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RAPID MOLECULAR CHARACTERIZATION OF VIRULENCE PATTERN IN CLINICAL ORIGINS METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* INCLUDING CYTOTOXIN PANTON VALENTINE LEUKOCIDIN

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Abstract

Panton-Valentine leukocidin (*luk-pv*) producing Methicillin Resistant *Staphylococcus aureus* (MRSA) is a serious health threating pathogen causes a skin and soft tissue infections. Accurate and rapid diagnosis of this pathogen determination the antibiotics resistance and virulence patterns is contributed significantly in effective treatment and improves the proceedings of infection control. In this study, single reaction of polymerase chain reaction technique applied to characterize the MRSA pathogen and determine the prevalence of its most important virulence genes including; *luk-pv*, *mec*A and *nuc* genes encoded for Panton-Valentine leukocidin, penicillin binding protein and thermo stable nuclease respectively, in addition to species-specific detection using 16S rRNA. Successful reactions were gathered in one multiplex reaction. In total 155 of different clinical samples were collected from main hospitals in Baghdad. The results showed variant profile of resistance against 18 antibiotics; complete resistance (100%) toward Methicillin and Oxacilline. High resistance to Ceftazidime (92.85%), Ampicillin (91%), Piperacillin (85.7%) and penicillin G (77%). Moderate resistance was to Tetracycline (67%), Cefotaxime (58.57%) and Clindamycin (34.28%). While low resistance was towards Tobramycin (14.28%), Vancomycin (17%), Gentamicin (18.57%), Levofloxacin (11.4%), Ciprofloxacin (4%), Chloramphenicol (9%), Erythromycin (14%), Rifampin (15.7%) and Amikacin (25.7%). Molecular screening using PCR technique showed 100%-228 bp, 91%-433bp, 100%-147bp and 85.7%-270bp towards 16S*rRNA*, *luk-pv*, *mec*A and *nuc* genes respectively. No clear correlation was observed between existence of pvl nuc and antibiotic pattern. Obviously, the *luk-pv* prevalence is significantly high in Baghdad. Optimization the monoplex PCR reactions in union multiplex reaction is succeed. Therefore, such sensitive and specific with no false positive result is reliable assay can be adapts for routine use in a diagnosis of MRSA pathogen.

Keywords **:** Multiplex PCR; Panton Valentine Leucocidin; MRSA; Baghdad

Introduction

Staphylococcus aureus is recognized as one of the most important bacterial pathogens causing a wide range of infections (Talebi-Satluo *et al.*, 2012), Specific group of *S. aureus* carrying staphylococcal cassette chromosome mec (SCCmec) characterized as Methicillin resistance *Staphylococcus aureus* (MRSA) has identified as a significant public health concern causing morbidity, mortality and long hospitalization (Talebi-Satluo *et al.*, 2012). Virulence factors have essential impact on MRSA pathogenicity and contributed in its spread. Panton-Valentine-Leucocidin (*luk-pv*) is one of these factors as a powerful cytotoxin forming pores for human mononuclear cells, disruption of leukocyte membranes and can cause cell death by apoptosis (Abdulrazaq *et al.*, 2014). The genetic material of bacteriophage has great contribution in the production of *luk-pv* cytotoxin (Shrestha *et al.*, 2014). MRSA strains those producing *luk-pv* are more virulent and highly transmissible strains than *luk-pv* negative. *luk-pv* cytotoxin encoded by *luk-pv* genes (*lukS-pv* and *lukF-pv*) that encodes *luk-pv* composed of two exoprotein subunits which are transcribed together and secreted separately to form a complete heptavalent leukocidin **(**Kaneko & Kamio, 2004; Prevost *et al.*, 1995). MRSA can be classified into community acquired (CA-MRSA) and hospital acquired (HA-MRSA) infections, which CA-MRSA show more-diverse clonal groups than HA-MRSA and belong to SCCmec type IV, V, or VI DNA element (Kloos & Wolfshohl, 1982; Fritz *et al.*, 2008) The pvl gene region characterizes as a significant virulence factor and conservative genetic marker of CA-MRSA strains (Monday & Bohach,1999). The reports had shown a high prevalence proportion 77% of *luk-pv* in CA-MRSA while it presents only 4% of HA-MRSA isolates (Yoong and Torres, 2013). The *luk-pv* gene can spread among strains through bacteriophage and plasmids transmission results gradually increased prevalence of *luk-pv* carrying strains (Bush *et al.*, 1995: Issa, 2019). Limited records on *luk-pv* prevalence has been reported in Baghdad the capital of Iraq. Therefore, applicable molecular diagnosis method was used to determine the *luk-pv* prevalence in addition to the main virulence factors and antibiotic resistance profile in clinical strains of MRSA.

