CHARACTERISATION OF NASAL COLONIZATION AND HAND CONTAMINATION OF FOOD HANDLERS WITH *STAPHYLOCOCCUS AUREUS* AND INVESTIGATION OF VIRULENCE AND RESISTANCE DETERMINANTS OF ISOLATES

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Characterisation of Nasal Colonization and Hand Contamination of Food Handlers with *Staphylococcus Aureus* and Investigation of Virulence and Resistance Determinants of Isolates

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Certificate of Originality

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

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Jeffery HO

Abstract

Investigation of staphylococcal food poisoning (SFP) outbreaks has frequently detected the same clone of *Staphylococcus aureus* from food handlers and the incriminated food, emphasising that *S. aureus* colonization of food handlers is of public health significance.

This study aimed to investigate nasal colonization and hand contamination rates with *Staphylococcus aureus* in food handlers employed in large scale catering establishments in Hong Kong over a ten year period. This was achieved by means of three related cross sectional studies, conducted before and after SARS, and in 2011. Risk factors for colonisation and temporal changes of prevalence of SE/ SEl genes circulating in *S. aureus* strains nasally colonizing food handlers were determined. Strains were characterised by *spa* typing to determine carriage status and track the route of transmission between workers in food establishments. The prevalence of *qac* genes in nasal isolates was investigated to determine if increased use of disinfectants in food establishments had selected for disinfectant tolerant strains.

Nasal swabs and hand imprint specimens were collected from food handlers working in large catering establishments in 2011 and isolates compared with those collected in the earlier studies. Risk factors were investigated by means of validated questionnaires completed in 2002 and 2011 and analysed by multivariate analysis. Staphylococcal enterotoxins (SE), *spa*, and antibiotic- and antiseptic-resistance genes were detected by PCR amplification followed by sequencing as appropriate.

Tracking of colonisation status and transmission to hands was performed by examination of *spa* types. Susceptibility to antibiotics was performed by disk diffusion and to antiseptics by determination of minimum inhibitory and bactericidal concentrations. Association between presence of SE genes, *qac* genes, antibiotic resistance, and *spa* types was investigated.

This study revealed that the prevalence of nasal colonisation and hand contamination with *S. aureus* in food handlers was significantly reduced after the SARS epidemic and the reduction appears to have been sustainable. There was a significant association between handling of raw meat and nasal carriage of *S. aureus* which was supported by the presence of specific clones previously associated with raw meat. Prevalence rates of classical SE genes remained stable while novel types varied over time. The prevalence of *qac* genes appeared to increase over time although this did not reach statistical significance and was found to be associated with the use of quaternary ammonium compound-containing disinfectants. The majority of hand contamination appeared to be attributable to cross-contamination from other persistently colonized co-workers.

The sustained reduction in nasal colonisation and the remarkable decrease in hand contamination rates over time emphasises the importance of hygiene campaigns in reducing spread of infectious agents. The higher colonization rate in raw meat handlers indicates that exposure to raw meat may increase infection risk. The frequent presence of SE- and *qac* genes in nasal isolates underscores the toxigenic potential of these strains and their ability to persist in the environment respectively.

This suggests that correct performance of environmental cleaning is an essential element in prevention of transmission and, when combined with efficient hand washing, could reduce transient nasal colonisation and hand contamination and thereby, the incidence of staphylococcal food poisoning.

Publications derived from this dissertation

Journal Articles

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Ho, J., O' Donoghue, M., Boost, M. (2014) Occupational exposure to raw meat: a newly recognized risk factor for *Staphylococcus aureus* nasal colonization amongst food handlers. *International Journal of Hygiene and Environmental Health.* 217, 347 – 353.

Ho, J., Boost, M., O' Donoghue, M. (2014) Sustainable reduction of nasal colonization and hand contamination with *Staphylococcus aureus* among food handlers, 2002 – 2011. *Epidemiology and Infection.* DOI 10.1017/ S0950268814002362.

Oral Presentation

Ho, J, Boost, M, O'Donoghue, M. (2015) Does the extensive use of QAC disinfectants select for enterotoxigenic *Staphylococcus aureus*? *Proceedings of 7th International Congress of the Asia Pacific Society of Infection Control.* Taipei, Taiwan. 26 – 29 March 2015.

Ho, J., O' Donoghue, M., Boost, M. (2013) Raw meat handling: an emerging risk factor for nasal colonization with *Staphylococcus aureus*? *Proceedings of 23rd European Congress of Clinical Microbiology and Infectious Diseases*. Berlin, Germany. 27 – 30 April 2013.

Poster Presentations

Ho, J, Boost, M., O'Donoghue, M. (2014) Does the superantigen profile of *Staphylococcus aureus strains isolated from persistent nasal carriers change? Proceedings of 7th International Congress of the Asia Pacific Society of Infection Control*. Taipei, Taiwan. 26 – 29 March 2015

Ho, J., Boost, M., O' Donoghue, M. (2014) Determination of source of staphylococcal hand contamination of food handlers using *spa* typing. *Proceedings of 15th Asia Pacific Congress of Clinical Microbiology and Infection.* Kuala Lumpur, Malaysia. 26 – 29 November 2014.

Ho, J., Boost, M., O' Donoghue, M. (2014) Temporal clonal changes of *S. aureus* isolates associated with healthy carriage, 2002 – 2011. *Proceedings of 6th International Infection Control Conference.* Wan Chai, Hong Kong. 1 – 3 August 2014.

Ho, J., O' Donoghue, M., Boost, M. (2013) Association between nasal colonization of food handlers with quaternary ammonium compound-resistance genes harbouring *Staphylococcus aureus* and disinfectant type. *Proceedings of 23rd European Congress of Clinical Microbiology and Infectious Diseases*. Berlin, Germany. 27 – 30 April 2013.

Ho, J., O' Donoghue, M., Boost, M. (2012) Are novel staphylococcal enterotoxins really genes novel? *Proceedings of 13th Asia Pacific Congress of Clinical Microbiology and Infection.* Beijing, China. 25 – 28 October 2012.

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
APC	Antigen Presenting Cell
AST	antimicrobial susceptibility test
BHI	Brain Heart Infusion Broth
BP	base pair
BURP	Based Upon Repeat Pattern
CC	Clonal Complex
ccr	cassette chromocome recombinases
CDC	centre for disease control
CFU	Colony Forming Unit
CHP	Centre for Health Protection
CI	Confidence Interval
ClfB	Clumping Factor B
CLSI	Clinical and Laboratory Standards Institute
CRP	C Reactive Protein
CV	Coefficient of Variation
DNA	Deoxyribose Nucleic Acid
Dnase	Deoxyribose Nuclease
EDTA	Ethylene Diamine Tetra Acetic Acid
egc	Enterotoxin Gene Cluster

ELISA	Enzyme Linked Immuno-sorbent Assay
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FNB	Fibronectin Binding Protein
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IgA	Immunoglobulin A
IgG	Immunoglobulin G
HIEC	Human Immune Evasion Cluster
IL	Interleukin
LA-MRSA	Livestock Associated Methicillin Resistant Staphylococcus aureus
LTA	Lipoteichoic Acid
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation Time of Flight
MBC	Minimal Bactericidal Concentration
MBL	Mannose Binding Lectin
MHA	Mueller Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimal Inhibitory Concentration
Mins	Minutes
MLSb	Macrolide-Lincosamine-Streptogramin B
mRNA	Messenger Ribonucleic Acid
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar

MSCRAM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MSSA	Methicillin Sensitive *Staphlococcus aureus*
OR	Odds Ratio
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PTSAgs	Pyrogenic Toxin Superantigens
PVL	Panton-Valentine Leukocidin
QAC	Quaternary Ammonium Compound
RNA	Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
SARS	Severe Acute Respiratoy Syndrome
SE	Staphylococcal Enterotoxin
SNP	Single Nucleotide Polymorphism
spa	Staphylococcal Protein A
ST	Sensitivity Testing
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TRIS	Tris(hydroxymethyl)aminomethane
TSS	Toxic Shock Syndrome
tsst-1	Toxic Shock Syndrome Toxin-1
WTA	Wall Teichoic Acid

Chapter 1 Literature Review

1.1 Introduction

Staphylococcus aureus is part of the commensal flora colonising human skin and mucosal surfaces. However, it can also be a pathogen responsible for a wide variety of infections that manifest in various ways depending on the infected site. The organism was firstly recognised by Sir Alexander Ogston from cases of sepsis and abscess (Lowy, 1998), and despite medical advances over the centuries, *S. aureus* remains as an important human pathogen responsible for considerable morbidity and mortality worldwide. Whether an encounter results in colonisation or infection is dependent upon the interaction between the host, the microbe and relevant environmental exposures. Considerable efforts have been made to identify important risk factors associated with colonisation in different populations. In the food industry, food handlers harbouring the organism may contaminate the food via incorrect practices. This review discusses *S. aureus* with respect to colonisation dynamics, staphylococcal food poisoning, and non-susceptibility to antibiotics and disinfectants.

1.2 Biology of *Staphylococcus aureus*

The bacterium *S. aureus* is a facultative anaerobe which belongs to the genus *Staphylococcus* which is in the family of *Staphylococcaceae*. This genus comprises at

least 41 species and 21 subspecies which share similarity in cell morphology, biochemical properties and genomic composition (Svec et al., 2010).

Staphylococci are gram positive cocci that measure between 0.5 to 1.5 µm in diameter. Although the organism may be present singly, in pairs or tetrads, it usually appears as clusters in stained preparations. Tzagoloff and Novick (1977) observed staphylococcal binary fission under phase contrast microscope and found that the organism divides in three perpendicular planes alternatively and that all daughter cells remain attached to the parental cell, hence, forming the clusters seen under the light microscope.

Most members of the genus *Staphyococcus* are catalase positive, non-motile, non-spore forming and unencapsulated except for *S. saccharolyticus* and *S. aureus* subsp. *anaerobius,* which are catalase negative. The production of a golden pigment, coagulase, and deoxyribonuclease together with the ability to ferment mannitol anaerobically allows *S. aureus* to be differentiated from other staphylococcal species.

Some *S. aureus* strains form a layer of polysaccharides outside their cell wall which is known as a microcapsule. These capsular polysaccharides render the organism resistant to phagocytosis (Kampen et al., 2005). The fact that most clinical isolates belong to particular capsular types has led to the consideration of using specific neutralizing antibodies to prevent staphylococcal infections (O'Riordan & Lee, 2004). This virulence factor will be discussed further in section 1.4.1.

The genome of *S. aureus* comprises approximately 2000 – 3000 kbp with 40% GC content. Genomic analysis not only allows novel staphylococcal species to be unambiguously identified, but also enables epidemiological investigations to be accurately conducted. DNA-DNA hybridization and nucleotide sequencing of RNA polymerase B gene (*rpoB*) and 16S rRNA gene are frequently employed to determine novel staphylococcal species (Futagawa-Saito et al., 2004; Trulzsch et al., 2007; De Bel et al., 2013). Methods used in epidemiological investigations will be discussed further in section 1.3.5.

Staphylococci produce a wide array of proteins which contribute to its pathogenic process. These can be classified into two groups: cell-surface bound and secretory proteins. At different stages of growth, the organism preferentially expresses different subsets of proteins. During the exponential-growth phase, surface proteins such as coagulase, protein A, elastin-binding protein, collagen-binding protein, fibronectin-binding protein, and clumping factor are actively synthesized to facilitate colonisation. These interact with human mucosal matrix molecules and hence are collectively known as microbial-surface components recognizing adhesive matrix molecules (MSCRAMM). Staphylococcal surface proteins share a number of structural similarities. These include the presence of an N-terminal secretory signal sequence, a hydrophobic membrane-spanning motif, a positively charged cytoplasmic domain, and an extracellular ligand-recognising motif (Lowy, 1998).

In the stationary phase, expression of secretory proteins such as nucleases, haemolysins, proteases, lipases, hyaluronidase and collagenase is up-regulated.

These enzymes turn the host cell matrix into nutrients which support bacterial growth. In addition, virulence factors such as exfoliative toxins, toxic shock syndrome toxin (tsst-1), and enterotoxins are also secreted at this stage (Lowy, 1998; Larkin et al., 2009). Strains harbouring tsst-1 are more likely to be invasive than those lacking this gene but tsst-1 positive strains are more important in infections in animals compared to human isolates (van Leeuwen et al., 2005).

Virtually all proteins produced by *S. aureus* are in one way or another involved in its pathogenic process in which the first and foremost step is to adhere to host surfaces, i.e. colonisation.

1.3 Human colonisation with *Staphylococcus aureus*

Colonisation with *S. aureus* refers to its presence in any anatomical site. In humans, the vestibulum nasi is believed to be the primary niche inhabited by the organism. To successfully colonise a host, the organism has to firstly adhere to cells, evade its immune defence, and then multiply *in situ.* Carriage is not only determined by the expression of bacterial surface proteins and relevant host receptors but is also modulated by exposure to various epidemiological risk factors.

Approximately one third of the general population has their skin and mucosa harmlessly colonised with *S. aureus* at any one time (van Belkum et al., 2009). Of these subjects, some people consistently carry the same strain while others at times harbour different strains. The organism is present in different anatomical sites with varied frequency across populations (Wertheim et al., 2005a). The observation of

increased risk for subsequent endogenous infection, particularly among persistent carriers who undergo repeated skin penetrating medical procedures, has led to considerable effort to find effective decolonisation strategies. However, such strategies are complicated by the emergence of multiple antibiotic resistance and, in recent years, increased tolerance to disinfectants in staphylococci. This section reviews epidemiological and biological aspects of human colonisation with *S. aureus*.

1.3.1 Classification of human carriage

In the early 1960s, human carriage with *S. aureus* was classified into four groups designated (1) Persistent, (2) Intermittent, (3) Occasional, and (4) Non-carriage (Williams, 1963). It was suggested that the occasional carriers referred to a subgroup of intermittent carriers from whom *S. aureus* was recovered less frequently. This four-group classification scheme has become less commonly employed by modern researchers. Eriksen et al. (1995) defined intermittent and occasional carriers as yielding a carrier index of 0.5 – 0.8 vs 0.1 – 0.4 respectively. A decade later, a three-group classification scheme was proposed by calculation of the carrier index, the proportion of consecutive nasal swab specimens positive for *S. aureus* (Nouwen et al., 2004).

The majority of longitudinal studies of human colonisation with *S. aureus* have distinguished three carriage patterns designated persistent carriage, transient carriage and non-carriage (Kluytmans et al., 1997; Wertheim et al., 2005a; Blumental et al., 2013; Chen et al., 2013; Muthukrishnan et al., 2013). A person

could be classified into one of the three groups based on the number of consecutive positive bacterial cultures over a given period. Approximately 60% of people are transient carriers who carry the organism intermittently in their life time whilst 20% each are persistent non-carriers or persistent carriers (Kluytmans et al., 1997). In a large scale longitudinal survey analysing nasal specimens, VandenBergh et al. (1999) successively isolated the same strain of *S. aureus* as confirmed by PFGE from staff members of a university hospital on two occasions eight years apart. This indicated that persistent carriers may harbour the same strain for almost a decade though the possibility of harbouring another strain or being non-carriers in-between sampling may be possible as a recent report has demonstrated that a persistent carrier can harbour different strains transiently while being colonised most of the remaining time by their resident strain (Muthukrishnan et al., 2013).

Due to problems of dropout, performing multiple sampling from the same individuals can be difficult. Nouwen et al. (2004) validated a “culture rule” which allows accurate identification of carriage status by provision of two consecutive nasal swab cultures at a one week interval. The negative predictive value was high (0.99). However, the positive predictive value was only 0.79. This suggested that this method might be more useful in terms of ruling out carriage rather than identifying carriage *per se*.

Classification of carriage status has varied between studies. VandenBergh et al. (1999) defined persistent carriage as yielding 80% positive out of 10 to 12 consecutive nasal swab cultures. The proportion of positive cultures was expressed

as the carrier index. In a more recent study, only individuals who repeatedly yielded *S. aureus* isolates of related *spa* types were considered as persistent carriers (Blumental et al., 2013). This lack of consensus in definition renders results from different studies less comparable.

Another characteristic seen in persistent carriers is that they harbour a greater bacterial load than intermittent carriers (Nouwen et al., 2004; van Belkum et al., 2009). It is believed that these persistent carriers play an important role in retention and transmission of *S. aureus* in the community because of this heavier load of the microorganism (Nouwen et al., 2004). Hence, they may be more likely to contaminate their working environment via their hands or skin scrapings from their forearms.

Recently, carriage status was suggested to be re-classified into persistent carriers and others based on the difference in antibody profile and bacterial elimination rate (van Belkum et al., 2009). A group of participants with known carriage status were decolonised and received artificial inoculation with a mixture of *S. aureus* strains. They were longitudinally followed up for 22 weeks. The organism survived significantly longer in persistent carriers (median> 154 days) as compared to intermittent (median= 14 days) and non-carriers (median= 4 days). In terms of antibody production, persistent carriers had a higher antibody titre against tsst-1 (IgG & IgA), SEA (IgA), ClfA (IgA) but a lower antibody titre against sasG. In this way, virulence factors are neutralised but the expression of adhesive proteins such as

sasG remain unaffected. This may partially explain why the organism is able to persist in some hosts without causing any harm.

The fact that transient carriers share considerable biological similarity with non-carriers has challenged the appropriateness of cross-sectional design in epidemiological studies on staphylococcal carriage where persistent and transient carriers are not differentiated. In this regard, the colonisation risk would either be overemphasized or underestimated.

1.3.2 Colonisation in humans

In the general population, *S. aureus* can be isolated in order of frequency from the nose (ca. 27%), hand (ca. 27%), pharynx (ca. 20%), perineum (ca. 22%) and intestine (ca. 20%) (Wertheim et al., 2005a; Acton et al., 2009) (Figure 1). Throat carriage of *S. aureus* is also common in healthy individuals (Nilsson & Ripa, 2005; Nguyen et al., 2014). Other colonisation sites, including the vagina and axillae are less commonly reported (Wertheim et al., 2005a). Rectal colonisation appears occur more frequently in seriously ill patients (Batra et al., 2008).

The nostrils (anterior nares) as the primary site for *S. aureus* survival in humans is substantiated by that the organism being isolated more frequently from the vestibule as compared to elsewhere inside the nasal cavity or nasal fossa (Collery et al., 2008; Blumental et al., 2013). The anterior nares are covered by stratified, keratinized, non-ciliated epithelium which is limited medially by the mucosal fold

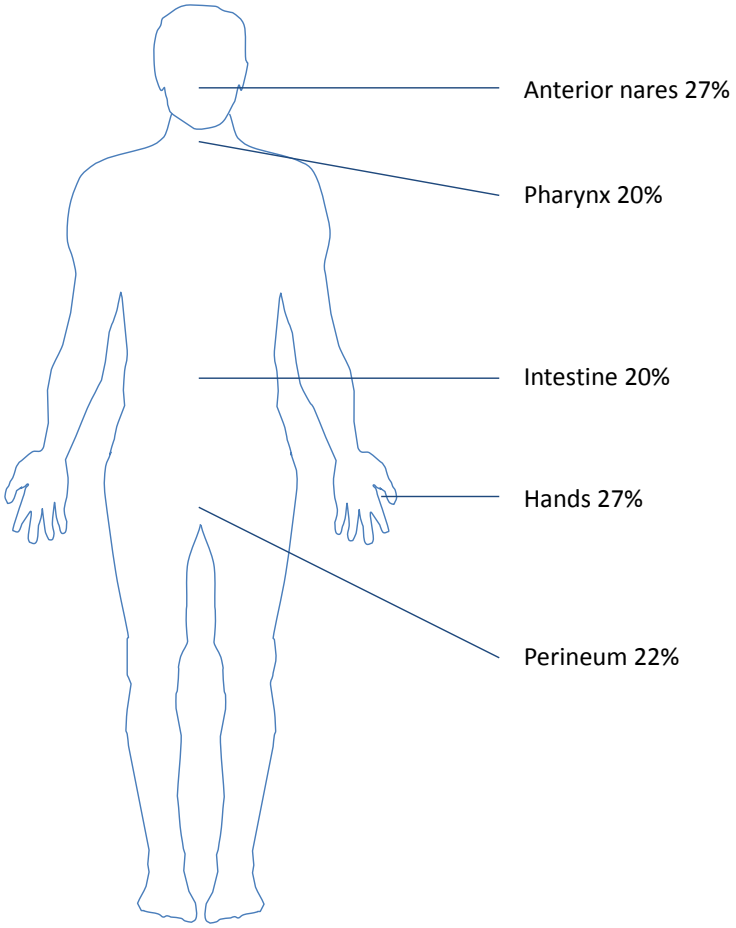
limen nasi and laterally by the wing of the nostrils ala nasi. The hairs inside the anterior nares do not possess erector muscles, and are specifically called vibrissae (Krstic, 1991).

Examination of nasal histological sections of human cadavers with positive nasal swab culture for *S. aureus* using anti-*Staphylococcus aureus* protein A specific antibodies clearly demonstrated that the presence of the organism is confined to the anterior nares as evidenced by the complete absence of the organism elsewhere (Ten Broeke-Smits et al., 2010). The immunologically stained histological sections further suggested that the bacteria are not only present on the surface of the nasal mucosa but also in the middle layer of the stratum corneum and deep in the shafts of hair follicles. The authors suggested that the bacteria residing in these hidden anatomical places may partially explain decolonisation resistance to mupirocin in some individuals. However, if its presence in nasal hair follicles plays a role in persistent carriage remains to be elucidated.

Although for decades the anterior nares have been well recognised as the primary reservoir from which the organism spreads to other body parts, solely intestinal carriage with *S. aureus* has also been reported (Lee et al., 1997; Bhalla et al., 2007). A systematic review examining the relationship between intestinal carriage and nasal carriage with *S. aureus* found that that individuals with both rectum and nares positive for *S. aureus* are at a four-fold higher risk for developing an infection compared to those who harbour the organism in the nares only and that only 60% of intestinal carriage can be attributable to nasal colonisation (R^2 = 0.6012) (Acton

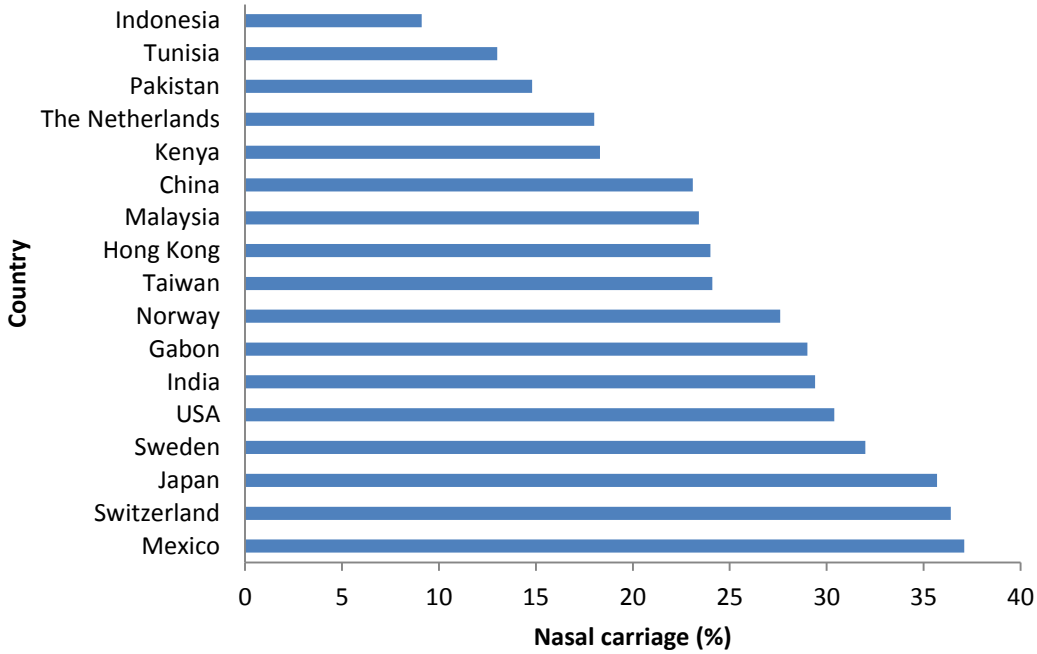
et al., 2009). Cultivation of nasal and pharyngeal swabs from children admitted to intensive care units for *S. aureus* found significantly more positives from pharyngeal (92.6%) than nasal specimens (63.1%) (Nakamura et al., 2010). These findings suggest that there may be more than one primary niche for the organism.

Figure 1. The frequency of isolation of *S. aureus* from different anatomical sites.



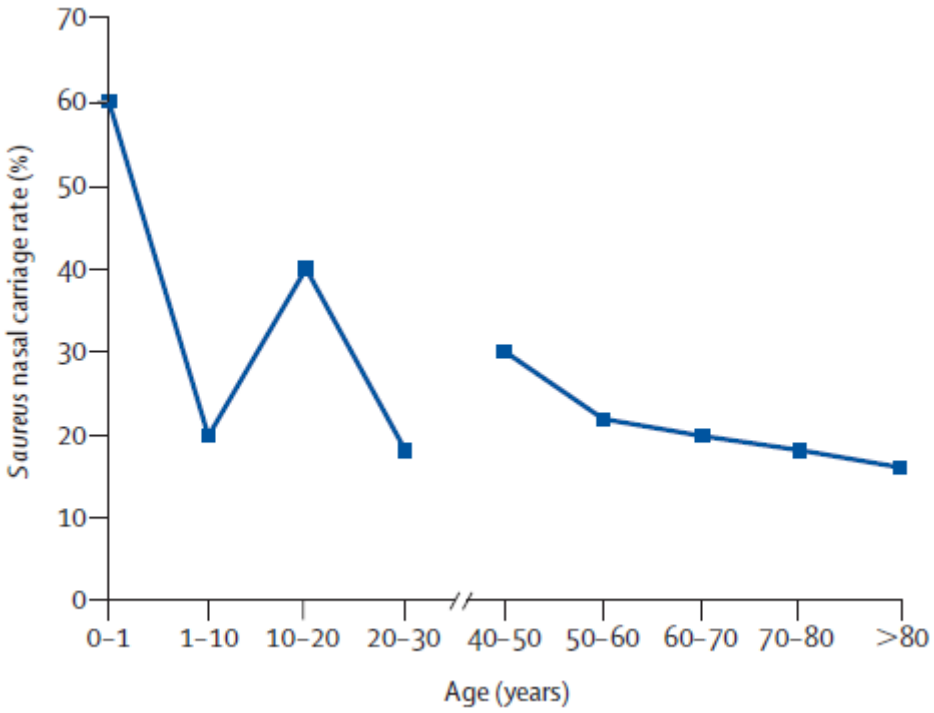
References: Wertheim et al. (2005); Acton et al. (2009).

