ISOLATION OF *STAPHYLOCOCCUS AUREUS* AND PROGRESSION OF PERIODONTAL LESIONS IN AGGRESSIVE PERIODONTITIS

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This work evaluates whether isolation and toxicity of subgingival *Staphylococcus aureus* strains correlate with progression of periodontal lesions and whether isolates are characterized by a specific genomic background. The study involved 165 subjects affected by generalized aggressive periodontitis. Three sets of samples of supragingival and subgingival plaque were obtained at 45-day intervals from active and non-active sites, to detect *S. aureus*. Susceptibility to antibiotics, the presence of 17 genes, genomic restriction profiles and multilocus sequence typing (MLST) were performed to characterize all isolates. *S. aureus* was detected in 37.6% of the subjects. Subgingival colonization rates were 66.1% and 12.9% for active and non-active sites, respectively ($P<0.01$). Supragingival and subgingival isolates were shown to be distinct by molecular genotyping and DNA fingerprint analysis. MLST showed that isolates were not genetically related and no sequence type was predominant in any of the two locations. These data demonstrate that *S. aureus* is associated with the progression of aggressive periodontitis and that a specific set of characters is necessary for the bacterium to colonize subgingival sites. Comparative analysis of genomic structure and genetic-related data suggest that the periodontal environment could promote genetic evolution of strains.

Staphylococcus aureus is a Gram positive bacterium colonizing several districts of the human body (1) and being able to evolve its own genome and adapt transcription of a wide array of genes devoted to pathogenicity and virulence in order to cause a number of opportunistic infections (2). Factors of virulence include surface proteins favoring colonization of different tissues and evasion of immune defenses of the host (3), membrane-damaging toxins and superantigens that can cause tissue damage, septic shock (4) and contribute to impairment and subversion of immunity of the host (5). In the last decade *S. aureus* has undergone rapid evolution that led to the emergence and diffusion of Methicillin Resistant *S. aureus* (MRSA), causing many different clinically relevant infections (6), initially mostly in hospital settings but later on also in the community, irrespective of the presence of predisposing factors.

Although once regarded mainly as a member of the commensal microbiota of humans that was able to cause damage in immunocompromised hosts alone, *S. aureus* is now emerging as a primary pathogen or as a cofactor in several pathologies where it was once

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believed to be only a spectator of a match played by other microorganisms against the host. Among sites that are frequently colonized by *S. aureus*, the oral cavity (7, 8) is actually receiving attention also as the reservoir for different types of infections (9). We have recently shown that patients affected by periodontal disease, host *S. aureus* in their mouth at higher rates than subjects who are periodontally healthy, and that *S. aureus* strains isolated from periodontally affected patients are more toxigenic than those isolated from healthy subjects (10). Those experiments were aimed at evaluating the effect of changing oral conditions on the status of *S. aureus* oral carrier and on the possibility that these conditions could promote the diffusion of severe systemic infections. Consequently we analyzed only supragingival plaque samples and could not evaluate the existence of any correlation between the isolation of *S. aureus* and the progression of periodontal disease.

Consequently, this work was aimed at evaluating whether the isolation of *S. aureus* from subgingival sites and their toxigenic potential correlates with progression of periodontal lesions and whether actively progressing periodontal sites are colonized by *S. aureus* strains characterized by a specific genomic background.

MATERIALS AND METHODS

Studied population

This was an observational study conducted on microbiological samples obtained from subjects attending private dental practitioners for periodontal problems in the period from March 2009 to May 2011. All patients were recruited from structures located in highly urbanized areas and consisted prevalently of subjects with high school or university degrees. Overall, 214 patients were initially selected basing on a diagnosis of generalized aggressive periodontitis assessed by an experienced clinician basing on the criteria outlined by the International Workshop for the Classification of Periodontal Diseases and Conditions (11), and according to the following inclusion/exclusion criteria: age ≤39 years, ≥20 teeth in the mouth, ≥30% of teeth with Periodontal Probing Depth (PPD) ≥ 5 mm with bleeding on probing, including at least one incisor and one first molar, evidence of recent progression of disease as compared to the last records reported on the periodontal chart, absence of systemic diseases or conditions known to affect antibacterial defenses, of diseases affecting soft tissues and epithelial barrier integrity, systemic or topic antimicrobial treatment or periodontal treatments in the previous 6 months, absence of pregnancy or breast feeding.

Enrolment in the study was then confirmed upon detection of active progression of at least one periodontal lesion as assessed by three serial evaluations of periodontal probing depth (PPD) at 45-day intervals. Accordingly, 165 of 214 patients (77.1%) were selected (mean age 32.3±3.8 years, ages ranging 18 to 39). Of these, 81 were males (mean age 32.4±3.6 years, ages ranging 21 to 39) and 84 were females (mean age 32.3±4.1 years, ages ranging 18 to 39) showing at least one active periodontal lesion during the period of observation, according to the criteria described below.

The nature of the study was explained to all subjects who met the inclusion criteria and they were asked to indicate their acceptance by voluntarily signing an informed consent complying with statements contained in the Helsinki Declaration.

