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RESEARCH ARTICLE

Cytotoxic activity of *Staphylococcus aureus* isolates from a cohort of Mexican children with cystic fibrosis

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Abstract

Background: *Cystic fibrosis (CF) is a genetic disease in which thick, sticky mucus is produced in the lungs (and other organs) that impairs ciliary clearance, leading to respiratory problems, increased chronic bacterial infections, and decreased lung function. Staphylococcus aureus is one of the primary bacterial pathogens colonizing the lungs of CF patients. This study aimed to characterize the genetic relatedness of S. aureus, its presence in children with CF, and its cytotoxic activity in THP1 cell-derived macrophages (THP1m).* **Methods:** *Genetic relatedness of S. aureus isolates from a cohort of 50 children with CF was determined by pulsed-field gel electrophoresis (PFGE). The VITEK® 2 automated system was used to determine antimicrobial susceptibility, and methicillin-resistance S. aureus (MRSA) was determined by diffusion testing using cefoxitin disk. The presence of mecA and lukPV genes was determined by the polymerase chain reaction and cytotoxic activity of S. aureus on THP1m by CytoTox 96® assay.* **Results:** *From 51 S. aureus isolates from 50 children with CF, we identified 34 pulsotypes by PFGE. Of the 50 children, 12 (24%) were colonized by more than one pulsotype, and 5/34 identified pulsotypes (14.7%) were shared between unrelated children. In addition, 3/34 pulsotypes (8.8%) were multidrug-resistant (MDR), and 2/34 (5.9%) were MRSA. Notably, 30/34 pulsotypes (88.2%) exhibited cytotoxicity on THP1m cells and 14/34 (41.2%) altered THP1m monolayers. No isolate carried the lukPV gene.* **Conclusions:** *Although a low frequency of MRSA and MDR was found among clinical isolates, most of the S. aureus pulsotypes identified were cytotoxic on THP1m.*

Keywords: *Staphylococcus aureus. Cytotoxicity. Macrophages. Methicillin-resistant Staphylococcus aureus (MRSA). Multidrug resistance.*

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Actividad citotóxica de Staphylococcus aureus provenientes de una cohorte de niños mexicanos con fibrosis quística

Resumen

Introducción: *La fibrosis quística (FQ) es una enfermedad genética en la que se produce moco espeso y pegajoso en los pulmones (y otros órganos), lo que conduce a problemas respiratorios, incremento de las infecciones bacterianas crónicas y disminución de la función pulmonar. Staphylococcus aureus es uno de los principales patógenos que colonizan los pulmones de los pacientes con FQ. El objetivo de este trabajo fue caracterizar la relación genética de S. aureus, su presencia en niños con FQ y su actividad citotóxica en macrófagos derivados de células THP1 (THP1m).* **Métodos:** *La relación genética de los aislados de S. aureus provenientes de una cohorte de 50 pacientes con FQ fue determinada por electroforesis en gel de campo pulsado (PFGE). La sensibilidad a los antimicrobianos se determinó mediante el sistema automatizado VITEK® 2, y la resistencia a la meticilina (SARM) mediante la prueba de difusión utilizando discos de cefoxitina. La presencia de los genes mecA y lukPV se determinó mediante reacción en cadena de la polimerasa, y la actividad citotóxica de S. aureus sobre células THP1m mediante el ensayo CytoTox96®.* **Resultados:** *A partir de 51 aislados de S. aureus provenientes de 50 niños con FQ se identificaron 34 pulsotipos por PFGE. De los 50 niños, 12 (24%) estaban colonizados por más de un pulsotipo y 5 de los 34 pulsotipos (14.7%) los compartían niños que no estaban relacionados. De los 34 pulsotipos, 3 (8.8%) presentaron multirresistencia (MDR) y 2 (5.9%) fueron SARM. Además, 30 pulsotipos (88.2%) fueron citotóxicos sobre células THP1m y 14 (41.2%) alteraron su monocapa. Ninguno de los pulsotipos presentó el gen lukPV.* **Conclusiones:** *Aunque se encontró una baja frecuencia de SARM y MDR en los aislados, la mayoría de los pulsotipos de S. aureus identificados fueron citotóxicos para células THP1m.*

