

# Determining the Presence of Pantone-Valentine Leukocidin PVL Virulence Gene and Methicillin-Resistant Gene *mecA* in *Staphylococcus aureus* Strains Isolated from Cream Puffs Sold by Confectionaries in Tehran by Multiplex PCR and Studying their Antibiotic Resistance

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## Abstract

*Staphylococcus aureus* is a major cause of food poisoning in the world that is created by consumption of contaminated food. Resistance to a variety of common and specific antibiotics is increasing. *Staphylococcus aureus* including PVL and gene *mecA* to heat pasteurization and many proteolytic enzymes are stable and can remain active for a long time in food samples. The purpose of this study was to isolate *Staphylococcus aureus* and identify virulence gene PVL and gene of methicillin resistance in bread pastry cream by multiplex PCR technique has been used. The study included 50 samples of bread pastry cream collected 23 cases (49%) *Staphylococcus aureus* isolates were detected. Antibiotic susceptibility testing by disk diffusion method according to CLSI guidelines was conducted. To identify and confirm *Staphylococcus aureus* virulence and resistance genes from multiple PCR assay was used. Antibiogram results showed that antibiotics are among the most sensitive to the antibiotic vancomycin, Tetracyclin, and doxycyclin hydrochloride respectively 100%, 100%, and 100%. Resistance to penicillin, cefixime, 65/3%, 56/5% more than other antibiotics was tested. Prevalence of methicillin resistance gene *mecA* in total 0% and PVL gene was not detected. Also, 16 rRNA genes in all samples were identified genus and species and confirmed. Different distribution of methicillin resistance gene in this study with other studies showing the potential risk of methicillin-resistant *Staphylococcus aureus* in the world. Therefore, early detection and timely resistant strains, in order to prevent the spread of resistance appears to be necessary.

**Keywords:** *Staphylococcus aureus*; Methicillin; Pantone valentine leukocidin; Multiplex-PCR

## Introduction

Some of *Staphylococcus* strains are pathogenic for humans and animals and some others lead to food spoilage [1]. Food is a suitable environment for bacterial growth to cause *Staphylococcus* poisoning. In human, this bacterium resides in the anterior nose and it is permanently found in 20%-40% of the human population and in 60% of people it is seen alternately. Thus people working in food preparation, processing, and distribution centers could possibly transfer this bacterium to food [2-4]. *Staphylococcus aureus* is one of the most common causes of bacterial food poisoning; moreover, it can cause dermal wounds and bumps and some of them can lead to quite dangerous difficult-to-treat hospital infections [5]. *Staphylococcus* food poisoning is the result of having *Staphylococcus*-contaminated food. It has diverse symptoms among which diarrhea and vomit are widely common [1]. Using immunologic methods and, molecular techniques such as PCR and Multiple PCR are suitable for detecting the bacterium with the toxin-coding gene [6]. On the other hand, *Staphylococcus aureus* is being globally resistant to common antibiotics which have introduced the serious problem of increased antibacterial resistance. PVL is a cellular toxin which was first detected in *Staphylococcus aureus* in 1930 and is produced by most *Staphylococcus aureus* strains [7]. This toxin acts against polymorphonuclear white cells and macrophages and increases the permeability of the cell membrane, therefore results in leaching leukocytes and tissue necrosis [8]. It is composed of two parts which are both leukocidin antigenic and can be converted to toxoid. Resisting against phagocytosis, this toxin increases the aggressive power of *Staphylococcus*. It is absorbed in blood circulation and it can engage some organs and lead to some clinical symptoms such as fever, low blood pressure, diarrhea, muscular pain, necrotizing pneumonia, and velutinous rashes [7,9]. *Staphylococcus aureus* resistance against methicillin is due to *mecA* gene which is coding a 78 kDa-penicillin-

binding-protein (PB2a) [10]. *Staphylococcus aureus* pathogens contain leukocidin Pantone valentine and they are resistant to methicillin, pasteurization, and many proteolytic enzymes, therefore they can remain active in food samples for quite a long time [8]. Nearly all countries around the world are involved in food problems. According to studies, 14%-40% of food-caused diseases are caused by *Staphylococcus aureus* [11]. Also, the *mecA* gene presence is mandatory to express the PVL gene and create resistance [9]. Regarding the importance of these two genes' existence in *Staphylococcus aureus* which can lead to increased pathogenicity, resistance to treatment, and infection transmission via food, this study was carried out with the aim of prompt detection of PVL virulence gene and determining the antibacterial resistance pattern of methicillin-resistant *Staphylococcus aureus* isolated from cream puff samples by multiplex PCR technique.

