Research in Veterinary Science 103 (2015) 54–59

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/yrvsc

Pattern characterization of genes involved in non-specific immune response in *Staphylococcus aureus* isolates from intramammary infections

Maria Mazzilli, Renata Piccinini, Federico Scali, Alfonso Zecconi *

Dept. Animal Pathology, Hygiene and Public Health, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form 7 September 2015
Accepted 9 September 2015

Keywords:
Staphylococcus aureus
Clonal complex
Immune evasion
Mastitis
Enterotoxins
Epidemiology

ABSTRACT

Staphylococcus aureus isolated from mammary gland are characterized by different genetic patterns. Ninety four isolates from 33 dairy herds were analyzed by the means of a microarray to investigate *S. aureus* virulence patterns and the distribution of genes believed to be involved in immune evasion. None of the 94 isolates considered were MRSA. However, 50% of the isolates belonged to complexes related to MRSA and to human diseases, while only about 25% of them can be considered as exclusively of bovine origin. The distribution of clonal complexes and the different gene patterns observed confirmed the presence of an influence of geographical localization. The assessment of the influence of genes related to immune evasion on quarter milk cell count showed as four of them showed to be significantly associated to an increase quarter milk SCC. These genes could be potential target for developing new vaccines against *S. aureus*.

1. Introduction

Staphylococcus aureus is able to colonize several tissues in both humans and animal species (Morgan, 2008; Weese, 2010). The importance of *S. aureus* as a pathogen increased furthermore, with the increasing frequency of involvement of methicillin-resistant *S. aureus* (MRSA) in severe human disease cases. MRSA has been isolated since many years (Barber, 1961) and cows and pigs emerged as MRSA reservoirs for human infection (LA-MRSA) (van Loo et al., 2007; Vanderhaeghen et al., 2010). Among animals, dairy cows represent an important host for *S. aureus*, and particularly mammary gland provides favorable conditions for infections. Once intramammary infections are established, relevant economic losses are generally observed (Halasa et al., 2007; Zecconi, 2010; Zecconi et al., 2006a). Furthermore, *S. aureus* isolates from mammary gland are characterized by different genetic patterns, and this variability is behind the differences observed in clinic, geographical and economic aspects of infections (Fijalkowski et al., 2012; Le Marechal et al., 2011; Zecconi et al., 2006a).

Despite the large array of immune defenses generally deployed by the host, *S. aureus* is able to survive in different hosts and tissues because of its impressive arsenal of virulence factors. These molecules may increase the severity of the infection, improving adhesion to the tissues, facilitating invasion, promoting immune evasion and impairing host defenses (Zecconi and Scali, 2013). Indeed, *S. aureus* can impair phagocytes activity through direct cytolysis of neutrophils (PMN), delay of migration or reduction of oxidative burst (Chavakis et al., 2007; DuMont et al., 2011; Peacock et al., 2002). Chemotaxis inhibitory protein (CHIPS) binds formyl peptide receptors and C5a receptors, hence it slow down PMN migration (Postma et al., 2004; Rooijakkers et al., 2006). Furthermore, chemotaxis may be delayed by Staphylococcal superantigen-like proteins (SSLs) 5, 7 10 and 11 (Bestebroer et al., 2007). SSL7 is able to concurrently bind Fc region of IgA and C5, therefore obstructing both recognition by phagocytes and complement activities (Langley et al., 2005; Laursen et al., 2010). Additionally, SSL7 seems to inhibit complement classical pathway via bounding Fc domain of IgG (Itoh et al., 2010). SSL10 showed to bind PMN C-X-C chemokine receptor type 4 (CXCR-4) (Walenkamp et al., 2009), and SSL11 seems to obstruct neutrophil adhesion to P-selectin-coated surfaces (Chung et al., 2007).

S. aureus have efficient defenses against complement system, too. Capsular polysaccharides 1, 5 and 8 are able to interfere with C3 or C3b deposition (Chavakis et al., 2007; O'Riordan and Lee, 2004). Staphylococcal complement inhibitor (SCIN) and extracellular complement-binding protein (Ecb) inhibits convertase enzyme and *S. aureus* surface protein E (SdrE) impairs complement regulator factor H (Cunnion et al., 2011; Sharp et al., 2012). Clumping factor A (ClfA) and extracellular fibrinogen-binding protein (Efb) not only permit adherence to specific host molecules, but also provide interferences with complement system. Indeed, ClfA binds complement regulator factor I (Hair et al., 2010) and Efb inhibits both C3 and C5 convertases (Jongerius et al., 2007).

* Corresponding author: Dept. Veterinary Sciences and Public Health, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy.
E-mail address: alfonso.zecconi@unimi.it (A. Zecconi).

http://dx.doi.org/10.1016/j.rvsc.2015.09.007
0034-5288/

Enterotoxins (SEs) should be included among the virulence factors that can modulate host immune response because of their well-known superantigen activities (Fraser and Proft, 2008). The number of these molecules identified in *S. aureus* genome steadily increased in recent years and actually at least 20 different SEs have been characterized (Pinchuk et al., 2010).

Several studies investigated roles of virulence factors in intramammary infections or as potential vaccine targets (Festa et al., 2013; Le Marechal et al., 2011; Li et al., 2013; Middleton, 2008; Piccinini et al., 2010; Tedeschi et al., 2009; Zecconi et al., 2005; Zecconi et al., 2006b). However, only few of them considered the factors involved in immune evasion (Piccinini et al., 2010; Scali et al., 2015; Seo et al., 2007; Zecconi et al., 2006b).

