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RESEARCH ARTICLE

Investigation of *Pseudomonas* Species in Chicken Drumstick Samples

Hayriye Yeşim CAN[1*]

[1]Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Hatay, Turkey

ABSTRACT

This study aimed to detect the contamination of refrigerated poultry meat with psychrotrophic bacteria, such as *Pseudomonas* species. *Pseudomonas* spp. can grow well in the skin and muscle of poultry meat by using carbonhydrates and amino acids at refrigeration temperature (4 °C). They are mainly responsible for the spoilage of poultry meat with their enzymatic activiy. For this purpose, a total of 107 chicken drumstick samples were analyzed for the presence of *Pseudomonas* species (*Pseudomonas fragi, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas lundensis, Pseudomonas aeruginosa*). Of the samples, 92.5% found to be contaminated with *Pseudomonas* spp. A total of 99 isolates were confirmed as *Pseudomonas* spp. by PCR, targeting the *16S rDNA* gene. Among the 99 isolates, 78.7% were identified as *P. fluorescens*, whereas *P. fragi*, *P. putida*, *P. lundensis*, and *P. aeruginosa* were not detected in the study. The findings show that *P. fluorescens* is the most prevalent species in refrigerated poultry meat, thus posing a potential risk for the spoilage of poultry meat.

Keywords: PCR, Poultry Meat, *Pseudomonas*, Spoilage

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Piliç Baget Örneklerinde *Pseudomonas* Türlerinin Araştırılması

ÖZ

Bu çalışmada, soğukta muhafaza edilen kanatlı etlerinin *Pseudomonas* türleri gibi psikrotrofik bakterilerle kontaminasyonunun belirlenmesi amaçlandı. *Pseudomonas*'lar soğuk muhafaza şartlarında (4 °C'de) kanatlı etindeki karbonhidratlar ile amino asitleri kullanarak deri ve kasta kolaylıkla gelişebilir. Enzimatik aktiviteleri ile kanatlı etlerinin bozulmasından esas olarak sorumludurlar. Bu amaçla, toplam 107 adet tavuk baget örneğinde *Pseudomonas* türlerinin (*Pseudomonas fragi, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas lundensis, Pseudomonas aeruginosa*) varlığı analiz edildi. Örneklerin % 92.5'inin *Pseudomonas* spp. ile kontamine olduğu bulundu. Toplam 99 izolat, *16S rDNA* geni hedef alınarak *Pseudomonas* spp. yönünden PCR ile doğrulandı. Çalışmada 99 izolatın %78,7'si *P. fluorescens* olarak identifiye edilirken, *P. fragi*, *P. putida*, *P. lundensis* ve *P. aeruginosa* tespit edilemedi. Bulgular, soğukta muhafaza edilen kanatlı etlerinde *P. fluorescens*'in en yaygın tür olduğunu ve dolayısıyla, kanatlı etinin bozulması için potansiyel bir risk oluşturduğunu göstermektedir.

Anahtar kelimeler: Bozulma, kanatlı eti, PCR, *Pseudomonas*

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ORCID ID; *HYC: 0000-0002-5191-6268.*
*Corresponding author e-mail: yesimcan@mku.edu.tr

INTRODUCTION

The people widely consume chicken meat due to being rich from essential amino acids, zinc, iron and group B vitamins, easily digestible besides being an economic animal protein source. Many factors beginning from production to consumption (the infections in the live birds, breeding, transporting, slaughtering, packaging, storage, and distribution conditions) affect the microbial flora of poultry meat (Okolocha and Ellerbroek 2005).

High post-rigor pH and water activity (a_w) values of poultry meat are risk factors for microbial spoilage (Allen et al. 1997). The competitive ability of the microorganisms that lead to food spoilage is good, and they cause structural changes in the food with their proteolytic and lipolytic enzymes. *Pseudomonas* spp. are the most commonly isolated psychrotrophic bacteria from food, and they lead to spoilage in foods stored at refrigeration temperatures by growing under aerobic conditions (Salvat et al. 1997, Geornaras et al. 1999, Mead 2004, Ercolini et al. 2007, Al-Rodhan and Nasear 2016, Caldera et al. 2016).