Palabras clave: *Staphylococcus aureus. Citotoxicidad. Macrófagos. Staphylococcus aureus resistente a la meticilina (SARM). Multirresistencia.*

Introduction

Cystic fibrosis (CF) is a genetic disorder that mainly affects the lungs but also the pancreas, liver, kidney, and intestine. This disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*)[1]. Mutations in *CFTR* alter the transport of chloride and sodium ions, HCO_3^- and water across the cell membrane in the airways.

Lung epithelia with impaired *CFTR* gene function produce thick, sticky mucus that clogs the airways and traps opportunistic bacteria, producing infections that cause inflammation, leading to decreased lung function, respiratory distress, and eventually respiratory failure[2].

During the first years of life, the airways of children with CF are rapidly colonized by non-typeable *Haemophilus influenzae* and *Staphylococcus aureus*[3], and progressively by *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex, which are the primary opportunistic pathogens associated with chronic infection and decreased pulmonary function[3]. In Mexico, most children with CF are colonized by *P. aeruginosa* and *S. aureus*[4]. Worldwide, airway colonization by *S. aureus* ranges between 30-50%[5].

S. aureus is a Gram-positive bacterium associated with establishing an inflammatory process in the lower respiratory tract, reducing lung function and contributing significantly to lung tissue damage[6]. The ability of these bacteria to produce a biofilm is associated with increased resistance to antibiotics *in vitro*[7]. In particular, methicillin-resistant *S. aureus* (MRSA) has been associated with accelerated deterioration of lung function and increased mortality[8]. Methicillin resistance is attributed to an alternate penicillin-binding protein (PBP2a or PBP2') encoded by the *mecA* gene[9]. *S. aureus* is a versatile bacterium with an arsenal of virulence factors, including Panton-Valentine leukocidin (PVL), which facilitates tissue adhesion and host cell injury[10]. PVL is often related to community-associated MRSA (CA-MRSA)[11], and its expression has been associated with severe infections, bacteremia, osteomyelitis, and necrotizing pneumonia[12]. PVL is a bicomponent pore-forming cytotoxin that causes leukocyte lysis[13]. This study aimed to characterize the genetic relatedness, presence of MRSA, and macrophage cytotoxic activity in clinical isolates of *S. aureus* from a cohort of children with CF in Mexico.

Methods

We conducted a descriptive study derived from a study published in 2020[4]. This study was approved by the Institutional Review Board of the Faculty of Medicine

of the Universidad Nacional Autónoma de México (Protocol FMED/CI/RGG/022/2016).

Bacterial isolates

Bacterial isolates were obtained from sputum samples collected from 50 pediatric patients attending the CF clinic of the Hospital Infantil de México Federico Gómez (HIMFG), Mexico City, Mexico, from August 2016 to January 2018[4]. Parents of children with CF who agreed to participate in this study signed a consent form authorizing the collection of sputum samples to identify bacterial pathogens. Patient samples were collected as part of routine hospital care. Each patient had appointments scheduled every 3 to 6 months, although some patients and their parents do not always attend their scheduled appointments at HIMFG.

Sputum samples or cough swabs were transported to our laboratory for processing. Sputum samples were dissolved weight/volume (1:1) in sputolysin (Merk-Millipore, Darmstadt, Germany) for 30 minutes at 37ºC. Dissolved sputum samples and cough swabs were used to inoculate salt and mannitol, chocolate, blood, MacConkey (DIBICO, State of Mexico, Mexico), and cetrimide (Becton Dickinson, New Jersey) agar media plates. Plates were incubated at 37ºC for 24 hours. Chocolate agar and blood agar plates were also incubated at 37ºC for 24 hours under microaerophilic conditions. Bacterial isolates were identified by standard microbiological methods[4]. All samples were stored at –70ºC until analysis.

