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Chlorhexidine whole-body washing of patients reduces methicillin-resistant *Staphylococcus aureus* and has a direct effect on the distribution of the ST5-MRSA-II (New York/Japan) clone

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Abstract

Purpose. Methicillin-resistant *Staphylococcus aureus* (MRSA) colonizes the skin of hospitalized patients and is associated with high morbidity and mortality. To prevent colonization and infection by *S. aureus*, better disinfection practices are required. Therefore, we evaluated the effect of chlorhexidine whole-body washing on hospital-acquired *S. aureus* infections among intensive care unit (ICU) patients in a tertiary hospital in Mexico.

Methodology. The study was conducted over 18 months to evaluate the effect of 2 % chlorhexidine gluconate (CXG) whole-body washing of ICU adult patients on chlorhexidine and antibiotic resistance, biofilm production and clonal distribution of *S. aureus* in a tertiary care hospital. Minimum inhibitory concentrations for CXG, antibiotic susceptibility and biofilm production by *S. aureus* isolates were determined. Pulsed-field gel electrophoresis, multilocus sequence typing (MLST) and PCR for Panton–Valentine leucocidin (PVL) were used for molecular typing of MRSA isolates.

Results/Key findings. We included 158 isolates. A reduction in antibiotic resistance in the study period was observed for clindamycin, levofloxacin, norfloxacin, oxacillin and trimethoprim/sulfamethoxazole. None of the isolates showed reduced susceptibility to CXG. Most of the isolates were non-biofilm producers (147/158). The most commonly identified clone was a descendant of the ST5-MRSA-II (New York/Japan) clone. This clone decreased during the intervention period and reappeared markedly in the post-intervention period. During the post-intervention period, two isolates were related with the clone ST8-MRSA-IV (also known as USA300).

Conclusion. Our findings suggest that the CXG bathing favored the reduction of healthcare-associated MRSA isolates and a temporary reduction of the predominant ST5-MRSA-II (New York/Japan) clone.

INTRODUCTION

Hospital-acquired skin infections due to a variety of bacterial species are common. Infection with methicillin-resistant *Staphylococcus aureus* (MRSA) is of particular interest because it is associated with substantial morbidity and mortality. Among the virulence factors for increased MRSA morbidity and mortality rates are biofilm production (occurrence rate, 68.3 %) [1] and the emergence, more than a decade ago, of community-acquired MRSA (CA-MRSA) strains in healthy persons without predisposing risk factors [2–5].

A limited number of MRSA lineages have emerged from the transfer of the staphylococcal cassette chromosome *mec* into successful methicillin-susceptible *S. aureus* (MSSA) clones. Using multilocus sequence typing (MLST), Enright *et al.* demonstrated that MRSA clones evolved from five different groups of related genotypes or clonal complexes, each arising from a distinct ancestral genotype [6–8]. The ST5-MRSA-II

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Keywords: chlorhexidine; HA-MRSA; MRSA; New York/Japan clone; USA300 clone.

Abbreviations: CA-MRSA, community-acquired MRSA; CXG, chlorhexidine gluconate; ICU, intensive care unit; MIC, minimal inhibitory concentration; MLST, multilocus sequence typing; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*; PFGE, pulsed-field gelelectrophoresis; PVL, Panton–Valentine leucocidin; ST, sequence type.

(or New York/Japan) clone has been shown to be particularly successful in establishing itself in Mexico [9, 10].

Chlorhexidine gluconate (CXG) has a broad activity against Gram-positive and Gram-negative bacteria [11]. Daily CXG bathing of intensive care unit (ICU) patients has been shown to reduce the risk of the acquisition of multidrug-resistant organisms and the development of hospital-acquired bloodstream infections [12]. A particular impact has been seen on infection and colonization by MRSA.

Daily CXG whole-body baths are associated with a decreased rate of colonization by MRSA and a lower rate of MRSA ventilator-associated pneumonia [13, 14].

Although it is known that CXG baths reduce MRSA acquisition [15], so far there is no evidence concerning the molecular behavior of *S. aureus* during the CXG baths.

The aim of this study was to evaluate the effect of 2 % CXG whole-body washing on chlorhexidine and antibiotic resistance, biofilm production and the clonal distribution of *S. aureus* among adult ICU patients in a tertiary care hospital.

