Research Square

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The Changing Landscape of Molecular Epidemiology of Staphylococcus Aureus Infections in Children.

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Abstract

Background: *Staphylococcus aureus* infections cause significant morbidity and mortality in children and adolescents. Aim of this study was to investigate the molecular epidemiology and antibiotic resistance of *Staphylococcus aureus* clinical isolates from children and adolescents.

Methods: All *S. aureus* isolates recovered from patients aged < 18 years, admitted to a referral hospital, with culture-proven invasive or non-invasive, community-associated or community-onset healthcare-associated or hospital-associated infections during the 4-year period from January 2015 to December 2018 were analyzed **for antimicrobial resistance, virulence genes,** pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)**.**

Results: Among 139 *S. aureus* clinical isolates, 16 (11.5%) were methicillin-resistant (MRSA) and 123 (88.5%) methicillin-susceptible (MSSA). MSSA infections increased significantly over time (2017-2018 vs. 2015-2016, 0R 3.32; 95%CI 1.18-8.96; p 0.03) along with increasing resistance to fusidic acid (OR 2.38; 95%CI 1.14-5.12; p 0.02) and in staphylococcal scalded skin syndrome prevalence (OR 3.24; 95% CI 1.10-8.36; p 0.03). A total of five sequence types (ST) were identified among 58 isolates that were analyzed by MLST. By PFGE typing, 22 pulsotypes were identified, whereas, PFGE type 1 classified as ST121 clone was the predominant (40/58,68.9%). MRSA were distributed into four pulsotypes and PFGE type C- ST80 was the most frequent. ST121 strains carried *fnbA* (40/40), *eta/etb* genes (29/40) and *lukF/S-PVL* genes in 3/40 cases. All ST121 exhibited high resistance percentage to fusidic acid and were increasingly resistant to mupirocin.

Conclusion: In our population, a CA-MSSA clone emerged, resistant to fusidic acid and increasingly resistant to mupirocin which belonged to the PFGE type 1, ST121 clone, harbored exfoliative toxins genes and was associated with rising trends of SSSS.

Introduction

Staphylococcus aureus is a common pathogen in children and adolescents and a cause of a broad spectrum of infections ranging from mild, often recurrent, skin and soft tissue to invasive infections, including bacteremia, necrotizing pneumonia, endocarditis, musculoskeletal infections and toxin-mediated disease [1].

The emergence of methicillin resistant *S. aureus* (MRSA) strains was first described in hospital settings and soon emerged in the community [2]. In recent years several studies have reported the emergence and predominance of methicillin-susceptible strains (MSSA) [2–5]. The capacity of *S. aureus* to cause infection is related to the expression of virulence factors and the production of a wide variety of toxins which include pore-forming toxins, phagocytosis inhibitors and superantigens [6].

To date, four serotypes of exfoliative toxins have been identified of which exfoliative toxins A (ETA) and B (ETB) are responsible for most human cases of toxin-mediated disease [7]. Exfoliative-protein producing

strains have been associated with localized epidermal infections such as bullous impetigo to generalized disease [8, 9] whereas, staphylococcal scalded skin syndrome (SSSS) is the most common toxin-mediated disease mainly affecting children younger than two years [9]. Toxic shock syndrome toxin (TSST) is one of the most important *S. aureus* superantigens which acts by stimulating release of IL-1, IL-2, TNF-α, and other cytokines and causes toxic shock syndrome [10].

Today, it is known that there are two fibronectin-binding proteins (FnBP A and B) encoded by the *fnbA* and *fnbB* genes which are involved in adherence and internalization of *S.aureus* into the host cell [11, 12]. Presence of both FnBPs has been associated with severe infections [13]. Panton-Valentine leukocidin (PVL) first described in 1932, is a toxin composed of two components, LukS-PV and LukF-PV, which lead to neutrophil lysis [13, 14]. PVL genes have been linked to community-onset MRSA disease worldwide [15].

While several studies have described the molecular characteristics and epidemiology of *S. aureus* strains in asymptomatic adult and children carriers [16, 17], little is known about the characteristics of *S. aureus* infections requiring hospitalization in childhood. Aim of this prospective cohort study was to investigate the epidemiology of *S.aureus* strains and focus on the molecular types, virulence genes and spectrum of infections in children and adolescents requiring in- hospital care.