Clinical evaluations

The periodontal and dental conditions of all patients and their compliance with inclusion criteria were evaluated separately by two skilled examiners. Compliance of periodontal conditions with inclusion criteria was clinically assessed in terms of Visible Plaque Index (VPI), Gingival Bleeding Index (GBI), PPD, and by the radiographic evaluation of the presence/absence of the crestal lamina dura, and the presence/absence of horizontal or vertical bone loss, measured as the distance of alveolar bone from the cementum-enamel junction. Each tooth was evaluated at six sites for VPI, GBI and PPD, separately and at 3 sites (mesial, central and distal) for alveolar bone levels (ABL). PPD was assessed to the nearest 1 mm using a William's color coded periodontal probe. Intra-oral standardized radiographs were obtained according to a long-cone paralleling technique.

Changes in periodontal conditions were evaluated in terms of VPI, GBI and PPD at the beginning of the study (T0), and at 45 (T45) and 90 (T90) days following first evaluation. Periodontal sites showing significant increase of PPD between T0, T45 and T90 were classified as active sites (AS), while the remaining ones were considered as non-active sites (non-AS).

Microbiological analyses

Samples of supragingival plaque were obtained at T0 from the mesial aspect of all teeth showing PPD≥4mm and stored at -80°C until processed for PCR assays to confirm/exclude the presence of *S. aureus*. Moreover, samples of supragingival and subgingival plaque were obtained at T45 from one AS and one non-AS for each

patient. Supragingival plaque samples were obtained by a sterile cotton swab and transported to the laboratory within 3 hours on ice in 0.5 ml of sterile Amies transport Medium (Difco, Milan Italy). Subgingival plaque samples were obtained, following careful removal of supragingival plaque, by 2 sterile nr.40 endodontic paper points placed for 60 seconds and then transferred together into 1.5 ml screw cap vials containing 0.5ml of sterile Amies transport fluid.

Upon receipt samples were vortexed for 2 minutes and divided into 2 aliquots (0.1 and 0.4 ml, respectively). The 0.1 aliquot was used for cultural microbiological analyses, and the 0.4 ml aliquot was stored at -80°C).

Cultural microbiological analyses

Samples for cultural microbiological analyses were processed for isolation and identification of *S. aureus* by standard methods. Briefly, a lapful of each sample was streaked for isolation onto Mannitol Salt Agar plates and incubated 48 h at 37°C. Samples yielding ≥10 identical mannitol positive colonies were analyzed by Gram staining, and assayed for catalase and coagulase production. Confirmatory species identification was obtained by a polymerase chain reaction (PCR) amplification based method detecting a 279bp specific fragment of the *nuc* gene encoding the *S. aureus* thermostable nuclease (12). The same PCR assay was used to detect the presence of *S. aureus* in supragingival and subgingival samples obtained from the selected sites at T0.

Susceptibility of *S. aureus* isolates to common antibiotics was tested by the method of Kirby-Bauer according to the criteria of the NCCLS (13). Isolates were classified as MRSA on the basis of resistance to oxacillin (≥4mg/ml) and positivity of the Penicillin Binding Protein (PBP2') latex agglutination test (Oxoid, Milan, Italy).

Pulsed-field gel electrophoresis (PFGE)

Fingerprints of chromosomal DNA restricted with *Sma*I endonuclease (Fermentas) were determined after separation of the DNA fragments by PFGE using a CHEF-DRII apparatus (Bio-Rad Laboratories) as described previously (14). The banding patterns were interpreted visually following published guidelines (15) and by means of the TotalLab 1D software (Nonlinear Dynamics), to construct a dendrogram of relatedness of strains by the UPGMA algorithm.

PCR assays for genotyping

Seventeen genetic determinants were assayed by PCR in genomic DNA purified from each *S. aureus* isolate as described previously (16); these included toxins (*sea*, *seb*, *sec*, *tst*, *eta*, *etb*, and PVL), adhesins (*clfA*, *cna*, *map/eap*, *sdrC*, *sdrD*, *sdrE*), and *agr* groups I to IV.

Multi-locus sequence typing

Multi-locus sequence typing (MLST) was performed as described previously(17) by amplifying and sequencing specific fragments of seven housekeeping genes (*arc C, aroE, glp, gmk, pta, tpi,* and *yqiL*). PCR products were assayed for purity by gel electrophoresis and purified with the NucleoSpin® Gel and PCR Clean-up purification system (Macherey-Nagel, GmbH, Düren, Germany). The purified PCR fragments were directly sequenced in service (BioFab Research, Rome, Italy). Allele numbers were assigned by using the MLST website (www.mlst.net) and sequence types (ST) were determined via the database (http://saureus. mlst.net/).

Partial Least Square Discriminant Analysis (PLS-DA) and other statistics

Evaluation of the significance of differences of single characters between AS and non-AS or between isolates from different sites for positivity of PCR assays of single genes were performed by the Fisher exact test or the c^2 test using the online resource available at http://quantpsy.org/ or by the Student's *t*-test available in the Microsoft Excel software. Differences yielding values of P in the range >0.01 to ≤ 0.05 were considered significant while differences yielding values of $P \leq 0.01$ were considered very significant. Results were approximated to the nearest second decimal value.

In order to evaluate whether the presence/absence of specific genes correlated with the origin of *S. aureus* strains from subgingival plaque of AS or supragingival plaque of non-AS, a 1/0 matrix was constructed based on results of PCR assays of adhesins and toxins. Logistic PLS-DA, included in SIMCA-P+ software (Umetrics AB, Umeå/Malmö, Sweden), was performed to assess the model predictability and gene importance in classifying strains. Data were automatically mean centered and unit variance scaled by the statistical software. Each gene was hierarchically classified based on a software-assigned variable importance (VIP) value. The variables with VIP value ≥1 were chosen as discriminatory for each strain belonging to class. Data were presented as a 3D score plot for each isolate, a 2D loading plot in which proximity of each variable to class centroid indicates relevance for inclusion into that class and a bar plot of VIP values for each studied character.

