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Molecular characterization of clonal lineage and Staphylococcal toxin genes from *S. aureus* in Southern Nigeria

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Background. The pathogenic role of *S. aureus* as causative agent of serious infections and food poisoning is on the increase. However, there are few reports on comprehensive analyses of toxins and staphylococcal enterotoxin (SE) genes in *S. aureus* in Africa. This study analyzed spa types and toxin genes in *S. aureus* obtained from our previous studies in Southern Nigeria.

Methods. Forty-seven non-duplicate *S. aureus* isolates were obtained from humans (n = 34) and poultry (n = 13) from previous studies in Southern Nigeria. The strains were analyzed for *mecA*, selected toxins genes (*sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser, seu*), TSST and lukS-PV/lukF-PV by PCR. Population structures of the strains were detected by Staphylococcal protein A (*spa*) typing.

Results. Twenty three percent of all isolates (47) carried the Panton-Valentine leukocidin (PVl) gene. Two MRSA were detected. Twenty different spa types were obtained, with the highest percentages, 17% belonging to *spa* type t091 was observed in 4 states from clinical, nasal and poultry samples while t069 is the most prevalent type in poultry. Eighty-nine percent of the all tested isolates harbored at least one staphylococcus enterotoxin. *Seo* was the most prevalent SE (34%) followed by *seg* (30%) and *sea* (21%), while toxic shock syndrome toxin (TSST), *seb*,*sec*,,*see, sej, sel, sem*, and *ser, seu* were absent in all strains. Spa type t355 was associated with the PVl and complete absence of all studied SE. *Sea, seq, seb, sek* were associated with spa type 069; t127 was associated with *sea* while *sep* was associated with spa type t091. There was coexistence of *seo/seg* and *sei/seg*.

Conclusions. We detected a high incidence of enterotoxins and PVl encoding genes in these potential staphylococcal reservoir. Specific toxin genes were observed in particular spa types.

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Abstract

Background: The pathogenic role of *S. aureus* as causative agent of serious infections and food poisoning is on the increase. However, there are few reports on comprehensive analyses of toxins

and staphylococcal enterotoxin (SE) genes in *S. aureus* in Africa. This study analyzed spa types and toxin genes in *S. aureus* obtained from our previous studies in Southern Nigeria.

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Results: Twenty three percent of all isolates (47) carried the Panton-Valentine leukocidin (PVl) gene. Two MRSA were detected. Twenty different spa types were obtained, with the highest percentages, 17% belonging to *spa* type t091 was observed in 4 states from clinical, nasal and poultry samples while t069 is the most prevalent type in poultry. Eighty-nine percent of the all tested isolates harbored at least one staphylococcus enterotoxin. *Seo* was the most prevalent SE (34%) followed by *seg* (30%) and *sea* (21%), while toxic shock syndrome toxin (TSST), *seb*,*sec*,,*see, sej, sel, sem*, and *ser, seu* were absent in all strains. Spa type t355 was associated with the PVl and complete absence of all studied SE. *Sea, seq, seb, sek* were associated with spa type 069; t127 was associated with *sea* while *sep* was associated with spa type t091. There was coexistence of *seo/seg* and *sei/seg*.

Conclusions: We detected a high incidence of enterotoxins and PVl encoding genes in these potential staphylococcal reservoir. *Spa* types are associated with specific toxin genes.

Keywords - Staphylococcal enterotoxins, spa type, virulence, Southern Nigeria, PVl

Background

Staphylococcus aureus is one of the most important human colonizers that can cause infectious diseases. Colonization of human nares by *S. aureus* is a source and risk factor for staphylococcal disease (Wertheim *et al*., 2004) and invasive staphylococci infection can have its source in strains occurring naturally in the host.