Methods

Design and population

This cohort study included all *S. aureus* isolates recovered from children and adolescents aged less than 18 years who were admitted for hospitalization in the University Hospital of Heraklion in Crete from 1stJanuary 2015 to 31stDecember 2018. The Hospital provides primary and secondary healthcare services to a population of approximately 306,000 and tertiary services to the largest island in Greece covering a population of 623,000 and includes general paediatric services (capacity 30 beds), neonatal intensive care services (25 beds), paediatric intensive care services (4 beds), paediatric oncology (12 beds) and paediatric surgery (22 beds) services. All isolates from January 2015 to December 2018 were identified and medical records were reviewed for demographic and clinical characteristics. Infections were classified into I) skin and soft tissue infections (SSTIs) such as impetigo, cellulitis, abscess, omphalitis, II) toxin-mediated disease (TMD); including staphylococcal scalded skin syndrome and toxic shock syndrome and III) invasive disease (INV) including septicaemia, pneumonia, osteomyelitis and septic arthitis.

Definitions

Isolates were included if culture type and site were consistent with *S. aureus* infection. Infections with positive cultures obtained from normally sterile fluids, including CSF, blood, and synovial fluid were

considered invasive infections. Isolates obtained from anatomic sites not indicative of infection were classified as colonization cultures and were excluded.

Infections and the recovered isolates were classified as community-associated (CA), community-onset healthcare-associated (COHA) and hospital-associated (HA) based on established clinical criteria [18]. To examine possible changes over time, we studied subsets of isolates collected during two different periods: 2015-2016 (period A) and 2017-2018 (period B).

Microbiology

The isolates were identified by conventional methods, including colony morphology, Gram staining, standard biochemical tests and by the automated Vitek 2 system (bioMérieux, Marcy l' Etoile, France). All *S. aureus* isolates were stored in brain heart infusion (BHI) broth with 20% glycerol at - 80^0 C for further testing.

Antimicrobial susceptibilities were determined for penicillin, oxacillin, erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, moxifloxacin, linezolid, teicoplanin, vancomycin, tetracycline, tigecycline, fusidic acid, mupirocin, rifampicin, fosfomycin, nitrofurantoin and trimethoprim/sulfamethoxazole (TMP-SMX) using the Vitek 2 system (bioMérieux), and the results were interpreted according to the 2018 Clinical and Laboratory Standards Institute (CLSI) criteria [19]. *S. aureus* ATCC 25923 and *S. aureus* ATCC 43300 were used as control strains. Isolates were phenotypically classified as methicillin-susceptible *S. aureus* (MSSA) or MRSA based on the cefoxitin disk diffusion test and the latex agglutination test for the detection of PBP2a (bioMerieux). Inducible resistance to clindamycin was tested by D-test as per CLSI guidelines [19]. *S. aureus* isolates that were resistant to cefoxitin were phenotypically classified as MRSA and verified by PCR for *mecA* or *mecC* gene carriage [20,21]. Genes encoding Panton-Valentine leukocidin (*lukS/lukF-PV*), toxic shock syndrome toxin-I (*tst*), exfoliative toxins (*eta, etb*), fibronectin binding proteins A and B (*fnbA*, *fnbB*) were investigated among 139 available isolates by polymerase chain reactions with specific primers, as previously described [22,23].

Strains were classified into pulsotypes by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA *SmaI* digests performed in a CHEF DR III apparatus (Bio-Rad, Richmond, CA). Patterns differing by less than seven bands were considered to belong to the same PFGE type, as described [24]. From the 139 *S. aureus* available, 58 representative strains from different pulsotypes and with variable toxins genes carriage were further characterized by MLST to sequence types (STs), based on the online MLST database (https://pubmlst.org/organisms/staphylococcus-aureus) [25,26].

Statistical analysis

For categorical variables, chi-square or two-tailed Fisher's exact test were conducted to calculate *p* values and odds ratios in order to identify differences in antimicrobial susceptibility and molecular characteristics of *S. aureus* isolates. For continuous variables, statistical significance of observed associations was evaluated using Mann–Whitney U-test. Antibiotic susceptibility trends and MLST trends across the 4-year period were analyzed using chi square for trend. The level of significance was set at *p* value <0.05. Statistical analysis was conducted using GraphPad Prism 8.4.3.