Pulsed-field gel electrophoresis

The bacterial relatedness of 50 clinical isolates of *S. aureus* was determined by pulsed-field gel electrophoresis (PFGE). Bacterial genomic DNAs were purified and prepared as described elsewere[14]. Genomic DNAs were digested with *Sma*I (Invitrogen) for 24 h and resolved by PFGE using a Gene Path system (BioRad® USA). Bacterial relatedness among *S. aureus* clinical isolates was determined according to the Tenover criteria and the use of the Dice coefficient, as previously described[4]. An isolate was considered a member of the same pulsotype when it had a > 85% correlation.

Antibiotic susceptibility testing

S. aureus isolates were tested for antimicrobial susceptibility to ciprofloxacin, levofloxacin, moxifloxacin, gentamicin, tigecycline, trimethoprim/sulfamethoxazole, oxacillin, erythromycin, clindamycin, linezolid, vancomycin, tetracycline, and rifampicin. The minimum inhibitory concentrations (MICs) were determined using the VITEK®2 system (bioMérieux®SA). Methicillin resistance was determined using the cefoxitin test by the disk diffusion method (Kirby-Bauer). Quality control and interpretation of results were performed according to Clinical and Laboratory Standards Institute (2019) guidelines[15]. *S. aureus* ATCC 43300, 25923, and USA300 were used as standard quality controls. The multidrug resistance (MDR) phenotype is defined as non-susceptibility to ≥ 1 agent from ≥ 3 antimicrobial categories[16].

DNA extraction and identification of *mecA* and *lukPV* genes by polymerase chain reaction (PCR)

S. aureus isolates were grown on salt and mannitol agar plates for 24 hours at 37ºC. An isolated colony was resuspended in 100 µL of MilliQ water and boiled for 10 minutes. The bacterial suspension was centrifuged at 10,000 rpm at 4ºC for 10 minutes. Supernatants containing genomic DNA were used for PCR reactions. Primers *mecA*(F): 5'-TGGCTATCGTGTCACAATCG-3' and *MecA*(R): 5'-CTGGAACTTGTTGAGCAGAG-3' were used for *mecA* amplification, whose amplification product is 310pb[17]. The *lukPV* gene was amplified with primer *LukPV*(F): 5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3' and *LukPV*(R): 5'-GCATCAACTGTATTGGATAGCAAAAGC-3', with an amplification product of 433pb[18].

PCR was performed as follows: we mixed 10 mM of each primer, 1X GoTaq® Green Master Mix, and 3 µL of DNA template extracted by boiling. The conditions for gene amplification were one cycle at 94ºC for 5 min, 30 cycles at 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 90 s with a final extension step at 72ºC for 5 min. PCR products were resolved on a 1% (w/v) agarose gel for one hour at 100 volts. The gel was stained with ethidium bromide (1 µg/mL) for 5 min and washed twice in deionized water. The gel was analyzed on a UV light transilluminator using Quantity One software (BioRad® USA).

Reagents, cells, and growth conditions

Fetal bovine serum (FBS) and RPMI-1640 cell culture media were obtained from Invitrogen, PBS and Luria-Bertani (LB) broth from Sigma-Aldrich, and salt and mannitol agar from DIBICO (State of Mexico, Mexico). Human THP1 cells were obtained from ATCC®

TIB-202™; 3x10^5 human THP1 cells were differentiated into macrophages (THP1m) using PMA 100 ng/mL (Sigma-Aldrich) for 24 hours[19]. Prior to infection, the medium was changed to RPMI medium with no antibiotics. THP1m cells monolayers were infected with *S. aureus* at an MOI of 50. To synchronize the infection, we centrifuged the cells at 1,200 rpm for 1 min and then incubated the plates for one hour. After infection, the cells were washed three times with PBS to remove extracellular bacteria; infected cells were returned to the incubator for an additional 24 h in RPMI medium supplemented with gentamicin (100 μg/mL).