The emergence of MRSA complicated the treatment of infections and increasing the focus on this pathogen. *S. aureus* has a broad spectrum of inherent virulence factors which can enhance infections ranging from mild skin infections to severe sepsis, pneumonia, osteomyelitis and endocarditis (Ayepola *et al*., 2015). The ability of *S. aureus* to successfully infect man is largely due to the expression of virulence factors which promote adhesion, acquisition of nutrients and evasion of host immunologic responses (Monday and Bohach, 1999) Staphylococcal enterotoxins and toxic shock syndrome toxin (TSST) are produced by *S. aureus* which enhances their status as important food-borne pathogens (Løvseth *et al*., 2004) because they can cause

food poisoning in humans. The toxins' genes in *S. aureus* encode different virulence factors which can promote the ability to cause infections in humans. This enhances *S. aureus'* pathogenicity; the toxins produced by the pathogen are responsible for toxin mediated diseases such as toxic shock syndrome and food poisoning . *Spa* typing of *S. aureus* strains provides information which can group isolates in clonal lineages. Clonal analyses can also provide useful insights into the virulence potential and nature of *S. aureus* populations (Kolawole *et al*., 2013).

Shittu *et al*., (2011) reported that *S. aureus* is the main etiological agent of many infections in sub-Saharan Africa and one of the most frequently encountered bacterial species in microbiology laboratories in Nigeria. To establish better infection control, it is important to understand the local epidemiology and clonal lineages of *S. aureus* in Nigeria. Ayepola *et al*. (2015) also stated that some virulence factors are highly prevalent in *S. aureus* isolated from infection but less frequently found in isolates from colonization in Nigeria. PVl can be implicated in skin and soft tissue infections and can also increase *S. aureus'* ability to cause severe infections in humans. Therefore, the objective of this study was to detect selected virulence factors genes and clonal lineages of *S. aureus* previously isolated from seven states of Southern Nigeria.

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Methods

Bacterial isolates

Forty-seven *S aureus* isolates used in this study were drawn from a larger staphylococcal collection from our previous studies to assess the rate of misidentification of *S. aureus* in 7 states (Oyo, Ogun, Osun, Lagos, Ekiti, Bayelsa, Rivers) of Southwestern Nigeria. Thirty four isolates from 6 states were from humans (previously isolated from nasal carriage in the community and clinical isolates) (Ayeni *et al*., 2014, Ayeni *et al*., 2015, Ayeni *et al*., 2017) while thirteen isolates from one State (Ogun) were co isolated with enterococci in a previous study on poultry (Ayeni and Odumosu, 2016). All *S. aureus* isolates from these previous studies were selected for this study.

95 **Identification of *S. aureus* strains by amplification of *femA* gene**

96 The DNA of all staphylococci isolates was extracted by QuickExtract^{TM} DNA extraction solution
97 (Epicentre, USA) according to the manufacturer`s instructions. One µl of extracted DNA was
98 used in PCR reaction in a total volume of 20 µl with 10 µl of 2-fold concentrated RedTaq Ready
99 Mix (Sigma, Germany), 7 µl PCR grade water, 1 µl of 10 pmol of *femA*-F
100 AACTGTTGGCCACTATGA and 1 µl of 10 pmol of *femA*-R CCAGCATTACCTGTAATC.
101 After an initial denaturation step (3 min at 92^{0}C), 30 cycles of amplification were performed as
102 follows: denaturation at 92^{0}C for 1 min, annealing at 56^{0}C for 1 min, and DNA extension at 72^{0}C
103 for 1 min with an increment of 2 s per cycle. The reaction was achieved with a final extension at
104 72^{0}C for 3 min (Vannuffel *et al*., 1995). The PCR product was analysed on agarose gel and
105 bands corresponding to 686-bp were recorded as positive for *femA*.