RESULTS

Clinical characteristics of active and non-active sites

During the course of the study, 214 patients affected by generalized aggressive periodontitis were

Table I. *Mean values of Visible Plaque Index (VPI), Gingival Bleeding Index (GBI) and Periodontal Probing Depth (PPD) detected at the beginning of the study (T0), 45 (T45) and 90 (T90) days following first evaluation at the selected actively progressing (AS) and non actively progressing (non-AS) periodontal sites.*

		AS	Non-AS	*P** AS *vs* non-AS
T0	VPI (±SD)	1.48 (±0.50)	1.49 (±0.50)	0.83
	GBI (±SD)	1.62 (±0.49)	1.61 (±0.49)	0.82
	PPD (±SD)	5.61 (±0.63)	5.61 (±0.59)	0.93
T45	VPI (±SD)	1.52 (±0.51)	1.49 (±0.50)	0.66
	GBI (±SD)	2.05 (±0.27)	1.62 (±0.49)	**≤0.01**
	PPD (±SD)	6.82 (±0.65)	5.61 (±0.59)	**≤0.01**
T90	VPI (±SD)	1.51 (±0.51)	1.49 (±0.50)	0.75
	GBI (±SD)	1.87 (±0.42)	1.60 (±0.49)	**≤0.01**
	PPD (±SD)	6.92 (±0.70)	5.61 (±0.59)	**≤0.01**

** Values of P calculated by performing Student's t-test. Bold characters indicate highly significant differences with P≤0.01.*

evaluated for 90 days at 45-day intervals to detect the presence of actively progressing periodontal lesions. During the observation period 165 subjects (84 females and 81 males) (77.1% of the recruited subjects) showed at least one actively progressing lesion and were consequently judged suitable for inclusion in the study. Males and females were comparable for age distribution (P=0.84). Sixty-seven of the 165 studied subjects (40.6%) were cigarette smokers; these included 33 of 84 females (39.3%) and 34 of 81 males (42.0%).

In these subject 165 AS and 165 non-AS were identified, taking care to select lesions showing initial clinical conditions that were as similar as possible (Table I). Results of repeated clinical characterization of these sites at T45 and T90 showed that AS underwent a very significant increase of values of GBI (P≤0.01 for T0 *vs* T45 and T45 *vs* T90) and PPD (P≤0.01 and P=0.16 for T0 *vs* T45 and T45 *vs* T90, respectively), while no significant difference was observed in VPI (P=0.51 and 0.91 for T0 *vs* T45 and T45 *vs* T90, respectively). No significant differences were observed in clinical parameters that were related to gender or cigarette smoking habit (data not shown).

Isolation of S. aureus from supragingival and subgingival sites

Microbiological samples of supragingival and subgingival plaque from both AS and non-AS were analyzed for the presence of *S. aureus*. Analysis of supragingival plaque samples showed that 62 of 165 subjects (37.6%) hosted *S. aureus* strains in their plaque. Of these 24 (38.7%) were cigarette smokers. A 100% correspondence was observed for positivity to *S. aureus* isolation in supragingival plaque samples between AS and non-AS. On the other hand,, the prevalence of *S. aureus* in subgingival plaque samples was significantly higher in AS as compared to non-AS (18.2% and 4.8% respectively, P≤0.01 at T45 and 14.5% and 4.8%, P≤0.01 at T90). Of the 30 *S. aureus* positive AS 9 (30.0%) belonged to cigarette smokers. When *S. aureus* positive subjects alone were considered, subgingival plaque samples

Table II. *Mean ages of patients and mean values of Visible Plaque Index (VPI), Gingival Bleeding Index (GBI) and Periodontal Probing Depth (PPD) detected at the beginning of the study(T0), 45 (T45) and 90 (T90) days following first evaluation at actively progressing periodontal sites divided according to results of microbiological analyses in S. aureus supragingival and subgingival positive (SSC), supragingival positive (SC) and negative (NC).*

		Mean value (±SD)			*P**		
		SSC	SC	NC	SSC *vs* SC	SSC *vs* NC	SC *vs* NC
	Age	32.3 (±3.7)	42.24(±4.53)	42.37(±3.79)	0.99	0.86	0.89
T0	VPI	1.49(±0.51)	1.43(±0.51)	1.49(±0.50)	0.66	0.98	0.64
	GBI	1.46(±0.50)	1.62(±0.50)	1.68(±0.47)	0.25	0.02	0.59
	PPD	4.39(±0.59)	4.81(±0.68)	4.65(±0.62)	**≤0.01**	0.02	0.29
T45	VPI	1.56(±0.50)	1.38(±0.50)	1.52(±0.52)	0.19	0.70	0.25
	GBI	2.20(±0.40	2.10(±0.30	1.98(±0.14	0.32	**≤0.01**	**≤0.01**
	PPD	6.10(±0.62)	5.90(±0.70)	5.69(±0.63)	0.27	**≤0.01**	0.16
T90	VPI	1.49(±0.51)	1.43(±0.51)	1.53(±0.52)	0.66	0.63	0.40
	GBI	1.71(±0.51)	1.90(±0.44)	1.92(±0.36)	0.14	**≤0.01**	0.84
	PPD	6.51(±0.51)	5.90(±0.70)	5.69(±0.63)	**≤0.01**	**≤0.01**	0.16

