



Antibiotic Resistance Pattern and Detection of *mecA* Gene in *Staphylococcus aureus* Isolated from Iranian Hamburger Samples

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HIGHLIGHTS

- Prevalence of *Staphylococcus aureus* isolated in handmade hamburgers was significantly higher than packaged ones.
- The highest antibiotic resistance was observed for penicillin G followed by oxacillin and tetracycline.
- Six *S. aureus* isolates which were evaluated for methicillin-resistance, contained the *mecA* gene.

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Acronyms and abbreviations

MIC=Minimum Inhibitory
Concentration
MRSA=Methicillin-Resistant
Staphylococcus aureus
PCR=Polymerase Chain Reaction

ABSTRACT

Background: Among the bacteria that cause food poisoning, *Staphylococcus aureus* is one of the most common causes of food poisoning worldwide. The aim of this study was to investigate the presence of *S. aureus* strains in Iranian hamburgers, analysis of their antibiotic resistance pattern, and molecular detection of *mecA* gene in isolated strains.

Methods: A total of 100 Iranian handmade (traditional) and packaged (factory-made) hamburger samples were investigated for the existence of *S. aureus*. The pattern of antibiotic resistance and the presence of *mecA* genes were investigated by disk diffusion and molecular methods, respectively. Data were statistically analyzed by SPSS software v. 24.

Results: The prevalence of *S. aureus* isolated in handmade hamburgers was significantly ($p=0.008$) higher than packaged ones. Most of 39 isolated *S. aureus* strains were susceptible to ciprofloxacin (31 isolate), chloramphenicol (27 isolate), and trimethoprim/sulfamethoxazole (37 isolate). The highest antibiotic resistance was observed for penicillin G followed by oxacillin and tetracycline. All isolates were found susceptible to vancomycin and gentamicin. Six *S. aureus* isolates which were evaluated for methicillin-resistance, contained the *mecA* gene.

Conclusion: The high presence of the *S. aureus* in Iranian hamburgers and the remarkable antibiotic resistance emphasize the need for policies which enforce hygienic practices within the food industry and fast food outlets.

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Introduction

Staphylococcus aureus is considered as one of the most important food-borne pathogens causing food poisoning (Gundogan et al., 2005). The bacterium is mainly isolated from foods of animal origin such as meat, dairy products,

and seafood (Soltan-Dallal et al., 2010a, 2015). Since the development of the Methicillin-Resistant *Staphylococcus aureus* (MRSA) in 1961, the currency of nosocomial infections and the deaths associated with its bacteria is

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increasing (Deng et al., 2015). Based on Clinical and Laboratory Standards Institute (CLSI, 2015) definition, MRSA are strains that show a Minimum Inhibitory Concentration (MIC) of ≥ 4 $\mu\text{g/ml}$ for oxacillin (Raygada and Levine, 2009). Most of the MRSA strains showed resistance or decreased sensitivity to other antibiotic agents which finally leads to spread of these pathogens. Methicillin resistance mainly is attributed to *mecA* gene. This gene encodes Penicillin-Binding Protein 2a (PBP2a) causing decreased binding affinity for the β -lactam antibiotics (Berger-Bächli, 1999). Despite the presence of the *mecA* gene in all MRSA strains, they may show different levels of resistance. Furthermore, other genetic elements have been postulated to be involved in the mechanism of resistance (Matsushashi et al., 1986). Resistance to ceftiofloxacin is found to be a surrogate marker of MRSA with high accuracy (Fernandes et al., 2005).

The consumption of foodstuffs with animal origin including meat products (e.g. hamburger) has increased remarkably all over the world. Therefore, contamination of hamburgers with *S. aureus* poses a great public health threat (Lee et al., 2005). Regular monitoring and analysis of the antibiotic resistance patterns of *S. aureus* strains isolated from different origins including meat products is crucial for final control of resistant strains.

Currently, antibiotic susceptibility testing by disk diffusion and antibiotic dilution methods are employed for identification of antibiotic resistance for staphylococci (Al-Zu'bi et al., 2004). However, there are some limitations with these methods including low discrimination rate and high dependence on growth conditions. Nowadays, molecular DNA-based methods such as Polymerase Chain Reaction (PCR) have been established as a useful alternative to traditional detection methods. Moreover, the *mecA* gene, the structural determinant encoding PBP2a, is considered as a useful molecular marker of putative methicillin resistance in *S. aureus* (Elhassan et al., 2015; Soltan-Dallal et al., 2010b).