Poultry meat is commonly consumed with the skin, and the initial microbial load of the skin is generally high. Although the initial microbial load in poultry meat varies depending on sanitary and hygienic practices, technical design, and cross-contaminations in the slaughterhouse, its high shortens the product's shelf life (Höll et al. 2016, Morales et al. 2016). While the initial microbial flora originating from live birds usually includes Gram-positive bacteria, they leave their place in Gram-negative bacteriae (*Pseudomonas*, *Flavobacteria, Acinetobacter, Moraxella*, and *Enterobacteriaceae*) in the final product over time. So, the final product may carry a risk for the presence of the microorganisms that cause spoilage like *Pseudomonas* spp. and pathogens such as *Salmonella, Campylobacter, Clostridium perfringens, Escherichia coli* O157, *Listeria monocytogenes*, and *Staphylococcus aureus*. The undesired levels of the abovementioned microorganisms in the final product indicate the insufficient sanitisation in the slaughterhouse, that hygienic practices are not cared during or after slaughter process, cross-contamination and recontamination in the plant. Because *Pseudomonas* spp. are usually destroyed at the scalding stage of the poultry slaughtering process (Mead 2004, Okolocha and Ellerbroek 2005, Wang et al. 2018). So, although the systems like HACCP that aims food safety in poultry production primarily target pathogen bacteria control, they also make a certain reduction in the level of spoilage-causing bacteriae. Thereby, the HACCP system positively contributes to shelf life of the product and public health (Mead 2004, Morales et al. 2016).

Hygienic practices during slaughtering, cold storage conditions, packaging techniques, post-rigor pH value of the product, species, and level of the psychrotrophic bacteria in the flora may be effective in spoilage of the poultry meat. The post-rigor pH value of the chicken leg is higher than that of the chicken breast, so spoilage can be faster here. *Pseudomonas* species may become dominant in the flora by quickly growing in chicken leg and breast meat. When the level of *Pseudomonas* reaches 10^7-10^8 cells per square centimeter, color change, off-odor, and slime formation occur. The unwanted metabolites that arise from protein degradation are reported to cause these changes (Allen et al. 1997, Nychas et al. 2008).

Usually, in fresh meat, the number of *Pseudomonas* is low and they grow during storage in cold and the number gradually increases. They easily compete with the other spoiler microorganisms in the flora as they can grow in cold and normal atmospheric conditions due to their oxidative properties. So, the spoilage in storage in the cold usually occurs from *Pseudomonas*. The frequently isolated *Pseudomonas* species in poultry stored in cold are reported *P. fragi, P. fluorescens, P. putida,* and *P. lundensis* (Geornaras et al. 1999, Ercolini et al. 2007, Nychas et al. 2008, Morales et al. 2016, Kumar et al. 2019).

Cold storage is the most commonly applied procedure for prolonging the shelf life of poultry meat. So, the presence and the level of psychrophile/psychrotrophic microorganisms that lead to spoilage in poultry meat have critical significance for determining shelf life. The present study was conducted to investigate *Pseudomonas* species (*P. fragi, P. fluorescens, P. putida, P. lundensis, P. aeruginosa*) in chicken drumsticks stored at refrigeration temperature (4°C) by using PCR method.

MATERIAL and METHODS

Sampling, Isolation and Identification of *Pseudomonas*

In the present study, 107 packaged chicken drumsticks belonging to different companies and sold in Hatay province consisted of the study sample. *Pseudomonas* isolation was done with the rinse method in the samples taken under aseptic conditions and brought to the laboratory under a cold chain. For pre-enrichment, each sample was put into sterile plastic bags and 100 ml Tryptic Soy Broth (Merck, 105459) was added. After the samples were shaken and washed during 1 min within the bag, the broth was closed and incubated at 30°C for 24 hours. One loop of culture suspension was taken from pre-enrichment broth and streaked on *Pseudomonas* Agar Base (Oxoid, CM0559) containing *Pseudomonas* CFC supplement (Cetrimide, Fucidin, Cephalosporin, Oxoid, SR0103).

