the dilutions so prepared, they were incubated for 24-48 hours at 30ºC (Marquart et al., 2005; Nicas and Iglewski, 1986). Thereafter, colonies with *Pseudomonas* suspect were planted in the special medium. The pyoverdin (fluorescein) pigment formed by *Pseudomonas* F(flo) agarda; *P.aeruginosa, P.fluorescens, P.putida* species were observed in the tube in yellow. Under the wood lamp and with UV light, only the pyoverdin pigment formation could be detected in the agar. In *Pseudomonas* P (tech) agar, only the pyocyanin pigment formed by *P.aeruginosa* was observed in blue in the tube with liquid medium (Koneman, 1997). Classical methods were employed to define the bacteria. As depending on the pigment formations, morphologies, and microscopic outlooks of the colonies reproducing in different mediums, the detected bacteria were pre-diagnosed as being *Pseudomonas*. In verifying the identification and naming the strains diagnosed as *Pseudomonas* through classical methods, BD BBL Crystal™(Becton Dickinson Microbiology Systems Cockeysville Md) Identification Systems Enteric/Nonfermenter ID system was used.

2.2. Determination of PLC activity of *Pseudomonas* bacteria by different methods

In this study, the PLC, which is one of the important virulence factors of *Pseudomonas* bacteria, was researches through two methods. In the first method, p-nitrophenyl phosphorylcholine (Sigma); and in the second method, egg tryptic soy agar medium were used. *P.aeruginosa* ATCC 27853 strain was used as positive control and the medium without bacterial strain was used as negative control**.**

Method 1: *Pseudomonas* bacteria were incubated for 24 hours at 37ºC in luria bertoni (LB) agar, then 5-6 colonies from each isolate were reproduced through incubation for 18 hours at 37°C in LB broth medium. Afterwards, 2 ml bacteria suspensions were taken and added in the tubes containing 30 ml LB broth medium and then supernatants were separated after 20-minute centrifuge in 1000 g. In our study, 10 µl supernatant was added in 90 µl NPPC solution. P-nitrophenyl phosphorylcholine (NPPC) was used as substrate (Table.1).

Table.1. NPPC solution consists of the above substances

NPPC	10 mM
Tris	250 mM
Glycerol	%60
ZnCl2	1.0 µM

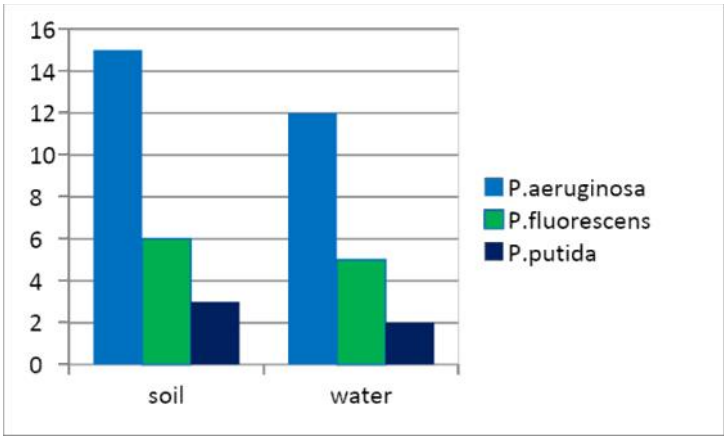
In the PLC research, sterile microplate was used. 10 µl bacterium supernatants and NPPC solution prepared as above were put in each well. After being incubated for 4 hours at 37°C the microplate so prepared was read at spectrophotometer (Versamax Tunable, Microplate Reader; Moleküler Devices®) at 405 nm absorbance value. One unit was defined in each milliliter of supernatant. Our study for PLC activity was made in parallel with the studies of Berka and others (Berka et al.,1981; Dubouix et al., 2004; Berk et al., 1987).

Method 2: PLC activities in bacteria were studies by using tryptic soy agar medium with 8% egg. After having been kept in ethyl alcohol for 24 hours, the eggs were broken with a sterile scissors and their yolks were put in a sterile flask with glass beads therein and mixed well. When the medium cooled down to 55ºC, the egg yolk so prepared was added. Then, it was poured in petri plates. Besides, one loop from each of the bacterium suspensions, which were prepared with distilled water according to Mc Farland 1, was inoculated to egg medium and kept for 24 hours at 37ºC. Petri dishes were reviewed after incubation. Colony diameter and the total diameter, which is formed by the precipitation zone around the colony and the colony diameter were measured. Where the value found accordingly had been equal to 1.00, PLC was considered negative, where it is below 1.00 PLC existence was considered positive
(Mohandas and Ballal, 2008).