The aim of present study was to investigate the presence of *S. aureus* strains in Iranian hamburger samples, analysis of their antibiotic resistance pattern, and molecular detection of *mecA* gene in isolated strains using PCR technique.

Materials and methods

Sample collection

A total of 100 hamburger samples were randomly purchased from small grocery, supermarkets, and sandwich shops in Tehran City, Iran. The samples consisted of 25 handmade (traditional) and 75 packaged (factory-made) hamburgers containing 30, 60, and 80% meat ingredient

($n=25$ for each type). The samples were placed in sterilized jars and cooling ice box (4°C) and shipped immediately to the laboratory under sterile conditions. Samples of hamburger were prepared in accordance with the International Organization for Standardization (ISO, 2003) under laboratory conditions.

Bacterial isolation and identification

Each sample (1 g) was weighed into sterile container diluted with 9 ml Giolitti-Cantoni broth (Merck, Germany) and homogenized in the container for 2 min. Ten ml of each sample incubated aerobically at 37°C for 24-48 h and spread-plated on Baird-Parker agar (BP; Merck, Germany) supplemented with egg yolk tellurite emulsion (50 ml/l) for incubation at 37°C for 24 h. The biochemical and cultural characters of colonies and cell morphology were investigated. Colonies of *S. aureus* appear dark gray to black, convex and shiny due to tellurite reduction. These colonies are normally surrounded by clear zones, a result of lecithinase activity. Typical colonies of *S. aureus* were submitted for the following tests: coagulase, catalase, DNase, acetoin production, and mannitol fermentation according to guidelines (Quinn et al., 2011). The reference strain used for microbiological analysis was *S. aureus* ATCC 25923.

Antibiotic susceptibility testing

Antibiotic susceptibility testing of *S. aureus* isolates was performed using the standard disk diffusion method on Mueller Hinton agar (Merck, Germany), as recommended by Clinical and Laboratory Standards Institute guidelines (CLSI, 2015) using *S. aureus* ATCC 25923 as a control strain. Briefly, Mueller Hinton agar media were inoculated with a suspension (equivalent to a 0.5 McFarland standard) of each *S. aureus* isolates. Disks (6 mm in diameter; Mast group LTD, Merseyside, UK) containing penicillin G (10 IU), ciprofloxacin (5 μg), erythromycin (15 μg), chloramphenicol (30 μg), clindamycin (2 μg), trimethoprim/sulfamethoxazole (25 μg), gentamicin (10 μg), oxacillin (1 μg), and tetracycline (30 μg) were placed on media. A sterile disk was used for each test as a negative control indicator. The plates were incubated at 37°C for 16 to 18 h; then the zones of growth inhibition were evaluated according to CLSI guidelines. Screening for MRSA strains and phenotype verification of *mecA* gene presence was performed by 30- μg ceftiofloxacin disk diffusion testing following the CLSI guidelines (CLSI, 2015). *S. aureus* strain COL (MRSA) used as control in this test. Furthermore, MICs were determined for all 39 isolates using Etest for vancomycin according to guidelines of CLSI (2015). The results of antibiotic susceptibility testing were classified into three groups; namely resistant, intermediate-resistant, and susceptible.

PCR detection of *mecA* gene

All MRSA strains were investigated for the presence of *mecA* gene PCR as a gold standard assay according to Sakoulas et al. (2001). DNA extraction of MRSA strains isolated from Iranian hamburger samples was carried out using TENT (Tris-EDTANaCl-TritonX100) methods according to the instructions (Unno et al., 2015). Amplification of the methicillin resistance gene *mecA* was performed using specific primer pair of *mecA*-F 5'-AAAATCGATGGTAAAGGTTGGC-3' and *mecA*-R 5'AGTTCTGCAGTACCGGATTTGC-3' based on Shahrzad et al. (2012) synthesized by Pishgam Co. (Tehran, Iran).