METHODS

Ethics statement

This research was performed according to the principles expressed in the Declaration of Helsinki with approval from the Ethics Committee of the Universidad Autónoma de Nuevo León (approval NM12-009). Informed consent was obtained from all patients.

Study design

The study was conducted at the Hospital Universitario Dr José Eleuterio González, a 460-bed tertiary care hospital in Monterrey, Mexico. This study was performed in the adult medical and surgical ICUs (a 20-bed combined unit). During the 1.5-year study period, we included a pre-intervention period (1 January to 30 June 2012), an intervention period (1 July to 31 December 2012) and a post-intervention period (1 January to 30 June 2013). We included one isolate per patient.

Selection of patients and whole-body washing

Exclusion criteria were a history of allergy to CXG, burns on more than 20 % of the total skin surface, and pregnancy. Only patients older than 18 years were included. Patients were subject to daily whole-body washing with 2 % CXG-impregnated wipes (Clorhexi-Wipes, G70 antisepsis, León, Guanajuato, Mexico). In the intervention period, enrolled patients received daily CXG baths during the complete ICU stay.

Staphylococcus aureus isolates

S. aureus from any specimen classified as a potential causative agent of infection according to standard procedures was included [16]. Microbiological identification was performed using Sensititre panels (TEK Diagnostic Systems, Inc., Cleveland, OH), according to the manufacturer's instructions. A coagulase tube test was carried out on all isolates.

Chlorhexidine and antibiotic susceptibility testing

Minimal inhibitory concentrations (MICs) for CXG were determined using the agar dilution method, as recommended by the Clinical Laboratory Standard Institute (CLSI) in documents M07-A10 and M100-S26 [17]. For CXG, the isolates with MIC values higher than the MIC_{90} value were classified as isolates with disinfectant-reduced susceptibility [18]. The antibiotic susceptibility of all isolates collected was determined using the broth microdilution method, as recommended by the CLSI [17], using Sensititre panels following the manufacturer's instructions. Antimicrobial testing included the determination of resistance to cefoxitin, clindamycin, erythromycin, levofloxacin, linezolid, nitrofurantoin, norfloxacin, oxacillin, penicillin, quinupristin/dalfopristin, teicoplanin, tetracycline, trimethoprim/sulfamethoxazole and vancomycin. Isolates were classified as MRSA when the MIC values for oxacillin and cefoxitin were $\geq$4 and $\geq$8, respectively [17].

Production of biofilm

Semi-quantitative determination of biofilm production was performed using crystal violet staining [19]. Isolates were tested in quadruplicate in two independent experiments carried out on different days. Isolates were cultured in trypticase soy broth supplemented with 1 % glucose in 96-well flat-bottom untreated polystyrene plates with low-evaporation lids. After staining with 0.1 % crystal violet, the biofilm was dissolved in 95 % ethanol, and the optical density at 595 nm (OD_{595}) was measured. Isolates with an $OD_{595} \leq 0.12$ were considered non-biofilm producers, weak biofilm producers were identified by $0.13 < OD_{595} < 0.24$, and an $OD_{595} \geq 0.25$ was interpreted as indicating strong biofilm producers. *S. aureus* ATCC 29213 (positive biofilm) and *S. hominis* ATCC 27844 (negative biofilm) were used as control strains.

Molecular typing

Pulsed-field gel electrophoresis (PFGE) was used for the molecular typing of MRSA isolates. Genomic DNA was prepared as described previously [20]. After digestion with *Sma*I endonuclease (New England BioLabs, Hitchin, UK), the DNA was separated in a CHEF-DRII apparatus (Bio-Rad, Birmingham, UK). Strains NCTC8325 and BK2464 (kindly provided by Professor Herminia de Lencastre from the Molecular Genetics Laboratory Institute de Tecnología Química e Biologica da Universidad Nova de Lisboa) and the USA300 strain were included as controls. The criteria of Tenover *et al.* were used to compare different clones [21]. PFGE gel images were analysed by visual inspection. Isolates showing identical or related PFGE patterns were considered to belong to the same clone. Clones were labelled with a capital letter (A, B, C, etc.) and related profiles were indicated by adding a number (A1, A2, B1, B2, etc.). The PFGE profiles of these strains were compared to control strains.

