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ABSTRACT

Staphylococcus aureus rappresenta uno dei principali agenti patogeni opportunisti per l'uomo capace di vivere sia come commensale sulla cute e sulla mucosa nasofaringea di portatori sani sia di causare, grazie alla sua capacità di produrre diversi fattori di virulenza, un ampia varietà di infezioni tra cui la Dermatite Atopica e la Fibrosi Cistica. Tale microrganismo è, inoltre, un importante patogeno in ambito veterinario in quanto associato a diverse infezioni tra cui le Mastiti Bovine.

Lo scopo del nostro lavoro è stato quello di analizzare la capacità di produzione di biofilm e il contenuto di geni di virulenza nonché le possibili associazioni geniche di tre differenti gruppi di *S.aureus* isolati rispettivamente dall'espettorato di pazienti affetti da Fibrosi Cistica (FC) (gruppo clinico), da lesioni cutanee, fosse nasali e cute sana di pazienti con Dermatite Atopica (DA) (gruppo clinico) e dall'epitelio ghiandolare mammario di bovini affetti da Mastiti Bovine (MB) (gruppo animale), al fine di determinare le loro similitudini/differenze in termini di profili di antibiotico-resistenza, capacità di produzione di biofilm, distribuzione e combinazioni di determinanti di virulenza e valutare se i microrganismi possano essere legati allo sviluppo di specifiche infezioni nell'ospite.

I nostri risultati sui saggi di antibiotico-resistenza mostravano in generale una prevalenza di ceppi Meticillino-Sensibili (MSSA) all'interno dei tre gruppi di *S.aureus* analizzati, mentre solo il 7.8% e il 7.14% di isolati Meticillino-Resistenti (MRSA) era presente rispettivamente in FC e in MB. Nel gruppo di *S.aureus* proveniente dalla FC, *agr*-I e II erano ugualmente distribuiti (29 and 30%, rispettivamente), mentre sia in DA (42%) che nel gruppo animale (MB) (89%), appariva evidente una prevalenza di microrganismi appartenenti all'*agr*-type I. Dall'analisi dei saggi di produzione di biofilm, venivano riscontrati molti ceppi produttori in DA (88%) e in FC (80.73%), rispetto al gruppo animale (MB) (64.28%), tutti appartenenti prevalentemente all'agr-type I. Una differente distribuzione dei geni cap5/8 si evidenziava all'interno degli stessi agr-genotype, ma solo tra gli isolati clinici. L'intero campione in studio possedeva, inoltre, un gruppo di geni di virulenza definiti "core" (*icaA, atl, sdrC, clfA/B*, e *spa* tra i geni di adesione e α- γ- δ-hl_s tra le tossine) presenti in tutti i ceppi analizzati

e uno di geni “accessori, (s*dr*E, *cna*, e *fnb*A tra i geni di adesione e *sea*, *sec*, *sed*, *seq*, *sek*, *sej*, *eta*, *tst,* e *luk*S/F tra le tossine) evidenziati solo in alcuni ceppi, con diversa distribuzione tra i due gruppi clinici (FC e DA) e quello animale (MB). Studi di associazioni geniche, mostravano una specifica distribuzione di geni accessori all’interno dei diversi agr-type, nonché importanti associazioni tra alcuni determinanti di virulenza e l’antigene capsulare solo nei gruppi clinici. Si evidenziava, inoltre una prevalenza di geni di adesione rispetto alle tossine che apparivano inoltre fortemente associati tra loro stessi e con uno o più geni codificanti tossine come *sea* e *tst* in FC; *sea, sec, sej, sek, seq*, e *tst* in DA.

In conclusione, i nostri dati supportano l’ipotesi che uno specifico background genico del microrganismo può essere strettamente associato alla capacità di *S.aureus* di colonizzare specifici distretti nell’ospite, come i polmoni nei pazienti affetti da FC, la pelle in DA e le ghiandole mammarie bovine nel caso della MB, definendo ceppi “infezione-specifici” capaci, dunque, di indurre precise malattie nell’ospite.

ABSTRACT

Staphylococcus aureus is a commensal and pathogenic organism, adapted to survive in many niches and causing, by the production of a large array of virulence factors, a wide variety of human and animal infections such as Atopic Dermatitis (AD), Cystic Fibrosis (CF), and Bovine Mastitis (BM).

The aim of our study was to analyze the biofilm-formation capacity and the virulence-gene content of three different *S.aureus* groups, collected from sputum of Cystic Fibrosis patients (CF clinical group), lesions or skin of patients affected by Atopic Dermatitis (AD clinical group) and from mammary-gland epithelium of cows affected by Bovine Mastitis (BM animal group) to determine their similarities/differences in terms of antimicrobial-resistance profiles, biofilm production ability, virulence determinant distribution and combinations, and to evaluate if the genetic background of the microrganisms could to be linked to a specific type of infection.

Our results show that all samples from the three groups studied were mainly constituted by Methicillin-Susceptible *S.aureus* (MSSA) strains, whereas Methicillin-Resistant *S.aureus* isolates (MRSA), were found only in the CF clinical group and the BM animal group, in percentages of 7.8% and 7.14%, respectively. In the CF clinical group, agr-I and II were equally distributed (29 and 30%, respectively) whereas, a prevalence of microrganisms belonging to agr-I genotype, in the AD clinical group strains (42%) and in the BM animal group (89%) was evident. Biofilm production assays showed a higher number of biofilm producer strains, belonging mainly to agr-I, in the AD (88%) and CF (80.73%) clinical groups compared to the BM animal one (64.28%). A different distribution of *cap*5/8 was found among the same agr-genotypes, only in both AD and CF clinical groups. All *S.aureus* strains possessed a chromosomal “core” of virulence genes (*icaA, atl, sdrC, clfA/B,* and *spa* adhesion genes, and α-toxin, γ-toxin, and δ-toxin genes) and an “accessory” virulence group, present in variable quantity (s*dr*E, *cna*, and *fnb*A adhesion genes, and *sea*, *sec*, *sed*, *seq*, *sek*, *sej*, *eta*, *tst,* and *luk*S/F toxins genes), prevalent in a different manner within all three groups. Studies of gene associations evidenced a specific distribution of accessory virulence determinants both among the four different agr-genotypes, and two capsular antigen types in the AD and

CF clinical groups. Moreover, the analysis of the associations among accessory virulence genes showed a prevalence of adhesin genes, strongly associated among themselves, and with one or more accessory toxin genes i.e. *sea* and *tst* in the CF clinical group; *sea*, *sec*, *sej*, sek, *seq,* and *tst* in the AD clinical group.

In conclusion, our data support the hypothesis that specific virulence backgrounds are strongly related to the capacity of *S.aureus* to colonize specific host districts, such as CF patient lung, AD patient skin and bovine mammary gland, defining "infection-specific" strains, able to induce diverse diseases.

INTRODUCTION

Staphylococcus aureus is an extremely versatile human pathogen that typically lives as a commensal in the human nose in 30-70% of the human population. However, once it contaminates a breach in the skin or mucous membranes, it goes on to infect other body tissues, causing diseases ranging in severity from minor skin infections to life-threatening ones, such as osteomyelitis, endocarditis, pneumonia, and bacteremia.