PCR amplification was carried out using a Primus 96 Plus thermal cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany). The reaction solution was prepared by 12.5 µl Master mix (Pishgam Biotech, Iran) and 10.1 µl distilled water. Then 2 µl template DNA was added separately to each reaction tube with a final volume of 25 µl/reaction. The thermal steps were as follows: initial 5 min for denaturation step at 95 °C for one cycle followed by 35 cycles of denaturation in 30 s at 95 °C, annealing in 30 s at 60 °C, and extension in 40 s at 72 °C. The final extension was done in 5 min at 72 °C. PCR product was assessed using 1% agarose gel electrophoresis alongside by a 100 bp DNA ladder. For all reactions, *S. aureus* ATCC 49476 (*mecA* positive) and ddH₂O were used as positive and negative controls, respectively. The expected amplicon was a fragment with 533 bp in length.

Sequence analysis

PCR products were purified using a QIAquick® PCR Purification kit (Qiagen, Germany) according to the manufacturer's instructions. Genome sequencing was performed for all confirmed MRSA isolates. Genomic sequencing was performed by Takapozist Co. (Iran) and after loading the genomic sequence in BLASTn program (<http://www.ncbi.nlm.nih.gov/BLAST/>) at National Center for Biotechnology Information (NCBI), it was compared with similar genomic sequences in GenBank database.

Statistical analysis

Data of this research were statistically analyzed by SPSS software version 24 (SPSS, Inc., Chicago, USA) using the analysis of variance and the Chi-square tests. Differences between the mean values were significant when $p < 0.05$.

Results

Isolation of *S. aureus* from hamburger samples

During the study, 39 *S. aureus* isolates were detected from hamburger samples (Table 1). The amount of *S. aureus* isolates was much higher than handmade burger samples (64%). As a result, there was a significant difference ($p=0.008$) in the frequency of *S. aureus* isolates between handmade and packaged hamburgers. Besides, burgers with different percentages of meat were significantly different in terms of *S. aureus* contamination ($p=0.037$). In contrast, there was no association between brand marks of hamburgers and contamination with *S. aureus* ($p=0.076$).

Antibiotic resistance of *S. aureus* isolates

All the isolates were tested against different antibiotics. The antibiotic resistance pattern of the isolates is shown in Table 2. Among 39 *S. aureus* isolates, most of strains were susceptible to ciprofloxacin (31 isolate), chloramphenicol (27 isolate), and trimethoprim/sulfamethoxazole (37 isolate). The highest resistance was observed for penicillin G followed by oxacillin. No vancomycin or gentamicin resistances were found among these 39 isolates. Only 6 isolates (15.38%) were resistant to cefoxitin. Resistance to cefoxitin is considered as a marker of MRSA stains. The MRSA strains were isolated equally from packaged (factory-made) and handmade (traditional) hamburgers.

Susceptibility to vancomycin

The results obtained by Etest showed that all the *S. aureus* isolates were susceptible to vancomycin with MICs varying from 0.5 to 2 µg/ml (Figure 1). According to CLSI (2015) guidelines, MIC concentrations $2 \leq$, 4-8, and ≥ 16 µg/ml were considered as susceptible, intermediate-resistance and resistant, respectively illustrated in Figure 1).

MRSA analysis of *S. aureus* isolates using PCR detection of *mecA* gene

In the present study, molecular detection of *mecA* gene, as a specific gene target of MRSA, was performed for all 39 *S. aureus* strains isolated from hamburger samples using PCR method. The result of PCR assay showed the band with 533 bp size (Figure 2) in 6 (15.38%) isolates. It confirms the presence of the *mecA* gene in the methicillin-resistant isolates.

