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Ana Isabel Rodrigues Pereira de Castro

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Microbiology

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Supervisor: **Professor Doutora Paula Cristina Maia Teixeira**

Co-Supervisor: **Doutora Joana Gabriela Laranjeira Silva**

November 2016

To my parents and my family

Abstract

Staphylococcus aureus is a pathogen responsible for skin infections and invasive diseases such as meningitis or pulmonary infection and staphylococcal food poisoning (SFP). The main goal of this study was to increase knowledge on *S. aureus* circulating in Portugal mainly concerning their virulence characteristics and antimicrobial resistance. According to EFSA-ECDC, Portugal is considered to have one of the highest levels of methicillin-resistant *S. aureus* (MRSA) from bacteraemia in Europe.

The frequency of occurrence of *S. aureus* from different origins was determined. Swab samples were collected from hands and nose of health care professionals and food handlers - noses and hands are niches where *S. aureus* are likely to be present in humans - and particularly noses of children (3 to 6 years). Presence of *S. aureus* in food samples was also determined. Collected isolates were further characterized phenotypically and genomically.

Nasal carriage of *S. aureus* was higher in children (48.6%) compared to health care professionals (39.6%) and food handlers (19.8%). The occurrence of *S. aureus* on the hands of health care professionals and food handlers was 8.9% and 11.1%, respectively. Globally, higher *S. aureus* occurrence rates were obtained for nasal carriage.

The first case of Methicillin-Resistant *Staphylococcus aureus* (MRSA) was reported in 1961. Since then, it has been considered the leading cause of nosocomial infections, responsible for causing serious morbidity and mortality rates, worldwide. Globally, the occurrence of MRSA strains was higher in hospital health care professionals; no MRSA strains were detected in food handlers, children presented a carriage of 9.7% of MRSA and low occurrence rates were detected in the analysed food samples (between 0.68 and 5.5 %).

Other virulence factors have been reported for *S. aureus* strains including antibiotic resistance and presence of enterotoxins, Panton-Valentine Leukocidin (PVL), haemolysins, exfoliative toxins and numerous factors involved in invasion of host cells among others.

In the present study, resistance to oxacillin, penicillin and ampicillin and to antibiotics of classes other than betalactams, namely ciprofloxacin, gentamicin, rifampicinn, vancomycin, tetracycline, erythromycin, nitrofurantoin and chloramphenicol was investigated. As expected, high levels of resistance to ß-lactams were observed. Tested

strains showed low resistance rates to gentamicin, chloramphenicol and rifampicin. Regardless of the source of isolates, resistance to erythromycin was evident.

Enterotoxin genes *sea* - *sej* and *tst* were evaluated by multiplex PCR. Although *sea* is considered the most prevalent enterotoxin gene reported all over the world, in the present study this was not verified. *tst* gene was detected in children, hospital health care professionals and food handlers. PVL genes were analysed in all the MRSA strains collected from various origins, but only one food strain showed the presence of these genes.

Typing by SCC*mec* has been performed and revealed the presence of type IV and V. SCC*mec* type IV is associated worldwide with PVL positive strains, but which was not verified in this study. PFGE typing was performed on *S. aureus* strains isolated from hands and from nose of the same individual of the health care professionals and food handlers; 60 and 30% of the individuals, respectively, presented the same *S. aureus* strain on hands and nose.

Resumo

Staphylococcus aureus é um patogénico responsável por infeções de pele e doenças invasivas tais como meningite ou infeção pulmonar e intoxicação alimentar estafilocócica. O principal objetivo deste estudo foi aumentar o conhecimento sobre *S. aureus* em circulação em Portugal, principalmente quanto às suas caraterísticas de virulência e resistência antimicrobiana. De facto, de acordo com EFSA-ECDC, Portugal é considerado um dos países da europa com maior incidência de bacteriemia por *Staphylococcus aureus* resistente à meticilina (MRSA)..

A ocorrência de *S. aureus* isolados em diferentes populações foi avaliada. Foram colhidas amostras por zaragatoas de mãos e nariz de profissionais de saúde e de manipuladores de alimentos e de nariz de crianças (3 a 6 anos) - o nariz e as mãos são nichos onde *S. aureus* pode estar presente nos humanos. A presença de *S. aureus* em alimentos foi também determinada. Os isolados recolhidos foram posteriormente caraterizados por médos fenotípicos e genotípicos. A ocorrência nasal de *S. aureus* foi mais elevada nas crianças (48,6%*)* do que nos profissionais de saúde (39,6%) e manipuladores de alimentos (19,8%). A ocorrência de *S. aureus* nas mãos dos profissionais de saúde e manipuladores de alimentos foi de, respetivamente, 8,9% e 11,1%. Globalmente, verificou-se uma maior ocorrência de *S. aureus* no nariz do que nas mãos.

O primeiro caso de estafilococos resistentes à meticilina (MRSA) foi reportado em 1961. Desde então, estes têm sido considerados a causa principal causa de infeções nosocomiais, responsáveis por elevadas taxas de morbilidade e de mortalidade em todo Mundo. Globalmente, a ocorrência de estirpes de MRSA foi maior para os profissionais de saúde; não foram detetadas estirpes de MRSA nas amostras recolhidas de manipuladores de alimentos, 9.7% das crianças eram portadoras de MRSA e uma baixa ocorrência foi observada para as amostras alimentares analisadas (entre 0,68 e 5,5%).

Outros fatores de virulência têm sido reportados em estirpes de *S. aureus* incluindo, entre outros, a resistência a antibióticos e a presença de enterotoxinas, Panton- Valentine Leukocidin (PVL), hemolisinas, toxinas exfoliativas, e numerosos fatores associados à invasão de células do hospedeiro.

No estudo presente, estudou-se a suscetibilidade à oxacilina, penicilina e ampicilina e a outros antibióticos de outras classes para além de beta lactâmicos, nomeadamente ciprofloxacina, gentamicina, rifampicina, vancomicina, tetraciclina, eritromicina, nitrofurantoína e cloranfenicol. No que respeita aos antibióticos betalactâmicos, e conforme esperado, foram observados níveis de resistência elevados. As estirpes testadas mostraram baixos níveis de resistência à gentamicina, ao cloranfenicol e à rifampina. Independentemente da origem, é de salientar a elevada resistência à eritromicina .

A presença de genes que codificam a produção das enterotoxinas *sea-sej* e *tst* foi avaliada por PCR multiplex. Apesar de *sea* ser considerado o gene mais prevalente no mundo, tal não foi verificado neste estudo. O gene *tst* foi detetado em crianças, profissionais de saúde e manipuladores de alimentos. Os genes que codificam a produção de PVL foram pesquisados em todas as estirpes de MRSA recolhidas de várias origens; a presença deste gene foi detetada apenas numa estirpe alimentar.

A tipagem por SCC*mec* foi realizada e revelou a presença dos tipos IV e V. SCC*mec* tipo IV é mundialmente associado a estirpes PVL positivo mas tal não foi verificado neste estudo. A tipagem por PFGE foi realizada para *S. aureus* isolados em simultâneo de mãos e de nariz, de um mesmo indivíduo; 60% dos profissionais de saúde e 30% manipuladores de alimentos apresentavam a mesma estirpe nas mãos e no nariz.

To the Direction Board of *Escola Superior de Biotecnologia* (ESB) of the *Universidade Católica Portuguesa* for accepting me as a post-graduate student and providing the necessary conditions to carry out this work.

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To parents of children that allowed sampling of their children and to all staff from kindergartens.

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Lastly, and most importantly, I wish to thank my parents, Isabel and Luís, and my Brothers Luís and Paulo for their endless love and for always encouraging me to pursue my dreams. They are my pillar, my joy and my guiding light. To them I dedicate this thesis.

LIST OF CONTENTS

Staphylococcus aureus is an opportunistic pathogen causing major problems in the food and medical fields. Since its discovery in the 19th century, *S. aureus* has been considered a major public health concern. It is responsible for nosocomial infections and staphylococcal food poisoning. As a commensal microorganism it has significant levels of carriage in the community.

Staphylococcus aureus may be present as part of the normal microbiota of humans and other animals, being carried on skin and nasal cavities of ca. 30% of the healthy human population. As a consequence and in contrast to other major types of food poisoning, humans play a major role in the transmission of *S. aureus* - transferred to foods, especially via the hands.

Staphylococcal food poisoning results from ingestion of enterotoxins, synthesized during growth in foods. Enterotoxin production is most common amongst *S. aureus* isolates of human origin. Occurrence of *S. aureus* intoxication is grossly under-reported and the importance consequently under estimated.

This work had as a global objective to increase knowledge on the *S. aureus* circulating in Portugal mainly concerning their virulence characteristics and antimicrobial resistance. In order to achieve this main goal, specific tasks were defined namely:

- To isolate *S. aureus* from food samples, food handlers and health care professionals at the same hospital;

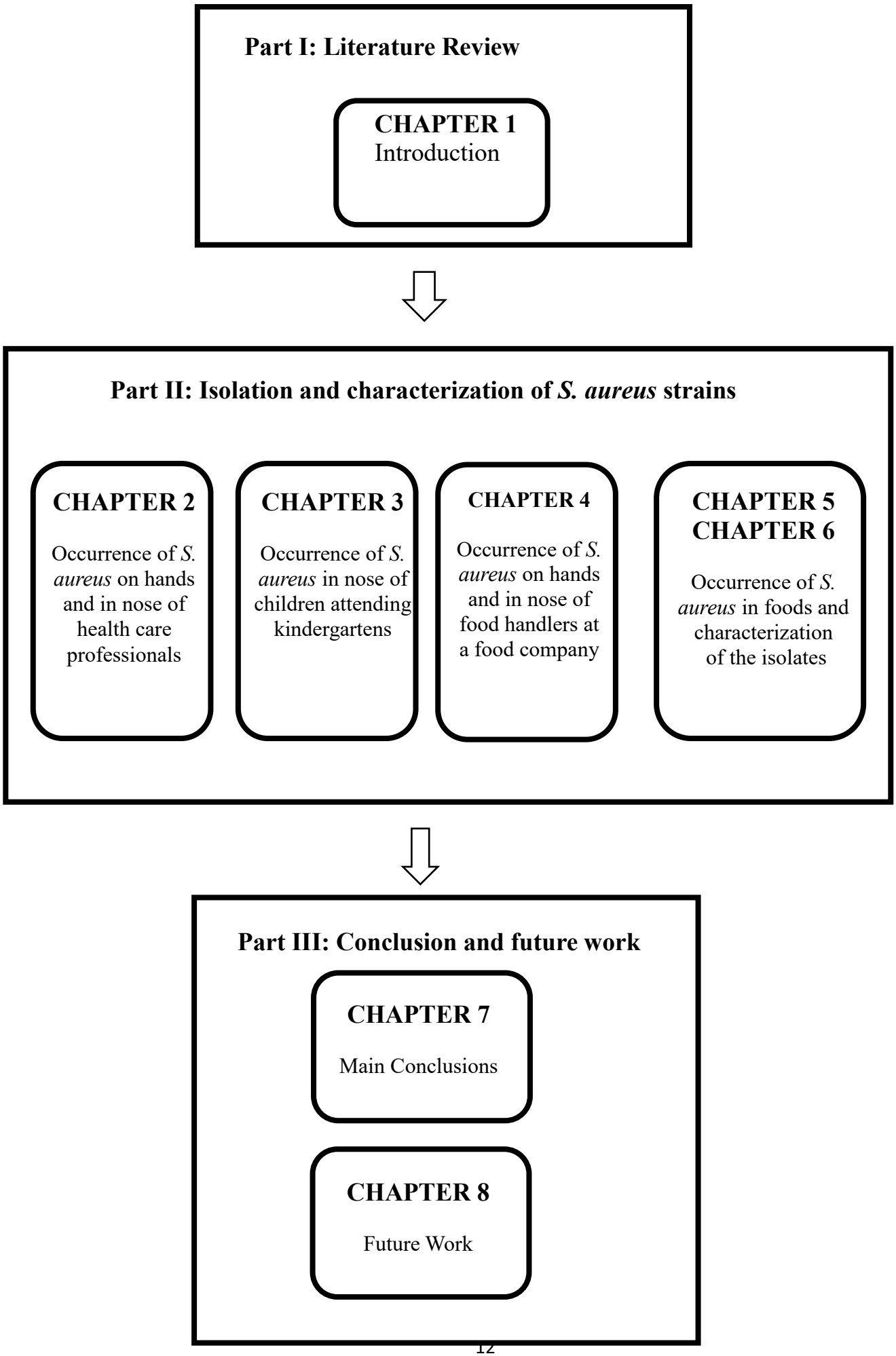
- To determine the genotypic and phenotypic characterization of the *S. aureus* isolates for the determination of MRSA strains;

- To characterize the isolates by genotypic and phenotypic methods concerning the presence of virulence factors and antimicrobial resistance:

- To compare the different strains of *S. aureus* isolates relative to the presence/absence of several virulence factors and to correlate their virulence profiles with their different origins;

- To determine potential clonal relationships of *S. aureus* strains recovered from the same source.

This thesis is structured in three parts comprising eight chapters.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Part I Consists of Chapter 1, in which a literature review is presented. The general characteristics and occurrence of *S. aureus* in various niches (humans, animals and food) are discussed in detail. The importance of *S. aureus* as a foodborne pathogen, including staphylococcal food poisoning outbreaks is discussed. Virulence traits of this bacterium that contribute to its pathogenicity, including antibiotic resistance are also considered. The importance of antibiotic resistance as a clinical and public health problem, highlighted the importance of Methicillin- Resistant *S, aureus* – MRSA strains in various environments. Moreover, to control propagation of this bacterium worldwide, alternatives to the use of antibiotics namely the use of plant extracts, bacteriocins and phages are discussed. In addition, the importance of preventing *S. aureus* presence in the food industry and in hospital settings is examined.

Part II comprises Chapters 2 to 6 in which the results of the prevalence / occurrence of *S. aureus* and MRSA, as well as antimicrobial resistance and some other virulence traits are presented: on health care professionals (Chapter 2), on children (Chapter 3), in food handlers (Chapter 4) and in food products (Chapters 5 and 6). The isolation was performed on selective media (BPA and MSA) and confirmation was based on phenotypic characteristics such as Gram staining, catalase and coagulation of rabbit plasma and DNAse production. Simultaneous detection of 16SrRNA and *nuc* (thermonuclease) genes confirmed the isolates to species level. All the confirmed *S. aureus* isolates were evaluated concerning the presence of *mec*A gene and further classified as MRSA and MSSA. Antimicrobial resistance to eleven antibiotics comprising eight different antibiotic classes was also evaluated. Other virulence traits were evaluated namely enterotoxins and toxic shock syndrome genes. For Panton-Valentine Leukocidin (PVL) genes the determination was performed only on MRSA strains. The importance of cross contamination of hand to nose or vice-versa (on health care professionals and food handlers) was studied through PFGE analysis. MRSA clonal relationship was observed among the MRSA strains collected from the same hospital. SCC*mec* typing was also performed.

Part III encompasses Chapter 7 in which the main conclusions of this study are presented and Chapter 8 with proposals for future work.

The work presented in this thesis comprises one book chapter submitted for publication in a peer-reviewed scientific book and five articles, four published in peer-reviewed scientific journals and one published in a peer-reviewed scientific acta namely:

Chapter 1

Ana Castro, Joana Silva, Paula Teixeira. 2016. *Staphylococcus aureus*- a food pathogen: virulence factors and antibiotic resistance. *Handbook of Food Bioengineering*, Elsevier (submitted).

Chapter 2

Pereira V., Lopes C., Castro A., Silva J., Gibbs P., Teixeira P. 2009. Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. *Food Microbiology*, 26, 278-82. doi:10.1016/j.fm.2008.12.008

Chapter 3

A Castro, C. Palhau, S. Cunha, S. Camarinha, J. Silva, P. Teixeira. 2016. Virulence and Resistance profile of *Staphylococcus aureus* Isolated from Food. *Acta Alimentaria* (accepted).

Chapter 4

Ana Castro, Carla Santos, Helena Meireles, Joana Silva, Paula Teixeira. 2015. Food handlers as potential sources of dissemination of virulent strains of *Staphylococcus aureus* in the community. *Journal of Infection and Public Health* 9, 153-160. doi:10.1016/j.jiph.2015.08.001

Chapter 5

Schmid H., Lôpo N, Castro A, Silva J, Teixeira P. 2012. Characterization of *Staphylococcus aureus* isolated from healthy children in Portugal. *Microbes in Applied Research, Current advances and challenges*, Edited by: A Mendez-Vilas (Formatex Research Center, Spain) 509-512. http://dx.doi.org/10.1142/9789814405041_0103

Chapter 6

Ana Castro, Norton Komora, Vânia Ferreira, Agostinho Lira, Margarida Mota, Joana Silva, Paula Teixeira. 2016. Prevalence of *Staphylococcus aureus* from nares and hands on healthcare professionals in a Portuguese Hospital. *Journal of Applied Microbiology* 121, 831-839. doi:10.1111/jam.13186.

Chapter 1

Paper submitted (in revision)

Ana Castro, Joana Silva and Paula Teixeira. ***Staphylococcus aureus* - a food pathogen: virulence factors and antibiotic resistance.** *Handbook of Food Bioengineering*, Elsevier.

Staphylococcus aureus - a food pathogen: virulence factors and antibiotic resistance

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Abstract

Staphylococcus aureus is an extraordinarily versatile pathogen responsible for staphylococcal food poisoning, hospital and community infections as well as toxic shock syndrome. *S. aureus* is considered the most effective foodborne bacterial pathogen that has ever evolved. The *S. aureus* metagenome contains 10's of genes encoding staphylococcal enterotoxins which are responsible for the clinical symptoms associated with staphylococcal food poisoning. *S. aureus* may be present in food products being a potential vehicle for transmission. Antibiotics are widely used not only in human but also in animal husbandry and other agricultural activities. The occurrence of multi-resistant strains in food(s) has been increasing; contaminated food is considered an important vehicle for antimicrobial resistance. Methicillin-resistant *S. aureus* (MRSA) were first recognized in animal infections in 1972 in milk from mastitic cows in Belgium. Animal associated MRSA infections in humans were first reported during 2003-2005 in Netherlands. Presently, it was reported that, livestock-associated MRSA CC398 in pork identifies a potential pathway from farms to the wider population through retail pork. MRSA commonly carry enterotoxin genes but there has been only one report of food intoxication due to MRSA. Antibiotic resistance associated to enterotoxins genes made *S. aureus* an evolving threat.

Introduction

Foodborne diseases are commonly associated with agents e.g. bacteria, vius, fungi, prions, parasites or chemicals that are present in contaminated food and water. At least 200 foodborne diseases are associated with food as a vechiche of contamination causing serious public health problems all over the world (WHO, 2015). These account for about 23 million cases of illness and 5000 deaths in Europe every year; diarrheal diseases cause the majority of these cases contributing with about 22 million cases and 3000 deaths annually (WHO, 2015). *Salmonella* spp., *Campylobacter* spp., *Clostridium perfringens*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, or viruses, such as norovirus are considered the cause of acute foodborne diseases (often called food poisoning, WHO, 2015).

Since 2003 it is mandatory to report foodborne outbreaks occurring in the European Union Member States (EU MS); in 2007, harmonized specifications on the reporting of foodborne

outbreaks at the EU level have been implemented (EFSA- ECDC, 2015a.). In 2014, a total of 5,251 foodborne outbreaks were reported by the 26 MS. Bacterial toxins (*i.e.* toxins produced by *Bacillus*, *Clostridium* and *Staphylococcus*) accounted for 16.1% of all outbreaks (EFSA- ECDC, 2015a). "Staphylococci" were identified as responsible for wound infections by the surgeon Sir Alexander Ogston in the 1880s (Ogston, 1984). *Staphylococcus aureus* was the name attributed to a strain showing colonies with pigmented appearance isolated by the German surgeon Anton J. Rosenbach (Rosenbach, 1984). Since then, *S. aureus* has attracted much attention as a cause of human infections and is still considered as one of the most important agents involved in nosocomial infections (Khan et al., 2015). Global spread and increasing resistance to antibiotics of this pathogen is still a major of concern. Methicillin-resistant *S. aureus* (MRSA) appeared in 1961 being responsible to cause serious morbidity and mortality rates in hospitals throughout the world. MRSA are known to be resistant to all the types of penicillin´s and lactams (Stefani and Goglio 2010). In fact, the high capacity of this agent to. acquire multi-antibiotic resistance continuous to be a paradigm for future chemotherapy against the multi-resistant pathogens. *S. aureus* carriage rate varies with geographic location, age, sex and body niches. In addition to staphylococcal infections, *S. aureus* is also responsible for food poisoning due to oral intake of enterotoxins present in foods (Johler et al., 2015a.). The fist description of staphylococcal poisoning was attributed to Vaughan (1984) - "Dryness of the throat, nausea vomiting, diarrhoea, nervous prostration, headache, and sometimes double vision. In short, the symptoms are those of a gastro-intestinal irritant, with marked secondary effects upon the nervous system. Not withstanding the alarming symptoms, recovery follows." - during an investigation of an outbreak of "cheese-poisoning" in Michigan that affected about three hundred individuals. Latter on, Dack et al. (1930) demonstrated that staphylococcal food poisoning was due to a filterable substance that maintained activity after being heated at 100° C for 30 minutes.

Staphylococcus aureus – general characteristics

Staphylococcus was firstly introduced by Ogston in 1883 for the group of micrococci causing inflammation and suppuration (Götz et al., 2006). Until the early 1970s, the genus *Staphylococcus* consisted of three species namely i) coagulase-positive species known as *S. aureus*, ii) coagulase negative species known as *S. epidermidis* and *S. saprophyticus* (Götz et al. 2006). Nowadays, 51 species and several subspecies are recognized (www.bacterio.net/staphylococcus.html; consulted in 7/6/2016).

Staphylococcus aureus consists of irregular clusters (*Staphyle*, bunch of grapes) with "spheres" with 0.5-1.0 µm in diameter and cells usually occur singly or in pairs. *S. aureus* are facultative anaerobic Gram-positive cocci, non-motile and non-spore forming bacteria. Catalase is produced by cells growing aerobically. As facultative anaerobes most strains produce acid aerobically and anaerobically from glucose, lactose, maltose and mannitol. Typical colonies are yellow to golden yellow in colour, smooth, raised, glistening, circular, entire and translucent and may obtain a size of 6-8mm in diameter on non-selective media used for propagation of staphylococci. The identification of this species is based on the production of coagulase which is produced by nearly all strains (http://www.bergeys.org/pubinfo.html). However, additional characters needed to be considered since some staphylococci strains show a variable expression of coagulase (http://www.bergeys.org/pubinfo.html).

As most strains of *S. aureus* produce a heat resistant nuclease, thermonuclease also known as TNase, might be used as an indicator for the detection of *S. aureus*. In addition, *S. aureus* can grow at NaCl concentrations up to 10% but with limitations at 15% (http://www.bergeys.org/pubinfo.html). Most strains grow at temperatures between 10 and 45ºC (optimum 30º-37 ºC) and pH values between 4.2 and 9.3 (optimum pH 7.0-7.5) (http://www.bergeys.org/pubinfo.html). With respect to water activity (a_w), *S. aureus* can tolerate values between 0.83 and 0.99. These characteristics enable *S. aureus* to grow and survive in a variety of environmental conditions including stressful environments (e.g. dry surfaces) for long periods (Valero et al., 2009). In addition, development of stress resistance in *S. aureus* after exposure to sublethal environmental conditions might occur and should be avoided in food-processing environments (Cebrián et al., 2010).