106 ***Spa* typing of *S aureus* isolates**

107 All *femA* positive isolates were further analysed by *spa* typing The polymorphic X region of the
108 *spa* gene was amplified in all isolates to a total volume of 20 µl comprising1µl of genomic
109 DNA, 10 µl of 2-fold concentrated RedTaq Ready Mix (Sigma, Germany), 7 µl PCR grade
110 water, 1 µl of forward primer1113F (5' –
111 TGTAAAACGACGGCCAGTTAAAGACGATCCTTCGGTGAG and 1 µl of reverse primer
112 spa 1514R CAGGAAACAGCTATGACCCAGCAGTAGTGCCGTTTGCTT were used in PCR
113 reaction according to protocols previously described (Schmid *et al*., 2013). PCR products were
114 run on agarose (1 %) gel electrophoresis previously stained with GelRed (BiotiumInc,
115 USA) and run at approximately 40 mAmp for 45 min. The PCR products were purified
116 with EXOSAP-IT (GEHealthcare, UK). Two microliters of the purified amplification products
117 were used for subsequent sequencing using the BigDye 3.1. Terminator sequencing kit (Applied
118 Biosystems, USA) and were finally analyzed on ABI Genetic Analyzer 3500Dx (Applied
119 Biosystems, USA). The chromatograms obtained were analyzed with the Ridom Staph Type
120 software version1.4; (RidomGmbH, Germany http://spa.ridom.de/index.shtml). *Spa* types were
121 deduced by the differences in number and sequence of spa repeats with BURP algorithm (Ridom
122 GmbH) and the Ridom Spa Server database (Montanaro *et al*., 2016).

123 **PCR amplification of *mecA/mecC* .**

124 PCR assay was performed for all confirmed *S. aureus* strains to amplify a region of *mecA* gene.
125 Primers were as follows: Fw, 5´TCACCAGGTTCAACY]CAAAA 3´; and Rv, 5´
126 CCTGAATCW] GCTAATAATATTTC 3 (García-Álvarez *et al.,* 2011). PCR reaction contained
127 20 µl reaction volume with 1µl of each primer, 10 µl mastermix and 7 µl of PCR grade water.
128 The PCR reaction consisted of an initial denaturation step at 95°C for 5 min; 40 cycles of
129 denaturing at 95°C for 30 seconds; annealing at 55°C for 45 s; extension at 72°C for 45 s; and a
130 final extension at 72°C for 10 min. PCR products were resolved by agarose (1%) gel

electrophoresis previously stained with GelRed (BiotiumInc, USA) and run at approximately 40 mAmp for 45 min.

Virulence factors detection.

Exfoliative toxins (*sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser, seu*), TSST and the lukS-PV/lukF-PV , encoding the Panton-Valentine leucocidin were detected by single PCRs in previously described protocols (Monday and Bohach, 1999, Jarraud *et al*., 1999, Orwin *et al*., 2001, Lina *et al*., 2003, Løvseth *et al*., 2004)

Results

Forty-seven *S. aureus* isolates (34 from human and 13 from poultry) were identified in this study as confirmed by *femA* gene amplification. Twenty different spa types were obtained. Most frequent *spa* type were t091(17%), t355 (17%). t091 was observed in 4 states from clinical, nasal and poultry samples while t069 is the most prevalent type in poultry. (Table 1, Fig I). Of the tested strains, two were MRSA (Table 2).

No distinctive difference of enterotoxin genes could distinguish between the human and poultry isolates Eighty-nine percent of all isolates harboured at least one staphylococci enterotoxin and 23% isolates had PVl. Only one of our nasal isolates harbour PVl while the remaining 12 lacked the gene. *Seo* was the most prevalent SE (34%) followed by *seg* (30%) and *sea* (21%). *Ser* was detected in one isolate while *seb*,*sec*,,*see, sej, sel, sem*, and *ser, seu* were not found in all strains. Several enterotoxin gene combinations were observed including isolates with a combination of two (n =4, 9%), three (n= 5, 11%), four (n= 13, 28%) and five (n= 2, 4%) different SE genes. There was coexistence of *seo/seg* and *sei/seg* toxins (Table 2, Fig II, Fig III).