S.aureus can also cause toxin-mediated diseases, such as toxic shock syndrome, staphylococcal scarlet fever, scalded-skin syndrome, and food poisoning (1). Thus, *S.aureus* can be isolated from different infection sites, such as, damaged skin and the anterior nares of Atopic Dermatitis (AD) patients. AD is a common inflammatory skin disease and it can follow a chronic course from infancy to adulthood (2). The pathogenesis of AD is multifactorial, and the mechanisms that contribute to this are the complex interactions among specific genes, environmental trigger factors and allergens, skin barrier dysfunction and abnormal systemic and local immune responses. Furthermore, the skin of AD patients exhibits a striking susceptibility to *S.aureus* colonization and infection that lead to a worsening of atopic eczema for superantigen exotoxin production such as SEA, SEB, SEC, and TSST-1 (3). Staphylococcal superantigens (SsAgs) may contribute to the persistence and exacerbation of allergic skin inflammation in AD patients because they mediate direct cross-linking of major histocompatibility complex class II (MHCII) molecules on antigen-presenting cells with T-cell receptors, inducing rapid production of interleukin-2 (IL-2) and tumor necrosis factor (TNF) and the subsequent expansion of a reactive T-cell population and keratinocytes. SsAgs also induce corticosteroid resistance of T cells contributing to difficulty in the management of AD because topical corticosteroids are the most common agents used for the treatment of this disease (4). *S.aureus* also colonizes the lung of cystic fibrosis (CF) patients, evoking a local intense host immune response, characterised above all by the presence of polymorph nuclear leukocytes. *S.aureus,* preferentially forming multicellular clusters within the viscous mucus, called biofilms, grows and persists in the airways of CF patients for months or even years despite appropriate anti-staphylococcal therapy. During chronic infection, pathogens will

experience changing selective pressures as they encounter new habitats and different co-infecting species and as they respond to medical interventions. In the short term, regulatory mechanisms allow the pathogen to quickly change its phenotype in response to the microenvironment; in the long term, instead, mutations or recombinations together with purging selection enforced by the changing environment, lead to shifts in the bacterial population. Adaptive strategies of *S.aureus* to the highly selective environment of the CF lung, are illustrated by the formation of biofilms, the occurrence of hypermutable strains, the down regulation of virulence genes and the manifestation of a heterogeneous bacterial population (5).

S.aureus is also one of the major veterinary pathogens associated with infections of animals, including ruminant mastitis, skeletal infections of chickens, dermatitis, and septicaemia of rabbits. In addition to their economic impact, animal strains of *S.aureus* pose a risk of human zoonoses and are therefore considered a potential threat to public health. Bovine mastitis (BM) caused by *S.aureus* is due to a combined effect of extracellular factors and toxin activity, together with the invasive properties of the strain such as adherence, biofilm formation, and resistant to phagocytosis. A slime layer, surrounding the majority of *S.aureus* strains causing mastitis, helps the microrganism in adherence and colonization of the bovine mammary gland epithelium. Mastitis is one of the most severe diseases in the dairy industry that impairs animal health and welfare, and is accompanied by decreased milk production, increased health care costs, higher culling rates and sometimes even death (6-8).

The adaptability and capacity of *S.aureus* to cause a wide variety of human and animal diseases and to occupy numerous niches cannot be explained by the action of a single virulence determinant, but it is likely that a number of factors act in combination during its infective process (9). This microrganism can, in fact, produce a large array of virulence factors, generally falling into two categories: secreted toxins that serve to either degrade host tissues or modulate the host immune system, and cell surface proteins involved in host cell adhesion, intracellular entry and immune system evasion (10). The wide-ranging pathogenic potential of different *S.aureus* strains is essentially due to the variation in the content of virulence and antibiotic resistance genes, encoded by accessory regions of the genome. These regions mostly consist of mobile

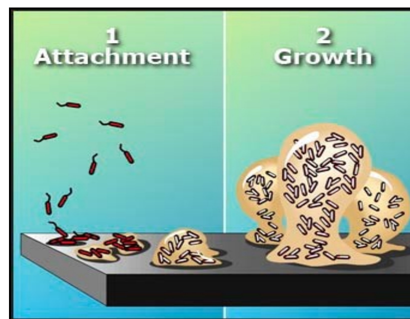
genetic elements (MGEs), such as plasmids, transposons, bacteriophages, pathogenicity islands, chromosomal cassettes, and genomic islands, which are frequently exchanged within and between lineages. Among the secreted virulence factors encoded by MGEs there are some superantigens that directly activate T-cells through by-passing normal antigen presentation and leading to a cytokine over-production, such as the toxic shock syndrome toxin-1 (TSST-1), and staphylococcal enterotoxin proteins (i.e. SEB, SEC, SEK, and SEL). These are carried in more than one staphylococcal pathogenicity island (SaPI) and are involved in severe human and animal diseases, such as, toxic shock syndrome toxinoses and bovine mastitis. SaPIs are 14–17 kb elements that are mobilized by bacteriophages and integrate at specific chromosomal locations defined by their integrase variants (1,11). Prophages have an important influence on *S.aureus* pathogenesis and niche adaptation as they often encode virulence loci that contribute to host/pathogen interactions. For example, phage-encoded SEA (enterotoxin A) can cause staphylococcal food poisoning (12), exfoliative toxin A (ETA), is responsible for the symptoms of localized scalded skin syndrome (SSS) (11,13), and the Panton-Valentine leukotoxin (PVL), a pore-forming toxin, is implicated in the pathogenesis of severe infections caused by Community-Acquired Methicillin-Resistant *S.aureus* (CA-MRSA) such as, furuncolosis, skin abscess formation, and necrotizing pneumonia; however, the exact role of this toxin in infection remains not totally clear and has produced contrasting data (14). Other SAgs are located on plasmids (*sed, sej*) (enterotoxin D, J) or on staphylococcal cassette chromosomes (SCCs) (*seh*) that also may contain antibiotic resistance genes such as *mec*A, encoding a modified penicillin binding protein (PBP2a), thus conferring resistance to methicillin and all β-lactam antibiotics. The presence, in the *S.aureus* genome, of the SCCmec elements has lead to the development of Methicillin-Resistant *S.aureus* strains (MRSA) associated to serious infections with difficult therapeutic treatment (11). In addition, the phenole-soluble modulin (PSMs) family that have a pro-inflammatory and cytolytic activity, are carried on the genomic island vSaγ (three families of genomic islands, named vSaα, vSaβ, and vSaγ, exist in the *S.aureus* genome and all are flanked by broken transposase genes) together with the *hla* (α-hemolysin) gene, capable of forming pores on the cell membrane, whereas virulence genes

encoding exoenzymes such as serine protease-like protein (*spl*), or leukotoxin D, and E (*lukD, lukE)* are located on the genomic island, vSaβ (11,15).

Besides the accessory region of the genome, constituted by MGEs, Lindsay *et al*. (16) have proposed two other categories of *S.aureus* genomic regions defined as the core genome and the core variable (CV) genome. The core genome is composed of genes conserved in all strains (c. 75%), including genes essential for growth and cell maintenance such as genes encoding molecules involved in metabolism, DNA and RNA synthesis, and replication. The CV genome is composed, instead, of genes predominantly conserved in strains sharing the same evolutionary history (c. 10%) such as numerous genes encoding extracellular virulence factors, such as protein A, which presents on microbial surfaces, inhibits antibody-mediated phagocytosis by blocking the Fc portion of IgG, and a large number of surface proteins known as "Microbial Surface Components Recognizing Adhesive Matrix Molecules" (MSCRAMMs) implicated in the host tissue adhesion and virulence regulatory proteins, involved in the modulation of colonization and infection processes (16). As many other invasive bacterial pathogens, *S.aureus* produces a capsular polysaccharide (CP), an extracellular virulence factor that enhances its resistance to clearance by innate host immune defences. Most clinical isolates of *S.aureus* are encapsulated and among the 11 serotypes of known CP, type 5 (CP5) and 8 (CAP8) seem to predominate (17). Among the MSCRAMMs, common adhesins are Atl (autolysin), implicated in primary attachment to surfaces, FnbA (fibronectin binding proteins A), SdrE, D (serine–aspartate repeats protein), ClfA, and B (clumping factors A, B) which mediate the attachment of bacterial cells to the host's extracellular matrix and contribute to platelet aggregation. Collagen binding protein (Cna) is also a MSCRAMM and is necessary for adherence of *S.aureus* to collagenous tissues and cartilage. Different *S.aureus* strains may have different constellations of MSCRAMMs and so may be predisposed to causing certain kinds of infections such as chronic diseases associated with the formation of communicating cell groups, known as biofilm (18-19).