Occurrence of *Staphylococcus aureus*

S. aureus in humans

Staphylococcus aureus is carried by a significant proportion of the population in skin and the nose (Kluytmans and Wertheim, 2005) but can also be found in various niches of the human body e.g. throat and perineum, the most common in the general adult (Muenks et al., 2016). Intestinal colonization, particularly in hospitalized individuals, has also been reported (Boyce et al., 2005). As found by Kluytmans and Wertheim (2005), *S. aureus* colonizes the

nares of approximately 50% of healthy adults, either persistently or intermittently. Endogeneous nasal colonisation is believed to be a common source of infection and a strong risk factor for subsequent colonisation; however, most carriers do not develop clinical disease (Kluytmans et al., 1997). *Staphylococcus aureus* carriage rates vary between different ethnic groups (with higher rates in white people and in men) and age. Colonisation with *S. aureus* was most common in persons aged <20 years. MRSA isolation from 5- to 9-year-old children tended to be higher than from other old age groups such as university students. Other determinants that have been associated with *S. aureus* carriage and population include: oral contraceptives use, smoking, crowding and healthcare exposure (Sollid et al., 2014).

While numerous surveillance programs have been producing information on resistance patterns and molecular types of *S. aureus* in hospitals, relatively little information is available on strains of this species that colonize healthy human populations. MRSA strains have a considerable capacity for spreading in the community, even when transmitted from children with SSTI (skin soft tissue infection) to healthy children in kindergartens. In the era of increasing CA-MRSA, physicians should be familiar with the spectrum of symptom´s, epidemiology and antibiotic susceptibility pattern of CA *S. aureus* in their local areas.

In a study performed by Lues and Van Tonder (2007), *S. aureus* was isolated from the hands of 88% of the population sampled. Human nasal or hand carriage of enterotoxigenic *S. aureus* during food processing is an important vehicle of contamination (Castro et al., 2015a; Hennekinne et al., 2010; Argudín et al., 2010). *S.aureus* outbreacks are in part of the responsibility of food handlers who carry enterotoxigenic. Hands of healthcare professionals are common vehicle for the transmission of healthcare-associated pathogens from patient to patient and within the healthcare environment (Allegranzi and Pittet, 2009).

S. aureus in animals

The occurrence of *S. aureus* among pets and veterinary personnel has already been reported by Drougka and his collaborators (2016) namely 36.3% and 38.9%, respectively. According to these authors, the prevalence of *S. aureus* was higher in younger companion animals. Petinaki and Spiliopoulou (2012) reported the presence of multiresistant *S. aureus* in pets

(cats and dogs), horses and marine mammals and, outbreaks among humans that had its origin in animals have already been recorded.

On the other hand, *S. aureus* is considered one of the most relevant pathogen causing clinical and subclinical, chronic mastitis in dairy animals being a major problem in the dairy industry (Wang et al., 2015). This agent was already associated to cows, goats and sheep mastitis (Wang et al., 2015; Xu et al., 2015).

Zhang et al. (2016), analysed 200 milk samples from cows suffering from mastitis and observed that 58 samples were positive for *S. aureus*. From those, 20% were methicillin-resistant *S. aureus*. Xing et al. (2016), determined the prevalence of *S. aureus* in goat milk powder processing plants. Out of 910 samples, 10.4% were positive for *S. aureus*. These authors, confirmed that cross-contamination of *S. aureus* exists in the analysed milk plant since, some isolates with the same PFGE patterns came from different processing stages.

Teramoto et al., 2016 studied the prevalence of *S. aureus* in poultry retail meat samples; globally, the prevalence of *S. aureus* were 25.0%, 14.29%, and 33.3% in retail poultry meats collected from farmers markets, organic and conventional retail supermarkets, respectively.

Verkade and Kluytmans (2014) reported a specific clone of multiresistant *S. aureus* CC398 that has spread extensively in livestock animals and in retail meat showing that people in contact with food production animals are at high risk of colonization. Although more research concerning this clone is needed, the routes of contamination might be related with the direct contact with animals, environmental contamination, and eating or handling contaminated meat.

Preventing the spread of *S. aureus* at the farm level reducing the exposure in the community and in categories related to farm animal industry (e.g., veterinarians, farmers, and farm workers) is mandatory and is actually a challenge (Carfora et al., 2016).

S. aureus in food

Staphylococcus aureus has been identified in numerous types of foods namely meat (Hanson et al., 2011, Pu et al., 2011, Jackson et al., 2013, Hadjirin et al., 2015), milk and raw-milk (Carfora et al., 2015; Jamali et al., 2015, Al-Ashmawy et al., 2016), fish (Vásquez-Sánchez

et al., 2012), retail food (Wang et al., 2014, Li et al., 2015) and ready-to-eat food (Li et al., 2015, Puah et al., 2016).

Arfatahery et al. (2015) reported 24.6% of seafood samples contaminated with *S. aureus*, from September 2013 to March 2014, in Iran. Jamali et al. (2015), analysed a total of 2650 samples from raw milk and detected 12.4% of positive *S. aureus* which 16.2% were multiresistant *S. aureus*. Rola and his collaborators (2015) showed that 62% of raw milk samples were positive for coagulase-positive staphylococci at levels between 1.0×10^0 and 1.0×10^5 cfu/ml and 29% of *S. aureus* contained SEs genes arguing that the presence of enterotoxigenic *S. aureus* on raw milk was due to its contamination from infected dairy animals, human handling, water and milking equipment.

Of the 2217 meat samples analysed, de Boer et al. (2009) reported that 11.9% were positive for the presence of multiresistant *S. aureus*, 10.6% from beef, 15.2% from veal, 6.2% from lamb and mutton, 10.7% from pork, 16.0% from chicken, 35.3% from turkey, 3.4% from fowl and 2.2 % from game. The presence of *S. aureus* on food from raw meat may be due to contamination during slaughtering, processing, storage and distribution (Hennekinne et al., 2012).

According food legislation on EU, food safety criteria only applies to staphylococcal enterotoxins for dairy products such as cheese, milk powder and whey powder (Commission Regulation (EC) No 2073/2005). SEs must not to be detected on 25 g of foodstuff (Commission Regulation (EC) No 2073/2005). Besides identification of *S. aureus* on various foods, this bacterium only produces sufficient amount of SEs to cause illness when cell numbers are greater than 10^5/g of food (Bhatia and Zahoor, 2007). To prevent reaching this threshold for enterotoxin production, enumeration of coagulase-positive staphylococci must be performed but by law only on milk and dairy products and seafood (Commission Regulation (EC) No 2073/2005). Cheeses made from raw milk or from milk that has undergone a lower heat treatment than pasteurization as well as ripened or unripened soft cheeses that has undergone pasteurization or a stronger heat treatment must follow process hygiene criteria (Commission Regulation (EC) No 2073/2005). The same applies to milk powder and whey powder. On seafood, shelled and shucked products of cooked crustaceans and molluscan shellfish follow also process hygiene criteria (Commission Regulation (EC) No 2073/2005). However, *S. aureus* is routinely enumerated in a wide variety of ready-to-eat foods as part of preventive approach and microbiological safety checks based on hazard

analysis and critical control point (HACCP) principles. For food business operators it is important that enterotoxins must be absent in their products during all food shelf-life. In fact, any food product that can support the growth of enterotoxin-producing *S. aureus* may be a potentially harmful for consumers if staphylococcal enterotoxins are produced causing a case or an outbreak of staphylococcal food poisoning.

In order to prevent growth of *S. aureus* and SE production, for example in dairy products, there are numerous processes on food processing e.g. heat treatment of milk, or inhibition using starter cultures, antagonistic effect of natural flora, concentration of salt, drop of pH, lowering temperature of processing and storage of cheese and/or minimizing the pressing time (Bianchi et al., 2013). However, SES are much more resistant to environmental effects and food-processing procedures than the staphylococcal bacterial cells (Bianchi et al., 2013) staying on final food product and could be a potentially hazard for consumers.

Staphylococcus aureus is an indicator of deficient hygiene of food and processing environment because it is a commensal organism of the skin and mucous membranes of humans (20-30% for persistent and 60% for intermittent colonization) particularly food handlers who by manual contact or through respiratory secretions contaminate food by enterotoxin-producing *S. aureus* carried in their noses or on their hands (Argudín et al, 2010). Human food contamination by *S. aureus* is mainly associated with inadequate handling of cooked or processed foods because it does not compete well with indigenous microbiota in raw foods (Argudín et al., 2010) followed by favorable environmental conditions for *S. aureus* growth during food storage and preparation (i.e. time and temperature abuse) (Valero et al., 2009).

S. aureus and clinical aspects – an overview

Staphylococcus aureus is a commensal organism colonising the skin, anterior nares and mouth of healthy humans and endogenous nasal colonisation is believed to be a common source of infection and a strong risk factor for subsequent colonisation; however, most carriers do not develop clinical disease (Gorwitz et al., 2008). Likewise, *S. aureus* is an extraordinarily and versatile pathogen responsible for hospital and community - acquired infections, staphylococcal food poisoning, as well as for the toxic shock syndrome

(Uhlemann et al., 2014; Song et al., 2015). Hospital-and community-acquired bacteremia occur, in some instances, when *S. aureus* enters wounds or damaged skin, and can cause abscesses, pneumonia, meningitis, endocarditis and septicaemia (EFSA, 2009). Skin infections are frequently assumed as community-acquired infections while hospital-acquired infections dominate on the lung. *S. aureus* pneumonia occurs in hospitalized patients with immune deficiencies or viral infections (Otto, 2014). Hospital - and community-acquired bacteremia has an overall population-based incidence rate of 15-40 cases per 100,000 population and year and is the second leading pathogen causing sepsis in industrialized countries (ECDC, 2013; Kaasch et al., 2014; Laupland et al., 2013). *Staphylococcus aureus* bacteremia (SAB) is associated with case fatality rates of approximately 15-25%, significant morbidity, frequent complications and imposes a significant burden on the healthcare system (Liu et al., 2013; Lundberg et al., 1998).

Hands of healthcare professionals are the most common vehicle for the transmission of healthcare-associated pathogens from patient to patient and within the healthcare environment (Allegranzi and Pittet, 2009). Hand hygiene is the leading measure for preventing the spread of antimicrobial resistance and reducing healthcare-associated infections (Allegranzi and Pittet, 2009). In addition to hand hygiene, contact precautions (masks, gloves), environmental decontamination, cleaning protocols as well as education and training (Calfee et al., 2008) are very important in the prevention and control of MRSA.

Staphylococcal food poisoning is one of the most prevalent causes of foodborne intoxication worldwide. It is typically self-limiting, presenting with violent vomiting following a short incubation period (Kérouanton et al, 2007). Within 2–6 h after ingestion of food containing staphylococcal enterotoxins (SEs), symptoms of acute gastroenteritis (nausea, abdominal cramps, diarrhea and fever) can be observed (Tranter et al., 1990). In addition, toxic shock syndrome (TSS) is also considered a pathogenic expression characterized by fever, erythema, hypotension, and multiple organ disorder and usually is associated with Toxic shock syndrome toxin (TSST-1) and staphylococcal enterotoxins (Todd et al., 1978).

MRSA strains

Despite the large amount of effective antistaphylococcal antibiotics, worldwide, MRSA are considered important pathogens and high morbidity and mortality rates have been reported

(Dias et al., 2016). Portugal has one of the highest levels of MRSA (>50%) in Europe (EFSA-ECDC, 2015). Methicillin resistance is mediated by *mecA* gene that encodes penicillin-binding protein 2a (PBP2a that shows decreased affinity for binding beta-lactam antibiotics). Recently, a new homologue of *mecA* gene designated as *mecC* gene (previously $mecA_{LGA251}$ gene) has been reported; however, little information about the frequency, epidemiology and possible transmission between livestock and humans is available (Petersen et al., 2013). A β-lactamase, encoded by *blaZ*, is also produced by MRSA strains being responsible for the decrease in the activity of β-lactam antibiotics.

Although initially reported as an hospital-acquired pathogen, MRSA have also been associated with community infections and with livestock-associated infections (VanEperen and Segreti, 2016). Except in southern Europe, the percentage of MRSA isolates appears to be stable, and even decreasing. Nevertheless, among all *S. aureus* isolates, this percentage remained above 25% in eight of the 28 EU/EEA reporting countries (ECDC, 2013). LA-MRSA (livestock-associated MRSA) have been found in i) pigs and in pigs production chain (Beneke et al., 2011); ii) dairy sheep (Carfora et al., 2016); iii) cows and in cow's milk (Chlotter et al., 2012); among others. LA-MRSA are reported as having lower transmissibility compared with other MRSA genotypes (not associated with livestock). Recently, Alen et al. (2016) reported the course of the LA-MRSA CC398 epidemic among patients of the University Hospital Münster. Overall, this strain emerged rapidly during the last decade, developed enormous sublineage diversity and contributed substantially to the total burden of MRSA colonization and infection at the hospital. These findings were also reported by Hadjirin et al. (2015) who reported that UK pig farms are a potential pathway for the transmission of LA-MRSA CC398 from livestock to humans. Recently, Bosch et al. (2016) studied the Next Generation sequencing data of 539 LA-MRSA isolates obtained from humans (n=206) and from nosocomial infections (n=333) and confirmed that transmission of LA-MRSA in Dutch healthcare facilities does occur between humans; therefore, a decision to discontinue the search and destroy policy for LA-MRSA should be taken with caution.

HA-MRSA are frequently associated with people who have been in hospitals or other health care settings and risk factors include the immunocompromised and the elderly, intensive care unit admission, surgery, dialysis, presence of invasive medical devices, history of broad-spectrum antibiotic use. These patients are older and have one or more comorbid conditions. HA-MRSA strains tend to cause pneumonia, bacteremia, and invasive infections

(David and Daum, 2010). With respect to CA-MRSA infections they commonly affect healthy younger patients who lack established risk factors and have significant skin and soft tissue infections as well as severe and invasive staphylococcal and musculoskeletal infections (David and Daum, 2010). CA-MRSA has been emerging and disseminated in many countries and is actively considered an important clinical pathogen. In fact, new successful lineages have been reported; the burden of CA-MRSA infections varies with the group of individuals and the region of the world. In addition, CA-MRSA strains are often more susceptible to 'traditional' antibiotics although this is considered a dynamic and changing situation that must be prevented and controlled (Skov et al., 2012). The prevalence of CA-MRSA in USA is higher than in Europe however, serious CA-MRSA infections in young and healthy individuals have been reported and considered a public health concern (Mera et al., 2011). Given the difficulty in establishing a clear delineation between CA-MRSA and HA-MRSA strains, a new category of MRSA infections - "health care-associated, community-onset" MRSA have been reported, molecular tools being the best way to distinguish them (Wang et al., 2015; Parisi et al., 2016).

Compared to methicillin-susceptible *S. aureus* (MSSA), MRSA strains are generally recognised as being responsible for higher morbidity and mortality rates, length of hospital stay, and costs (VanEperen and Segreti, 2016). Nevertheless, MSSA strains are also responsible for epidemics cases especially in neonates (Williams et al., 2011) and some of them might have resistance to vancomycin and daptomycin increasing the risk for further complications (San-Juan et al., 2016).

Staphylococcal food poisoning –Outbreaks

The diagnosis of SFP is established by the detection of staphylococcal enterotoxin (SE) in food consumed by patients and is identical regardless of whether intoxication is caused by MRSA or MSSA (EFSA, 2009). However, there are other factors that support the diagnosis: (i) the presence of enterotoxin together with large numbers of organisms in *vomitus* (ii) the presence of enterotoxin producing *S. aureus* in the studied food ($>10^6$/g) (iii) the presence of *S. aureus* strain in faeces of affected patients following intoxication (EFSA, 2009). In some cases, confirmation of SFP is not possible by presence of *S. aureus* enterotoxigenic strains because the organism is only rarely isolated in implicated food and enumeration of

CPS (Coagulase Positive Staphylococci) is difficult. In addition, staphylococcal enterotoxins are resistant to environmental conditions (freezing, drying, heat treatment and low pH) that easily destroy the enterotoxin-producing strain (Hennekinne et al., 2012). Hence, conclusive diagnosis is mainly based on the demonstration of SEs in the food.

Studies on SEs began from the analysis of *S. aureus* strains involved in outbreaks. The first well-documented report that clearly identified SES as a cause of SFP outbreaks was done in 1930 (Dack et al., 1930). Over time, several outbreaks concerning *S. aureus* have been reported, worldwide. Table 1 illustrates some recently reported outbreaks.

Fetsch et al. (2014) reported a *S. aureus* food-poisoning outbreak associated with the consumption of ice-cream. Since none of the employees carried the outbreak strain, according to these authors, either the equipment used for the production of the ice-cream or a contaminated ingredient was the most likely introduction source. Solano and his collaborators (2011) described an outbreak that occurred in 2011 at a Barcelona sports club. They observed at least 8 *S. aureus* strains that showed the same profile and staphylococcal protein A (spa) type (t008) revealing that the source of transmission of the contamination was the same.

Table 1. Recent *Staphylococcus aureus* food poisoning outbreaks.

Year	Implicated Food	SE detected	Number of cases	Country	Local of outbreak	Reference
2007	Thermized goat milk cheese	*egc* cluster	5 (4 hospitalized)	Switzerland	Store	(Johler et al, 2015a)
2011	Macaroni and fresh tomato	SEA and SED	42 (20 hospitalized)	Spain	Restaurant Summer school	(Solano et al, 2013)
2012	"Kerala matta" rice	unknown	46	India	Nursing hostel	(Basavegowda et al, 2014)
2013	Ice-cream	SEA	13 (7 hospitalized)	Germany	Christening party at hotel	(Fetsch et al, 2014)
2013	Pancakes with minced chicken	SEA, TSST-1, *egc* cluster	12 (4 hospitalized)	Germany	Wedding celebration catering	(Johler et al, 2013)
2014	semi-hard goat cheese	*egc* cluster	5	Switzerland	Store	(Johler et al, 2015)
2014	raw-milk cheese	SEA and SED	14 (including 10 children)	Switzerland	Swiss boarding school	(Johler et al, 2015b)
2014	Packaged meals	SEA and SED	27 (4 hospitalized)	Australia	Tourists travelling	(Fletcher et al, 2015)

According to the most recent European Food Safety Authority report, in 2014, 12 member states reported 393 food-borne outbreaks caused by staphylococcal toxins representing 7.5% of all outbreaks (EFSA, 2015). There was a small increase when compared with former years. The overall reporting rate in the EU was 0.12 per 100,000 population, the majority of

cases occurring in France. In total, 2 deaths occurred and 264 persons were hospitalized (EFSA-ECDC, 2015). In the same year it several food vehicles were reported as being related with outbreaks caused by staphylococcal toxins in the EU (EFSA, 2015). Mixed foods caused the major number of outbreaks with 9 among 31 total outbreaks (29.0%). Broiler meat and pig meat had equal numbers of outbreaks (3) followed by cheese, dairy products, fish and fish products, vegetables and juices and bakery products with 2 outbreaks each. With the exception of vegetables and fruits or drinks including bottled water, all outbreaks came from protein-rich food which is in accordance with the study of Crago and his collaborators in Canada (Crago et al, 2012). In 2000 in Japan, a large outbreak concerning *S. aureus* was studied; 13,420 victims were reported and the food vehicle was powdered skim milk (Asao et al., 2003). In 2002, Jones et al., firstly reported an outbreak caused by community-acquired MRSA stains responsible of gastrointestinal illness. In this case study, a food handler, a food specimen, and three ill patients were culture positive for the same toxin-producing strain of MRSA. More recently, Kassis et al. (2011) reported an outbreak of CA-MRSA skin infections among health care workers in a cancer center.

Presence of virulence factors on *Staphylococcus aureus*

S. aureus virulence factors -an overview

Staphylococcus aureus strains possess a variety of virulence factors which are responsible for the potential of a given strain to cause disease. With respect to staphylococci a given virulence factor may have several functions in pathogenesis and also multiple virulence factors may be responsible for the same function (Table 3). The virulence factors can be divided into structural and secreted products. The structural products are surface proteins, called “microbial surface components recognizing matrix molecules” (MSCRAMMs) that mediate adherence to host tissues (Gordon and Lowy, 2008). These proteins bind molecules such as collagen, fibronectin and fibrinogen and different MSCRAMMs may adhere to the same host-tissue component (Gordon and Lowy, 2008). The most common staphylococcal proteins anchored in the cell wall are proteins with affinity to fibrinogen (i.e. clumping factors A and B, encoded by the *clfA* and *clfB* genes, respectively), fibronectin (*fnbA*), collagen *(cna*), sialoprotein (*bbp*), elastin (*ebpS*) and adhesins with unknown function (*sdrC*

and *sdrE*) (Jonsson et al., 1991). Another MSCRAMM is protein A. Most clinical isolates present the SpA (staphylococcal protein A) that is encoded by the *spa* gene and is associated with the disruption of the humoral immune response in mice (Pauli et al, 2014) . SpA contains four or five immunoglobulin-binding domains capable of binding the Fc of IgG antibodies; binding of Fc on the cell surface of staphylococci has long been recognized as a strategy to mask underlying surface antigens and inhibit opsonophagocytic killing of staphylococci by PMNs (Kim et al, 2012). Also SpA binds to Fab of Variable Heavy 3 (VH3) idiotype antibodies (Pauli et al, 2014). These facts demonstrate stimulation of B lymphocyte proliferation provoking their clonal expansion and subsequent death (Kim et al, 2012). The VH3-family of immunoglobulin idiotypes represents the largest portion of VH genes in B cell populations in humans suggesting a mechanism of *S. aureus* immune evasion by depletion of the B cell repertoire (Pauli et al, 2014).

The antiphagocytic microcapsule production (i.e. type 5 or 8 frequently found in most clinical isolates) is known to enable evasion of the host immune system during an infection (Gordon and Lowy, 2008).

The secreted products by *S. aureus* consist of numerous enzymes and toxins that interfere directly with the host. During infection, secretion of enzymes such as proteases, lipases and elastases that enable invasion and destruction of host tissues and metastization to other sites (Gordon and Lowy, 2008). In addition, nucleases produced by *S. aureus* can interfere negatively with the antibacterial activity of neutrophils (Otto, 2014). However, these mechanisms are poorly understood in *S. aureus* pathogenesis (Otto, 2014).

The *S. aureus* toxins are divided into: (i) membrane-damaging toxins (ii) toxins that can interact with the receptor function, and (iii) enzymes that are secreted (Otto, 2014). The first ones act on cytoplasmic membranes causing pore formation leading to the removal of vital molecules and metabolites, and are therefore considered to be cytolytic. These toxins could be receptor mediated such as hemolysins and leukotoxins whether they act on red or/and white blood cells or non-receptor mediated such as PSMs (phenol-soluble modulins; Otto, 2014). Hemolysin - α is a well-known *S. aureus* toxin of 33 kDa, with pore-forming and pro-inflammatory properties (Otto, 2014; Grumman et al, 2014). Hla facilitates the efflux of mono- and di-valent ions (Otto, 2014). It is lytic to red blood cells and a series of leukocytes but not neutrophils (Otto, 2014). Other toxins structurally similar to α -toxin, are bi-component toxins such as Panton-Valentine Leukocidin or PVL (lukS and lukF proteins),

Leukocidins lukDE and lukAB and gamma-hemolysin (Hlg A, Hlg B and Hlg C; Otto, 2014). The bi-component (hetero-oligomeric) pore-forming leukotoxins can lyse cells such as monocytes, macrophages and neutrophils which are considered important for *S. aureus* immune evasion. Gamma-hemolysin is produced by virtually every strain of *S. aureus*.