For isolates with PFGE patterns similar to MRSA USA300, the presence of Panton–Valentine leucocidin (PVL) was detected by PCR [22], with USA300 as a control. MLST was performed as previously described [23]. The assignment of alleles and sequence types (ST) was performed using software from the Multi Locus Sequence Typing website, available at www.mlst.net.

Statistical analyses

Data were analysed using the statistical program SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA). The correlation between the MIC values for CXG and antimicrobial agents and the production of biofilm were determined using Spearman's rank correlation test. One-way ANOVA with *post hoc* Holm–Sidak tests was performed to compare the MIC of CXG, PFGE types and biofilm production. $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Patients and isolates collected

The study enrolled 327 participants, of whom 158 had an *S. aureus*-positive culture. The mean age was 43.7 years ±SD 17.9, with a range of 17–85, and 111 were male and 47 were female. A clinical summary of 156 patients is shown in Table 1 [we were unable to obtain clinical data from two patients (females)]. The mean length of stay for the patients was 23.5 days, and most patients with *S. aureus*-positive cultures had received cephalosporins (41.67 %). Specimens were isolated from 149 respiratory samples [tracheal aspirate (*n*=121), bronchial lavage (*n*=26) and sputum (*n*=2)], 4 blood samples, 3 wounds, 1 cerebrospinal fluid sample and 1 urine sample. The isolate distribution was: 61 isolates (41 MRSA and 20 MSSA) in the pre-intervention period, 52 isolates (29 MRSA and 23 MSSA) during the intervention period and 45 isolates (12 MRSA and 33 MSSA) in the post-intervention period.

Minimal inhibitory concentrations of chlorhexidine and antibiotics

The distribution of the MIC of CXG and antibiotics for the *S. aureus* isolates is shown in Table 2. None of the isolates showed reduced susceptibility to CXG. The MIC_{50} and MIC_{90} did not change for clindamycin, erythromycin, linezolid, norfloxacin, penicillin, quinupristin/dalfopristin, teicoplanin, tetracycline, trimethoprim/sulfamethoxazole or chlorhexidine throughout the study. A decrease in the MIC_{50} value was detected for levofloxacin (from ≥ 2 in the pre-intervention period to ≤ 1 as of the intervention period). A reduction in antibiotic resistance was detected for clindamycin (P=0.042), levofloxacin (P=0.013), norfloxacin (P=0.022), oxacillin (P=0.003) and trimethoprim/sulfamethoxazole (P=0.592). There was no vancomycin and linezolid resistance. No correlation was found the antibiotic resistance and the MIC for CXG. Overall, a significant reduction in the frequency of MRSA isolates as an effect of the CXG bathing (P=0.003) was detected.

Table 1. Clinical data of included patients

Characteristic	Total (*n*=156*)
Male gender, *n* (%)	111 (71.2)
Reason for ICU admission	
Neurosurgery, *n* (%)	53 (34)
Multiple trauma, *n* (%)	11 (7.1)
Medical, *n* (%)	62 (39.7)
Other surgery, *n* (%)	30 (19.2)
Comorbidities	
Chronic renal insufficiency, *n* (%)	9 (5.8)
Neoplasia, *n* (%)	13 (8.3)
Hospitalization >72 h in the last 12 months *n* (%)	14 (9.0)
Length of stay in days, mean (±SD)	23.5 (4–72)
Length of stay before isolation in days, mean (±SD)	6 (2–26)
Length of stay after isolation in days, mean (±SD)	15 (1–56)
Days of central venous catheter, mean (±SD)	10.5 (2–39)
Days of bladder catheter, mean (±SD)	11(2–39)
Days of mechanical ventilation, mean (±SD)	10 (2–39)
Antibiotics received before isolation	
Cephalosporin *n* (%)	65 (41.7)
Fluoroquinolones *n* (%)	14 (9.0)
Vancomycin *n* (%)	24 (14.5)
Carbapenems *n* (%)	27 (17.3)
Others *n* (%)	26 (16.7)
Days of antibiotic use before isolation, median (range)	5 (2–25)
Number of antibiotics before isolation, mean (range)	2 (1–4)

*We obtained clinical data from 156 patients.

Biofilm production

Only 5/158 (3.16 %) of the isolates were strong biofilm producers, with four of these being collected during the intervention period. Six isolates (3.8 %) were weak biofilm producers and 147/158 (93.0 %) isolates did not produce biofilm (Fig. 1). No significant differences in biofilm production were observed between isolates collected in the pre-intervention period and those collected during the intervention period (P=0.168).