Cytotoxicity assays

Supernatants from THP1m and THP1m colonized with *S. aureus* were used to quantify cytosolic enzyme activity of lactate dehydrogenase (LDH; Promega, Madison WI, USA). The following formula determined the percentage of LDH activity:

$$\% \text{ of release} = \frac{(\text{experimental LDH activity} - \text{spontaneous LDH activity})}{(\text{maximal LDH activity} - \text{spontaneous LDH activity})} \times 100\%$$

Results

Presence and genotyping of S. aureus isolates from CF pediatric patients

Of the 50 pediatric patients studied, the most frequently isolated bacterial pathogen was *P. aeruginosa,* followed by *S. aureus*[4]. *S. aureus* was isolated from 26/50 patients, from whom we obtained 51 isolates (Figure 1). Chromosomal analysis by PFGE yielded 34 patterns (pulsotypes) (Table 1). With the 34 patterns, we were able to identify nine clusters (designed as I-IX), which differed by approximately 85% in PFGE band similarity (Figure 1). The cluster with more members (thirteen) was cluster II. The results showed that nine patients (CF001, CF010, CF011, CF013, CF014, CF016, CF019, CF022, and CF029) were chronically infected (the same pulsotype was identified in two or more samples for two or more months) (Figure 2). Twelve patients were colonized with different pulsotypes: eleven with two different pulsotypes and one with three different pulsotypes (Table 1). Patients CF008 (colonized with *Sau*25) and CF014 (colonized with *Sau*06 and *Sau*14) died during the study period.

Table 1. *S. aureus* pulsotypes isolated from Mexican children with cystic fibrosis

Patient	Sample			
	1st	2nd	3rd	4th
CF001	*Sau*01	*Sau*01	*Sau*01	—
CF003	x	*Sau*12	*Sau*29	—
CF004	x	Sau02	—	—
CF006	*Sau*03	*Sau*17	*Sau*32	—
CF007	*Sau*04	x	—	—
CF008[a]	x	*Sau*25	—	—
CF009	x	*Sau*15	x	x
CF010	*Sau*05	*Sau*05	*Sau*31	—
CF011	x	*Sau*19	*Sau*19	—
CF012	x	x	*Sau*22	—
CF013	x	*Sau*04	*Sau*04	*Sau*28
CF014[a]	*Sau*06	*Sau*14	*Sau*14	—
CF016	*Sau*07	*Sau*07	*Sau*07	—
CF019	*Sau*08	*Sau*16	*Sau*16	*Sau*16
CF022	*Sau*09	*Sau*09	*Sau*31	—
CF025	x	*Sau*18	—	—
CF028	x	*Sau*20	*Sau*26	—
CF029	*Sau*11	*Sau*11	—	—
CF030	x	*Sau*21	*Sau*30	—
CF032	*Sau*13	—	—	—
CF034	*Sau*01	*Sau*14	—	—
CF036	*Sau*17	—	—	—
CF041	x	*Sau*24	*Sau*27	—
CF042	*Sau*23	—	—	—
CF048	*Sau*10	*Sau*33	—	—
CF049	x	*Sau*34	—	—

[a]Patient died.
X, sample with no S. *aureus* isolate; —, no sample available.