Panton-Valentine Leukocidine or PVL is a *S. aureus* toxin frequently associated with skin and soft tissue infections (SSTIs) like abscess formation or furunculosis and also severe necrotizing pneumonia (Grumman et al, 2014; Watkins et al, 2012). These diseases are frequently associated as occurring outside the hospital setting (Grumman et al. 2014). In fact, since PVL was first associated with SSTIs in 1932 by Panton and Valentine, numerous studies also associated PVL-producing strains with chronic or recurrent SSTIs and with CA-MRSA (Watkins et al, 2012). However, this issue is controversial regarding the pathogenic role of PVL (Watkins et al, 2012; Grumman et al, 2014). In addition, it has been noted that lukDE and lukGH, similar to PV, are also expressed by the majority of CA-MRSA (Grumman et al. 2014). However, the relative contribution to community-acquired SSTI and necrotizing pneumonia remains unknown (Grumman et al, 2014).

Toxins non-receptor mediated, such as PSMs are small amphipathic peptides with detergent-like properties (Otto, 2014). One of these is hemolysin δ (Hld) which is reported as having multiple functions in the pathogenesis of *Staphylococcus*; it contributes to atopic dermatitis by inducing cell degranulation (Otto, 2014). PSMs as well as α-toxin are produced by most *S. aureus* strains in contrast to many bi-component leukocidins (Otto, 2014). The PSMα peptides are responsible for neutrophil lysis after phagocytosis.

Toxins that interfere with receptor function (other than membrane damaging) are enterotoxins. Enterotoxins are secreted toxins of ~20-30 kDa that interfere with gastro-intestinal function and typically cause emesis and diarrhoea (Otto, 2014). They are considered superantigens (SAgs). Even at low concentrations, they stimulate human T-cells (Grumman et al, 2014). SEs, as SAgs act on dependence on Vβ elements of T-cell receptors and in association with the histocompatibility complex on antigen-presenting cells, activating a vast number of T cells (Hu and Nakane, 2014). After that, a cytokine release and systemic shock is observed (Hu and Nakane, 2014). Originally, the SAgs of *S. aureus* were termed staphylococcal enterotoxins (SEs) because they elicit vomiting and diarrhoea after oral uptake, the hallmarks of *S. aureus* food poisoning (Grumman et al, 2014). However, some of the SAgs recently identified, lack the emetic properties being considered a

difference in their superantigenicity (Grumman et al, 2014). In 2004, Lina and collaborators defined that only those toxins that were classified as emetic should be designated as SEs. Other toxins that lack the emetic capacity or have not been tested in the model after oral administration, should be designated "staphylococcal enterotoxin-like" (SEl) Sags to indicate that their potential role in SFP has not been confirmed (Lina et al, 2004). So far, 24 different staphylococcal SAgs have been described: the staphylococcal enterotoxins A-E, G-J and R-T (SEA-SEE, SEG-SEJ, SER-SET), the staphylococcal enterotoxin-like toxins K-Q and U-X (SE*l*K-SE*l*Q, SE*l*U-SE*l*X and TSST-1 (Grumman et al, 2014). They are encoded mainly by mobile genetic elements (MGEs) such as bacteriophages, plasmids, *S. aureus* pathogenicity islands (SaPI) and transposons (Grumman et al. 2014). The numerous locations for SE /SEl genes and TSST-1 as well as biological characteristics are described in Table 2. The emetic activity was determined on a non-human primate model (*Macaca mulatta*) to develop human-like enterotoxigenic disease and house musk shrew, *Suncus murinus* as a small animal model used for emetic response to various emetic drugs (Hu and Nakane, 2014).

Table 2. Major characteristics of Staphylococcal enterotoxins and staphylococcal enterotoxins-like toxins and TSST-1

Toxin	Genetic element	Molecular Weight (kDa)	Super-antigenic activity	Emetic activity[a, *] Monkey[b]	Suncus[c]
SEA	Prophage	27.1	+	25	0.3
SEB	Chromosome, SaPI, plasmid	28.4	+	100	10
SEC1	SaPI	27.5	+	5	NE
SEC2	SaPI	27.6	+	NE	1000
SEC3	SaPI	27.6	+	<50	NE
SEC bovine	SaPI	27.6	+	-	-
SEC sheep	-	27.5	+	-	-
SEC goat	-	27.6	+	-	-
SED	Plasmid (pIB485)	26.9	+	NE	40
SEE	Prophage	26.4	+	NE	10
SEG	*egc*, Chromosome	27.0	+	160-320	200
SEH	Transposon	25.1	+	30	1000
SEI	*egc*, Chromosome	24.9	+	300-600	1
SElJ	Plasmid (pIB485, pF5)	28.6	+	NE	NE
SElK	SaPI	25.3	+	NE	NE
SElL	SaPI	24.7	+	Not emetic	NE
SElM	*egc*, Chromosome	24.8	+	NE	NE
SElN	*egc*, Chromosome	26.1	+	NE	NE
SElO	*egc*, Chromosome	26.8	+	NE	NE
SElP	Prophage	26.7	+	NE	50

	(Sa3n)				
SElQ	SaPI	25.2	+	Not emetic	NE
SER	Plasmid (pIB485, pF5)	27.0	+	<100	<1000
SES	Plasmid (pF5)	26.2	+	<100	20
SET	Plasmid (pF5)	22.6	+	<100	1000
SElU	*egc*, Chromosome	27.2	+	NE	NE
SElV	*egc*, Chromosome	27.6	+	NE	NE
SElX	Chromosome	19.3	+	NE	NE
SEF or TSST	SaPI	22.0	+	Not emetic	-

+: positive reaction; NE: not examined; -: no results available; * adapted from Hu and Nakane (2014). µg/animal; [b.] Oral administration; [c.] Intraperitoneal administration

SEs and SEls are short secreted proteins, biochemically and structurally similar, soluble in water and saline solution (Hu and Nakane, 2014). SEs possess extraordinary stability in denaturing conditions, such as heat and low pH, and resistance to most proteolytic enzymes, such as pepsin or trypsin (Grumann et al, 2014; Bhatia and Sahoor, 2007). SEs are not completely destroyed by mild cooking, only with decreased potency by prolonged boiling or autoclaving, and ability to keep their activity in the digestive tract after ingestion (Bhatia and Sahoor, 2007; Hu and Nakane, 2014).

Toxic shock syndrome toxin (TSST), commonly in *S. aureus* superantigen with 22kDa, also lacks emetic properties (Grumman et al, 2014). TSS is characterized by high fever, rash, desquamation, vomiting, diarrhoea and hypotension, frequently resulting in multiple organ failure. In TSS, *S. aureus* is usually localized, either at mucosal sites (vagina or nasophryx) or abscesses, but the released SAgs act systemically, triggering large number of T-cells to produce massive amounts of pro-inflammatory cytokines (Grumman et al, 2014).

The secreted enzymes by *S. aureus* are responsible for the degradation of the host molecules or for interfering with the host metabolism (Otto, 2014). As examples it is possible to find

toxins ETA, ETB, ETC encoded by *eta, etb, etc* genes, respectively (Grumman et al, 2014). These toxins are responsible for cleaving the desmosomal cadherins of the superficial skin layers leading to a syndrome known as staphylococcal scalded skin (SSSS), a severe skin disease with rash, blisters, and severe lesional damage.

S. aureus can form biofilms (slime) on host and on foreign materials like catheters, prosthetic joints and environmental or food-processing surfaces, enabling it to persist by evading host defences and antimicrobials (Watkins et al, 2012; Gordon and Lowy, 2008; Vázquez-Sánchez et al, 2013). Biofilms are surface-attached communities of cells encased in an extracellular polymeric matrix where enclosed cells are dormant and are refractory to antibiotic therapy (Watkins et al, 2012). Biofilm formation occurs in multiple steps: starting with adherence of the bacteria through MSCRAMMs and followed with proliferation of the bacteria and accumulation into a biofilm through intercellular adhesion (Watkins et al, 2012). The transition between planktonic and biofilm stages is promoted by polysaccharide intercellular adhesion (PIA) encoded by the *ica* ADBC operon however, this transition through quorum sensing is encoded by another operon, the *agr* (Watkins et al, 2012).

Clinically, endovascular infections, bone and joint infections and prosthetic-device infections are particularly initiated by MSCRAMMs (Gordon and Lowy, 2008). Once *S. aureus* adheres to host tissues or prosthetic materials it is able to grow and persist in various ways (Gordon and Lowy, 2008).

Regulation of expression of Staphylococcal virulence factors is observed to reduce undue metabolic demands; only occurring when required by the bacterium (Gordon and Lowy, 2008). A critical role in the regulation of Staphylococcal virulence is attributed to the accessory gene regulator (*agr*) a quorum-sensing system. Hence, during infection it is necessary to express early MSCRAMM proteins (during logarithmic growth) to facilitate initial colonization of tissue sites and later (stationary phase) to secrete toxins to facilitate the spread of the bacterium (Gordon and Lowy, 2008).

Antibiotic resistance

Antibiotic resistance has become a major clinical and public health problem. Antibiotics such as beta (β)-lactams (penicillins and cephalosporins, carbapenems), aminoglycosides

and macrolides were first used to treat Staphylococcal infections (Ito et al., 2003). As stated above, methicillin, a form of penicillin, was introduced to counter the increased resistance of *S. aureus* to (β)-lactams. Penicillin and other beta-lactams act as substrate analogs preventing cell wall synthesis (Nour et al. 2005). Thus, in the presence of a beta-lactam, the sensitive bacteria have a weakened wall and are unable to resist osmotic shock (Nour et al. 2005). Beta-lactam antibiotics bind irreversibly to proteins called penicillin-binding proteins (PBPs) that catalyze the transpeptidisation reaction that cross-links the peptidoglycan of the bacterial cell wall (Chambers, 1997). Four major PBPs, PBPs 1,2,3,4, are produced by both susceptible and resistant strains of *S. aureus* (Chambers, 1997). The PBPs 1-3 which have affinity for most beta-lactam antibiotics, are essential for cell growth and for the survival of susceptible strains and so binding of beta-lactams by these PBPs is

Table 3. *Staphylococcus aureus* virulence factors and associated clinical syndromes (Adapted from Gordon and Lowy, 2008).

Selected factors	Genes	Function of virulence	Associated clinical syndromes	Reference
Proteases, lipases, nucleases	-	Involved in tissue invasion/penetration	Tissue destruction and metastatic infections	Otto, 2014
MSCRAMMs (protein with affinity to fibrinogen, fibronectin, collagen, sialoprotein elastin, other adhesins)	*clfA, clfB, fnbA, cna, bbp, ebpS, sdrC, sdrE*	Involved in adherence to host tissues	Endovascular, bone and joint and prosthetic-device infections	Gordon and Lowy, 2008
Biofilm accumulation (PIA)	*ica* locus	Involved in persistence	Evasion host defences and antimicrobials	Watkins et al, 2012
Membrane-damaging toxins – hemolysins (α), leukocidins (PVL, ɣ-toxin, lukDE and lukAB)	*Hla, lukDE, lukAB, Hlg lukS-*P*V* and *lukF-PV*	Destruction of host defences	SSTIs, abscess formation and necrotizing pneumonia	Grumman et al, 2014
Toxins not membrane-damaging (PSMs)	*psm- α* gene cluster	Destruction of host defences		(Otto, 2014)
SAgs toxins (enterotoxins, TSST-1)	*sea-q, tst*	Stimulation of host immune defences	SFP, TSS	Grumman et al, 2014
Secreted enzymes (exfoliative toxins)	*eta, etb, etc*	Cleavage of skin tissue	SSSS	Grumman et al, 2014

lethal (Chambers, 1997). In methicillin-resistant cells, the *mec*A gene encodes PBP 2a which with its low affinity for binding beta-lactam antibiotics, substitutes the essential functions of high affinity PBPs at concentrations that are otherwise lethal (Chambers, 1997).

The *mec*A gene is located on the Staphylococcal cassette chromosome (SCC), a mobile genetic element, designated as SCC*mec* (Hanssen and Sollid, 2006). SCC*mec* (21-67kb) is inserted at the *attB* site of MRSA strains (Plata et al, 2009; Becker et al, 2014). SCC*mec* elements are classified according to the type of recombinase they carry and their general genetic composition (Hanssen and Sollid, 2006). Three basic components comprise SCC*mec*: the *mec* gene complex, the *ccr* gene complex and the joining regions, (J) regions (Becker et al, 2014). The *mec* gene complex comprises the structural *mec*A gene itself and when present, its regulatory genes (the transcriptional repressor MecI, the sensor/transducer MecR1) and the insertion sequence, IS431mec (Becker et al, 2014). The SCC recombinase genes (*ccrAB* and *ccrC*) encode site-specific integrases. They catalyze the integration or excision of the entire SCC*mec* into the *orfX* locus at its 3'-end of the staphylococcal genome (Becker et al, 2014). The joining regions, (J) regions, vary in length and composition and contain diverse resistance, virulence and other (pseudo-) genes (Becker et al, 2014).

Until now eleven SCC*mec* types have been described in *S. aureus*, ranging in size between 20 to 60 kb (Shore and Coleman, 2013). Each SCC*mec* type is designated by a Roman numeral with a unique combination of the *mec* and *ccr* gene complex (IWG-SCC, 2009; http://www.sccmec.org). To date, in MRSA, four classes of *mec* gene and seven of *ccr* gene complexes have been described (Shore and Coleman, 2013).

Another mechanism involved in resistance to beta-lactams is production of beta-lactamase, an inducible enzyme encoded by the *blaZ* gene carried by a plasmid (Nour et al. 2005). This enzyme hydrolyses penicillin G and its structural analogues (Nour et al. 2005). Expression of *blaZ* is controlled by two genes, *blaR1-blaI* upstream and transcribed in the opposite direction *blaZ* (Nour et al. 2005). The expression of PBP 2a depends on at least two regulator systems acting at the transcriptional level: the gene system *mecI* and *mecR1* located upstream of the *mec*A gene and the *bla1* and *blaR1* system located upstream of the *blaZ* gene of penicillinase (Nour et al. 2005). The *mecI-mecR1* system exerts transcriptional repression on *mec*A greater than that exerted by the *blaI-blaR1* system. It seems that the majority of MRSA have a non-functional *mecI-mecR1* system either by deletion of these genes either by

point mutation. The *blaI-blaR1* system then takes control of the *mec*A gene inducing the transcription (Nour et al. 2005).

Methicillin and resistance to other beta-lactams in Staphylococci is also due to *mec*B and *mec*C (*mec*A gene homologous) and not only to the *mec*A gene (Becker et al, 2014). *mec*B is present in a non-staphylococcal species (*Micrococcus* species) and it is possibly an explanation for the origin of *mec*-based beta-lactam mechanism (Becker et al, 2014). *mec*C (previously $mecA_{LGA251}$ gene) has been associated with its integration in SCC*mec* type XI and with its occurrence in livestock and humans (Becker et al, 2014). At the moment, all isolates were detected in Europe (Becker et al, 2014).

Recently, new data suggest that penicillin susceptibility may be in a period of renaissance (Cheng et al., 2016). These authors observed that more than one-quarter of patients with MSSA bacteremia potentially could be treated with parenteral penicillin, which may offer pharmacokinetic advantages over other beta-lactam drugs and potentially improved outcomes.

Recently, Kong and his collaborators (2016) reported a synergetic effect between CPZ (Chlorpromazine) and β-lactam antibiotics that affect the susceptibility of MRSA strains to β-lactam antibiotics by perturbation of the cell membrane of MRSA.

Vancomycin is the antibiotic used in severe/invasive infections by MRSA strains. Alternative therapies have been reported, namely treatment with linezolid in pneumonia and severe skin cases and daptomycin for MRSA bacteremia and endocarditis as well as glycopeptides (Farrell et al., 2014). In addition, other alternatives to vancomycin have also been studied such as ceftaroline, ceftobribole, dalbavancin, oritavancin and tedizolid (VanEperen and Segreti, 2016). Fang et al., 2016 reported a 100% vancomycin susceptibility rate of MRSA strains (n=743) collected from Stockholm, Sweden in 2014. Nevertheless, resistance to vancomycin by MRSA strains has becoming a problem and was recently described (Sievert et al., 2006; Liu et al., 2011; Melo-Cristino et al., 2013). Rare in the beginning but with a considerable susceptibility nowadays, vancomycin resistant and intermediate vancomycin resistant *S. aureus* (VRSA and VISA, respectively) strains have been reported (Howden et al., 2010; Saravolatz et al., 2010). The irrational use of vancomycin to treat infections caused by MRSA, the immune status of the patient, the medical and surgical procedures and the prevalence of healthcare workers infected with

MRSA, might explain this tendency and enhancement (Howden et al., 2010; Castro et al., 2016b).

Oritavancin, a semisynthetic lipoglycopeptide, demonstrated excellent activity against VRSA, VISA and MRSA strains and was recently approved for the treatment of acute Gram-positive skin and skin structure infections (Saravolatz et al., 2010; Brade et al., 2016).

Biocontrol and *S. aureus*

Alternatives to antibiotics have been discussed, namely the use of plant extracts, bacteriocins and phages in *S. aureus* therapy. Horváth et al. (2016) suggested as a promising solution to minimize/control the rising antibiotic resistance problem, the combination of plant secondary metabolites and antibiotics. The effect of bacteriocins against *S. aureus* MR23, a clinically isolated MRSA strain, has been recently reported by Okuda et al., (2016). Nisin A was shown to be effective against the MRSA strain and other staphylococcal biofilms. Nagao (2009) also discussed the potential of Nisin against MRSA and VRSA strains. Aunpad and Na-Bangchang (2007) described a novel bacteriocin, pumilicin 4, produced by *B. pumilus* that has potential for use as an alternative antibacterial agent for the treatment of infection with MRSA and VRE. The use and the efficacy, safety, and commercial viability of phages to treat *S. aureus* infections using the commercially available phage SATA-8505 was addressed by Pincus et al. (2015). These authors reported that SATA-8505 effectively controlled *S. aureus* growth and reduces bacterial viability both *abscess* and in a skin infection mouse model. This killing effect, however, was not observed when phage was cultured in the presence of human whole blood.

Bacteriophages, regarded as natural antibacterial agents in food, are a promising tool for the biocontrol of pathogenic bacteria however, the correlation between phage resistance and lysogeny in *S. aureus* strains was found to be weak (Gutiérrez et al., 2016). Previously, García et al., (2009) showed that inhibition of *S. aureus* by phages was dependent on the food matrix. Bueno et al. (2012) reported phage inactivation of *S. aureus* in fresh and hard-type cheeses. Two obligate lytic bacteriophages were tested, vB_SauS-phi-IPLA35 and vB_SauS-phi-SauS-IPLA88. At the end of ripening, 1.24 log CFU/g of the staphylococcal strain was still detected in test cheeses whereas 6.73 log CFU/g was present in control

cheeses. Miao and his collaborators (2016) studied the effect of peptide F1, a novel antimicrobial produced by *Lactobacillus paracasei subsp. tolerans* FX-6, against the pathogen *S. aureus*. They observed a bactericidal effect represented by bacterial damage of the cell membranes and by the disruption of the genomic DNA leading to rapid cell death.

The activity of five bacteriocins produced by *Bacillus thuringiensis* against *S. aureus* associated with bovine mastitis, was evaluated by Barboza-Corona et al. (2009); all the tested isolates of *S. aureus* showed susceptibility to the five bacteriocins synthesized: morricin 269, kurstacin 287, kenyacin 404, entomocin 420 and tolworthcin 524.

Recently, Shi et al. (2017), reported the combination of nisin and cinnamaldehyde that could not only be used as a promising naturally sourced food preservative, but also to reduce the problem of bacterial resistance.

According to Manikprabhua et al. (2016), good antimicrobial activity was observed in the metabolite produced by the novel species *S. mesophila* MPKL 26, synthesized silver nanoparticles, against multi drug resistant *S. aureus*. Also, silver nanoparticles produced from *Trichoderma harzianum* (an agriculturally beneficial fungus), were tested against *S. aureus* and a reduction of bacterial growth was found, depending on the dose administered (Ahluwalia et al., 2014).

The role of probiotics in the prevention and treatment of clinical MRSA infections was reviewed by Sikorskaa and Smoragiewicz (2013). They considered that the effects of probiotics were mediated both by direct cell competitive exclusion as well as production of acids or bacteriocin-like inhibitors. Nevertheless, many strains of lactobacilli and bifidobacteria isolated from a variety of sources inhibited the growth of *S. aureus* and clinical isolates of MRSA *abscess*.

Fan et al (2016) obtained a recombinant endolysin of *S. aureus* bacteriophage IME-SA called trx-SA1 and observed that this recombinant endolysin could effectively treat dairy cow mastitis and might be an alternative treatment strategy for infections.

Preventing *S. aureus* – other than the use of antibiotics

Strict infection control strategies to prevent the spread of multidrug-resistant outbreaks are costly, difficult to implement and require human and laboratory resources. The estimation of the costs is challenging and lack of information is a concern. Birgand et al. (2015) studied 13 outbreak cases in which 5 were related to MRSA. According to these authors, costs associated with strict measures are highly variable varying with the organism and the location. They also state that the loss of income due to the interruption of admissions represents the main cost to prevent these outbreaks.

Concerning the clinical trials, Chauveaux (2015) referred that impeccable surgical technique and operating room behavior, are essential to prevent surgical-site infections (e.g. laminar airflow and high-efficiency particulate air filters and body exhaust suits). Kelly et al. (2016) studied the recent WHO Guidelines on Hand Hygiene in Health Care and observed improvements in the decrease in methicillin-resistant *S. aureus* infection rates; 23 inpatient units over a 33-month period were studied. Prophylactic vaccination to prevent *S. aureus* infections, are actively being pursued in healthcare settings (Jansen et al., 2013); however, no vaccine approach has been successful thus far.

With respect to the food industry, new food preservation technologies are increasingly gaining interest, namely high pressure processing. Recently, Baptista et al. (2016), reported that monomeric proteins such as staphylococcal SE are not affected by HPP, and strains with SE appear to be more efficiently inactivated than those without.

Pulsed electric fields inactivation was studied by Wang et al. (2016). *S. aureus* changed its membrane fluidity via fatty acids to become less or more fluid and PEF inactivation was shown to be dependent on membrane structure, field strength and treatment time.

Gustafson et al. (2014) reported the need to study *S. aureus* growth in food utilizing the modern “omics” tools. According to them, maintaining appropriate holding temperatures by handlers is imperative and consumers must know how to store and cook food properly. In addition, they also agree that novel production and packaging procedures might prevent enterotoxin production.

Finally, and more focused on the animal trials, a number of potential vaccines are in development and in investigation (Spellberg and Daum, 2012; Schukken et al., 2014). The

efficacy of vaccination on *S. aureus* and coagulase-negative staphylococci intramammary infection dynamics in 2 dairy herds has recently been investigated (Schukken et al., 2014). These authors demonstrated that vaccine efficacy was dependent upon the age group of the animals and on the farm management practices, emphasizing that vaccine utilization will need to be combined with excellent milking procedures, culling of known infected cattle, and other management procedures to effectively reduce incidence and duration of infection. Host immune response to *S. aureus* vaccination needs additional investigations, including how differences among *in vivo* models may influence vaccine development (Scali et al., 2015).

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Chapter 2

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ABSTRACT

Staphylococcus aureus represents a public health challenge worldwide. The aim of this study was the characterization of different food isolates of *S. aureus* on the basis of their production of enterotoxins, hemolysins and resistance to antibiotics. A total of 148 coagulase-positive staphylococcal strains isolated from different food origins were identified to the species level. By multiplex PCR, 69% of the isolates were shown to be enterotoxigenic (SEs); the most common were *sea seg*, *sea seg sei* and *seg sei*. According to CLSI [CLSI, Clinical and Laboratory Standards Institute, 2007. Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement. CLSI document M100-S15. Clinical and Laboratory Standards Institute, Wayne, PA], 38% of the isolates were resistant to oxacillin (≥6 μg/mL; MRSA positives) but only 0.68% showed the presence of *mecA* gene. 70 and 73% of the *S. aureus* strains were resistant to β-lactams, ampicillin and penicillin, respectively. The virulence pattern was demonstrated to be origin and strain dependent. These findings emphasise the need to prevent the presence of *S. aureus* strains and SEs production in foods.