Results

Clinical characteristics

We identified 139 *S. aureus* clinical isolates from children and adolescents with invasive and non-invasive infections (102 CA, 20 HA, 17 COHA, 77 boys, median age 2.02 years, mean age 3.8 years). Sixteen (11.5%) isolates were MRSA and 123 (88.5%) were MSSA. Skin and soft tissue infections (SSTIs) were predominant (94/139,67.6%), followed by toxin-mediated disease (TMD 23/139,16.5%, all 23 cases were SSSS) and invasive disease (INV 22/139,15.8%). MRSA isolates did not differ regarding age, gender, clinical characteristics, PICU admission as compared to MSSA (Table 1). A total of 52 isolates belonged to children with comorbidities or prolonged hospital-stay, including neonates (38), children with cystic fibrosis (7), children admitted to PICU (6) and one child with malignancy. HA and COHA-isolates were more commonly isolated from these children (OR 2.8; 95% CI 1.27-5.78; p 0.01).

Table 1

Clinical characteristics of children admitted with *S. aureus* infection

	Total (%) N=139	MRSA (%) N=16	MSSA (%) N=123	OR (95% CI)	p value
Gender (male)	77 (55.3)	11 (68.7)	66(53.6)	1.9 (0.68-5.14)	0.29
Age (yr)	2.0	2.0	1.9		0.59 [a]
Manifestations					
SSTI	94 (67.6)	14 (87.5)	80 (65)	3.7 (0.91-17.19)	0.09
TMD	23 (16.5)	0 (0)	23 (18.7)	0.00 (0.00-1.13)	0.00
INV	22 (15.8)	2 (12.5)	20 (16.2)	0.73 (0.15-3.30)	>0.99
Admission to PICU	6 (4.3)	1 (6.2)	5 (4)	1.6 (0.12-13.40)	0.52

[a]Mann- Whitney *U* test was used to analyze differences between groups.

PICU: Paediatric Intensive Care Unit; MRSA: methicillin-resistant *S. aureus;* MSSA: methicillin-susceptible *S. aureus;* SSTIs: Skin and soft tissue infections; TMD: Toxin mediated disease; INV: Invasive disease

Antibiotic resistance profiles

Of 139 *S. aureus* strains, 93.5% were resistant to penicillin, 35.9% to fusidic acid, 7.2% to mupirocin, 22.3% to tobramycin, 18.7% to erythromycin, 16.5% to clindamycin and 9.3% to tetracycline. Among 26 erythromycin-resistant strains, prevalence of constitutive ($cMLS_B$), inducible ($iMLS_B$) and MS resistance phenotypes were 53.9%, 34.6%, and 11.5%, respectively. Resistance to clindamycin and tetracycline were significantly higher among MRSA isolates (Table 2). Low percentages of resistance to gentamicin, ciprofloxacin, moxifloxacin, levofloxacin, fosfomycin and trimethoprim/sulfamethoxazole were observed (2.1%, 5%, 5.5%, 5,2%, 3.3% and 3.6% respectively). None of the *S. aureus* strains were resistant to vancomycin, rifampicin, linezolid or teicoplanin. All phenotypically cefoxitin-resistant isolates were also oxacillin-resistant, carrying *mecA* and were characterized as MRSA.

Methicillin resistance declined significantly over the study period (period A:18.3% *vs*. period B: 6.3%, OR 3.32; 95% CI 1.18-8.96; p 0.03). An increasing trend of fusidic acid and mupirocin resistance was observed (fusidic acid resistance x^2test for trend=5.69, df=1, p 0.01; mupirocin resistancex^2 test for trend =25.76, df=3, p <0.0001) along with a rise in SSSS (x^2test for trend TMD vs SSTIs/INV, =5.6, df=1, p 0.01). Resistance to clindamycin, tobramycin, erythromycin and levofloxacin also increased though not significantly.

Table 2

Antibiotic resistance rates of MRSA and MSSA strains.

	Total N (%)	MRSA (%) N:16	MSSA (%) N:123	OR (95% CI)	p value
Penicillin G	130 (93.5)	16(100)	114 (92.6)	NA	0.59
Erythromycin	26 (18.7)	6(37.5)	20 (16.2)	3.09(0.96-9.26)	0.07
Clindamycin	**23 (16.5)**	**6 (37.5)**	**17 (13.8)**	**3.74(1.13-11.61)**	**0.02**
Fusidic acid	50 (35.9)	9 (56.2)	41 (33.3)	2.57(0.91-6.73)	0.09
Gentamicin	3 (2.1)	1 (6.2)	2 (1.6)	4.03(0.26-35.71)	0.30
Tobramycin	27 (22.3)	1 (7.7)	26 (24.1)	0.26(0.02-1.61)	0.29
Levofloxacin	7 (5.1)	2 (12.5)	5 (4.1)	3.31(0.60-18.36)	0.18
Rifampicin	0 (0)	0 (0)	0 (0)		>0.99
Tetracycline	**13 (9.3)**	**6 (37.5)**	**7 (5.6)**	**9.93(2.76-36.94)**	**0.001**
Tigecycline	0 (0)	0 (0)	0 (0)		>0.99
TMP/SMX*	5 (3.6)	2 (12.5)	3 (2.4)	5.71(0.93-29.23)	0.10
Nitrofurantoin	0 (0)	0 (0)	0 (0)		>0.99
Mupirocin	10 (7.2)	0 (0)	10 (8.1)	0.00(0.00-2.75)	0.60
Vancomycin	0 (0)	0 (0)	0 (0)		>0.99
Linezolid	0 (0)	0 (0)	0 (0)		>0.99
Teicoplanin	0 (0)	0 (0)	0 (0)		>0.99