Some toxins were associated with particular spa types. 88% of t355 (n=8) isolates obtained from two different locations had PVl and were characterized by complete absence of SE. *Sea, seq, seb, sek* were associated with spa type 069 (obtained from 2 locations).All t127 isolates carried *sea* while *sep* was associated with spa type t091 and was the only SE gene that all t091 strains except 1 isolate carried

Discussion

We report spa types and virulence factors of *S. aureus* isolates that have been previously collected in Southern Nigeria. This study reports predominant of t091 spa types of *S. aureus* isolates gotten from different locations and sources in Southern Nigeria. Species-confirmation of

189 the 47 *S. aureus* isolates was done by *femA* gene amplification. Vannuffel *et al*., (1995)
190 confirmed that *femA* expression is a unique feature of *S. aureus*, allowing its specific detection.
191 O'Malley *et al*., (2015) reported that MRSA exist in clinical and community settings in Nigeria.
192 We report a relatively low detection of *mecA/mec* in the current study although several authors
193 have reported high phenotypic detection of MRSA in Nigeria (Onanuga *et al*., 2005, Ayeni *et al*.,
194 2014). The two detected MRSA isolates were PVl negative. Lack of PVl in Nigerian MRSA
195 strains was also previously reported by Kolawole *et al*., (2013).

196 We observed a relatively high rate of PVl positive isolates which is in line with other studies
197 from Africa. Sub-Saharan Africa is observed to be a PVl endemic region showing PVl
198 prevalence among MSSA isolates. O'Malley *et al*. (2015) indicated that 40% (23/57) of MSSA
199 isolates are PVl positive with no PVl positive MRSA therefore they study concluded that PVl
200 positive isolates are most often seen in MSSA. Shittu *et al*., (2011) also reported high proportion
201 of PVl positive isolates among MSSA (40%) in Nigeria. However, in other region of the world,
202 it was reported that MSSA rarely harbor PVl gene (Becker *et al*., 2017).

203 Molecular typing technologies such as *spa* typing provide information which enable the
204 grouping of individual isolates in clonal lineages (Kolawole *et al*., 2013). Twenty spa types were
205 found in this study with the highest percentages belonging to t091. This is different from other
206 studies from Nigeria where t064 have higher prevalence. . Kolawole *et al*., (2013) reported 24
207 spa types with the most frequent spa types being t064, t084, t311, and t1931. Also spa type t064
208 is the most common spa type among HIV positive patients in Nigeria (Olalekan et al., 2012).
209 Shittu *et al*., (2011) reported a total of 28 spa types with the predominant spa type identified as
210 t084 among the MSSA isolates, while t451, t008, t002 and t064 were observed in Southwest
211 Nigeria. These studies, however, were confined to Southwestern Nigeria while a study by
212 O'Malley *et al*., (2015) which involved nasal carriage from Southwestern and Southeastern
213 Nigeria reported spa types t091 and t355, which we also found in our study. Our study locations
214 were also Southwestern and South-South parts of Nigeria and some isolates were from nasal
215 carriage. Therefore, location and site of isolation may be an important factor in types of *spa*
216 found in a study. Interesting, t091 was seen in all three sources of isolates in this study i.e. nasal,
217 clinical and poultry sources. It also spread across widely spaced locations in 4 states of Southern
218 Nigeria and consistently seen even in the small number of isolates used in this study. The
219 predominant *spa* type t091 reported in this study has recently been reported in Germany (Becker
220 *et al*., 2017) and Poland (Ilczyszyn *et al*., 2016) while t355 have been recently reported in
221 Uganda (Asiimwe *et al*., 2017) and Italy (Basanisi *et al*., 2017).