## Materials and Methods

### Collection and isolation of samples

50 samples of cream puffs were accidentally collected from 200 confectionaries throughout north, south, east, west, and center of

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Tehran and delivered to the laboratory. These samples were grown on blood agar, mannitol salt agar, Baird Parker agar, and chrome agar staph aureus cultures and incubated in 37°C for 24 hours. After identifying and confirming the presence of the bacterium, ultimately 23 strains of *Staphylococcus aureus* among 50 samples were isolated.

### Antibiogram test

In order to do the antibiogram test, the Kirby-Bauer disc diffusion method was used based on CLSI instruction [12]. In order to equate turbidity standard of NIM McFarland, a number of colonies were removed by an inoculation needle and dissolved in sterile physiologic serum. Then it was cultured on Muller-Hinton agar, after that, antibiotic discs were placed on cultures within standard distances and incubated in 37°C. The results were read after 24 hours [12]. In order to carry out the test methicillin antibiotic discs (5 µg), vancomycin (30 µg), oxacillin (1 µg), penicillin (10 µg), tetracycline (30 µg), doxycycline hydrochloride (40 µg), and Cefixim (5 µg) were purchased from HiMedia Laboratories. To check the quality control of the tests, *Staph aureus* standard strain ATCC25923 was used as the positive control.

### DNA extraction

At this stage, the isolated bacteria were cultured in Luria-Bertani broth medium, and then DNA was extracted according to instructions by Cina Pure DNA KIT-PR881614.

### Multiplex-PCR

The initial denaturation stage was 90°C for 5 minutes, next 90°C denaturation for 40 seconds, 58°C binding phase for 1 minute, 72°C expansion phase for 2 minutes (40 cycles), and 72°C final expansion for 7 minutes. Primers used in this test are listed in Table 1 [9]. The mixtures used for reactions were as follows: distilled water 10.7 µL, 1X

PCR buffer 1.25 µL, MgCl<sub>2</sub> 1.25 µL, dNTP mix (5 Mm) 0.2 µL, 1.5 µL of each primer, 0.1 µL of Ta polymerase, and 2.1 µL of DNA sample were prepared in a final volume of 25 µL. Multiplex-PCR test was carried out in TECHNE. In order to check Multiplex-PCR product, samples were transferred to 1% agar gel and were studied in BIORAD DOC gel after being dyed. Data were analyzed through SPSS software (version 13, SPSS Inc., Chicago, IL) and applying descriptive statistical tests (Figures 1 and 2).

### Results

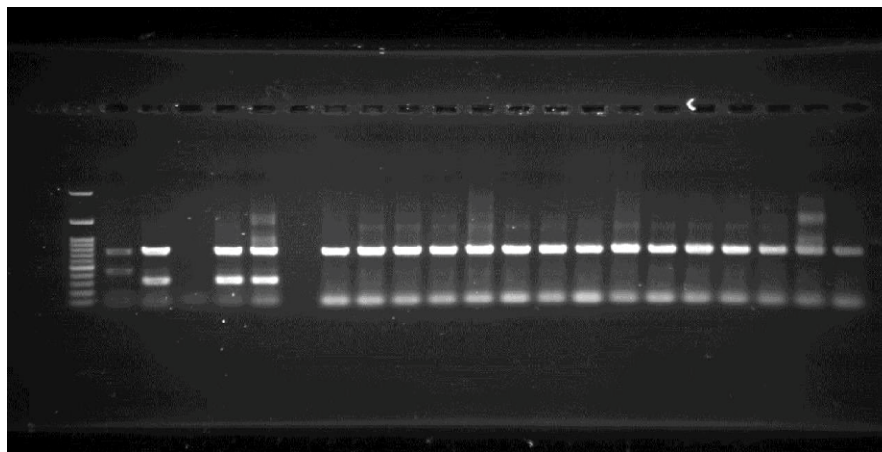
Of the 50 cream puff samples in this study, 23 *Staphylococcus aureus* strains (49%) were isolated. Strains' varying sensitivity to antibiotics is mentioned in Table 2. The highest sensitivity respectively belonged to vancomycin (100%), doxycycline hydrochloride (100%), tetracycline (100%), and methicillin (95.6%). Penicillin 65.3% and cefixime 56.5% were respectively reported to have the highest resistance. 16s rRNA was selected to identify and confirm the presence of *Staphylococcus aureus* bacterium. All the 23 samples which were verified by biochemical tests, were certainly proved. The frequency of *mecA*, Luk-PV, and 16s rRNA are reported in Table 3.