Molecular characteristics

Thirteen isolates (9.4%) harboured *lukS/lukF-PV*, 27 (19.4%) *eta*, six (4.3%) *etb*, 13 (9.4%) *tst*, 125 (89.9%) *fnbA* and 21 (15.1%) *fnbB* genes. *tst* and *lukS/lukF-PV* genes were more common among MRSA as compared to MSSA (*tst* OR 4.22; 95% CI 1.26-14.6; p 0.04, *lukS/lukF-PV* OR 6.53; 95% CI 1.93-22.7; p 0.007); *eta* was more common among isolates causing TMD as compared to those causing INV/ SSTIs (OR 3.5; 95% CI 1.36-9.29; p 0.01) and among MSSA (OR 0.00; 95% CI 0.00-0.91; p 0.04). No significant differences were found among CA/COHA/HA strains. No significant difference in *fnbA, fnbB, and etb* positive rates was found between MRSA and MSSA and no significant difference was identified regarding *etb* gene carriage among *S.aureus* strains causing TMD and SSTIs (OR 0.219, 95% CI 0.04-1.01; p 0.08]. Of 23 *S. aureus* strains isolated from skin lesions of children with SSSS, 11 (47.8%) were shown to carry at least one exfoliative gene (*eta* or *etb*).

By PFGE, MSSA were classified into 20 pulsotypes including one to 40 strains each (type 1: 40 strains, type 2: 16 strains, types 3, 5 and 6: eight strains each, type 4: seven strains, type 16: five strains, type 17: four strains, types 7, 8, 11, 12, 13, 15 and 18, : three strains each, type 10: two strains, types 9 and 14: one strain each, types C and H: one strain each); MRSA belonged to four pulsotypes; type A: four strains (ST30), B: four strains (ST239), C: five strains (ST80) and H: three strains (ST225). Multilocus sequence typing identified five sequence types among 58 tested isolates (Table 3). Among 42 MSSA isolates that were tested, all strains but two belonged to pulsotype 1/ ST121.Type C/ST80 and type H/ST225 included one MSSA strain each. Regarding MRSA strains, five strains belonged to type C/ ST80; four strains belonged to type A/ ST30; four to type B/ST239 and three MRSA strains belonged to type H/ ST225. Isolates harbouring *eta* or *etb* genes all belonged to PFGE type1-ST121 clone.

Table 3

Molecular characteristics of 58 *S. aureus* strains characterized by MLST. Among hospitalized children with *S. aureus* infection, isolates belonged to five clones with distinct virulence genes patterns.

Sequence type	Total (%)	*lukS/F (%)*	*eta (%)*	*etb (%)*	*tst (%)*	*fnbA (%)*	*fnbB (%)*
ST121	40(69)	3(7.5)	27(67.5)	6(15)	0(0)	40(100)	3(7.5)
ST80	6(10.3)	6(100)	0(0)	0(0)	0(0)	5(83.3)	0(0)
ST239	4(6.9)	0(0)	0(0)	0(0)	0(0)	3(75)	0(0)
ST225	4(6.9)	0(0)	0(0)	0(0)	0(0)	4(100)	0(0)
ST30	4(6.9)	4(100)	0(0)	0(0)	3(75)	3(75)	0(0)

Characteristics of the dominant MSSA ST121 clone versus other clones

The pulsotype 1, sequence type 121 clone (ST121) dominated (40/58, 68.9%). This ST121 clone rarely carried PVL and *tst* genes (3/40 and 0/40 respectively) but harboured at least one epidermolysin gene (*eta or etb*, 29/40,72.5%) and genes that facilitate adherence to the epithelium (*fnbA*, 40/40) causing mainly SSTIs (26/40), SSSS (13/40) and one case of invasive disease. All ST121 strains were MSSA (100%), increasingly resistant to fusidic acid (52.5%, fusidic acid resistance: x^2 for trend analysis= 7.54, p 0.006), resistant to tobramycin (43.7%) and exhibited rising trends of mupirocin resistance ($x^2$for trend analysis: 14.79, p 0.0001).