** Values of P calculated by performing Student's t-test. Bold characters indicate highly significant differences with P≤0.01. Underlined characters indicate significant differences with P in the range ≤0.05 to >0.01.*

resulted overall positive in 66.1% of AS compared to only 12.9% in non-AS ($P \leq 0.01$). Thirteen out of the 30 *S. aureus* positive AS at T45 were positive also at T90, while 11 AS that were *S. aureus* negative at T45 were positive at T90. All of these sites showed further progression of the lesion between T45 and T90. The overall prevalence of *S. aureus* in the 90 days of observation was 24.8% for AS and 4.8% for non-AS ($P \leq 0.01$).

Analysis of clinical parameters detected at T0, T45 and T90 at AS divided according to positivity or negativity to the isolation of *S. aureus* from either supragingival and/or subgingival plaque, showed the existence of significant differences (Table II). Sites were divided in 3 groups, namely: i) sites found *S. aureus* positive both supragingivally and subgingivally (SSC), ii) sites found *S. aureus* positive only supragingivally (SC) and iii) *S. aureus* negative sites (NC). Although subjects in the 3 groups were comparable in respect to age and sites showed comparable values of VPI at all times (Table I), very significant differences ($P \leq 0.01$) were observed in both GBI and PPD between SSC and NC ($P \leq 0.01$ for GBI and PPD both at T45 and at T90) (Table II). Moreover, very significant differences existed in GBI at T45 between SC and NC ($P \leq 0.01$) and in PPD both at T0 and T90 between SSC and SC ($P \leq 0.01$) (Table II). Significant differences ($P \leq 0.05$) were observed in both GBI and PPD at T0 between SSC and NC (Table II).

Characterization of S. aureus isolates

Overall, 62 *S. aureus* strains were isolated during the study. Antibiotic susceptibility patterns and genotypic profiles showed that isolates from supragingival and subgingival sites at both AS and non-AS were 100% identical and consequently that each *S. aureus* positive subject hosted a single strain. Of the 62 isolates that were further characterized, 21 (33.9%) were isolated from supragingival plaque (SUP isolates) of non-AS, while the remaining 41 (66.1%) were isolated from both supragingival and subgingival plaque (SUB isolates) of AS. Although resistance to betalactamic antibiotics was widely

Table III. *Prevalence of resistant strains among the 62 S.aureus isolates from subgingival plaque of active sites (SUB) and supragingival plaque of non-active sites (SUP).*

	Prevalence of resistant isolates (%)			*P** SUB *vs* SUP
Antibiotic	Overall	SUB	SUP	
Penicillin	85.5	90.2	76.2	0.25
Oxacillin	11.3	9. 8	14.3	0.68
Ciprofloxacin/norfloxacin	4.8	7.3	0.0	0.99
Levofloxacin	4.8	7.3	0.0	0.99
Gentamycin	9.7	12. 2	4.8	0.65
Tobramycin	1.6	0.0	4.8	0.34
Erithromycin	24.2	21.9	28.6	0.75
Clindamycin	8.1	9. 8	4.8	0.65
Tetracycline	4.8	4. 9	4.8	1.00
Fosfomycin	6.4	4. 9	9.5	0.60
Nitrofurantoine	1.6	2.4	0.0	1.00
Rifamycin	1.6	0.0	4.8	0.34
Mupirocine	6.4	0.0	19.0	**≤0.01**

** Values of P calculated by performing Fisher exact test. Bold characters indicate highly significant differences with P≤0.01.*

diffused among the 62 isolates (85.5% of isolates was resistant to at least one betalactamic antibiotic) only 7 of 62 isolate (11.3%) were shown to be MRSA being both oxacillin resistant and PBP2a positive (Table III). Excluding penicillins, the 62 *S. aureus* isolates showed limited resistance to the tested antibiotics, erythromycin being the one with the highest prevalence of resistant strains (24.2%) (Table III). No isolate was resistant to Moxifloxacin, Linezolid, Vancomycin, Teicoplanin, Tigecycline, Sulfomethoxazole, and Quinopristin/Dalfopristin, while resistance to the remaining tested antibiotics ranged 1.6 to 9.7%.

No significant difference was observed in the distribution of resistances between SUP and SUB isolates, with the exception of Mupirocine (Table III). The relevance of such a difference is nevertheless low, due to low prevalence of resistant strains and scarce relevance of this antibiotic for periodontal treatment.

Genomic DNA fingerprinting of S. aureus isolates

Analysis of genomic DNA *Sma*I restriction profiles obtained from the 62 *S. aureus* isolates showed the absence of recurrent fingerprints (Fig. 1). Analysis of the 62 fingerprints enabled to evaluate the genomic relatedness of isolates distinguished as SUP and SUB. The resulting dendrogram showed that the 62 isolates were grouped in 3 main clusters (A, B and C), consisting respectively of 17, 16 and 29 isolates (Fig.1). Cluster A consisted mainly of SUP isolates (11 of 17, 64.5%), while both cluster B and C consisted mainly of SUB isolates (11 of 16, 68.7%, P=0.084 and 24 of 29 82.8%, $P \leq 0.01$, respectively).

