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Pseudomonas aeruginosa Detection Methods from Fish Samples

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Abstract - A total of 70 fish samples were randomly purchased from different butchers and supermarkets brands in Samsun Province, Turkey in 2018, and analyzed for present of *Pseudomonas* (*P.) aeruginosa.* For that purpose, *P. aeruginosa* isolation was carried out in conventional culture technique; briefly, under aseptic condition 10 g fish samples were transferred into a sterile polyethylene bag and 90 ml of peptone water (PW-Oxoid CM 00099) broth was added. The mixture was homogenized and prepared decimal dilution up to 10^{-6}. Following that, the broth was plated onto Pseudomonas CN Selective Agar [Oxoid SR 102E, suppl. Pseudomonas Agar base-(Oxoid CM 0559)] (EN ISO 13720) using spread plate technique and the plates were incubated aerobically for 24-48 h at 37 °C. After the incubation, up to five susceptible colonies grown on the Pseudomonas CN Selective Agar were subcultured onto Tryptone Soya Agar plates (TSA-Oxoid-CM0131-L21). The presumptive *P. aeruginosa* colonies were tested with the Gram staining, oxidase (Oxoid BR 64) and catalase test. In addition, the colonies were streaked onto Endo Agar Base (Oxoid, CM0479, suppl. BR0050), for confirmation of the isolates for being *P. aeruginosa* at molecular levels, two types of genes were detected in the isolates- *oprL* and PA-SS (16 S rDNA) genes using PCR assay. In conclusion, we obtained 100 *Pseudomonas* spp. isolates. Of those, 65 isolates were identified as a *P. aeruginosa* using classing culture technique. However, 30 out of 65 isolates were confirmed at molecular levels. Between two genes regions, although only *oprL* gene detected in the 30 isolates, PA-SS (16 S rDNA) gene was not detected in any of the isolates.

Keywords: *Pseudomonas aeruginosa, fish, oprL gene, PA-SS (16 S rDNA) gene*

1. INTRODUCTION

Pseudomonas spp. genus is a large and complex heterogeneous group of organisms and belongs to the Pseudomonadaceae family. The genus composes of over 255 species [1]. These species are metabolically diverse and nutritionally heterogeneous. Some species of this genus are useful for plants and are used as biological healing and biocontrol agents, while other members of the genus are pathogens for plants and animals. The bacterium is also one of opportunistic microorganisms for humans. *Pseudomonas (P.) aeruginosa* species take place in *Pseudomonasa* genus, and contains 13 different subgroup. These subtypes; *P. aeruginosa, P. alcaligenes, P. anguilliseptica, P. caeni, P. citronellolis, P. flavescens, P. jinjuensis, P. mendocina, P. nitroreducens/multiresinivorans* group, *P. oleovorans/pseudoalcaligenes* group, *P. cf. pseudoalcaligenes, P. resinovorans* and *P. straminea* [2].

P. aeruginosa is also a prevalent bacterium that can take place in normal human flora, rarely causes disease in healthy individuals, but can cause serious infections if host defense is weakened. Therefore, the bacteria are considered opportunistic pathogens. *P. aeruginosa* can adapt quickly to adverse environmental conditions, including aquaculture environment and the bacterial adaptation is not dependent on nutrients [3], [4]. Some *Pseudomonas* species such as *P. aeruginosa, P. fluorescens, P. angulliseptica* and *P. putida* are commonly found in aquatic environment. In the fish species, they cause bacterial hemorrhagic septicemia, bacterial fin and gill disease, darkness of the skin, detached scale, abdominal ascitis and exophthalmia bacterial scarlet fever, pike fish scarlet disease, carp ascites and trout red mouth [5], [6], [7], [8], [9]. In the infections caused *P. aeruginosa,* virulence factors particularly related cell expressed out of which play an important role. The bacterium has higly genetic variations and capable of adaptation in different environmental conditions [2]. The such diseases cause economic losses because of mortality, expensive treatment, loss of the opportunity to sell the fish and contraction of zoonotic diseases by the handler [4].

P. aeruginosa is a common and opportunistic, Gram negative bacillus that leads to different clinical infections and severe infectious disease. The bacterium can cause infections at very low concentrations [10].