Other MLST types were rare. ST80 clone belonged to pulsotype C, was PVL-positive (100%,6/6) and *mecA*-positive (83.30%, 5/6), carried *fnbA* gene (83.3%, 5/6) and no epidermolysin genes. ST80 isolates were highly resistant to tetracycline (5/6,83.3%) and fusidic acid (3/6,50%). ST80 demonstrated a

significant decline over the study period with rising trends of ST121 (ST121 vs ST80, x^2test for trend=4.08, df=1, p 0.04) (Fig.1). Antibiotic resistance did not differ significantly among ST121 and ST80 strains, except for a statistically significant higher resistance to methicillin and tetracycline in ST80 isolates (methicillin: ST121 vs ST80: 0% vs 83.3%, p <0.0001, tetracycline; ST121 vs ST80: 28.5% vs 71.4%, p <0.0001).

Discussion

This is the first study of clinical, microbiological and molecular characteristics of *S. aureus* in hospitalized children and adolescents younger than 18 years in the study area. We have demonstrated the decline of MRSA among hospitalized children with invasive and non-invasive infections and the predominance of one CA-MSSA clone that has outweighed MRSA strains in both community- and hospital- associated infections. This new CA-MSSA clone is highly resistant to fusidic acid, tobramycin and increasingly resistant to mupirocin, belongs to PFGE type 1, ST121, carries the *fnbA* gene and in two-third of cases carries at least one gene encoding epidermolysins (*eta* or *etb*). This dominant clone is associated mainly with skin and soft tissue infections and staphylococcal scalded skin syndrome and less commonly with invasive disease.

ST121 clone was rarely reported before 2008 [27, 28]. The first case of ST121 positive infection in adults was described in 2007 in France [29] and thereafter, ST121 has been identified in 10–30% of isolates in Europe, Africa and Asia [30, 31]. In Greece, the emergence of a new community-associated MSSA ST121 clone was reported for the first time in 2017 [32]. This clone was resistant to mupirocin, tobramycin and fusidic acid and carried *eta/etb* genes encoding epidermolysins, causing SSTIs and SSSS [32, 33]. The present study has shown the dissemination of a community-associated MSSA, ST121 clone with similar molecular characteristics that has led to increasing numbers of SSTIs and SSSS in the study area.

In our study, we noticed a three-fold increase in the number of SSSS cases from 2015 to 2018 parallel to the rise of ST121 clone. Increasing cases of SSSS have also been reported globally [33, 34]. Children are known to be highly susceptible to *S. aureus* toxin-mediated disease, which is attributed to their immature immune system, reduced renal clearance of toxins and the fact that children are common carriers of *S. aureus* [7]. The epidermolytic activity of *S. aureus* isolates associated with SSSS has been linked to the activity of exfoliative toxins A and B [34]. In the present study, ST121 strains harbored one of the *eta/etb* genes in 72.5% of the cases, which can explain the predominance of SSTIs and SSSS in this population.

The emerging ST121, methicillin-susceptible, PVL-negative clone described here carries fibronectin-binding protein genes (mainly *fnbA*) and *eta* and *etb* genes which contribute significantly to its high virulence. Our results are consistent with previous studies reporting low rates of PVL genes in ST121 isolates and recognizing PVL-negative ST121 *S. aureus* as an emerging cause of severe morbidity [15, 30, 35]. Our findings also support that *S. aureus* infections are usually community-acquired in children, whereas nosocomial pathogens are more common in hospitalized patients with underlying conditions, such as children with cystic fibrosis, with malignancies and neonates.

Why MRSA declines and MSSA strains takes over? The reasons for replacement of MRSA strains by MSSA strains have not been fully understood [36]. Specific host factors, differences in virulence factors, in antibiotic resistance and the ability to cope with environmental stress may play a role. The dominant MSSA-ST121 clone varies in virulent genes to the previous ST80-MRSA clone, being negative for PVL, positive for exfoliative toxin genes and *fnB* genes [37]. Antibiotic resistance might be an additional factor in MRSA replacement. In previous studies, the impact of SCC*mec* elements on the replication of *S. aureus* cells was identified as a possible factor in MRSA replacement being sometimes associated with a higher glucose consumption [38, 39]. As the MSSA-ST121 clone gradually replaces the previous dominant CA-MRSA ST80 clone, changes in the resistance patterns are also observed. High rates of tobramycin and fusidic acid resistance were observed in the emerging ST121 clone conferring additional advantage in the niche. Traditionally, methicillin resistance has been associated with worse outcomes and higher admission rates to PICU [40]. In this study, MRSA and MSSA did not differ significantly as to disease severity suggesting that the newly spread ST121 clone is equally virulent.