222 It has been observed that prevalence of enterotoxin genes differs greatly depending on the
223 geographic affiliation and the population structure tested (Kolawole *et al*., 2013). In this study,
224 *seo* gene was the most prevalent followed by *seg* .89% of the all tested isolates harbor at least
225 one staphylococcal enterotoxin. This is a high occurrence and has implication in public health.
226 Staphylococcal enterotoxins may induce T-cell stimulation resulting in systemic illness such as
227 toxic shock syndrome and food poisoning. Peck *et al*. (2009) also reported significant differences

and higher prevalence of selected enterotoxin genes in *S. aureus* isolates obtained from blood compared to nasal isolates (7.2% blood vs. 30.5% nasal). The clinical significance of SE cannot be overemphasized. Argudín *et al.,* (2010) stated that staphylococcal food poisoning results from the consumption of foods containing sufficient amounts of preformed enterotoxin and its real incidence is probably underestimated due to misdiagnosis, and improper laboratory examination with the control of social and economic importance.

Seo and *sei* were found in association with *seg* in this study. Previous studies have reported associations of *seg* and *sei.* Kolawole *et al*., (2013) reported that the most frequent SE genes detected were *seg/sei* (41.0%) while Loncarevic *et al*., (2005) stated that 27.9% of 215 isolates harbored *seg* and *sei* . Rosec and Gigaud (2002) reported that 80.6% of 155 isolates harbored *seg* and *sei*. Kim *et al*., (2011) reported that *sec, seg, sei, sel, sem, sen, seo*, were associated with genomic islands thereby probably responsible for their observed combined occurrence. Some *S. aureus* strains in this study also had several enterotoxin gene combinations, from a combination of two to a combination of five different SE genes.

Some toxins were observed in specific spa types. Most t355 spa types had PVl genes in contrast to other *spa* types where there was complete absence of PVl genes. t355 is also characterized by complete absence of all investigated SE. *Sea, seq, seb, sek* were observed in *spa* type 069. All t127 carried *sea* gene while *sep* gene was seen only in spa type t091 and that is the only SE gene that all t091 strains carried, except 1 isolate. These *S. aureus* strains were isolated from different locations across Nigeria, yet the spa types consistently displayed the presence or absence of a particular virulence gene. This information could be useful in predicting virulence toxins a particular strain of *S. aureus* likely carries once the spa type is known. However, further representative studies with larger sample sizes are needed to confirm this. Shittu *et al*., [6] also reported association of some toxin genes (*seh* and *etd)* with a sequence type (ST25).

Conclusions

This study reports predominance of t355 and t091 spa types of *S. aureus* from different locations in Nigeria. A relatively high rate of PVl-positive isolates was found. In this study, *seo* was the most prevalent followed by *seg* while 89% of the all tested isolates harbour at least one staphylococci enterotoxin. *Seo* and *Sei* are found in association with *Seg* in this study. Interestingly, some toxins were seen only in specific spa types.

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Table 1 (on next page)

Prevalence of spa types in different locations in Southern Nigeria

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1 Table 1:- Prevalence of spa types in different locations in Southern Nigeria

State	Region	Source	Number	spa type (no)	Predominant spa type
Bayelsa	South-South	Nasal	13	t084 (1)	t091 (3)
				t091 (3)	t127 (3)
				t1045 (2)	
				t127 (3)	
				t939 (1)	
				t311 (1)	
				t786 (1)	
				t1154 (1)	
Oyo	South-West	Clinical	4	t091 (2)	t091 (2)
				t127 (1)	
				t008 (1)	
Rivers	South-South	Clinical	1	t127 (1)	t127 (1)
Osun	South-West	Clinical	4	t355 (2)	t355 (2)
				t537 (1)	
				t091 (1)	
Ekiti	South-West	Clinical	7	t355 (6)	t355 (6)
				t1931 (1)	
Lagos	South-West	Clinical	5	t1095 (2)	t1095 (2)
				t069 (1)	
				t1045 (1)	
				t021 (1)	
Ogun	South-West	Poultry	13	t069 (4)	t069 (4)
				t091 (2)	
				t14223 (1)	
				t095 (1)	
				t292 (1)	
				t939 (1)	
				t318 (1)	
				t050 (1)	
				t1171 (1)	

2

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Table 2 (on next page)

:Spa types and enterotoxin gene profiles of S. aureus isolatesfrom Southern Nigeria.