A biofilm is a sessile microbial community in which cells are attached to abiotic or biotic surfaces and generally possess an altered growth and gene expression profile compared to planktonic (free-living) bacteria. Biofilm formation develops in two steps:

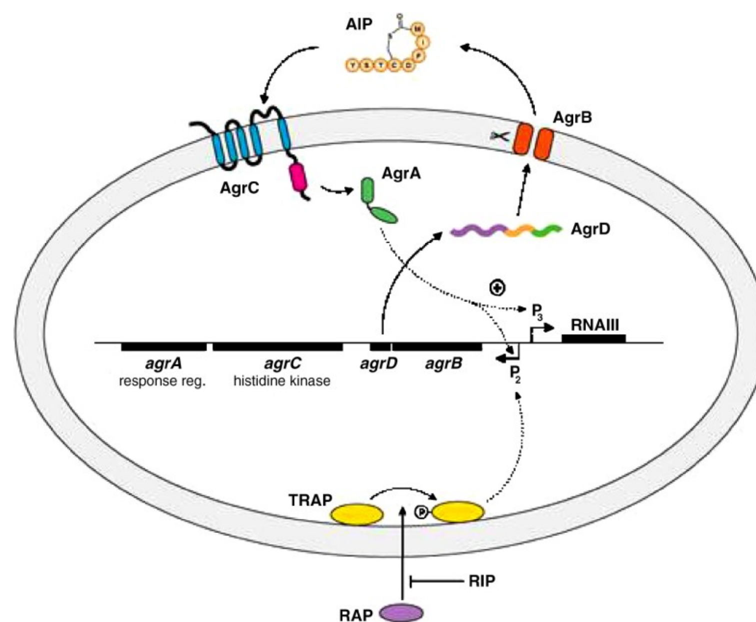
(1) a primary attachment onto polymeric surfaces mediated, in part, by cell-wall associated adhesins including MSCRAMMs; (2) cell-cell proliferation to form a multilayered biofilm mediated by production of extracellular factors such as the polysaccharide-intercellular-adhesin (PIA) produced by the co-operation of proteins encoded by an operon called *ica*-operon (inter-cellular-adhesin) (20).



Since one of the most notorious biofilm characteristics is their extraordinary resistance to antimicrobial killing, biofilm-associated infections, such as, endocarditis, osteomyelitis, implanted device–related infections, and even some skin infections, have special clinical relevance as they are generally resistant to antibiotic therapy and clearance by host defenses (21).

Furthermore, to cause so many human diseases and to colonize different ecological niches within the host, *S.aureus* has developed a quorum-sensing system that via cell-to-cell communication controls the regulation of a multitude of virulence factors including biofilm. The staphylococcal accessory gene regulator quorum-sensing system, known as *agr* (accessory-gene regulator), in a cell density-dependent manner and by a secreted auto-inducing peptide (AIP), decreases the expression of several cell-surface proteins and increases the expression of many secreted virulence factors in the transition from late-exponential growth to stationary phase *in vitro*. The *agr*-locus is composed of two divergently transcribed units, RNAII and RNAIII, the transcription of which is driven by the P2 and P3 promoters, respectively. The RNAII contains the four genes *agr*BDCA. The *agrB* and *agrD* gene products are engaged in the AIP production. AIP binds to a transmembrane protein, AgrC, which acts as the sensor kinase of the bacterial two-component regulatory system *agr.* Upon binding to the AIP, AgrC activates the response regulator, AgrA, a DNA-binding protein, which in turn induces

the transcription of RNAII and RNAIII, the latter is the effector molecule of the *agr*-locus, which acts as both a regulatory RNA and the messenger RNA for the *hld* gene, encoding the δ-toxin (22). The sequence of the AIP and its receptor AgrC, define four *S.aureus agr*-groups (I, II, III, IV). Distinct secreted AIPs, activate their specific receptors while inhibiting activation of AgrC in strains of other specificity groups. This intraspecies cross-inhibition is a form of bacterial interference that does not result in mutual growth inhibition but, rather, in the blocking of virulence gene expression that can condition the possibility of colonizing specific infection sites (skin, nasal mucosa, lung, urinary and genital mucosa) and contributes to the development of specific diseases (23). The reasons for this association between *agr* group and infection type are not yet clear, but a better understanding of this phenomenon may contribute to our understanding of the epidemiology of staphylococcal diseases.



Schematic diagram of quorum-sensing systems of *S. aureus*. The gene locus for the *agr* system is shown in *black* and contains two divergent transcripts, RNAII and RNAIII, driven by the P2 and P3 promoters, respectively (Matthew R *et al*.. Analytical and Bioanalytical Chemistry Vol. 387 Issue 2).

AIM OF THE STUDY

Our goal was to analyzed the antimicrobial-resistance profile, the biofilm-formation ability and the virulence-gene content of three different *S.aureus* groups, collected from sputum of Cystic Fibrosis (CF) patients (CF clinical group), lesions or skin of patients affected by Atopic Dermatitis (AD) (AD clinical group) and from mammary-gland epithelium of cows affected by Bovine Mastitis (BM) (BM animal group) in order to determine the antibiotypes and pathotypes of staphylococci isolated from different clinical syndromes (i.e antimicrobial-resistance, biofilm production, *agr*-membership, antigen capsular type, and virulence determinant distribution and combinations) to better understand the correlation between strains possessing a particular antibiotype and/or pathotype and the onset of a specific disease.

MATERIALS AND METHODS

Bacterial isolates

The samples were collected from three different sources: 109 isolates from sputum of CF patients (CF clinical group); 91 isolates from lesions or skin of patients affected by AD (AD clinical group); 56 isolates from BM (BM animal group).

All strains were grown on MSA (Mannitol–Salt–Agar, Oxoid, Basingstoke, UK), identified by coagulase test and the Api-Staph System (Bio-Merieux, Marnes-la-Coquette, France) and maintained at -80°C until used for successive experiments.

Antimicrobial susceptibility test

Susceptibility tests of the samples were performed by the disk diffusion method following the Clinical and Laboratory Standards Institute guidelines (CLSI) (24). The following drugs were tested: oxacillin, gentamicin, ciprofloxacin, levofloxacin, erythromycin, co-trimoxazole, clindamycin, rifampin, and chloramphenicol. The methicillin-susceptible *S.aureus* strain ATCC 29213 was used as a control for antimicrobial susceptibility tests.

agr-locus genotyping

agr-typing was performed by PCR amplification of the hyper-variable domain of the agr locus using oligonucleotide primers specific for each of the four major specificity groups as described by Shopsin B *et al.* (25).

Biofilm production

The biofilm-forming ability was tested by a spectrophotometric quantitative assay. Each strain was grown in Tryptone Soy broth (Oxoid), with the addition of 0.25% glucose (TSBG). These assays were performed in microtitre plates as described previously (26,27). Each reported value was the average of 12 measurements at 490nm (±SD). Values ≥0.12 were regarded as biofilm positive and values of >0.4 were considered strong producers, 0.4–0.2 were medium producers and <0.2 were

considered weak producers. These absorbance values were a species-related modification of previously published values (28).

Virulence gene content and capsular type

The virulence gene content, including adhesion genes (*spa*, *cna, fnb*A, *sdr*C *clf*A, *clf*B, *sdr*E, *atl,* and *ica*A) and toxin genes (*hla*, *hld*, *hlg*, *sea, sej, sec, sed, sek, seq, eta*, *luk*S/F, and *tst*), was determined by using both conventional PCR and multiplex PCR as previously published (29). Both methods were performed using primers designed on the deposited gene sequences shown in Table 1.