Table 1: Frequency of *Staphylococcus aureus* strains isolated from packaged and handmade hamburgers

Samples type	Sample size	No. (%) of <i>S. aureus</i> strains
Handmade	25	16 (64)
Packaged (30% meat)	25	7 (28)
Packaged (60% meat)	25	11 (44)
Packaged (80% meat)	25	5 (20)
Total	100	39 (39)

Table 2: Antibiotic resistance of *Staphylococcus aureus* isolates (n=39) obtained from Iranian hamburger samples

Antibiotic	Resistant No (%)	Intermediate No (%)	Susceptible No (%)
Penicillin	28 (71.92)	0 (0.00)	11 (28.20)
Ciprofloxacin	2 (5.12)	6 (15.38)	31 (97.48)
Erythromycin	11 (28.20)	17 (43.58)	11 (28.20)
Clindamycin	11 (28.20)	20 (51.28)	8 (20.51)
Chloramphenicol	1 (2.56)	11 (28.20)	27 (69.23)
Cefoxitin (as marker of MRSA)	6 (15.38)	0 (0.00)	33 (84.61)
Trimethoprim/sulfamethoxazole	1 (2.56)	1 (2.56)	37 (94.87)
Vancomycin	0 (0.00)	0 (0.00)	39 (100)
Tetracycline	15 (38.46)	4 (10.25)	20 (51.28)
Gentamicin	0 (0.00)	0 (0.00)	39 (100)
Oxacillin	23 (58.97)	0 (0.00)	16 (41.02)

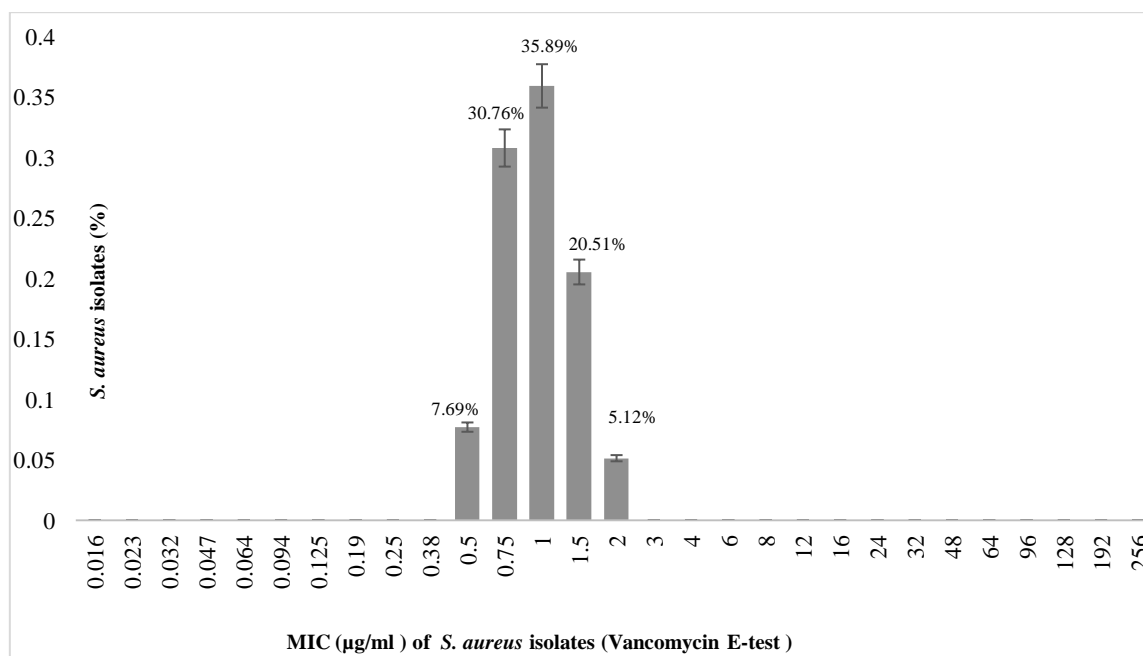


Figure 1: *Staphylococcus aureus* isolates with different Minimum Inhibitory Concentration (MIC) ranges for vancomycin