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		Toxins															
Isolate	spa	pvl	A	O	M	Q	N	K	P	L	B	G	R	U	I	H	Total
S. aureus FA001	t355	+															1
S. aureus FA002	t537			+	+					+		+					4
S. aureus FA003	t355																0
S. aureus FA004	t355	+															1
S. aureus FA005	t355	+															1
S. aureus FA006	t1931	+															1
S. aureus FA007	t355	+															1
S. aureus FA008	t355	+															1
S. aureus FA009	t355	+															1
S. aureus FA010	t355	+															1
S. aureus FA012	t1045			+	+							+					3
S. aureus FA013	t021	+		+								+		+			4
S. aureus FA014*	t069		+			+		+			+						4
S. aureus FA015	t1095			+	+							+					3
S. aureus FA016	t1095			+	+												2
S. aureus FA017*	t069		+			+		+			+						4
S. aureus FA018*	t069		+			+		+			+						4
S. aureus FA019*	t14223																0
S. aureus FA020*	t095			+	+					+		+					4
S. aureus FA021*	t091								+								1
S. aureus FA022*	t069		+			+		+			+						4
S. aureus FA023*	t091								+								1
S. aureus FA024*	t292			+	+						+	+	+				5
S. aureus FA025*	t939			+	+							+					3
S. aureus FA026*	t318	+		+								+		+			4

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S. aureus FA027*	t069		+			+		+			+						4
S. aureus FA028*	t050			+	+							+					3
S. aureus FA029*	t1171																0
S. aureus FA031	t091								+								1
S. aureus FA034	t084																0
S. aureus FA035	t091			+					+								2
S. aureus FA036	t1045			+								+			+		3
S. aureus FA037	t1045			+			+					+			+		4
S. aureus FA039	t127	+	+			+		+								+	5
S. aureus FA040	t939			+			+					+			+		4
S. aureus FA041	t311			+			+					+			+		4
S. aureus FA043	t127		+													+	2
S. aureus FA044**	t786																0
S. aureus FA045	t091								+								1
S. aureus FA046	t091								+								1
S. aureus FA047	t127		+													+	2
S. aureus FA048	t091								+								1
S. aureus FA049	t127		+														1
S. aureus FA050	t1154			+			+					+			+		4
S. aureus FA051	t127		+														1
S. aureus FA052	t008								+								1
S. aureus FA053	t091								+								1
No		11	10	16	8	6	4	6	9	2	6	14	1	2	5	3	
%		23	21	34	17	13	9	13	19	4	13	30	2	4	11	6	

Note: *=Poultry Isolates

**=MRSA

%=- % occurrence of each SE and PVl

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Figure 1(on next page)