Analysis of clonal relatedness

All strains of *S. aureus* (*n*=158) were analysed by PFGE; 82 strains were MRSA and 76 strains were MSSA. MSSA strains were detected in all three periods, but were more frequent in the post-intervention period (*n*=33) than in the intervention (*n*=23) and pre-intervention (*n*=20) periods. The opposite was observed for MRSA strains, which were less abundant in the post-intervention period (*n*=12) than in the intervention (*n*=29) and pre-intervention (*n*=41) periods (Table 3). PFGE analysis separated the *S. aureus* strains into 24 different patterns (A–W; Fig. 2). PFGE pattern C and subtypes predominated in this study; these patterns were related to the ST5-MRSA-II (New York/Japan) clone. Profiles C and C34 were the most frequent; pattern C was detected in only MRSA strains in all three periods.

Table 2. Distribution of chlorhexidine and antibiotic MIC_{50} and MIC_{90} values (μg ml^{-1}) for *S. aureus* isolates

Period		Antibiotic*												
		CLI	ERY	LEV	LZD	NIT	NOR	OXA	PEN	SYN	TEC	TET	SXT	VAN	CXG
Pre-intervention	MIC_{50}	≥2	≥4	≥2	≤4	≤32	≥8	≥4	≥2	≤1	≤8	≤2	≤2/38	2	8
	MIC_{90}	≥2	≥4	≥2	≤4	64	≥8	≥4	≥2	≤1	≤8	≤2	≤2/38	4	32
	% R	68.7	68.7	56.7	0	1.5	58.2	65.7	91	3	4.5	3	7.5	0	NA
	% S	31.3	31.3	43.3	100	98.5	41.8	34.3	9	97	95.5	97	92.5	100	NA
Intervention	MIC_{50}	≥2	≥4	≤1	≤4	≤32	≥8	≥4	≥2	≤1	≤8	≤2	≤2/38	2	8
	MIC_{90}	≥2	≥4	≥2	≤4	≤32	≥8	≥4	≥2	≤1	≤8	≤2	≤2/38	2	32
	% R	74.1	72.2	52.3	0	0	55.5	55.5	83.4	1.5	1.5	3	4.5	0	NA
	% S	25.9	27.8	47.7	100	100	44.5	44.5	16.6	98.5	98.5	97	95.5	100	NA
Post-intervention	MIC_{50}	≥2	≥4	≤1	≤4	≤32	≥8	1	≥2	≤1	≤8	≤2	≤2/38	2	8
	MIC_{90}	≥2	≥4	≥2	≤4	64	≥8	≥4	≥2	≤1	≤8	≤2	≤2/38	4	32
	% R	34.8	67.4	34.8	0	1.5	34.8	32.6	86.9	1.5	4.5	3	3	0	NA
	% S	65.2	32.6	65.2	100	98.5	65.2	67.4	13.1	98.5	95.5	97	97	100	NA

MIC_{50} and MIC_{90}: minimal inhibitory concentration (μg ml^{-1}) that inhibits 50 and 90 % of isolates, respectively; % R and % S: percentage of isolates resistant and susceptible, respectively; this classification was according to the cut-offs proposed by the CLSI.