Figure 2. Prevalence of nasal carriage with *S. aureus* in different countries.



References: Zhang et al. (2011); Sollid et al. (2013).

Figure 3. Changes of *S. aureus* nasal carriage rate with respect to age.



Reference: Wertheim et al. (2005).

Though much attention has been given to nasal colonisation *per se*, the association between hand carriage and nasal colonisation is noteworthy (Wertheim et al., 2006). Hand carriage particularly in food handlers is of public health concern because the organism can produce enterotoxins which cause food poisoning. It was suggested that hand carriers are of two types, those who carry the organism superficially on skin and those who harbour the organism deep inside glandular structures (Williams, 1963). They may be differentiated from each other by hand disinfection followed by putting on sterile gloves the inner surface of which are subsequently pressed directly onto agar plates. Growth of staphylococci indicated "deep carriers" as the organism is liberated from the glands during sweating (Williams, 1963). However, no published studies of hand carriage have differentiated "deep carriers" from "superficial carriers".

The prevalence rate of *S. aureus* nasal carriage varies geographically, with respect to demographics such as age and gender in addition to an array of modifiable factors. How these factors may help determine *S. aureus* nasal colonisation will be discussed in details in section 1.3.3.

Cross sectional studies have reported nasal carriage rates ranging from 9.1 % to 37.1 % (Figure 2). Kluytmans et al. (1997) attributed the wide range of prevalence seen in cross sectional studies to differences in the quality of sampling and in cultivation methods whereas the relatively lower prevalence reported in recent studies compared to those of the past might be a result of improved hygiene over time.

The frequency of carriage changes with age (Figure 3). The highest rate is observed in new-born infants from whom a carriage rate of up to 70% was reported. In a study where the epidemiological relationship of the cocci from mothers and their new-borns was investigated by *spa* typing revealed that 90% of the *S. aureus* isolates from the neonates were acquired from their mothers' nares. Interestingly, infants born to carrier mothers are not only at risk for acquiring maternal strains but are also predisposed to be colonised with *S. aureus* of non-maternal origins, indicating that host factors may play an important role in acquisition of the cocci in infants. The authors concluded that horizontal transfer of the organism from mother to new-born might be significant in contributing to the high prevalence seen in new-born babies (Sangvik et al., 2011). This finding is in concordance with reports from elsewhere (Peacock et al., 2003). Interestingly, older studies found that mothers did not contribute significantly to their infants' acquisition of *S. aureus* (Williams, 1961; Williams, 1963). Only 10% of 502 babies harboured *S. aureus* of the same phage type as their mothers. Instead, the new borne presumably acquired their coloniser strains from nurses, other babies, the environment and other household contacts. In addition, colonisation of the umbilicus was found to precede nasal carriage of the organism in infants (Williams, 1963). In a study conducted by Matussek et al. (2007) who investigated the source of *S. aureus* among infants born in three Swedish maternity units, a total of 218 infants including their parents and staff members who worked in the maternity units were sampled. Swabs from nostrils, fingers and ears were collected from parents and health-care providers. For

infants, the umbilicus instead of the fingertip was swabbed. Samples from the hospital environment and the air were also collected. Through *spa* typing, this study found that in 25% of the cases, infants acquired their strains from either their mother or father but 30% of them were colonised with strains carried by staff members. Interestingly, 45% of the colonised infants harboured a *spa* type which did not match *S. aureus* from any of the environmental samples, air samples or those from staff members.

The carriage rate decreases in infants through to toddlers. Thereafter, the prevalence remains as low as 20%. By the age of five, the rate approximates to that of adult carriage rate. The colonisation rate surges in adolescence but declines gradually afterwards. Persistent carriage is more common in children than in adults. A shift from persistent carriers to transient or non-carriers usually occurs in adolescence (Williams, 1963; Kluytmans et al., 1997; Wertheim et al., 2005a).

Investigations of the nasal colonisation rate with *S. aureus* in relation to gender have revealed variable results. In the United States, screening of 9622 persons found that males exhibited a 70% increased risk of being colonised with the organism (Kuehnert et al., 2006). However, a recent study comparing the carriage rate between healthcare workers and the general population found that women are at 54% higher risk of *S. aureus* carriage and that the risk further increased to 86% for women who rear children (Olsen et al., 2013). Further insight was provided by the same research team that *S. aureus* of *spa* type t012 was significantly more

common in females than in males (Sangvik et al., 2011), indicating that bacterial determinants may be involved.

One of the factors which may affect bacterial growth is climate, for instance, temperature and humidity. Although older studies fail to demonstrate any seasonal pattern in relation to carriage/ infection rate (Williams, 1963), modern time-series investigations have suggested a seasonal pattern regarding colonisation and infection with *S. aureus* in which MRSA colonisation rate and staphylococcal skin and soft tissue infection rates are relatively higher in summer and autumn. Colonisation with *S. aureus* tends to be higher in spring than in winter (Spaulding et al., 2013).

Other factors associated with increased risk of colonisation with the organism include obesity (Herwaldt et al., 2004; Botelho-Nevers et al., 2014), diabetes mellitus (Smith & O'Connor, 1966; Tamer et al., 2006), history of cardiovascular accident (Herwaldt et al., 2004), undergoing continuous peritoneal dialysis (Nouwen et al., 2006), positivity for human immunodeficiency viruses (Melles et al., 2008) and having chronic skin conditions including eczema and psoriasis (Andersen et al., 2013).

Prevalence of *S. aureus* carriage is indeed a complex outcome which is a consequence of interactions between age, gender, ethnicity, underlying health conditions, medical treatment undergone, climate change, and genetic factors yet to be identified.

1.3.3 Determinants of colonisation

Determinants can be considered in three aspects: human factors, bacterial factors and environmental factors. In order to successfully colonise the nostrils, the organism has to firstly come into contact with the nose, adhere to the epithelium, evade the host immune system and proliferate *in situ*.

The versatility of the organism as both a commensal and a pathogen has led to speculation that *S. aureus* strains of clinical and carriage origins may be genetically different. However, studies have demonstrated that pathogenic and carriage strains shared considerable genetic similarity (Melles et al., 2004; Lamers et al., 2011). Further analyses of nasal isolates from persistent carriers found that in carriage strains genes encoding surface molecules responsible for adhesion (clfB, isdA, fnbA, atlA, eap, tagO, tarK) and proteins for immune evasion (sak, chp, spa) were up-regulated whereas those encoding toxins were down-regulated (Burian et al., 2010). These findings suggested that colonisation is determined at the transcription level instead of by the genetic make-up *per se*.

Comparison of mutant and wild type strains in their ability to colonise animals and humans revealed several molecules, predominantly surface proteins, are involved in colonisation of the human nasal epithelium. Of these, clumping factor B (ClfB), iron-regulated surface determinant protein A (IsdA), wall teichoic acid (WTA), and serine-aspartic acid repeat proteins (SdrC, SdrD, SdrE) have received most attention

(O'Brien et al., 2002; Koreen et al., 2004; Wertheim et al., 2008; Corrigan et al., 2009; Hemen et al., 2013).

Use of *in vitro* models showed that the ClfB protein of *S. aureus* is one of the molecules responsible for adhesion to the nasal epithelium. This protein ClfB interacts with type I cytokeratin CK10 expressed on squamous epithelial cells and type II cytokeratin CK8 on simple epithelial cells (O'Brien et al., 2002; Dharod et al., 2009), as well as with the extracellular matrix protein loricrin (Mulcahy et al., 2012). In the presence of anti-ClfB antibodies, both ClfB mutant strains and wild-type strains of *S. aureus* adhered poorly to cytokeratin (O'Brien et al., 2002). A human colonisation model in which volunteers were inoculated with a *S. aureus* single locus ClfB (-) mutant and its corresponding wild type strain, mutant strains were eliminated significantly faster than the wild-type counterparts. Whilst a portion of wild type strains remained colonising the participants, all mutant strains were eliminated at the end of the study (Wertheim et al., 2008).

IsdA protein has also been shown to bind to cytokeratin 10 as well as to cornified envelope associated proteins. The cornified envelope is a proteinaceous layer covering keratinised corneocytes on squamous epithelium and is predominantly loricrin. Replacement of loricrin with bovine serum albumin significantly reduced adhesion to immobilised proteins *in vitro*, indicating the interaction between loricrin and IsdA was specific.

The significance of WTA, encoded by *tagO* gene, to nasal colonisation has been investigated. The *tagO* locked out *S. aureus* mutants were unable to colonise cotton rat nares (Weidenmaier et al., 2004). Surface proteins are linked to peptidoglycan by sortase enzyme (*SrtA*). Comparison of *S. aureus* mutants of *tagO* and *srtA* loci revealed that *tagO* and *srtA* mutants were rapidly eliminated from cotton rat nares (Weidenmaier et al., 2008; Weidenmaier et al., 2012).

The serine-aspartate proteins (SdrC, SdrD, SdrE) also contribute to adherence to nasal epithelial cells. An *in vitro* experiment where nasal squamous cells from healthy volunteers were incubated with *L. lactis* expressing proteins IsdA, ClfB, SdrC, SdrD and SdrE individually and in combination found that presence of SdrE alone showed no significant difference in terms of colonisation ability compared to the counterpart lacking any adhesive proteins expression. Effect on adherence of these proteins in order of importance was SdrC, ClfB, IsdA and SdrD (Corrigan et al., 2009).

Evidence has suggested *S. aureus* does not only survive on the surface of the epithelial cells. Investigation of nasal biopsy specimens of the anterior part of the middle turbinate from patients suffering from recurrent rhinosinusitis revealed that *S. aureus* survives inside epithelial, glandular and myofibroblastic cells where the organism is likely to avoid host immune response and seeds for future infection (Deurenberg et al., 2009).

Inside the nares, several other bacterial species co-exist with *S. aureus* which, therefore, has to compete for space and nutrient in order to successfully establish

colonisation in the host. Competitiveness between *S. aureus* strains was recognized long before the introduction of molecular characterization (Shinefield et al., 1974). The microbiota community in a person appears to be stable over time. Analyses of normal flora in human nares using 16S-rRNA sequence comparison revealed a difference between healthy individuals and hospitalised patients. The former group is predominantly colonised by *Propionibacterium spp.* and *Corynebacerium spp.* which are under-represented in the latter group whose isolation rate of *S. aureus* and *S. epidermidis* was much higher. In addition, colonisation with *S. epidermidis* is inversely correlated with *S. aureus* (Aspiroz et al., 2010).

As early as the 1980s, particular phenotypes of histocompatability antigens (HLA) were suggested to be associated with carriage status. Lymphocytotoxicity assays revealed that several HLA phenotype combinations, notably of DR1-A11 and DR1-B7 protected the host against carriage whilst that of DR3-DR5 increased colonisation risk. However, conflicting results were obtained between the two groups of subjects, the laboratory workers and patients, which might be the consequence of the majority of the patients suffering from immune disorders which were important confounders for the observed HLA phenotypic differences (Kinsman et al., 1983).

The contribution of human factors to colonisation was further evaluated by Nouwen et al. (2004) who artificially inoculated a mixture of *S. aureus* strains into predefined persistent carriers and non-carriers. The persistent nasal carriers were initially decolonised with mupirocin treatment in prior to inoculation. The bacteria survived in the anterior nares significantly longer in persistent carriers than in non-

carriers. Interestingly at the end of the experiment, 50% of the persistent carriers carried a foreign strain which was not included in the initial inoculums whereas the remaining half returned to carriage with their original resident strain. To elucidate if bacterial interference protected non-carriers from being colonised, the elimination rate of artificially inoculated *S. aureus* in non-carriers with mupirocin treatment prior to inoculation was compared to that in non-carriers without receiving mupirocin. Both of the groups eliminated *S. aureus* efficiently. The author concluded that the avoidance of colonisation in non-carriers were not due to mupirocin-susceptible resident bacteria (Nouwen et al., 2004). Considering the same mixture of bacteria was used, the different outcomes were likely to be determined by host factors.

The use of genome sequencing has identified carriage associated single nucleotide polymorphisms (SNP). These variations are thought to be linked with adhesive properties of host receptors and immune modulation. Epithelial cells express pattern recognition receptors such as toll-like receptors (TLR) and nucleotide-binding oligomerization domain-like receptors (NOD), which recognize pathogen associated molecular patterns on the bacterial surface and in response stimulate the production of cytokines and antimicrobial peptides (Sollid et al., 2013). Therefore, changes which alter the structure of TLR may influence host recognition of *S. aureus* and hence influence carriage risk. Single nucleotide polymorphism of TLR2 leading to single amino acid substitution (Arg753Gln) increased the colonisation risk in infants by three-fold (OR=2.91, 95% CI 1.17 – 7.26). Mannose

binding lectin (MBL) is an acute phase protein produced in the liver in response to infection. The variant MBL genotypes elevated the risk by 75% (OR=1.75, 95% CI 1.09 – 2.81) (van Belkum et al., 2007a; Vuononvirta et al., 2011).

One group of important anti-microbial peptides responsible for mucosal defense are the defensins. To date, at least three groups of defensins, namely α- β- and θ- defensins, have been distinguished. α- and β- defensins which are encoded by at least eight genes, are found in humans whereas θ- defensins are found in monkeys. The copy number of defensin genes varies across individuals (Ganz, 2003). This has led a hypothesis that the level of expression of the antimicrobial defensin may differ and hence determine carriage status. However, comparison between persistent carriers, transient carriers and non-carriers found no significant difference in the expression level of β- defensin 3 encoding gene (*DEFB103*) regardless of the number of gene copies present (van Belkum et al., 2007a; Fode et al., 2011). Polymorphism of defensin genes has been suggested to be associated with persistent carriage of *S. aureus* (Nurjadi et al., 2013).

In a study of SNP of genes encoding inflammatory mediators, interleukin 4 (IL-4), complement factor H (CFH) and C-reactive protein (CRP), the IL4-524 C/C genotype was identified as a contributing factor to persistent carriage (OR=2.52, 95% CI 1.0 – 6.2) whereas the CRP haplotypes 1184C, 2042C, 2911C were found to be more common in non-carriers but did not reach statistical significance. CFH did not appear to be a contributing factor for colonisation, however, individuals with CFH

C/C haplotype exhibited a 17% reduced risk for developing staphylococcal boils (OR=0.83, 95% CI 0.67 – 1.0) (Emonts et al., 2008).

Glucocorticoid plays an important role in immune suppression. It suppresses pro-inflammatory cytokine production and induces expression of anti-inflammatory genes. Polymorphism of the glucocorticoid gene is associated with cortisol sensitivity. Studies of glucocorticoid gene polymorphism determined that haplotype 3 (G-A-C-G) homozygosity reduced the risk of persistent carriage by 68% (OR=0.32, 95% CI 0.13 – 0.82) (van den Akker et al., 2006).

The linkage of polymorphism in the vitamin D receptor (VDR) gene to susceptibility to several infectious diseases such as tuberculosis, hepatitis B and HIV led to a search for its contribution to *S. aureus* nasal colonisation. The Rotterdam elderly study screened 1547 participants (≥ 55 years old) for VDR gene polymorphism and nasal carriage but failed to identify any association (Claassen et al., 2005). However, in patients with type 1 diabetes, homozygosity of *Taq1* T allele was associated with nearly three-fold increased risk for persistent carriage with *S. aureus* (Panierakis et al., 2009).

Although the genetic influence on *S. aureus* nasal carriage is evident, persistent carriage does not appear to be heritable. In a Danish study involving 617 middle- and old- aged twin pairs, the concordance rate of nasal carriage did not differ between monozygotic twins and dizygotic twins. Using the ADE (additive genetic effects, genetic dominance, environment) model, the heritability of carriage status

was estimated to be 29.6% (95% CI 0% - 62.8%) (Andersen et al., 2012). In a familial aggregation study of Amish family members living in different households, persistent colonisation did not preferentially occur in the same family. The authors suggest that in contribution to nasal colonisation, environmental exposure and acquired host factors are likely to be more important than host genetic determinants (Roghmann et al., 2011).

Epidemiological studies demonstrated that demographics such as age, gender, and ethnicity are contributing factors to nasal carriage. An older person (≥ 65 years old) is generally less likely to be colonised but carriers in this population tend to harbour methicillin resistant *S. aureus* strains (Mainous et al., 2006). A relatively higher risk for carriage among men has been observed in multiple studies (Van Duynhoven et al., 2005; Kuehnert et al., 2006; Olsen et al., 2012; Andersen et al., 2013). Interaction between gender and ethnicity was suggested in a study in which higher carriage risk was observed for non-hispanic whites regardless of gender difference. However, only male Mexican-American but not female were at a higher risk (Gorwitz et al., 2008). Table 1 summarizes epidemiological findings of risk factors associated with *S. aureus* nasal carriage.

Table 1. Epidemiological studies on risk factors associated with *S. aureus* nasal carriage.

Putative risk/ protective factor	N[a]	Country	OR (95% CI)	Reference
Hormonal contraceptive use	1180	Germany	1.88 (1.29 – 2.75)	Zanger et al. (2012)
Antibiotic use in 6 months	247	Japan	5.75 (2.12 – 15.59)	Tamer et al. (2006)
DM[b] patients using insulin	247	Japan	3.32 (1.39 – 7.91)	Tamer et al. (2006)
Non-hispanic caucasian men	9622	USA	1.70 (1.4 – 2.0)	Kuehnert et al. (2006)
Female gender	2196	Denmark	0.72 (0.59 – 0.88)	Andersen et al. (2013)
Psoriasis	2097	Denmark	1.73 (1.16 – 2.58)	op. cit.
Atopic diseases[c]	2196	Denmark	1.36 (1.11 – 1.68)	op. cit.
Live or work in farms	2174	Denmark	2.05 (1.35 – 3.11)	op. cit.
Pet ownership	2160	Denmark	1.25 (1.02 – 1.54)	op. cit.
Oral contraceptive use	346	Malaysia	4.92 (1.12 – 21.67)	Choi et al. (2006)
Ex-smokers	346	Malaysia	2.61 (1.08 – 6.32)	op. cit.
Current smokers	3789	Norway	0.64 (0.49 – 0.84)	Olsen et al. (2012)
Serum 25(OH)D[d] $\geq$75 mmol/L	3789	Norway	0.54 (0.35 – 0.84)	op. cit.
Partner is a carrier	84	The Netherlands	5.20 (1.10 – 24.52)	Mollema et al. (2010)
Work in healthcare industry	1302	Norway	1.54 (1.09 – 2.19)	Olsen et al. (2013)
Obesity	4066	USA	1.3 (1.1 – 1.5)	Gorwitz et al. (2008)

[a]Number of subjects, [b] DM denotes diabetes mellitus, [c] Atopic diseases include asthma, atopic dermatitis and allergy,

[d]25(OH)D denotes 25-hydroxyvitamin D

Various studies have shown that current smokers exhibit a reduced risk for *S. aureus* nasal colonisation (Herwaldt et al., 2004; Mainous et al., 2006; Olsen et al., 2012). This is consistent with a finding that living with a family member who is a smoker can also reduce one's own risk for colonisation (OR=0.78, 95% CI 0.65 – 0.95) (Mainous et al., 2006). This may partially be explained by a recent *ex vivo* study that reported the nasal epithelial cilia beat significantly more frequently in smokers (Zhou et al., 2009). Other potential mechanisms included bactericidal activity of cigarette smoke and immune-boosting effects related to smoking-triggered hypoxia (Wang et al., 2009; Sollid et al., 2013). However, a study found a higher prevalence of *S. aureus* carriage amongst smokers (Durmaz et al., 2001).

A prospective cohort study found that 47% MRSA carriers transmitted their strains to household contacts. The transmission risk increased with more family members in a household. The author suggested this may be the result of overcrowding. The risk for spreading is not even for all relationships (siblings, parents-chid, partner, others). For a partner relationship, the risk went up to five-fold. People with eczema were found to be more likely to spread the organism. The author attributed the higher transmissibility of these subjects to the fact that eczema sites are usually not covered by wound dressing which enhanced contamination of the environment where other contacts may acquire the organism (Mollema et al., 2010).

Increased carriage risk for *S. aureus* including MSRA has been reported in prisoners, sportsmen, military personnel and the homeless. Possible explanations included frequent skin-to-skin contact between individuals, potential abrasions and poor

personal hygiene (Campbell et al., 2004; Kazakova et al., 2005; Mukherjee et al., 2014).

Frequent skin puncture also increases risk for colonisation in subjects including intravenous drug abusers and patients undergoing surgical procedures, peritoneal dialysis, haemodialysis and/or insulin therapy. The penetration of skin exposes fibronectin to which *S. aureus* may bind and subsequently establish nasal colonisation (Kluytmans et al., 1997).

Use of antibiotics in the last six months appears to be a predisposing factor for MRSA colonisation in diabetic patients (OR=5.75, 95% CI 2.12 – 15.59) (Tamer et al, 2006). However, screening of 9622 healthy individuals in the United States for *S. aureus* carriage identified receiving antibiotic treatment in 30 days as a protective factor for *S. aureus* carriage (OR=0.63, 95% CI 0.45 – 0.88) (Mainous et al., 2006).

Factors which modulate the immune system also play an important role in *S. aureus* nasal colonisation. For instance, use of contraceptives, infection with human immunodeficiency viruses (HIV), existing atopic diseases, serum 25-hydroxyvitamin D (Williams et al., 1999; Olsen et al., 2012; Zanger et al., 2012; Oliva et al., 2013).

Working and/or living environment is also an important factor. Female healthcare workers have a 54% increased risk (OR = 1.54, 95% CI 1.09 – 2.19) for *S. aureus* nasal colonisation as compared to non-health care female workers in the Norwegian population (Olsen et al., 2013). However, a recent cross sectional survey comparing the carriage rates amongst nurses and the general population in Hong

Kong did not reveal any significant difference (Zhang et al., 2011). Living in pig farms increased carriage of livestock associated methicillin-resistant *S. aureus* by nine to 40-fold (van den Broek et al., 2008).

These risk factors associated with carriage have been predominantly investigated in different groups of patients. Whether these risk factors are relevant to nasal carriage in food handlers have not been well investigated. Numerous studies of nasal carriage in food handlers did not include risk factors (Simsek et al., 2009; Saeed & Hamid, 2010; Dagnew et al., 2012; Ferreira et al., 2014). A recently published cross-sectional study of nasal carriage and enterotoxigenicity of *S. aureus* in food handlers failed to identify any risk factors for carriage, including gender, age and smoking, in this population (El-Shenawy et al., 2013). These findings were in contrast with those reported elsewhere (Mainous et al., 2006; Olsen et al., 2012; Andersen et al., 2013). The inconsistency might have been attributed to the fact that not all known risk factors associated with carriage were considered. Therefore, subsequent investigations of risk factors for nasal colonisation in food handlers should take all known risk factors into account. These minimized overestimation or underestimation of risks which might otherwise imposed by the presence of confounders which were not taken into account.

1.3.4 Sources of *Staphylococcus aureus*

Molecular analysis has revealed that *S. aureus* is of two origins: humans and animals. The organism belonged to either human lineages (ST1, ST5, ST8, ST12, ST15, ST22,

ST25, ST30, ST45, ST51) or animal lineages (ST9, ST97, ST130, ST151, ST188, ST398, ST771, ST873) (Feil et al., 2003). Sporadically, infection with strains of animal origin occurs in humans (ST97, ST188, ST398) and of human origin in horses (ST1, ST8, ST22) (Spoor et al., 2013). Basically, the animal and human clusters are phylogenetically closely related but certain genes contributing to human colonisation (*fnbA, fnbB* and *coa*) and mobile genetic elements are likely absent in *S. aureus* of animal origins. Genes (*scn, chp, sak*) encoding staphylococcal complement inhibitor, chemotaxis inhibitory protein and staphylokinase respectively are important for human immune evasion are also missing in animal-associated isolates (Ghaznavi-Rad et al., 2010).

The potential spread of live-stock associated *S. aureus* (LA-MRSA) from animals to humans as a new source of community acquired MRSA epidemics is of public health concern. Since the first case of human infection with LA-MRSA of porcine origin recognised in the Netherlands (Voss et al., 2005), swine MRSA predominantly ST398 in humans were subsequently reported in other European countries, Malaysia, the United States and Singapore (Bischoff et al., 2006; Kuhn et al., 2007; Kim et al., 2008). In Hong Kong, ST398 *S. aureus* has been detected in two cases of bacteraemia, indicating this clone is potentially invasive (Ip et al., 2005). However, it was not known if these patients worked in the live-stock related industries. Case-control studies demonstrated that operatives such as pig farmers who worked closely with live-stock animals had a twelve times increased risk for acquisition of the organism (Weidenmaier et al., 2012).