Antimicrobial susceptibility of S. aureus pulsotypes isolated from CF children

One of the 34 *S. aureus* pulsotypes identified was used to determine the antimicrobial susceptibility pattern to ten different classes of antibiotics: glycylcyclines, oxazolidinones, glycopeptides, folate pathway

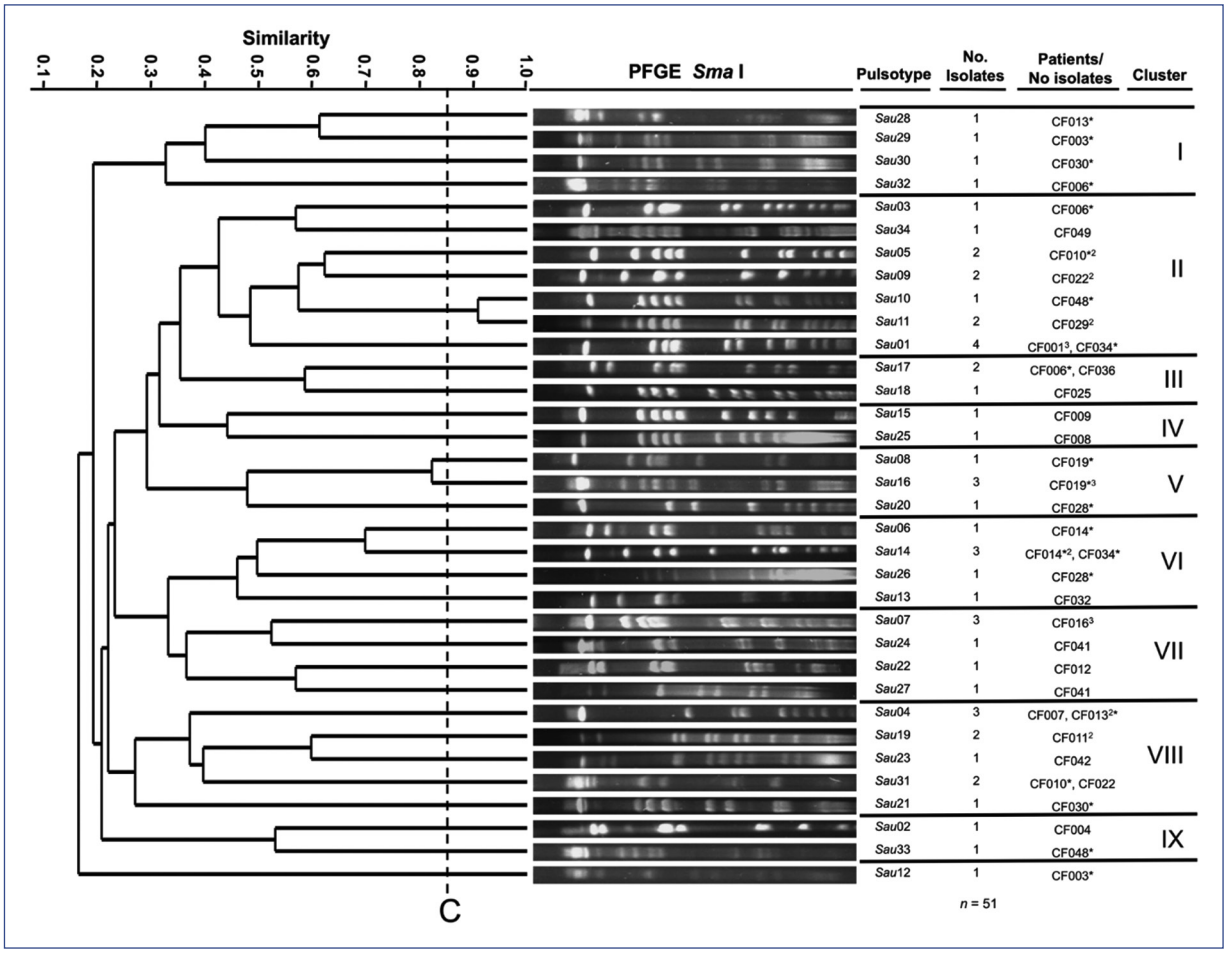

Figure 1. Dendrogram generated from PFGE analysis of 51 *S. aureus* isolates from pediatric CF patients. A representative PFGE profile of each pulsotype was used to construct the dendrogram. Each pulsotype frequency (number of isolates) is indicated, and each row indicates the patient in which the pulsotype was identified. Patients colonized by more than one pulsotype are indicated (*). A superscript indicates the frequency of each pulsotype identified per patient. The dotted line indicating 85% similarity was used to determine the cluster designation (I-IX).