The Multiplex PCR comprised Mix1: *fnb*A, *ica*A, and *sdr*E; Mix2: *sej, and atl*; Mix3: *hlg*, *hla*, *hld*, *sea,* and *eta*; and Mix4: *cna*, *clf*A, and *clf*B. The identification of the capsular polysaccharide types 5 and 8 was performed with specifically designed primers (see tab.1) on the variable segments of the deposited gene sequences of the cap locus. Amplification mixes contained the following final concentrations: 10mM Tris-HCl pH 8.8, 1.5mM $MgCl_2$, 150mM KCl and 0.1% Triton X-100, 1U of Taq-polymerase (DyNAzymeTMII DNA polymerase, Finnzymes, Oy), 200mM each deoxynucleoside triphosphate and 100ng of genomic DNA.

PCR and Multiplex PCR conditions were an initial 5 min denaturation step at 94°C, followed by 30 cycles of 1 min at 93°C, 1 min at annealing temperature, and 1 min at 68°C, with a final annealing step of 10 min at 68°C. All PCR products were run on 1% agarose gels and stained with SYBR green (0.4 µg/mL).

Multi Locus Sequenced Typing

The sequences of the seven housekeeping genes used for MLST, corresponding to the allelic profile *arc*C-*aro*E-*glp-gmk-pta-tpi-yqi*L, were compared with the MLST database http://mlst.zoo.ox.ac.uk sequences (30). All STs described in the study were deposited on the MLST website and were compared with the major international *S.aureus* STs published.

Statistical analysis

Differences in virulence determinant distribution between groups were assessed by using the chi-square test. P values <0.05 were considered statistically significant.

RESULTS

Antimicrobial susceptibility

The antimicrobial susceptibility tests demonstrated that all three samples were constituted mainly of Methicillin-Susceptible *S.aureus* strains (MSSA), together with a broad susceptibility level to CIP, LEV, E, DA, CN, CL, RD, and SXT (data not shown). Methicillin-Resistant *S.aureus* strains (MRSA), only in the CF clinical group and the BM animal group, in percentages of 7.8% and 7.14%, respectively, were found (31).

Agr-groups and capsular antigen type determination

agr-group typing showed that, in the CF clinical group, agr-I and II were equally distributed (29 and 30%, respectively) whereas in the AD clinical group, a low prevalence of microrganisms belonged to agr-I (42%). A strong agr-I membership (89%) was found in the BM animal group. Capsular antigen determination analyses evidenced that, c*ap*8 prevailed on *cap*5 in the CF clinical group (56%) and with a high distribution (79%) in the AD clinical group whereas, an equal *cap*8/5 distribution was evident in the BM animal group (Tab.2).

Virulence gene content

The conventional PCR and multiplex PCR for virulence gene content determination revealed the presence of a common core of virulence genes, corresponding to those for adhesion, i.e. *ica*A, *atl*, *sdr*C, *clf*A/B, and *spa*, and to those for toxigenicity *hl*-s genes (α-toxin, γ-toxin, and δ-toxin) in more than 90% of *S.aureus*, indipendently from their origin, and a group of accessory virulence determinants, including adhesins and toxins, present in variable quantities in all *S.aureus* groups studied.

Our data highlight a significantly different distribution in the accessory adhesion genes (*fnb*A, *sdr*E, and *cna*) among the studied groups. In particular, all the BM animal group isolates presented *fnb*A, whereas in AD and CF clinical groups, this was detected in percentages of 79.12 and 46.78%, respectively ($p<0.001$). *sdr*E was found in 62.63% and 60.7% of isolates in the AD clinical and BM animal groups respectively, whereas, in the CF clinical group it was presented only in 39.45% ($p<0.05$). *cna* was detected in

53.84% of the AD clinical group, 40.36% of the CF clinical group and 3.57% of the BM animal group ($p<0.001$) (Tab.3).

The analysis of the accessory toxin gene distribution showed that, in *S.aureus* strains isolated from CF patients, *sea* (19.3%) and *tst* (18.3%) genes were more abundant than *sej* (8.25%), *eta* and *luK*S/F (both 2.75%) ($p<0.001$). In *S.aureus* belonging to AD patients, the *tst* gene was found in 59% of the sample, *sek* and *seq* genes in 50%, *sea*, *sec*, and *sej* in a percentage ranging from 20-24%, *sed* in 11% and *eta* and *luK*S/F in 4.4% of the isolates. Furthermore, MLST analyses demonstrated that, strains harbouring *luk*S/F, *tst*, *se*q, and *se*k enterotoxin genes were prevalently of ST5, strains harbouring *sea*, *se*q, *se*k, *sej*, *sed*, *tst,* and *luk*S/F were prevalently of ST45, whereas isolates without *luk*S/F but with enterotoxins (*sea*, *sec*, and *seq*), *tst*, and *eta* were of ST109 (data not shown).

In the BM animal group, our data reveal that *sej* was widely distributed in 54% of the isolates, *luk*S/F in 39.3%, *sed* and *tst* genes in 25 and 21.4% respectively, *sec*, *seK,* and *seq* were present in 14.3 and 7.14% respectively, whereas *sea,* and *eta* were never detected (Tab.3).

Biofilm production

The assays of biofilm production showed that, a higher number of biofilm producer strains were present in the AD (88%) and CF (80.73%) clinical groups with respect to the BM animal group (64.28%) (Fig.1A). In addition, both the CF and AD clinical groups isolates exhibited a higher number of medium biofilm producers (mean of OD_{490} values=0.4) than the BM animal group strains (mean of OD_{490} values=0.2) (Fig.1B). Moreover, in all *S.aureus* groups studied, most biofilm producer strains were members of the agr-I group. Specifically, a significant percentage (89%) both in the CF clinical and BM animal groups was observed with respect to the AD clinical group (42%). Finally, some biofilm producer strains of different agr-genotypes were less present than agr-I (Fig.1A).

Virulence gene association

The study of the virulence gene association revealed a different distributions of *cap*5 and *cap*8 genes among the same agr-genotypes, in both CF and AD clinical groups. In fact, *cap*5 appeared to be more abundant in the CF clinical and BM animal agr-I isolates (68.7 and 56%, respectively) than *cap*8, that was predominant, instead, in the AD agr-I (82%). As concerns the agr-II group, in the CF clinical group no capsular antigen gene was strongly prevalent (51.5% for *cap*8 and 48.4% for *cap*5), whereas in the AD agr-II, *cap*5 isolates prevailed (63.1%). In the entire sample, both clinical (CF and AD groups) and animal (BM group), agr-III and agr-IV, when present, were constituted mainly of *cap*8 isolates (78.26-100%).

The study of the associations between agr-genotypes and virulence determinant content showed, in the CF and AD clinical groups, a specific distribution of accessory virulence genes among the four different agr-genotypes. In particular, *sej* and *sdr*E were associated with agr-II (although a *sej* marked prevalence between AD agr-I and II was not found); *sea* and *tst* with agr-III (even if a prevalence of *sea* in the agr-I or III in the CF clinical group and *tst* in agr-III and IV in the AD clinical group was not shown); *cna*, *eta* and *luk*S/F with agr-IV (Tab.4).

Furthermore, an association between capsular antigens and the distribution of virulence genes in both CF and AD clinical groups, was found. In fact, *cap*8 was associated with *sea*, *cna* and *eta* genes, whereas *cap*5 with *sej* and *sdr*E. No correlation was found between *cap*8/5 and virulence determinants in the BM animal group (data not shown).

The analyse of the content and the association of accessory virulence genes in the all three groups studied also highlights a prevalence of adhesin genes (*cna*, *fnb*A and *sdr*E), consequently, strongly associated with each other, as well as with one or more accessory toxin genes i.e. *sea* and *tst* in CF isolates; *sea*, *sec*, *sej*, sek, *seq* and *tst* in the AD strains.

In particular, 62.3% of the CF *S.aureus* isolates contained associations of accessory virulence determinants constituted by the association of only two genes in 45% of the strains, and of three or four virulence determinants in 13.7 and 3.6%, respectively. It is important to note that *sea* and *tst* were present in only six isolates (9%).