Hence, early detection is critical for treating *P. aeruginosa* infection. In these terms, conventional *P. aeruginosa* detection methods are based on the biological characteristics of the bacterium (Gram-negative or positive, oxidase, catalase, acetamidase, arginine dihydrolase and pyocyanin etc.). The classic cuture technique needs to long time. In addition, sometimes the technique gives rise to false negative or false positive results because of the *P. aeruginosa*'s large genome and heterogenous. To rapid detection especially clinical isolates, automated identification systems are used in the hospital' laboratory. Although the systems have been clinically used to identify a variety of microbial species, these systems have a low rate of accuracy in the identification of *P. aeruginosa* [11], [12], [13]. Therefore, scientists have long been committed to establishing a rapid and sensitive detection method for *P. aeruginosa* [10]. Then, conventional PCR is widely utilized for its developed procedure to obtain consequence of reliability and stability. To *P. aeruginosa* detection, PCR-based assays have also developed progressively. For this aim, different kind of genes are uses for the molecular confirmation or detection aims [14], [15]. For instance, researchers reported that the PCR method targeting *exotoxin A* gene was detected in 57 positive samples out of 364 total samples, whereas the conventional culture method only detected in 36 positive samples. These results indicate that the *exotoxin A* gene-based PCR method had higher sensitivity [15], Another study, [16] used a multiplex real-time (RT) PCR assay targeting the 16S rRNA and *gyrB* genes for detection *P. aeruginosa* in CF patients. They concluded that the method and used two genes enabled detection of *P. aeruginosa* in CF patients within a shorter period. Besides the genes, number of specific genes have been discovered such as *ecfX*, *gyrB*, *algD*, GDP mannose, *oprL* and *fliC* like that [16], [17], [18], [19], [20], [21]. To success of conventional PCR, specificity is critical, but is also the most important cause of failure in PCR detection. Therefore, many researchers have investigated the specificity of different *P. aeruginosa* genes [17], [18].

P. aeruginosa's outer membrane protein plays an important role in the adaptation of the bacteria to the environment. The presence of this specific outer membrane protein also plays an important role in the hereditary resistance of *P. aeruginosa* to many antibiotics (efflux transport system or membrane selectivity) [17]. The *oprL* gene encodes the structural membrane lipoprotein of *P. aeruginosa*. It also is used for the detection of *P. aeruginosa* in clinical and other samples by PCR at the species level [17] or by RT-PCR method [16], reported that they design and clone two molecularly characterized outer membrane lipoprotein genes; these are *oprI* and *oprL* genes [23], [20], [16], [21]. Because the *oprI* gene is specific for fluorescent pseudomonas [17], [24] and *oprL* is specific for *P. aeruginosa*. So, *oprL* gene was used for the identification of *P. aeruginosa* from clinical isolates with *oprI* gene by using multiplex-PCR method. In their study, they have tested 20 different fluorescent positive *Pseudomonas* spp., According to their study results, while the two genes were detected in only clinic and environmental origin *P. aeruginosa* isolates (n=250), *oprI* gene was detected also fluorescent positive *Pseudomonas.*

Previous findings have shown that as few as 10–100 bacilli are capable of colonizing the intestine of critically ill or immunocompromised patients [25]. Therefore, early and accuracy detection of *P. aeruginosa* is particularly important. Today, generally, classic culture technique remains the most commonly applied method for detecting *P. aeruginosa*, but this method is time-consuming and susceptible to inconsistent results due to large genome and heterogenous of the bacterium. To address these issues, researchers have developed various assays, each with their own advantages and disadvantages. One of the developed method is PCR [26]. Therefore, the aim of this study was to isolate and identificate of *P. aeruginosa* isolates from fish samples, sold in Samsun province-Turkey, by using classic culture technique and confirmed at the molecular level by using PCR method targeted both *oprL* and PA-SS-16S rDNA genes.

II. MATERIALS and METHOD

A. Sample Collection

In the present study a total of 30 *P. aeruginosa* isolates were obtained. The 30 colonies were used as a material. For that, a total of 70 fish samples were randomly purchased from different butchers and supermarkets brands (up to 2 times each brand) in Samsun Province, in Turkey in 2018, and then transferred under cold chain to the laboratory and tested immediately for *P. aeruginosa.*

B. P. aeruginosa Isolation and Identification

P. aeruginosa isolation was carried out in conventional culture technique; briefly, under aseptic condition 10 g fish samples were transferred into a sterile polyethylene bag and 90 ml of peptone water (PW-Oxoid CM 00099) broth was added. The mixture was homogenized and prepared decimal dilution up to 10^6. Following that, the broth was plated onto Pseudomonas CN Selective Agar [Oxoid SR 102E, suppl. Pseudomonas Agar base-(Oxoid CM 0559)] (EN ISO 13720) using spread plate technique and the plates were incubated aerobically for 24-48 h at 37 °C. After the incubation, up to five susceptible colonies grown on the Pseudomonas CN Selective Agar were subcultured onto Tryptone Soya Agar plates (TSA-Oxoid-CM0131-L21).