There are several method to classify *S. aureus* (Tenover et al., 1994), and the one most frequently used is MLST (Enright et al., 2000) which allow to define clonal complexes and, consequently their epidemiological characteristics (Enright et al., 2000; Monecke et al., 2011). While this latter method is very useful to define cluster of isolates, it does not allow identifying the potential association of specific genes with infection characteristics. The availability of a diagnostic microarray that includes the analysis of about 170 distinct genes and their allelic variants, including the ones related to methicillin resistance (MRSA), allows to investigate *S. aureus* virulence patterns and, in addition, the role of specific virulence factors.

Therefore, in order to achieve further information on *S. aureus* epidemiology, potentially useful for vaccine development, we designed a study aimed to identify clonal complexes of *S. aureus* isolates from Italian dairy cows defined by MLST and the relationship between genes believed to be involved in immune evasion and in inducing changes in quarter milk somatic cell counts.

2. Materials & methods

2.1. Sampling and somatic cell counts

Quarter milk samples were collected from lactating cows from 33 herds enrolled in a contagious mastitis control program based on routine sampling of cows to determine if they were infected, followed by segregation of positive ones (Zecconi, 2006; Zecconi et al., 2003). Quarters were sampled following the procedures described by NMC (Hogan et al., 1999) and were analyzed by standardized procedures (Hogan et al., 1999). The isolates were presumptively identified as *S. aureus* according to the following scheme: Gram-positive cocci, hemolytic on blood agar, catalase positive, and coagulase positive in 4–24 h. The presumptive identification was confirmed by API ID32 Staph (BioMerieux, Marcy L'Etoile, F). Isolates were stored at −80 °C in Microbank Bacterial Preservation System (Thermo Fisher Scientific Inc., Waltham MA, USA) until needed.

Quarter milk somatic cells (SCC) were counted using Bentley Somacount 150 (Bentley, USA), following standardized procedures (I.D.F., F.I.L., 1995).

2.2. Isolates

Ninety-four isolates from 33 different dairy herds were considered, and isolates from intramammary infection cases (*S. aureus* positive and absence of clinical signs such as milk alteration and quarter swelling) were considered for each herd, based on phenotype characteristics. Practically, to select isolates representative of each herd, they were recovered from each colony having a different phenotype appearance (i.e. color, hemolysis). This procedure led to select two or three isolates from each herd.

Forty-two isolates were recovered from herds located in Northern Italy (NIt), while 52 from herds in Southern Italy (SIt). After thawing, isolates were cultured on blood agar plates with 5% bovine blood, and then submitted for genotyping.

2.3. Genotyping analysis

Genotyping of staphylococcal DNA was performed both by DNA microarrays based on Alere StaphyType DNA microarray (Alere Technologies Gmbh, Jena D) and by MLST procedure. The microarray covers approximately 170 distinct genes and their allelic variants for a total of 334 target sequences including species markers, SCCmec, capsule and agr group typing markers, resistance genes, exotoxins, and MSCRAMM genes (Monecke et al., 2007, 2008). The array comprised the probes in twofold redundancy. Genomic target DNA was amplified in a linear manner and labeled as previously described (Monecke et al., 2007, 2008). The Iconoclust software package (Alere Technologies Gmbh, Jena, Germany) were used combined with a defined script according to the procedure described by Monecke et al. (2007, 2008). Clonal complex was defined by standardized procedure based on MLST genotyping (Enright et al., 2000).

2.4. Data collection, classification and statistical analyses

Clonal complexes distributions were analyzed by FREQ procedure of SAS 9.4 software (SAS Institute, Cary NC USA), applying χ^2 test on frequency tables produced. The relationship between gene patterns and quarter milk somatic cell counts was performed by General liner models (GLM) procedure of SAS 9.4 software (SAS Institute, Cary NC USA), using SCC as a response variable in a model that included all the considered genes as factors and without interactions. p value threshold for statistical significance was set at 0.05.

3. Results

3.1. Isolate characteristics

Isolates were characterized by both MLST and microarray. This latter technique showed as all the isolates carried specific *S. aureus* genes: protein A (*spa*), coagulase (*coa*), IgG-binding protein (*sbi*), and thermostable extracellular nuclease (*nuc*) as expected (Monecke et al., 2007, 2008).

None of the isolates were MRSA, being negative for methicillin-resistance regulatory protein, signal transducer protein MecR1, homolog of xylose repressor and others related genes included in the array, while 52.1% of them carried genes involved in penicillin resistance.

When isolates were grouped in clonal complexes (CC) by MLST, 12 CC including 88 isolates were identified, while 6 isolates could not be included in any group. Only 5 CC included 5 or more isolates, while the other seven had 4 or less isolates each. Therefore, for further statistical analysis, the complexes were grouped in 7 groups (Table 1). Out of them, 5 had more than 5 isolates each (CC 1, 8, 97, 479 and 705), one including all the other clonal types (OTH) and the last one included the unclassified isolates (UNC). Within the OTH isolates, it should be emphasized that four of them were classified as CC398, which is a lineage that includes livestock-associated (LA) *S. aureus* strains able to cause diseases in humans (Monecke et al., 2011). When distribution of CC

Table 1
Distribution of clonal complex frequencies among 94 *S. aureus* isolates considered.

Clonal complex	Frequency (%)	Group
CC1	5 (5.3%)	CC1
CC8	45 (47.9)	CC8
CC97	12 (12.8%)	CC97
CC479	5 (5.3%)	CC479
CC705	11 (11.7%)	CC705
Other types (5, 20, 101, 398)	10 (10.6%)	OTH
Unclassified	6 (6.45)	UNC