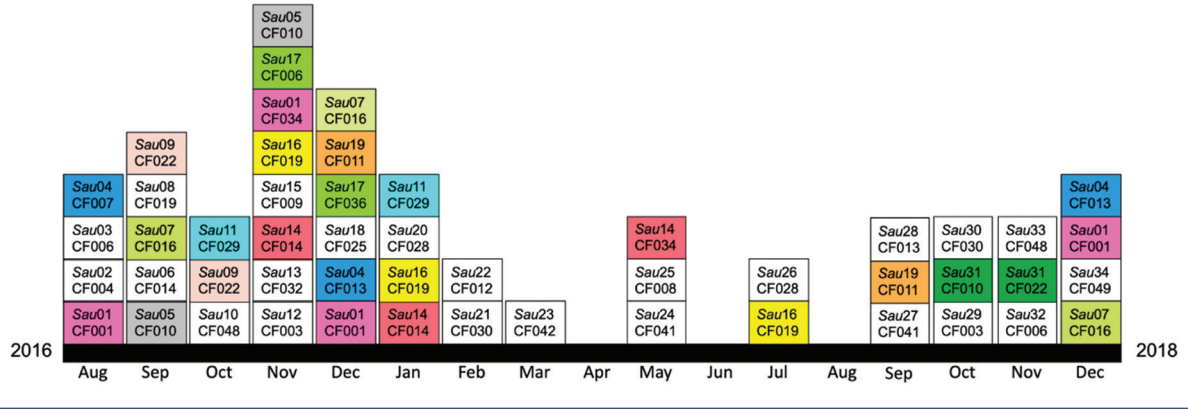

Figure 2. *S. aureus* pulsotypes were identified from 2016 to 2018. White boxes indicate a single pulsotype; colored boxes indicate a clone detected twice or more times.

Table 2. Antibiotic susceptibility for 34 *Staphylococcus aureus* pulsotypes of pediatric patients with cystic fibrosis

Antibiotic family	Antibiotic	Breakpoints (µg/mL)			Susceptible n (%)	Intermediate n (%)	Resistant n (%)
		S	I	R			
Aminoglycosides Lincosamides	Gentamicin	≤ 4	8	≥ 16	30 (88.2)	2 (5.9)	2 (5.9)
	Clindamycin	≤ 0.5	1-2	≥ 4	27 (79.4)	1 (2.9)	6 (17.7)
Penicillins Ansamycins	Oxacillin (MRSA)	≤ 2	—	≥ 4	30 (88.2)	0 (0.0)	4 (11.8)
	Rifampicin	≤ 1	2	≥ 4	31 (91.2)	0 (0.0)	3 (8.8)
Macrolides	Erythromycin	≤ 0.5	1-4	≥ 8	27 (79.4)	0 (0.0)	7 (20.6)
Tetracyclines	Tetracycline	≤ 4	8	≥ 16	27 (79.4)	0 (0.0)	7 (20.6)
Fluoroquinolones	Ciprofloxacin	≤ 1	2	≥ 4	29 (85.3)	2 (5.9)	3 (8.8)
	Levofloxacin	≤ 1	2	≥ 4	31 (91.2)	2 (5.9)	1 (2.9)
	Moxifloxacin	≤ 0.5	1	≥ 2	33 (97.1)	0 (0.0)	1 (2.9)
Folate pathway inhibitors	Trimethoprim/ sulfamethoxazole	≤ 2/38	—	≥ 4/76	34 (100.0)	0 (0.0)	0 (0.0)
Glycopeptides Oxazolidones	Vancomycin	≤ 2	4-8	≥ 16	31 (91.2)	0 (0.0)	3 (8.8)
	Linezolid	≤ 4	—	≥ 8	34 (100.0)	0 (0.0)	0 (0.0)
Glycylcyclines	Tigecycline	≤ 0.25	—	—	34 (100.0)	0 (0.0)	0 (0.0)