Genotyping of S. aureus strains

Among the 62 *S. aureus* strains isolated during

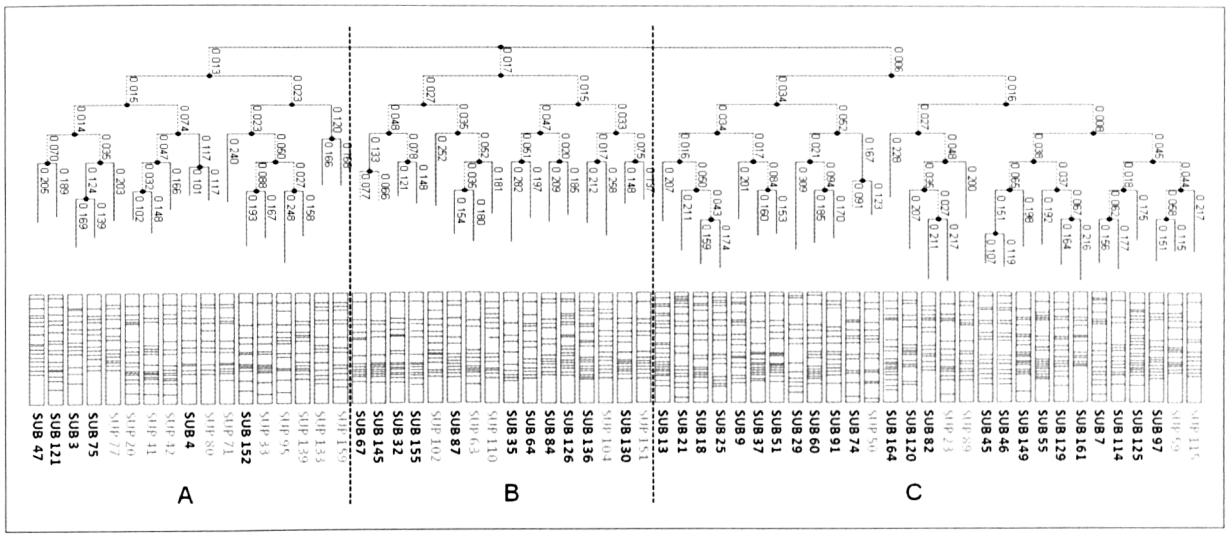

Fig. 1. *Dendrogram constructed following analysis of SmaI restriction profiles of the 62 S. aureus isolates from subgingival plaque of active sites (SUB) and supragingival plaque of non-active sites (SUP). Dotted lines evidentiate subdivision in 3 clusters (A, B, and C).*

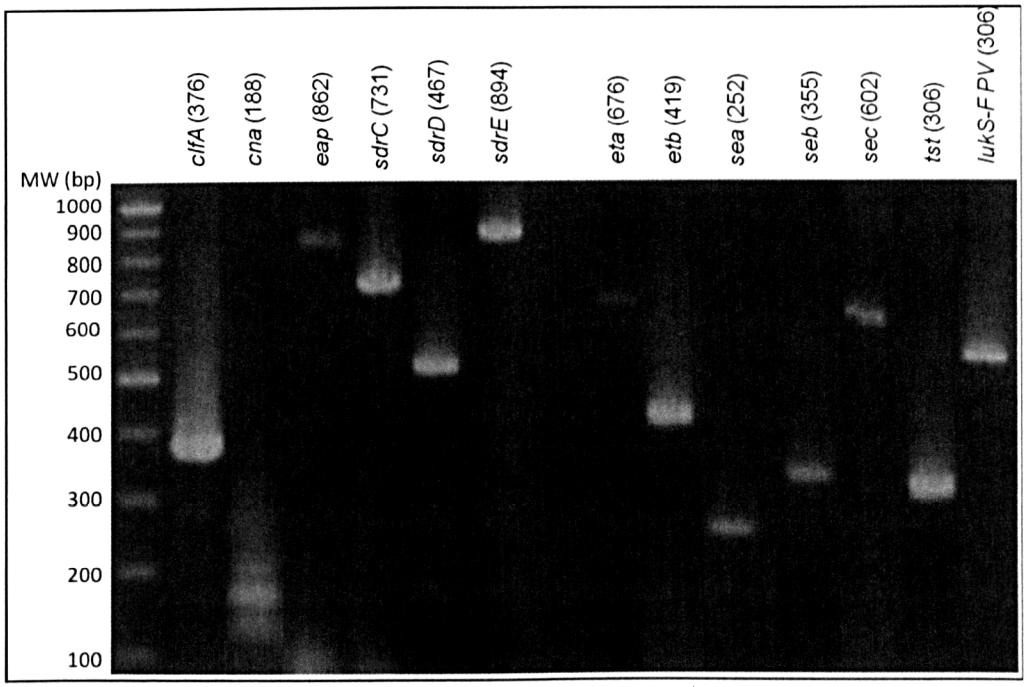

Fig. 2. *Separation by 1.5% agarose gel electrophoresis of the specific amplification products of the 13 genes studied for genotyping. The image is a collection of amplification products obtained in different separations performed in standardized conditions. Lane 1 is the separation profile of 1 kb DNA ladder (molecular weights are indicated on the left).*

the study, 32 (51.6%) were *agr*I, 16 (25.8%) were *agr*II, 10 (16.1%) were *agr*III, and 4 (6.5%) were *agr*IV. No statistically significant difference was observed in the distribution of *agr* groups between SUB and SUP isolates.