Strengths of this prospective study were the analysis of *S. aureus* isolates causing infection restricted to the paediatric population in a long period of time. One limitation of this study is that it represents a single-center cohort. However, this represents the largest tertiary reference center in Crete and analogous data have not been previously published in this area.

In conclusion, we have shown here that the majority of *S. aureus* infections are caused by a small number of major clones and in recent years, the previously dominant ST80 clone has been replaced by ST121 as the main cause of an increasing number of SSTIs and SSSS in our region. Awareness of this disseminating clone and its resistance patterns is essential and further characterization of its genetic background and its role in disease pathogenesis will improve the understanding of the unique characteristics of this clone.

Declarations

Ethics approval:

The study protocol was approved by the local Ethics Committee (approval number 17702).

Consent for publication:

Not applicable

Availability of data and material:

All data used and analysed in the current study will be made available on reasonable request

Competing interests:

The authors declare that they have no competing interests

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Authors' contributions:

EG and IS contributed to the study design; MT, NG, SM and IS contributed to data collection and analysis. The first draft of the manuscript was written by MT and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

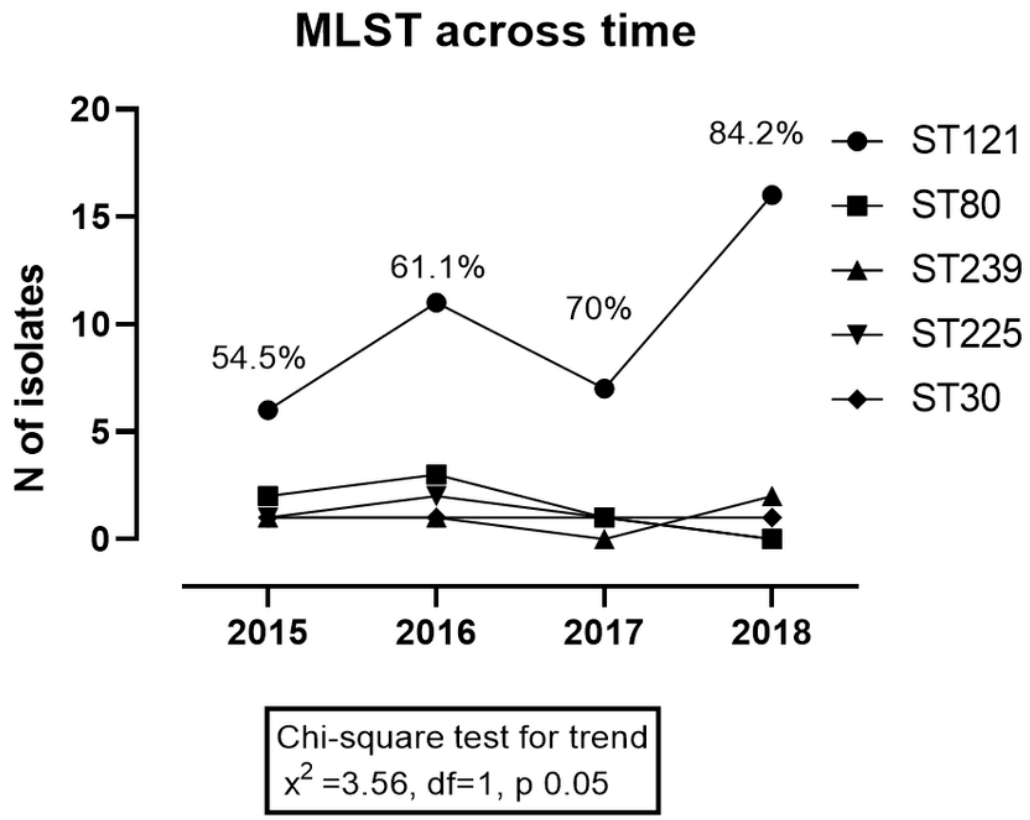

Figure 1

Trend analysis of MLST types of S. aureus strains in hospitalized children Graphpad Prism 8.4.3 was used to create the figure