Frequency of Spa Types in 47 S. aureus Isolates

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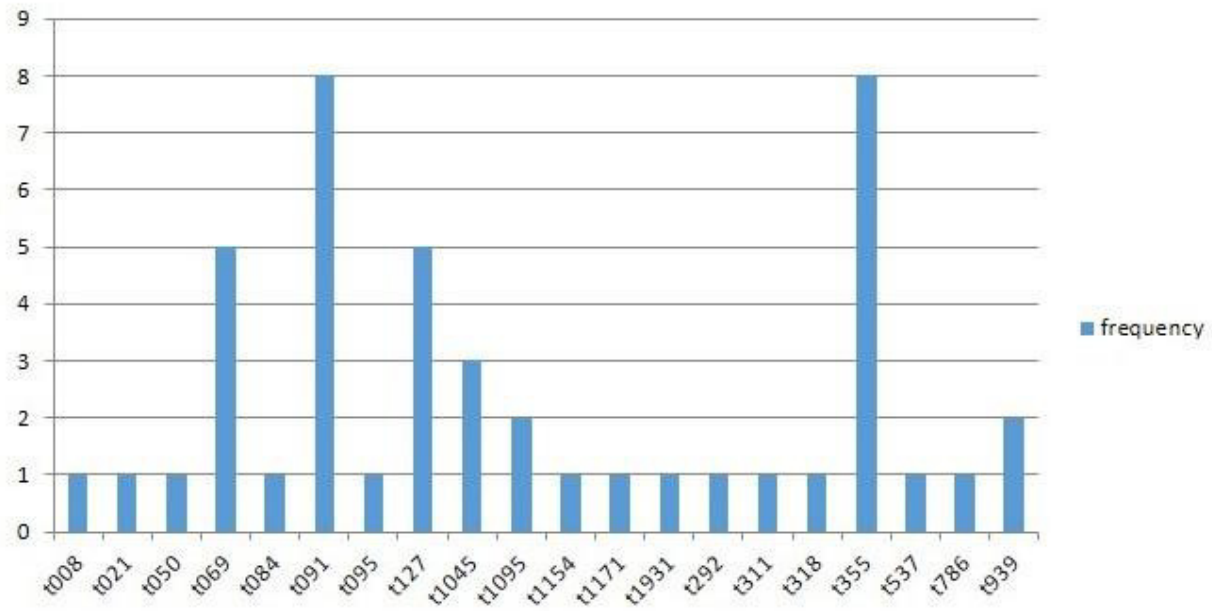

Fig 1: Frequency of spa types occurrence in 47 *S. aureus* isolates

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Figure 2(on next page)

Prevalence of Staphylococci Enterotoxins in Studied Isolates

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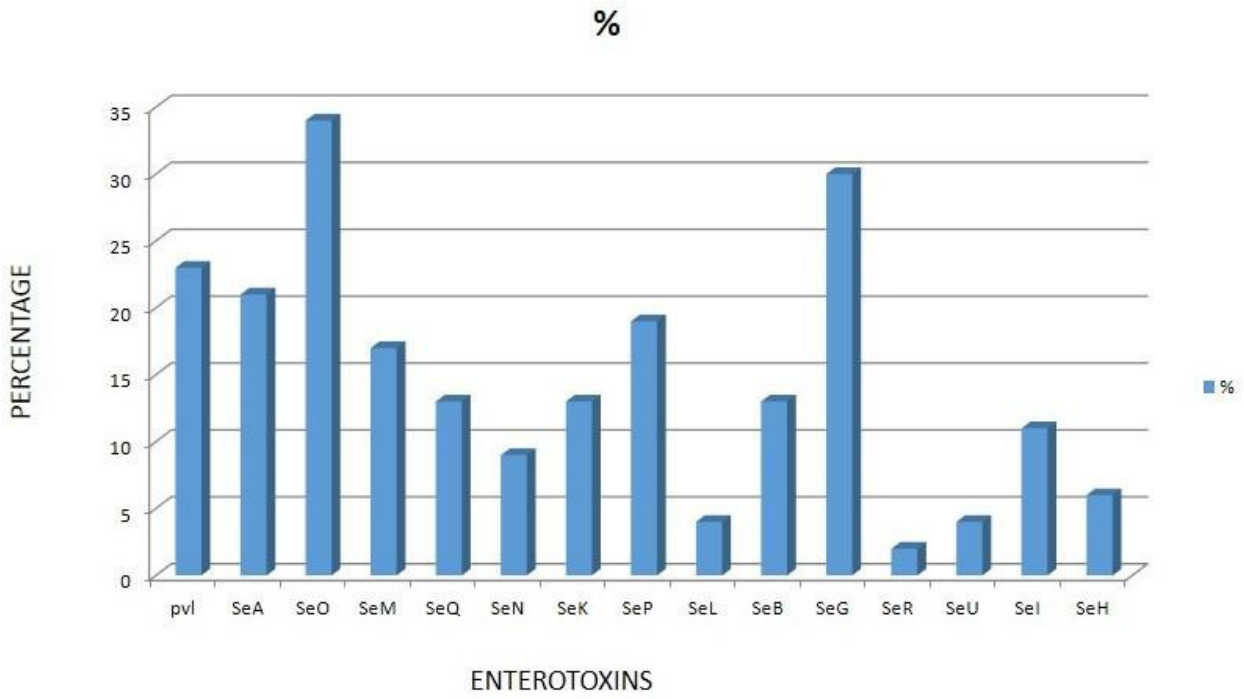