Materials and Methods

Clinical samples collection: A total of 155 samples were collected from different clinical specimens from hospitalized patients attended specific hospitals (26 % of the total was from Al-Kindi, 24% from Al -Zaafaraniyah General Hospital, 30 % from the Medical city, 20 % from Al-Karkh hospital) in Baghdad during the period from December 2018 the end of April 2019. Seventy isolates were obtained from the following sources: C.S.F (n=2), ear (n= 7), wounds (n=13), H.V.S (n=5), burn. (n=17), Abscess. (n=10), Urine (n=13) and Blood (n=3).The isolates were identified by means of routine tests: colony morphology and pigment formation on selective medium (mannitol salt agar), catalase test, oxidase reaction, coagulate test. Identification was confirmed via Api staph, VITEK 2((bioMerieux).

Antimicrobial susceptibility: The Antibiotic susceptibility testing was carried out for all the isolates on Mueller-Hinton method using disk diffusion method (CLSI, 2018) to measure zones of inhibition against slandered concentrations for the following antibiotics:

(30µg-AK, Amikacin) (10µg-GM, Gentamicin), (30µg-CAZ, Ceftazidime) (5µg-Lev, Levofloxacin), (30µg-VA, Vancomycin), (5µg-Cip, Ciprofloxacin), (30µg-C, Chloramphenicol), (1µg-OX, Oxacillin), (5µg-Me, Methecilline), (10µg-Am, Ampicillin), (100µg-PRL, Pipercilline), (10µg-PG, pencillineG) (30µg-E, Erythromycin), (2µg-CD, Clindamycin), (30µg-CX, Cefoxitin), (15µg-AZM, Azithromycin), (5µg -RA, Rifampin), (10µg -TOB, Tobramycin).

Molecular detection:

A: DNA was extracted from activated pure culture of studied strains using Bacterial DNA extraction Kit (Geneaid). Detection of DNA bands using Agarose gel electrophoresis (1%). Monoplex and multiplex PCR amplification of (16Sr*RNA*) for the detection of *Staphylococcus spp.* was performed on all tested strains of and primer for *nuc, mec*A, *and luk-pv.* The primers used in this study are shown in Table (1).

Table 1 : Primers sequences used in this study.

Primer	Primer sequence (5-3)	Product length (bp=base pairs)	Reference
16SrRNA	F-GTAGGTGGCAAGCGTTATCC	228	(Monday & Bohach,1999)
	R-CGCACATCAGCGTCAG		
mecA	F -GTGAAGATATACCAAGTGATT	147	Zhang *et al.*,2005)
	R -ATGCGCTATAGATTGAAAGGAT		
nuc	F-GCGATTGATGGTGATACGGTT	270	(Brakstad *et al.*,1992)
	R-AGCCAAGCCTTGACGAACTAAAGC		
luk-pv	F -ATCATTAGGTAAAATGTCTGGACATGATCCA	433	(McClure *et al,* 2006*)*
	R -GCATCAAGTGTATTGGATAGCAAAAGC		

F, forward; R, reverse

B: PCR amplification: Phenotypically characterized *S. aureus* isolates were subjected for molecular identification through single PCR amplification of species-specific *16SrRNA* gene with expected size 288bp. Positive strains were subjected to the detection of *mecA*, *nuc* and *luk-pv* genes with expected size of amplified regions: 147bp, 270bp and 433bp, respectively. All reaction mixtures were set up 25 µl reaction mixtures as follows: 12.5 µl ready to use PCR master mix mixture (Promega) containing approximately 10 ng of template DNA and 1 µl (10 pmol) of each primer. The rest volume completed with deionizer water. The mixtures were subjected to the following thermal cycling parameters in a Thermocycler (Applied BioSystem): Initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification with 94°C for 45 sec, annealing at 50°C, for 1Min for the following genes 16S *rRNA*, mecA, nuc and 57 *luk-pv* respectively, extension at 72°C for 1 Min, and a final extension at 72°C for 6 min. The PCR products were analyzed on a 2% agarose gel. Combinations of the four target genes in one reaction was designed and performed as Multiplex PCR. Annealing temperatures were optimized using 50°C.