It was speculated that swine ST398 MRSA initially originated from humans as MSSA which, during the human-to-pig transfer, lost the human immune evasion cluster (HIEC) genes and acquired those for methicillin- and tetracycline resistance (Price et al., 2012). A high prevalence rate of LA-MRSA colonisation has been observed in pigs in Asia (ST9) and in Europe (ST398) (Guardabassi et al., 2009; Ho et al., 2012; Shambat et al., 2012; Bennett et al., 2013). Food animals after slaughter and retail meats are also frequently contaminated with the staphylococci (Feil et al., 2003; Kahl et al., 2005; Kitai et al., 2005; Boost et al., 2013b).

In Asia, the presence of LA-MRSA was first documented in pig carcasses in Hong Kong (Guardabassi et al., 2009; Ho et al., 2012). This ST9-LA-MRSA has reduced susceptibility to a wider range of antibiotics, including vancomycin, as compared to the European clone ST398 (Kwok et al., 2013). Gene sequencing of these isolates suggested that the multiple resistance determinants originated from *Enterococcus* (Wendlandt et al., 2014). Further investigation of the presence of *S. aureus* carriage in live pigs in Southern Chinese pig farms which supply the majority of live pigs to Hong Kong detected the same clone of ST9 MRSA (Boost et al., 2012). This ST9 clone appeared to be readily transferrable to butchers who are responsible for routine cut up of the slaughtered animals (Boost et al., 2013a). This poses an additional risk for colonisation and infection with the organism for workers in animal husbandry, slaughterhouses and perhaps food handlers who regularly handle contaminated raw meat. A recent local survey indicated that up to 47% retail meat is contaminated with MRSA, including LA-MRSA (Boost et al., 2013b), suggesting that

this may be an important source of MRSA for food handlers. Contamination of pigs with *S. aureus* has been documented (Armand-Lefevre et al., 2005). However, the risk for colonisation with *S. aureus* in raw meat handlers has not been investigated.

Regarding isolates of human origin, there have been numerous studies on MRSA but MSSA has received inadequate attention. In general, MSSA isolates are more genetically diverse compared to their resistant counterparts (Aires de Sousa et al., 2005; Ghasemzadeh-Moghaddam et al., 2011; Hennekinne et al., 2012). Characterisation of MSSA isolates from carriage and community-onset infection in Taiwan revealed that ST1 (t127, t2457), ST6 (t701), ST12 (t160, t213), ST188 (t189) and ST 97 (t267, t359) were the predominant lineages found in MSSA infection. Of these, ST188 (t189) represented 21% of all MSSA-infection cases studied. These included urinary tract infection, spondylitis, paraspinal abscess, septic arthritis, pneumonia, epidermal cyst, and wound infection. The lineage ST188 (t189) represented the predominant lineage in clinical MSSA isolates collected in both 2002 and 2006. ST188 (t189) MRSA has also been reported as a causative agent for bacteraemia in Hong Kong (Ip et al., 2014). Compared to MSSA isolates causing infection, the lineages from carriage isolates were found to be even more diverse and included CC1, CC6, CC8, CC12, CC15, CC25, CC30, CC45, CC59, CC97, CC121 and CC398 (Chen et al., 2012). Screening for carriage of MRSA upon admission to Hong Kong hospitals revealed that predominant MRSA strains belonged to ST5, ST45, and ST239 (Luk et al., 2014). Regardless of the origin, concomitant analysis of clinical

and carriage isolates suggested that all *S. aureus* lineages are potentially pathogenic (Melles et al., 2004).

There is evidence showing that MRSA can survive on environmental surfaces for more than five days. Fung and Cairncross (2007) compared survival rate of MRSA on different environmental surfaces (glass, wood, vinyl, plastic, and cloth) and found that MRSA survived longest on plastic and vinyl and shortest on wood. The number of CFU/mL recovered from surfaces which were stored in a higher relative humidity (45 – 55%) was significantly higher than those held in a lower humidity (16%). The environment in kitchens is usually humid as a result of intensive cooking in an enclosed area. This environment may facilitate the growth of staphylococci including MRSA.

1.3.5 Laboratory investigations of nasal colonisation dynamics

1.3.5.1 Specimen collection and bacterial identification

Colonisation is traditionally determined by swabbing the anterior nares followed by cultivation and biochemical tests. The success rate for recovery of the organism is dependent on several factors such as the type of transport medium, incubation time and culture medium chosen. It was shown that isolation rate can be maximized by using charcoal swabs, including broth enrichment with 6.5% sodium chloride, and storing in Stuart's transport medium for seven days in prior to plating out. Incubating the mannitol salt agar (MSA) for four to seven days can increase recovery rate by 11.5% (Eriksen et al., 1994). The authors suggest the use of

charcoal swabs in Stuart's transport medium followed by cultivation on MSA in order to obtain a reasonable estimate of colonisation rate. More recently a study without an enrichment step showed the use of nylon flocked swabs achieved a 97.1% sensitivity of *S. aureus* detection (95% CI 93.6 – 100), as compared to 74.3% sensitivity of using traditional rayon swabs (95% CI 63.8 – 84.8) in terms of both the number of positive specimens and the CFU per swab (Verhoeven et al., 2010).

The introduction of a chromogenic agar *Sa Select* (Bio-rad) in 2006 has shortened the time required for staphylococcal identification. The sensitivity and specificity of this chromogenic agar were evaluated by comparing to MSA using 322 *S. aureus* clinical isolates of various origins (Daurel & Leclercq, 2007). Considering pink colonies appearing on the chromogenic agar after 24 hour incubation, the sensitivity for detection of *S. aureus* reached 98% using specimens in comparison to 90.8% for MSA although the sensitivity of the latter will slightly increase to 94.7% after 48 hour incubation. The authors concluded that the use of *Sa Select* (Bio-rad) could shorten the working time and omit complementary coagulase test.

Another issue which concerns specimen collection is who should be responsible for sample taking. A study which compared the isolation rate of *S. aureus* between nasal/pharyngeal swab samples collected by the participants and by investigators showed high concordance (Cohen's kappa = 0.85, 95% CI 0.74 – 0.96) in positivity. The authors concluded that self-sampling can be time saving and increase response rate particularly in community-based studies. However, this study was limited by

the fact that all subjects were nursing staff who presumably were better at sampling themselves than the general public (van Cleef et al., 2012).

Sampling nasal or nasopharyngeal areas requires a skilled person and the procedure may not be well-tolerated especially by children. Evaluation of the use of paper tissues in which subjects blew their noses into a tissue paper to collect samples from 66 children aged below four years old provided promising results in comparison with samples of a nasal swab and a nasopharyngeal swab (Weidenmaier et al., 2004). Isolation of *S. aureus* and other upper respiratory tract infection associated pathogens such as pneumococci, *Haemophilus* and *Moraxella* from the tissue paper blow was highly concordant (80 – 97%) with traditional methods of nasal swabs for *S. aureus* and nasopharyngeal swabs for other organisms.

Identification of *S. aureus* commences with a pure culture. Initial rule-out screening is based on Gram stain morphology, catalase reaction, coagulase formation and colony morphology on non-selective agar. Although in research laboratories, house-keeping genes are usually amplified to confirm bacterial identity, an accurate prior phenotyping can eliminate unnecessary workload and minimize costs. A combination of DNase, mannitol salt agar and coagulase test was shown to increase sensitivity and specificity of *S. aureus* detection to 75% and 100% respectively (Kateete et al., 2010). Identification with commercially available latex agglutination kits also correlates well with tube coagulase test (Papasian et al., 1999). The occasional ambiguous results of biochemical tests can be resolved by molecular

methods which by means of PCR amplification of several house-keeping genes allow identification of *S. aureus.* These include genes encoding thermostable nuclease (*nuc*), staphylococcal protein A (*spa*), ribosomal RNA (*16S-rRNA*), factors essential for expression of methicillin resistance (*femA, femB*) and extracellular adherence protein (*eap*) (Hussain et al., 2008; Crossley et al., 2009).

1.3.5.2 Typing of *S. aureus*

The ability to differentiate different strains of *S. aureus* is of utmost importance in terms of outbreak investigations and understanding bacterial population dynamics which is useful for long term epidemiological studies. For patients with recurrent staphylococcal infections, comparison of their strains allows differentiation of being re-infected with the same strain or by a different strain. In public health laboratories, typing enables tracking of food poisoning outbreak sources (Lindsay et al., 2013).

To objectively evaluate the discriminatory power of a bacterial typing method, Simpson's index of diversity (SID), calculated as a probability of assigning a different type for two strains which are not the same, can be used (Hunter, 1990; van Belkum et al., 2007). A modification of SID called epidemic index (1 – SID) has been applied to indicate the extent of similarity of strains collected in a sample (Blumental et al., 2013).

The earliest method established to type a *S. aureus* strain was bacteriophage typing which was in use for more than 30 years for strain typing before it was largely

replaced by pulsed field gel electrophoresis (PFGE) in the mid-1990s considering the poor reproducibility of the former method (Bannerman et al., 1995). Currently, there are two common approaches for typing *S. aureus* strains: (1) macro-restriction of chromosome and (2) nucleotide sequencing.

PFGE is a macro-restriction technique which separates restriction enzyme digested DNA fragments of the bacterial chromosome. The most common restriction enzyme used is *SmaI.* However, the enzyme target sites in live-stock originated clone CC398 are methylated which obscured *SmaI* cleavage Therefore, other restriction endonucleases such as ApaI and XmaI have to be used (Bens et al., 2006). PFGE has the highest discriminatory power amongst all methods described to date (SID=0.984) (Holmes et al., 2010). Interpretation of the restriction pattern follows the guidelines proposed by Tenover et al. (1995) who considered patterns differing by only one to four bands as closely related. The high discriminatory power allows differentiation of transient carriers from persistent carriers using repetitive samples. Though PFGE is very useful in long-term epidemiological studies, this method has its own limitations which include poor reproducibility, difficulty of inter-laboratory comparison, long turn-around time, high costs and lack of a common internationally accepted nomenclature for each type. These shortcomings can be overcome by sequencing-based typing techniques (Lindsay et al, 2013).

Multi-locus sequence typing (MLST) which is based on single nucleotide polymorphism of the sequence of 450 to 500bp defined internal fragments of seven housekeeping genes, namely carbamate kinase (*arcC*), shikimate dehydrogenase

(*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*) (Enright et al., 1999). Each sequence variant is assigned with a distinct number and the combination of seven numbers is defined as a sequence type (ST). If two isolates share the same sequence for five out of the seven loci, they are defined as belonging to the same clonal complex (CC) (Lindsay et al., 2013).

Sequencing of seven loci can be rather time consuming, labour intensive and likely to be limited by funding and other resources. Thereby, a single locus sequence typing method based on the polymorphic region X of staphylococcal protein A (*spa*) gene has been developed. Sequence analysis of clustered ALFP markers suggested that genetic diversity among *S. aureus* clusters is primarily caused by point mutation rather than by large scale deletions or insertions, supporting the usefulness of single nucleotide polymorphism in determining clonal relatedness (Melles et al., 2004). The use of *spa* typing in epidemiological investigation was further evaluated in various large collections and showed high concordance with other typing methods and high discriminatory power similar to PFGE (SID= 0.97 to 0.98) (Frenay et al., 1996; Mathema et al., 2008; Hallin et al., 2009). Considering its rapidity, reproducibility and unambiguity, *spa* typing is perhaps the best alternative to PFGE.

Spa typing is performed by firstly amplifying the variable region of *spa* gene using PCR followed by analyzing the sequence of the amplified products. A *spa* type can then be assigned by the order of the repeat sequence (Harmsen et al., 2003). Related *spa* types can be clustered into *spa*-clonal complexes (*spa*-CC) according to

the algorithm based upon repeat pattern (BURP) using the software Ridom Staphtype (Mellmann et al., 2007). Unfortunately, the software may not be always available because of limited resources. Ruppitsch et al. (2006) proposed a visual analysis scheme for clustering strains with related *spa* repeat successions into complexes without using the software. Although, under this manual classification, designations of new *spa* types may not be assigned, comparison of strains by *spa* repeat succession remains feasible. Recently, a free softare for *spa* identification called DNAGear has been introduced but remains underused (Al-Tam et al., 2012).

Direct repeat unit (*dru*) typing is another single-locus sequence typing method but it applies exclusively to methicillin resistant staphylococci. This sequence based typing method targets the 40bp variable number of tandem repeat region downstream of the *mec*A gene (Goering et al., 2008).

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is another MRSA typing method which is based mainly on the combination of *mec* and *ccr* gene variation to assign a SCC*mec* type. To date, eight *ccr* and five *mec* gene complex types comprising 11 SCC*mec* types have been distinguished (Turlej et al., 2011). The *mec* complexes carry different insertion sequences and antibiotic/ heavy metal resistance genes. A minimum of two multiplex PCR are needed for the identification of SCC*mec* type (Zhang et al., 2005; Kondo et al., 2007).

Evaluation of the use of a recently introduced bacterial identification method, MALDI-TOF mass spectrometry, in an attempt to sub classifying *S. aureus* strains

below species level was unsuccessful because bacterial strains belonging to different complexes did not produce visually distinct peak patterns (Lasch et al., 2014).

1.4 Pathogenesis of *S. aureus* infection

The organism *S. aureus* is responsible for a range of human infections and intoxications ranging from those that may subside spontaneously to life threatening disease. For instance, the adjusted mortality rate of staphylococcal bacteraemia can be as high as 83.3% (van Hal et al., 2012). The most common infection foci are seen in skin and soft tissues, heart valves, intravascular devices and bones (Lowy, 1998).

The wide range of disease severity has led to a question of whether bacterial factors determine virulence. Comparison of 829 *S. aureus* carriage isolates to 225 clinical isolates originating from blood culture, deep-seated soft tissue infection and impetigo by amplified fragment length polymorphism (AFLP) has shed light on this issue. Twenty one clinically isolated MRSA strains of unknown origin and two reference strains were also included. These strains were categorised into five AFLP clusters in which each cluster concomitantly embraced carriage isolates, invasive isolates and multi-drug resistant isolates. This suggested that virtually all lineages of the organism are capable of causing invasive infections. However, it appeared that certain clones are more likely to be associated with particular clinical manifestations. For instance, the AFLP group comprising CC121 was overrepresented by impetigo isolates and 39% *S. aureus* strains isolated from cases of abscesses and arthritis

were PVL-positive compared to 2.1% positive of blood culture isolates (Melles et al., 2004).

In this section, current understanding of staphylococcal virulence factors is reviewed.

1.4.1 Virulence factors in pathogenesis

Virulence factors involved in staphylococcal disease process can be classified into three categories, namely (1) capsular polysaccharides, (2) cell-wall anchored proteins, (3) exotoxins and (4) associated regulators.

1.4.1.1 Capsular polysaccharides

To date, at least 11 distinct serotypes of microcapsular polysaccharide have been identified, of which type 5 and 8 represent 25 – 50% of clinical isolates in several countries although a single predominant serotype 336 is reported in Germany (von Eiff et al., 2007). Except for the rare serotypes 1 and 2 (initially named as M and Smith diffuse *S. aureus* strains respectively) which form mucoid colonies, other capsular serotypes form non-mucoid colonies which are indistinguishable from non-capsule forming strains. These capsular polysaccharides are among the most important virulence factors that help resist phagocytosis. The antiphagocytic action can be neutralized by specific capsular antibodies (type 5 and 8) as demonstrated in an *in vitro* model, leading to exploration of its potential for vaccine development (O'Riordan & Lee, 2004; Kampen et al., 2005).

1.4.1.2 Cell-wall anchored proteins

Cell-wall anchored proteins are extracellular structures which are linked to the staphylococcal cell wall. On the basis of differences in structural motifs, four classes of cell-wall anchored proteins are distinguished, namely (1) microbial surface component recognizing adhesive matrix molecules (MSCRAMMS), (2) near iron transporter motif (NEAT) protein family, (3) three-helical bundle family and (4) G5-E repeat family. These are involved in host cell adhesion and immune evasion (Foster et al., 2014).

1.4.1.3 Exotoxins

Staphylococcal exotoxins represent a group of proteins which are secreted by bacterial cells. Some of these proteins turn host tissues into nutrient that support staphylococcal growth. These include lipases, proteases, nucleases, collagenase, lipaess, hyaluronidase and haemolysins (α-, β-, γ- and δ-). Other exotoxins such as toxic shock syndrome toxin (*tsst-1*) and staphylococcal enterotoxins are collectively known as pyrogenic toxin superantigens (PTSAgs) and are responsible for specific clinical symptoms due to their pyrogenic, superantigenic and endotoxin-toxicity enhancing nature (Dinges et al., 2000).

1.4.1.4 Virulence associated regulators

To date, two main molecular systems responsible for regulating expression of staphylococcal virulence factors have been described. These are accessory gene

regulator (*agr*) and staphylococcal accessory regulator (*sar*). During the late exponential to stationary phase of growth, the *agr* represses the transcription of cell wall proteins but upregulates the expression of exotoxins. The *sar* loci encode an RNA binding protein called sarA. This protein is responsible for the stability of the mRNA transcript encoding protein A and collagen-binding protein during exponential phase of growth (Morrison et al., 2012; Agra et al., 2013).

The *agr* operon comprises four genes designated *agrA to D* whose expression was controlled by activation of a single promotor induced by an autoinduction peptide. The auto induction peptide is encoded by *agrD* which is activated in reponse to phosphorylation of agrA protein by inducing peptide binding. This is accompanied by upregulated transcription of RNAIII which subsequently increases the expression of exotoxins including a number of enterotoxins (Novick, 2003; Yarwood & Schlievert, 2003; Asad & Opal, 2008; Singh & Ray, 2014). For instance, the enterotoxins encoded by *seb, sec, sed, sel* and *ser* are *agr*-dependent and therefore produced in the post-exponential growth phase (Derzelle et al., 2009; Hennekinne et al., 2012). Other enterotoxin genes such as *sea, see, seh, sej, sek, sep* and *seq* are independent from *agr* regulation and are therefore efficiently expressed even when bacterial numbers are low (Zhang et al., 1998; Lis et al., 2012a). The expression patterns for SES and SET remain unknown. Interestingly, the expression of egc encoded toxin genes *seg, sei, sem, sen, seo* and *selu* decreases as the organism grows (Derzelle et al., 2009).

Variations in the *agr* locus distinguished four agr types designated I to IV (Novick, 2003). A strain with a given agr type produces auto-inducing peptides which suppress the growth of other strains with different agr types (Asad & Opal, 2008). Agr type I is more frequently seen in healthy carriage and food (Bibalan et al., 2014). In consistent with this, more than half of the isolates colonising food handlers in a cross sectional survey in Kuwait belonged to agr type I (Udo et al., 2009). Comparison of the expression of various exotoxins *in vivo* by agr deficient mutants and wild type strains in animal models suggested that agr negative strains were less virulent in models of arthritis, endocarditis and obscesses (Novick, 2003). However, whether a functional agr is essential in causing food poisoning has not been investigated.

1.4.2 Human diseases

Staphylococcal diseases fall mainly into two categories, infection and intoxication. Infections are usually initiated with a micro wound of the skin whereby the organism gains entry into the body. For most immunocompetent individuals, the infection tends to be localized. Of these, skin and soft tissue infection and wound infection are the most common. However, for patients receiving invasive medical procedures or with pre-existing medical conditions leading to altered immune defect, metastatic infection foci such as bacteraemia, endocarditis, pneumonia or osteomyelitis may develop (Crossley et al., 2009).

Staphylococcal intoxication refers to toxin-mediated disease of which staphylococcal food poisoning (SFP) is the most well-known. It is caused by ingestion of the staphylococcal enterotoxins preformed in the food matrix instead of the organism *per se.* Although *S. aureus* is usually destroyed by cooking, its toxins may survive heating. Staphylococcal enterotoxins are stable entities which retain their emetic activity after boiling for 30 minutes and passing through the stomach when the gastric acid level is likely to be above pH 2.0 after ingestion with other food (Bergdoll & Wong, 2006).

1.5 Staphylococcal Enterotoxins

Staphylococcal enterotoxins (SEs), which belong to the PTSAg group, are superantigenic exotoxins produced by *S. aureus,* which are associated with staphylococcal food poisoning. So far, at least 22 types of SEs (SEA to SE*V*) and their variants (SEC1, SEC2, SEC3, SE*U*2) have been recognized (Munson et al*.*, 1998; Orwin et al*.*, 2001; Omoe et al*.*, 2002; Orwin et al*.*, 2003; Omoe et al*.*, 2004; Omoe et al*.*, 2005; Fraser & Proft, 2008; Ono et al*.*, 2008; Argudín et al*.*, 2010). Nomenclature is based on the degree of similarity in terms of enterotoxin gene sequence and amino acid sequence. Enterotoxin genes with 90% similarity should be designated as variants of the earlier identified enterotoxin gene (Lina et al*.*, 2004). In terms of nucleotide sequence, genes encoding SE variants are very similar. For instance, *sec*1, *sec*2 and *sec*3 exhibit more than 97% sequence homology (Hsiao et al*.*, 2003) whereas *selu*2 differs from *selu* by only a 15 nucleotide base insertion at position 206 of the *selu* gene (Thomas et al*.*, 2006).

Enterotoxin types A to E, described in the 1970s, have been extensively studied and are collectively known as classical SEs. The remaining SEs are called novel SEs. Emetic activity has been demonstrated in animal models for SEA through SES, indicating that the presence of these enterotoxins is of considerable public health significance (Hu et al., 2003; Ono et al., 2008; Omoe et al., 2013). SEs are divided into five groups with respect to the percentage of amino acid sequence similarity, (Le Loir et al., 2003; Larkin et al., 2009; Argudin et al., 2010). The first group comprises SEA, SED, SEE, SEJ, SEN, SEO, SEP and SES. The second group comprises SEB, SEC, SEG, SER and SEU. The third group includes SEI, SEK, SEL, SEM and SEQ. The remaining two groups each contain a single member, namely SET and SEH respectively (Arguidin et al., 2010).

Different strains of enterotoxigenic *S. aureus* can produce different combinations of enterotoxin types, or enterotoxin profile, and frequently several enterotoxins co-exist. Staphylococcal enterotoxins are actively produced during the logarithmic phase of staphylococcal growth and during the transition from exponential to stationary phase. A recent report has shown that 70% of SE genes harboured by *S. aureus* are transcribed, indicating that most of the enterotoxin types that a strain harbours will be expressed (Cunha & Calsolari, 2008).

1.5.1 Biological characteristics

Ingestion of SE in 0.1 to 100 microgram quantities can result in intoxication syndrome. SE remains intact after boiling at 100^{o}C for one hour or exposure to pH

greater than two which is the usual acidity in the stomach after eating. In the human stomach, only overnight fasting can produce a pH of two (Larkin et al., 2009). The development of allergic rhinitis and asthma are also highly associated with staphylococcal SE production. It has been found that people with the above conditions have significantly higher levels of IgE antibodies to SEA, SEB, SEC, SED and TSST-1 ($p = 0.029$) (Rossi & Monasterolo, 2004). The authors showed that sensitization to SE was associated with a significantly higher level of eosinophil cationic protein, which is a marker for clinical severity of allergy, suggesting that the presence of SE may worsen clinical severity of allergic rhinitis.

The mechanism of SE induced emesis remains unclear. It has been suggested that the vagus nerve in the abdominal viscera is stimulated and impulses transmitted to the vomiting centre in the brain leading to emesis (Sugiyama & Hayama, 1965). A recent study using a house musk shrew model revealed that after vagotomy or administration of 5-hydroxytryptamine synthesis inhibitor, administration of staphylococcal enterotoxin A could not induce emesis. The author concluded that SEA could induce massive release of serotonin in the intestine and that the vagus nerve is part of the emesis pathway involving emesis triggered by staphylococcal enterotoxin A (Hu et al., 2007). Although the emetic pathway of other staphylococcal enterotoxins have yet been elucidated, it is likely that they share similar physiologic pathways due to similar amino acid sequence and three dimensional folding.

Superantigens (SAgs) are groups of protein exotoxins produced by some microorganisms which can elicit an abnormally overwhelming immune response (Petersson et al., 2004). Microorganisms that can produce SAgs range from viruses to bacteria and fungi. SAgs have been reported in *Yersina pseudotuberculosis*, *Clostridium perfringens, Mycoplasma arthritidis,* Epstein Barr virus, Rabies virus, Herpes virus and *Candida albicans* (Llewelyn & Cohen, 2002; Larkin et al., 2009,). The most thoroughly characterized bacterial SAgs produced by *S. aureus* are SEs, implicated in food poisoning.

Conventionally, the human immune response to microbial antigens is to process them by antigen presenting cells (APC) such as monocytes into small peptide fragments which are subsequently presented along with membrane bound major histocompatibility complex (MHC) class I or II on the surface of the APC. The presented peptides are highly specific in immunogenicity as solely $CD4^+$ T-cells are activated and, hence, upon encounter with an antigen, only approximately 0.001% of T cell subsets would respond and produce cytokines (Llewelyn & Cohen, 2002). *S. aureus* carriers have a lower incidence of death from bloodstream infections than non-carriers which may be attributed to the highly specific neutralizing antibody response against superantigens present in their colonizing strains (Holtfreter et al., 2009; van Belkum et al., 2009). It was shown that individuals having higher antibody titre against tsst-1, one of the staphylococcal superantigens, were less likely to develop septic shock. Using a murine model, development of septic shock was avoided in animals treated with superantigen toxoids (Holtfreter et al., 2007). This

may help to explain the lower mortality from staphylococcal infections observed in colonized individuals compared to those not carrying the organism (van Hal et al., 2009).

However, SAgs including SE do not demonstrate such specificity and, in the majority of cases, they directly cross link the β-chain variable region of the T-cell receptor (Vβ TCR) and APC, although particular SEs, such as SEH, alternatively bind to the Vα domain (Saline et al., 2010). Each SAg is able to bind to more than one Vβ repertoire, thereby, more than 25% of T cell subsets will be activated (Petersson et al., 2004; Fraser & Proft, 2008; Pinchuk et al., 2010).