3. Results

In the study, total 43 *Pseudomonas* isolates were obtained from the soil and water samples taken from the areas. In the study, 15 *P.aeruginosa*, 6 *P.fluorescens*, 3 *P.putida* were investigated in total 24 *Pseudomonas* isolates obtained from the soil samples taken in Bilecik and its villages. Further, in the study, 12 *P.aeruginosa*, 5 *P.fluorescens*, 2 *P.putida* were investigated in total 19 *Pseudomonas* isolates obtained from the water samples taken from brooks and the lake in the region (Table.2.).

Table.2. Distribution of *Pseudomonas* species isolated from nature samples



The existence of PLC, which plays an important role in the virulence of *Pseudomonas* bacteria was researched. For this purpose, PLC activity was positive in 28 (65.11 %) of 43 natural samples according to method 1; and negative in 15 (34.89 %). In addition, in 24 soil isolates, 16 (66.67 %) was found positive; while in 19 water isolates 12 (63.15 %) were found positive. In the study conducted according to method 2, PLC activity was found 26 (60.46%) of 43 natural isolates positive, while 17 (39.54%) were found negative. In 24 soil isolates, 15 of them (62.50 %) was found positive, while in 19 water isolates 11 of them (57.89 %) was found positive (Table.3).

Table.3. Phospholipase C (PLC) (%) existence in soil and water isolates

Samples	Method 1	Method 2
Soil	66.67	62.50
Water	63.15	57.89

Table.4. Statistical evaluation of *Pseudomonas* bacteria according to PLC activity

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	12,866[a]	1	,000		
Continuity Correction[b]	10,601	1	,001		
Likelihood Ratio	13,091	1	,000		
Fisher's Exact Test				,001	,001
Linear-by-Linear Association	12,567	1	,000		
N of Valid Cases	43				
a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 5,58.					
b. Computed only for a 2x2 table					

In the analysis made in our study, chi-square test was used. There is a significant difference since the *p* value between methods used in determining PLC activity is less than 0.01 ($P<0.01$) (Table.4).

4. Discussion

Pseudomonas bacteria are moving and opportunistic pathogen microorganisms. While generally isolated from soil and water, they are isolated from plant and animal samples too. These microorganisms are commonly found in the nature. Although they do not generally result in diseases in healthy people, they bring about serious infections with the impact of various virulence factors that they release when the defense system is damaged and the immune response is insufficient (Pollack, 1995; Todar, 2004). In the study conducted by they, it was observed that the PLC production originating from *Pseudomonas* brings about urinary system infections more than blood, lung, and other samples (Berka et al., 1981) On the other hand, they found that PLC levels are higher than trachea, urinary tract, and wound isolates in comparison with other zones (Hamood et al., 1996). In a study, they compared the strains isolated from patients with bacteremia with the strains isolated from soil and water in terms of enzyme production. They found that the PLC enzyme production in *P.aeruginosa* bacteria, which are isolated from environment samples, is as high as the *P.aeruginosa* bacteria, which are isolated from clinical samples, and stated that there is no significant difference between them in terms of enzyme production (Nicas and Iglewski, 1986).

In the named *Pseudomonas* isolate, the PLC activity was found positive (65.11 %) in 43 natural samples according to method 1. In the study conducted according to method 2, rate in environmental isolates (60.46%) was positive. A similarity has been observed between the results of our study and the results of the studies performed by Nicas and others. Existence of PLC in *Pseudomonas* bacteria is very important for the nature. Biochemical and phenotypic similarities between *P. aeruginosa* strains taken from clinical, water and soil, have shown that they are the same species. They even stated that *P. aeruginosa* is a good habitat for water and soil (Schroth et al., 2018).

5. Conclusion

Phospholipase is stunning enzyme found in nature samples as well as in clinical isolates. It is among many important virulence factors produced by Pseudomoas bacteria. Further research on the phospholipase activities of *Pseudomonas* species isolated from samples taken from natural sources will contribute to this area.

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Conflicts of interest: The authors declare no conflict of interest

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