Results and Discussion

1. Isolation: A total number of 155 clinical samples (swabs) were collected from various patients and hospital staff in different units in four hospitals in Baghdad. The results included 70 positive samples of swabs diagnosed as *S. aureus* from the following different sources: 17 burns, 13 Wounds, 14 urine, 9 abscess and boils, 7 ear, and 5 swabs of vaginal (H.V.S), 3 Blood and 2 CSF. Diagnosed isolates was determined according to conventional cultural and microscopic characteristics was grape-like clusters of blue colour under microscopic examination (Subhankari, 2011**;** Benson, 2002). On mannitol salt agar yellow colony indicated a mannitol salt fermenting (Jayasundara**,** 2014; Bush & Jacoby, 2010). Also Identification of *S. aureus* by API Staph System (Kloos, & Wolfshohl, 1982) and Vitek 2 automated system (bioMerieux, France). (Ligozzi,2002). Confirmed isolates identified as SA# for further experiments.

2. Antibiotic Sensitivity: The resistance pattern to the 18 antimicrobials tested is shown in table 2 according to Clinical Laboratory Standards Institute (CLSI) guidelines. According to the results, isolates had the lowest rate resistant to Ciprofloxacin (4%). This shows that this antibiotic the last choice of therapy for these infections. According to the results, isolates had the lowest rate resistant to Ciprofloxacin, Cephalosporin antibiotics are even more resistant to β-lactamases, although some β-lactamases have an increased affinity for cephalosporins (Simon and Sanjeev, 2007). Developed the second generation of penicillins to counteract the resistance of antibiotics, which includes Methicillin. Oxacilline, a synthetic semi-synthetic penicillin resistant to the enzyme Pencillinase (Al-Taai *et al.*, 2017). The MRSA prevalence was 100%. Antimicrobial susceptibility testing revealed a high resistance rate (91%, 85% 92%) towards Ampicillin, Piperacillin and Ceftazidime respectively. Study conducted by researcher indicated that the rate of isolation resistance clinical *S. aureus* of Ciprofloxacin was 23% which is unlike the current study (Bush *et al.*, 1995). Cephalosporin antibiotics are even more resistant to β-lactamases, although some β-lactamases have an increased affinity for cephalosporins (Issa, 2019), Results showed that the most effective antagonists against isolated clinical MRSA isolates Wounds and burns were Ciprofloxacin and Vancomycin This is consistent with result (Shekarabi *et al.*, 2017), The lack of resistance to Vancomycin is due to its lack of *vanA* and *vanB* gene(Zhang *et al.*, 2004), indicated that resistance to *S. aureus* clinical isolates was 43% for Gentamicin contrasting study while 13% for Chloramphenicol which is similar to the current study (Bush *et al.*,1995).

Table 2: Percentages of antimicrobial susceptibility rate of 70 *S.aureus* isolates against 18 antimicrobial agents

Antibiotics	*Staphylococcus aureus*						Chi-Square -χ^2
	S		I		R		
	No.	%	No.	%	No.	%	
Amikacin (AK)	34	48.57	18	25.7	18	25.7	7.62 **
Pencillin G (PG)	16	22.8	0	0	54	77	12.04 **
Methecillin (ME)	0	0	0	0	70	100	15.00 **
Ampicillin (AM)	6	8.57	0	0	64	91	14.38 **
Piperacillin (PRL)	10	14	0	0	60	85.7	14.02 **
Oxacillin (OX)	0	0	0	0	70	100	15.00 **
Ceftazidime (CAZ)	5	7	0	0	65	92.85	14.52 **
Cefotaxime (CTX)	29	41	0	0	41	58.57	11.69 **
Levofloxacin(LEV)	62	88.57	0	0	8	11.4	13.86 **
Ciprofloxacin (CIP)	66	94	1	1.4	3	4	14.57 **
Rifampin(RA)	59	84	0	0	11	15.7	14.33 **
Vancomycin(VA)	58	82.85	0	0	12	17	13.26 **
Erthromycin(E)	45	64	15	21	10	14	10.63 **
Tetracycline (TE)	21	30	2	2.85	47	67	9.53 **
Chloramphenicol(C)	58	82.85	3	4.28	9	12.8	13.30 **
Gentamicin (GM)	53	75.7	4	5.7	13	18.57	12.76 **
Tobramycin(TOB)	57	81.42	3	4.28	10	14.28	13.29 **
clindamycin (CD)	42	60	4	5.7	24	34.28	10.87 **