Presence of putative virulence determinants in S. aureus strains

Analysis of distribution of the studied genes among the 62 *S. aureus* isolates by specific PCR methods (Fig. 2) evidenced that several genes

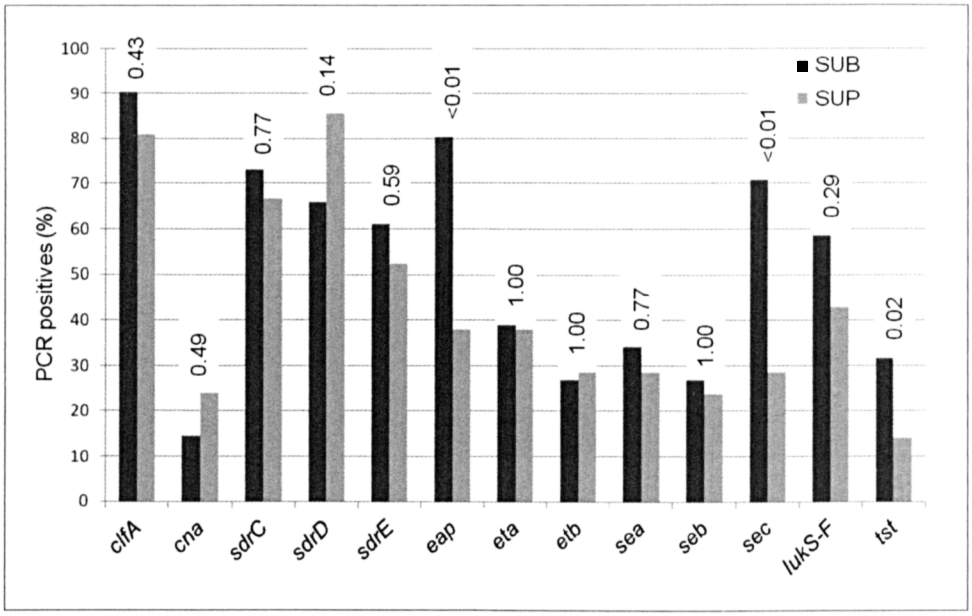

Fig. 3. *Prevalence of 13 virulence related genes among the 62 S. aureus isolates from subgingival plaque of active sites (SUB) and supragingival plaque of non active sites (SUP) assessed by specific PCR assays. Values of P for significance of differences between SUP and SUB for each gene are reported above the bars.*

encoding for adhesins (*clfA, sdrC, sdrD, sdrE*) and toxins (*sec, lukS-F*) had overall prevalence rates higher than 50% in the studied strains. Genes encoding for the adhesin Eap, the superantigen toxin Sec and the exotoxin TSST were significantly more prevalent among SUB isolates than among SUP isolates (Fig. 3), while no significant differences were observed for the other studied genes. Overall, genes encoding for the superantigen enterotoxins (*sea, seb, sec*) were shown in 100% of SUB isolates and in 66.7% of SUP isolates (P=0.04).

Characterization by PCR of the *SCCmec* cassette for the 7 oxacillin resistant, PBP2a positive isolates confirmed the presence of the *mecA* gene and demonstrated that all isolates hosted a type IV cassette (3 were IVc, 2 were IVa and the remaining were IVb and IVd).

Analysis of genetic relatedness by MLST

The 62 *S. aureus* isolates were classified according to sequence data in 17 different sequence types (ST) (Fig. 4). ST30, ST45 and ST938 were the most frequent types among the 62 isolates. No statistically significant difference was observed in the prevalence of STs between SUP and SUB isolates (Fig. 4).

Analysis of overall genetic diversity between SUP and SUB isolates

The PLS-DA model was constructed with an overall predictability of 90.3%, with a Fisher probability of 1.2×10^{-9}. Predictability for classification of an isolate was 95.1% for SUB and 80.9% for SUP. Cumulative genetic characters possessed by the examined isolates allowed a good separation between SUB and SUP (as evident from the score 3D-plot in Fig. 5). Five of the 13 studied genes contributed mainly to separation (VIP≥1) and namely *sec, eap, tst, sdrD* and *cna* in decreasing order of relevance (bar plot in Fig. 5). Analysis of the loading plot representing the hierarchical classification of genes according to variable importance in discriminating SUB from SUP isolates, showed that genes *tst*, *eap* and *sec* were associated with SUB isolates, while *sdrD* and *cna* with SUP isolates (loading plot in Fig. 5).

DISCUSSION

S. aureus is receiving increasing attention as an opportunistic pathogen of primary importance, that has gained the ability to cause serious invasive infections even in young and healthy adults, both in