On the contrary, in the AD clinical *S.aureus* group, 90.1% of the sample presented gene associations. In particular, associations of two or three virulence genes were found in 17% of strains, 14.6 % of isolates presented associations of four virulence determinants, and 12.2 % of five virulence genes. Six virulence gene associations were present in 19.5% of this sample, seven-eight associated genes in 6.1% and nine gene associations in 7.3%. Furthermore, a strong association among *seK-seq* and *tst* in 27 isolates (32.9%), *sea* with *tst* in 16 isolates (19.5%), *sej* with *sek-seq* in 16 isolates (19.5%), and *sea* and *sek*/q in 15 isolates (18.3%) was found. All the toxin genes were associated in combinations ranging from 3 to 6 but obviously in a lower percentage than the associations of two genes, as shown in Tab.5.

In the BM animal group, all isolates (100%) presented gene associations, in particular 25% of the sample showed associations between two genes, 32.14% between three genes, 28.6 % between four genes, 10.7% between five genes and 3.6% between six genes but no toxin gene combination was relevantly found (Tab. 5).

DISCUSSION

The antibiotic-resistance profiles, biofilm production ability, agr-membership, antigen capsular type and virulence gene distribution and combinations, represent the aspects of *S.aureus* that, as is well known, acting in variable combination during the infective process, define the pathogenic potential of the microrganism. In particular, agr-membership, as proposed by Wright JS et al. (32), defines the biology of the microrganism and consequently, conditions the possibility to colonize specific infection sites (skin, nasal mucosa, lung, urinary, and genital mucosa) by a mechanism of microbial cross-inhibition. Antibiotic-resistance facilitates the survival of the microrganism in the presence of antimicrobial molecules, biofilm lifestyle confers protection from the host immune system and antimicrobial agents, which may not eradicate it (33), the antigen capsular type plays an important role in bacterial evasion of host immune surveillance, thereby conferring virulence to the pathogen, whereas the abundant presence, and in variable combinations, of specific virulence genes can represent the basis of bacterial pathogenicity (34).

The presence, in the all three groups studied, of a small quantity of MRSA strains, and more in general, of isolates with a broad susceptibility level to all antimicrobial tested, shows that methicillin-resistance and/or antibiotic-resistance is not involved in the selection of specific *S.aureus* strains.

Moreover, our data clearly indicate that CF *S.aureus* is a heterogeneous clinical group of isolates that do not have a prevalent agr-grouping and antigen capsular type (CP), leading us to consider that these two characteristics have no impact on the colonization dynamics of *S.aureus* in CF patients, as previously observed by other authors (5).

CF clinical group also presents a high number of biofilm producer strains (80.73%) with a good capacity to form biofilm (mean OD_{490} values = 0.4) and are members mainly of the agrI-genotype (89%). The capacity to form a thick biofilm is correlated to the presence in the *S.aureus* genome of numerous tissue adhesion genes such as *ic*a-operon genes (responsible for PIA production), and *atl*, as well as adhesive core genes (*spa*, *clf*A/B, and *sdr*C) and accessory adhesin genes (*sdr*E, *cna* and *fnb*A).

Moreover, the accessory toxin genes were detected in a lower quantity than adhesion genes in CF *S.aureus* clinical isolates, showing also, a poor distribution in this sample. A low presence of strains with associated toxin accessory determinants (62.3%) in combinations of not more than two superantigen toxin genes, was found. Moreover, PVL-leucocidin, a phage gene, was detected only in 2.75% of the strains studied. The abundance of MSCRAMM encoding genes with different functions, such as, receptors in the human host, surface proteins, polysaccharide intercellular adhesin and capsular polysaccharides, confer strong adhesive properties to the sample, strongly supporting the hypothesis that biofilm is the major virulence factor responsible for the persistence of lung chronic infection of CF patients, involving microrganisms that are difficult to treat by conventional antimicrobial therapy.

AD clinical *S.aureus* are a more homogenous group of isolates among which agr-I genotype (42%) and the CP8 type (79%) predominate. Thus, it would seem that CP8 can confer more protection from the host immune system in AD patients. Moreover, a great number of biofilm producer strains was found (88%), belonging to agr-I (42%), with medium ability to form a thick biofilm ($O.D._{490}$=0.4), and all harbouring the *ic*a-operon and the *atl* gene. In the AD clinical group, a diffuse presence of adhesin was detected, both core adhesive genes (*spa*, *clf*A/B, and *sdr*C) and accessory adhesin determinants (*sdr*E, *cna* and *fnb*A) showing good tissue adhesive properties.

The AD clinical group further possess an enhanced toxigenicity attributable to a very high abundance of different accessory toxin genes, encoding superantigens, i.e. *sek, seq* and *tst* found in at least 50% of the sample or *sea, sec, sed* and *sej* in 11-24.2%. Moreover, a very high percentage of strains with associated toxin accessory genes (90.1%) was found as well as the presence of strains harbouring combinations of superantigen toxin genes ranging from 2 to 9 i.e. *sek, seq* and *tst* in 33% of the sample*;* *sea, sek* and *seq* in 18.3%, *sea* and *tst* in 19.5%. *PVL*-leucocidin was also detected, in this case, in a low percentage of the sample (4.4%). Thus, the abundance of superantigen encoding genes in the AD clinical group together with its well-known strong biofilm production ability (2)*,* acting in concert, represent two characteristics closely linked to a their ability to cause persistent, chronic, and severe infections in which superantigen toxins can serve as allergens that stimulate a massive specific IgE

response in AD patients. Furthermore, the skin of AD patients can represent a source of potentially highly virulent *S.aureus* that can spread in the community and from this source to hospital settings.

Due to all the characteristics described above, AD strains are almost community-acquired strains. Our results demonstrate that strains harbouring *luk*S/F, *tst*, *seq* and *sek* are prevalently of ST5 and those harbouring *sea*, *seq*, *sek*, *sej*, *sed*, *tst* and *luk*S/F are prevalently of ST45, whereas isolates without *luk*S/F but with enterotoxins (*sea*, *sec*, *seq*) *tst* and *eta* are of ST109. This is in agreement with results obtained by Yeung M et al. (35).

The BM animal group is a more uniform group of strains, in which agr-I was predominant (89%), in agreement with other authors (36,37) but none observed a prevalence of a specific capsular antigen type. With respect to the CF and AD clinical groups, the BM animal one has few biofilm producers (64.3%), with a weak ability to form it ($O.D._{490}$= 0.2), in agreement with Vasudevan P *et al*. (38), Crampton *et al.* (39). Moreover, all strains posses the ica-operon, *atl*, *fnbA* and a high percentage of *sdrE*, but only few strains (3.57%) present collagenase (*cna*). This could explain the low percentage of biofilm producers and their weak production, considering that *cna* has been associated, by different studies, with biofilm or slime production on prostheses, in arthritis, and in nasal carriers (18).

Our BM *S.aureus* are more toxigenic than biofilm producers, presenting an important content of enterotoxin genes i.e. *sej, sed* and *sec* in 54, 25 and 14.3% of the sample, respectively, in agreement with other authors (40). 21.4% of the isolates harboured the *tst* gene, and the *lukS/F* gene was found in a high percentage of isolates (39.3%), in agreement with other data (41). It is important, moreover, that enterotoxin A (*sea*), a typical toxin of human *S.aureus*, and exfoliatin A (*eta*) were never detected. Our results emphasize that toxigenicity is the prevalent aspect of the virulence of animal isolates and the large diffusion in BM animal group of several toxin genes i.e. enterotoxins, *tst* and *PVL*, suggests that these infections could represent a risk of milk contamination with thermostable enterotoxins.