Despite considerable differences existing in amino acid sequences among different superantigens produced by *S. aureus*, they exhibit similar three-dimensional folding and are highly associated with inflammatory diseases. These include toxic shock syndrome, atopic dermatitis, seasonal rhinitis, asthma, chronic bronchitis and staphylococcal food poisoning (Fraser & Proft, 2008).

Staphylococcal Toxic Shock Syndrome (TSS) was first reported in the 1970s. Initially, the etiologic toxin TSST-1 was falsely identified as enterotoxin F which was subsequently found to be non-emetic. Outbreaks of TSS involved menstruating women who used a highly absorbent commercial brand of tampons, Rely tampons (Todd et al., 1978). At that time, *S. aureus* was isolated from the vagina of all cases of menstrual TSS. Laboratory examination revealed that 94% of *S. aureus* strains

isolated from confirmed TSS cases harboured tsst-1, as compared to only 4% of *S. aureus* strains from non-TSS cases (Bergdoll, 1981).

TSS manifests with a brief prodrome illness characterized by high fever, vomiting, diarrhea, abdominal tenderness and erythematous rash, followed by hypotensive shock, respiratory failure and renal failure. These syndromes were associated with the presence in the bloodstream of toxic shock syndrome toxin 1 (tsst-1) produced by *S. aureus*. Tsst-1 is a superantigen which induces a massive release of cytokines and chemokines, in particular interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α), which further recruit lymphocytes to the foci of superantigen-lymphocyte interaction and, hence, more cytokines will be released by lymphocytes *in situ.* This cytokine storm induces vasodilation, disseminated coagulation and hence hypotensive shock. Bloodstream culture for *S. aureus* during the acute phase of illness is usually negative. Positive culture is usually obtained from foci of infection elsewhere, such as skin lesions. These indicate that toxic shock syndrome is an intoxication syndrome rather than a septicaemic condition (Fraser & Proft, 2008).

1.5.2 Genetic basis

SEs are encoded by genes present in plasmids, prophages or pathogenicity islands. Two plasmids involved with the presence of SEs, pIB485 and pF5, have been well characterized. pIB485 harbours SED, SEJ and SER, whilst pF5 harbours SER, SES, SET

and SEJ. These plasmids were identified in 1997 in a food poisoning outbreak in Japan (Zhang et al., 1998; Omoe et al., 2004).

Enterotoxin genes demonstrate a certain degree of genetic linkage. Jarraud et al. (2001) found that SEG and SEI coexisted in all clinical isolates examined in his study. The coexistence of SEG and SEI has also been documented in other studies which later proved to be a result of genetic linkage of the respective genes (Omoe et al., 2002; Naik et al., 2008). Further examination of the DNA sequence flanking the region of SEG and SEI revealed another three enterotoxins designated SEM, SEN and SEO. These enterotoxin genes are co-transcribed and present in the same operon collectively called an enterotoxin gene cluster (*egc*), which has been suggested to be a result of genetic recombination which favours the formation of new enterotoxin genes which allows *S. aureus* to stimulate different T cell subsets.

The cluster *egc* appears to be present in virtually all *S. aureus* strains isolated from clinical specimens including skin and soft tissue infections, pneumonia, toxic shock and abscesses of various organs. This cluster also seems to be associated with nasal carriage (57%), suppurative infections (67%) and toxic shock conditions (92%) (Jarraud et al., 2001).

Genetic recombination in *egc* seems to be a frequent event. Soon after the documentation of *egc* in 2001, a new enterotoxin SEU was identified in the operon as a result of such recombination (Letertre et al., 2003). Up to now, 4 variants of the *egc* cluster have been reported. All enterotoxin gene clusters so far reported are

carried on the chromosome. Pseudogenes are only present in the original *egc,* later designated as *egc*1, habouring five enterotoxin genes (*seg, sei, sem, sen, seo*). *egc*2 additionally harbours *selu.* The same enterotoxin gene profiles are observed in *egc*2 and *egc*3 except that slight variations of amino acid sequence (<10%) exists in *egc*3, which is common in food but not yet observed from human and animal isolates. The genotypes in *egc*4 are considerably different from other operons. It harbours *seg, seo, selv, selu2* and *sen* with deletion of two widely disseminated genes *sei* and *sem* (Collery et al*.,* 2009). Table 2 summarizes the main characteristics of staphylococcal enterotoxins.

Distribution of SE genes have been thoroughly investigated in clinical isolates (Efuntoye et al., 2003; Flemming et al., 2007; de Oliveira et al., 2011; Nhan et al., 2011; Vasconcelos et al., 2011; Garcia et al., 2013; Calderwood et al., 2014). However, only a few studies have determined their distribution in samples from healthy carriage (Lim et al., 2012; Argudin et al., 2013). In Hong Kong, there are no reports concerning the distribution of these determinants in *S. aureus* carriage isolates in food handlers.

Table 2. Properties of staphylococcal enterotoxins.

Enterotoxin	Emetic activity	Genetic location	References
SEA	yes	Prophage	Betley et al., 1988
SEB	yes	transposon, plasmid	Johns et al., 1988
SEC variants	yes	pathogenicitiy islands, plasmids, prophages	Marr et al., 1993
SED	yes	Plasmid	Zhang et al., 1998
SEE	yes	defective phage	Couch et al., 1988
SEG	yes	egc1, egc2, egc3, egc4	Omoe et al., 2002
SEH	yes	pathogenicitiy islands	Omoe et al., 2002
SEI	yes	egc1, egc2, egc3	Omoe et al., 2002
SEJ	yes	Plasmid	Zhang et al., 1998
SEK	yes	pathogenicitiy islands	Orwin et al. , 2001
SEL	yes	pathogenicitiy islands	Orwin et al. , 2003
SEM	yes	egc1, egc2	Jarraud et al., 2001
SEN	yes	egc1, egc2, egc3, egc4	Jarraud et al., 2001
SEO	yes	egc1, egc2, egc3, egc4	Jarraud et al., 2001
SEP	yes	Prophages	Omoe et al., 2005
SEQ	yes	Prophages	Chiang et al., 2008
SER	yes	Plasmids	Ono et al., 2008
SES	yes	Plasmids	Ono et al., 2008
SET	yes	Plasmids	Ono et al., 2008
SE/U	nd	egc2, egc3	Litertre et al., 2003
SE/U2	nd	egc4	Thomas et al., 2006
SE/V	nd	egc4	Thomas et al., 2006

nd = not determined

1.5.3 Laboratory detection of enterotoxigenicity

Immunological and nucleic acid based methods are two approaches to detect staphylococcal enterotoxins. Immunological methods require the use of monoclonal antibodies which specifically bind to expressed SE. Nucleic acid based methods employ PCR to detect SE genes or corresponding mRNA transcripts (Bergdoll & Wong, 2006).

Antibodies specific to enterotoxin types are used to capture enterotoxin produced *in vitro.* Several enzyme linked immunosorbent assays available today are able to detect classical enterotoxin SEA to SEE at levels of less than 1ng per gram of food. Some of the commercially available kits are able to differentiate enterotoxin serotypes (SET-EIA, RIDASCREEN) whereas others do not (TECRA, TRANSIA) (Su & Wong, 1997; Ostyn et al., 2011).

The RIDASCREEN kit is able to detect SEA, SEB, SEC, SED and SEE individually. It is based on monoclonal antibody capture technology and is able to detect the presence of as low as 0.2 ng/ml of enterotoxin (Park et al., 1994; Su & Wong, 1997). SET-EIA kit uses a mixture of polyclonal antibodies to detect SEA, SEB, SEC and SED without differentiation between serotypes.

Despite the convenience of commercially available immunological kits, these methods may not always detect enterotoxigenic *S. aureus* strains because SE expression is dependent on a range of variables such as pH and temperature. In

addition, due to similarity of various SEs, other SE types may cross react with antibodies producing false positive results (Cunha & Calsolari, 2008).

At present, nucleic acid based methods are most frequently used for detection of SEs. These include conventional polymerase chain reactions (PCR) and real time PCR (RT-PCR). PCR allows the detection of all enterotoxin genes present in isolates, whether it is expressed or not. The expression of enterotoxin genes may differ across foods. Factors such as sodium concentration, the amount of glucose presence, and the proportion of valine, arginine and cystine in food matrix affects the amount of enterotoxin expression from staphylococci (Regassa & Betley, 1993; Le Loir et al., 2003). Failure to detect excreted toxin in one food does not mean that a particular strain of *S. aureus* has no risk for causing food poisoning. Instead, it may express clinically important enterotoxins when it is transferred to other food surfaces. In this regard, PCR can identify virtually all potentially enterotoxigenic *S. aureus* strains by detection of enterotoxin genes instead of the excreted enterotoxin proteins (Monday & Bohach, 1999; Chiang & Chang, 2006; Chiang et al., 2008). A good correlation between ELISA and PCR for detection of enterotoxigenicity has been demonstrated, favouring the use of PCR in determining enterotoxigenicity (Hait et al., 2014).

1.6 Staphylococcal food poisoning and the role of food handlers

Staphylococcal food poisoning (SFP) outbreaks caused by staphylococcal enterotoxins (SEs) have been reported in many countries and lead to considerable economic losses for the food industry (Sockett, 1993). Whilst in general SFP is self-limiting, it can be severe amongst the young and the elderly as well as the immunocompromised (Naik et al., 2008). Occasionally but importantly, a potentially fatal form of cholera-like enterocolitis associated with enterotoxigenic strains of *S. aureus* can develop. In a case described by Lieverse et al. (2001), a patient was initially diagnosed with *Clostridium perfringens* associated necrotic colitis after endoscopy examination, however, *S. aureus* was subsequently cultivated from the intestinal biopsy specimen. Metronidazole was then given but the patient died within 2 weeks. Further investigations revealed that the *S. aureus* strain isolated produced staphylococcal enterotoxin A (SEA).

Enterotoxins are proteins produced by certain strains of *S. aureus* during their growth in food. It has been demonstrated that approximately 50% of *S. aureus* isolates from humans produce enterotoxins (Imanifooladi et al., 2007). Although the organism may be killed by heat during the cooking process, enterotoxins are highly resistant to degradation. They are insensitive to heat and extremes of pH which renders them resistant to gastric acid and proteolytic enzyme activity in the stomach. The presence of staphylococcal enterotoxins in food does not change the texture of food or give it an unfavourable taste (Murray, 2005; Principato et al.,

2009). Without being noticed, contaminated food may be consumed by individuals leading to food poisoning.

SFP is a form of intoxication resulting from ingestion of food contaminated with one or more preformed enterotoxins in the food matrix. Clinical manifestations include hypersalivation, abdominal cramps, nausea and vomiting with or without diarrhoea. Fever, rash and symptoms mimicking systemic infection are usually absent (Dinges et al., 2000; Murray, 2005). The incubation period ranges from 30 minutes to 8 hours with an average of 2 to 4 hours, depending on the amount of enterotoxin ingested, the susceptibility of the host to SEs, and the general health status of the host. In Japan, cases of glomerulonephritis associated with staphylococcal enterotoxin superantigenicity have been documented. Patients infected with enterotoxigenic *S. aureus* developed glomerulonephritis due to a cytokine storm induced by SEs (Siu, 2010).

Medical management involves mainly supportive therapy such as oral fluid rehydration and symptomatic relief strategies including administration of antispasmodic and antiemetic medications. For individuals that are particularly susceptible to dehydration such as children and the elderly, hospitalization and intravenous fluid replacement may be needed.

Differential diagnosis of SFP can be made by the absence of fever and short incubation period as distinguishable from other gastrointestinal infections caused by *Salmonella* and *Vibrio* where fever is usually present.

1.6.1 Staphylococcal enterotoxins

Distribution of genes encoding staphylococcal enterotoxins or enterotoxin-like proteins has been investigated in *S. aureus* isolates of food, animal and human clinical origins from several sources. Tables 3 to 5 summarize the distribution of SE/ SEl genes from these sources. However, there has been little attention to their distribution in carriage strains which may differ considerably from clinical isolates.

Table 3. Studies of SE or SEl genes in *S. aureus* isolated from food.

Types of food	Country	Major findings	Reference
Ready-to-eat food	Thailand	Reverse latex agglutination revealed that 20% *S. aureus* isolates (n=20) produced enterotoxins (SEA and/or SEB).	Chomvarin et al., 2006
Milk	Brazil	68.4% *S. aureus* isolates were positive for at least one SE gene. The SE genes in order of frequency were *sea* (41%), *seg* (28.2%), *sec* (20.5%), *sed* (12.8%), *seb* (7.7%) and *see* (5.1%).	Rall et al., 2008
Dairy products	Turkey	ELISA revealed 2.8% of the isolates (n=80) produced enterotoxins. The distribution was in order of frequency: SEA (1.6%), SEB (0.46%), SEC (0.23%) and SED (0.46%).	Ertas et al., 2010
Bovine raw milk	Korea	77.3% *S. aureus* isolates carried at least one SE gene.	Hwang et al., 2010
Deli products, bakery products, fresh cheeses	Italy	PCR revealed 49% of the isolates carried at least one SE gene whilst only 16.3% of these were confirmed to be enterotoxigenic by reverse passive latex agglutination.	Di Giannatale et al., 2011
Ready-to-eat food	Korea	51.3% *S. aureus* isolates carried at least one SE gene. The major SEl genes were *sek, sem, sen* and *seq* (22.1% each). Others were *seo* (13.6%), *seu* (7.8%), *sep* (5.2%), *sel* (4%) and *ser* (1.3%).	Oh et al., 2011
Cake, dairy products, meat	Indonesia	*seb-sec* is the most common genotype (4/11). Only one (1/11) food specimen contained the newly described *seg* gene.	Salasia et al., 2011

Table 3. Continued.

Types of food	Country	Major findings	Reference
Bakery, dairy products, sea food, meat, eggs	Spain	58.3% *sea* and 50% *seb* harbouring *S.aureus* expressed the corresponding toxins. Simultaneous production of two classical enterotoxins was detected. The SE gene profile was in order of frequency: *sea* (38.7%), *sed-selj* with or without *ser* (22.9%), *seb* (12.9%), *sec* (16.1%), *seh* (9.7%), *sell* (9.7%), *selp* (9.7%), *selk-selq* (6.5%).	Arguidin et al., 2012
Chicken carcasses	Switzerland	All isolates of CC12 harboured *seb,* all isolates of CC5 and CC45 harboured *seg, sei, selm, seln, selo* and *selu.*	Ebner et al., 2013
Ice-cream	Turkey	Of the 12 *S. aureus* positive for corresponding SE genes, 75% (SEB), 8.3% (SED), 8.3% (SEB + SED) and 8.3% (SEA + SEB + SED) expressed their enterotoxins as determined by latex agglutination test.	Gucukoglu et al., 2013
Meat, raw milk, dairy products	China	Enzyme linked fluorescent assay (detecting SEA to SEE) revealed 43.7% *S. aureus* isolates were enterotoxigenic.	Hu et al., 2013
Chicken meat	Brazil	62% of the coagulase positive staphylococcal isolates were *S. aureus* 8% were *S. hyicus*, 10% each were *S. intermedius, S. delphini, and S. schleiferi.* A total of 70% of the isolates harboured at least one classical SE gene.	Martins et al., 2013[a]

Table 3. Continued.

Types of food	Country	Major findings	Reference
Raw milk and cheese	Brazil	Of the egc-borne SE genes (*seg, i, m, n, o, u*) investigated, *sei* present in 97.8% of the isolates. Of these, 53.3% were present in combination with *seg.* A complete set of egc was detected in 40% of the isolates.	Vicosa et al., 2013
Milk and dairy products	Italy	53% positive for one or more SE genes. The most common SE gene was *ser* (28%) followed by *sed* and *selj* (25% each). A total of 35 different SE gene profiles were detected out of 481 isolates.	Bianchi et al., 2014
Ready-to-eat food	China	55.5% of the isolates were positive for at least one SE gene. Newly described SE genes *seg, sei, selj* were not found. Common SE genes were *sed* (25.8%) and *sea* (19.5%).	Xing et al., 2014

[a] Studies in which coagulase positive staphylococci were included.

Table 4. Studies of SE or SEl genes in *S. aureus* isolated from humans.

Source	Major findings	Reference
Clinical	84.7% *S. aureus* isolates harboured at least one SE gene. The *sea* gene was present singly in 60.7% isolates.	Efuntoye et al., 2003
Clinical	Of 198 *S. aureus* isolates, 114 produced enterotoxin A (36), B (20), C (19), D (68) and E (2) *in vitro.*	Flemming et al., 2007
Clinical	86% *sea* through *sed* positive *S. aureus* strains produced the corresponding enterotoxins as assessed by mRNA RT-PCR.	de Oliveira et al., 2011
Clinical	The presence of *sea, sek, seq* was linked to the *spa* type t037.	Kim et al., 2011
Clinical	29.6% *S. aureus* isolates harboured *seb* and/or *sec.* No significant association between the presence of enterotoxin genes and the severity of infection.	Nhan et al., 2011
Clinical	*S. aureus* endocarditis isolates were more likely to harbor *sea, sed, see* and *sei* genes. CC30 isolates were more lilel to harbor *sea, see,* and *seg.*	Nienaber et al., 2011
Clinical	60% *S. aureus* isolates harboured at least one of the SE gene tested *see, seg, seh* and *sei.*	Vasconcelos et al., 2011
Clinical and carriage	Most SE genes (*sea, seb, sec, seg, sei*) did not differ significantly between clinical isolates and carriage isolates except for *seh* which was more common in clinical than carriage isolates.	Lim et al., 2012
Clinical	52.6% of *S. aureus* isolated from patients with atopic dermatitis were positive for *sea.* Of these *sea* positive strains, 42.1% additionally harboured *tsst-1* gene.	Na et al., 2012
Carriage	Comparison of prevalence of SE/SEl genes in 2004-2006 and 1997 – 2002 found significant differences for *sea, seb, sec, sed, seg, seh, sei, selj, sell, selm, seln, selo, selp, ser, selu*).	Argudin et al., 2013

Table 4. Continued.

Source	Major findings	Reference
Clinical	Of the *S. aureus* isolated from tropical pyomyositis, the presence of enterotoxin genes (*seg, sei, sem, sen, seo, sed, sej, ser*) was linked to the spa type t078.	Garcia et al., 2013
Clinical	Colonisation with *sep* harbouring MRSA increased the risk of MRSA bacteremia.	Calderwood et al., 2014

Table 5. Studies of SE or SEl genes in *S. aureus* isolated from other sources.

Source	Major findings	Reference
Poultry and humans	Only one out of 34 poultry isolates contained SE gene (*sec*) whereas more than 51% human isolates were positive for at least one SE gene. The *sea* (12.2%), *seb* (2.4%), *sec* (22%) and *sed* (24.4%) were present. None yielded *see.*	Hazariwala et al., 2002
Handles of handheld shopping baskets	12% *S. aureus* isolates produced enterotoxin B, which was significantly higher than that of supermarket workers (2%) and another independently collected clinical specimens (4%)	Mizumachi et al., 2010
Cows with subclinical mastitis	The *sea, seb, see* genes were absent in all *S. aureus* isolates tested. The *sec* and *sed* genes accounted for 6% and 2.4% of the isolates.	Oliveira et al., 2011
Food industry surfaces	6.1% of samples (442) yielded *S. aureus.* Of these isolates, 90% were positive for *sea, seg, seh* or *sei* gene. The prevalence of SE genes was *sea* (63%), *seg* (40%), and *sei* (77%). The *seb, sec, sed* and *see* genes were absent in all isolates.	Gutierrez et al., 2012
Buffaloes and small ruminants	*S. aureus* isolated from buffaloes were likely to harbour *sea, sec, sed, seg, sei, selj, sell* whilst those from small runminants were linked to *sec* and *sell.*	Cremonesi et al., 2013
Insects	The *sea* gene was present in 21.8% isolates	Oliveira et al., 2013

A great diversity of SEs has been found in outbreak investigations, staphylococcal enterotoxin A (SEA) either alone or in combination with other types of enterotoxin being the most frequently identified. In France where SFP is the second most common cause of food borne illness, SEA was detected in 87% of *S. aureus* strains isolated from 31 food poisoning outbreaks during 1981 to 2002, followed in order of frequency by SED, SEG, SEH and SEI (Kerouanton et al., 2007). SEA harbouring *S. aureus* was recovered from 11 people in an outbreak affecting 14 students attending a Japanese university festival (Kitamoto et al., 2009). SEA was also believed to be a cause in a food poisoning outbreak in Japan (Haga et al., 2007). However, in another outbreak affecting 40 school children in Austria, *S. aureus* strains harbouring both enterotoxin A and D were isolated. It was suggested that enterotoxins, which remained intact in milk after pasteurization were responsible for the outbreak. The primary source remained uncertain but enterotoxigenic strains were isolated from workers (Schmid et al., 2009). In fact, dairy products appear to be an important reservoir for staphylococcal enterotoxins in addition to chicken and ham (Chomvarin et al., 2006; Principato et al., 2009; Hwang et al., 2010; Hu et al., 2012). In another post-food poisoning outbreak study conducted by Nema et al. (2007), a single type of enterotoxin, SEB, was found in all samples, reflecting the possibility of a single source contamination. In recent years, enterotoxin intoxication involving non-A non-B SEs has been increasingly reported, probably because more toxin types have been identified and included for epidemic investigations (Molenda et al., 2008). For example, SEE was firstly reported to be involved in six food poisoning outbreaks in France (Ostyn et al., 2010).

Absence of the *see* gene has been reported in several studies. Ferry et al. (2005) collected 80 *S. aureus* isolates from septic patients and found all were negative for *see* gene. Absence of the *see* gene has also been observed by authors who investigated 147 strains collected from various food sources including meat, meat products, raw milk, dairy products, bakery products and ready-to-eat food over seven cities in Marmara region of Turkey (Aydin et al., 2011). Macro-restriction digestion analysis of these food isolates resulted in a high variety of patterns which demonstrated 69% to 100% homology. It indicated that the absence of *see* gene was possibly a universal phenomenon, at least for strains of food origin. Consistent with the observation, a study in Portugal failed to detect the *see* gene in 148 *S. aureus* strains isolated from raw meat, cheeses, bovine mastitis, raw cow milk, bread, kitchen surfaces swab and pie (Pereira, 2009). In Poland, 50 *S. aureus* strains isolated from food were negative for *see* gene (Bania et al., 2006). Studies on clinical isolates in Georgia (n=41), Jordan (n=100) and China (n=108) also confirmed the rarity of *see* gene (Hazariwala et al., 2002; Naffa et al., 2006; Xie et al., 2011). To date, there are no reports on the distribution of SE genes among food handlers in Hong Kong.

1.6.2 Epidemiology of SFP

In the United States, an estimated 241,148 (90% CI: 72,341 – 529,417) domestically acquired SFP cases occurred annually between 2000 and 2008. Amongst all notified food poisoning cases, *S. aureus* was the fifth most common bacterial causative agent responsible for acute gastroenteritis in the period. Although the case fatality rate remained less than 0.1%, a proportion of sufferers (6%) required hospitalization. As it is believed there are far more SFP cases than are documented because of the self-limiting nature of the illness

leading to the majority of infected individuals not seeking for medical attention. Even for those who consult a physician, the causative agents are not usually identified. The authors therefore recommended a multiplication of the estimated incidence of SFP by a factor of 29.3 for under-diagnosed cases and by 25.5 for under reported cases (Scallan et al., 2011).

In Japan, an outbreak occurred in a hotel in Ishikawa Prefecture affecting 10 people. *S. aureus* strains with indistinguishable PFGE patterns were isolated from a vomit specimen, three food specimens (sashimi, frozen crab and pickled radish), finger swabs of the cooks and a refrigerator handle swab (Kuramoto et al., 2006). In other outbreaks at a community festival and a university festival, 20 and 74 people were involved respectively (Haga et al., 2007; Kitamoto et al., 2009). Apart from sporadic outbreaks, a massive outbreak in Kansai affecting more than 13,000 people has been documented (Asao et al., 2003). According to the most updated information provided by the Ministry of Health, Labour and Welfare of Japan, *S. aureus* remains the second most important bacterial agent responsible for food poisoning after *Salmonella* (ISD, 2009).

In France, characterization of 178 coagulase-positive staphylococci recovered from SFP outbreaks (1981 – 2002) indicated that 82% of these were human biotypes (Kerouanton et al., 2007). The first report of SFP caused by SEE was noted in 2009 upon investigation of six food poisoning outbreaks involving unpasteurized milk derived soft cheese (Ostyn et al., 2010).

In Austria, where milk products were implicated in causing SFP outbreaks involving more than 1025 children in 10 neighbouring schools, investigations revealed that consumption of

milk, cacao milk, or vanilla milk provided by dairy X was associated with a 37.8 fold higher risk (95% CI 2.3 – 116.5). The presence of enterotoxins A and D were detected in the left over milk in the affected schools (Schmid et al., 2009).

In a large gathering involving 8000 individuals in Brazil, 4000 people experienced SFP symptoms after a meal. Of these sufferers, 396 required hospitalization and 81 were admitted to intensive care units. Of the patients admitted to ICU, 16 developed multi-system shock and died during hospitalization. The food preparers were subsequently found to be positive for enterotoxigenic *S. aureus* (Do Carmo et al., 2004).

In India, consumption of a snack called “Bhalla” was implicated in a food poisoning incident affecting more than 100 individuals. All enterotoxigenic *S. aureus* isolates originated from the incriminated food and the organism from the corresponding sufferers produced SEB and SED (Nema et al., 2007).

In Hong Kong, more than 100 food poisoning outbreaks associated with *S. aureus* occur annually. From 2001 to 2009, there were a total of 910 outbreaks involving 3,049 individuals (Figure 4). This accounts for 18.5% of all bacterial food poisoning outbreaks in the region. An estimate of 47% SFP outbreaks occur in food premises followed by homes (39%). The most commonly associated food types were Chinese style soy-sauced meat (lo-mei) and roasted meat (siu-mei) (Department of Health, 2010).