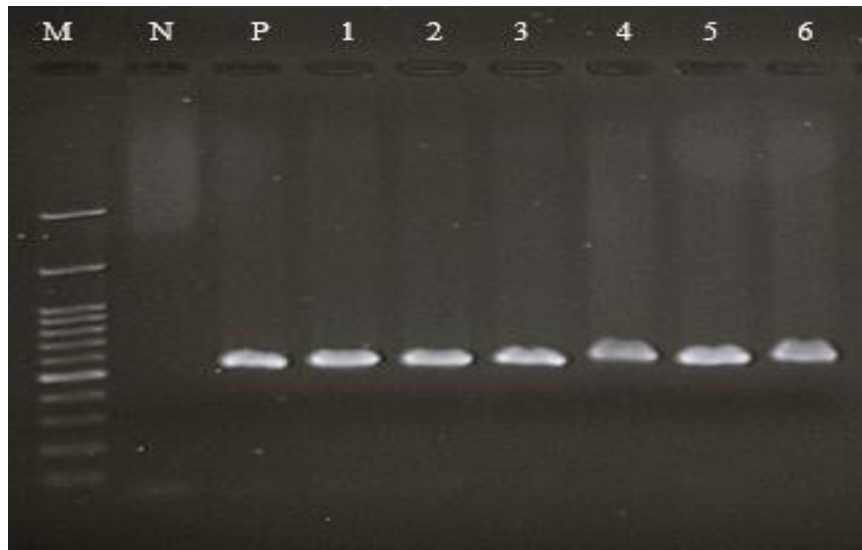


Figure 2: Agarose gel electrophoresis analysis of PCR amplification products of *mecA* gene, extracted from *Staphylococcus aureus* strains isolated from packaged and handmade hamburger samples. Lane M: 100 bp DNA ladder, lane N: negative control, lane P: positive control (*S. aureus* strain COL; 533 bp), Lane 1-6: positive isolates for *mecA* gene

Discussion

S. aureus is involved in a nosocomial infection and food-borne illness. Human acts as a carrier and may contribute to the spread of this bacteria (Aycicek et al., 2005; Shahraz et al., 2012; Shawish and Al-Humam, 2016; Soltan-Dallal et al., 2016). *S. aureus* is considered as a main cause of food poisoning following ingestion of staphylococcal enterotoxins (Hennekinne et al., 2012; Sharifi-Yazdi et al., 2016). The food contamination with *S. aureus* commonly occurs by workers who have skin lesions containing *S. aureus*, or by sneezing or coughing (Gundogan et al., 2005). In the present study, high percentage of *S. aureus* contamination (39%) was found in handmade and packaged hamburgers marketed in Iran. Similar studies in Turkey showed *S. aureus* contamination in 53% of meat and chicken (Gundogan et al., 2005) and 61% of raw milk, pasteurized milk, and ice cream samples (Gundogan et al., 2006). Another study in Brazil revealed that 68% of raw hamburgers and 14% of sandwiches are contaminated with *S. aureus* (Contreras et al., 2015). Similarly, Shahraz et al. (2012) reported 25% contamination with *S. aureus* in hamburgers in Tehran, Iran. High level of contamination with *S. aureus* in hamburger samples poses a serious health hazard to the community and emphasizes requirement for the more efficient hygiene practice to decrease the level of contamination in meat products.

Consumption of ready-to-cook meat products such as hamburger has been dramatically increased all over the world. It is delicious and enjoyable with an easy consumption method (Nejad et al., 2014). Approximately, five billion hamburgers are consumed by Americans each year. Lipid oxidation and microbial contamination during the maintenance period are considered as two important factors, affecting the quality of this food material (Prayson et al., 2008). Foods contaminated with *S. aureus* after preparation are considered a significant food threat. Because of the processing manner, there are no other microbes in them. A wide variety of factors can affect the hygiene of hamburgers, including hamburger composition (protein, water, lipids and etc.), storage condition, the age and physiological phase of bacterium, time and temperature of freezing period, cooking process, and handling after cooking (Shahraz et al., 2012). In present study handmade hamburgers showed higher contamination which emphasizes that human involvement in preparation and processing may be an important risk factor. Packaged hamburgers are frozen shortly after their processing during storing and distribution until they are shipping for customer or prepared for serving. During freezing, cold shock gradually decreases bacterial population in hamburger. Nowadays, handmade unpacked hamburgers are sold in some food stores. There is no

clear history regarding the quality of their processing which makes them as a health hazard to the community. According to Iranian National Standard for meat products, Number 2304, hamburgers are classified in three groups based on red meat content as follows: 30%, 60-74%, and 75-95%. A hamburger with less than 60% red meat contains vegetable protein mainly soy protein, while others are not permitted (INSO, 2016). Furthermore, we found that hamburgers with 60% meat were associated with higher level of contamination. Hamburgers with 60% meat contain more non-meat ingredients such as fillers and binders which in combination with meat made an ideal environment for bacterial growth. Some common filler ingredients used in hamburgers are egg, whey powder, and powdered milk. In contrast, hamburgers with 80% meat contain minimum amount of filler and binding ingredients. Furthermore, 30% hamburgers usually use plant originating proteins such as soybean and gluten which are not suitable for bacterial growth (INSO, 2016).