In this study, a possible important association between agr-group, capsular type and virulence genes in all *S.aureus* groups was investigated. In particular, these

associations were only detected in FC and AD clinical strains where, *sej* and *sdrE* were associated with agr-II; *sea* and *tst* with agr-III; *cna, eta* and *lukS/F* in agr-IV. Antigen capsular type 8 was also associated with *sea, cna* and *eta*, whereas *cap5* was associated with *sej* and *sdrE*. Frequent and "multiple" associations of toxin genes were found in the AD clinical group, in which superantigen enterotoxin genes (*sea*, *sec*, *sed*, *sej*,*seq*, *sek*) were associated among themselves and with *tst*, *eta* and *luk*S/F in addition to adhesin genes. On the contrary, the frequency of isolates harbouring plasmid related genes i.e. *sed* and *sej*, confirmed that these two genes cannot always be identified together, as previously published (40,42,43). These strains can represent a vast "reservoir" of virulent determinants harboured within MGEs that, by horizontal gene transfer, probably under microbial competition or antimicrobial selective pressure, can promote dissemination of genes encoding both virulence factors and antibiotic resistance molecules playing a key role in bacterial adaptability and survival (11). Moreover, a large presence of strains harbouring superantigen genes, often also associated with serious staphylococcal diseases (including TSS, purpura fulminans and necrotizing pneumonia) contribute to the insurgence of always more serious community and hospital staphylococcal disease.

Conclusion

Our data support the hypothesis that the specific virulence backgrounds of the studied *S.aureus* are strongly related to the capacity of this microrganism to colonize specific host districts, defining "infection-specific" strains, able to induce specific and diverse diseases.

REFERENCES

[1] Lindsay J.A. and. Holden M. *Staphylococcus aureus*: superbug, super genome?. Rev. Trends in Microbiology. Vol.12 No.8 August 2004.

[2] Kim D.W., Park K.D., Kim T.H., Lee S.J, Kim J. Are there predominant strains and toxins of Staphylococcus aureus in atopic dermatitis patients? Genotypic characterization and toxin determination of *S. aureus* isolated in adolescent and adult patients with atopic dermatitis. J Dermatol. 2009, 36(2):75-81.

[3] Lin Y.T., Wang C.T., Chiang B.L. Role of Bacterial Pathogens in Atopic Dermatitis. Clinic Rev Allerg Immunol 2007, 33:167-177

[4] Iwatsuki K., Yamasaki O., Morizane S., Oono T. Staphylococcal cutaneous infections: Invasion, evasion and aggression. J of Dermatol Sc 2006, 42 203-214.

[5] Goerke C., Wolz C. Adaptation of *Staphylococcus aureus* to the cystic fibrosis lung.. Inter J of Med Microb 2010, 300 520-525. Mini Rev.

[6] Bergonier D., de Crémoux R., Rupp R., Lagriffoul G., Berthelot X. Mastitis of dairy small ruminants. Vet Res 2003, 34(5): 689-716. Review.

[7] McCullagh J.J., McNamee P.T., Smyth J.A., Ball H.J. The use of pulsed field gel electrophoresis to investigate the epidemiology of *Staphylococcus aureus* infection in commercial broiler flocks. Vet Microbiol 1998, 63(2-4): 275-81.

[8] Hermans K., Devriese L.A., Haesebrouck F. Rabbit staphylococcosis: difficult solutions for serious problems. Vet Microbiol 2003, 91(1): 57-64.

[9] Peacock S.J., Moore C.E., Justice A., Kantzanou M., Story L., Mackie K., O'Neill G., and P. J. Day N. Virulent Combinations of Adhesin and Toxin Genes in Natural Populations of *Staphylococcus aureus*. Infect and Imm 2002, p. 4987-4996. Vol 70, No. 9.

[10] Benton B.M., Zhang J.P., Bond S., Pope C., Christian T., Lee L.,. Winterberg K.M,. Schmid M. B., and. Buysse J.M. Large-Scale Identification of Genes Required for Full Virulence of *Staphylococcus aureus.* Journ of Bacter 2004, p. 8478–8489 Vol. 186, No.24.

[11] Malachowa N. and Deleo F.R. Mobile genetic elements of *Staphylococcus aureus*. Cell Mol Life Sc 2010, 67:3057-3071. Review.

[12] Betley M.J. and Mekalanos J.J. Staphylococcal enterotoxin A is encoded by phage. Science 1985, 229(4709) 185-7.

[13] Yamasaki et al. Clinical manifestations of staphylococcal scalded-skin syndrome depend on serotypes of exfoliative toxins. J Clin Microb 2005, 43(3) 1890-3

[14] Lina G., Piemont Y., Gamot F.G., Bes M., Etienne J. Involvement of Panton-Valentine Leukocidin-Producing *Staphylococcus aureus* in Primary Skin Infection and Pneumonia. Clin Infect Dis 1999, 29:1128-32.

[15] Lindsay J.A. Genomic variation and evolution of *Staphylococcus aureus*. Int J of Med Micr 2009.

[16] Lindsay J.A., Moore C.E, Day N.P., Peacock S.J., Withey A.A. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. J Bacter 2006, 188(2): 669-67.

[17] LuongT.T and Lee C. Overproduction of Type 8 Capsular polysacharide Augments *Staphylococcus aureus* Virulence. Inf and Imm 2002, p. 3389-3395 Vol.70, No.7.

[18] Switalski, L., Patti, J., Butcher, W., Gristina, A., Speziale, P. A collagen receptor on *Staphylococcus aureus* strains isolated from patients with septic arthritis mediates adhesion to cartilage. Mol. Microbiol. 1993, 7 99–107.

[19] Feng Y., Chen C.J., Su L.H., Hu S., Yu S., Chiu C.H. Evolution and pathogenesis of Staphylococcus aureus: lessons learned from genotyping and comparative genomics. FEMS 2007. Review.

[20] Gotz F. *Staphylococcus* and biofilms. Mol Microb 2002, 43:1367-1378.

[21] del Pozo JL, Patel R**.** The challenge of treating biofilm-associated bacterial infections. Clin Pharmacol Ther 2007 Aug;82(2):204-9.

[22] Kong K.F., Vuong C., Otto M. Staphylococcus quorum sensing in biofilm formation and infection. Int Journ of Med Microb 296(2006) 133-139. Review.

[23] Goerke C., Kummel M., Dietz K., Woltz C. Evaluation of Intraspecies Interference Due to *agr* Polymorphism in *Staphylococcus aureus* during Infection and Colonization. Jour of Infect Dis 2003; 188:250-6.

[24] Clinical and Laboratory Standards. Performance standards for antimicrobial susceptibility testing. Nineteenth informational supplement. Document M100-S19. Wayne PA: Clinical and Laboratory Standard Institute; 2010.

[25] Shopsin B., Mathema B., Alcabes P., Said-Salim B., Lina G., Matsuka A., Martinez J., Kreiswirth B.N. Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. J Clin Microbiol 2003; 41(1): 456-9.

[26] Christensen G.D., Simpson W.A., YoungerJ.J., Baddour L.M., Barrett F.F., Melton D.M. and Beachey E.H. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985; 22: 996-1006.

[27] Ziebuhr W., Krimmer V., Rachid S., Löbner I., Götz F., and Hacker J. A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis*: evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element IS*256*. Mol Microbiol 1999; 2**:** 345-356.

[28] Cafiso V., Bertuccio T., Santagati M., Campanile F., Amicosante G., Perilli M.G., Selan L., Artini M., Nicoletti G., Stefani S. Presence of the ica operon in clinical isolates of *Staphylococcus epidermidis* and its role in biofilm production. Clin Microbiol Infect 2004; 10(12): 1081-8.

[29] Stefani S., Bongiorno D., Cafiso V., Campanile F., Crapis M., Cristini F., Sartor A., Scarparo C., Spina D., Viale P. Pathotype and susceptibility profile of a community-acquired methicillin-resistant *Staphylococcus aureus* strain responsible for a case of severe pneumonia. Diagn Microbiol Infect Dis 2009; 63(1): 100-4.