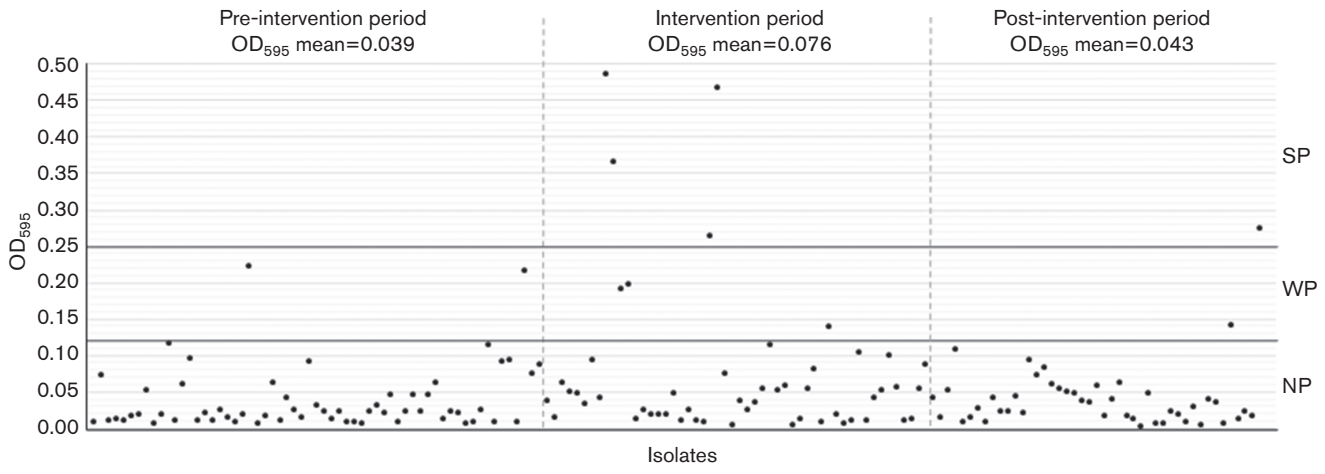
*CLI, clindamycin; ERY, erythromycin; LEV, levofloxacin; LZD, linezolid; NIT, nitrofurantoin; NOR, norfloxacin; OXA, oxacillin; PEN, penicillin; SYN, quinupristin/dalfopristin; TEC, teicoplanin; TET, tetracycline; SXT, trimethoprim/sulfamethoxazole; VAN, vancomycin; CXG, chlorhexidine.

Profile C34 was present in both MRSA and MSSA. Furthermore, during the post-intervention period, two isolates (PVL+ and ST8) were related to the ST8-MRSA-IV (USA300) clone (Table 3).

The highest clonal diversity was identified during the intervention period. The ST5-MRSA-II (New York/Japan) clone decreased significantly in the intervention period, but recovered in the post-intervention period.

Among the MSSA strains during the post-intervention period, there was a predominance of subtypes of the ST5-MRSA-II (New York/Japan) clone (20/33), where only 13 isolates (patterns D, E, F1, I, T, U, V and X) were not related to this clone (Fig. 2). Patterns D, E, F1 and I were also present in the preceding periods.

In the pre-intervention period (41 MRSA isolates), there was a marked predominance (37/41; 90.4 %) of the ST5-MRSA-II-New York/Japan clone (pattern C and subtypes). In the intervention period, 15/29 (51.8 %) of the isolates belonged to the ST5-MRSA-II New York/Japan clone, and in the post-intervention period, most MRSA strains (10/12; 83.3 %) belonged to the New York/Japan lineage. As can be seen, the ST5-MRSA-II New York/Japan clone decreased



OD_{595}: Optical density at 595 nm per isolate during study periods, NP: non-producers, WP: weak producers, SP: strong producers.

Fig. 1. Biofilm production of isolates among the three study periods.

Table 3. Distribution of PFGE patterns for MRSA and MSSA isolates in the study periods

Clones	Pre-intervention period		Intervention period		Post-intervention period	
	MRSA (*n*=41)	MSSA (*n*=20)	MRSA (*n*=29)	MSSA (*n*=23)	MRSA (*n*=12)	MSSA (*n*=33)
A, A1, B, C43, C3, C27, C41, R1	1					
C*	17		10		5	
C4, C25, C50, D, F1, I, T, W						1
C6, C27, D1, D2, G2, L1, M1, N, Ñ		1				
C14	2				2	
C28, C42, C43, C45, C46, D, F, F1, G, G1, H, R			1			
C34*	14		5		3	12
C34, C44, F1, I				2		
C47		3				
C48						3
C49, U, V						2
D, G		2				
I		4				
E			2			4
E, F				3		
F2, G2, H, J, K, O, P, Q, S				1		
CA-MRSA					2	

*Pattern C and subtypes represent strains of MRSA descendants of ST5-MRSA-II (New York/Japan) clone.

during the intervention period and recovered markedly in the post-intervention period (Fig. 3).