1. Introduction

Staphylococcus aureus is a common pathogen associated with serious community and hospital acquired diseases and has for long been considered as a major problem of Public Health (Pesavento et al., 2007). Some strains of this organism can cause food-poisoning by production of enterotoxins (SEs) when growing in foods; SEs have been divided into different serological types initially SEA through SEE and later the existence of new types of SEs have also been reported (Monday and Bohach, 1999; Omoe et al., 2005; Chiang et al., 2006; Chiang et al., 2008).

Most of the nosocomial *S. aureus* infections are caused by methicillin-resistant *S. aureus* (MRSA) strains and have become a widely recognized cause of morbidity and mortality throughout the world (Ardic et al., 2006; Pesavento et al., 2007; Ho et al., 2008). In addition, MRSA strains resistant to quinolones or multiresistant to other antibiotics have been emerging, leaving a limited choice for their control (Mee-Marquet et al., 2004; Nejma et al., 2006; Pesavento et al., 2007).

Several virulence factors implicated in the pathogenesis of *S. aureus* strains, have been described in the literature (Kérouanton et al., 2007; Normanno et al., 2007; Vancraeynest et al., 2007) such as thermonuclease, hyaluronidase, lipases and hemolysins (Sandel and McKillip, 2004; Kuroda et al., 2007), which are involved in tissue invasion of the host cells. Perhaps the most notable virulence factors associated with this microorganism are the heat-stable enterotoxins (SEs), that cause the sporadic food-poisoning syndrome or food-borne outbreaks, and the toxic shock syndrome toxin 1 (TSST-1), which diminishes the immune response of a colonized host (Tsen et al., 1998; Martin et al., 2003; Sandel and McKillip, 2004; Kérouanton et al., 2007; Vancraeynest et al., 2007). On the other hand, most of the severe *S. aureus* infections are due to the cumulative effects of several virulence determinants (Nejma et al., 2006).

The aim of the present study was the characterization of different *S. aureus* isolates collected from different food origins. This characterization was based on the ability of the isolates to produce and to express staphylococcal enterotoxins, on the antibiotic susceptibilities in order to determine the presence of MRSA strains, and on the presence of other virulence factors. Furthermore, the relationship between the different origins of the isolates and the ability of these isolates to produce virulence factors was also evaluated.

2. Materials and methods

2.1. Bacterial strains and media

From 2006 to 2008, different food products, mainly from the north of Portugal, were submitted to a routine microbiological lab

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(ESBUCP, Porto) for analyses of the presence of coagulase-positive staphylococci on Baird-Parker agar with rabbit plasma fibrinogen (BPA + RPF, bioMérieux, France). Two hundred and six colonies showing the typical appearance of coagulase-positive staphylococci were tested for Gram staining, catalase, coagulase and thermostable DNAse activity (Gündoğan et al., 2006). After this screening, 148 presumptive *S. aureus* isolates from raw meat ($n = 15$), traditional Portuguese fermented meat products ($n = 65$), cheeses ($n = 9$), bovine mastitis (from raw milk samples; $n = 18$), raw cow's milk ($n = 20$) and other food products ($n = 21$) were stored in cryovials at −80 °C in Tryptone Soy Broth (TSB, Pronadisa, Spain) plus 30% v/v glycerol for further characterization. Working cultures were prepared by streaking directly from the cryovials onto Tryptone Soy Broth (TSB, Pronadisa) and incubating at 37 °C for 24 h.

2.2. DNA isolation for PCR

DNA template used for PCR analysis was isolated from the strains by the guanidine-isothiocyanate extraction method (Aires de Sousa et al., 1996).

2.3. Identification by multiplex PCR

The isolates were identified to the species level according to the multiplex PCR developed by Zhang et al. (2004). According to this PCR assay the presence of the target 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific) and *mecA* (a determinant of methicillin resistance) was determined. *S. aureus* DSM 11729 was used as a positive control for gene *mecA*, *Staphylococcus epidermidis* DSM 20044 as a negative control for gene *nuc* and *S. aureus* ATCC 29213 as positive control for targeting 16S rRNA and *nuc* gene and negative for gene *mecA*.

2.4. Detection of enterotoxin production

S. aureus strains were studied for their ability to produce enterotoxins according to the VIDAS methodology and for the presence of the enterotoxin genes by multiplex PCR (Zhang et al., 2004).

2.4.1. VIDAS methodology

The enzyme-linked fluorescent assay (ELFA) using the automated VIDAS instrument was used for the specific detection of Staphylococcal enterotoxins (Staph enterotoxin II, SET 2, bioMérieux) according to the instructions of the manufacturer. In this test, complementary monoclonal and polyclonal antibodies directed to the 5 different staphylococcal enterotoxins SEA, SEB, SEC, SED and SEE are used for the capture and detection process without distinguishing individual toxins. One isolated colony of *S. aureus* was cultured in BHI broth for 24 h at 37 °C. The culture was centrifuged at 7000 *g* for 10 min at 4 °C and 500 µL of the supernatant was then added to the initial VIDAS strip wells and further analysed by the automated method.

2.4.2. Multiplex PCR

The detection of staphylococcal enterotoxin genes in staphylococcal isolates was determined according to Løvseth et al. (2004) for the detection of enterotoxin genes A–E and G–J (Table 1). The amplification of the target 16S rRNA gene was included as the internal control. As positive controls, different strains of *S. aureus* kindly supplied by Prof. Løvseth (National Veterinary Institute, Norway) were used: R2102/00 for *sec*, *seg*, *sei* genes; R4571/00 for *sec* gene, FRI572 for *seg*, *sei* genes; 3169 for *sec*-bovine, *sed*, *sej* genes; FRI472 for *sed*, *seg*, *sei*, *sej* genes; R5371/00 for *sea*, *seg*, *seh*, *sei* genes; R963/00 for *sed*, *seg*, *sei*, *sej* genes; R5460/00 for *seb*, *seg*, *seh*, *sei* genes; FRI913 for *sea*, *sec*, *see* genes; FRI445 for *seg*, *sei* genes; R4071/00 for *seb* gene; R4774/00 as a negative control. The mixes were submitted to a program performed on a thermocycler (Mycycler, BioRad) with an initial denaturation step at 94 °C for 10 min, 31 amplification cycles each with 1 min at 95 °C, 45 s at 62 °C and 1 min at 72 °C followed by an additional extension step of 10 min at 72 °C. PCR products supplemented with ethidium bromide were resolved by electrophoresis in 2% w/v agarose (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 50 V, for 3 h, using 100–1000 bp ladder molecular size markers (BioRad) as standards. DNA patterns were visualized on a UV transilluminator (Gel Documentation System 2000, BioRad).

Table 1
Primers used in this study for detection of SE genes, and target 16S rRNA gene.

Primer[a]	Primer sequence (5′–3′)	Amplified product size (bp)	Multiplex PCR reaction mixture no.[b]
sea forw.	GCA GGG AAC AGC TTT AGG C	521	1
sea rev.	GTT CTG TAG AAG TAT GAA ACA CG		
seb–sec forw.	ACA TGT AAT TTT GAT ATT CGC ACT G	667	1
seb–sec rev.	TGC AGG CAT CAT ATC ATA CCA A		
sec forw.	CTT GTA TGT ATG GAG GAA TAA CAA	284	1
sec rev.	TGC AGG CAT CAT ATC ATA CCA A		
sed forw.	GTG GTG AAA TAG ATA GGA CTG C	385	2
sed rev.	ATA TGA AGG TGC TCT GTG G		
see forw.	TAC CAA TTA ACT TGT GGA TAG AC	171	2
see rev.	CTC TTT GCA CCT TAC CGC		
seg forw.	CGT CTC CAC CTG TTG AAG G	328	2
seg rev.	CCA AGT GAT TGT CTA TTG TCG		
seh forw.	CAA CTG CTG ATT TAG CTC AG	359	1
seh rev.	GTC GAA TGA GTA ATC TCT AGG		
sei forw.	CAA CTC GAA TTT TCA ACA GGT ACC	466	2
sei rev.	CAG GCA GTC CAT CTC CTG		
sej forw.	CAT CAG AAC TGT TGT TCC GCT AG	142	1
sej rev.	CTG AAT TTT ACC ATC AAA GGT AC		
16S rRNA forw.	GTA GGT GGC AAG CGT TAT CC	228	1 and 2
16S rRNA rev.	CGC ACA TCA GCG TCA G		

[a] forw., forward; rev., reverse.
[b] According to Løvseth et al. (2004).

2.5. Antibiotic susceptibility test

The minimal inhibitory concentrations (MICs (µg/mL)) for *S. aureus* strains were determined by the agar dilution method described in the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). The inoculum was prepared from an overnight culture on TSA plates, by suspension in sterile Ringer's

solution in order to obtain turbidity equivalent to 0.5 McFarland standard. The antibiotics investigated were penicillin G (Sigma, Spain), ampicillin (Fluka, Spain), vancomycin, nitrofurantoin, erythromycin, chloramphenicol (Sigma) and oxacillin (BioChemica, Spain); rifampicin, gentamicin, tetracycline and ciprofloxacin were kindly supplied by Labesfal, Portugal. The MIC was determined in Muller Hinton agar (MH, bioMérieux) plus 2% w/v of NaCl in the case of oxacillin, in cation-adjusted MH for penicillin G and ampicillin and in MH to test the other antibiotics investigated. *S. aureus* ATCC 29213 was plated as a control. For each antibiotic susceptibility determination, at least two independent experiments were performed.

2.6. Other virulence factors

Gelatinase activity was detected using a medium with 12% w/v of gelatine (10 g/L Yeast extract, 15 g/L Tryptone, 120 g/L gelatine from bovine skin; Sigma). After overnight growth on TSA, cultures were transferred to tubes containing 4 mL of medium containing gelatine. The tubes were incubated at 30 °C for seven days. If the bacteria did not produce gelatinase the medium remained solid, while the presence of sufficient gelatinase turned the medium liquid even when placed in the refrigerator.

The hemolytic test was performed on blood agar plates (COS, Columbia agar plus 5% v/v sheep blood plates; bioMérieux). The strains were streaked onto the plates and incubated at 37 °C for 1–2 days. The presence or absence of zones of clearing around the colonies was interpreted as β-hemolysis (positive) or γ-hemolysis (negative) activity, respectively. Greenish zones around the colonies were interpreted as α-hemolysis.

For each virulence factor tested, at least two independent experiments were performed.

3. Results and discussion

3.1. Bacterial strains and media

One hundred and forty seven strains were confirmed to be *S. aureus* when the gene *nuc* and the target 16S rRNA were observed to be present simultaneously. The genotypic identification was totally in concordance with the results obtained for the phenotypic characterization namely, the Gram, Catalase and coagulase test and the presence of DNAse activity.

The gene *mecA* which has been reported to be responsible for methicillin resistance (Zhang et al., 2004; Bagcigil et al., 2007; Zaraket et al., 2007; Zhang et al., 2008) was determined during the genotypic identification step. According to our results, only 0.68% (1/148) of the isolates showed the presence of the gene *mecA* (from bovine mastitis).

3.2. Detection of enterotoxin production

3.2.1. VIDAS test

The VIDAS test for enterotoxin production was performed for all the strains; 40% of the tested strains were enterotoxigenic and were mainly isolated from fermented sausages (raw materials and fermented meat products; Table 2). Few of the strains originally isolated from cases of bovine mastitis and from raw milk were demonstrated to be enterotoxigenic by the VIDAS test (1 and 3 isolates, respectively). This methodology is known to be rapid and easy to perform but only detects the expression of staphylococcal enterotoxins SEA–SEE and cannot detect the SEs that have more recently been described by several authors (Kérouanton et al., 2007; Lawrynowicz-Paciorek et al., 2007; Chiang et al., 2008). In addition, this methodology only gives a positive or a negative result concerning the expression of the SE toxins A–E and does not differentiate between them.

3.2.2. SE production and se genes detection

The results of the multiplex PCR analysis of all the 148 strains of *S. aureus* are shown in Table 2. One or more *se* genes were carried by 69% of the isolates; 12% of these isolates possessed one kind of *se* gene, and the remaining 88% more than one *se* gene. Eleven *se* genotypes were observed, the most commonly detected were *sea seg*, *sea seg sei* and *seg sei* with 26, 23 and 25% respectively. The isolates collected from cheeses, raw cow's milk and bovine mastitis, showed a lower incidence of *se* genes. On the other hand, the isolates obtained from fermented meat products showed higher incidence of types of enterotoxins.

The frequent detection of *sea*, *seg* and *sei* genes among *S. aureus* taken from a variety of sources, has already been demonstrated as having an association with food-poisoning outbreaks (Omoe et al.,

Table 2
Enterotoxin genes distribution among *S. aureus* isolates.

	Fermented meat products	Raw meat	Cheeses	Bovine mastitis	Raw cow's milk	Other food products[a]	Total
Total	65 (100%)	15 (100%)	9 (100%)	18 (100%)	20 (100%)	20 (100%)	148 (100%)
VIDAS							
se negative	26 (40%)	6 (40%)	8 (89%)	17 (94%)	17 (85%)	14 (70%)	88 (60%)
se positive	39 (60%)	9 (60%)	1 (11%)	1 (6%)	3 (15%)	6 (30%)	59 (40%)
Genotype							
se negative	16 (25%)	2 (13%)	1 (11%)	10 (56%)	13 (65%)	4 (20%)	46 (31%)
se positive	49 (75%)	13 (87%)	8 (89%)	8 (44%)	7 (35%)	16 (80%)	101 (69%)
sea seg	22 (44%)	1 (7.7%)	–	–	–	3 (19%)	26 (26%)
sea seg sei	17 (34%)	4 (30.8%)	–	–	–	2 (13%)	23 (23%)
seg sei	4 (8%)	4 (30.8%)	5 (62.5%)	5 (63%)	3 (43%)	4 (25%)	25 (25%)
sec-bovine	1 (2%)	–	–	–	2 (29%)	–	3 (3%)
seg	–	3 (23%)	–	2 (25%)	–	6 (38%)	11 (11%)
sea seg seh	4 (8%)	–	–	–	–	–	4 (4%)
sec-bovine *seg*	1 (2%)	–	–	1 (12%)	2 (28%)	–	2 (2%)
seg sei sed	–	–	2 (25%)	–	–	–	2 (2%)
sec	–	–	1 (12.5%)	–	–	–	1 (1%)
sea sec	–	–	–	–	–	1 (5%)	1 (1%)
sed seg sei seh	1 (2%)	1 (7.7%)	–	–	–	–	1 (1%)

–; Not detected.
[a] Other food products (cheeses, bread, kitchen surfaces swab and pie).

2002; Cha et al., 2006; Kérouanton et al., 2007). Our results are in agreement with the study performed by Lawrynowicz-Paciorek et al. (2007) which demonstrated that 74% (39/53) of the isolates of *S. aureus* collected in Poland in the years 2004–2005, from various food products, were enterotoxigenic.

The results obtained for VIDAS SET methodology and for *se* genes detection were compared according to the presence of *sea–see* genotypes determined by PCR. There was a correlation of 80% between the toxin types and the presence of respective genes. Concerning the other 20% of the isolates, one of these conditions was observed: the enterotoxin was expressed during growth but the gene was not detected (6%); the gene was present and no expression was observed (4%) and the VIDAS was positive for the expression of SEA–SEE but the gene detected by PCR was another one (10%). Vernozy-Rozand et al. (2004) described differences in the specificity and in the sensitivity of the assays for the detection of staphylococcal enterotoxins from foods.

3.3. Antibiotic susceptibility test

Food is an important factor for the transfer of antibiotic resistances. Such transfer can occur by means of antibiotic residues in food, through the transfer of resistant food-borne pathogens or through the ingestion of resistant strains of the original food microflora and resistance transfer to pathogenic microorganisms (Khan et al., 2000; Pesavento et al., 2007). *S. aureus* strains are known to be frequently resistant to antibiotic therapy due to their capacity to produce an exopolysaccharide barrier and because of their location within microabscesses, which limit the action of drugs (Gündoğan et al., 2006).

The antimicrobial resistance profile of the tested *S. aureus* strains to different antibiotics was analysed; 15% of the isolates were sensitive to all the tested antibiotics and 51% of the strains were shown to be intermediate (according to CLSI, 2007) and/or resistant to at least 3 antibiotics (data not shown). The isolates collected from bovine mastitis and from raw cow's milk were demonstrated to be the most sensitive to the tested antibiotics (data not shown). No resistance to nitrofurantoin, vancomycin and ciprofloxacin was found. A small percentage of the isolates demonstrated resistance to rifampicin, gentamicin, erythromycin, chloramphenicol, and tetracycline (Table 3). Also resistance to β-lactams such as ampicillin, penicillin and oxacillin was evident 70, 73 and 38% respectively. Previous studies have already discussed the resistance of *S. aureus* to β-lactams (Gündoğan et al., 2005; Kérouanton et al., 2007; Pesavento et al., 2007).

According to CLSI (2007) it was possible to conclude that 38% of the strains were potentially MRSA (MIC $\geq$ 6 μg/mL for oxacillin). However, these results were significantly different from those obtained by PCR, in which the gene *mecA* was detected in only 0.68% of the isolates. According to the CFSPH (2006) report, the definition of MRSA when using antibiotic can overestimate methicillin resistance. Despite the standardized recommendations for the susceptibility testing of MRSA, Lee et al. (2004) demonstrated that some of the isolates that did not carry *mecA* gene were considered phenotypically resistant to oxacillin (MRSA) and that the phenotypic expression of resistance can vary depending on the cellular growth conditions.

In the present work, 19% (28/148) of the tested isolates were both enterotoxigenic and oxacillin positives.

A correlation was observed between the antibiotic resistance profile of the strains isolated from cases of bovine mastitis and from the raw cow's milk; in general, these specific isolates were demonstrated to be resistant to penicillin and ampicillin and more sensitive to the other antibiotics (data not shown) possibly as a result of treating mastitis cases with β-lactams. In the case of isolates from fermented sausages, a higher overall resistance to antibiotics was observed (again possibly from using antibiotics as growth promoters in animal feed).

Table 3
Antimicrobial susceptibility of *S. aureus* isolates.

	No (%) of *S. aureus* isolates ($n = 148$)		
	% S	% I	% R
Erythromycin	52 (35%)	89 (60%)	7 (5%)
Gentamicin	136 (92%)	9 (6%)	3 (2%)
Tetracycline	147 (99.3%)	–	1 (0.7%)
Chloramphenicol	(127) 85.8%	19 (12.8%)	2 (1.4%)
Ciprofloxacin	(145) 98%	3 (2%)	–
Rifampicin	(147) 99.3%	–	1 (0.7%)
Ampicillin	44 (30%)	NA	104 (70%)
Penicillin	40 (27%)	NA	108 (73%)
Oxacillin	92 (62%)	NA	56 (38%)
Vancomycin	133 (90%)	15 (10%)	–
Nitrofurantoin	141 (95%)	7 (5%)	–

–, Not detected; S, Sensitive; I, Intermediate; R, Resistant; NA, Not applied.

3.4. Virulence factors

Except for 4 isolates (3%), all were gelatinase positive; 81% were demonstrated to be β-hemolytic, 8% were α-hemolytic, 11% were γ-hemolytic. Recently, El-Jakee et al. (2008) reported that 92.3% of the *S. aureus* isolates (from different sources) were positive for the gelatinase test; 89.7% were hemolytic in sheep blood agar and that 10.3% were non-hemolytic. These results are in agreement with the present study.

4. Conclusion

S. aureus is well established as a clinical and epidemiological pathogen; in this study it was demonstrated that the potentially pathogenic role of *S. aureus* as a food-borne pathogen should not be neglected. Antibiotic-resistant isolates might be transmitted to humans by the consumption of food products containing such resistant and multiresistant bacteria and that the use of antibiotics as growth promoters in animal husbandry, especially of those commonly used for both human and animal care, should be avoided (Swann, 1969; Wise, 2007).

In conclusion, these findings highlight the high potential risk for consumers in the absence of strict hygienic and preventative measures to avoid the presence of *S. aureus* isolates and SEs production in foods, emphasising the need for improved hygiene practices during food processing and also during the distribution and consumption of the final food products.

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Chapter 3

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Virulence and Resistance profile of *Staphylococcus aureus* Isolated from Food

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Short title: Virulence and Resistance profile of *S. aureus*

Abstract

Staphylococcus aureus is considered a global community and health care pathogen responsible for Staphylococcal Food Poisoning. The aim of this study was to characterize several isolates of *S. aureus* recovered from different food products concerning enterotoxin genes and other virulence factors including antimicrobial resistance. In 2009, a total of 78 coagulase-positive staphylococci from 1454 food samples were identified to species level; 73 were confirmed as *S. aureus*. 5.5% of the *S. aureus* isolates were resistant to oxacillin, 52.0% showed resistance to erythromycin and 45.2% to tetracycline. Multidrug resistance was observed in 33.3% of the isolates (resistance to three or more antibiotics of different classes). SCC*mec* types IV and V were detected among Methicillin-resistant *S. aureus* (MRSA). One MRSA isolate was *pvl* positive. 52.0% of food isolates were shown to be enterotoxigenic; *egc* (63.0%), *secbov* (44.7%) were the main detected SEs. *tst* gene was also detected in food isolates. The present work demonstrates the presence of virulent *S. aureus* (collected in 2009), in foods.

Keywords: *Staphylococcus aureus* food isolates; MRSA; SEs; antibiotic resistance

Introduction

Staphylococcus aureus is an extraordinarily versatile pathogen responsible for staphylococcal food poisoning, hospital- and community-acquired infections as well as for the toxic shock syndrome (SONG et al., 2015).

Staphylococcus aureus can be present in different foods such as raw milk and dairy products (JAMALI et al., 2015), fishery products (VÁZQUEZ-SÁNCHEZ et al., 2012), meat products (BORTOLAIA et al., 2016) among others. Staphylococcal food poisoning has been reported worldwide and is associated with oral intake of enterotoxins present in foods (JOHLER et al., 2015). Staphylococcal toxins were responsible for 7.5% of the total foodborne outbreaks reported to EFSA in 2014 (EFSA-ECDC, 2015a).

Staphylococcal enterotoxins are represented by a group of thermostable gastrointestinal protease-tolerant single chain exoproteins; at least 23 different SEs/SEls have been reported (GRUMANN et al., 2014). Another toxin, TSST-1, the toxic shock staphylococcal toxin

lacks emetic activity and is known to be responsible for toxic shock syndrome (OTTO, 2014). Other virulence factors such as the presence of PVL (Panton–Valentine Leukocidin) and hemolysin-α, exfoliative toxins, thermonuclease, hyaluronidase and lipases are involved in tissue invasion of the host cells by *S. aureus* (GRUMANN et al., 2014).

Antibiotics are widely used not only in human but also in animal husbandry and other agricultural activities (KLUYTMANS, 2010). The occurrence of multi-resistant strains in foods has been increasing; contaminated food is considered an important vehicle for *S. aureus* antimicrobial resistance (EFSA-ECDC, 2015b). On the other hand, methicillin-resistant *S. aureus* (MRSA) strains are emerging in foods (PARISI et al., 2016).

The purpose of this study was to characterize *S. aureus* strains previously collected from several food products regarding their resistance to antibiotics and virulence factors.