### Discussion

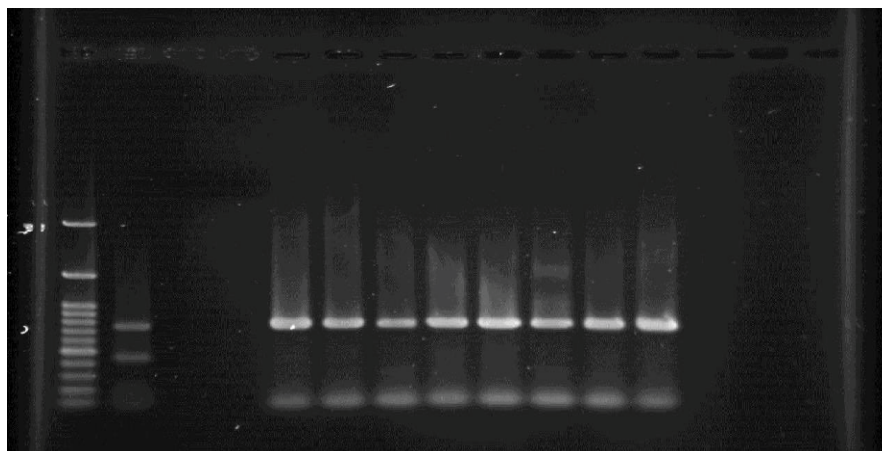
*Staphylococcus* food poisoning encompasses most of the bacterial food poisonings. According to reports, 14%-40% of all cases attribute food-borne diseases to this bacterium [11]. In a study by Little et al., during 2004-2005 in England it was shown that providing suitable sanitation can prevent bacterial growth. Moreover, cheese made from raw milk has more *Staphylococcus aureus* bacteria than cheese made out of pasteurized milk [13]. In the present study, the highest contamination was related to cream puff samples with 23 *Staphylococcus aureus* bacteria which have the highest frequency among microorganisms and because

Primer	Primer Sequence (3'-5')	Target gene	Product length (bp)
<i>Staph756F</i>	AACTCTGTTATTAGGAAGAACA	16s rRNA	756
<i>Staph750R</i>	CCACCTTCCTCCGGTTTGTCACC	16s rRNA	756
<i>Luk-PV-1</i>	ATCATTAGGTAAAATGTCTGGACATGATCCA	<i>LukS/F-PV</i>	433
<i>Luk-PV-2</i>	GCATCAA GTGTATTGGAT AGCAAAA GC	<i>LukS/F-PV</i>	433
<i>mecA1</i>	GTAGAAATGA CTGAA CGTCCGATAA	<i>mecA</i>	310
<i>mecA2</i>	CCAATCCACATTGT TTCGGTCTAA	<i>mecA</i>	310

**Table 1:** Primers sequence to perform Multiplex-PCR.



**Figure 1:** Multiplex PCR results: left to right ladder 100 bp, positive standard strain-negative control-samples 1-10 have 16s rRNA gene (756 bp) and regarding virulence genes Pantone Valentine leukocidin PVL (433 bp) and methicillin resistant gene *mecA* (310 bp) they are zero.



**Figure 2:** Multiplex PCR results: left to right ladder 100 bp, positive standard strain-negative control-samples 16-3 have 16s rRNA gene (756 bp) and regarding virulence genes Pantone Valentine leukocidin PVL (433 bp) and methicillin resistant gene *mecA* (310 bp) they are zero.

Antibiotics	Sensitivity (%)	Average sensitivity (%)	Resistance (%)
Doxycycline	23 (100)	-	-
Penicillin	8 (34/7)	-	15 (65/3)
Tetracycline	23 (100)	-	-
Oxacillin	18 (78/2)	1 (4/4)	4 (17/4)
Cefixime	7 (30/4)	3 (13/1)	13 (56/5)
Vancomycin	23 (100)	-	-
Methicillin	22 (95/6)	1 (4/4)	-

**Table 2:** Sensitivity of the isolated strains to various antibiotics through disc diffusion based on numbers (%).

%	Frequency	Gene Type
0	0	<i>mecA</i>
0	0	<i>Luks/F-PV</i>
100%	23	<i>16s rRNA</i>