Plates were incubated at 30°C for 24 hours. The oxidase positive, straw-coloured colonies were selected and suspended into tubes containing 4-5 ml of Brain Hearth Infusion broth (Oxoid, CMO225). The broth culture was incubated at 30°C for 18-24 hours under aerobic conditions.

For DNA extraction, 1 ml of broth culture was taken to eppendorf tube and centrifuged at 6000xg for 2 min. Supernatant was thrown and the following procedures were applied in order as instructed in the commercial kit (GF-1 Bacterial DNA Extraction, GF-BA-100, Vivantis). The obtained DNAs were stored at -20°C.

Verification of Isolates as *Pseudomonas* spp., and *Pseudomonas aeruginosa*

According to the amplification protocol described by Spilker et al. (2004), PCR was done by using primer pairs for amplifying the 618 bp (genus- specific; *Pseudomonas* spp.) and 956 bp (species-specific; *P. aeruginosa*) 16S rDNA gene sequence (Table 1). Two µl of DNA extract was taken and added to 23µl PCR mixture (2.5µl of 10xPCR buffer, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 1 U *Taq* polymerase, 1 µl of each primer at 10 µM concentration, and 2 mM $MgCI_2$) and a total of 25 µl mixture was subjected to amplification.
Amplification conditions included initial denaturation at 95°C for 2 min, 25 cycles of denaturation at 94°C for 20 sec, primer annealing at 58°C for 20 sec, and primer extension at 72°C for 40 sec in a thermal cycler (Boeco, Germany), and final extension at 72°C for 1 min.

Molecular Identification of *Pseudomonas* Species

The obtained isolates were identified by PCR with regard to *Pseudomonas fluorescens, Pseudomonas fragi*, *Pseudomonas putida*, *Pseudomonas lundensis* by using specific primer pairs described by Al-Rodhan and Nasear (2016), Ercolini et al. (2007), Morales et al. (2016). For the verification of isolates for *P. fluorescens*, a total of 25 µl of PCR mix (1 µl of each primer, 12.5 µl DreamTaq Green Master Mix (K1081, Thermo Scientific), and 5 µl of template DNA) was subjected to amplification. Amplification conditions included initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 45 sec, primer annealing at 59°C for 45 sec, primer extension at 68°C for 2 min, and final extension at 72°C for 5 min (Al-Rodhan and Nasear, 2016). Isolates were analyzed with multiplex PCR for *P. fragi, P. putida* and *P. lundensis*. For this purpose, a total of 20 µl PCR mix (0.4 µl of each primer, 1.2 µl reverse primer, 10 µl DreamTaq Green Master Mix (Thermo Scientific), 1 µl of template DNA) was prepared. For multiplex PCR, amplification conditions included initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, primer extension at 72°C for 1 min, and final extension at 72°C for 7 min (Morales et al. 2016).

Positive Control

Pseudomonas fluorescens ATCC 13525 was used as positive control in the study.

Electrophoresis and Imaging Procedure

The amplified DNAs were loaded to 1.5% agarose gel and subjected to electrophoresis procedure at 100 V for 50 min (CS-300V, England). At the end of this procedure, specific amplicons for *Pseudomonas* were analyzed at UV-transulliminator (UVP, USA) with the aid of positive control and DNA Ladder (Bioatlas, Estonia).