1. Materials and methods

During 2009, seventy-eight presumptive colonies of coagulase-positive staphylococci were collected in routine analysis from several food companies (1454 food samples) in a microbiological lab (CINATE, Porto). Confirmation and presumptive identification of *S. aureus* were performed by Gram staining, presence of catalase and coagulase, growth on Mannitol Salt Agar (Pronadisa, Spain). DNAse activity, on DNase agar (Pronadisa) and thermostable DNAse activity were also detected according to CASTRO et al. (2016). Presumptive *S. aureus* isolates were thereafter stored in cryovials at -80 ºC in Tryptone Soy Broth (TSB, Pronadisa) plus 30% (v/v) of glycerol for further characterisation. All the assays were performed as previously presented by CASTRO et al. (2016) including the identification to species level namely multiplex PCR with 16S rRNA and *nuc* and the detection of MRSA strains with *mecA* gene. Antibiotic susceptibility testing and detection of enterotoxin genes were determined by agar dilution and PCR, respectively. Detection of Panton-Valentine leucocidin genes was performed only on MRSA strains by PCR. Finally, *SCCmec* typing of MRSA was performed as described by BOYE et al. (2007). Control strains for five types of SCC*mec* were kindly supplied by Prof. Keiichi Hiramatsu (Juntendo University, Tokyo, Japan: Type I (NCTC 10442), Type II (N315), Type III (85/2082), Type IV (JCSC 4744) and Type V (Wis).

2. Results and Discussion

2.1. *S. aureus isolates and mecA* gene

Among the 1454 food samples, seventy-eight samples were positive for the presence of presumptive colonies of coagulase-positive staphylococci. Of those, 73 isolates were confirmed to be *S. aureus* (*nuc*+ and 16S rRNA+ detected simultaneously). *S. aureus* isolates were recovered from raw meat (n=3), raw fish (n=10), fermented and cured meat products (n=5), cheese (n=8), milk (n=14), pastry (n=7), bakery (n=2), seafood (n=3), ready-to-eat (n=19) and vegetables (n=2). *S. aureus* has already been isolated in similar products namely: meat (HADJIRIN et al., 2015), milk and raw-milk products (CARFORA et al., 2015; JAMALI et al., 2015), fish products (VÁSQUEZ-SÁNCHEZ et al., 2012), retail food products (WANG et al., 2014) and ready-to-eat food (LI et al., 2015). 5.5% of the food isolates (4/73) were classified as MRSA as the gene *mecA* was detected; one strain recovered from a fermented meat product, two from ready-to-eat and one from a pastry product. The occurrence of MRSA strains in food samples varies between the food product and the place of isolation. Recently, JAMALI and his collaborators (2015) detected 16.2% of MRSA strains in milk and dairy products in Iran. In Italy, CARFORA et al. (2016) demonstrated that 5.6% of MRSA strains were present in food from retail meat (pork and beef) in USA. 1.4% of MRSA strains were detected in ready-to-eat foods (WANG et al., 2014). No MRSA strains were reported in powdered infant formula in China (WANG et al., 2014) and fishery products in Spain (VÁSQUEZ-SÁNCHEZ et al., 2012).

2.2. Antimicrobial resistance

High resistance to antibiotics was detected among the *S. aureus* food isolates (83.6%, 90.4%, 52.0% and 45.2% with respect to penicillin, ampicillin, erythromycin and tetracycline (Table 1). All the isolates were sensitive to vancomycin, gentamicin and nitrofurantoin. It is globally accepted that antibiotic resistance in food isolates is due to the widespread usage of antibiotic (KLUYTMANS, 2010).

Of the *S. aureus* isolates resistant to oxacillin (5.5%), only one was *mecA* positive (isolate 709) showing a MIC of 32 µg/mL. The other three MRSA strains did not show oxacillin resistance besides the presence of gene *mecA*. Three other isolates (*mecA* negative strains) were shown to be borderline oxacillin-resistant *S. aureus* strains (MICs varying from 1 to 8 µg/mL). These types of isolates have already been detected in foods (BYSTRÓN et al., 2010). 33.3% of *S. aureus* isolates were multi-resistant (resistant to ≥3 antibiotics of different classes); 30.1% (22/73) and 3.2% (2/73) were resistant to three and four antibiotics, respectively. Among food isolates only one strain was susceptible to all the eleven antibiotics investigated (data not shown).

2.3. MRSA characterization

The detection of MRSA is based usually (with exception of *mecC*) on the presence of *mecA* gene in *S. aureus*. All MRSA were resistant to beta-lactams as an inherent characteristic due to presence of *mecA* gene (Table 2). The location of *mecA* gene is within the large chromosomal element known as the SCC*mec* (WATKINS et al., 2012). Only one isolate 704 (Table 2) was non-typeable by SCC*mec* typing. The other MRSA isolates presented SCC*mec* type IV and V (Table 2). Generally, these SCC*mec* types are the most prevalent types on food; MRSA isolates SCC*mec* type IV were found in hamburgers in Spain (ARGUDÍN et al., 2012) and type V in raw meats in UK (HADJIRIN et al., 2015).

2.4. Virulence factors

2.4.1. Enterotoxin genes detection

Staphylococcal food poisoning (SFP) results from the consumption of foods containing sufficient amounts of (one or more) enterotoxins (SE). Not all strains of *S. aureus* were enterotoxigenic; 52.0% presented at least one of the tested enterotoxins (38/73) and 13 SEs/TSST gene arrangements were found among food isolates; 52.6% (20/38) that carried three or more SEs/*tst* genes (Table 3). 90.6% of Methicillin-sensitive *S. aureus* (MSSA) presented more than one SE gene (data not shown). The percentage of MSSA isolates harboring two, three, four and five SEs/*tst* genes were 34.4%, 12.5%, 31.3% and 9.4%,

respectively (data not shown). Moreover, one MSSA isolated from milk presented seven SEs genes. In contrast, MRSA strains showed a lower presence of enterotoxin genes (Table 2). In agreement with previous findings (ARGUDÍN et al. 2012) *egc* was the most prevalent (63.0%). *secbov* was the most prevalent classical enterotoxin gene (44.7%). An outbreak due to *egc* has already been described (JOHLER et al., 2015). *tst* gene, the marker for TSST-1 (Staphylococcal Toxic Shock Syndrome) was detected (associated with others SEs) in 21.0% of the isolates. Similar results were obtained by ARGUDÍN et al. (2012) and ALIBAYOV et al. (2014) namely 25.8 and 30%, respectively. Absence of *tst* genes has already been reported by PU et al. (2011) for retail meats in the USA. Toxic shock syndrome toxin (TSST) is responsible for TSS, characterized by high fever, rash, desquamation, vomiting, diarrhoea and hypotension, frequently resulting in multiple organ failure (GRUMANN et al., 2014). SEs, as superantigens (SAgs) selectively activate a vast number of T cells and interfere with-gastro-intestinal function and typically cause emesis and diarrhoea (OTTO, 2014). SEs possess extraordinary stability to denaturing conditions, such as heat and low pH, and resistance to most proteolytic enzymes, such as pepsin or trypsin (GRUMANN et al., 2014).

2.4.2. PVL genes detection and hemolysins

Another virulence determinant, PVL-encoding genes (*lukS-PV and lukF-PV*), was investigated only in the case of MRSA strains. PVL, likely staphylococcal enterotoxins are *S. aureus* virulence determinants that are widely distributed in many European countries and it is possible to recover *S. aureus* isolates with PVL from clinical, animal and food sources (HU et al. 2015; VERKADE and KLUYTMANS, 2014). *pv*l positive MRSA isolates had been detected in some animals (pigs, poultry, cattle; VERKADE and KLUYTMANS, 2014) and in foods (*i.e*. in raw and processed food commodities in Shanghai and in meat in the United States; SONG et al., 2015 and HANSON et al., 2011, respectively). The data in the literature concerning food isolates and pvl is scarce. Most of the studies concerning this virulence factor were performed with clinical isolates. In the present study, one MRSA isolated from a Portuguese traditional fermented meat product was detected. To the authors' knowledge there are no reports concerning the detection of *pvl* genes in *S. aureus* collected from food products in Portugal. PVL is a cytotoxin that causes leukocyte destruction and tissue necrosis (WATKINS ET AL, 2012) associated with severe skin and soft tissue

infection and necrotizing pneumonia (LINA et al., 1999). The presence of *pvl* genes represents an increment of virulence of MRSA food isolates.

Hla (α-haemolysin) is probably the best-known toxin of *S. aureus* with pore-forming and pro-inflammatory properties (OTTO, 2014). It is lytic to red blood cells and a series of leukocytes but not neutrophils (OTTO, 2014). In the present study, Hla was present in 6.9% of *S. aureus* isolates. Hlg (ɣ -haemolysins) contrary to PVL is inflammatory but not necrotic in the rabbit skin model and is produced by more than 99% of *S. aureus* clinical strains (LINA et al., 1999). In contrast to our results (presence in 17.8% of *S. aureus* isolates), on retail foods in China none of *S. aureus* isolates had *hlg* gene (LI et al. 2015).

3. Conclusion

S. aureus is routinely detected and/or enumerated in a wide variety of ready-to-eat foods as part of preventive approaches and microbiological safety checks based on hazard analysis and critical control point principles. In the present study, the characterization of *S. aureus* isolated from food samples was evaluated. Globally, it was demonstrated that foods might be an important source of dissemination of antibiotic resistant and virulent strains of *S. aureus*. Since the isolates present here are from the year 2009, it would be interesting to collect and study more recent isolates and further understand if the occurrence of MRSA and the pathogenicity of *S. aureus* varies from year-to-year.

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Table 1. Antimicrobial susceptibility of *S. aureus* isolates.

Antibiotic	Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Penicillin	12 (16.4)	a.*	61 (83.6)
Ampicillin	7 (9.6)	a*.	66 (90.4)
Oxacillin	69 (94.5)	a*.	4 (5.5)
Chloramphenicol	21 (28.8)	51 (69.8)	1 (1.4)
Ciprofloxacin	58 (79.5)	11 (15.0)	4 (5.5)
Erythromycin	25 (34.2)	10 (13.7)	38 (52.0)
Gentamicin	73 (100)	0 (0.0)	0 (0.0)
Rifampin	71 (97.3)	0 (0.0)	2 (2.7)
Vancomycin	73 (100)	0 (0.0)	0 (0.0)
Nitrofurantoin	73 (100)	0 (0.0)	0 (0.0)
Tetracycline	36 (49.3)	4 (5.5)	33 (45.2)

N, number of isolates; *,Antibiotic with no described value for the intermediate MIC

Table 2. MRSA strains collected from food samples

Isolate	**Origin**	**Resistance profile**	**SEs genes**	**SCC*mec* type**	***pvl***
301	Fermented meat product	Pen, Amp, Eri	*secbov*	IV	+
528	Ready-to-eat	Pen, Amp, Eri	*secbov*	V	-
704	Pastry	Pen, Amp	*seg, sei*	-	-
709	Ready-to-eat	Pen, Amp, Eri, Tetra, Oxa	-	V	-

Pen – Penicillin, Amp – Ampicillin, Eri - Erythromycin, Tetra - Tetracycline, Oxa - Oxacillin

Table 3. Distribution of enterotoxins among *Staphylococcus aureus*

Enterotoxin genes profile	N (%)
Secbov	5 (13.2)
seg, sei	9 (23.7)
secbov, seg, sei	4 (10.5)
secbov, seg, sei, tst	3 (7.9)
secbov, tst	4 (10.5)
seh, seg, sei	3 (7.9)
seh, sea, seg, sei	4 (10.5)
sea, sej, seb, seg, sei	1 (2.6)
sej, seb, sed, seg, sei	1 (2.6)
sec, sej, sed, seg, sei	1 (2.6)
seb, sed, seg, sei	1 (2.6)
secbov, seh, sea, sej, sed, seg, sei	1 (2.6)
sec, seg, sei, tst	1 (2.6)

N, number of isolates

Chapter 4

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Food handlers as potential sources of dissemination of virulent strains of *Staphylococcus aureus* in the community

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KEYWORDS
Staphylococcus aureus;
Food handlers;
Hands and nose carriage;
Antimicrobial resistance;
Enterotoxin genes

Summary Food handlers may constitute a reservoir of virulent strains of *Staphylococcus aureus* and may be vehicles of their transmission to food.

One hundred and sixty-two volunteers were assessed for the presence of *S. aureus* on the hands and in the nose. *S. aureus* was isolated by routine procedures, and the isolates were tested for susceptibility against a panel of nine antimicrobial agents. The isolates were further characterized by SmaI-PFGE profiling and the presence of virulence factors.

Results: The prevalence of *S. aureus* was 19.8% in the nose and 11.1% on the hands; 6.2% of the individuals carried *S. aureus* both in their noses and hands, and three individuals had the same strain (PFGE type) in the nose and on the hands. Although 82% of the isolates were resistant to at least one antibiotic, none demonstrated the presence of either *mecA* gene or resistance to oxacillin (none identified as MRSA). Sixty-eight percent of the isolates from the nose and hands possessed enterotoxin genes.

This study revealed a high prevalence of antibiotic resistance and virulence determinants among the isolates, including not only classical and novel enterotoxin genes but also major virulence factors such as *tst*. Potential dissemination of these strains in the community is a matter of concern.

Introduction

Staphylococcus aureus is one of the most important species in the field of food microbiology and has been considered a foodborne hazard for a

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long time. In 2013, 386 staphylococcal outbreaks were reported by the EFSA, representing 7.4% of all outbreaks reported in the European Union [1]. Staphylococcal food poisoning, gastroenteritis with emesis and with or without diarrhea [2] characterized by a short incubation period, typically 2—4 h [3], is caused by the ingestion of food containing preformed enterotoxins. Not all strains are capable of producing staphylococcal enterotoxins [4], but up until now, 22 SEs have been described, 11 of them with known emetic action [5].

S. aureus can colonize the skin and the anterior nares of individuals and is carried by a significant proportion of the population [6]. As found by Kluytmans and Wertheim [6], *S. aureus* colonizes the nares of approximately 50% of healthy adults, either persistently or intermittently. In a study by Lues and Van Tonder [7], *S. aureus* was isolated from the hands of 88% of the population sampled. Human nasal or hand carriage of enterotoxigenic *S. aureus* during food processing is an important source of food contamination with *S. aureus* [5,8]. In fact, food poisoning outbreaks associated with post-process contamination of foods with *S. aureus* are in part the responsibility of food handlers who carry enterotoxigenic staphylococci in their nares or on their skin [7].

In recent decades, the increasing prevalence of antimicrobial-resistant *S. aureus* is receiving widespread attention. Strains of methicillin-resistant *S. aureus* (MRSA) are of particular concern given that they represent a significant cause of morbidity and mortality throughout the world. Methicillin-resistant *S. aureus* are resistant to all available penicillins and other β-lactam antimicrobial drugs [9]. Trends for the period 2009—2012 were calculated for 28 countries. Statistically significant increasing trends were observed for four countries, including Portugal, where in 2012, the percentage of MRSA isolates was greater than 50% [10].

Since Kluytmans et al. [11] described the first fatal foodborne outbreak of MRSA, food microbiologists now consider the possibility of foods as vectors of antimicrobial-resistant strains.

To identify MRSAs, the detection of the presence of the *mecA* gene and consequent resistance to methicillin is important not only in food isolates but also on food handlers who contribute to the cross-contamination of food products.

The combination of enterotoxin genes and the *mecA* gene could provide us with information about the presence of resistant strains in foodborne diseases and also the importance of food as a vehicle for antimicrobial resistance.

The purpose of this study was to evaluate the prevalence of *S. aureus* among healthy individuals working in a food company and to characterize isolates regarding their resistance to antibiotics and virulence factors. A potential clonal relationship between isolates from the nose and hands of the same individuals was also investigated.

Material and methods

Staphylococcus aureus sampling

One hundred and sixty-two volunteers from a food company were assessed for the presence of *S. aureus* on their hands and in their nose (a total of 324 samples were recovered). The definition of the sample was one of convenience and included 103 women and 59 men. This company sells food to numerous clients all over Portugal; raw meat is chopped and used within the company for further processed meat-containing foods or is sold to local shops.

The specimens were collected using a cotton-tipped swab previously moistened with sterile Ringers solution. The anterior nares were sampled by rotating the swab tip in both nostrils. Swabs were then spread onto Baird-Parker Egg Yolk Tellurite Medium (LabM, Bury, United Kingdom) and incubated aerobically at 37 °C for 48 h. Characteristic colonies were sub-cultured on Mannitol Salt Agar (MSA; Pronadisa, Madrid, Spain) incubated aerobically at 37 °C for 24 h. Presumptive *S. aureus* colonies on MSA (yellow colonies with yellow zones, Gram-positive, catalase positive, coagulase positive and DNase positive) were streaked on Tryptone Soy Agar (TSA; Pronadisa) before being stored at −80 °C in Brain Heart Infusion (BHI; LabM) broth containing 30% (v/v) glycerol.

DNA extraction

DNA was extracted from single colonies on TSA using the guanidine-isothiocyanate method [12]. DNA was quantified spectrophotometrically at 260 nm and 280 nm.

Identification of isolates by multiplex PCR

PCR multiplex to detect the simultaneous presence of 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific) and *mecA* (determinant of methicillin resistance) genes was performed according to Zhang et al. [13]. *Staphylococcus aureus* DSM 11729 was used as a positive control for the gene *mecA*, *Staphylococcus epidermidis* DSM

20044 as a negative control for the gene *nuc* and *S. aureus* ATCC 29213 as a positive control for targeting 16S rRNA and the *nuc* gene and as a negative control for the gene *mecA*.

Detection of staphylococcal enterotoxin genes by multiplex PCR

The detection of enterotoxin genes, *sea* to *sej* and *tst*, was performed by multiplex PCR according to Løvseth, Loncarevic and Berdal [14]. The amplification of the target 16S rRNA gene was included as the internal control. As positive controls, different strains of *S. aureus* kindly supplied by Prof. Løvseth (National Veterinary Institute, Norway) were used: R2102/00 for the *sec*, *seg*, and *sei* genes; R4571/00 for the *sec* gene, FRI572 for the *seg* and *sei* genes; 3169 for the *sec*-bovine, *sed*, and *sej* genes; FRI472 for the *sed*, *seg*, *sei*, and *sej* genes; R5371/00 for the *sea*, *seg*, *seh*, and *sei* genes; R963/00 for the *sed*, *seg*, *sei*, and *sej* genes; R5460/00 for the *seb*, *seg*, *seh*, and *sei* genes; FRI913 for the *sea*, *sec*, and *see* genes; FRI445 for the *seg* and *sei* genes; R4071/00 for the *seb* gene; and R4774/00 as a negative control.

A 5-μl aliquot of DNA was added to a 20-μl PCR mixture containing 0.3 μM of each primer except 16S rRNA and *sei* (0.1 μM) and 12.5 μl Kapa 2G Fast master mix (Grisp, Porto, Portugal). Amplification was carried out as follows: an initial denaturation step at 95 °C for 3 min; 30 cycles at 95 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s; and a final extension step at 72 °C for 1 min. The PCR products were resolved in 2% (w/v) agarose gels (1× Tris Acetic Acid, EDTA) at 60 V (constant voltage) for 3 h and visualized in a transilluminator.

Antibiotic susceptibility testing by agar dilution

The minimal inhibitory concentrations (MICs; μg/mL) for *S. aureus* isolates were determined by the agar dilution method described in the guidelines of the Clinical and Laboratory Standards Institute [15]. The inoculum was prepared from a 24 h culture on TSA by suspension in sterile Ringer's solution to obtain turbidity equivalent to the 0.5 McFarland standard. The antibiotics investigated were penicillin G, chloramphenicol (both obtained from Sigma, Steinheim, Germany), oxacillin (Bio-Chemica, Billingham, UK), rifampin, gentamicin, tetracycline, erythromycin and ciprofloxacin (all kindly supplied by Labesfal, Tondela, Portugal) and Vancomycin (Fluka, Steinheim, Germany). The MICs were determined in Mueller-Hinton agar (MH; bioMérieux, Marcy l'Etoile, France) plus 2% (w/v) NaCl in the case of oxacillin, in cation-adjusted MH for penicillin and ampicillin and in MH for the other antibiotics investigated. *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as controls. For each antibiotic susceptibility determination, at least two independent experiments were performed.

Detection of other of virulence factors

The production of hemolysin was evaluated on blood agar plates (COS, Columbia agar plus 5% (v/v) sheep's blood; bioMérieux). The isolates were streaked onto the plates and incubated at 37 °C for one to two days. The presence or absence of zones of clearing around the colonies was interpreted as β-hemolysis (positive) or gamma-hemolysis (negative) activity, respectively. Greenish zones around the colonies were interpreted as α-hemolysis [16].

Lipase activity was assessed as described by Tiago et al. [17]. A positive reaction was indicated by opacity around the colonies.

Gelatinase activity was assessed according to Tiago et al. [17].

For each virulence factor tested, at least two independent experiments were performed.

DNA-macrorestriction by pulsed-field gel electrophoresis (PFGE)

PFGE typing of the isolates was performed as previously described by Chung et al. [18] using the restriction enzyme SmaI (ThermoScientific, New York, USA) and *Salmonella enterica* ser. Braenderup H9812 as a standard and a CHEF Mapper XA (Bio-Rad, Laboratories, Hercules, CA, USA). PFGE image analysis and similarity clustering were performed with GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was done by the unweighted pair group method with average linkages (UPGMA), using the Dice coefficient to analyze the similarities of the banding pulsotypes.

Results

Fifty *S. aureus* isolates were recovered from the hand and nose samples of 162 individuals (Supplementary Table). Nearly one-quarter (24.7%, 40/162) of the individuals were *S. aureus* carriers; 60% of these (24/40) were female, and 40% (16/40) were male (Supplementary Table). Nasal carriage was found in 19.8% and hand carriage was found

Table 1 Hand and nasal carriage of *Staphylococcus aureus* among food handlers.

	Number of positive (%)
Hands	18 (11.1)
Nose	32 (19.8)
Hands and nose (simultaneously)	10 (6.2)
Hands but not nose	8 (4.9)

in 11.1% of the individuals; 6.2% had *S. aureus* in both their hands and nose. Eight individuals (4.9%) had *S. aureus* on their hands but not in their nose (Table 1).

Supplementary Table related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiph.2015.08.001.

PFGE analysis was performed only for those isolates collected from the nose and hands of the same individual. Eighteen of these isolates were distributed among 15 PFGE types, and two were non-typeable (2095H and 2095N; Supplementary Table). In three individuals, the same strain was found in the nose and on the hands (Fig. 1).*seg* and *sei* were the most prevalent genes in the isolates recovered from both the nose (82.6%) and hands (70%), followed by the *tst* gene, which was recovered from 39.1% to 40% of noses and hands, respectively (Supplementary Table and Table 2). For all of the isolates, an association between the *seg* and *sei* genes was observed (Table 2).

Table 2 Arrangements of enterotoxin genes profiles of *Staphylococcus aureus* isolated from hands and nose of food handlers.

	N (%)
Nasal isolates	
secbov, seg, sei	6 (30.4)
seg, sei	3 (13.0)
sea, seg, sei, tst	2 (8.7)
seh, seg, sei, tst	2 (8.7)
tst	2 (8.7)
sej, tst	1 (4.3)
sea, seh, seg, sei	1 (4.3)
seg, sei, tst	1 (4.3)
sea, tst	1 (4.3)
sea, sej, seg, sei, sed	1 (4.3)
sec, seg, sei	1 (4.3)
sea, sec, seg, sei	1 (4.3)
sea, seh	1 (4.3)
Hands isolates	
secbov, seg, sei	3 (33.3)
sej, tst	2 (22.2)
seg, sei	2 (22.2)
seh, seg, sei, tst	1 (11.1)
seb	1 (11.1)
sec, seg, sei, tst	1 (11.1)

Table 3 Antibiotic sensitivity pattern of the *Staphylococcus aureus* isolates recovered from hands and nose of food handlers.