Occurrence of SFP in Hong Kong appears to follow a seasonal pattern. Incidents have been noted more frequently during July to September and December to February. During 2001 to 2009, the accumulated incidence of SFP in February alone was more than double that in

March and April together (Department of Health, 2010). Reasons accounting for these variations remain unknown. These might be related to particular variations in climate including humidity and temperature in Hong Kong favouring the production of SEs. The Chinese New Year festival in early February may also account for the increased incidence. During the festival, foods are prepared in advance and left at ambient temperature for long time which may provide opportunities for the formation of enterotoxins. The common SFP incriminated food items, chinese-style roast meats and soy-sauced meats, are also popular in the festival (Department of Health, 2010). A recent cross sectional survey in Hong Kong found that 50% retail roast meats were contaminated with *S. aureus* of which 18% of the isolates were entertoxigenic (Young et al., 2014).

1.6.3 Food handlers implicated in food contamination

In the majority of staphylococcal food poisoning outbreaks, cross-contamination, improper handling of food and contamination by food handlers are the major contributing factors (Bergdoll and Wong, 2006; Department of Health, 2010). Ready-to-eat food may be cross-contaminated by raw ingredients, especially those of animal origin, if they are stored close together. Indirectly, inadequate cleaning of food preparation surfaces can also play a role (Greig et al., 2007). Improper handling of food such as prolonged display of foods at room temperature, slow cooling and failure to maintain hot/cold chain for food storage can allow residual bacteria to proliferate and subsequently release toxins (Todd et al., 2008a).

Evidence shows that foods implicated in SFP outbreaks are mainly multiple-ingredient products requiring hand contact (Greig et al., 2007). A wide variety of sources leading to SFP outbreaks have been demonstrated including single food handlers and environmental surfaces (Todd et al., 2007).

An investigation of an SFP outbreak in Hong Kong in 2006 concluded that an outbreak attributable to traditional Chinese soy-sauced meat and roasted meat affected 65 persons in 22 districts involved food preparation surfaces. The same *S. aureus* strain was isolated from the incriminated food, the food preparation surfaces and nasal specimens of the food handlers (Department of Health, 2010).

Persistent *S. aureus* carriers likely contaminate their hands. They can also pass the organism to others in contact with them or to environmental surfaces. Food handlers

carrying enterotoxigenic *S. aureus* in their noses and/or on their hands are therefore considered to be a source of food contamination. Evidence shows that, in the absence of good personal hygiene, food handlers carrying *S. aureus* in their noses can contaminate the working environment and food with the organism (Todd et al., 2008a, Todd et al., 2008b). Food can be contaminated when food is improperly handled. These include cross contamination of cooked and raw food on kitchen surfaces, not allowing sufficient time for cooked food to cool down before refrigeration, constantly putting hot food into refrigerators leading to a rise in refrigeration temperature favouring the growth of microorganisms, inadequate cleaning of kitchen surfaces and utensils, and prolonged exposure of food on buffet tables at room temperature. All of these situations create a favourable environment for growth of *S. aureus* and production of enterotoxins (Greig et al., 2007). The contribution of food handlers in contributing to food poisoning outbreaks is depicted in Figure 5.

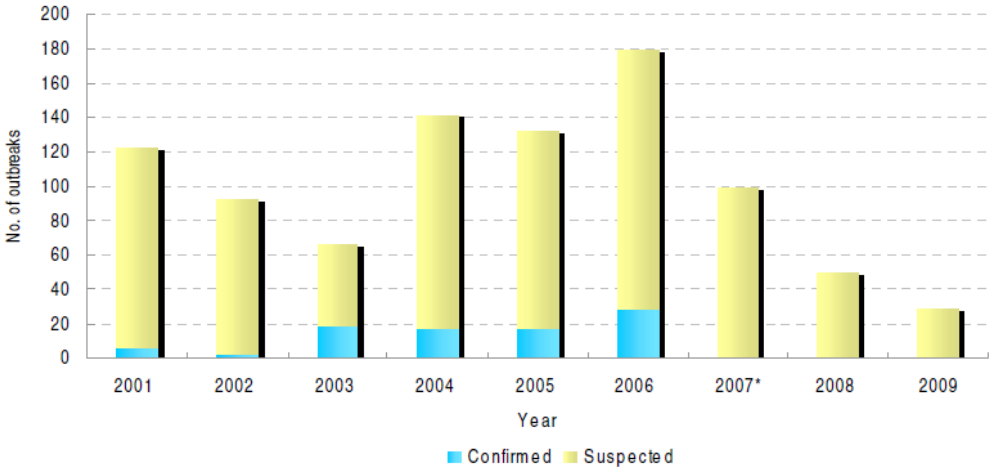
Identical bacterial strains carrying the same types of enterotoxin have been isolated from both patients and food handlers upon investigation of staphylococcal food poisoning outbreaks, indicating the risk of food contamination by catering workers (Udo et al., 1999; Do Carmo et al., 2004; Greig et al., 2007; Haga et al., 2007; Schmid et al., 2009), although absence of the incriminated strains carried by food handlers has also been reported (Fetsch et al., 2014). Supporting food handlers as a source of food poisoning, Kerouanton et al. (2007) revealed that 84% of *S. aureus* strains associated with staphylococcal food poisoning were with a human biotype. Touching of the nose can transfer the microorganism to the hands which can then contaminate food, utensils and surfaces on which food is

manipulated. *S. aureus* is capable of producing and secreting enterotoxins *in vitro* (Marino et al., 2011).

Carriage with *S. aureus* in food handlers have been studied in different settings and countries (Table 6). Most of these studies investigated only nasal carriage rate without taking hand contamination into account (Figueroa et al., 2002; Gunduz et al., 2008; Simsek et al., 2009; Udo et al., 2009; Rall et al., 2010; Saeed & Hamid, 2010; Dagnew et al., 2012). Hands are important vectors which transfer *S. aureus* from noses to food being handled. Although there were studies investigating carriage rates from more than one anatomical site (de Jonge et al., 2010; El-Shenaway et al., 2014), the prevalence rate of these anatomical sites was not reported separately making interpretation difficult.

The majority of the studies did not detect the production of SEs or the presence of relevant genetic determinants (Andargie et al., 2008; de Jonge et al., 2010; Saeed & Hamid., 2010; Simsek et al., 2010; Zaglool et al., 2011; Dagnew et al., 2012; Sospedra et al., 2012). Only a few studies investigated the production of enterotoxin SEA through SED by ELISA (Hatakka et al., 2000; Loeto et al., 2007). Of two studies in which enterotoxin genes were detected, less than half of the known SE determinants were included (Udo et al., 2009; Rall et al., 2010). The presence of antiseptic-tolerant staphylococci in food handlers has been briefly investigated in a study focused on MRSA (Ferreira et al., 2014).

Figure 4. Incidence of Staphylococcal food poisoning outbreaks in Hong Kong (2001 – 2009)



Reference: Centre for Health Protection, Hong Kong, 2010.

Figure 5. Diagram depicting the role of food handling in *S. aureus* transmission

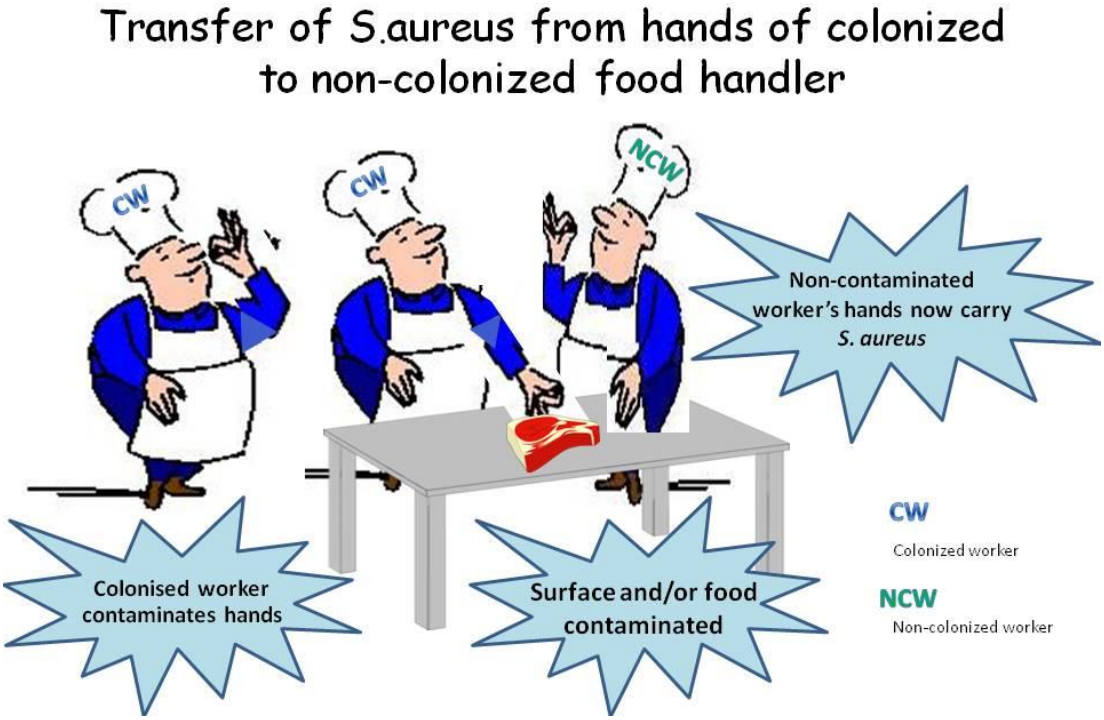


Table 6. Studies on nasal and hand carriage with *S. aureus* in food handlers

Setting	Country	Subjects (n)	[a]Prevalence (%)		Reference
			Nose	Hands	
Flight catering	Finland	N: 111[b] H: 117[c]	29	6	Hatakka et al. (2000)
Restaurants	Chile	102	34	N/A	Figueroa et al. (2002)
Institutions	Botswana	200	44.6	30.9	Loeto et al, (2007)
University	Ethiopia	127	N/A	16.5	Andargie et al. (2008)
Restaurants	Turkey	8,895	0.77	N/A	Gunduz et al. (2008)
Restaurants	Turkey	299	21.2	N/A	Simsek et al. (2009)
Restaurants	Kuwait	250	53.2	N/A	Udo et al. (2009)
Hospital	Brazil	70	15.7	2.9	Borges et al. (2010)
Restaurants	Turkey	25	10.4	7.5	Cepoglu et al. (2010)
Hospital	The Netherlands	89	(33) [d]	(33)[d]	de Jonge et al. (2010)
Kitchens	Brazil	68	22.1	N/A	Rall et al. (2010)
Restaurants	Sudan	259	21.6	N/A	Saeed and Hamid (2010)
Hospital	Saudi Arabia	200	N/A	17.5	Zaglool et al. (2011)
University	Ethiopia	200	20.5	N/A	Dagnew et al. (2012)
Restaurants	Spain	227	N/A	8.4	Sospedra et al. (2012)
Food plants	Egypt	200	31.0	(38) [e]	El-Shenaway et al. (2013, 2014)
Hospital	Brazil	140	29.3	50	Ferreira et al. (2014)

[a] Prevalence of *S. aureus* carriage

[b] Number of specimens from nares

[c] Number of specimens from hands. Prevalence rates were estimated on the presence of coagulase positive staphylococci

[d]Authors did not specify the carriage rate (whether from noses or hands)

[e]Skin carriage rate (hands or face).

1.6.4 Hygiene behaviour and incidence of food poisoning

The number of food poisoning outbreaks reported to the Department of Health (DH), Hong Kong increased from 2001 to 2006 except in 2003 during which a significant drop was noted followed by a gradual decrease from 2007.

In recent years, public attention about infectious diseases has shifted from food poisoning to concern about epidemics of respiratory infections, in particular SARS, and new strains of influenza A viruses. The emergence of the SARS epidemic in 2003 considerably raised public awareness of personal hygiene and led to the use of personal protective equipment (surgical masks, aprons, disposable gloves) in the food industry in Hong Kong. It also demonstrated the importance of one of the conventional hygiene measures – hand washing. Indeed, hand washing has long been recognised as an effective way of preventing communicable diseases. Surveys conducted immediately after the SARS epidemic revealed that the general population in Hong Kong started developing more healthy behaviours such as participating in more exercise and adopting more personal hygienic measures as compared with the pre-SARS period (Lau et al., 2005; Lau et al., 2006). It was noted that three months after the SARS epidemic, more than 70% of people washed their hands more frequently and wore a mask in public places. Notably, respondents who were female, aged 50 or above, university graduates, or married tended to comply more with personal hygienic measures (Lau et al., 2005).

The change of behaviour among food industry workers at that time might lead to lowering of the cross-contamination rate, and possibly incidences of food poisoning. According to

Centre for Health Protection in Hong Kong (2010), the number of food poisoning cases dropped significantly in 2003 compared to previous years. From 2001 to 2002, a total of 1300 food poisoning outbreaks were noted. In 2003, there were only 450 food poisoning outbreaks reported. This remarkable change could have been attributed to the hygienic measures adopted including wearing gloves and masks among food handlers, lowered the risk of food contamination. The figure went up again from 2004 to more than 800 outbreaks per year though not reaching previous levels. Although personal hygiene standards of the general public considerably improved after the SARS epidemic, it remains uncertain if these practices can be permanently sustained without an imminent threat.

1.7 Emergence of Antibiotic resistance in *S. aureus*

There are at least three antibiotic resistance mechanisms recognised in staphylococci. They are (1) intrinsic resistance, (2) *de novo* mutation and (3) acquisition of resistance determinants (Tong et al., 2012). Intrinsic resistance refers to the ability of a bacterial species to be innately resistant to a particular antibiotic class. This insensitivity is encoded by chromosomal genes, and hence is transferred vertically to all progeny cells. Microorganisms undergo evolution and mutation from generation to generation. Even prior to the introduction of antimicrobial agents, antibiotic resistant *S. aureus* could have been present as a result of mutation. Bacterial mutation rate is estimated to be approximately 10^{-8} indicating that, in the absence of selective pressure, one mutant would be formed in 10^{8} bacterial cells (Crossley et al., 2009).

Without antibiotics, these resistant strains have no particular survival advantages and they have to compete with sensitive strains for nutrients and niches. However, the introduction of antibiotics kills susceptible strains leaving resistant bacteria to grow. Over time, resistant strains become the predominant form of the species. Acquisition of resistance determinants is mediated by horizontal transfer of plasmids, transposons or other mobile genetic elements via conjugation, transduction and transformation. Due to the large amount of information, only antimicrobial classes relevant to this study were reviewed.

1.7.1 Resistance to cell wall inhibitors

Cell wall synthesis inhibitors are the major class of antibiotics used for treatment of staphylococcal infections worldwide, of which beta-lactams are the single most important class of anti-staphylococcal agent in human medicine.

Resistance to β lactams such as penicillins and cephalosporins in *S. aureus* is mediated by two main mechanisms: (1) hyperproduction of β lactamases and (2) acquisition of the *mec*A or *mecC* gene.

β-lactamases hydrolyse the β-lactam ring, which is the functional group of penicillins and cephalosporins. The enzyme, is encoded by *blaZ* gene, is harboured by approximately 90% *S. aureus* isolates (Olsen et al., 2006). After the recognition of this enzyme, a β-lactamase stable penicillin, methicillin, was introduced. This drug remains intact after exposure to β-lactamases. However, in the 1970s, resistance to methicillin emerged in *S. aureus* (MRSA). This resistance was mediated by the *mec*A gene which codes for an altered drug target site,

namely penicillin binding protein 2a, which exhibits abnormally low affinity to β lactam agents, thereby allowing transpeptidation to continue. The *mecA* gene is regulated by *mec*R1 and *mec*I. In the absence of β lactam agents, the *mecA* gene is repressed by *mec*I. Binding of beta lactams to staphylococcal surface protein *mec*R1 leads to serial proteolytic events leading to de-repression of *mecA* gene (Crossley et al., 2009).

In recent years, a *mecA* homologue called *mecC* has been identified from humans and cows (Garcia-Alvarez et al., 2011). This gene also codes for an altered penicillin binding site. The distribution of the *mecC* gene is not clearly understood but it appears to be linked to three clonal complexes (CC130, CC49 and CC1943) and does not harbour additional resistance determinants to non β-lactam antibiotics (Deplano et al. 2014).

Both the *mec*A and *mecC* genes are situated in a transposon called the staphylococcal cassette chromosome *mec* (SCC*mec*). So far, 11 types of SCC*mec* elements documented (Zhang et al., 2009). SCC*mec* is classified on the basis of the *mec* complex and *ccr* complex. Except for type VII which is associated with canine staphylococci, all of the other SCC*mec* types have been reported in human MRSA isolates (Shore & Coleman, 2013).

1.7.2 Resistance to protein synthesis inhibitors

Because of the structural differences between bacterial and eukaryotic ribosomes, most protein synthesis inhibitors act by either binding to the 50S ribosomal subunit (macrolides, lincosamides, streptogramins, chloramphenicol, oxazolidinones) or the 30S ribosomal subunit (tetracyclines, aminoglycosides). Others bind to enzymes such as isoleucyl-tRNA

synthetase (mupirocin) and elongation factor G (fusidic acid) which are involved in staphylococcal protein synthesis (Ruimy et al., 2010; Tong et al., 2012).

Resistance to macrolide-lincosamide-streptogramins (MLS) is largely mediated by degradation enzymes. In staphylococci, cross-resistance to macrolide, lincosamide and streptogramin B is usually mediated by the production of a methylase encoded by *erm* genes. Co-resistance to macrolides and streptogramin B is conferred by the *msrA* gene which encodes an efflux protein (Li & Nikaido, 2009). Different combinations of *msrA* and *erm* genes have been reported in human carriage *S. aureus* isolates (Lozano et al., 2011). Sole resistance to lincosamide is encoded by *Inu(A)* and *Inu(B)* genes which encode for lincosamide nucleotidyltransferase. Isolates solely resistant to streptogramin A, encoded by *vat(A), vat(B)* and *vat(C)*, and streptogramin B, encoded by *vgb*(A) and *vgb*(B) have also been documented (Wendlandt et al., 2013).

Methylation of an adenine residue at position 2503 of 23S rRNA contributes to cross resistance to lincosamide, streptogramin, oxazolidinones, phenicols and pleuromutilins (Witte & Cuny et al., 2011). This is conferred by a plasmid borne *cfr* gene. *S. aureus* harbouring *cfr* has been reported in human clinical isolates as well as in animal isolates which demonstrated an elevated MIC to linezolid from 16 to 32 mg/L (Morales et al., 2010).

Tetracycline resistance in staphylococci is mainly mediated by (1) active efflux which is encoded by *tet(K)* and *tet(L)* genes and (2) ribosomal protection *tet(M)* (Crossley et al., 2009). Non-susceptibility to aminoglycosides is a result of enzymatic modification. This is

achieved by acetylation-phosphorylation (*aacA-aphD*), adenylation (*aadE*) and phosphorylation (*aphA3*) (Crossley et al., 2009).

Fusidic acid resistance is mediated by *fusA* which encodes single point mutation in the drug target or by *fusB* and *fusC* genes which encode ribosomal protection proteins. Clinical staphylococcal isolates resistant to fusidic acid mainly carry *fusB* or *fusC* genes (87%) while the remainder are due to *fusA* encoded mutation (McLaws et al., 2011).

1.7.3 Resistance to nucleic acid syntheses inhibitors

Fluoroquinolones and rifampicin are two common anti-staphylococcal agents which interfere with DNA and RNA synthesis respectively. Resistance to these agents are caused by mutations of genes encoding enzymes involved in DNA replication, topoisomerase IV (*grlA* and *grlB*) or gyrase (*gyrA* and *gyrB*) (Crossley et al., 2009). These regions are collectively called quinolone resistance determining regions (QRDR). Mutations of these regions do not produce the same tolerance effect to all quinolones. Ciprofloxacin is least effective against *S. aureus* QRDR mutants whereas moxifloxacin retains activity (Schmitz et al., 1998).

Rifampicin is an inhibitor of DNA-dependent RNA polymerase. In staphylococci, resistance to this agent is caused by mutations of the *rpoB* gene. High levels of rifampicin resistance in *S. aureus* have been reported in clinical isolates from wounds, blood and respiratory samples. The rate is significantly higher in MRSA than in MSSA (Villar et al., 2011).

1.7.4 Resistance to other anti-staphylococcal agents

The combination of sulfamethoxazole and trimethoprim works synergistically to inhibit folic acid synthesis in *S. aureus*. Sulfonamides compete with para-aminobenzoic acid for dihydropteroate synthetase whereas trimethoprim inhibits dihydrofolate reductase which is essential for the conversion of dihydrofolate to tetrahydrofolate. Notably, high level of resistance to trimethoprim is mediated by plasmid borne *dfrA* gene which encodes a dihydrofolate reductase with reduced affinity to trimethoprim (Crossley et al., 2009).

1.8 Emergence of reduced susceptibility to disinfectants

Unlike antibiotic resistance, the fairly recent emergence of disinfectant tolerance in staphylococci has received far less attention. There is evidence showing that *S. aureus* is becoming less susceptible to disinfectants including chlorhexidine, triclosan and other QAC compounds (Smith et al., 2008; Marino et al., 2011; Zhang et al., 2011). These biocides are widely used in hospitals and the food industry for hand washing and decontamination of environmental surfaces. With the emergence of reduced susceptibility to biocides, *S. aureus* may survive cleaning and pose a threat on food safety.

Studies have suggested genes encoding antibiotic resistance and increased disinfectant tolerance may be co-selected. Initially the genetic linkage of beta lactam resistance determinants and the presence of *qacB* was demonstrated in the plasmid pST6 carried by *S. epidermidis*. The plasmid pST6 was subsequently proven to be transferrable to *S. aureus* by transformation (Sidhu et al., 2001).

In practice, the recommended concentration of antiseptics for use provided by manufacturers is usually up to 1000 fold higher than the MICs of isolates harbouring *qac* genes. However, in situations when disinfectants are topped up, surfaces are heavily contaminated or in the presence of biofilms, the disinfectant could be rendered ineffective (Smith et al., 2008). This sub-bactericidal concentration would favour growth of *S. aureus* which are tolerant to the relevant compounds. The ability to survive disinfection could promote dissemination of enterotoxigenic *S. aureus.*

The ability to survive in low levels of disinfectants is attributable to the quaternary ammonium compound genes (*qac*) which are found on transmissible plasmids in staphylococci. Microorganisms harbouring *qac* genes have increased tolerance to disinfectants in which QACs are the active ingredient. Although an association has been reported by multiple authors, the relationship between *qac* genes to disinfectant tolerance remains unclear. Although the presence of *qac* genes may only result in modest increase in MIC, these may nevertheless allow bacteria to survive under inappropriate uses of disinfectants such as high dilution or failure to rinse cleaning cloth completely. It has been reported that 41% to 56% of antiseptic resistant staphylococci harboured *qac* genes (*qacA, qacB, or smr*) (Noguchi et al., 1999; Zmantar et al., 2011). The *qac* genes encode integrated membrane efflux pumps which allow the host microorganism to pump out QAC disinfectants which would otherwise be lethal to the cell. More recently, nanotechnology has demonstrated the bactericidal action of QAC is mediated by cell wall disruption and cytoplasmic leakage (Bragg et al., 2014).

Studies on disinfectant resistance have largely focused on clinical isolates of *S. aureus,* particularly MRSA. In Japan, screening for the presence of *qacA* and *smr* in 98 MRSA isolates recovered from various clinical sources revealed that 71 strains harboured genes for antiseptic resistance. Of these, *qacA* and *smr* were detected in 10 and 20 strains respectively (Noguchi et al., 1999). Screening of MRSA isolates from 11 Asian countries revealed that 41.6% carried *qacA/B* (372/894) and 1.9% (16/856) harboured *smr* (Noguchi et al., 2005).

The prevalence of antiseptic resistance genes varies considerably across countries. Of 65 clinical MRSA isolates tested in a Japanese study, 52.3% harboured *qacA* gene. All these isolates were resistant to acriflavin, benzalkonium chloride, and benzalkonium chloride (Sekiguchi et al., 2004). In Brazil, the *qacA/B* gene was present in 80% of the MRSA isolates tested (Miyazaki et al., 2007). However, in the United Kingdom, only 8.3% MRSA yielded *qacA/B* but 44.2% harboured *smr* (Vali et al., 2008). In Canada, 2% *qacA/B* and 7% *smr* genes were detected from 334 MRSA isolates from intensive care units (Longtin et al., 2011). In the mid-Atlantic region of the United States, only five out of 493 MRSA isolates from seven hospitals harboured *qacA/B* (McGann et al., 2011). Co-existance of *qacA/B* and *smr* is rarely detected, screening of 497 *S. aureus* isolates revealed only five strains (1%) concomitantly carried both determinants. However, whether they were linked in the same plasmid remains unknown (Mayer et al., 2001). In China, 40% *S. aureus* isolated from metro stations were *qac* positive (Zhou & Wang, 2013).

In Hong Kong, the distribution of *qac* genes have been investigated in staphylococcal isolates collected from automated teller machines and from carriage isolates of nurses and

the general public (Zhang et al., 2011a; Zhang et al., 2011b). The presence of *qac* genes was overrepresented in MRSA isolates compared to MSSA.

