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Pseudomonas aeruginosa Detection Methods from Ground Beef and Chicken Meat Samples

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Abstract - In this study, chicken and ground beef samples purchased from supermarkets and butchers in Samsun Province were analyzed for presence of *Pseudomonas (P.) aeruginosa*. In this case, classical culture technique were applied with molecular techniques. Isolation was performed on Pseudomonas CN Selective Agar [Oxoid SR 102E, suppl. Pseudomonas Agar base-(Oxoid CM 0559)] (EN ISO 13720). The plates was 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, namely the *oprL* and PA-SS (16 S rDNA) gene region, were performed by a single-targeted PCR assay. As a result, 140 isolates were obtained and identified as *P. aeruginosa* using classic culture technique, and 50 out of 140 isolates were identified *P. aeruginosa* isolates using PCR technique. Between two genes regions, although only *oprL* gene detected in the 50 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 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].

The bacterium has higly genetic variations and capable of adaptation in different environmental conditions [2]. *P. aeruginosa* is coomonly isolated from different kinds of environment, and they able to colonized various anatomical sites such as animals and humans [2], [5], [6]. The bacterium may be also lead to food poisoning and has many virulence factors which holded responsible for severe infections in human and animals [7], [8].

P. aeruginosa is a common and opportunistic and often implicated in nasocomial infections [9], [10], [11]. The bacterium can cause infections at very low concentrations [12]. 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* [13], [14], [15]. Therefore, scientists have long been committed to establishing a rapid and sensitive detection method for *P. aeruginosa* [12]. 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 [16], [17]. 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 [17], Another study, [18] 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 [18], [19], [20], [21], [22], [23]. 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 [19], [20].

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). The *oprL* gene encodes the structural membrane lipoprotein of *P. aeruginosa*. It is also used for the detection of *P. aeruginosa* in clinical and other samples by PCR at the species level [19] or by RT-PCR method [18], reported that they design and clone two molecularly characterized outer membrane lipoprotein genes; these are *oprI* and *oprL* genes, [22], [18], [23]. Because the *oprI* gene is specific for fluorescent pseudomonas [19], [25] 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 [26]. 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 [19;26]. Therefore, the aim of this study was to isolation and identification of *P. aeruginosa* isolates from chicken meat and ground beef 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 this study, chicken and ground beef samples purchased from supermarkets and butchers in Samsun Province were analyzed for presence of *P. aeruginosa*. For these purposes, the classic culture and molecular techniques were applied.

B. P. aeruginosa Isolation and Identification

P. aeruginosa isolation was carried out in conventional culture technique; briefly, under aseptic condition 10 g ground beef or chicken meat 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

Template DNA was extracted from whole organisms by boiling methods [28]. 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 [19], [29]. For 16S rDNA detection, the primer was used according to Spilker et al. [30]. The 16S rDNA primers targeted species-specific signature sequences in 16S rDNA variable regions 2 and 8 (V2 and V8), respectively [30]. 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	[19]
F GGGGGATCTTCGGACCTCA R TCCTTAGAGTGCCCACCCG	16 S rDNA (PA-SS)	956 bp	[30]

III. RESULTS

In the present study, 140 isolates were obtained and identified as *P. aeruginosa* using classic culture technique, and 50 out of 140 isolates were identified *P. aeruginosa* isolates using PCR technique. Between two genes regions, although only *oprL* gene detected in the 50 isolates, PA-SS (16 S rDNA) gene was not detected in any of the isolates. In that way, the 50 *oprL* gene positive isolates were evaluated as *P. aeruginosa* isolates.

IV. DISCUSSION

P. aeruginosa is of a many different virulence factors. The virulence factors can increase bacterial pathogenicity besdes infection severity. Despite the importance of knowledge about them, these factors are not more characterized at level of strains derived from food products. 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. There have been many studies according to the subject around the World [18], [19],[20], [30], [31], [32], [33]. One of the studies, Anuj et al. [20] reported that of the 91 clinical and environmental isolates tested, 62 (n=35 clinical and 27 environmental) were identified as *P. aeruginosa* by the API 20NE kit and exhibited positive results in all 6 *P. aeruginosa* PCR assays (PAduplex, *ecfX*-PCR, *gyrB*-PCR, *oprL*-PCR, ETA-PCR and Pa16S-PCR). A further 21 environmental isolates were characterized as a non-*P. aeruginosa* species by the API 20NE kit and provided negative results in all 6 *P. aeruginosa* PCR assays. They observed false-positive results for 2 clinical isolates in the Pa16S-PCR assay and for 4 environmental isolates by the *oprL*-PCR assay. A false-negative result was observed in the ETA-PCR assay, and 1 isolate was misidentified as *Chromobacterium violaceum* by the API 20NE kit. Another study was reported by Quin et al. [18]. They examined 200 Gram-negative clinical isolates from cystic fibrosis (CF) respiratory tract specimens and compared identification by biochemical testing and real-time PCR. For real-time assay, they tested multiple different target sequences using a standardized combination of biochemical testing and molecular identification, including 16S rRNA partial sequencing and *gyrB* PCR and sequencing as a "gold standard." Of 50 isolates easily identified phenotypically as *P. aeruginosa*, all were positive with PCR primers for *gyrB* or *oprI*, 98% were positive with *exotoxin A* primers, and 90% were positive with *algD* primers. Of 50 *P. aeruginosa* isolates that could be identified by basic biochemical testing, 100% were positive by real-time PCR with *gyrB* or *oprI* primers, 96% were positive with *exotoxin A* primers, and 92% were positive with *algD* primers. For isolates requiring more-extensive biochemical evaluation, 13 isolates were identified as *P. aeruginosa*; all 13 were positive with *gyrB* primers, 12 of 13 were positive with *oprI* primers, 11 of 13 were positive with *exotoxin A* primers, and 10 of 13 were positive with *algD* primers. A single false-positive *P. aeruginosa* result was seen with *oprI* primers. The best-performing commercial biochemical testing was in exact agreement with molecular identification only 60% of the time for this most difficult group. They concluded that real-time PCR with a combination of two target sequences appears to be the optimum choice for identification of atypical *P. aeruginosa* and for non-*P. aeruginosa* gram-negative isolates.

Spilker et al. [30] reported that they tested 42 culture collection strains (including 14 *P. aeruginosa* strains and 28 strains representing 16 other closely related *Pseudomonas* species) and 43 strains that had been previously identified as belonging to 28 nonpseudomonal species also recovered from CF patient sputum. Based on these 85 strains, the specificity and sensitivity of both assays were 100%. To further assess the utility of the PCR assays, we tested 66 recent CF sputum isolates. The results indicated that preliminary phenotypic testing had misidentified several isolates. The 16S rDNA sequence was determined for 38 isolates.

Benie et al. [33] reported that firstly, the *Pseudomonas* spp. isolates (n=225) 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 (n=204) 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 [19] or by RT-PCR method [18] for the detection of *P. aeruginosa* in clinical and other samples at the species level.

De Vos et al. [19] reported that they designed and cloned two outer membrane lipoprotein genes which designed according to molecular characterization. These are *oprI* ve *oprL* genes [24], [22]. From two genes, *oprI* of these genes is specific for fluorescent pseudomonas [19]. 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 fish origin *P. aeruginosa* isolates, PA-SS 16S 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, chicken meat and ground beef 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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