Antibiotic	Sensitive	Intermediate	Resistant
	N (%)		
Penicillin	26 (52.0)	[a]	24 (48.0)
Oxacillin	50 (100.0)	[a]	0 (0.0)
Tetracycline	48 (96.0)	0 (0.0)	2 (4.0)
Ciprofloxacin	37 (74.0)	3 (6.0)	10 (20.0)
Erythromycin	34 (68.0)	0 (0.0)	16 (32.0)
Gentamicin	49 (98.0)	0 (0.0)	1 (2.0)
Rifampin	46 (92.0)	0 (0.0)	4 (8.0)
Chloramphenicol	41 (82.0)	8 (16.0)	1 (2.0)
Vancomycin	(100)	0 (0.0)	(0.0)

[a] Antibiotic with no described value for the intermediate MIC.

The antibiotic resistance profile of the *S. aureus* isolates is presented in Table 3. Eighteen percent of the isolates were sensitive to all of the antibiotics investigated. Forty-eight percent and 26% of the isolates were resistant to two or three antibiotics of different classes, respectively. One isolate was resistant to five antibiotics, and another was resistant to six. Forty-eight percent of isolates were resistant to penicillin, 32.0% to erythromycin and 20.0% to ciprofloxacin. Four isolates were resistant to rifampin, two were resistant to tetracycline, one was resistant to gentamicin, and another was resistant to chloramphenicol. All of the isolates were sensitive to vancomycin and oxacillin (Table 3). The gene *mec*A was absent among all of the isolates (data not shown).

β-Hemolysin, γ-hemolysin, α-hemolysin, lipase and gelatinase were identified in 66%, 12%, 22%, 82% and 88% of isolates, respectively (Supplementary Table).

Discussion

The prevalence of *S. aureus* in the nose of the workers at the food company investigated (19.8%) is in accordance with the mean nasal colonization of healthy adults (20—30%) who are persistent carriers [6]. Similar values have been reported by other authors [19,20]. Nevertheless, other authors had reported higher [21—24] and lower prevalences [25]. A lower prevalence of *S. aureus* was found on the hands (11.1%) than in the nose of the food handlers. This is consistent with the findings of previous studies [21,22]. The reported prevalence of *S. aureus* on the hands of food handlers is highly variable. While values similar to or lower

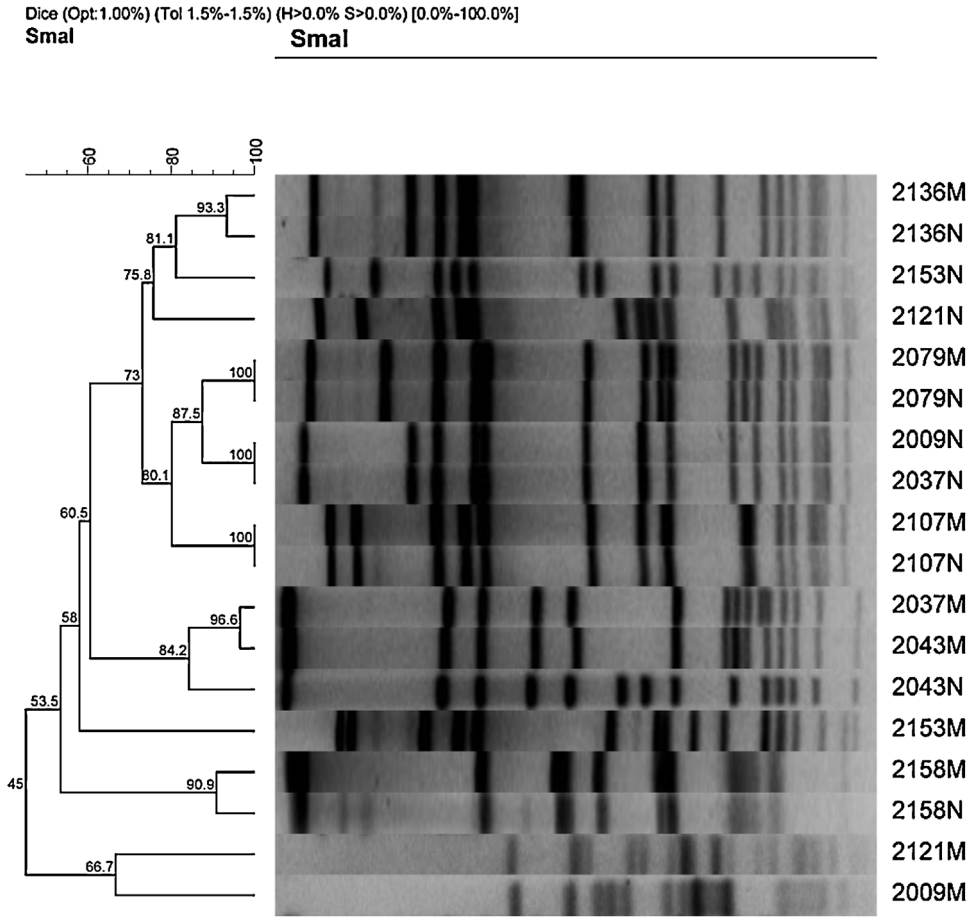

Figure 1 Dendrogram of *Staphylococcus aureus* isolates recovered from the hands and nose of the same individual.

than those found in this study have been reported [21,26], prevalences higher than 50% have also been reported [7,24,27,28]. A high carriage level had been found for food handlers working in hospitals [24,27] where *S. aureus* is highly disseminated.

Ten individuals carried *S. aureus* both nasally and on their hands, and three of these individuals had the same strain in their nose and hands. The presence of *S. aureus* on the hands but not in nose and the detection of isolates in the nose and hands of the same individual with different PFGE types indicate that hand contamination may result from sources other than the individual [21]. Food is naturally contaminated, at least temporarily, when raw or even through exposure during processing or refrigeration to temperatures that allow the growth of *S. aureus*. Although food handlers are usually the main source of food contamination in food poisoning outbreaks, equipment and environmental surfaces can also be sources of contamination with *S. aureus* [29].

Eighty-two percent of the isolates were resistant to at least one antibiotic, and 48%, 26% and 8% were resistant to one, two and three or more antibiotics of different classes, respectively. None demonstrated the presence of either the *mecA* gene or resistance to oxacillin. MRSAs have frequently been isolated from health professionals [30], although the isolation of MRSAs from food handlers is rare [22,23,25]. The rate of penicillin resistance was lower in our study relative to that found for isolates recovered from foods commercialized in Portugal [31] and also from food handlers in other studies [22,24,25]. In contrast, higher percentages of isolates resistant to ciprofloxacin and to erythromycin were observed. Working in hospitals seems to be a risk factor for the carriage of resistant strains by food handlers, as demonstrated in the study by Ferreira et al. [24]. The high prevalence of antibiotic resistance among food handlers and the carriage of multidrug-resistant strains highlights the growing problem of antibiotic resistance in the ''healthy'' community.

S. aureus produces a wide array of cell surface and extracellular proteins (proteases, lipases and cytotoxins such as hemolysins — alpha, beta,

gamma and delta) involved in virulence that enable it to invade and destroy host tissues and metastasize to other sites [2]. *Staphylococcus aureus* Hlb (gene of beta-hemolysin) also plays an important role in skin colonization by damaging keratinocytes, in addition to its well-known hemolytic activity for erythrocytes [32]. The results from this study are consistent with those observed in previous studies. Among the *S. aureus* isolated from the oral cavity, 100% produced gelatinase, 77% lipase, 59% beta-hemolysins and 41% alpha-hemolysins [33]. Saising et al. [34] reported that 65.6% of the strains isolated from acne lesions were lipase positive, and Wu et al. [35] showed that 80% of *S. aureus* isolated from corneal ulcers produced gelatinase. Beta-hemolysin is secreted by certain strains of *S. aureus*, especially strains isolated from corneal infections [33].

S. aureus produces a wide variety of toxins including staphylococcal enterotoxins (SEs; SEA to SEE, SEG to SEI, SER to SET) with demonstrated emetic activity and staphylococcal-like (SEl) proteins, which are not emetic in a primate model (SElL and SElQ) or have yet to be tested (SElJ, SElK, SElM to SElP, SElU, SElU2 and SElV). SEs and SEls have been traditionally subdivided into classical (SEA to SEE) and new (SEG to SElU2) types. Each of these toxins is known to have potent effects on the cells of the immune system, but many of them have other biological effects as well. Their primary function *in vivo* may be to inhibit host immune responses to *S. aureus* [2]. SEs are the causative agents of staphylococcal food poisoning resulting from the ingestion of contaminated food. Due to their extraordinary stability in denaturing conditions, such as heat and low pH levels, SEs are not completely destroyed by mild cooking or the digestion of food in the stomach. Nausea, emesis, abdominal pain or cramping and diarrhea ensue after a short incubation period. The disease is usually self-limiting [36].

The majority (71.9% and 55.6%, respectively) of isolates recovered from the nose and hand samples possessed enterotoxin genes. A high prevalence of enterotoxigenic isolates recovered from food handlers has been reported by several studies [19,22,37]. Although *sea* is the most commonly reported enterotoxin gene [19,37], this was not observed in the present study in which *seg* and *sei* were the most prevalent enterotoxin genes and were associated with all of the isolates. This association has been previously reported [19] and is justified by the fact that they belong to an operon of the *egc* enterotoxin gene cluster, which contains five enterotoxin genes (*seg*, *sei*, *sem*, *sen*, and *seo*) [38]. The *tst* genes were also highly prevalent. *tst* genes have been previously detected in isolates recovered from food handlers [22,37], though normally in lower percentages. TSST-1-producing *S. aureus* was detected for the first time on a food service worker's hand by Sospedra et al. [39]. TSST-1 was the first marker identified for Staphylococcal Toxic Shock Syndrome (TSS), which is an acute and potentially fatal illness that is characterized by a high fever, diffuse erythematous rash, desquamation of the skin one to two weeks after onset (if not fatal before this time), hypotension, and the involvement of three or more organ systems [36].

Food has to meet high food safety and food quality standards. Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Points (HACCP) systems are applied to improve the microbial safety and quality of food. However, even with the best control measures in place, a food product may still pose a risk to the consumer. The presented data have certain limitations, as the population sampled in this study is not representative of the food handler population as a whole in Portugal. Nevertheless, the food company analyzed followed all of the measures referred to above, and yet, the food handlers' hands were contaminated with enterotoxigenic and antibiotic-resistant *S. aureus* strains.

Conclusion

This study revealed a high prevalence of antibiotic resistance and virulence determinants, including not only classical and novel enterotoxin genes but also major virulence factors such as *tst*, in the studied population, which is one of the major sources of contamination/recontamination of food with *S. aureus* during processing. Potential dissemination of these strains in the community is a matter of concern.

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Competing interests

None declared.

Ethical approval

Not required.

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Supplementary Table: Detailed information on isolates of *Staphylococcus aureus* recovered from hands and nose of food handlers

Isolate[a]	**Sex**	Antibiotic Resistance	Enterotoxin genes profile	Hemolysins	Lipase	Gelatinase
2009N	F	Cip	-	A	+	+
2009H		Pen, Eri	-	B	+	+
2015H	F	Pen, Eri	*secbov, seg, sei*	A	+	-
2037N	F	-	-	A	+	+
2037H		Pen, Eri	*sec, seg, sei, tst*	A	+	-
2043N	F	-	*secbov, seg, sei*	B	+	-
2043H		-	*sej, tst*	B	+	-
2046N	F	Pen	*seg, sei, tst*	A	-	+
2050H	M	-	-	B	+	-
2065N	M	Pen	*sea, seg, sei, tst*	B	-	+
2067N	M	Pen	-	B	+	+

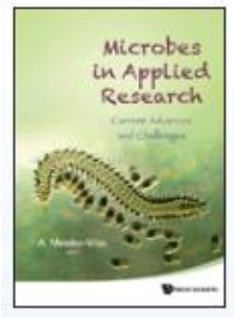
2068N	F	Pen	*seg, sei*	B	+	+
2074N	M	Pen	*sea, seg, sei, tst*	B	+	+
2079N*	M	Pen, Eri	-	B	+	+
2079H*		Pen, Eri	-	B	+	+
2083N	M	Pen	*secbov, seg, sei*	B	+	+
2084N	M	Eri	-	A	+	+
2088H	F	Pen, Eri	-	Γ	-	+
2095H	M	-	*secbov, seg, sei*	B	+	+
2095N		Cip	-	B	+	+
2098H	F	Pen, Eri,	*secbov, seg, sei*	Γ	+	+
2101N	F	Pen	*secbov, seg, sei*	A	+	+
2105H	F	Eri	*seg, sei*	B	+	+
2107H	F	-	*seg, sei*	B	+	+
2107N		-	*seg, sei*	B	+	+
2109N	F	Pen, Cip	*seh, seg, sei, tst*	A	-	+

2110N	M	Pen	*tst*	B	-	+
2112H	M	Pen	*seh, seg,sei, tst*	A	-	+
2113N	F	Pen	*sea, tst*	B	+	+
2120H	F	-	*sej, tst*	Г	-	+
2121N	F	Pen, Tet	-	B	+	+
2121H		Pen	-	Г	+	+
2123N	M	Pen, Eri	*sec, seg, sei*	B	+	+
2128N	M	Pen, Eri	-	Г	-	-
2132N	F	Pen	*tst*	B	+	+
2134N	F	Pen	*sej, tst*	B	+	+
2135H	F	Pen	-	Г	+	+
2136H*	M	Pen	-	B	+	+
2136N*		Pen	-	B	+	+
2140N	M	Eri	*sec, sea, seg, sei*	A	+	+
2149N	M	Pen, Cip	*seg, sei*	B	-	+

2150N	M	Pen, Eri, Cip	*secbov, seg, sei*	B	+	+
2151N	F	Pen	*seh, seg,sei tst*	A	+	+
2152N	F	Pen, Eri	*secbov, seg, sei*	B	+	+
2153N	F	-	*sea, seh*	B	+	+
2153H		Cip, Rif, Tet	*seb*	B	+	+
2154N	F	Eri	*sea, sej, seg, sei, sed*	B	+	+
2158H	F	Pen, Eri, Chl, Cip, Rif	-	B	+	+
2158N		Pen, Eri, Gen, Cip, Rif, Tet	*secbov, seg, sei*	B	+	+
2159N	F	Pen	*sea, seh, seg, sei*	B	+	+

[a]Isolates with the same code belong to the same individual; N – nose, H - hands; M – male, F – Female; +, positive result; -, negative result; Cip (Ciprofloxacin), Chl (Chloramphenicol), Eri (Erythromycin), Pen (Penicillin), Rif (Rifampicin), Tet (Tetracycline). *Same PFGE profile of isolates recovered from hands and nose of the same individual . *Same PFGE profile of isolates recovered from hands and nose of the same individual

Chapter 5



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Characterization of *Staphylococcus aureus* isolated from healthy children in Portugal

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The prevalence of *Staphylococcus aureus* carriage among healthy children from four kindergartens located in the North of Portugal and antibiotic resistance of the isolates were evaluated. Nasal swabs were collected from 296 healthy children aged 3 to 6 year. 144 were carriers of *S. aureus*; 15% of the strains were multidrug resistant. Resistance to gentamicin, chloramphenicol, rifampicin, oxacillin, nitrofurantoin, tetracycline, erythromycin, ampicillin and penicillin were determined to be 1.5, 2.2, 2.2, 9.7, 19, 34.1, 73.3, 84.4 and 91.1%, respectively. All the strains were sensitive to vancomycin. Our study reveals a high prevalence of healthy children carrying *S. aureus* (49%), including MRSA (9%), in the nasal cavity.

Keywords *Staphylococcus aureus*; susceptibility to antibiotics; MRSA; characterization.

Introduction

Staphylococcus aureus can be carried by healthy humans; up to 20% of the population carry *S. aureus* in the nose, with no symptoms and are considered to be colonised [1]. However, *S. aureus* is well known as an important agent of nosocomial and community-acquired infections in many European Countries [2, 3]. Infections range of mild (i.e. skin infections) to severe (i.e. pneumonia, deep abscesses, endocarditis, phlebitis, mastitis and meningitis) and, in some cases fatal (necrotizing pneumonia, severe sepsis and necrotizing fasciitis) [4].

Antibiotics are commonly used in prophylaxis and treatment of *S. aureus* infections. The percentage of infections caused by methicillin resistant *S. aureus* (MRSA), increased between 1997 and 2007 from 47.9 and 64.7%, respectively, in intensive care units (ICU) in the United States [5]. MRSA isolates that are resistant to other antibiotics i.e. vancomycin are being emerging [6, 7]. With the increase of staphylococcal resistance to methicillin, vancomycin is often a treatment of choice in infections with MRSA [7].

Methicillin resistance is mediated by an acquired penicillin binding protein, PBP2a, a peptidoglycan transpeptidase encoded by the *mec*A gene that has low affinity for beta-lactams. Thus, when the four native peptidoglycan synthetases (penicillin-binding proteins 1, 2, 3 and 4) are bound and inactivated by beta-lactams, PBP2a can still affect cell-wall synthesis. The *mec*A gene is harboured on the staphylococcal chromosomal cassette *mec* (SCC*mec*), a genetic element that integrates site specifically into the *S. aureus* chromosome [8].

Community-acquired MRSA infections have been increasing worldwide in the last years. Risk factors for community-acquired MRSA include recent and prolonged hospitalization, stay in an ICU, chronic diseases, prior exposure to antibiotics, surgery and contact with patients colonized or infected with MRSA [9].

The carriage of MRSA among young healthy children that are not considered as a traditional risk group for MRSA has been increasingly reported [1, 10, 11]. Oguzkaya-Artan *et al*. [1] reported that 18% of 200 children (5 to 7 years old) were asymptomatic carriers of *S. aureus* and that 5.6% of the carried strains were MRSA.

The purpose of this study was to evaluate the prevalence of *S. aureus* carriage among healthy children from four kindergartens located in the North of Portugal, characterize isolates regarding their resistance to antibiotics, and investigate and the proportions of MRSA in this niche of Portuguese children population.

Materials and Methods

This study was performed in 4 kindergartens located in the north of Portugal, during the year 2008. In total 296 children from both genders and aged from 3 to 6 years participated in this study. Parents were asked fill a brief questionnaire concerning antibiotic usage in the past 3 months and presence of any disease or infection.

A cotton swab from the nasal cavity of each child was collected and streaked on Baird-Parker Agar (BPA; Pronadisa), supplemented with egg yolk-tellurite emulsion (EY, BioRad), maintained at 4ºC, and immediately transported to the laboratory and incubated at 37ºC for 24/48h. Each characteristic colony was platted in Triptyc Soy Agar (TSA, Pronadisa) and further incubated at 37ºC for 24h. Pure cultures were then stored at -80ºC in Triptyc Soy Broth (TSB, Pronadisa) plus glycerol (30% v v^{-1}) for further identification. Confirmation was carried out by Gram staining, testing for the production of coagulase and catalase and fermentation of mannitol. The isolates were identified to the species level according to the multiplex PCR developed by Zhang *et al*. [12].

According to this PCR assay the presence of the target 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific) and *mecA* (a determinant of methicillin resistance) was investigated. *S. aureus* DSM 11729 was used as a positive control for gene *mecA*, *S. epidermidis* DSM 20044 as a negative control for gene *nuc* and *S. aureus* ATCC 29213 as positive control for targeting 16S rRNA and *nuc* gene and negative for gene *mecA*. DNA template used for PCR analysis was isolated from the strains by the guanidine-isothiocyanate extraction method [13]. The minimal inhibitory concentrations (MICs (μg mL^{-1}) for *S. aureus* strains were determined by the agar dilution method described in the guidelines of the Clinical and Laboratory Standards Institute [14]. The inoculum was prepared from an overnight culture on TSA plates, by suspension in sterile Ringer's solution in order to obtain turbidity equivalent to 0.5 McFarland standard. The antibiotics investigated were penicillin G, ampicillin, chloramphenicol (all obtained from Sigma) and oxacillin (BioChemica); rifampicin, gentamicin, tetracycline and ciprofloxacin were kindly supplied by Labesfal, Portugal. MICs were determined in Muller Hinton agar (MH, bioMérieux) plus 2% w v^{-1} NaCl in the case of oxacillin, in cation-adjusted MH for penicillin G and ampicillin and in MH for other antibiotics investigated. *S. aureus* ATCC 29213 was plated as a control. For each antibiotic susceptibility determination, at least two independent experiments were performed.

Results and Discussion

One hundred and forty-four children (144/296; 48.6%) were carriers of *S. aureus* (positive for the presence of gene *nuc* and the target 16S rRNA, simultaneously). In a concomitant study in the south of Portugal investigating a larger sample only 17.4% of the children carried *S. aureus* [15].

Antibiotic sensitivity patterns are summarized in Figure 1. Of the 144 strains, 91.1% were resistant to penicillin, and 84.4% to ampicillin, which is consistent with previous studies [11, 16, 17, 18] 73% of the isolates were resistant to erythromycin. This is in accordance with previous works that reported 100%, 97% and 76.1% of erythromycin resistance [19, 16, 17] respectively. However, others authors, reported that the patterns of resistance to erythromycin are very low i.e. 16.7% [1]. Almost all of the studied *S. aureus* isolates were sensitive to chloramphenicol, rifampicin, gentamicin and ciprofloxacin and 100% were susceptible to vancomycin.

This is consistent with the data reported in other studies, in children from Turkey, Korea, Greece and Taiwan [1, 11, 17, 20]. 61.5% of the isolates were susceptible to tetracycline. This higher sensitivity to tetracycline was already described by other authors i.e. 100% [11], 73.9% [17] and 91.7% [1]. Finally, 9.7% of the isolates were oxacillin-resistant, which is a value similar to that previously found in Japan [21].