[30] Cassone M., Campanile F., Pantosti A., Venditti M., Stefani S. Identification of a variant "Rome clone" of methicillin-resistant *Staphylococcus aureus* with

decreased susceptibility to vancomycin, responsible for an outbreak in an intensive care unit. Microb Drug Resist 2004; 10: 43–9.

[31] Cafiso V., Bertuccio T., Spina D., Campanile F., Bongiorno D., Santagati M., Sciacca A., Sciuto C., Stefani S. Methicillin resistance and vancomycin heteroresistance in *Staphylococcus aureus* in cystic fibrosis patients. Eur J Clin Microbiol Infect Dis 2010; 29(10): 1277-85.

[32] Wright J.S. 3rd, Traber K.E., Corrigan R., Benson S.A., Musser J.M., Novick R.P. The agr radiation: an early event in the evolution of staphylococci. J Bacteriol 2005; 187(16): 5585-94.

[33] Donlan R.M. and Costerton J.W. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clinical Microb Reviews 2002; 15(2): 167-193.

[34] Strauss E.J., Falkow S. Microbial pathogenesis: genomics and beyond. Science 1997; 276(5313): 707-12.

[35] Yeung M., Balma-Mena A., Shear N., Simor A., Pope E., Walsh S., McGavin M.J. Identification of major clonal complexes and toxin producing strains among *Staphylococcus aureus* associated with atopic dermatitis. Microbes Infect 2011; 13(2): 189-97.

[36] Buzzola F.R., Alvarez L.P., Tuchscherr L.P., Barbagelata M.S., Lattar S.M., Calvinho L., Sordelli D.O. Differential abilities of capsulated and non-capsulated *Staphylococcus aureus* isolates from diverse agr groups to invade mammary epithelial cells. Infect Immun 2007; 75(2) :886-91.

[37] Vautor E., Abadie G., Pont A., Thiery R. Evaluation of the presence of the bap gene in *Staphylococcus aureus* isolates recovered from human and animals species. Vet Microbiol 2008; 127(3-4) :407-11.

[38] Vasudevan P., Nair M.K., Annamalai T., Venkitanarayanan K.S. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet Microbiol 2003; 92(1-2): 179-85.

[39] Cramton S.E., Gerke C., Schnell N.F., Nichols W.W., Götz F. The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect Immun 1999; 67(10): 5427-33.

[40] Zecconi A., Cesaris L., Liandris E., Daprà V., Piccinini R. Role of several *Staphylococcus aureus* virulence factors on the inflammatory response in bovine mammary gland. Microb Pathog 2006; 40(4): 177-83.

[41] Rainard P., Corrales J.C., Barrio M.B., Cochard T., Poutrel B. Leucotoxic activities of *Staphylococcus aureus* strains isolated from cows, ewes, and goats with mastitis: importance of LukM/LukF'-PV leukotoxin. Clin Diagn Lab Immunol 2003; 10(2): 272-7.

[42] Zhang S., Iandolo J.J., Stewart G.C. The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (sej). FEMS Microb Lett 1998; 168(2): 227-33.

[43] Zschöck M., Kloppert B., Wolter W., Hamann H.P., Lämmler C.H. Pattern of enterotoxin genes *seg*, *seh*, *sei* and *sej* positive *Staphylococcus aureus* isolated from bovine mastitis. Vet Microbiol 2005; 108(3-4): 243-9.

TAB. 1 PRIMERS AND THEIR SEQUENCES USED IN THE STUDY

Primer	Sequence	Length (bp)	Annealing T°	Concentration (µM)	Control strains	Reference
Multiplex PCR						
eta up	5′-ACTGTAGGAGCTAGTGCATTTGT-3′	190	55	0.4	ATCC BAA-44	Jarraud et *al.* (2002)
eta dw	5′-TGGATACTT TTGCTATCTTTTTCATCAAC-3′			0.4		
hld up	5′-CTG AGT CCA AGG AAA CTA ACT CTA C-3′	220	55	1.2	NCTC 8325	Stefani et *al.* (2009)
hld dw	5′-TGA TTT CAA TGG CAC AAG AT-3′			1.2		
hlg up	5′-GCC AAT CCG TTA TTA GAA AAT GC-3′	938	55	0.4	NCTC 8325	Lina et *al.* (1999)
hlg dw	5′-CCA TAG ATG TAG CAA CGG AT-3′			0.4		
hla up	5′-AGG TTC CAT ATT GAT GAA TCC TG-3′	737	55	0.8	NCTC 8325	Stefani et *al.* (2009)
hla dw	5′-CCA TAG TAA TAA CTG TAG CGA AGT CTG-3′			0.8		
sea up	5′-GAA AAA AGT CTG AAT TGC AGG GAA CA-3′	560	55	0.8	FRI 913	Jarraud et al. (2002)
sea dw	5′-CAA ATA AAT CGT AAT TAA CCG AAG GTT C-3′			0.8		
cna dw	5′-GGT AAG GAA GAA GTG AAC GGG-3′	508	55	0.4	MW2	Stefani et *al.* (2009
cna up	5′-TCT CTG CTT TGT CTA CTG GTG TTG-3′			0.4		
sdrE up	5′-CAG TAA ATG TGT CAA AAG A-3′	767	50	0.8	COL	Peacock et *al.* (2002)
sdrE dw	5′-TTG ACT ACC AGC TAT ATC-3′			0.8		
icaA up	5′-CAT TGA ACA AGA AGC CTG ACA-3′	1060	50	0.4	NCTC 8325	Stefani et *al.* (2009)
icaA dw	5′-ATG CGA CAA GAA CTA CTG CTG C-3′			0.4		
atl up	5′-TAT TTG AGC AAC TTG GTT TAA TGT C-3′	470	50	1.2	NCTC 8325	Stefani et *al.* (2009)
atl dw	5′-CGG CTT ATC AAT GGT TCC TTG-3′			1.2		
fnbA up	5′-CAC AAC CAG CAA ATA TAG-3′	1362	50	0.8	NCTC 8325	Peacock et *al.* (2002)
fnbA dw	5′-CTG TGT GGT AAT CAA TGT C-3′			0.8		
clfA up	5′-CGA CAC AAT CAT CAT CAA CAA ATG-3′	476	55	1.2	NCTC 8325	Stefani et *al.* (2009)
clfA dw	5′-ATA ACC TGC TTG GTG CGG ATA-3′			1.2		
clfB up	5′-GCA ATA CCA CTA CAA CAG AGC CAG-3′	317	55	1.2	NCTC 8325	Stefani et *al.* (2009)
clfB dw	5′-AGC ATC AGC AGC ATT TAC TAC GC-3′			1.2		
sej up	5′-CTC CCT GAC GTT AAC ACT ACT AAT AA-3′	641	50	0.4	NRS 156	Becker et *al.* (2003)
sej dw	5′-TTG TCT GGA TAT TGA CCT ATA ACA TT-3′			0.4		
Conventional PCR						
spa up	5′-GGTACATTACTTATATCTGGTGGCG-3′	1142	56	0.4	NCTC 8325	This work
spa dw	5′-TTCTTATCAACAACAAGTTCTTGACC-3′			0.4		
lukS up	5′-ATC ATTAGGTAAAATGTCTGGACATGATCCA-3′	433	50	0.4	ATCC 49775	Lina et *al.* (1999)
lukF dw	5′-GCA TCA ACT GTA TTG GAT AGA AAA GC-3′			0.4		
tst up	5′-GCC CTT TGT TGC TTG CGA C-3′	548	50	0.4	NRS 111	Stefani et *al.* (2009)
tst dw	5′-TTT CCA ATA ACC ACC CGT TT-3′			0.4		
cap5 up	5′-ATG ACG ATG AGG ATA GCG ATA-3′	838	50	0.4	NCTC 8325	Stefani et *al.* (2009)
cap5 dw	5′-TTC TCG GAT AAC ACC TGT TGC-3′			0.4		
cap8 up	5′-GCA ATA CCA CTA CAA CAG AGC CAG-3′	1100	50	0.4	NRS 103	Stefani et *al.* (2009)
cap8 dw	5′-TTC CAA TTA CAT CAT TTC TAT AAG C-3′			0.4		
sdrC up	5′-CGAACATTGATATTGCGATTGAT-3′	187	55	0.4	NRS 1	Stefani et *al.* (2009)
sdrC dw	5′-GGTAATCTTACAGAACCTGGACGG-3′			0.4		
sec up	5′-CTCAAGAACTAGACATAAAAGCTAGG-3′	271	55	0.4	NRS 1	Stefani et *al.* (2009)
sec dw	5′-TCAAAATCGGATTAACATTATCC-3′			0.4		
sed up	5′-CTAGTTTGGTAATATCTCCTTTAAACG-3′	319	55	0.4	NRS 1	Stefani et *al.* (2009)
sed dw	5′-TTAATGCTATATCTTATAGGGTAAACATC-3′			0.4		
sek/q up	5′-TTAAGTGTCTCAAATAGTGCCAGCG -3′	449	55	0.4	USA300	This work
sek/q dw	5′-ATAGTGTTTTGCTTACCATTGACCC-3′			0.4		