The presumptive *P. aeruginosa* colonies were tested with the Gram staining, oxidase (Oxoid BR 64) and catalase test. In addition, the colonies were streaked onto Endo Agar Base (Oxoid, CM0479, suppl. BR0050), For further analysis, the isolates were kept at

-80 °C in cryovials containing 10% (w/v) glycerol in Brain Heart Infusion broth (BHI; CM0225, Oxoid).

C. Detection of P. aeruginosa Using PCR Assay

DNA extraction from the isolates was performed by using boiling method. In addition, *P. aeruginosa* isolates were confirmed by the presence of the species-specific *oprL* and 16S rDNA genes for PA-SS region. For that purpose, a single target PCR technique was applied according to the methodologies of [17], [27]. For 16S rDNA detection, the primer was used according to [22]. The oligonucleotide primers and product sizes are listed in Table 1. *P. aeruginosa* (ATCC 15692) and *E. coli* ATCC 25922 were used as the positive and negative control, respectively.

Table 1. The Sequences of Primers and PCR Product Size of *OrpL* ad 16S rDNA Genes

Oligonucleotide Sequence (5'-3')	**Amplified**	**Products (bp)**	**Reference**
F-ATGGAAATGCTGAAATTCGGC- R CTTCTTCAGCTCGA CGCGACG	*oprL* gene	504 bp	[17]
F GGGGGATCTTCGGACCTCA PA-SS-R TCCTTAGAGTGCCCACCCG	16 S rDNA (PA-SS)	956 bp	[22]

III. RESULTS

In the present study, the isolation, identification and molecular confirmation procedures were applied for the detection of *P. aeruginosa*. Based on the analyzed results, *Pseudomonas* spp. were detected in a total of 100 isolates of the 70 fish samples. Of 100 *Pseudomonas spp.* isolates, *P. aeruginosa* was detected in the 30 isolates. The 30 isolates were tested mentioned above material methods section.

For the confirmation of the isolates being *P. aeruginosa* at molecular levels, we used two different gene regions; OprL and PA-SS (16S rDNA). Although *oprL* gene detected 30 isolates, 16S rDNA was not able to detect in any isolates. In that way, the 30 *oprL* gene positive isolates were evaluated as *P. aeruginosa* isolates.

IV. DISCUSSION

Bacterial infections are one of the biggest threats to wild and aquaculture fish. *P. aeruginosa* is one of the most important pathogens responsible for infections with high mortality in fish. Besides this, it is evaluated as a responsible of the degradation agents of fish and fish and other animal products [4]. For this reason, accurately detection and determination of virulence factors and antibiotic resistance properties of *P. aeruginosa* originated from clinic or other sources of samples is very important.

Due to importance of human and animal health of this bacterium, identification of *P. aeruginosa* is very important, but this may be problematic due to its large phenotypic variations. Therefore, in order to confirmation of the clinical isolates in terms of being *P. aeruginosa*, molecular based on detection techniques involved in detection of different gene regions can be applied. Studies on this subject, there have been some researches from different parts of the world. In the studies, there have been used some different genes belonged to *P. aeruginosa* species specific [17], [18], [4], [28], [21]. In one of the these studies [18], a total 62 (of which 35 clinics and 27 environmental origin) *P. aeruginosa* isolates, which confirmed by using API System firstly, were also detected by using PA-duplex-PCR (*ecfX* and *gyrB*) and *P. aeruginosa* monoplex PCR methods (*ecfX*-PCR, *gyrB*-PCR, *oprL*-PCR, *ETA*-PCR and PA-16S-PCR). According to analyses results, both of *ecfX* and *gyrB* genes were detected in the isolates by using duplex RT-PCR. They reported that the use of the two genes together can prevent false positive results. They also reported that two clinical isolates gave false positives by PA 16S-PCR and four environmental isolates by *oprL*-PCR method and one isolate by ETA-PCR. These false results mainly due to cross-reaction of *P. aeruginasa* with *Chromobacterium violaceum*. However, the researchers reported that although they obtained these results, there was limited sequence information about these genes in the literature and the GenBank. Therefore, the researcher did not believe that these results supported only single-targeted use. In contrast, [29] reported that sequencing variances is a more serious problem affecting molecular diagnostic methods. [27], [16] reported that these two genes are safe in identifying *P. aeruginosa* at species level.