The origin of *qac* genes remains unclear. An early study commenced with screening staphylococci isolated from poultry plants, red meat plants, baguettes and chicken carcasses for phenotypic resistance to benzalkonium chloride followed by species identification failed to detect any *S. aureus.* The most commonly isolated species were *S. epidermidis* and *S. saprophyticus* (Heir et al., 1999a). However, further characterization of the plasmid pST6 isolated from *S. epidermidis* of the same collection revealed similar genetic organization as those of plasmids harboured by *S. aureus* of clinical origin (Sidhu et al., 2001). The *smr* gene initially described in clinical and food isolates was found to be carried on a large plasmid in *S. aureus* of bovine origin (Bjorland et al., 2001). However, screening for resistance determinants (*qacA/B, smr, qacG, qacJ*) of staphylococci in cattle/ goat herds detected only one *S. aureus* isolate positive for *qac* gene. The overall high prevalence of disinfectant resistant staphylococci in cattle and goat herds (21% and 10% respectively) was mainly attributable to *S. haemolyticus* and *S. warneri* (Bjorland et al., 2005). The presence of *qacG, qacH* and *qacJ* has been reported in staphylococci of human clinical (*S. haemolyticus*) and animal origins (*S. cohnii, S. warneri, S. hominis, S. delphini*) (Bjorland et al., 2005; Correa et al., 2008). In Hong Kong, screening of 237 *S. aureus* isolates and 604 coagulase negative staphylococci associated with human carriage for *qacG, qacH* and *qacJ* found that these genes were only present in 0.5 to 2% of the isolates (Ye et al., 2011).

The presence of disinfectant resistance appears to be linked with methicillin resistance. Comparison of MRSA and MSSA isolates revealed a 1.5- to 3-fold higher MIC and 2- to 4-fold higher MIC to chlorhexidine and QAC respectively (Suller et al., 1999). In a Dutch study including MSSA and MRSA isolates originated from 24 different European hospitals found a significantly higher prevalence for *qacA/B* in MRSA (63%) than in MSSA (12%) but not for *smr* (6.4% in MRSA and 5 in MSSA) (Mayer et al., 2001). Screening of 237 *S. aureus* isolates collected from nurses and general public in Hong Kong revealed a three-fold higher risk for harbouring *qacA/B* and *smr* with the presence of *mecA* gene (OR=22.9, 95% CI 1.8 – 4.8), indicating nursing as an associated occupational risk for acquisition of *qac* gene positive *S. aureus* (Zhang et al., 2011).

Staphylococci with elevated minimal inhibitory concentration (MIC) to QACs have been reported in the food industry. The most commonly isolated species were *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* harbouring *qac*A and/or *qac*B (Heir et al., 1999a; Marino et al., 2011). However, the presence of *qac* genes in MSSA, including that of carriage origin, remains under investigated.

1.9 Conclusions

This review has shown that risks for nasal colonisation with *S. aureus* vary across populations. The recent emergence of live-stock associated strains in animal husbandry workers and reports of high contamination rate of retail meats in Hong Kong has led to concerns about carriage in food handlers, and if, as they are regularly exposed to raw meat,

they would exhibit an increased risk for *S. aureus* colonisation, including strains of animal origins.

Investigations of staphylococcal food poisoning outbreaks have on multiple occasions isolated the same *S. aureus* strains from noses or hands of food handlers and the incriminated food items, indicating that nasal colonisation and hand contamination with the organism is of public health concern. In 2003, the SARS epidemic resulted in enhanced hygiene measures in the food industry where workers started using gloves and masks in routine practice. According to surveillance data from the Department of Health of Hong Kong, the incidence of staphylococcal food poisoning appeared to be inversely correlated with the surge of the SARS epidemic in 2003. The occurrence of the epidemic was followed by stricter enforcement of hygiene especially in the food industry. However, the effect of change in hygiene practice on staphylococcal nasal colonisation rate is unknown. The long term effect of the changes in food handling practices on nasal carriage and hand contamination has not been investigated.

Staphylococcal food poisoning is caused by the ingestion of enterotoxins produced by the staphylococci when they multiply in food. To date, there are at least 20 staphylococcal enterotoxins and enterotoxin-like proteins described in the literature. Whilst there are numerous studies on classical enterotoxins (sea through see), our knowledge of the newly described enterotoxins is rather limited. Currently, no study with sufficient sample size has described the prevalence of the extended array of genes encoding staphylococcal enterotoxins and staphylococcal enterotoxin-like proteins in carriage isolates of *S. aureus* from food handlers.

Quaternary ammonium compound based disinfectant is widely used in both the food industry and hospitals. Increased tolerance to this compound is mediated by *qac* genes. Most studies on the occurrence of *qac* genes are conducted in the healthcare settings. No study has investigated the prevalence of *qac* genes in carriage isolates of food handlers.

Thus a large scale study of *S. aureus* carriage in food handlers and characterization of these isolates could help provide insight into risks for carriage, and effects of changes in practice on nasal colonization and hand contamination, whilst also providing isolates for characterization for virulence and antimicrobial resistance.

Chapter 2 Aims and Objectives

The aims of this study were to; investigate staphylococcal colonisation of food handlers over time, in particular with respect to the long term effect of the implementation of hand washing and hygiene reinforcement during and after the epidemic of the Severe Acute Respiratory Syndrome, and to characterize presence of genes for enterotoxins and reduced antiseptic susceptibility. Food handlers working in catering establishments were chosen because *S. aureus* remains the second most important bacterial food poisoning agent in Hong Kong and continues to be a significant cause of food poisoning worldwide. It is common for local Hong Kong residents to eat out frequently and buffet meals are particularly popular in this region. Consequently, most outbreaks are associated with restaurants and large-scale catering premises.

In order to achieve these aims, the following objectives were established.

(1) *to compare the rates of nasal colonisation and hand contamination with S. aureus amongst food handlers in Hong Kong before, and immediately after, the Severe Acute Respiratory Syndrome (SARS) epidemic (in 2002 and 2003) with those in 2011*.

A large group of food handlers had been sampled twice, before and after the SARS outbreak but the isolates were not fully characterized at that time. However, as they

had been kept in – 80^{o}C freezer, this allowed a unique comparison of a recent sampling to these historical isolates. In the 2003 sample performed after the outbreak, when hygiene measures including the use of gloves and masks were strictly reinforced in the food industry, a remarkable reduction in carriage rate was noted. In order to determine if the change had been sustained, the nasal and hand carriage rates were compared between samples collected in 2002 and 2003 and that in 2011. In order to estimate the extent to which changes in nasal colonization rates would have impacted on the hand contamination rates, correlation between nasal colonization and hand contamination rates was investigated.

(2) *to identify risk factors for nasal colonisation with S. aureus in food handlers.*

Risk factors which might influence colonization, including types of food handled and experience in the food industry, in addition to previously reported risk factors, were investigated. Preparation of Chinese food in enclosed kitchens creates high temperature and humidity. Thereby, occupational exposure to this environment could predispose workers to unknown risk factors which may favour colonization. In addition, the recent emergence of livestock-associated MRSA in food animals and in human workers in contact with livestock, as well as the high contamination rate with this organism in raw meat has become an area of concern. Contamination of meat suggested that food handlers regularly exposed to raw meat may exhibit an increased risk for carriage of *S. aureus,* possibly of livestock origin. Risk was estimated by comparing carriage rates of food handlers who regularly handled raw

meat to those who did not and the clonal origin of the isolates determined by *spa* typing. A validated questionnaire was employed to determine risk factors for carriage of *S. aureus.*

(3) *to determine the prevalence and distribution of genes encoding staphylococcal enterotoxins (SE) and staphylococcal enterotoxin-like proteins (SEl) in all nasal isolates and compare their prevalence between isolates collected at different time periods*.

Carriage of enterotoxigenic *S. aureus* in food handlers is a risk factor for staphylococcal food poisoning outbreaks. The isolation of *S. aureus* with no known enterotoxin types in food poisoning outbreaks and the ability of novel SEs in triggering emesis in primate models have highlighted the possible role of novel enterotoxins in food poisoning. However, studies of *S. aureus* isolates in food handlers have largely focused on classical enterotoxins whilst the abundance of novel enterotoxin types remains under investigated. In order to determine the importance of these new SE/ SEl types, this study aimed to determine the prevalence and distribution of SE/ SEl genes in all nasal isolates. Retrospective detection of newly reported SE/ SEl genes in historical isolates (2002 and 2003 samples) could allow the determination of the actual novelty of these newly reported genes.

(4) *to compare the prevalence and distribution of genes encoding disinfectant tolerance (qac) in nasal isolates and to determine if there is any association between the presence of these genes and the type of disinfectant use in establishments.*

QAC compounds are commonly used in both the healthcare and food industry for disinfection purposes. Whilst the presence of *qac* genes has been investigated in clinical isolates and in healthcare workers, isolates from the food industry have received little attention. The presence of disinfectant tolerance genes in the food industry is of public health concern because non-susceptible strains may survive routine disinfection leading to subsequent growth. Experimental studies have suggested that exposure of planktonic *S. aureus* cells to sub-lethal concentration of QAC compounds would select QAC resistant strains. However, whether routine use of QAC disinfectants in real working environments would select for resistant strains has not been investigated. Determination of presence of *qac* genes could also allow for investigation of association of these genes with other virulence determinants.

(5) *to determine spa types and their relationships with genes encoding enterotoxigenicity and disinfectant tolerance.*

Determination of *spa* types and analyses of association with other determinants could identify if the presence of these virulence genes is related to the genetic background (*spa* type) of a strain. The knowledge of this correlation may allow the

use of *spa* type as a predictor of food poisoning risk, by means of amplication of respective genes and statistical analysis of association.

(6) *to investigate transmission between carriers and non-colonised subjects with hand contamination.*

Few cross-sectional studies have investigated the transmission between nasally colonised subjects and hand contamination of their co-workers in the same workplace. By *spa* typing of nasal and hand isolates, the relative importance of persistent and transient nasal carriers leading to hand contamination of co-workers could be determined.

(7) *to determine antibiotic susceptibility including methicillin resistance characterization.*

Antibiograms can serve as preliminary indicators of clonal relatedness allowing streamlining of the choice of molecular typing and efficient use of time and resources. Knowledge of antibiotic resistance could elucidate the relationship between antibiotic and antiseptic resistance. Characterization of methicillin resistance determinants, enables identification of the sources of MRSA nasally carried by food handlers and may reveal if LA-MRSA was colonizing those exposed to meat as has been reported in butchers.

Chapter 3 Methods

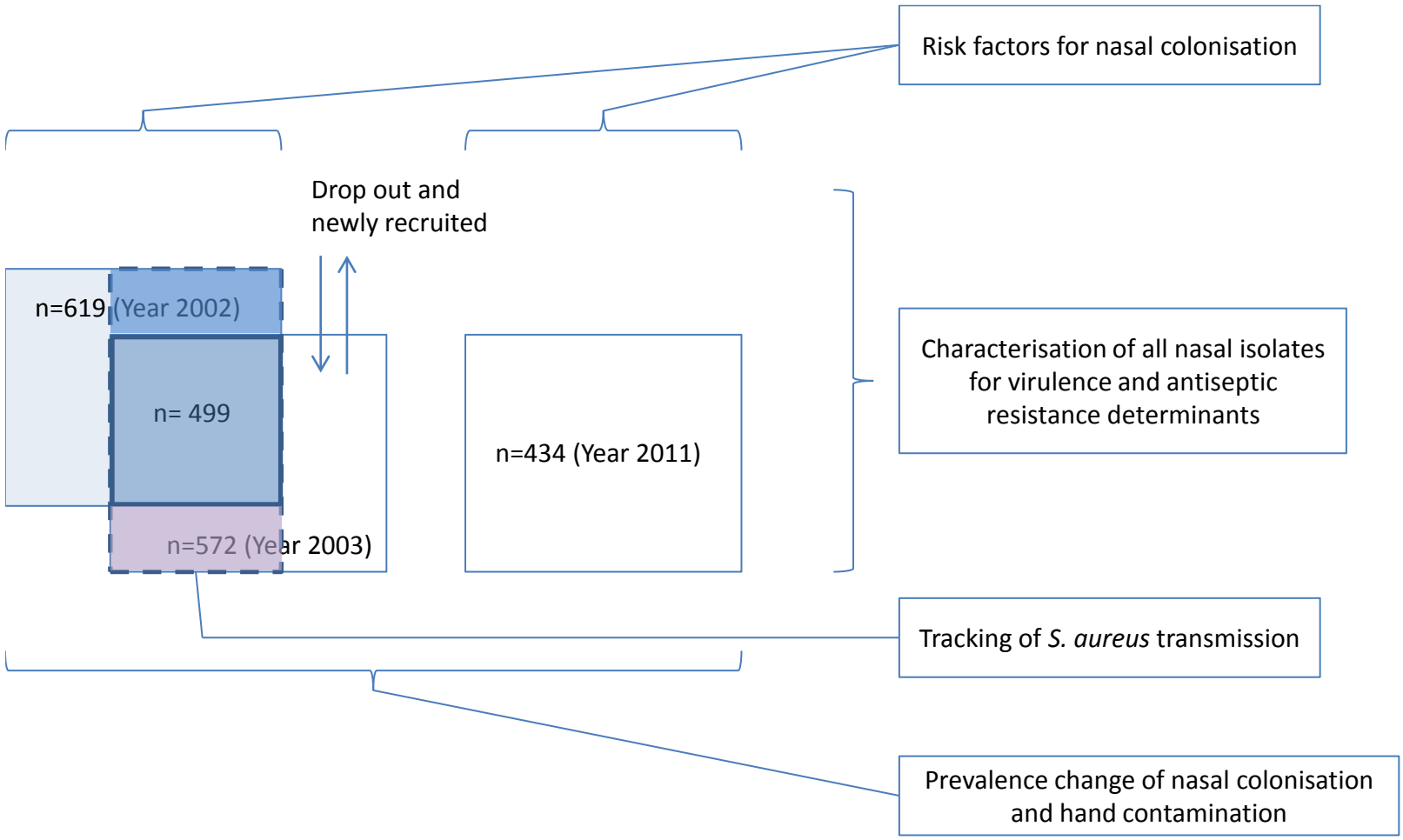
3.1 Study Design

This study compared the carriage of *S. aureus* and presence of virulence determinants in the isolates collected from food handlers over eight years in Hong Kong by use of a series of related cross sectional studies. Samples collected from the same groups in 2002 and 2003 were compared with a cross sectional study in 2011. The first sample was collected immediately before the SARS epidemic, and the second, two months after the outbreak. The third sample was collected eight years after the epidemic. On both the first and the second occasions, nasal and hand imprint specimens were collected from food operatives for a survey on nasal colonisation and hand contamination rates.

The initial study was not planned to collect samples before and after SARS for *S. aureus* screening. It started in 2002 as a longitudinal study to examine carriage patterns of food handlers in Hong Kong. The emergence of SARS outbreak in 2003 prevented the intended outcomes to be possible and funding was insufficient to continue at that time. Changes in frequency of food poisoning prompted a new research question leading to the current study. The project was initially funded by an internal research grant from School of Nursing, The Hong Kong Polytechnic University. *S. aureus* isolates from both these collections were stored in at -80^{o}C since isolation and had not been characterised. These historical isolates provided a unique opportunity for comparison of carriage dynamics and changes in microbial

virulence over the last decade. In the most recent sample collection, food handlers were recruited and asked to complete a short questionnaire, provide a nasal swab specimen and a fingerprint impression. Specimens from food preparation surfaces were collected upon agreement with relevant caterers. Firgure 4 summarised the design of this study.

Figure 6. Study design.



3.2 Sample Size Determination

To determine the change of prevalence of *S. aureus* carriage among food handlers, a proper sample size was essential. The sample size required for nasal swab and hand imprint samples was determined using the standard formula (Chow et al., 2008) for comparing two proportions as shown below.

$$n = \left[\frac{Z_\alpha}{2} + Z_\beta\right]^2 \times \frac{P_1(1-P_1) + P_2(1-P_2)}{(P_1 - P_2)^2}$$

where Z_α and Z_β equal to 1.96 for 95% confidence and 0.84 for 80% power, respectively.

P_1 equals 0.35 based on 35% carriage rate and P_2 0.23 based on 23% carriage rate.

Using the formula, it was calculated that at least 221 specimens would be required to determine the change of prevalence from 35% to 23% in order to achieve 95% confidence interval with 80% power. Inclusion of 499 samples increased power to 99.8%. In order to have sufficient isolates to allow for investigation of virulence factors, including staphylococcal enterotoxin genes, a larger sample was recruited with an aim to isolate approximately 100 *S. aureus* from nasal samples.

3.3 Questionnaire

In order to identify risk factors for *S. aureus* carriage, all participants recruited in 2002 and 2011 were requested to complete a questionnaire at the time of sampling. The details of the questions are provided in the Appendices 6 – 9. The 2011 questionnaire was translated into Chinese by the researcher and back translated by an independent translator to check for accuracy. Questions regarding demographics, health-related behaviours, presence of illness, exposure to health-care facilities, known risk factors for *S. aureus* carriage, compliance to hygiene practices and experiences in the food industry were included. Content validity was determined by inviting two experts in the field to rate the relevance of each of the questions and determine the presence of any leading questions. The validity of the questionnaire was calculated as in Table 7. The questionnaire used in this study scored 0.95. A score of more than 0.8 is considered as acceptable (Polit et al*,* 2006). In order to assess the reliability of the questionnaire, participants at the first establishment were asked to repeat filling in the questionnaire two weeks after sampling.

In 2011, the questionnaire was expanded and greater emphasis was put on hygiene practices and other risk factors for carriage as studies published in the interim have indicated the importance of these factors particularly with respect to community associated MRSA which had not been noted in the community in HK prior to 2005 and made a notifiable disease in 2007.

3.4 Sample Collection

3.4.1 Subjects

Food handlers were defined as workers who work on either full time or part time basis in a kitchen in contact with food and involved in its preparation for consumption. In 2002 and 2003, a total of 619 and 527 food handlers respectively were recruited from 15 catering establishments which included local supermarkets, college canteens, and centralized kitchens for local hospitals and a major sports facility. Only supermarket employees whose job duties were to prepare ready-to-eat food and/or raw food were recruited. A total of 499 participants were sampled on both occasions (264 from supermarkets, 80 from college canteens, 57 from centralised kitchens for hospitals and 98 from a centralised kitchen for major sports facility) (Table 8). In the 2011 study, a total of 434 food handlers were sampled from six large catering establishments. These included hotel kitchens and caterers serving educational institutions, local hospitals, and a major sports facility. Centralized kitchens and the major sports facility were sampled on all three occasions. Details regarding the number of food handlers sampled and caterers in each type of facility are shown in Table 8.

Supervisors of catering establishments were asked to give permission for sampling specimens from food handlers by our researchers. Supervisors who agreed to participate invited individual workers to be sampled in our study. All samples were

collected two hours after commencement of a shift. They were instructed not to wash their hands in prior to hand plating.

3.4.2 Confidentiality and ethical considerations

In order to provide privacy to participants, names of participants were not included on questionnaires. Names were recorded on consent forms which were kept separately from other data. All completed questionnaires were anonymously coded to allow matching with corresponding specimens and stored in a locked filing cabinet which was only accessible by relevant investigators. The procedures involved in this study were in compliance with the ethical standards of human experimentation as described in the declaration of Helsinki, 1975. Ethical approval was obtained from the University Human Subjects Ethics Sub-committee. Each participant in all three samplings was given a brief introduction of the study and signed a written consent.

Table 7. Validity of the questionnaire used in this study.

		Expert Rater no. 2		
		Rated 1 or 2[a]	Rated 3 or 4[b]	Total
Expert Rater no. 1	Rated 1 or 2[a]	0	1	1
	Rated 3 or 4[b]	0	19	19
	Total	0	20	20
		S-CVI[c] = 19/20 = 0.95		

[a]Ratings of 1, 2 = irrelevant, neutral

[b] Ratings of 3, 4 = relevant, very relevant

[c]S-CVI = score for content validity index

Table 8. Distribution of subjects and sites sampled.

Type of workplace	Number of food handlers recruited (number of establishments sampled)		
	2002	2003	2011
College canteen	80 (5)	80 (5)	93 (2)
Centralized kitchen for hospitals[c]	103 (2)	58 (1)	40 (1)
Centralized kitchen for sports facility[c]	130 (1)	125 (1)	98 (1)
Others	306 (7)[a]	264 (7)[a]	203 (2)[b]
Total	619 (15)	527 (14)	434 (6)

[a]Supermarkets

[b]Hotels

[c]These facilities were visited on all three occasions.

3.4.3 Collection of nasal swabs

Sterile transport swabs (Medical Wire and Equipment Co. Ltd., Corsham, Wilts, UK) which had been moistened with sterile normal saline before sampling, were gently inserted into one nostril of the subject and rotated against the nasal septum. This procedure was the same for 2002, 2003 and 2011. The squamous epithelium on the septum has been shown to be the main colonisation site in carriers (Cole et al, 2001). For consistency in sampling, all swabs were collected by the researcher or another trained member of the research team. Subjects were not asked to self collect in order to ensure the proper anatomical sites were swabbed. Specimens were processed in the laboratory within four hours. The flow of specimen processing is depicted in Figure 5.

3.4.4 Fingerprint impression

Mannitol Salt Agar (MSA) (Oxoid, Basingstoke, UK) plates were used to collect fingertip impression specimens for 2002, 2003 and 2011. The medium has a high salt content (7.5% sodium chloride) which suppresses most bacterial growth other than staphylococci. The presence of mannitol in the medium allows differentiation of mannitol-fermenting *S. aureus* from other staphylococci. Upon sampling, the lid was removed and five fingertips were pressed directly onto the agar under the guidance of the researcher. This aimed to identify hand contamination. Specimens were

collected from the dominant hand (Figure 7). The lid was then replaced and the agar plates delivered to the laboratory for processing within four hours.

Figure 7. Flowchart depicting specimens processing

Specimens: nasal swabs, direct fingertip imprint

↓

Enrichment in BHI with 5% NaCl (nasal swabs only)

↓

Cultivation on *Sa* Select (2011 nasal sample)

MSA (2002 and 2002 nasal samples, all hand imprint samples)

↓

Species identification

(Latex agglutination, detection of *femA* gene)

↓

Screening for phenotypic resistance to methicillin

(Disc diffusion using cefoxitin and oxacillin)

↓

SCC*mec* typing with *mecA* detection

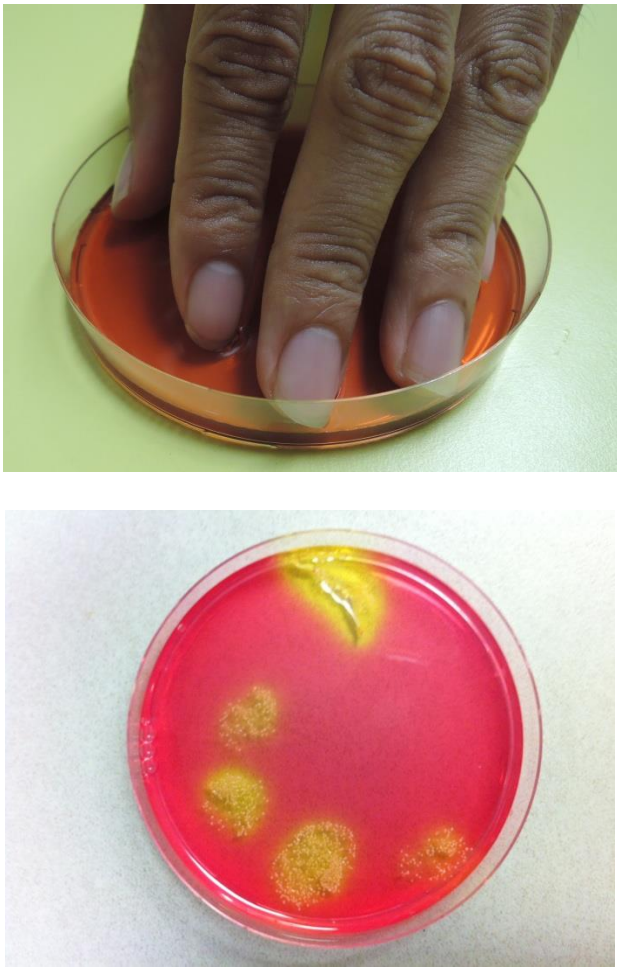
(For methicillin resistant strains only)

↓

Phenotypic and Genotypic characterisation

(Antibiotic resistance phenotypes, *spa* typing, SE/SEl genes, *qac* genes)

Figure 8. Direct fingerprint impressions on mannitol salt agar (MSA) (Upper) and MSA plates after 24 hour incubation at 37°C (Lower).



3.5 Bacterial Identification and Characterization

3.5.1 Isolation of *Staphylococcus aureus*

Nasal swabs were enriched in 10mL Brain Heart Infusion broth (BHI) (Oxoid) supplemented with 5% sodium chloride (NaCl) and incubated at 37^{o}C for 24 hours. The addition of sodium chloride was to increase salinity in order to enhance the growth of salt tolerant staphylococci and suppress the growth of gram negative bacteria (Crossley et al., 2009).

For samples collected in 2002 and 2003, 10µL inoculum was taken from the enriched broth using a standardized disposable inoculation loop and plated onto MSA agar (Oxoid) which was incubated at 37^{o}C for 24 hour in ambient air. For samples collected in 2011, 10uL inoculum was taken from the enriched broth by standardized disposable inoculation loop and plated onto an Sa *Select* (Bio-rad, Hemel Hempstead, UK) chromogenic agar plate which was incubated at 37^{o}C for 24 hour. This selective medium was introduced into commercial market in 2007 and contained specific substrates which detect phosphatase activity of *S. aureus.* Previous evaluation of this selective agar showed no non-specific coloration appeared after overnight incubation (Decoster & Dehecq, 2010). Pilot testing comparing *Sa* Select (Bio-rad) with Chromagar (Biomerieux) and Brilliance Staph 24 agar (Oxoid) showed that *Sa* Select had least non-specific growth.

From chromogenic Sa *Select* agar, pink to orange colonies indicative of *S. aureus* were taken for further biochemical analysis. MSA was used for hand imprint specimens collection as this is more selective against Gram negative organisms that may be contaminating the fingers. Yellow colonies were subsequently picked for further investigations. Pure colonies were obtained by successive subculture and stored in BHI with 20% glycerol in the -80C freezer.