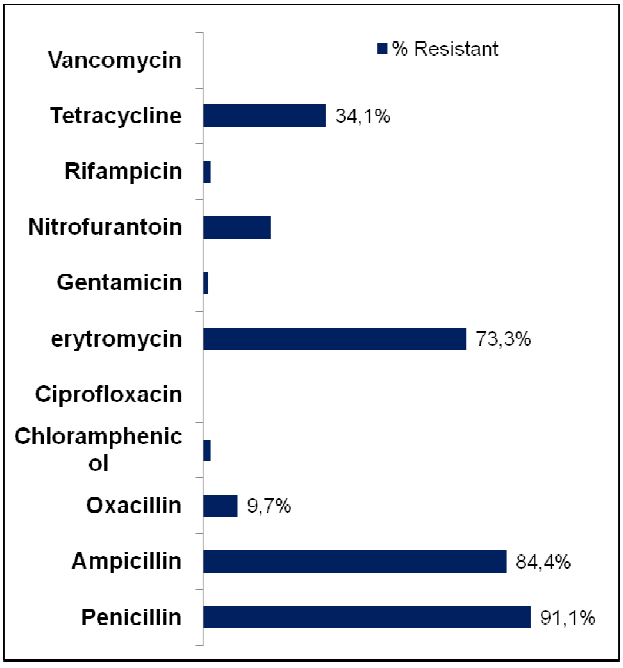

Figure 1: Pattern of antibiotic resistant *S. aureus* isolates

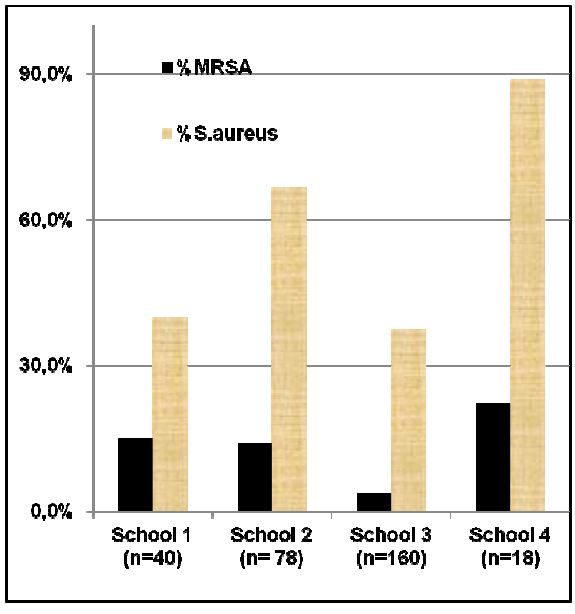

Figure 2: Percentage of *S. aureus* and MRSA isolated in each kindergarten

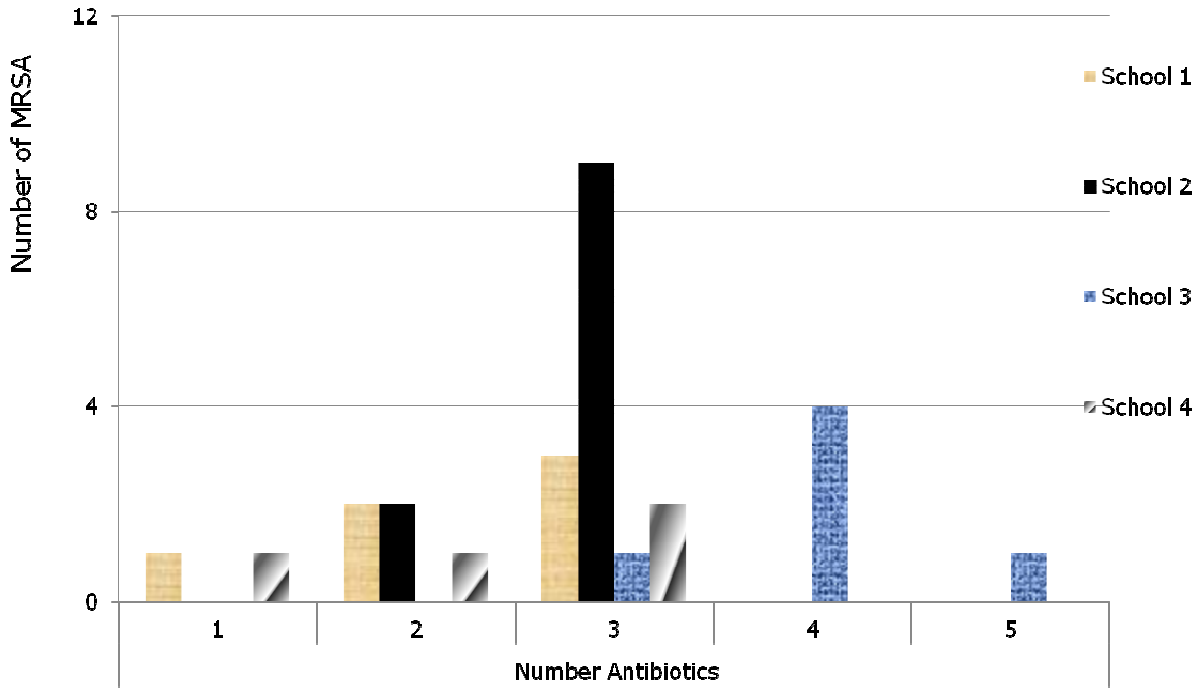

Figure 3: Multidrug resistance patterns of MRSAs

The gene *mecA* was present in 18.8% (27/144) of the isolates. 9% (13/144) of the isolates, although carrying this gene were not oxacillin resistant. These isolates are considered MRSA by the Clinical and Laboratory Standards Institute [13]. *Staphylococcus aureus* strains that are positive for *mecA* but phenotypically susceptible to oxacillin have been reported worldwide [22, 23] In the study of Lencastre et al. (2010) only 0.53% of the isolates were resistant to oxacillin.

As shown in Figure 2 a high variability was observed in the results obtained in the different kindergartens concerning both *S. aureus* and MRSA nasal colonisation. School 4 had the highest percentage of *S. aureus* nasal colonisation 88.9% (16/18) and the highest percentage of MRSA among *S. aureus* isolates 25% (4/16). School 3 had the lowest percentage of *S. aureus* nasal colonisation 37.5% (60/160) and the lowest percentage of MRSA among S. aureus isolates 10% (6/60).

Figure 3 represents the number of MRSA isolates resistant to antibiotics other than oxacillin. the Resistance to three antibiotics among MRSA (other than oxacillin) was a common pattern to all schools. School 3 had a pattern of resistance to five antibiotics. These data are not in agreement with previous studies reporting that CA-MRSA which is generally susceptible to most antibiotics classes except β-lactams [24]. In our study in addition to resistance to β-lactams, isolates also demonstrated resistance to erythromycin and tetracycline, a multiresistance profile typically observed for hospital acquired MRSA- HA-MRSA.

Conclusions

Our results demonstrate that in certain kindergartens a high carriage of MRSA exists among healthy children. Therefore continuing surveillance is needed to more accurately assess the prevalence and epidemiology of community-acquired infection and to develop strategies that will improve therapy and infection control.

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Chapter 6

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ORIGINAL ARTICLE

Prevalence of *Staphylococcus aureus* from nares and hands on health care professionals in a Portuguese Hospital

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Prevalence of *Staphylococcus aureus* from nares and hands on health care professionals in a Portuguese Hospital

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Keywords
antimicrobial resistance, enterotoxin genes, hand carriage, health care professionals, methicillin-resistant *Staphylococcus aureus*, nasal carriage, pulsed-field gel electrophoresis.

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Abstract

Aims: The main goal was to estimate the prevalence of methicillin-resistant *Staphylococcus aureus* on hands and in nose of health care professionals.
Methods and Results: Detection of *Staph. aureus* on hands or in the nose of 169 individuals was performed. Nasal and hand carriage was found in 39·6 and in 8·9% respectively. About 17·2% of the individuals were carriers of methicillin-resistant *Staph. aureus* (MRSA) in the nose and 4·7% on hands. The majority of nasal MRSA were resistant to β-lactams, erythromycin and ciprofloxacin. All nasal MRSA were SCC*mec* type IV and Panton-Valentine leukocidin (PVL) negative. One MRSA isolated from hand was SCC*mec* type V. About 75·6% of MRSA isolates presented the same or closely related restriction patterns. Sixty per cent of *Staph. aureus* from hands and from noses from the same individual were the same strain.
Conclusions: MRSA nasal carriage was high considering healthy health care professionals but in accordance with high level of MRSA infection in Portugal. Isolates recovered in this study seemed to be different from major clones previously isolated in other Portuguese hospitals.
Significance and Impact of the Study: These findings may have implications on the knowledge of healthy health care workers as vehicles of MRSA infections among the community. Presence of several virulence factors may contribute to increased pathogenesis in case of infection.

Introduction

Staphylococcus aureus is a common cause of infection in the hospital and the community (Baron and Tenover 2012). Global spread and increasing resistance to antibiotics of this pathogen requires numerous actions of prevention and infection control. Methicillin-resistant *Staph. aureus* (MRSA) are a particular cause of concern, given that they represent a significant cause of morbidity and mortality in many hospitals throughout the world. Methicillin-resistant *Staph. aureus* are resistant to all available penicillin's and other -lactam antimicrobial drugs (Stefani and Goglio 2010).

Portugal has one of the highest levels of MRSA (more than 50% of *Staph. aureus* bacteraemia isolates were MRSA) in Europe (ECDC 2015). Initially confined to hospital environments, causing hospital acquired MRSA (HA-MRSA) infections—one of the leading causes of nosocomial infections—nowadays MRSA transmission and infections are common among people who live in the community without risk factors (CA-MRSA; Deurenberg *et al.* 2007). The *mecA* gene is harboured on the staphylococcal chromosomal cassette *mec* (SCC*mec*), a genetic element that integrates site specifically into *Staph. aureus* chromosome (Chambers 1997). There are at least 11 SCC*mec* types (types I–XI) in *Staph. aureus*, which differ in structural organization and genetic content (www.sccmec.org/, access June 2015).

As reviewed by Wertheim *et al.* (2005), *Staph. aureus* colonizes the nares of approx. 50% of healthy adults,

either persistently or intermittently (Wertheim *et al.* 2005). Endogeneous nasal colonization is believed to be a common source of infection and a strong risk factor for subsequent colonization (Wertheim *et al.* 2004, 2005). Since health care workers are at the interface between hospitals, long-term care facilities, and nursing homes on the one hand and the community on the other, they may serve as reservoirs, vectors, or victims of MRSA cross-transmission (Albrich and Harbath 2008). Hands of health care professionals are the most common vehicle for the transmission of health care-associated pathogens from patient to patient and within the healthcare environment (Allegranzi and Pittet 2009). Hand hygiene is the leading measure for preventing the spread of antimicrobial resistance and reducing health care-associated infections (Allegranzi and Pittet 2009). In addition to hand hygiene, contact precautions (masks, gloves), environmental decontamination, cleaning protocols as well as education and training (Calfee *et al.* 2008) are very important in the prevention and control of MRSA.

The purpose of this study was to evaluate the prevalence of *Staph. aureus* and MRSA carriage on hands and in the nose among healthy health care workers (HCWs) of a central hospital in the North of Portugal, and to characterize isolates regarding their resistance to antibiotics and virulence factors. A potential clonal relationship between isolates was also investigated.

Materials and methods

Staphylococcus aureus sampling

One hundred and sixty-nine health care professional volunteers (doctors, nurses, auxiliaries, dieticians, administratives) working in the medicine and surgery services of a central hospital in Porto (Portugal) were analysed for the presence of *Staph. aureus* in nose and on hands (Table S1). These were healthy individuals with no symptoms of skin or respiratory infection. A questionnaire was performed at the time of collection considering sex, age, function, service, service time and antibiotics taken in the previous 3 months.

Specimens were collected using a sterile cotton-tipped swab previously moistened with sterile Ringers solution. The anterior nares were sampled by rotating the swab tip in both nostrils. Swabs were then spread onto Baird-Parker Egg Yolk Tellurite Medium (LabM, Bury, UK) and incubated at 37°C for 48 h. Characteristic colonies were isolated on Mannitol Salt Agar (MSA; Pronadisa, Madrid, Spain). Presumptive *Staph. aureus* colonies on MSA (yellow colonies with yellow zones, Gram-positive, catalase positive, coagulase positive and DNase positive) were streaked on Tryptone Soy Agar (TSA; Pronadisa) before being stored at −80°C in Brain Heart Infusion (BHI; LabM) broth containing 30% (v/v) glycerol.

DNA extraction

DNA was extracted from single colonies on TSA by the guanidine-isothiocyanate method (Aires-de-Sousa *et al.* 1996). DNA was quantified spectrophotometrically at 260 and 280 nm.

Identification of isolates by multiplex PCR

PCR multiplex detecting simultaneous presence of 16S rRNA (*Staphylococcus* genus specific), *nuc* (*Staph. aureus* species specific) and *mecA* (determinant of methicillin resistance) genes was performed according to (Zhang *et al.* 2004). *Staphylococcus aureus* DSM 11729 was used as a positive control for gene *mecA*, *Staphylococcus epidermidis* DSM 20044 as a negative control for gene *nuc* and *Staph. aureus* ATCC 29213 as positive control for targeting 16S rRNA and *nuc* gene and negative for gene *mecA*.

Antibiotic susceptibility testing by agar dilution (MIC)

Minimal inhibitory concentrations (MICs; $\mu g\ ml^{-1}$) for *Staph. aureus* isolates were determined by the agar dilution method described in the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2007). The antibiotics investigated were penicillin G, ampicillin, chloramphenicol, nitrofurantoin (all obtained from Sigma, Steinheim, Germany), oxacillin (BioChemica, Billingham, UK), rifampin, gentamicin, tetracycline, erythromycin and ciprofloxacin (all kindly supplied by Labesfal, Tondela, Portugal) and vancomycin (Fluka, Steinheim, Germany). MICs were determined on Mueller-Hinton agar (MH; bioMérieux, Marcy l'Etoile, France) plus 2% (w/v) NaCl in the case of oxacillin, on cation-adjusted MH for penicillin and ampicillin and on MH for the other antibiotics investigated At least two independent experiments were performed. *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as controls.

Detection of β-lactamases

To those isolates that are *mecA* negative and presenting a MIC for oxacillin of 4 $\mu g\ ml^{-1}$, β-lactamases were detected using nitrocefin dicks (Fluka; Pitkälä *et al.* 2007).

Presence of virulence factors

Production of haemolysin was evaluated on blood agar plates (COS, Columbia agar plus 5% (v/v) sheep blood; bioMérieux). Isolates were streaked onto the plates and incubated at 37°C for 1–2 days. The presence or absence of zones of clearing around the colonies was interpreted as β-α- or γ-haemolysis activity respectively (Semedo *et al.* 2003). Lipase and Gelatinase activity was assessed according to Tiago *et al.* (2004). Two independent experiments were performed for each.

Detection of enterotoxin genes

Detection of enterotoxin genes, *sea* to *sej* and *tst*, was performed by multiplex PCR according to Løvseth *et al.* (2004). Amplification of the target 16S rRNA gene was included as the internal control. Different strains of *Staph. aureus* kindly supplied by Professor Løvseth (National Veterinary Institute, Norway), were used as positive controls: R2102/00 for *sec*, *seg*, *sei* genes; R4571/00 for *sec* gene; FRI572 for *seg*, *sei* genes; 3169 for *sec-bovine*, *sed*, *sej* genes; FRI472 for *sed*, *seg*, *sei*, *sej* genes; R5371/00 for *sea*, *seg*, *seh*, *sei* genes; R963/00 for *sed*, *seg*, *sei*, *sej* genes; R5460/00 for *seb*, *seg*, *seh*, *sei* genes; FRI913 for *sea*, *sec*, *see* genes; FRI445 for *seg*, *sei* genes; R4071/00 for *seb* gene; and, R4774/00 as a negative control.

DNA-macrorestriction by pulsed-field gel electrophoresis typing

Pulsed-field gel electrophoresis (PFGE) typing of isolates was performed as previously described by Chung *et al.* (2000) with minor modifications using the restriction enzyme *Sma*I (ThermoScientific, New York, NY) and *Salmonella enterica* subsp. *enterica* serovar Braenderup H9812 as standard and a CHEF Mapper XA (Bio-Rad, Laboratories, Amadora, Portugal). The electrophoresis conditions had an initial switch time of 4·0 and a final switch time of 40·0 s and a run time of 20·5 h. PFGE image analyses and similarity clustering were performed with the GELCOMPAR software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was done by the unweighted pair group method with average linkages (UPGMA), using the Dice coefficient to analyse the similarities of the banding pulsotypes.

SCC*mec* typing

All MRSA isolates were typed by SCC*mec* as previously described (Milheiriço *et al.* 2007). Control strains for five types of SCC*mec* were kindly supplied by Professor Keiichi Hiramatsu (Juntendo University, Tokyo, Japan: Type I (NCTC 10442), Type II (N315), Type III (85/2082), Type IV (JCSC 4744) and Type V (Wis).

Detection of Panton-Valentine leukocidin genes

All MRSA isolates were analysed for the presence of Panton-Valentine leukocidin (PVL) genes with conditions previously described by Holmes *et al.* 2005;. Control strains (HDES 57 and HDES 26) were kindly provided by Professor Hermínia de Lencastre (Laboratório de Genética Molecular, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa (UNL), Oeiras, Portugal, and Laboratory of Microbiology, The Rockefeller University, New York, NY; Conceição *et al.* 2010).

Statistical analyses

Statistical Package for Social Sciences (IBM SPSS STATISTICS, ver. 23.0, Lisbon, Portugal) software was used. The Chi-Square test for independence was applied to the categorical variables under study. For quantitative and continuous variables, the dependence was assessed by the Pearson correlation coefficient.

Results

Prevalence of *Staphylococcus aureus* and MRSA carriage

Eighty-two *Staph. aureus* isolates were recovered after sampling on hands and in nose of 169 individuals working in medicine (57 isolates) and surgery (25 isolates). Nasal carriage (39·6%) was significantly higher ($P < 0·001$) than hand carriage (8·9%). Six per cent of the individuals carried *Staph. aureus* in both hands and nose and 3% carried *Staph. aureus* only on hands (Table 1). Being a carrier of *Staph. aureus* was negatively correlated with previous intake of antibiotics ($P < 0·01$). According to the European Antimicrobial Resistance

Table 1 Hand and nasal carriage of *Staphylococcus aureus* among health care professionals

	Number of positive (%)	Number (%) MRSA	Number (%) BORSA	Number (%) MSSA
Hands	15 (8·9)*	8 (4·7)	0 (0·0)	7 (4·1)
Nose	67 (39·6)†	29 (17·2)	3 (1·7)	35 (20·7)

MRSA, methicillin-resistant *Staphylococcus aureus*; BORSA, borderline oxacillin-resistant *Staph. aureus*.

**Staphylococcus aureus* was isolated only from hands on five occasions (2 MSSA and 3 MRSA).

†*Staphylococcus aureus* was isolated from both hands and nose from 10 individuals (5 MSSA and 5 MRSA).

Surveillance System (EARS)-net protocol (EARS-Net 2012), 17·2 and 4·7% of the individuals, respectively, were MRSA carriers in nose and on hands as the presence of *mecA* gene was detected by PCR (Table 1). Being a carrier of MRSA was correlated with the function performed ($P = 0{\cdot}018$; 26·0 and 18·2% of the nurses and auxiliaries, respectively, were MRSA carriers).

Antibiotic resistance of MRSA and MSSA isolates

As demonstrated in Table 2 and Table S2, there was a high percentage of resistance of MRSA and MSSA (Methicillin-Sensitive *Staphylococcus aureus*) isolates to the β-lactams penicillin, and ampicillin. Although resistance to erythromycin and to ciprofloxacin was observed for both MRSA and MSSA isolates, the highest percentage of resistance was found for MRSAs. About 45·1% (37/82) of the isolates were *mecA* gene positive; two of these were sensitive to oxacillin (Table S2). Three isolates were *mecA* gene negative and oxacillin-resistant (MIC ≥ 4 μg ml^{-1}; Table S2) and were classified as borderline oxacillin-resistant *Staph. aureus* (BORSA; Livermore 1995). These showed the presence of betalactamases. The concordance between the presence of *mecA* gene with oxacillin resistance was of 94·6% (35/37).

Virulence determinants

Results for virulence determinants investigated are presented in Table S2. Enterotoxin genes, either alone or in combination, were detected for 81·7% of the isolates. A higher diversity of enterotoxin genes was observed for MSSA isolates *tst* genes were detected in 3·2 and 51·5% of the MRSA and MSSA enterotoxigenic isolates respectively (Table S2). All isolates were PVL negative. With the exception of isolate 139M SCC*mec* type V, all the other isolates were SCC*mec* type IV.

Clonal relationship between isolates

PFGE typing of 20 *Staph. aureus* isolated from ten individuals that carried this organism in nose and on hands is presented in Fig. 1. Six individuals (i.e., 4II, 12, 81, 116, 122, and 148) presented indistinguishable PFGE types for isolates collected from both locations.

PFGE typing of 37 MRSA isolated from 34 individuals that carried this organism in nose ($n = 27$), hands ($n = 4$), and both locations ($n = 3$) is presented in Fig. 2. Several clusters were observed, however, a major cluster (A), comprising 50% of the MRSA isolates (13 and 6 isolates collected from individuals working in the medicine and surgery services respectively) was identified.

Discussion

To the author's knowledge, this is the first report on the prevalence of *Staph. aureus* and MRSA hand and nose carriage, simultaneously, among healthy health care professionals in Portugal. Moreover, only a few studies have been performed elsewhere (Albrich and Harbath 2008; Dulon *et al.* 2014). About 39·6% of healthy health care workers carried *Staph. aureus* in the nasal cavity; higher than (Olsen *et al.* 2013; Conceição *et al.* 2014) and lower than (van Vugt *et al.* 2015) the prevalence that had been reported. As previously observed by (Tammelin *et al.* 2003) in this study, hand carriage (8·9%) was lower than nasal carriage. For the 6% of individuals that carried both hands and nose *Staph. aureus*, 60% carried the same strain in hands and in nose according to PFGE analysis. Similar results (50%) were previously obtained in a

Table 2 Antibiotic sensitivity pattern of the *Staphylococcus aureus* isolates recovered from hands and nose of health care professionals

	Sensitive, *n* (%)		Intermediate, *n* (%)		Resistant, *n* (%)	
Antibiotic	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA
Penicillin	0 (0·0)	5 (11·1)	*	*	37 (100)	37 (88·1)
Ampicillin	2 (5·4)	6 (13·3)	*	*	35 (94·6)	36 (85·7)
Oxacillin	2 (5·4)	42 (100)	*	*	35 (94·6)	0 (0·0)
Ciprofloxacin	6 (16·2)	33 (78·6)	0	1 (2·4)	31 (83·8)	8 (19·0)
Erythromycin	7 (18·9)	17 (40·5)	0	14 (33·3)	30 (81·1)	11 (26·2)
Tetracycline	37 (100)	41 (97·6)	0	0	0	1 (2·4)
Gentamicin	37 (100)	41 (97·6)	0	0	0	1 (2·4)
Nitrofurantoin	37 (100)	42 (100)	0	0	0	0
Rifampicin	36 (97·3)	41 (97·6)	0	1 (2·4)	1 (2·7)	0
Chloramphenicol	19 (51·4)	31 (73·8)	17 (45·9)	11 (26·2)	1 (2·7)	0
Vancomycin	37 (100)	42 (100)	0	0	0	0

MRSA, methicillin-resistant *Staphylococcus aureus*.

*Antibiotic with no described value for the intermediate minimal inhibitory concentration.