For the determination and identification of *P. aeruginosa* based on molecular levels, *toxA* and *algD* genes were also detected in *P. aeruginosa* susceptible isolates. In the [16], [27]'s studies, the two genes were used for molecular confirmation of the isolates.

In generally, after the conventional culture technique- because the isolates have wide range of heterogeneity [30], we have to use molecular technique for obtained more accuracy results and prevent false positive results. In another study, there has been used another *P. aeruginosa* specific gene for confirmation of the isolates being as *P. aeruginosa* [28]. In their study, [28] reported that, firstly, the *Pseudomonas* spp. isolates were isolated from the samples and confirmed at a species levels. Then, whether the isolates were being *P. aeruginosa* or not, they detected *rpoB* genes in the isolates. As a result, 99.5 % of the isolates were containing the gene and therefore, the isolates were evaluated as *P. aeruginosa*. They also reported that after the identification of *P. aeruginosa* at the species levels, it is necessary to applied molecular assay technique due to the heterogeneity of the bacterium as

well. In conclusion, according to their results, they advised that *rpoB* gene can be used successfully due to high molecular identification ratio properties for the confirmation of the *P. aeruginosa* isolates.

The *oprL* gene encodes the structural membrane lipoprotein of *P. aeruginosa*. This gene is also used by using PCR assay [17] or by RT-PCR method [16] for the detection of *P. aeruginosa* in clinical and other samples at the species level.

De Vos et al. [17] reported that they designed and cloned two outer membrane lipoprotein genes which designed according to molecular characterization. These are *oprI* ve *oprL* genes [23], [20]. From two genes, *oprI* of these genes is specific for fluorescent pseudomonas [17]. Then, they developed multiplex-PCR method using two *oprL* genes for the identification of *P. aeruginosa* from clinical isolates. In their study, they analysed 20 different fluorescent isolates. All of which, the two genes were detected in 250 clinic and environmental origin isolates which belong to 20 different fluorescent pseudomonas. *Oprl* gene was detected also in other fluorescent isolates. The two genes were not detected in the non-*P. aeruginosa* isolates (n=15). The lowest determination level of *P. aeruginosa* was also 10^2 bacteria/ml. In our study, *oprL* gene was detected in 30 *P. aeruginosa* isolates which obtained and confirmed phenotypic methods. In contrast, in our study the other *P. aeruginosa*-specific gen PA-SS (16 S rDNA) was not detected in any of the isolates. But, that two genes (*oprL* and PA-SS 16 S rDNA) were detected in the control isolate (ATCC 15692). In another our study, just as the study results, while *oprL* gene was detected in chicken meat and ground beef origin *P. aeruginosa* isolates, PA-SS 16 S rDNA was not detected in the isolates, except that control isolates (ATCC 15692). The similar results were reported by [4]. They reported that *Pseudomonas* spp. was confirmed in 29 out of 49 water and different fish origin isolates according to Gram stain, morphological properties, and other biochemical test results. After that, confirmation of the isolate at molecular levels, *oprL* gene detected in the isolates. So, the isolates were evaluated as *P. aeruginosa*. They also concluded that the gen can be very useful for determination of the *P. aeruginosa* isolates.

In conclusion, fish samples contaminated by *P. aeruginosa*. This is risk assocated with fish health and consumers. As a result, according mentioned above study, PCR method could be successfully applied by investigating of verifying suspected *P. aeruginosa* isolates. *oprL* gene can also used for this aim. According to the results of these study mentioned above, correct identification and characterization of *P. aeruginosa* can only be achieved by combining cultural, biochemical and molecular tests. In addition, the high molecular identification rates have shown that genomic studies are needed to confirm the exact taxonomic position of *P. aeruginosa.* For the boost the sensivity, one of other gene mentioned above may detect as well as *oprL* genes.

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