TAB.2 PERCENTAGE OF *AGR*-TYPE AND CAPSULAR ANTIGEN IN THE *S.AUREUS* GROUPS STUDIED

	AGR-GROUP DISTRIBUTION (%)				CAPSULAR TYPES DISTRIBUTION (%)	
SAMPLE	agr-I	agr-II	agr-III	agr-IV	*cap5**	*cap8**
CF GROUP	29	30	17	24	44	56
AD GROUP	42	21	29	8	21	79
ANIMAL GROUP	89	0	10.7	0	50	50

* ($p<0.05$)

TAB.3 DISTRIBUTION OF ACCESSORY ADHESIN GENES AND TOXIN GENES IN THE *S.AUREUS* GROUPS STUDIED

	ACCESSORY ADHESIN GENES (%)			ACCESSORY TOXIN GENES (%)							
				ENTEROTOXINS ($p<0.001$)							
Sample	*sdrE* ($p<0.05$)	*cna* ($p<0.001$)	*fnbA* ($p<0.001$)	*sea*	*sec*	*sed*	*sej*	*sek/q*	*eta*	*luk*S/F ($p<0.001$)	*tst* ($p<0.001$)
CF GROUP	39.45	40.36	46.78	19.3	ND*	ND*	8.25	ND*	2.75	2.75	18.3
AD GROUP	62.63	53.84	79.12	22	24.2	11	20	50	4.4	4.4	59.3
ANIMAL GROUP	60.7	3.57	100	0	14.3	25	54	7.14	0	39.3	21.4

***ND: not determined**

TAB.4 ASSOCIATIONS BETWEEN *AGR*-GENOTYPES AND ACCESSORY VIRULENCE GENES IN THE *S.AUREUS* GROUPS STUDIED

	CF GROUP (%)				AD GROUP (%)				ANIMAL GROUP (%)	
	agr-I	*agr*-II	*agr*-III	*agr*-IV	*agr*-I	*agr*-II	*agr*-III	*agr*-IV	*agr*-I	*agr*-III
cap5	68.7	48.4	11.1	30.7	18	63.1	0	0	56	0
cap8	31.2	51.5	88.8	78.2	82	37	100	100	44	100
sea	31.2	3	33.3	15.38	2.6	26.3	46	28.5	0	0
sej	3.2	21.2	0	3.8	23	21	15.4	14.3	56	33.3
tst	22	9	38.8	11.5	36	63	84.6	85.7	24	0
*luk*S/F	3.12	0	0	7.6	2.5	0	3.8	14.3	32.14	7.14
eta	0	0	0	11.5	0	10.2	0	28.5	0	0
cna	25	9	61	94.6	54	31.5	61.5	85.7	0	33.3
*fnb*A	44	48.5	38.8	54	77	94.7	69.2	85.7	100	100
*sdr*E	47	57.5	11.1	27	74.3	89	34.6	28.5	56	100

TAB.5 ACCESSORY VIRULENCE GENE ASSOCIATIONS IN THE *S.AUREUS* GROUPS STUDIED

	Total (%) Isolates with associated accessory genes		**Total (%) isolates presenting from 2 to 9 associated accessory virulence genes**								**Frequency of the most recovered combinations of toxin genes among all isolates**		
	Number of isolates	Frequency (%)	2 genes	3 genes	4 genes	5 genes	6 genes	7 genes	8 genes	9 genes	Combination	number of isolates	Frequency (%)
CF GROUP	66/109	62.3	45	13.7	3.6	-	-	-	-	-	*sea, tst*	6	9
AD GROUP	82/91	90.1	17	17	14.6	12.2	19.5	6.1	6.1	7.3	*sea, tst*	16	19.5
											sec, tst	12	14.6
											sed, tst	10	12.1
											sej, seq, sek	16	19.5
											seq, sek, tst	27	32.9
											sea, seq, sek	15	18.3
											sec, seq, sek	12	14.6
											sec, seq, sek, tst	10	12.2
											sea, sec, seq, sek	9	11
											sed, seq, sek, tst	8	9.7
											*sej, seq, sek, luk*S/F	7	8.5
											sea, sec, seq, sek, tst	7	8.5
											sea, sec, sed, seq, sek, tst	2	2.4
											sea, sec, seq, sek, tst, eta	2	2.4
											sea, sed, sej, seq, sek, tst	2	2.4
ANIMAL GROUP	56/56	100	25	32.14	28.6	10.7	3.6	-	-	-	No frequent associations were found		

FIG.1 BIOFILM PRODUCTION (%) AND PREVALENCE OF THE *AGR*-GROUPS (A), QUANTIFICATION (MEAN OF OD_{490} VALUES) (B) IN THE *S.AUREUS* GROUPS STUDIED

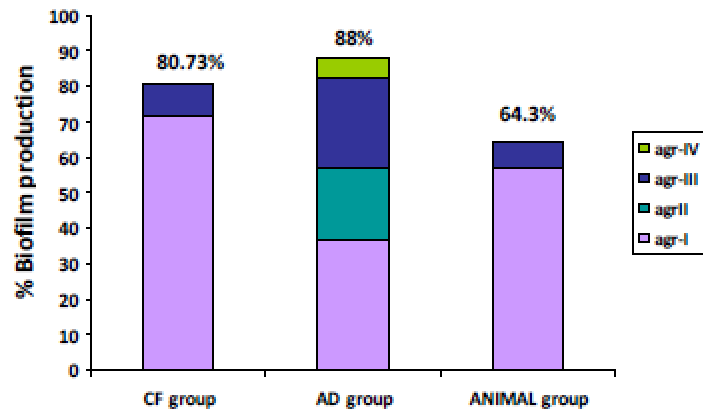


Fig. 1A

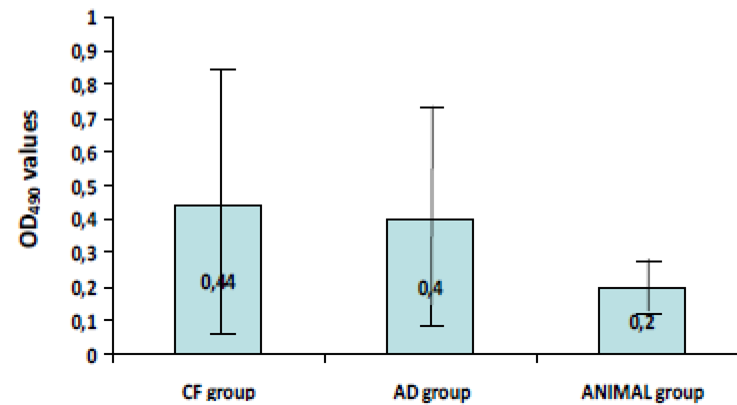


Fig. 1B