DISCUSSION

Healthcare-associated infections are a major problem in hospitals. CXG whole-body washing has been proposed to reduce colonization and infection by some pathogens, especially *S. aureus*. In this study, we evaluated the effect of 2 % CXG whole-body washing on the antibiotic resistance, biofilm production and clonal distribution of *S. aureus* among ICU patients in a tertiary care hospital. Antibiotic resistance diminished significantly for clindamycin (from 69 to 35 %), levofloxacin (from 57 to 35 %) and norfloxacin (from 58 to 35 %). Interestingly, in the pre-intervention period, 65.7 % of the isolates were oxacillin-resistant and in the post-intervention period this percentage reduced to 32.6 %. This result indicates a significant reduction in the frequency of MRSA isolates as a result of the CXG bathing (P=0.003).

To highlight the importance of this effect, we compared these data to the prevalence of MRSA in the ICU in 2011 (62 %) [24] and 2014 (32.14 %) (data not published). As can be seen, the MRSA frequency was maintained after CXG bathing. Importantly, although the CXG baths were stopped after the intervention period, MRSA colonizations have been low ever since.

Our report confirms previous research on the usefulness of CXG bathing to reduce MRSA frequency [15, 25]. Remarkably, there was no increase in resistance for any of the antibiotics evaluated against *S. aureus* in this study.

In general, most of the isolates were non-biofilm producers. Some reports show a moderate biofilm production by *S. aureus*. For example, a study in India revealed that 55 % of isolates produced biofilm, with 76.6 % of MRSA producing biofilm in comparison to 38.8 % of MSSA [25]. Furthermore, an association between biofilm formation and the special molecular characteristics of MRSA has been reported. Among 126 isolates, 68.3 % were biofilm producers (5 strong producers and 81 weak producers) and these were more likely to be multidrug-resistant than non-biofilm-forming isolates [1]. In our study, there was no correlation between biofilm production and antibiotic resistance, and most of the isolates were non-biofilm producers. We hypothesize that the low frequency of this virulence factor may have had a role in the reduction of the MRSA percentages.

A few MRSA pandemic clones have spread worldwide, and the ST5-MRSA-II New York/Japan clone has established itself in Mexico [9]. In our study, we detected a remarkable diversity of clones, but two clones predominated, both descendants of the ST5-MRSA-II New York/Japan clone. Interestingly, the ST5-MRSA-II New York/Japan clone decreased during the intervention period, but this displacement was temporal because the clone reappeared strongly in the post-intervention period. This singularity may be relevant to the predominance of this pandemic clone. To our knowledge, this phenomenon has not previously been reported. The ST5-MRSA-II New York/Japan clone has been reported in five hospitals in this city apart from ours [10].