Fig II: Prevalence of staphylococcal enterotoxins in studied isolates

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Figure 3(on next page)

Association of Enterotoxins with Spa Types

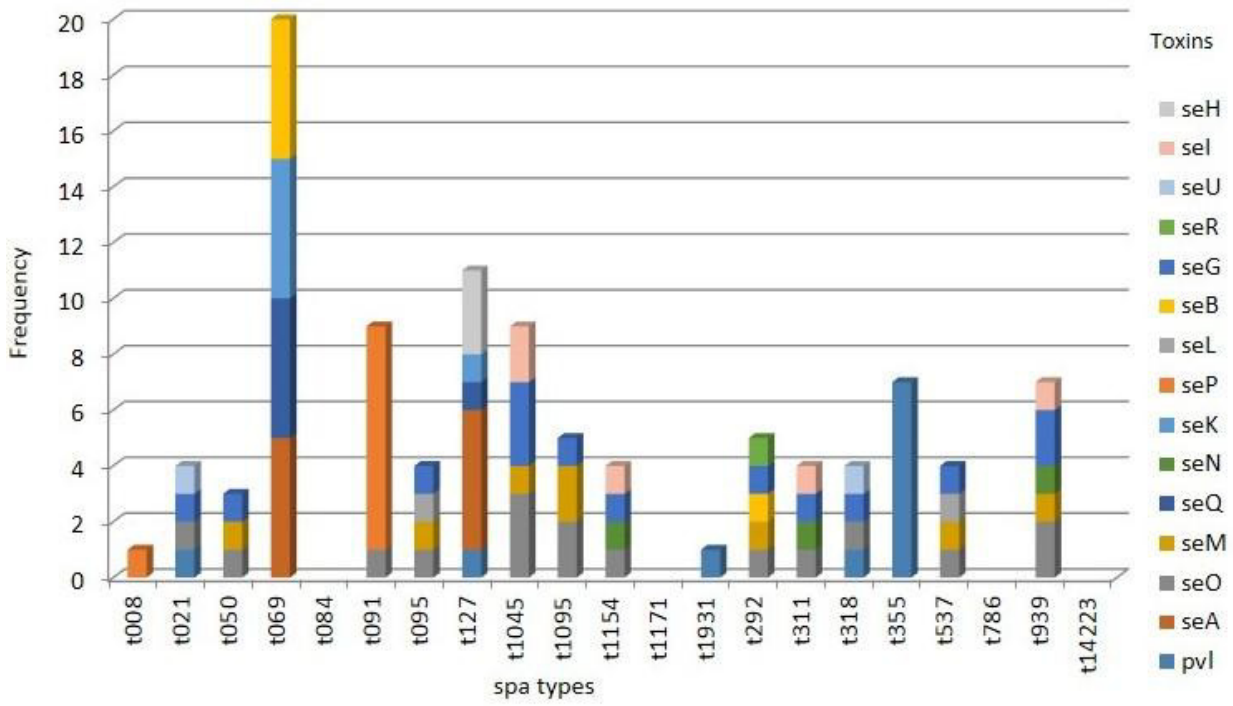

Fig III: Association of enterotoxins with spa types