**Table 3:** Frequency distribution based on *mecA*, *Luks/F-PV*, *16s rRNA* genes in food samples.

of using dairy products in the confectionary industry it is significant. Therefore, this issue is of particular importance in people who carry this bacterium and are continuously in close contact with food since they can contaminate food. As a result, people who carry *Staphylococcus aureus* in their nasal tract are considered a source of distributing contamination [14]. One of the important issues in treating diseases is the resistance of pathogenic bacteria to antibiotics. Antibiotic resistance can be inherent or acquired. In inherent resistance, the natural or wild cell can inhibit antibiotic and is chromosomally originated. While the acquired resistance is due to being exposed to different factors and converting sensitive strains to resistant strains. Today the increased resistance to an antibiotic is a global issue which is due to increased and uncontrolled consumption of medications and unfortunately, Iran is no exception to this issue. Bacteria can easily transfer resistant genes which are on mobile genetic elements (plasmids, transposons, and integrons) from one bacterium to another [15]. Therefore because of high consumption of foods like fruit juice, and in particular dairy products, among the general population, they probably play an important role in transferring medical resistance [16]. PVL gene is a contagious factor which is methicillin-resistant with *Staphylococcus aureus* infections. Its discovery is time taking and depends on its medium. Considering the surveys, the frequency percentage of PVL is higher in methicillin-resistant strains. Therefore, in the current study with respect to the results of disc tests and comparing them with the

standard pattern, it was revealed that majority of the isolated and surveyed samples were sensitive to methicillin and PVL gene was not expressed in respective strains. This proves that the *mecA* gene has to be present for the PVL gene to be expressed and resistance to be built. In addition, Martinez et al. examined the *Staphylococcus aureus*-infected children. Antibiotic sensitivity was determined via the disc diffusion method. They finally come to this conclusion that the PVL gene was more often identified by a molecular method in methicillin-resistant children than other children who were sensitive to methicillin [17]. In Bentzmann et al., [18] studied the effects of *Staphylococcus aureus* with the PVL gene and *Staphylococcus aureus* without PVL gene in causing necrotizing pneumonia. Bentzmann et al., [18] pointed out that PPSA *Staphylococcus aureus* causes more tissue damages to epithelial tissues in comparison with PNSA. In studies carried out by Enany et al., in Egypt [19], it was found out that in spite of the global distribution of PVL gene presence in methicillin-resistant *Staphylococcus aureus* (acquired resistance), this gene was not detected in Egypt and PVL is a good marker for detecting these strains. In the present study, non-isolation of PVL gene in food samples can be a verification of results by Bentzmann and Enany. Thus it seems that the present study with the possibility of examining the presence or absence of PVL gene, in comparison with the isolated strains obtained from other parts of the country, is a suitable factor for studying the geographical distribution of these strains. In a study by Mokhtari et al., [20] studied antibiotic

residual in raw milk tanks. In this survey which was carried out on 79 raw milk collecting tanks from four regions of Iran, 32.9% of the samples contained antibiotic. In another study by Khakpoor et al., [14] on local cheese in West Azerbaijan, it was revealed that 7.05% of traditional cheese samples were contaminated with *Staphylococcus aureus*. In a study by Pereira et al., [21] on *Staphylococcus aureus* isolated from various foods, 38% were reported to be methicillin-resistant strains. A study by Soltan et al. in different regions of Tehran indicated that methicillin-resistant strains numbers in *Staphylococcus aureus* samples isolated from various foods were significant. Therefore how these resistant strains are distributed via foods is important and is worth considering [22]. In the present study methicillin resistance by disc, diffusion was 0% and the frequency of the *mecA* gene was reported to be 0% which is significant in comparison with other studies and both responses are consistent. Considering that *Staphylococcus aureus* is transferred by carries and the methicillin-resistant gene is zero in this study, therefore these carries have not been efficiently cured which indicates that the issue of resistance has not been introduced yet. Thus a suitable course of treatment can eradicate the problem. On the other hand, failing to observe personal hygiene is totally unacceptable. It is highly recommended that these individuals be treated and sampling be done again, so we can examine if this resistance has been caused or not.

## Conclusion

The prominent point is that the resistance level to methicillin among *Staphylococcus aureus* strains in this study has been different from some other figures reported in other studies either in Iran or worldwide. These discrepancies can be due to a different distribution of methicillin-resistant gene in various locations or they can result from their different identification methods. However, the common subject in all these surveys is that methicillin-resistant strains are widely spread throughout the globe which indicates the potential hazard of methicillin-resistant *Staphylococcus aureus* worldwide. In order to decrease the antibiotic resistance two factors; need to be taken into account; excessive consumption of antibiotics and ease of spreading the resistant gene. According to the findings of this study, the isolations from cream puffs displayed the resistance pattern to penicillin as well as cefixime. Therefore, food can probably play a significant role in transferring medical resistance.

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