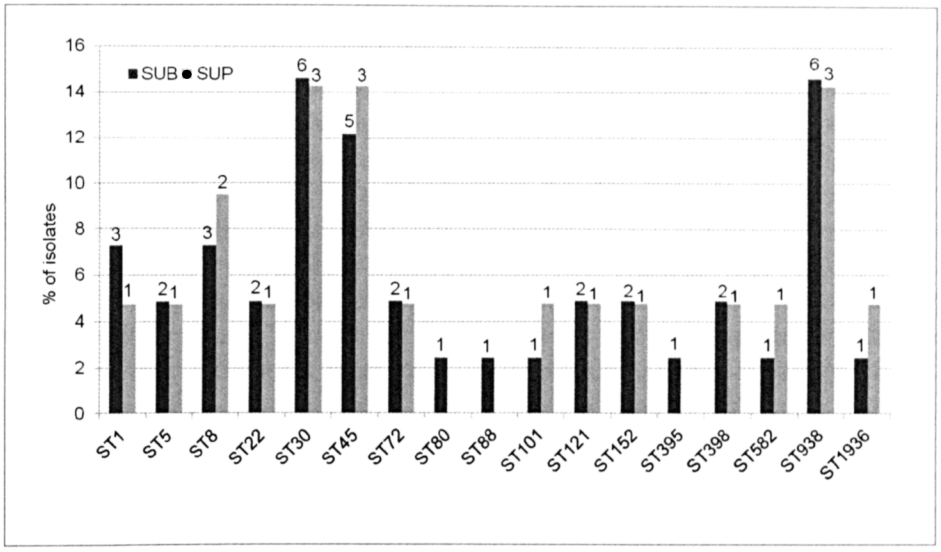

Fig. 4. *Prevalence of different sequence types (ST) among the 62 S. aureus isolates from subgingival plaque of active sites (SUB) and supragingival plaque of non active sites (SUP) assessed by MLST. The number of isolates for each ST in the two groups is reported above the bars.*

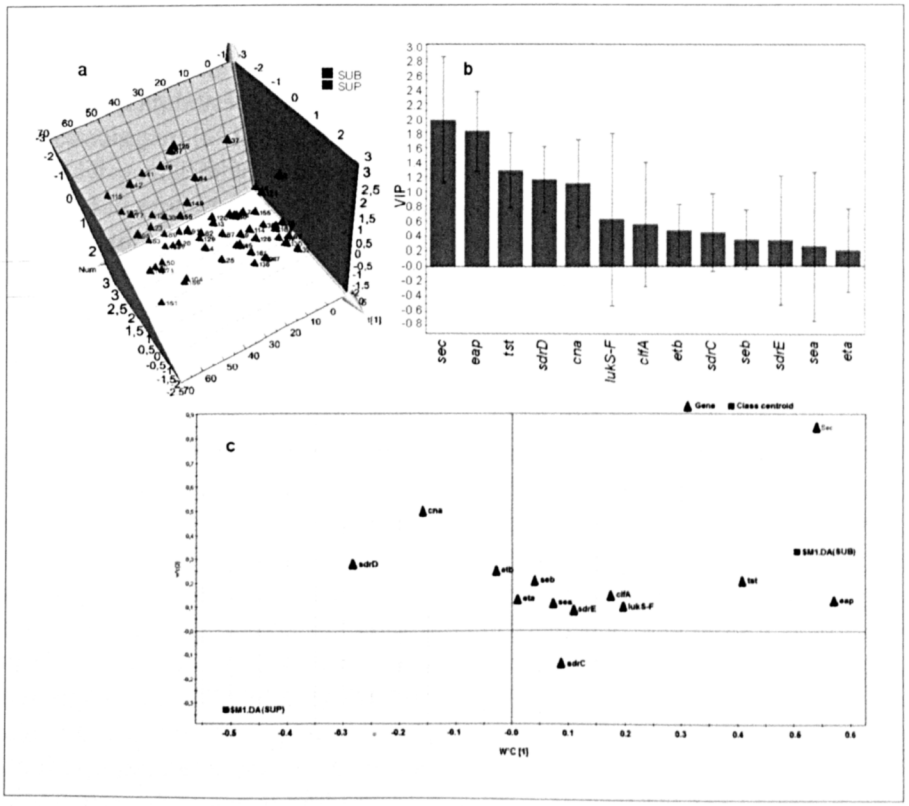

Fig. 5. *Summary of principal results of PLS-DA for comparison of the 62 S. aureus isolates from subgingival plaque of active sites (SUB) and supragingival plaque of non active sites (SUP), based on cumulative results of PCR genotyping.* ***a)*** *score-3D-plot representing distribution of the isolates according to presence of specific characters and correlation with the classification of isolates as SUP and SUB.* ***b)*** *bar plot representing values of variable importance (VIP) in classifying isolates in each of the two classes, calculated for each studied gene.* ***c)*** *loading plot representing the hierarchical classification of genes, according to their VIP value in discriminating between SUP and SUB isolates.*

hospital settings and in the community (6). Thanks to genomic plasticity this bacterium has gained the ability of being easily transmitted among humans (18) and to colonize several niches of the host (19, 20). In parallel it evolved and widely diffused drug resistant genotypes (21), that constitute a great menace to treatment options. Recent studies have shown that, under the pressure of hostile environments, characterized by the presence of drugs and high concentrations of inflammatory mediators, *S. aureus* has gained the capacity to induce genome mutations at high frequencies, thus enhancing its possibility to adapt to these environments (22-24).