** (P<0.01).

our results are compatible with those obtained by Researcher (Brady *et al.*, 2007) as they observed that all isolates were resistant to Oxacilline and Methicillin and other β-lactam antibiotics,

While Al-Hossainy (2007), showed that VRSA were 20% among *S. aureus*, the result is close to our results, This results disagreed with finding of Al-Geobory (2011) in that the rate of resistant to vancomycin was 2.27%. Where the VRSA isolate among S. aureus is isolates 4 out of 50 (8%) (Mohammed, 2011).

3. DNA extraction: Total DNA was extracted from 70 *S. aureus* isolates using genomic DNA Extraction Kit (Geneaid). Extracted DNA was confirmed as bands by gel electrophoresis as shown in figure (1). DNA concentration and purity were measured by Nanodrop spectrophotometer, all the isolates had DNA concentration between (50-80 ng/µl) with purity about 1.7-2.

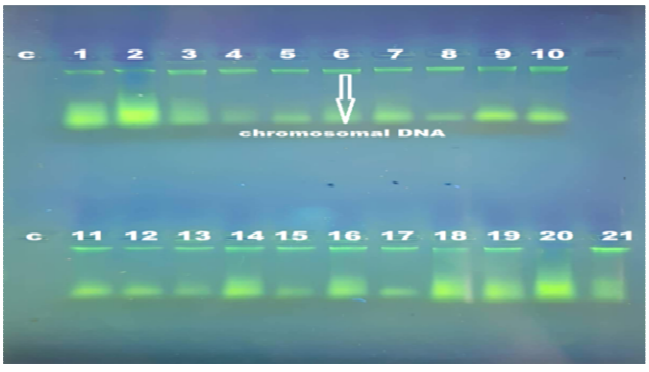

Fig. 1: Agarose gel electrophoresis of extracted DNA of different *Staphylococcus aureus* isolates . Lane (1-21): DNA of strains SA1-SA21, Lane C: Negative control. (1% Agarose, 75 V/ 30 min).

4. Molecular identification: Phenotypically positive isolates of *S. aureus* were confirmed using the specific initiator of the 16S *rRNA* gene by PCR using thermal cycler. The results showed all bacterial isolates belong to *S. aureus* (figure 2-A). The 16S *rRNA* PCR assay has been shown to be able to successfully identify multiple types of bacteria in multiple sample types Matsuda *et al.*, 2007. According to study conducted by Al–Alak and Qassim (2016), the detection of *16S rRNA* by PCR is rapid and reliable method as they observed that using *16s rRNA* confirmed all the 126 staphylococcal isolates as *S. aureus.*

Use as Khudhr **(**2016); El-Hadedy &El-Nour, (2012**)** PCR carried out using *16S rRNA* gene as a specialist. for *S. aureus*. In this method for identifying and confirmed all the staphylococcal isolates as *S. aureus*.

Methicillin resistant phenotypic identification of the *S. aureus* isolates was confirmed by the PCR detection of the *mec*A gene. The results of the detection of *mec*A 70/70 gene (100%) coded for anti-methicillin resistance gave a positive result for the gene as shown in figure (2-B), AL-Hasnawi, *et al*. (2013) found that the proportion of *mecA* (100%) was an approach to the study, Researchers Ghaznavi-Rad and Ekrami, (2018) also reported the rate of clinical MRSA isolates That gave a positive result for the *mecA* gene was 100% as well, is a similar result with the current results, While In a study conducted by the researcher Rahama *et al.* (2017) found that 85.37% of *mecA* gene is not similar to the current study,

The results of the detection of the *nuc* 61/70 (85.70%) gene encoded for thermal cores gave a positive result of the genetic (figure 2-C). Existence of *nuc* gene in MRSA strains is 100% (Alagely, 2016:, Brakstad *et al.*, 1992) Found all isolates give positive result to *nuc* gene, However, there is study showed MRSA strains with negative *nuc* explaining this due to mutation in primer-annealing site or partial deletion in the gene. (Klaassen *et al.*, 2003).