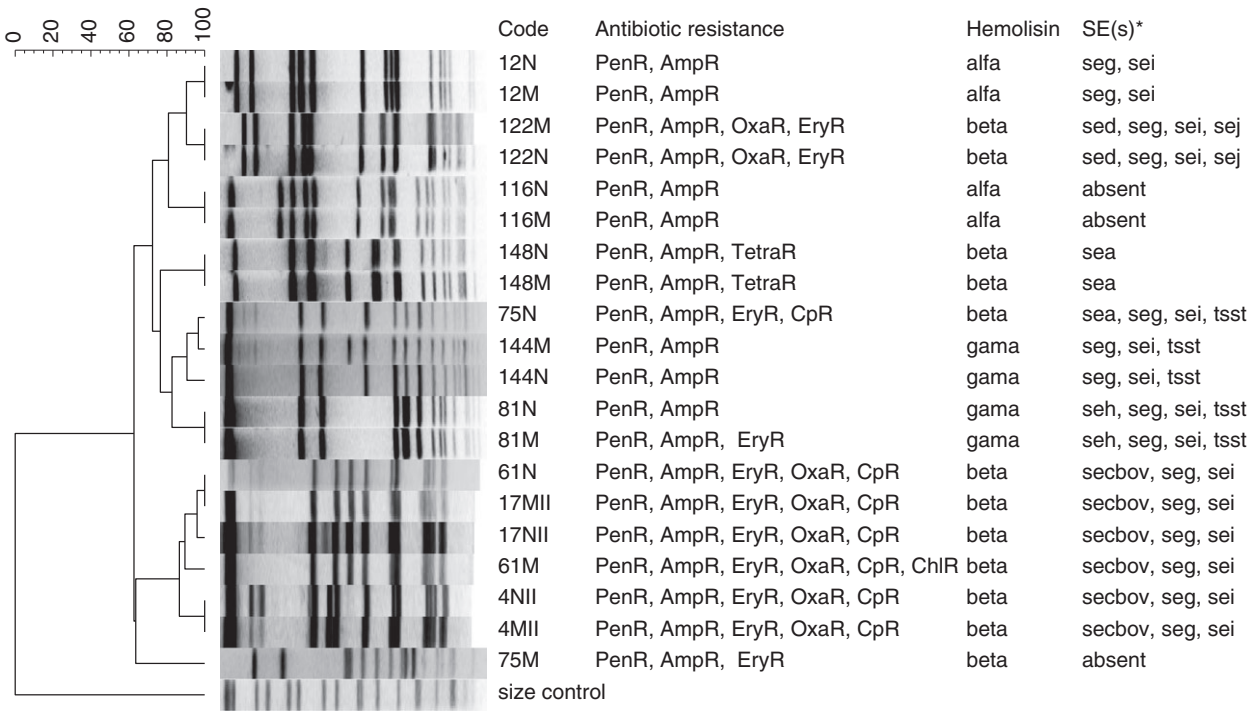


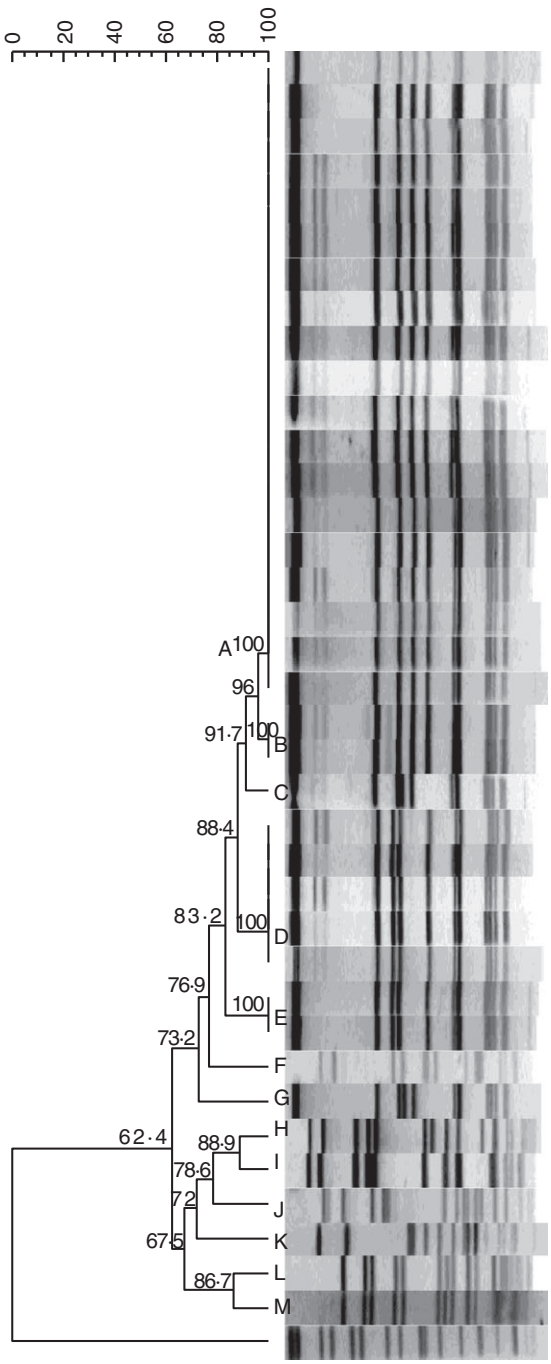
Figure 1 Dendrogram showing *SmaI* macrorestriction types of 20 isolates from 10 individuals that carried *Staphylococcus aureus* simultaneously on nose (N) and hands (M). Pen, Penicillin; Amp, Ampicillin; Oxa, Oxacillin; Ery, Erythromycin, Cp, Ciprofloxacin; Tetra, Tetracycline; Chl, Chloramphenicol; Rifa, Rifampicin; R, resistant; SE, Staphylococcal enterotoxin.

Swedish hospital (Tammelin *et al.* 2003). This suggests cross contamination between nose and hands although contamination from patients or environment cannot be excluded. About 17·2 and 4·7% of healthy health care workers were MRSA carriers, respectively, in nasal cavity and on hands. Although higher prevalence has already been reported (ex. 18·7%; Conceição *et al.* 2014), MRSA nasal carriage found in this study was higher than that reported in other studies (0·2–14·5%; Dulon *et al.* 2014) including a study in another Portuguese hospital (4·8%; Amorim *et al.* 2009). According to these authors, there are few studies from countries with very low or very high prevalence. It has been demonstrated that carriage rates among HCWs are higher than among community members without known risk factors (Salgado *et al.* 2003). It is important to point out that that prevalence of MRSA in Europe is highly variable: 0% in the north to over 50% in southern European countries (EARSS, 2007). Hand carriage was similar to that previously reviewed and reported (4·6%) by Albrich and Harbath (2008). However, it is important to highlight that MRSA carriage on hands has been less investigated than nasal carriage (Albrich and Harbath 2008; Cimolai 2008). Nevertheless, hands of healthcare personnel have been generally considered critical vectors for transmission of MRSA (Cimolai 2008). As previously reported by other authors and reviewed by Dulon *et al.* (2014), nurses were the group of professionals who demonstrated the highest prevalence of MRSA. This has been attributed to the high contact of nurses with patients (Olsen *et al.* 2013; Dulon *et al.* 2014).

With the exception of one MRSA isolate that was SCC*mec* type V, all the other isolates were type IV. These types are associated with CA-MRSA, strongly associated with PVL and considered less resistant to non-β-lactam antimicrobial agents (DeLeo *et al.* 2010). In the present study, both MRSA and MSSA isolates were PVL negative. Most of the MRSA isolated in several hospitals in different areas of Portugal harboured SCC*mec* type IV and were PVL negative (Amorim *et al.* 2007; Aires-de-Sousa *et al.* 2008).

As Aires-de-Sousa *et al.* (2008), we also found a strain characterized as SCC*mec* type V. These authors hypothesized that these strains might have been imported from the community. As reviewed by Chatterjee and Otto (2013), an increasing number of reports worldwide indicate that CA-MRSA strains are gradually replacing HA-MRSA strains in hospitals.

Considering antibiotic resistance, simultaneous resistance to penicillin and ampicillin, oxacillin, erythromycin and ciprofloxacin was the most frequent resistance



Code	Antibiotic resistance	SE(s)*
54N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
61M	PenR, AmpR, EryR, OxaR, CpR, ChlR	secbov, seg, sei
80N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
9NII	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
10NII	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
6NII	PenR, AmpR, EryR, RifaR, CpR	secbov, seg, sei
12NII	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
17MII	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
134N	PenR, AmpR, OxaR, CpR	secbov, seg, sei
126N	PenR, AmpR, EryR, OxaR, CpR	seg, sei
22N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
94N	PenR, EryR, OxaR, CpR	secbov, seg, sei
140N	PenR, AmpR, OxaR, CpR	secbov, seg, sei
73N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
72N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
124N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
61N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
69N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
18N	PenR, AmpR, OxaR, EryR, CpR	secbov, seg, sei
149M	PenR, AmpR, OxaR, CpR	secbov, seg, sei
17NII	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
29N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
4NII	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
4MII	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
49N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
59N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
53N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
51N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
47N	PenR, AmpR, EryR, OxaR, CpR	secbov, seg, sei
13MII	PenR	absent
30N	PenR, AmpR, OxaR, CpR	secbov, seg, sei
60N	PenR, AmpR	seg,sei, tsst
122M	PenR, AmpR, OxaR, EryR	sed, seg, sei, sej
14NII	PenR, AmpR, OxaR, EryR, CpR	absent
75M	PenR, AmpR, EryR	absent
139M	PenR, AmpR, OxaR, EryR	absent
78N	PenR, AmpR, OxaR, EryR	absent
Size standard		

Figure 2 Dendrogram showing *SmaI* macrorestriction types of 37 methicillin-resistant *Staphylococcus aureus* isolates collected from hands and noses of health workers and HDES57 (PVL positive control). N, nose; M, hands; Pen, Penicillin; Amp, Ampicillin; Oxa, Oxacillin; Ery, Erythromycin, Cp, Ciprofloxacin; Chl, Chloramphenicol; Rifa, Rifampicin; R, resistant; SE, Staphylococcal enterotoxin.

profile detected for MRSA; only two isolates were resistant to β-lactams only. Most of the MSSA isolates demonstrated simultaneous resistance to penicillin and ampicillin. High percentage of isolates resistant to erythromycin and ciprofloxacin among MRSA isolates SCC*mec* type IV was previously reported (Conceição *et al.* 2010).

Enterotoxin genes were detected in 83·8% (31/37) of MRSA and in 78·6% (33/42) of MSSA; enterotoxins profile of MSSA was more diverse than of MRSA. Although

sea has been the most common reported enterotoxin gene (Rall *et al.* 2010) this was not observed in the present study. *seg* and *sei* were the most prevalent enterotoxin genes and were associated in all the isolates. Similar results were previously reported by (Becker *et al.* 2003).

Although the roles that enterotoxins play in skin and soft tissue infections are not known, they have been shown to influence the development of secondary staphylococcal infections due to their pro-inflammatory effects that trigger increased inflammation (Skula *et al.* 2010). TSST-1 was the first marker identified for Staphylococcal Toxic Shock Syndrome (TSS) which is an acute and potentially fatal illness that is characterized by a high fever, diffuse erythematous rash, desquamation of the skin 1–2 weeks after onset hypotension, and involvement of three or more organ systems (Grumann *et al.* 2014) *tst* genes were highly prevalent in MSSA isolates (48·5%). On the contrary this gene was found in only one MRSA isolate; Becker *et al.* (2003) reported a prevalence of *tst* genes in nasal isolates of 22·4%. 75·6% the MRSA isolates were distributed through four closely related PFGE clusters; this probably explains the low diversity observed in the antibiotic resistance and enterotoxin genes profile in this group of isolates.

Isolate HDES57, previously demonstrated (Conceição *et al.* 2010) to show the molecular characteristics of the highly internationally disseminated EMRSA-15 clone (Moore and Lindsay 2002), was not included in any of these major clusters. However, this clone has been systematically considered the major clone circulating in Portuguese hospitals (Amorim *et al.* 2007; Aires-de-Sousa *et al.* 2008; Conceição *et al.* 2010).

Isolates recovered in this study, in a hospital not included in previous studies, seemed to be different from major clones previously isolated in Portugal, namely the New York/Japan clone (SCC*mec* type II) and the Brazilian MRSA (SCC*mec* type IIIA; Aires-de-Sousa *et al.* 2008).

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Conflict of Interest

The authors have no conflict of interests to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Characterization of the sample population (n = 169)

Table S2 *Staphylococcus aureus* profile concerning antibiotic resistance and virulence factors

Table S1 Characterisation of the sample population (n=169).

Factor		**%**
*Sex	Female	82.8
	Male	17.2
Function	Nurse	45.6
	Auxiliary	26.0
	Doctor	21.9
	Administrative	4.7
	Dieticians	1.8
*Service time	<1 year	16.0
	1-5 years	56.8
	5-10 years	11.2
	>10 years	16.0
Antibiotic taking in the	Yes	18.9
past three months	No	81.1
*Service	Medicine	71.6
	Surgery	28.4
*Age	Median	35.4 years
	Minimum	21 Years
	Maximum	68Years

* There was no association between age, sex, working service or service time and carriage of *S. aureus* ($p>0.01$) or MRSA ($p>0.01$)

Table S2: *Staphylococcus aureus* profile concerning antibiotic resistance and virulence factors

MRSA isolates	Antibiotic resistance	haemolysis	gelatinase	lipase	SE(s)
22N, 29N, 51N, 54N, 61N, 72N, 73N, 80N, 94N, 124N, 9NII, 17NII, 17MII	Pen, Amp, Oxa, Ery, Cp	β	+	+	*secbov, seg, sei*
18N	Pen, Amp, Oxa, Ery, Cp	α	+	+	*secbov, seg, sei*
30N, 134N, 140N, 149M	Pen, Amp, Oxa, Cp	β	+	+	*secbov, seg, sei*
78N	Pen, Amp, Oxa, Ery	α	-	+	-
47N, 14NII	Pen, Amp, Oxa, Ery, Cp	β	-	-	-
49N, 59N, 4NII, 4MII	Pen, Amp, Oxa, Ery, Cp	β	+	-	*secbov, seg, sei*
126N	Pen, Amp, Oxa, Ery, Cp	α	-	+	*seg, sei*
53N	Pen, Amp, Oxa, Ery, Cp	α	-	-	*secbov, seg, sei*
10NII, 12NII	Pen, Amp, Oxa, Ery, Cp	β	-	+	*secbov, seg, sei*

6NII	Pen, Amp, Oxa, Rifa, Ery, Cp	β	+	+	*secbov, seg, sei*
60N	Pen, Amp Oxa	α	+	+	*seg,sei, tst*
69N	Pen, Amp, Oxa, EryR, CpR	β	-	-	*secbov, seg, sei*
61M	Pen, Amp, Oxa, Chl, Ery, Cp	β	+	+	*secbov, seg, sei*
139M	Pen, Amp, Oxa, Ery	γ	-	-	-
75M	Pen, Amp, Ery	β	-	+	-
13MII	Pen	γ	-	-	-
122M	Pen, Amp, Oxa, Ery	β	+	+	*sed, seg, sei, sej*

MSSA isolates	**Antibiotic resistance**	**haemolysis**	**gelatinase**	**lipase**	**SE(s)**
37N	Pen, Amp	β	+	+	*secbov, seg, sei, tst*

83N	Pen, Amp	β	+	+	*seg, sei*
152N	Pen, Amp	β	+	-	*seh, seg, sei, tst*
52N	Pen, Amp	α	+	+	*seh, seg, sei, tst*
38N	Pen, Amp	α	-	-	*seh, seg,sei, tst*
81N	Pen, Amp	γ	+	+	*seh, seg, sei, tst*
107N, 91N	Pen, Amp	α	-	+	*seg, sei, tst*
114N	Pen, Amp	β	-	+	*seg, sei, tst*
67N	Pen, Amp	α	+	-	*sea,seg,sei, tst*
12N, 12M	Pen, Amp	α	+	+	*seg, sei*
108N	Pen, Amp	β	+	+	*secbov, seg, sei*
76N	Pen, Amp	γ	+	+	*seg,sei*
116N, 116M, 70M	Pen, Amp	α	-	+	-
141N	Pen, Amp	α	+	+	*secbov, seg, sei*
11N	Pen, Amp, Ery	α	+	+	*secbov,seg,sei, tst*
34N	Pen, Amp, Ery	α	-	+	*sea,seg,sei, tst*

58N	Pen, Amp, Ery	β	+	+	*secbov*
122N	Pen, Amp, Ery	β	+	+	*sed, seg, sei, sej*
63N	Pen, Amp, Ery	α	-	+	*seh, seg, sei*
71N	Pen, Amp, Cp	γ	-	+	*seg,sei*
103N	Pen, Amp, Cp	γ	+	+	*sea, seg,sei, tst*
57N	Pen, Amp, Cp	α	+	+	*sea, seg,sei*
26N	Pen, Amp, Cp	α	+	-	*sea, seg,sei, tst*
85N	-	β	+	-	-
10N	Pen, Amp	β	+	+	-
33N	-	α	+	+	*seg,sei*
144N	Pen, Amp	β	+	+	*sea, seg,sei, tst*
75N	Pen, Amp, Ery, Cp	β	+	-	*sea, seg, sei, tst*
101N	Amp	β	-	+	*seb*
82N	Pen, Amp, Ery, Cp	β	+	+	*secbov, seg, sei*
133N	Pen	α	-	-	-

131N	Amp, Ery	α	-	-	-
146N	Pen, Ery	α	-	+	-
148N, 148M	Pen, Amp, Tetra	β	+	-	*sea*
144M	Pen, Amp	γ	+	+	*seg, sei, tst*
81M	Pen, Amp, Ery	γ	-	+	*seh, seg, sei, tst*
41M	Ery	γ	-	-	-

BORSA isolates	**Antibiotic resistance**	**haemolysis**	**gelatinase**	**lipase**	**SE(s)**
42N	Pen, Amp, Oxa, Tetra	β	-	+	*seh, seg, sei*
130N	Pen, Amp, Oxa	α	-	+	*seh, seg,sei, tst*
137N	Pen, Amp, Ery, Oxa	β	+	+	*seb, seg, sei*

N, nose; M, hands; +, positive result; -, negative result; Pen, Penicillin; Amp, Ampicillin; Oxa, Oxacillin; Ery, Erythromycin, Cp, Ciprofloxacin; Tetra, Tetracycline; Chl, Chloramphenicol; Rifa, Rifampicin; SE, Staphylococcal enterotoxin

Chapter 7

Main Conclusions

Main Conclusions

Worldwide, *S. aureus* is considered a public health problem and a ubiquitous organism found in hospital, community and livestock environments. Nasal and hand carriage of *S. aureus* are important vehicles of dissemination of this pathogen. Considering that endogenous nasal colonisation is a risk factor for developing disease, a special highlight was made to this issue in this work.

In this study, the occurrence and further characterization of *S. aureus* isolated from healthy individuals - health professionals, food handlers and children- were studied. *Staphylococcus aureus* nasal occurrence in hospital health care professionals and in children was 39.6% and 48.6%, respectively, much higher than that observed for food handlers (19.8%). Nasal *S. aureus* carriage in children was the highest among the studied populations and was dependent on kindergarten; two out of four of analysed kindergartens showed an occurrence of *S. aureus* of more than 60%. Risk factors for nasal carriage such as age and exposure to environment were confirmed in this study. The occurrence of *S. aureus* on the hands of health care professionals and food handlers was respectively 8.9% and 11.1%. Globally, higher *S. aureus* occurrence was obtained for nasal carriage. For the 6% of the health care professional individuals that carried *S. aureus* in both hands and nose, 60% carried the same strain. For the 6.2% of food handler individuals that carried *S. aureus* in both hands and nose, 30% carried the same strain. This result shows the importance of possible contamination routes by *S. aureus*.

Methicillin-Resistant *S. aureus* was the first form of antibiotic resistance discovered in *S. aureus* in 60's decade. Since then, a large importance has been given to MRSA especially as a nosocomial pathogen. Recently, their importance in the community had been noted. In order to highlight community occurrence in Portugal this study was performed. Nasal occurrence of MRSA was higher (17.2%) in health care professionals than in children (9.7%), while food handlers presented no MRSA. Considering health care profession, nurses were the group of hospital health care professionals who showed the highest prevalence of MRSA. The occurrence of MRSA on hands of health care professionals was 4.7%. Globally nasal carriage of MRSA was higher than hand carriage. Considering children it was demonstrated

that in one kindergarten (School 4) a high carriage (25%) of MRSA strains exists among healthy children. There was no homogeneous distribution of MRSA among different kindergartens.

Food analysis in two years (2007 and 2010) revealed low levels of MRSA, 0.68% and 5.5%, respectively. This observation is in accord with the worldwide reports; although an increase of MRSA strains between the two sampling years was observed.

Antibiotic resistance to other antibiotics other than encoded by *mec*A gene, was performed. Although, some ß-lactams (penicillin, ampicillin and oxacillin) was also tested. Concerning the ß-lactams and, as expected, high resistance levels were observed. *S. aureus* strains presented low levels of resistance to gentamicin, chloramphenicol and rifampicin. No resistance to vancomycin was detected. Regardless of the source, resistance to erythromycin was evident. Among food isolates, some differences between the antibiotic resistance profile were observed in different years: in 2010, the resistance to erythromycin and tetracycline was higher compared to the isolates collected in 2007. This fact might be related to the type of food samples. Globally, all community isolates were demonstrated to be simultaneously resistant to antibiotics of different classes.

One virulence factor tested was presence of staphylococcal enterotoxin (SEs) genes. Not all the tested *S. aureus* were enterotoxigenic but an occurrence higher than 50% of was observed. Regardless of the source *egc* cluster was the most prevalent. Except for the food samples collected in 2007, *secbov*. *seg* and *sei* profile was detected in all the tested groups/origins. Although *sea* is considered the most prevalent enterotoxin reported all over the world, in the present study this was not verified. The prevalence of *sea* was around 13.2 to 21.2% from the various origins with exception of food isolates 2007 (53.5%). All origins presented enterotoxigenic strains confirming this virulence factor occurs not only in food isolates.

TSST-1 has a virulence marker known as *tst* gene that was detected in children, hospital health care professionals and food handlers, 24.6%, 39% and 39.3%, respectively. There is a predisposition for carriage of *tst* by *S. aureus* isolates from all origins. Food can also carry *S. aureus* strains with *tst* usually associated with clinical isolates.

Presence of PVL genes was analysed in some MRSA strains collected but only one food strain showed the presence of these genes. SCC*mec* typing revealed the presence of type IV and V (for MRSA from hospital and food samples). SCC*mec* type IV is associated with PVL positive strains worldwide. Although the majority of Hospital strains were SCC*mec* type IV they were PVL negative, which is in accordance with other studies in Portugal. According to PFGE typing a major clone predominated among the isolates collected from health care professionals at the same hospital. Isolates recovered in health care professionals in this hospital differed from the major clones previously isolated in Portugal in other hospitals.

This study revealed a high occurrence of antibiotic resistance and virulence determinants, including not only classical and novel enterotoxin genes (*seg* to *sej*) but also major virulence factors such as *tst*, in the studied population. Dissemination of these strains in the community is a matter of considerable concern.

Chapter 8

Proposals for future work

Future Work

Concerning the present work, several questions might be suggested for future research namely:

- Most of the *Staphylococcus aureus* isolates characterized in this study were collected in 2007. It would be important, as a future work, to update the generated information performing new samplings and further isolations and characterizations. This new information should be further compared with the presented information in order to understand the evolution/tendency and the consistency of the present study.

- The PVL detection should have been performed for all the *S. aureus* isolates including the MSSA; those results would be important to understand the overall virulence capacity of these strains.

- New typing techniques could have been implemented and developed in order to compare our results with the ones reported worldwide namely: *spa* typing and MLST. These techniques are being reported by other authors as capable of classifying community, hospital- and livestock-associated isolates. It could also been implemented phage typing with human, bovine and poultry phages.

- Although *mecC* MRSA are currently rare, the presence of *mecC* gene should have been tested. In fact, *mecC* gene is reported as a new homologue of *mecA* gene and was firstl reported in 2011. This gene might be important in the misdiagnosed as methicillin-sensitive *S. aureus*. *mecC* MRSA have now been reported in several European countries and have been isolated from different host species.

- To date, at least 11 types of SCC*mec* have been identified for MRSA. It would be interesting as a further study, to test the presence of those 11 types in our isolates. All SCC*mec* carry the *mec* and the *ccr* gene complex. SCC*mec* in *S. aureus* are classified

into different types based on the combination of *mec* and *ccr*, which share variations, five classes in *mec* and eight in *ccr*.

- New enterotoxins such as SER-SET and SElK-Q and SelU-X could have been studied.

- The susceptibility of the *S. aureus* isolates to disinfectants frequently used in the food industry and in hospitals should also be evaluated. In another perspective, some biocontrol strategies could be studied in order to prevent/reduce the occurrence of *S. aureus* and MRSA strains. As an example, plant extracts, propolis, bacteriocins, phages and others.