Nowadays, antibiotic resistant *S. aureus* strains are considered as a major healthcare problem as they cause numerous nosocomial and community-acquired pathological infections. There are some reports of resistant *S. aureus* transmission by contaminated milk, meat, and retail chicken products (Gündoğan et al., 2006). In the present study, 71% and 58% of *S. aureus* strains obtained from hamburgers were resistant to penicillin G and oxacillin, respectively. Penicillin and oxacillin are of the commonly used antibiotics for treatment of infections in humans and animals. Overuse of these antibiotics leads to occurrence of resistance. Furthermore, administration of antibiotics to food-producing animals for therapeutic purposes, use of antibiotics with poor activity or inappropriate administering of dosage level are main factors contributing to the antibiotic resistant (Al-Zu'bi et al., 2004). Previous studies showed different rate of resistance to antibiotics. A study on *S. aureus* strains isolated from hamburgers distributed in Tehran, Iran revealed that resistance to methicillin was 89% (Shahraz et al., 2012). Acco et al. (2003) showed that 70% of strains of *S. aureus* isolated from Brazilian food handlers were resistant to penicillin. In several countries, however, large-scale studies revealed that resistance of the microorganism to penicillin is nearly 60% (Contreras et al., 2015). Antibiotic resistance may limit our therapeutic options and so, restrictive policies are needed to prevent further health problems.

The presence of high levels of contamination in hamburgers as well as their high levels of resistance to antibacterial agents shows that the existence of scientific and specific guidelines to prevent increased contamination and resistance is essential to maintain hygiene at different levels from production to consumption. Establishing a

microbial resistance monitoring system is one of the measures that must be taken in every country. If disease monitoring is usually available, treatment failure is identified in the early stages, in which case a decision to change the medication. Although the strains were resistant to different antibiotics, they were completely susceptible to vancomycin and gentamicin. Vancomycin is considered as a drug of choice for MRSA treatment in humans. Clinicians should carefully reconsider the use of vancomycin-based combination therapies for the treatment of infection due to methicillin-resistant *S. aureus* (Deresinski, 2009).

Following elevated incidence and spread of MRSA, development of fast and sensitive laboratory methods is crucial for the immediate identification of multiple-antibiotic-resistant MRSA. Previously, DNA-based detection of antibiotic resistance genes was performed by PCR methods for the gene targets of *mecA* (Shahraz et al., 2012). Finding of *mecA* gene is regarded as a major evidence for the detection of MRSA isolates as observed in present study. Microbiological susceptibility testing was performed using cefoxitin for phenotypic identification of MRSA strains.

The comparison between standard disk diffusion, as a phenotypic method, and PCR, as a genotypic method, were previously discussed in some other studies (Al-Zu'bi et al., 2004; Anand et al., 2009; Baddour et al., 2007; Boşgelmez-Tınaz et al., 2006; Mathews et al., 2010; Mimica et al., 2007; Shahraz et al., 2012; Soltan-Dallal et al., 2010b; Swenson et al., 2005; Velasco et al., 2005).

Conclusion

The high presence of the *S. aureus* in Iranian hamburgers and the remarkable antibiotic resistance emphasize the need for policies which enforce hygienic practices within the food industry and fast food outlets. Although all *S. aureus* strains were susceptible to vancomycin and gentamicin, the *S. aureus* strains have shown resistance to one or more antibiotics causing important therapeutic implications in the future. More restrictive policies on the use of antibiotics in animals, Good Manufacturing Practices (GMP), and Hazard Analysis Critical Control Point (HACCP) systems in food industries are recommended to overcome current problems.

Author contributions

N.M.B. and M.M.S.D. designed the study; N.M.B. and Z.R. conducted the experiments; M.M.S.D. and M.R.P. analyzed the data; N.M.B. wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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