In order to detect the presence of *luk-pv* gene (*luk-pv*) and determination the prevalence of it among MRSA clinical isolates, single-plex polymerase chain reaction for each DNA extracted sample have been used. The results of the detection of the *luk-pv* 64/70 (91.40 %) gene encoded for Leukocidin gave a positive result (figure 2-D). The found researcher

Kareem, (2016) in Baghdad Also the results have been revealed (6.55%) of MRSA isolates har boring of pvl gene that the percentage of pvl gene which is a little percentage compared to the current study

In found Kandala *et al.* (2017). The ratio (*luk-pv*) is 53.48% which is moderate, Al-Hassnawi, *et al.*, 2013 found that the percentage of *luk-pv* (79%) gave a positive result which is comparable to the current results, The researcher Rahama *et al.* (2017) carries the gene *luk-pv* 31.71% which is a small result for the study, For this study, the *luk-pv* gene was more prevalent. Where *luk-pv* was the gene carried by methicillin-resistant *Staphylococcus aureus* It is a convergent result with Aghmiyuni *et al.* (2016). The study showed a relatively high prevalence of *luk-pv* in *Staphylococcus aureus* in India city.

All PCR products have been confirmed by analysis of the bands on gel electrophoresis and by comparing their molecular weight with 100 bp DNA Ladder.