Table 1. Primers used in this study

Gene	Primer	Sequence (5'- 3')	Target	Annealing temp (°C)	Size (bp)	Reference
16S rDNA	F1	GACGGGTGAGTAATGCCTA	*Pseudomonas spp.*	54	618	Spilker et al. (2004)
	R1	CACTGGTGTTCCTTCCTATA				
	F2	GGGGGATCTTCGGACCTCA	*P. aeruginosa*	58	956	
	R2	TCCTTAGAGTGCCCACCCG				
16S rDNA	F3	TGCATTCAAAACTGACTG	*P. fluorescens*	59	850	Al-Rodhan and Nasear (2016)
	R3	AATCACACCGTGGTAACCG				
carA	F4	CGTCAGCACCGAAAAAGCC	*P. fragi*	60	370	Ercolini et al. (2007), Morales et al. (2016)
	R	TGATGRCCSAGGCAGATRCC				
	F5	ATGCTGGTTGCYCGTGGC	*P. putida*	60	230	
	R	TGATGRCCSAGGCAGATRCC				
	F6	TGTGGCGATTGCAGGCATT	*P. lundensis*	60	530	
	R	TGATGRCCSAGGCAGATRCC				

RESULTS

In the study, *Pseudomonas* spp. were detected in 99 out of 107 chicken drumstick samples (92.5%) (Figure 1). In total, *P. fluorescens* was isolated from 78 samples (72.8%), and it was found to that it constituted the dominant flora for *Pseudomonas* (Figure 2). The other *Pseudomonas* species (*P. aeruginosa, P. fragi, P. putida,* and *P. lundensis*) were not detected in the samples.

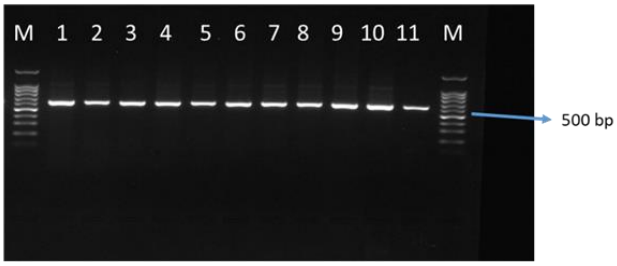

Figure 1. PCR analysis of *Pseudomonas* spp. isolates [M: 100 bp DNA marker, 1-11: *Pseudomonas* spp. Positive isolates (618 bp)]

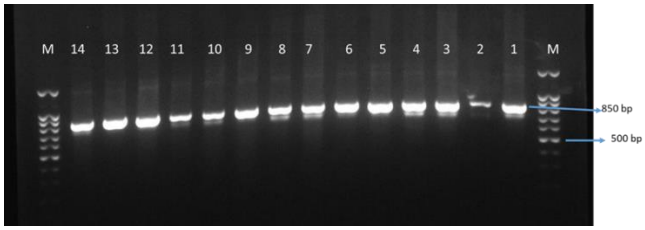

Figure 2. PCR analysis of *Pseudomonas fluorescens* isolates [M: 100 bp DNA marker, 1: Positive control (*Pseudomonas fluorescens* ATCC 13525), 2-14: Some of *P.fluorescens* positive isolates (850 bp)]

DISCUSSION and CONCLUSION

Globally, it is estimated that about 1/3 of the food produced for human consumption is lost or wasted each year (FAO, 2011). Food's becoming unavailable to introduce to consumers due to microbial or chemical spoilage is an important food loss (Morales et al., 2016). Meat and meat products account for approximately 21% of food losses in Europe and North America (Nychas et al., 2008). According to the world meat projection, poultry is the second most consumed meat worldwide and is expected to rank first by 2022 (OECD-FAO, 2013). In this context, the fact that chicken meat is generally consumed with its skin, mainly due to its nutritional content, and that the post-rigor pH and aw value are suitable for microbial growth limits the shelf life of poultry even under cold storage conditions. Poultry is susceptible to spoilage, and storage temperature is critical for spoilage. However, cold storage conditions of poultry create the typical microflora of poultry by bringing the microorganisms that grow in this condition in the foreground (Höll et al., 2016; Morales et al., 2016).
P. fluorescens was predominantly isolated from the chicken drumstick samples in the present study. Similarly, Morales et al.(2016) detected *P. fragi* and *P. fluorescens* most in chicken breast meat. Phenotypically, *P. fluorescens* was found to show proteolytic, lipolytic and lecithinase activity, while *P. fragi* was found to produce mainly proteolytic enzymes. As the result of the study, the researchers reported that there are important phenotypical and genotypical differences among *Pseudomonas* species from poultry meat. Differently, Caldera et al. (2016) isolated *P. fragi* and *P. putida* most from meat products. Also, when they have investigated the enzymatic activities of their isolates at 5°C and 25°C, they have detected that 30% of them showed proteolytic activity at 5°C, whereas 10% showed proteolytic activity at 25°C.