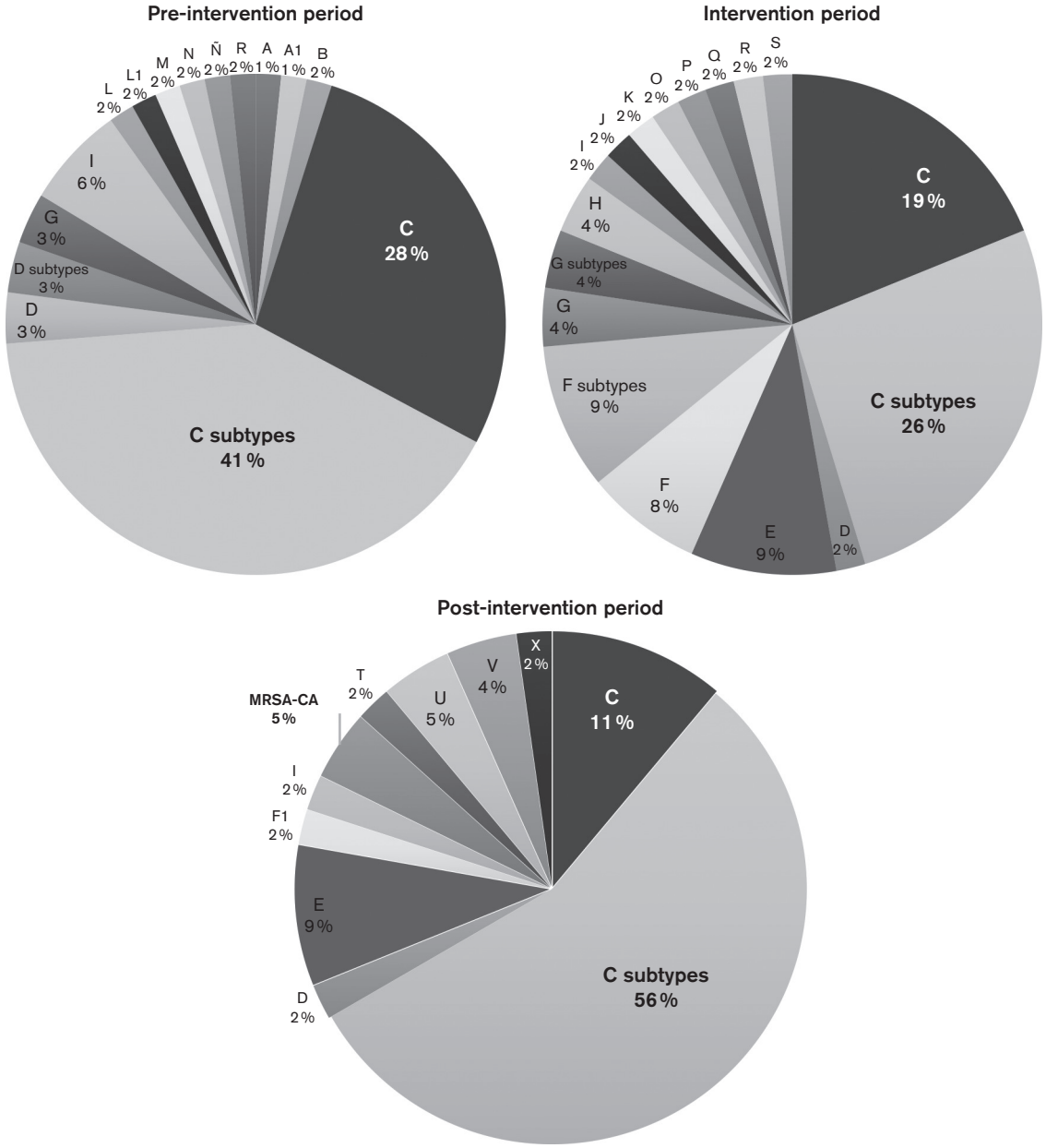


Fig. 2. Distribution of PFGE patterns for *S. aureus* isolates in the study periods.

In this study, two isolates from the post-intervention period were revealed to be CA-MRSA and related to the ST8-MRSA-IV (USA300) clone. This finding suggests that USA300 isolates were acquired under CXG pressure during the hospital stay rather than while in contact with the community. The ST8-MRSA-IV (USA300) clone predominates in the United States [26] and has been reported in our city [27]. In our hospital, we provided active surveillance of MRSA and, based on finding the CA-MRSA (USA300) in our hospital, we will implement molecular surveillance of CA-MRSA.

In our study, a duration of stay of 23.5 days was detected, and this is considered to be a long stay in the hospital. We were not surprised, because the length of hospital stay has been reported as a risk factor associated with MRSA acquisition during hospitalization (odds ratio 1.04, 95 % confidence interval 1.03–1.06, $P<0.001$) [28].

A potential bias of our study is the fact that the number of patients in each study period was different. This difference may reflect a difference in the number of patients seen in the hospital during the three periods.

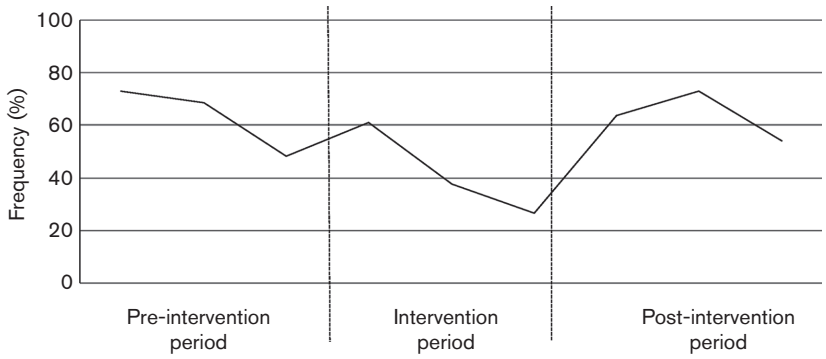


Fig. 3. Changes in the distribution of ST5-MRSA-II New York/Japan clone during the study periods.

In conclusion, our results suggest that CXG bathing favoured the reduction of MRSA isolates by the selection of MSSA and temporarily reduced the frequency of the ST5-MRSA-II (New York/Japan) clone.

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Conflicts of interest
The authors declare that they have no competing interests.

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