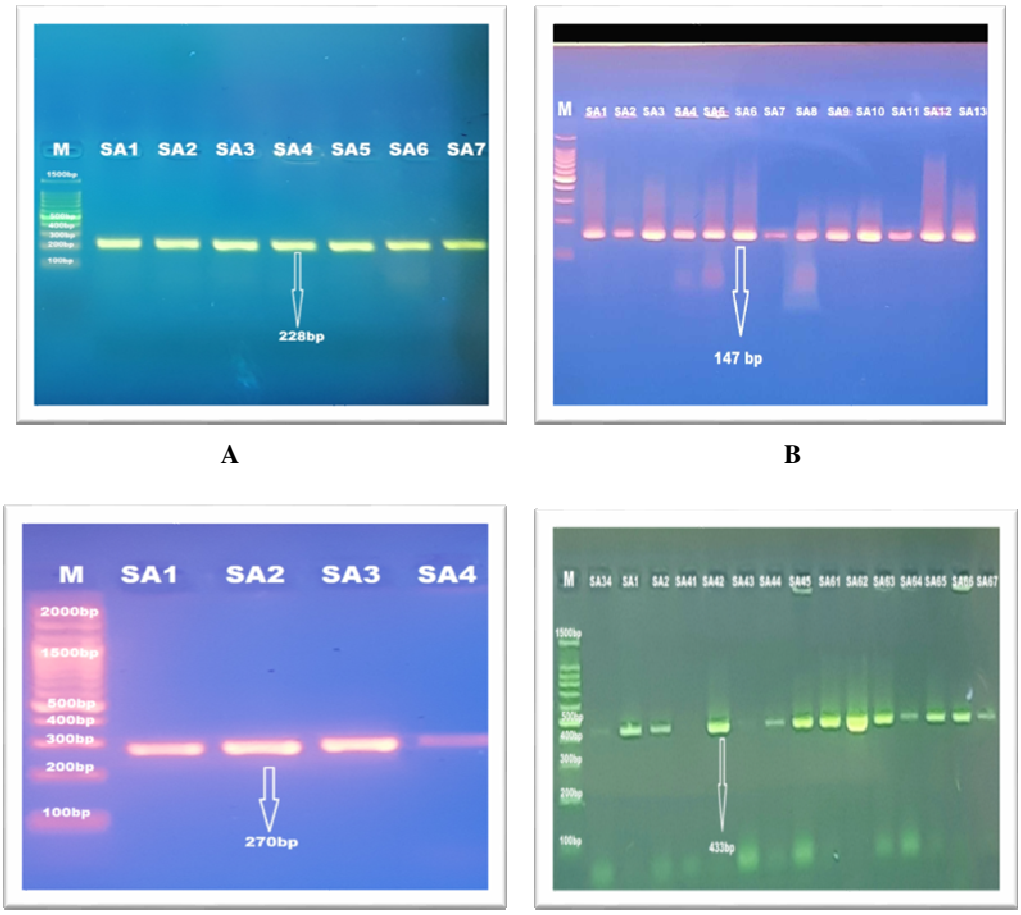

Fig. 2 : Gel electrophoresis of PCR products for four targeted genes in *Staphylococcus aureus.* (2% agarose gel and at 75V for 90 min) :

(A)- PCR Amplified Products for*16S rRNA* Gene. Lane (M): l00bp ladder, lanes (1-7), strains SA1-SA7 Positive Result with expected band (228 bp).

(B)- Agarose gel electrophoresis of PCR products for the resistance genes *mecA*. Lane M: 100bp DNA ladder; lanes (1-13) DNA of strains SA1-SA13. Result with Positive Bands of 147bp

(C)- Agarose gel electrophoresis for detection the *nuc* gene. Lane M: 100bp DNA ladder; lanes (1-4), strains SA1-SA4 Result with Positive Bands of 270bp

(D)- Agarose gel electrophoresis of PCR products, for the resistance genes *luk-pv* Lane M:100bp DNA ladder; lanes (SA1, SA2, SA42, SA44, SA45, SA61, SA62, SA63, SA64, SA65, SA66, SA67), Positive and line (SA34, SA41, SA43) Negative , Result with Positive Bands of 433 bp.

In this study, rapid and easy PCR assay was designated to diagnosis MRSA strains those carrying virulence factors through combine the four single reactions of PCR in union multiplex assay. The Optimization is depends on select the optimum annealing temperature (55°C) using gradient option thermal cycler. Similar thought was conducted by (Strommenger *et al.,* 2008).

Developed a new multiplex PCR assay for detection of Panton-Valentine leukocidin virulence genes and simultaneous discrimination of methicillin-susceptible from -resistant staphylococci, this assay is simple, rapid, and accurate and offers the potential for prompt detection of newly emerging CA-MRSA (McClure *et al.*, 2006).

The significant spread of MRSA and the introduction of these highly pathogenic virulent isolates represent increasing health threats. Thus, sensitive and specific with no false positive result is reliable assay can be adapts for routine use in a diagnosis of MRSA pathogen might be good tool to accurate diagnosis and determine the correct treatment resulted in good management.

Fig. 3 : Figure (4-13): Gel electrophoresis for PCR products by ethidium bromide, PCR multiplex to detect four genes (*16srRNA, MecA, Nuc, Luk-pv*), Note in line (1,2,4,5,7,8,9, 11) of *nuc* genes: positive and line (3,6,10) of *nuc* genes: negative, as for line (1-11) (appearance of genes *16srRNA ,MecA* and *Luk-pv* : Positive (*Staphylococcus aureus* (2% Agarose ,75Vol for 2 hours).

This study demonstrates the *luk-pv* gene is highly prevalent (91%) among MRSA strains in Baghdad governorate patients spread. Multiplex PCR is highly recommended to be used to detect genes as one approach to identifying MRSA in routine diagnostic laboratory for its rapid and precise diagnostic properties suggested it can be replaced to traditionally assays. A lower prevalence of *luk-pv* has been reported from other parts of world (5% in France, 4.9% in UK, 8.1% in Saudi Arabia, and 14.3% in Bangladesh) (Lina *et al.*, 1999; Holmes *et al.*, 2005; Afroz *et al.*, 2008) reflecting the significant variation in prevalence of *luk-pv* among geographical areas and communities. Kaur *et al*., from India, have reported overall 62.85% prevalence of *luk-pv* among MRSA and MSSA (MRSA: 85.1% and MSSA: 48.8%), Johnsson *et al*. (2004) detected *luk-pv* gene in one isolate (1%) from 65 patients with *S. aureus* bacteraemia, in two (2.19%) isolates from 91 patients with cutaneous infections, and in four (7.27%) isolates from 55 patients with respiratory tract infections.

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