Glove juice methods are the recommended standard according to the American Society for Testing and Materials (ASTM) standards. However, these were not employed in this study because of limited budget available for this project. In addition, the glove juice method was not used in 2002 and 2003 samples, which were initiated by other researchers. In order to mimimize variations that might otherwise be resulted from different methods employed, the same method “finger print” as for 2002 and 2003 collections was applied to 2011 survey. This allows direct comparison of carriage rates between 2002, 2003 and 2011. Once again, deep hand carriage assessment was not assessed because of minimal resource as it would require performance of glove juice methods twice for each subject.

3.5.2 Identification of *Staphylococcus aureus*

Bacterial identity was initially determined by phenotypic tests which included catalase reaction, Gram stain, DNase production and rapid latex agglutination (Staphaurex-Plus, Remel, USA) (Crossley et al., 2009).

The catalase reaction was performed by placing a drop of 3% hydrogen peroxide onto a glass slide followed by emulsifying one colony from pure overnight culture in it using a sterile wooden stick. The formation of bubbles indicated the presence of catalase which is a characteristic of staphylococci. Strains of *E.coli* and *S. aureus* were used as negative and positive controls respectively.

DNase production was detected by overnight incubation of the tested organism on DNase agar (Oxoid) followed by flooding the plate with 1N HCl. The appearance of a clear zone surrounding the colonies indicated the organism produced deoxyribonuclease which hydrolyzed DNA.

The latex agglutination test targeted fibrinogen binding protein and staphylococcal protein A which are present on the bacterial cell surface of *S. aureus.* The sensitivity and specificity were 99.4% and 95.5% respectively (Papasian & Garrison, 1999). Some other staphylococcal species such as *S. hyicus, S. intermedius* group*, S. lugdunensis* and *S. schleiferi* may give positive results. However, these species are rarely encountered in human isolates and could be ruled out by subsequent molecular characterisation.

Species identity was confirmed by amplifying the *fem*A gene, which encodes a 48kDa protein involved in cell wall metabolism and is present in all *S. aureus* strains (Methrotra et al., 2000). Isolates showing positive reactions were then stored in BHI supplemented with 20% glycerol in a freezer (-80^{o}C) for subsequent testing.

Positive (ATCC 25923 *S. aureus*) and negative (ATCC 12228 *S. epidermidis*) controls were included for DNase production, latex agglutination reaction and amplification of *femA* gene by PCR.

3.5.3 Antibiotic susceptibility testing

Disc diffusion testing was performed following the CLSI guidelines (2012) except for fusidic acid which was based on EUCAST clinical breakpoints v3.1 (2013).

A bacterial suspension was prepared using normal saline and adjusted to 0.5 MacFarland by measuring optical density at 625 nm using a light spectrophotometer. Absorbance in a range of 0.08 to 0.13 was considered as acceptable. The bacterial suspension was then swabbed evenly onto a Mueller-Hinton agar plate (20mL in 90mm petri dish for CLSI; 25mL in 90mm petri dish for EUCAST) which was poured on a level surface by Mediaclave (Integra-Biosciences, CH) to standardise its thickness. Drug impregnated discs were then placed on the plates which were subjected to incubation at 35^{o}C for 24 hours. The zone sizes were measured using the Mastascan Elite (Mast Group Ltd., Bootle, UK) which was monitored during use to ensure correct optical estimation of the zone sizes.

For strains displaying the phenotype of erythromycin sensitive and clindamycin resistant, a D-test was performed by placing erythromycin disc and clindamycin disc 15mm apart on MHA. Inducible clindamycin resistance is confirmed by the presence of a “D” zone surrounding the clindamycin disc.

ATCC 25923 *S. aureus* was used as quality control measure to ensure the potency of the antibiotic impregnated discs. This organism should be sensitive to all antibiotics tested. Antibiotics tested in this study are listed in Table 9.

Table 9. Antibiotics tested in this study.

Antibiotic	Disc Content	Zone diameter (mm)		
		S	I	R
Cefoxitin[a]	30µg	≥ 22	–	≤ 21
Chloramphenicol[a]	30µg	≥ 18	13 – 17	≤ 12
Ciprofloxacin[a]	5µg	≥ 21	16 – 20	≤ 15
Clindamycin[a]	2µg	≥ 21	15 – 20	≤ 14
Erythromycin[a]	15µg	≥ 23	14 – 22	≤ 13
Fusidic Acid[b]	10µg	≥ 24	-	≤ 23
Gentamicin[a]	10µg	≥ 15	13 – 14	≤ 12
Imipenem[a]	10µg	≥ 16	14 – 15	≤ 13
Linezolid[a]	30µg	≥ 21	-	≤ 20
Oxacillin[a]	1µg	≥ 13	11 – 12	≤ 10
Penicillin G[a]	10U	≥ 29	-	≤ 28
Quinupristin-dalfopristin[a]	15µg	≥ 19	16 – 18	≤ 15
Rifampicin[a]	5µg	≥ 20	17 – 19	≤ 16
Tetracycline[a]	30µg	≥ 19	15 – 18	≤ 14
Trimethoprim-sulfamethoxazole[a]	25µg (1.25/23.75µg)	≥ 16	11 – 15	≤ 10

[a]CLSI 2012

[b]EUCAST v3.1

3.5.4 Detection of methicillin resistance in *S. aureus*

Phenotypic resistance to methicillin was determined by testing susceptibility to cefoxitin (30µg) and oxacillin (1µg) using standard disc diffusion according to guidelines provided by Clinical and Laboratory Standards Institute (CLSI, 2012). Use of cefoxitin and oxacillin simultaneously increased sensitivity of methicillin resistance detection (Jain et al., 2008). The testing procedure was performed as described in 3.5.3.

Isolates with zone sizes equal or smaller than 10mm surrounding the oxacillin (1ug) disc and/or equal or smaller than 21mm surrounding the cefoxitin (30ug) disc were considered as phenotypically resistant to methicillin (CLSI, 2012). DNA was extracted from these isolates according to procedures described below in 3.5.5 to detect the presence of the *mec*A gene encoding methicillin resistance by PCR. The *mec*A gene was simultaneously amplified in a multiplex PCR targeting *ccr* genes (Multiplex 1) as described by Kondo et al (2007). Multiplex 1 and 2 were performed to identify *ccr* gene (type 1 to 5) and *mec* complex (class A to C) respectively. TaKaRa *Ex Taq* hot start version (TaKaRa, Tokyo, Japan) was used in order to avoid non-specific amplification which might otherwise result from non-specific binding before a thermal cycle begins. According to the supplier information, 10X *Ex Taq* buffer contained 20mM $MgCl_2$ which was therefore not added separately. Preparation of the reaction mixture is described in Table 10. After the PCR reaction, amplicons were visualized by ethidium bromide stain which was incorporated in a 1.5% agarose gel

(First Base, Kuala Lumpur, Malaysia). Corresponding positive controls were included in each PCR run: NRS100 (SCC*mec* I, *ccr*1, class A *mec*), NRS2 (SCC*mec* II, *ccr*2, class A *mec*), NRS65 (SCC*mec* III, *ccr*3, class A *mec*), NRS123 (SCC*mec* IV, *ccr*2, class B *mec*), ATCC 2094 (SCC*mec* V, *ccr*5, class C *mec*). Double distilled water was used in place of DNA template for the negative control.

Table 10. PCR parameters for SCC*mec* typing.

PCR mastermix (Multiplex 1)			
Component	Working concentration	Volume (µL)	Final Concentration
Ex Taq Buffer	10X	5	1X
dNTPs	2mM	5	0.2mM
mA1	10µM	0.5	0.1µM
mA2	10µM	0.5	0.1µM
α1	10µM	0.5	0.1µM
α2	10µM	0.5	0.1µM
α3	10µM	0.5	0.1µM
βC	10µM	0.5	0.1µM
α4.2	10µM	0.5	0.1µM
β4.2	10µM	0.5	0.1µM
γF	10µM	0.5	0.1µM
γR	10µM	0.5	0.1µM
Ex Taq	5U/µL	0.5	2.5U
ddH_2O	-	32.5	-
DNA	10ng/µL	2	0.4ng/µL

PCR conditions (Multiplex 1)		
No. of cycles	Temperature ^oC	Duration
1	94	2 min
30	94	2 min
	57	1 min
	72	2 min
1	72	2 min

Table 10. Continued.

PCR mastermix (Multiplex 2)			
Component	Working concentration	Volume (µL)	Final Concentration
Ex Taq Buffer	10X	5	1X
dNTPs	2mM	5	0.2mM
mI6	10µM	0.5	0.1µM
IS7	10µM	0.5	0.1µM
IS2	10µM	0.5	0.1µM
mA7	10µM	0.5	0.1µM
Ex Taq	5U/µL	0.5	2.5U
ddH_2O	-	35.5	-
DNA	10ng/µL	2	0.4 ng/µL

PCR conditions (Multiplex 2)		
No. of cycles	Temperature ^oC	Duration
1	94	2 min
30	94	2 min
	60	1 min
	72	2 min
1	72	2 min

Table 10. Continued.

Nucleotide sequences of primers used in SCC*mec* typing	
Designation	5’ → 3’ sequence
mA1	TGCTATCCACCCTCAAACAGG
mA2	AACGTTGTAACCACCCCAAGA
α1	AACCATATCATCAATCAGTACGT
α2	TAAAGGCATCATGCACAAACACT
α3	AGCTCAAAAGCAAGCAATAGAAT
βC	ATTGCCTTGATAATAGCCITCT
α4.2	GTATCAATGCACCAGAACTT
β4.2	TTGCGACTCTCTTGGCGTTT
γF	CGTTATTACAAGATGTTAAGGATAAT
γR	CCTTTATAGACTGGATTATTCAAAATAT
mI6	CATAACTTCCCATTCTGCAGATG
IS7	ATGCTTAATGATAGCATCCGAATG
IS2	TGAGGTTATTCAGATATTTCGATGT
mA7	ATATACCAAACCCGACAACTACA

Kondo et al., 2007

3.5.5 Extraction and purification of total genomic DNA

Total genomic DNA was extracted by inoculating bacterial colonies into 400µL lysis buffer (Appendix 1) to give a turbidity equivalent to a 2.0 McFarland standard by visual comparison with a standard suspension. The mixture was incubated with agitation for 40 minutes at 37^{o}C. Lysate was then subjected to 10 minutes boiling followed by 10 minute chilling on ice. The solution was centrifuged at 13,000 *x g* for 5 minutes and supernatant transferred to a new Eppendorf tube for purification.

One mL aliquot of chilled phenol-chloroform-isoamyl alcohol (25:24:1) (USB, Cleveland, OH, USA) was added to the tube containing DNA extract. The solution was mixed thoroughly by inversion and centrifuged at 11,000 rpm for 20 minutes. The aqueous layer was recovered into a new Eppendorf tube in which purification using phenol-chloroform-isoamyl alcohol was repeated again as described above. After centrifugation, 1mL absolute ethanol was added to precipitate DNA. Samples were kept in the - 20 ^{o}C freezer for 25 minutes and DNA pelleted by centrifugation at 10,000g for 20 minutes. The supernatant was discarded and pellets air-dried in a biosafety cabinet for 30 minutes. The pellet was resuspended in 50 µL Mili-Q water and its purity determined by obtaining the ratio of absorbance at wavelength 260nm to that at 280nm using Nano-Drop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA extracts with a single peak at 260nm that fell into the ratio of 1.8 to 2.0 were considered as pure and kept in -20^{o}C freezer for use in subsequent PCR reactions.

3.5.6 Detection of genes encoding staphylococcal enterotoxins and SE-like proteins

Genes encoding staphylococccal enterotoxins (*sea* - *set*) and enterotoxin-like exoprotein (*selu*) were individually detected by PCR. All PCR reactions were performed using GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). PCR reagents [Five-fold concentrated polymerase synthesis buffer; 25mM magnesium chloride ($MgCl_2$); deoxynucleotriphosphates (dNTPs); Thermostable DNA polymerase (Taq)] were purchased from Promega Corporation (Madison, WI, USA) and primers synthesized by Invitrogen Hong Kong Limited. Bacterial strains harbouring respective genes were obtained either from the Network on Antimicrobial Resistance in *S. aureus* (NARSA, USA) or kindly provided by Prof K Hiramatsu, Juntendo University (Japan), and used as positive controls. All PCR reactions were performed in a 25µl mixture in which 1µl total genomic DNA template (50ng/µL) was used. Details on PCR primers and running conditions are described in Table 11.

Table 11. PCR parameters for detection of SE or SEl genes.

PCR mastermix			
Component	Working concentration	Volume (µL)	Final Concentration
Buffer	5X	5	1X
$MgCl_2$	25mM	1.5	1.5mM
dNTPs	2mM	2.5	0.2mM
Primer Fw	10µM	1	0.4µM
Primer Rv	10µM	1	0.4µM
Taq Polymerase	5U/µL	0.2	1U
ddH_2O	-	12.8	-
DNA	50ng/µL	1	2ng/µL

According to manufacturer's instruction (Promega reaction buffer, M791A).

Table 11. Continued.

Target	Primer sequence (5’ – 3’)	Target	Primer sequence (5’ – 3’)
sea	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	*sei*	CAACTCGAATTTTCAACAGGTAC CAGGCAGTCCATCTCCTG
seb	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	*sej*	CATCAGAACTGTTGTTCCGCTAG CTGAATTTTACCATCAAAGGTAC
sec	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	*sek*	CACAGCTACTAACGAATATC TGGAATTTCTCAGACTCTAC
sed	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	*sel*	CATACAGTCTTATCTAACGG TTTTCTGCTTTAGTAACACC
see	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	*sem*	CTTGTCCTGTTCCAGTATC ATACGGTGGAGTTACATTAG
seg	CGTCTCCACCTGTTGAAGG CCAAGTGATTGTCTATTGTCG	*sen*	CTTCTTGTTGGACACCATCTT GAAATAAATGTGTAGGCTT
seh	CAACTGCTGATTTAGCTCAG GTCGAATGAGTAATCTCTAGG	*seo*	AAATTCAGCAGATATTCCAT TTTGTGTAAGAAGTCAAGTGTAG

Table 11. Continued.

Target	Primer sequence (5’ – 3’)	Target	Primer sequence (5’ – 3’)
sep	ATCATAACCAACCGAATCAC	*ses*	TTCAGAAATAGCCAATCATTTCAA
	AGAAGTAACTGTTCAGGAGCTA		CCTTTTTGTTGAGAGCCGTC
seq	TCAGGTCTTTGTAATACAAAA	*set*	GGTGATTATGTAGATGCTTGGG
	TCTGCTTGACCAGTTCCGGT		TCGGGTGTTACTTCTGTTTGC
ser	AGATGTGTTTGGAATACCCTAT	*selu*	ATTTGCTTTTATCTTCAT
	CTATCAGCTGTGGAGTGCAT		GGACTTTAATGTTTGTTTCTGAT

References:

Monday et al., 1999 (*seg – j*),

Mehrotra et al., 2000 (*sea – e*),

Chiang et al., 2006 (*sek – m*),

Chiang et al., 2008 (*sen – r* and *selu*),

Ono et al., 2008 (*ses* and *set*).

Table 12. Positive control strains used for detection of SE/ SEl genes.

Strain	Source	Positive for SE/SEl genes
NRS123	NARSA[a]	*seo, seq*
Fukuoka 5	Juntendo[b]	*sej, ser, ses, set*
FRI137	Juntendo	*sec, seg, seh, sei, sel, sen, selu*
NRS111	NARSA	*sea, see*
NRS70	NARSA	*sem*
NRS158	NARSA	*seb*
ATCC6538	ATCC[c]	*sek*
NRS382	NARSA	*sed*
NRS2	NARSA	*sep*

[a]NARSA: Network on antimicrobial resistance in *Staphylococcus aureus.*

[b]Juntendo: Juntendo University, Japan.

[c]ATCC: American type culture collection.

3.5.7 Typing of staphylococcal protein A polymorphic X region (*spa*X)

The *spa* gene encodes IgG Fc-binding staphylococcal protein A which is specific for *S. aureus.* The polymorphic X region of *spa* gene comprises of a variable number of 24 bp repeats which allows the identification of clonal spread (Lindsay et al., 2013). Though *spa* gene is present in *S. pseudintermedius*, this homolog is not amplifiable with primers targeting *spa* genes from *S. aureus* (Moodley et al., 2009). The polymorphic X region of *spa* gene was amplified by conventional PCR using the conditions described in Table 13. PCR amplicons with visible bands in 1.5% agarose gel were diluted to 7ng/µL and sent to the Centre for Genomic Sciences, The University of Hong Kong for sequencing. The nucleotide sequence was then compared with published repeats in the Ridom database (http://spa.ridom.de/repeats.shtml). Each repeat pattern was then matched with described *spa* types (http://spa.ridom.de/spatypes.shtml).

Table 13. PCR parameters for amplification of *spa* gene.

PCR Mastermix			
Component	Working concentration	Volume (µL)	Final Concentration
Buffer	5X	10	1X
$MgCl_2$	25mM	3	1.5mM
dNTPs	2mM	5	0.2mM
spa Fw	10µM	1	0.2µM
spa Rv	10µM	1	0.2µM
Taq Polymerase	5U/µL	0.3	1.5U
ddH_2O	-	28.7	-
DNA	50ng/µL	1	1ng/µL

PCR conditions		
No. of cycles	Temperature ^oC	Duration
1	80	5 min
35	94	45 sec
	60	45 sec
	72	90 sec
1	72	10 min

Nucleotide sequence for primers in *spa* gene amplification	
Designation	5’ → 3’ sequence
spa Fw	TAAAGACGATCCTTCGGTGAGC
spa Rv	CAGCAGTAGTGCCGTTTGCTT

Harmsen et al, 2003.

3.5.7.1 Clustering of *spa* types

Isolates with (1) number of *spa* repeat equals or longer than five and (2) cost distance less than or equals four were grouped into the same *spa* clonal complex (*spa*-CC) by visual analyses of repeat organisation according to the algorithms previously described as having identical order of small sequence repeat units with variations as deletion, duplication or single nucleotide polymorphism (Mellmann et al., 2007; Ruppitsch et al., 2006). Clustering was performed by firstly weighting the beginning and the last repeats and each gap or mismatch between these was scored one point. The total score represents the cost distance (Figure 9). Clustering of *spa* types into complexes avoids false discrimination of two related strains as unrelated due to the inherently frequent recombination of the *spa* loci. Manually grouped *spa* types have high concordance (95.7%) with automated grouping using Ridom software (Mellmann et al., 2007).

Figure 9. Example illustrating *spa* type clustering.

spa type	Repeat succession							
t189	r07	r23	r12	r21	r24	r33	r22	r17
t213	r07	r23	r12	r21	r17	-	-	r34
Cost distance = 3	B	0	0	0	1	1	1	E

B and E stands for beginning and ending repeats, respectively.

3.5.7.2 Calculation of molecular clock and Simpson's index of diversity (SID)

Of the isolates from participants who were nasally positive for *S. aureus* on two occasions (in 2002 and 2003), the molecular clock of the *spa* loci was calculated as the number of mutations divided by time in months (Blumental et al., 2013). This was to differentiate subjects harbouring different strains from those carrying isolates with point mutations in the *spa* loci.

Simpson's index of diversity (SID) was calculated according to the following formulae to compare the diversity of *spa* types detected in different populations at different times. The index represents a ratio of the total number of all *spa* types to the total number of strains collected in a sample population previously described (van Belkum et al., 2007a). The total number of strains collected in a sample is denoted by *N.* The total number of *spa* types detected in a sample is designated as *S.* The number of strains belonging to *j*th type is represented by n_j .

$$\text{SID} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$$

3.5.8 Plasmid extraction for detection of antiseptic resistance genes

A single colony was inoculated into 10mL BHI which was incubated overnight in shaking incubator at 37°C. 1.5mL of this overnight culture was transferred to an Eppendorf tube and centrifuged at 760 x g at 4 °C for 10 minutes. The supernatant was discarded and 400µL lysis buffer as described in section 3.4.4 was added. The

suspension was incubated at 37^{o}C for one hour with agitation. Following this, 300 µL TENS solution was added and mixed by slowing inverting the tubes 10 times followed by adding 150µL potassium acetate (KoAc) and mixed immediately again by slowly inverting the tubes 10 times. Preparation of reagents is described in Table 2.8. Samples were then kept on ice for 20 minutes and centrifuged at 3,500 x g for 20 minutes at 4^{o}C. The supernatant was transferred to a new Eppendorf tube to which 1mL 100% ethanol freshly taken from the – 20^{o}C freezer was added. It was mixed by inverting 14 times and kept on ice for one hour. The mixture was then centrifuged at 3,500 x g for 5 minutes at 4 ^{o}C and the supernatant discarded. To each sample, 1 mL of 70% ethanol was added without mixing followed by centrifugation for 10 minutes at 3,500 x g and discarding of the supernatant. Samples were air dried at room temperature for at least one hour and then suspended in 50 µL Mili-Q water. The concentration of DNA was measured by Nano-Drop 2000 Spectrophotometer as described in section 3.4.4. Plasmid extracts were stored in the – 20^{o}C freezer for subsequent PCR reactions.

3.5.9 Detection of genes encoding reduced susceptibility to quaternary ammonium compounds

Genes encoding reduced susceptibility to quaternary ammonium compounds (*qacA/B, smr, qacG, qacH, qacJ*) were individually amplified by PCR. These reactions were performed using the plasmid DNA extract. Due to the significant homology of the sequences of *qacA* and *qacB*, sequencing was performed to differentiate *qacA* from *qacB.* Type strains TS77 and L20 (Kindly provided by Professor K. Hiramatsu, Juntendo University, Japan) were used as positive controls for *qac*A/B and *smr* respectively (Noguchi, 2005). Respective control strains for *qac*G, *qac*H and *qac*J were kindly provided by Dr J. Bjorland from Norwegian School of Veterinary Science. These control strains were clones of respective genes transformed into RN4220 *S. aureus* recipient cells using staphylococcal vector pSK265 (Bjorland et al., 2003; Heir et al., 1998; Heir et al., 1999b). Details regarding PCR reaction setup are provided in Table 14.

Table 14. PCR parameters for detection of *qac* genes.

PCR mastermix			
Component	Working concentration	Volume (µL)	Final Concentration
Buffer	5X	5	1X
$MgCl_2$	25mM	1.5	1.5mM
dNTPs	2mM	2.5	0.2mM
Primer Fw	10µM	1	0.4µM
Primer Rv	10µM	1	0.4µM
Taq Polymerase	5U/µL	0.2	1U
ddH_2O	-	12.8	-
DNA	50ng/µL	1	2ng/µL

PCR conditions				
		Temperature (^oC) x Time (second)		
Target	No. of cycle	Denaturation	Annealing	Extension
qacA/B	30	95 x 30	53 x 30	72 x 60
smr	20	94 x 20	53 x 20	72 x 20
qacG	20	95 x 60	48 x 60	72 x 60
qacH	30	95 x 30	55 x 30	72 x 60
qacJ	20	95 x 60	48 x 60	72 x 60

All reactions were initiated with 96^{o}C for 3 mins and ended with 72^{o}C for 5 mins

Table 14. Continued.

Nucleotide sequence of primers used in PCR detection of *qac* genes		
Designation	5’ → 3’ sequence	Reference
qacA/B Fw	GCAGAAAGTGCAGAGTTCG	Noguchi et al., 2006
qacA/B Rv	CCAGTCCAATCATGCCTG	
smr Fw	GCCATAAGTACTGAAGTTATTGGA	Noguchi et al., 2006
smr Rv	GACTACGGTTGTTAAGACTAAACCT	
qacG Fw	CAACAGAAATAATCGGAACT	Bjorland et al., 2005
qacG Rv	TACATTTAAGAGCACTACA	
qacH Fw	CAATAGTCAGTGAAGTAATAGGCAGTG	Bjorland et al., 2005
qacH Rv	TGTGATGATCCGAATGTGTTT	
qacJ Fw	CTTATATTTAGTAATAGCG	Bjorland et al., 2005
qacJ Rv	GATCCAAAAACGTTAAGA	

3.5.10 Phenotypic detection of reduced susceptibility to QAC compounds

All isolates harbouring *qac* genes were tested for phenotypic susceptibility to chlorhexidine (CHX) and benzylkonium chloride (BC) which were purchased from Sigma-Aldrich Ltd. (USA). The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by macro-dilution method. The testing concentration ranged from 0.5 to 128 mg/L.

Isolates were sub-cultured onto tryptic soy agar (TSA) and incubated at 37^{o}C overnight. For each sample, ten sterile bijou bottles were needed. Firstly, 2 mL of Mueller Hinton broth (MHB) was transferred into the first bottle and 1mL each for the remaining nine bottles. Secondly, 10mL MHB was transferred to a sterile universal bottle. This was for subsequent bacterial suspension preparation.

To prepare stock solution for BC, 50mg BC was dissolved in 1mL sterile water in a sterile Eppendorf tube. This would give a final concentration of 50µg/µL. A volume of 10.24 µL (512/50) was added to the first bottle with 2mL MHB, giving final concentration of 256mg/L. This was then diluted in a series of two-fold dilution. The MHB with BC was not added to the last tube which was used as a positive control. For each batch of MIC testing, a negative control was included using 1mL of MHB. This was done to check if there was cross-contamination which might have arisen from repetitive pipetting from the same MHB bottle.

To prepare stock solution for CHX, 250 µL of 20% chlorhexidine digluconate solution was added to 750 µL sterile water to give a final concentration of 50 µg/ µL. The remaining procedures were the same as for BC.

To prepare bacterial suspensions for testing, 4mL sterile NS was added into a sterile universal bottle. Colonies from fresh overnight culture were inoculated into the bottle. The suspension was then adjusted to 0.5 McFarland using light spectrophotometer. The acceptable range for absorbance was 0.08 – 0.13 at 625nm. To ensure the whole process from preparing bacterial suspension to commencement of incubation be completed in 15 minutes, two samples were handled at a time.