In addition, the packaging method also determines the spoilage of poultry meat. Höll et al. (2016) used a modified atmosphere packaging (MAP) method with two different gas mixtures (65% N_2 + 35% CO_2; 80% O_2 + 20% CO_2) for the packaging of chicken breast meats. The samples were packaged in both atmospheric conditions and incubated for 14 days at 4°C and 10°C. At the end of the eighth day, *Pseudomonas*, *Carnobacterium* and *Brochothrix thermosphacta* were found to form the dominant flora in the samples packed with the MAP method with high O_2 content. However, in the MAP method with low O_2 content, the dominant species responsible for spoilage were found to be *Carnobacterium, Serratia* and *Yersinia* at 4°C, while it was found to be *Hafnia alvei* at 10°C. Consequently, researchers recommend packaging poultry with MAP with a high O_2 content, due to its being less harmful and as pathogenic bacteria such as *Yersinia* cannot grow. Another advantage of packaging poultry with MAP with a high O_2 content is it inhibits the growth of an important food pathogen, *Campylobacter jejuni*, which poses a risk for poultry meat (Rajkovic et al., 2010). Usually, poultry is packaged in conditions where the CO_2/N_2 concentration is high and the O_2 concentration is reduced. Because oxymyoglobin formation and preservation of bright red color are not required in poultry meat as it is required for red meat (Sante et al., 1994). Besides, *Pseudomonas*, which is one of the most important spoilage factors in poultry meat is very sensitive to high CO_2 concentration, thus inhibiting their growth (Höll et al., 2016).

Different decontamination practices in poultry can increase the microbiological quality and shelf life of products by creating different levels of inhibition on both pathogenic and spoilage bacteria (Mead, 2004). In this context, Okolocha and Ellerbroek (2005) applied decontamination by spray or dip method with acid and alkaline agents (1% lactic acid and 10% trisodium phosphate) in poultry carcasses. The authors have reported that decontamination with the immersion method was more effective, and an average of 0.2-2.4 logarithmic reduction in *Pseudomonas* number was obtained as a result of

storage at 4°C for 6 days. They also stated that the sensoric evaluations were also positive by the consumers, and accordingly, with acid or alkali applications within the legal limits, poultry can be stored at 4°C for at least 6 days, preserving its quality.

Biofilm formation by *P. fluorescens* is also an important problem for the poultry industry, and this event can cause cross-contamination throughout the production process. Next time, this bacterium can also get resistance against disinfectants used in the poultry plant (Geornaras et al., 1999; Wang et al., 2018; Kumar et al., 2019).

In conclusion, the present study has revealed that the prevalence of *Pseudomonas* spp. is quite high in chicken drumstick samples stored at refrigeration temperature (4°C) and *P. fluorescens* was found to be the dominant species. *Pseudomonas* species except for *Pseudomonas aeruginosa* have not been included in food safety management systems as they do not cause any infection in humans. However, *Pseudomonas* spp. are primarily responsible for microbial spoilage in poultry samples kept in cold and distributed under cold chain, which is extremely important in terms of the quality and shelf life of poultry in today's global conditions. On the other hand, the presence of *Pseudomonas* in poultry may facilitate the growth of *Campylobacter jejuni* due to the microaerophilic environment that they create. This is an adverse event and thereby, *Pseudomonas* as an important spoilage bacterium that should not be overlooked by focusing only on pathogen bacteriae in the poultry production chain.

Conflict of interest: --

Ethical Approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k).

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