MRSA, methicillin-resistant *Staphylococcus aureus* S, susceptible; I, intermediate; R, resistant.
*Breakpoints were obtained from the Clinical and Laboratory Standards Institute (2019).

antagonists, fluoroquinolones, tetracyclines, macrolides, ansamycins, penicillins, lincosamides, and aminoglycosides (Table 2). We detected 3/34 (8.8%) MDR pulsotypes, and a low rate of resistance to vancomycin (8.8%), moderate resistance to tetracycline and erythromycin (20.6%), and clindamycin (17.7%). All the pulsotypes were susceptible to tigecycline, linezolid, and trimethoprim/sulfamethoxazole (Table 2). We found only two MRSA pulsotypes (5.9%): *Sau*08 and *Sau*16, both isolated from patient CF019. *Sau*16 was isolated three times at different times during sample collection (Figure 1 and Table 1). We identified both pulsotypes (*Sau*08 and *Sau*16) carrying the *mecA* gene by PCR (data not shown). These results also revealed that the two pulsotypes were MRSA with an MDR phenotype.

Pulsotypes of S. aureus induced cytotoxicity in human THP1-derived macrophages

The ability of *S. aureus* to induce cytotoxicity in human THP1 monocytes differentiated into macrophages (THP1m) was evaluated. The results showed that 30/34 (88.2%) pulsotypes were cytotoxic (> 10% of cytotoxicity) (Figure 3), 8/34 (23.5%) were highly cytotoxic (> 50% of cytotoxicity), and 14/34 (41.2%) were able to disrupt the THP1m monolayers (data not shown). To determine whether the cytotoxic effect was associated with pulsotypes carrying the *PVL* gene, we amplified the *lukPV* gene by PCR. The results showed that none of the analyzed pulsotypes carried the *lukPV* gene (data not shown).

Discussion

The lower airways of children with CF are rapidly colonized by *S. aureus* and non-typeable *H. influenzae*[5]. However, the pulmonary bacterial microbiome gradually changes with the emergence and persistence of *P. aeruginosa* during adolescence and adulthood[5]. The eradication of *S. aureus* in the lower respiratory tract has been compromised by the emergence of MRSA[20]. The World Health Organization (WHO) has declared priority level 2 in identifying new antibiotics to combat MRSA. To date, MRSA is usually associated with the community- and hospital-acquired infections. In CF, the occurrence of MRSA has been associated with a more rapid decline in lung function[21]. In a previous study, 44% of children with CF (22/50) were