Although the oral cavity is a well known site of *S. aureus* colonization (7, 8, 25), the significance of *S. aureus* oral carriage is still a matter of debate. Recent reports suggested that *S. aureus* is possibly involved also in the pathogenesis of periodontal lesions (26-28) although the existence of a correlation between disease progression and specific genetic characters was never investigated. Recently we demonstrated that the presence of periodontitis significantly influences *S. aureus* oral carriage rates (10). Moreover, we showed that *S. aureus* isolates from periodontitis affected patients are more toxigenic than isolates from healthy subjects. Although these observations were suggestive of a possible role of *S. aureus* in the pathogenesis of periodontal lesions, the experimental plan, limiting analyses to supragingival plaque, did not enable us to draw any conclusion on this matter. To investigate this aspect we consequently planned this study aiming to compare the prevalence of *S. aureus* in supragingival and subgingival plaque samples of patients affected by generalized aggressive periodontitis. Moreover, we intended to evaluate whether isolation of *S. aureus* from subgingival sites correlates with active progression of periodontal lesions and whether *S. aureus* isolates from subgingival sites had specific genetic/genomic traits.

Results confirmed that the prevalence of *S. aureus* in the supragingival plaque of periodontitis patients is high but, although associated with increased inflammation of colonized sites, it is not predictive of disease progression. This observation suggests that secreted factors of this microorganism could cooperate to amplify inflammation and tissue damage by diffusing into periodontal tissues and eventually favoring other pathogens. The present hypothesis is in accordance with our previous data (10) showing that *S. aureus* supragingival isolates from periodontitis patients show a significantly higher prevalence of genes encoding for superantigen toxins. In fact, also in this study, genes encoding for superantigen toxins and exfoliative toxins are possessed by about 90% of SUP isolates.

More interestingly, our data demonstrate that the isolation of *S. aureus* from subgingival plaque is strongly associated with disease progression. In fact, when both AS and non-AS are considered, 49 subgingival samples resulted *S. aureus* positive; of these 41 (83.7%) were AS and only 8 (16.3%) were non-AS ($P<0.01$). The existence of an association between the presence of *S. aureus* in the subgingival microbiota and activity of the lesion is further supported by the observation that *S. aureus* positive active sites show higher levels of inflammation and periodontal destruction as compared to *S. aureus* negative active sites. Overall these observations suggest that *S. aureus* can participate in the pathogenesis of periodontal lesions in different ways.

Supragingival colonization seems to depend upon characteristics of the host, which in about 37% of the cases is susceptible to it. In these cases *S. aureus* possibly colonizes the majority of supragingival sites and eventually cooperates to disease by secreted factors that make host defences dysfunctional.

According to our findings, a subset of *S. aureus* isolates is able to colonize subgingingival sites. This ability appears to be the consequence of specific surface characteristics; in fact, PLS-DA showed that SUB and SUP isolates are different in their armamentarium of adhesins. The majority of SUB isolates possess the Eap adhesin. Moreover, SUB isolates are characterized by the presence of two exotoxin encoding genes, *tst* and *sec*. These 3 genes have already been recognized as important virulence factors in a variety of severe staphylococcal infections (29) and are possibly involved in disregulating local immunity towards classic periodontal pathogens and amplifying tissue damage by specific molecular mechanisms.

According to analysis of DNA fingerprinting, obtained by PFGE of *Sma*I restrictions of chromosomal DNA, SUP and SUB isolates have distinct, although highly variable, genomic

structures. These data, together with the observation that a specific set of genes seems to be required to *S. aureus* to play a pathogenic role in aggressive periodontitis would suggest that these strains could be evolutionary related. On the other hand, analysis of genetic relatedness of isolates by MLST, indicated that the 62 studied isolates were poorly related and that, although some lineages were more frequent (i.e. ST30, ST45 and ST938), their distribution between SUP and SUB isolates was casual.

These data suggest that, the ability to colonize subgingival sites and cause damage is not the consequence of a process of selection of pre-existing strains, but instead the consequence of an adaptive process, as for other infections with a prolonged clinical duration (22-24).

Although the prevalence of cigarette smokers among the studied patients of both sexes were significantly higher than those reported in the same geographic area for the general population of comparable age (see report on smoking habit in Italy from the Istituto Superiore di Sanità, available online at http://www.iss.it/binary/fumo/cont/Rapporto_annuale_sul_fumo_anno_2010.pdf) (40.6% *vs* 21.7%, $P<0.01$), in accordance with data suggesting that smoke is a factor of risk for aggressive periodontitis (30), cigarette smoking was not correlated with a higher prevalence of *S. aureus*, indicating that cigarette smoking does not promote colonization of periodontal sites by *S. aureus*. Unavailability of specific records on habits and social/educational status of the studied patients limits the possibility to evaluate the possible role of these variables on the isolation of *S. aureus* from AS of aggressive periodontitis.

Further studies will be necessary to better elucidate the importance of specific virulence factors of *S. aureus*, and the influence of environmental, genetic and behavioural factors (28) in the role played by this microorganism in the pathogenesis of periodontal lesions and their influence on virulence mechanisms of other known periodontal pathogens.

S. aureus strains isolated during this study showed low prevalence of antibiotic resistant phenotypes, including the oxacillin resitant MRSA one. These data, including data of SCC*mec* typing of MRSA isolates, are in accordance with the communitary origin of these isolates (6, 17), and suggest that antibiotic therapy could efficiently contribute to eradication of subgingival *S. aureus*.

Overall, these data indicate that a significant number of patients affected by aggressive periodontitis are colonized both supragingivally and subgingivally by highly toxigenic strains of *S. aureus*. This situation is associated to a higher probability of disease progression and may constitute a risk for the general health of patients, making detection of *S. aureus* colonization and its eradication a desirable option in the clinical management of these patients, particularly in light of molecular evidence of genetic *in situ* evolution of *S. aureus* in the inflamed periodontal environment.

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