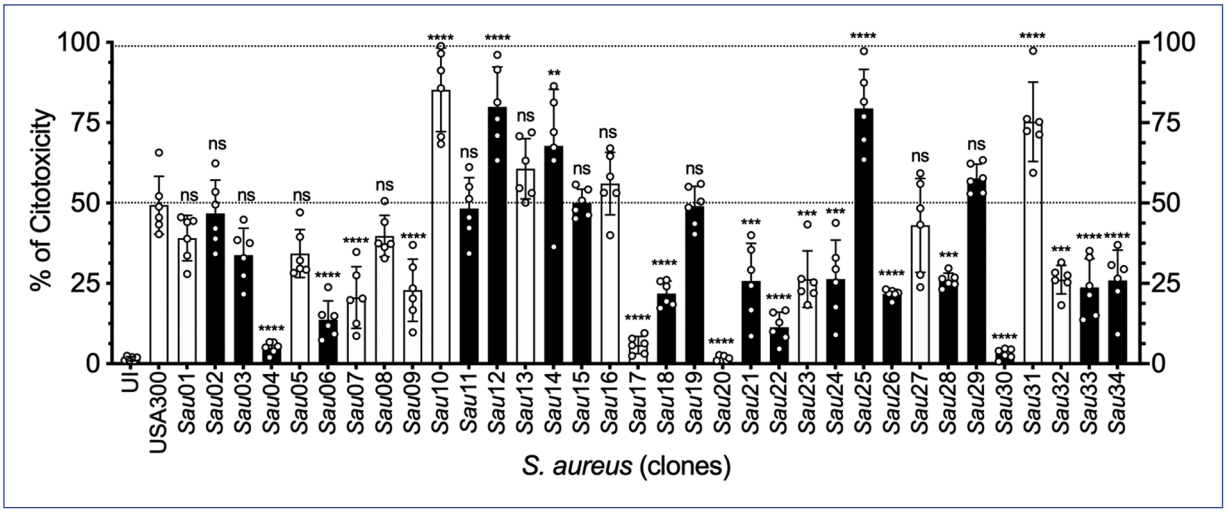

Figure 3. Induction of cytotoxicity by *S. aureus* isolates in THP1macrophages: 5 x 10^5 THP1 monocytes were differentiated into macrophages with 100 ng/mL of PMA for 24 h. Cells were infected with *S. aureus* isolates at MOI of 100 for 30 min. Once infected, cells were washed and incubated for 24 h. Supernatants were used to quantify macrophage cell death (cytotoxicity). Results were obtained from three independent experiments, each in duplicate (n = 6). Data were plotted as the mean ± SD and analyzed by one-way ANOVA and Dunnett's multiple comparisons in relation to *S. aureus* -USA300. **p < 0.01; ***p < 0.001; ****p< 0.0001; ns: non-significant.

colonized by *P. aeruginosa* and *S. aureus*[4]. In the present study, we identified 34 unrelated pulsotypes, of which 2/34 were MRSA. In particular, the *Sau*16 pulsotype was consistently isolated three times in patient CF019 during one year. We also isolated the genetically related *Sau*08 pulsotype (group V) in this patient, suggesting a genetic evolution. Analysis of clinical isolates of *S. aureus* by whole-genome sequencing suggests possible genetic evolution and spread[22]. In this study, we identified that pulsotypes *Sau*08 and *Sau*16 are MRSA with a consistent MDR phenotype. We also identified the persistence of *S. aureus* in eight patients. Persistence and long-term carriage of *S. aureus* are often associated with specific phenotypes, including small colony variants, increased antimicrobial resistance, and biofilm-formation[23]. Furthermore, MRSA colonization is more frequently associated with individuals carrying the ΔF508 mutation[24]. The patient CF019 from whom we isolated both MRSA pulsotypes carried the ΔF508 mutation[4].

S. aureus contains several virulence factors that promote host tissue damage[25-27]. Toxic shock syndrome toxin (TSST-1) and PVL are two important secreted virulence factors[12]. Our results showed that none of the *S. aureus* pulsotypes tested carried the *lukPV* gene. It has been demonstrated that PVL expression is not sufficient to induce cell death[28], suggesting the presence of additional virulence factors that contribute to pathogenesis[27].

We determined that 88.2% of the isolates induced cell death in THP1-derived macrophages and that 14/34 (41.2%) of the tested pulsotypes could alter the integrity of the THP1m monolayers. *S. aureus* α-toxin is a secreted virulence factor involved in the disruption of epithelial cell monolayers[29], suggesting that this virulence factor could alter THP1m monolayers, a hypothesis that we will address in the future.

In conclusion, in this study, we demonstrated a low presence of MRSA and MDR and a high frequency of *S. aureus* pulsotypes with the ability to induce cytotoxicity in THP1-derived macrophages.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author has this document.

Conflicts of interest

The authors declare no conflict of interest.

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