To prepare bacterial suspension- antiseptic containing medium mixture, 10 µL 0.5 McFarland bacterial suspension was added into 10mL sterile MHB. One millilitre of this bacterial suspension in MHB was added into each antiseptic containing broth. These were then subjected to incubation at 35^{o}C for 16 hours. The MIC of an isolate was the lowest concentration of antiseptic which gave invisible growth.

To determine MBC, 10 µL of overnight mixture without visible growth (Bacterial suspension + antiseptic containing MHB) was mixed with 90 µL neutralising broth (Dey-Engley broth, Sigma-Aldrich, USA) and held at room temperature for 10 minutes. This mixture was then spread onto nutrient agar plates which were subsequently incubated at 37^{0}C for 16 hours. The plate with the lowest

concentration showing no growth was considered as the MBC. All MIC and MIC testing was performed in duplicate (Zhang et al., 2011).

3.5.11 Analyses of PCR products

All PCR products were resolved in 1.5% agarose gel (1st Base) using a 1kb Plus ladder (Invitrogen Hong Kong Ltd, Tai Po Industrial Estate, HK) as a marker for comparison. PCR products for SE genes amplified by different pairs of primers were sequenced to confirm their identities. For *qacA/B* PCR products, the amplicons were sequenced using the *qacA/B* forward primer and sequence compared with Genebank database.

3.6 Statistical Analyses

Risk factors for *S. aureus* colonisation, including risks associated with harbouring *qac* and/or SE positive isolates, were initially identified by univariate analysis using Pearson's Chi-squared test. Variables with a *p*-value less than or equal to 0.1 were adjusted by backward logistic regression for confounding factors. In order to minimise type I error which might otherwise arise from multiple comparison, Bonferroni correction was applied such that variables with a *p*-value less than or equals to 0.003 were considered as statistically significant.

Linear-by-linear association was employed to determine the *p*-value for trends in odds ratios among stratified groups.

Association between categorical variables were investigated using the chi-square test whereas those with a count less than five were analysed by Fisher's exact test. Co-existence propensity between pheno- and genotypes was analysed by the chi-square test. Bonferroni's correction was applied whenever appropriate. Otherwise, a *p*-value ≤ 0.05 was considered as statistically significant. All statistical tests were performed using the Statistical Package for Social Sciences version 19.0 (SPSS, Chicago, IL, USA).

Chapter 4 Research Findings

In this chapter, the results will be presented in eight sections with respect to the previously defined research aims: to investigate the sustainability of the reduction in staphylococcal colonisation rates amongst food handlers in Hong Kong after the SARS epidemic and to characterise carriage isolates for genes encoding enterotoxins and reduced antiseptic susceptibility. Section 4.1 compares the baseline characteristics of the two populations sampled in 2002 and 2011. Section 4.2 demonstrates the sustainable reduction of colonisation rates of food handlers. Section 4.3 presents risk factors associated with nasal colonisation with *S. aureus* in food handlers. Sections 4.4 and 4.5 describe the presence of genetic determinants encoding enterotoxins and antiseptic tolerance and associated risks. Section 4.6 identifies the temporal clonal changes in *S. aureus* colonising these healthy workers over a ten year period. Section 4.7 discloses the route of staphylococcal transmission in food premises. Section 4.8 shows the prevalence of antibiotic resistance in carriage isolates.

4.1 Baseline characteristics.

The characteristics of the 2002 and 2011 sample populations were comparable in terms of age distribution, years of experience in the catering industry and the proportion of meat handlers. In 2002, the mean age (± SD) of the population was 41.4 ± 9.8 with an average experience of 4.3 ± 1.8 years. In 2011, the mean age was

42.6 ± 6.7 whereas the years of experience was 4.9 ± 1.5 years. The proportion of meat handlers ranged from 57% to 67%. Though there were more males and smokers in the 2011 sample, further analysis revealed that these were associated with each other ($p< 0.001$). The baseline characteristics of the two populations were compared by Pearson's chi-squared test. Results are shown in Table 15.

Table 15. Baseline characteristics of the sample population 2002 and 2011.

	Percentage of subjects		*p-value*[a]
	2002	2011	
Gender			
Male	50.5	66.4	0.03
Female	49.5	33.6	
Age			
≤40	38	43.5	0.47
>40	62	56.5	
Smoking			
Yes	8.9	30.4	< 0.001
No	91.1	69.6	
Years of experience			
≤ 5 year	54	44	0.21
> 6 years	46	56	
Handling raw meat			
Yes	67	57	0.19
No	33	43	

[a] Pearson chi squared test.

4.2 Prevalence of nasal colonisation and hand contamination with *S. aureus.*

A total of 217 (35.1%) subjects were nasally colonised in 2002, 124 (23.5%) in 2003 and 99 (22.9%) in 2011. The nasal colonisation rates on the second and third occasions were significantly lower than on the first occasion ($p< 0.001$). Hand contamination was detected in 255 (41.2%) subjects on the first occasion but was significantly reduced to 61 (11.6%) on the second with a further significant reduction to 16 (3.7%) on the third. Over the three sample periods, a significant trend of decrease was noted for both the nasal colonisation rates ($\chi^2= 21.3$, df= 1, $P_{trend}< 0.001$) and hand carriage rates ($\chi^2= 234$, df= 1, $P_{trend}< 0.001$) (Table 16). Following further stratification by workplace type, the decreasing trend remained significant except for the nasal carriage rate observed among food handlers working in the centralised kitchen for hospitals, which was approaching statistical significance (Table 16).

Although there were considerable differences in the nature of establishment sampled, no significant variation in the rates of nasal carriage between staff working in the different catering facilities sampled in 2002 ($p= 0.31$), 2003 ($p= 0.76$) or 2011 ($p= 0.86$) was detected (Table 16). Further analyses showed that the reduction of nasal carriage rate from 2002 to 2003 was greater in establishments other than supermarkets. The nasal colonisation rate amongst supermarket workers reduced by only 5.2% whereas an overall 19.9% decrease of nasal carriage rate was observed in workers from other establishments ($p= 0.002$) (Table 17).

In 2002, the hand carriage rates of seven establishments (AS, CS, FS, FW, HF, HS, SS) were above the overall mean hand carriage rate. In 2003, all establishments with hand carriage rates above the mean were supermarkets. The 25% hand carriage rate reduction observed amongst supermarket workers was significantly lower than 56% amongst food handlers from other establishments ($p< 0.001$) (Table 17).

In the case of the reference establishment PY, the first two samples were collected before the SARS epidemic i.e. before the implementation of hygiene measures in local food premises. The nasal colonisation rates in PY remained stable over the two periods whereas the hand carriage rates varied from 15.5% (2002) to 17.8% (2003) (Table 17).

Food workers from the establishment JC were sampled on three occasions. Both the nasal and hand carriage rates decreased significantly over the two periods: from 40% (2002) to 26.4% (2003) and further to 24.5% in 2011. The hand contamination rates reduced from 27.7% (2002) to 8% (2003) and 3.1% (2011) (Table 18).

Plotting the reduction of hand contamination rates against nasal colonisation rates did not identify any statistical correlation (Spearman correlation coefficient= 0.25, p= 0.39) (Figure 10). Non-parametric Spearman correlation was used for analysis because the data was not normally distributed.

Table 16. Temporal changes of nasal and hand carriage rates by workplace.

Site		Number of carriers (%)			χ^2	P_{trend}[a]
		2002	2003	2011		
Canteens	Nasal	31/80 (38.8)	16/80 (20)	19/93 (20.4)	7.1	**< 0.001**
	Hand	60/80 (75)	3/80 (3.8)	2/93 (2.2)	113.9	**< 0.001**
Hospital	Nasal	39/103 (37.9)	9/58 (15.5)	11/40 (27.5)	13.8	0.06
	Hand	28/103 (27.2)	3/58 (5.2)	6/40 (15)	6.6	**0.01**
Sport facility	Nasal	52/130 (40)	33/125 (26.4)	24/98 (24.5)	6.8	**0.009**
	Hand	36/130 (27.7)	10/125 (8)	3/98 (3.1)	30.2	**< 0.001**
Others[b]	Nasal	95/306 (31)	66/264 (25)	45/203 (22.5)	5.2	**0.02**
	Hand	131/306 (42.8)	45/264 (17.1)	5/203 (2.5)	116.2	**< 0.001**
Overall	Nasal	217/619 (35)	124/527 (23.5)	99/434 (22.9)	21.3	**< 0.001**
	Hand	255/619 (41.2)	61/527 (11.6)	16/434 (3.7)	234	**< 0.001**
		p= 0.31[c]	*p*= 0.76[c]	*p*= 0.86[c]		

[a] Chi-squared test for trend, df=1.

[b] Refers to supermarkets and hotels.

[c] Comparison of prevalence between establishments. Bold face indicates statistical significance.

Table 17. *S. aureus* carriage rates of food handlers by establishment (2002/2003).

Site	Nasal carriage Percentage of positive (N)			Hand carriage Percentage of positive (N)		
	2002	2003	% change[c]	2002	2003	% change[c]
Overall	35.7± 10.7 [a]	23.5± 11.8	-12.2	41.2± 54.3	11.6± 16.8	- 29.6
	p= 0.31[d]	*p*= 0.76[d]		***p*< 0.001[d]**	*p*= 0.045[d]	
			Supermarkets			
FL	23.5 (34)	30.8 (26)	+7.3	29.4 (34)	26.9 (26)	-2.5
FW	27.4 (62)	29.6 (54)	+2.2	69.4 (62)	11.1 (54)	-58.3
HF	33.3 (30)	31.5 (28)	-1.8	66.7 (30)	21.4 (28)	-45.3
KO	37.3 (67)	21.2 (66)	-17.6	34.3 (67)	19.7 (66)	-14.6
LP	27.5 (51)	13.9 (43)	-13.6	29.4 (51)	16.3 (43)	-13.1
MO	30.3 (33)	25.0 (24)	-5.3	9.1 (33)	12.5 (24)	+3.4
SS	37.9 (29)	30.4 (23)	-7.5	58.6 (29)	13.0 (23)	-45.6
	$\bar{x}$ = 31.03	$\bar{x}$ = 26.06	$\bar{x}$ = -5.19	$\bar{x}$ = 42.41	$\bar{x}$ = 17.34	$\bar{x}$ = -25.14
	SD = 5.39	SD = 6.53	SD = 8.68	SD = 22.71	SD = 5.29	SD = 24.18
			Other establishments			
AS	57.1 (7)	28.6 (7)	-28.5	100 (7)	0 (7)	-100
CS	32.5 (40)	17.5 (40)	-15.0	75.0 (40)	5 (40)	-70.0
FS	55.6 (18)	22.2 (18)	-33.4	77.8 (18)	5.6 (18)	-72.2
HS	20.0 (10)	20.0 (10)	0	80.0 (10)	0 (10)	-80.0
KS	40.0 (5)	20.0 (5)	-20.0	20.0 (5)	0 (5)	-20.0
NH	44.8 (58)	15.5 (58)	-29.3	36.2 (58)	5.2 (58)	-31.0

Table 17. Continued.

JC	40.0 (130)	26.4 (125)	-13.6	27.7 (130)	8.0 (125)	-19.7
	$\bar{x}$ = 41.42	$\bar{x}$ = 21.45	$\bar{x}$ = - 19.97	$\bar{x}$ = 59.53	$\bar{x}$ = 3.4	$\bar{x}$ = - 56.13
	p= 0.22[e]	*p*= 0.56[e]	***p*= 0.002**[e]	*p*= 0.08[e]	***p*= 0.003**[e]	***p*< 0.001**[e]
			Reference establishment[b]			
PY	28.9 (45)	28.9 (45)	0	15.5 (45)	17.8 (45)	+2.3

[a]The overall prevalence rate is expressed as mean prevalence ± 2SD.

[b]Excluded in the comparison of change in prevalence of carriage investigation as the second sample was collected from this establishment before the SARS epidemic.

[c]Percentage of change was calculated by subtracting the carriage rate of 2002 from that of 2003.

[d]Comparison of carriage rates across different establishments by Fishers' exact test.

[e]Comparison of carriage rates or rate reduction between supermarkets and other establishments by Pearson Chi-squared test. Bold face indicates statistical significance.

Table 18. Carriage rates of food handlers in the establishment sampled on three occasions.

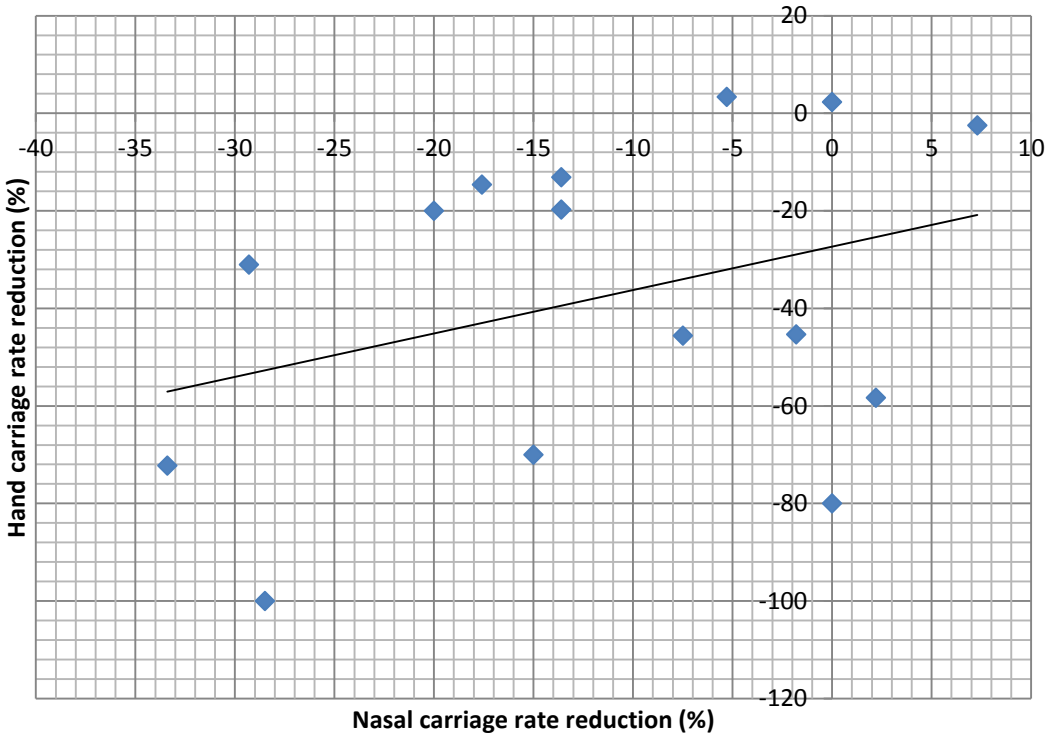
Site	Carriage	Percentage of positive (number of subjects sampled)			P_{trend}[a]
		2002	2003	2011	
JC	Nares	40.0 (130)	26.4 (125)	24.5 (98)	**0.009**[b]
	Hands	27.7 (130)	8 (125)	3.1 (98)	**0.007**[c]

[a] Comparison of carriage rates across time periods using Chi-squared test for trend. Statistical significance was set at $p \leq 0.05$.

[b] $\chi^2 = 6.8$, df=1.

[c] $\chi^2 = 11.4$, df=1.

Figure 10. Correlation of nasal and hand carriage rate reduction.



Spearman correlation r = 0.25, *p*= 0.39

4.3 Risk factors for nasal colonisation of food handlers with *S. aureus.*

For the 2002 sample, univariate analyses revealed handling cooked meat as a risk factor for nasal colonisation (p= 0.044, OR 1.42, 95% CI 1.01 – 1.99) whilst contact with raw meat almost reached significance (p= 0.062, OR 1.38, 95% CI 0.98 – 1.94) (Table 19). Of the total 151 colonised workers handling meat, 82 (54%) were in contact with both cooked and raw.

Risk factors approaching significance (age, gender, handling cooked meat and handling raw meat) were further analyzed using multiple logistic regression. After adjustment, age and handling cooked meat appeared to be significant risk factors for nasal colonisation (Table 20).

For the 2011 sample, univariate analyses revealed that raw meat handling (OR=2.7, 95% CI: 1.7 – 4.5) and male gender (OR=1.6, 95% CI: 1.1 – 2.3) were associated with increased risk for nasal colonisation (Table 21). Factors including gender, raw meat handing, hand washing after handling raw meat, recent antibiotic use and acne were further analysed by backward logistic regression which showed raw meat handling as the only independent risk factor for colonisation (Table 22). Trend analysis demonstrated a positive relationship between frequency of raw meat handling (never, sometimes, always) and colonisation status ($P_{trend} < 0.001$). "Always" was defined as more than three days per week while "sometimes" referred to one to two days per week. Compared to non-exposed workers, food handlers who sometimes

handled raw meat had a two-fold higher risk for colonisation while those always exposed had a nearly four-fold increased risk for colonisation (Table 23).

Table 19. Univariate analyses of risk factors for nasal carriage with *S. aureus* in 2002.

Variable	Number of subjects (%)		*p* value	χ^2	OR
	Carriers	Non-carriers			(95% CI)
Gender					
Male	95 (38.9)	149 (61.1)	0.08	2.8	1.36
Female	110 (31.9)	235 (68.1)			(0.95 – 1.95)
Smoking					
Yes	18 (34.6)	34 (65.4)	0.58	0.2	1.19
No	81 (30.7)	183 (69.3)			(0.61 – 2.34)
Age					
≤ 40	39 (41.2)	46 (58.8)	0.08	11.4	1.84
> 40	60 (43.2)	79 (56.8)			(0.98 – 2.63)
Years of experience as a food handler					
≤ 1 year	18 (40.0)	27 (60)	0.184	4.84	-
2 – 3 years	52 (28.7)	129 (71.3)			
4 – 5 years	32 (34.0)	62 (66)			
≥ 6 years	103 (38.2)	167 (61.8)			
Handled raw meat regularly at work					
Yes	114 (38.4)	183 (61.6)	0.062	3.2	1.38
No	91 (31.1)	202 (68.9)			(0.98 – 1.94)
Handled cooked meat					
Yes	119 (38.5)	190 (61.5)	**0.044**[a]	3.7	1.42
No	86 (30.6)	195 (69.4)			(1.01 – 1.99)
Handled vegetables					
Yes	109 (36.7)	188 (63.3)	0.33	0.79	1.18
No	96 (32.9)	196 (67.1)			(0.83 – 1.69)

Table 19. Continued.

Variable	Number of subjects (%)		*p* value	χ^2	OR (95% CI)
	Carriers	Non-carriers			
Handled dairy or creamy desserts					
Yes	41 (36.6)	71 (63.4)	0.67	0.12	1.11
No	164 (34.3)	314 (65.7)			(0.70 – 1.73)
Handled bakery					
Yes	64 (37.2)	108 (62.8)	0.42	0.48	1.16
No	141 (33.7)	277 (66.3)			(0.79 – 1.71)
Handled rice, congee, or noodles					
Yes	80 (35.7)	144 (64.3)	0.69	0.09	1.07
No	125 (34.2)	241 (65.8)			(0.74 – 1.54)
Handled salad					
Yes	35 (41.7)	49 (58.3)	0.15	1.73	1.41
No	170 (33.6)	336 (66.4)			(0.86 – 2.32)

[a]The difference is statistically significant.

[b]Degree of freedom for Chi-squared test= 1 except for years of experience (df= 3).

Table 20. Multivariate analyses of risk factors for *S. aureus* nasal carriage in 2002.

Variable	*p* value	aOR	95% CI
Age	**0.012**	1.03	1.01 – 1.04
Gender	0.186	1.18	0.86 – 1.61
Handled raw meat	0.177	1.22	0.85 – 1.76
Handle cooked meat	**0.023**	1.49	1.06 – 2.11

aOR, adjusted odds ratio; CI, confidence intervals, bold face denotes statistical significance.

Table 21. Univariate analyses of risk factors for nasal carriage with *S. aureus* (2011).

Factor	Number of subjects (%)		*p* value	χ^2	OR (95% CI)
	Carriers	Non-carriers			
Gender					
Male	76 (26.4)	212 (73.6)	**0.013**[a]	5.6	1.6 (1.1 – 2.3)
Female	23 (15.8)	123 (84.2)			
Smoking					
Yes	27 (20.5)	105 (29.5)	0.44	0.4	0.8 (0.5 – 1.4)
No	72 (23.8)	230 (76.2)			
Age					
≤ 40	45 (23.8)	144 (76.2)	0.75	0.1	1.1 (0.7 – 1.8)
> 40	54 (22.0)	191 (88.0)			
Years of experience as a food handler					
≤ 1 year	6 (28.6)	15 (71.4)	0.52	2.3	N/A
2– 3 years	14 (29.2)	34 (70.8)			
4– 5 years	29 (23.8)	93 (76.2)			
≥ 6 years	50 (20.6)	193 (79.2)			
Handled raw meat regularly at work					
Yes	74 (29.8)	174 (70.2)	**<0.001** [a]	16	2.7 (1.7 – 4.5)
No	25 (13.4)	161 (86.6)			
Daily working hour					
≤ 8 hour	28 (25.5)	82 (74.5)	0.44	0.4	1.2 (0.7 – 2.1)
≥ 9 hour	71 (21.9)	253 (78.1)			
Use of gloves at work					
Yes	92 (23.6)	298 (76.4)	0.34	0.9	1.6 (0.7 – 4.2)
No	7 (15.9)	37 (84.1)			

Table 21. Continued.

Factor	Number of subjects (%)		*p* value	χ^2	OR (95% CI)
	Carriers	Non-carriers			
Hand washing after handling raw meat					
Yes	97 (23.7)	313 (76.3)	0.13[b]	N/A	1.9 (0.4 – 8.5)
No	2 (8.3)	12 (91.7)			
Hand washing after touching faces or noses					
Yes	98 (23.3)	323 (76.7)	0.16[b]	N/A	3.1 (0.5 – 20)
No	1 (7.7)	12 (92.3)			
Hand washing after smoking					
Yes	76 (24.2)	238 (75.8)	0.26	1.25	1.3 (0.8 – 2.3)
No	23 (19.2)	97 (80.8)			
Acne					
Yes	14 (32.6)	29 (67.4)	0.11	2.6	1.8 (0.9 – 3.4)
No	85 (21.7)	306 (78.3)			
Allergies					
Yes	9 (25.7)	26 (74.3)	0.67	0.18	1.2 (0.5 – 2.6)
No	90 (22.6)	309 (77.4)			
Dermatitis					
Yes	2 (15.4)	11 (84.6)	0.74[b]	N/A	0.7 (0.2 – 2.4)
No	97 (23.0)	324 (77.0)			
Rhinitis					
Yes	21 (23.9)	67 (76.1)	0.79	0.07	1.1 (0.6 – 1.9)
No	78 (22.5)	268 (77.5)			
Other chronic diseases					
Yes	4 (16.7)	20 (83.3)	0.32	N/A	0.7 (0.3 – 1.8)
No	95 (22.6)	315 (77.4)			

Table 21. Continued.

Factor	Number of subjects (%)		*p* value	χ^2	OR (95% CI)
	Carriers	Non-carriers			
Antibiotic use in the last three months					
Yes	20 (30.8)	45 (69.2)	0.10	2.8	1.4 (0.9 – 2.2)
No	79 (21.4)	290 (78.6)			
Hospitalization in previous 12 months					
Yes	5 (23.8)	16 (76.2)	0.97	N/A	1.5 (0.5 – 2.3)
No	94 (22.8)	319 (77.2)			
Pet ownership					
Yes	6 (18.8)	26 (81.2)	0.57	0.3	0.8 (0.4 – 1.7)
No	93 (23.1)	309 (76.9)			
Living with a family member who is a health care worker					
Yes	6 (28.6)	15 (71.4)	0.52	0.4	1.5 (0.6 – 4.1)
No	93 (22.5)	320 (77.5)			

[a]The difference is statistically significant, [b] Fishers' exact test

Table 22. Multivariate analyses of risk factors for nasal carriage with *S. aureus* (2011).

Variable	*p* value	aOR	95% CI
Gender	0.210	1.4	0.8 – 2.5
Acne	0.150	0.6	0.3 – 1.2
Handled raw meat	**0.002**	1.6	1.2 – 2.0
Previous use of antibiotics	0.075	0.6	0.3 – 1.1
Hand washing after contact raw meat	0.240	0.4	0.1 – 1.8

aOR, adjusted odds ratio; CI, confidence intervals.

Table 23. Level of exposure to raw meat and risk for nasal colonisation (2011).

	S. aureus carriage (%)		OR (95% CI)	*p* value[a]
	Yes	No		
Always[b]	31 (36.5)	54 (63.5)	3.7 (2.0 – 6.8)	**< 0.001**
Sometimes[c]	43 (26.4)	120 (73.6)	2.3 (1.4 – 4.0)	**0.002**
Never	25 (13.4)	161 (86.6)	1.0	1.0
	P_{trend} <0.001			

[a] Comparison of "always" and "sometimes" versus "never group"

[b] At least three days a week.

[c] One to two days per week.

4.4 Prevalence and distribution of genes encoding SE/SEl in nasal *S. aureus* isolates.

The presence of one or more SE/SEl genes was detected in 82.9% (95% CI 77.4 – 87.4), 82.4% (75.1 – 87.8) and 79.8% (70.9 – 86.5) of *S. aureus* nasal isolates in 2002, 2003 and 2011 respectively. There was no significant difference in the overall prevalence of SE/SEl positive *S. aureus* nasal isolates between the three samples (*p*=0.79). One sample of each *sea* to *selu* amplicon was sequenced and the sequence compared to GenBank database.

Chi-squared test for trend revealed that the prevalence of *sej, sem, sen, seo, seq, ses* and all classical SE genes remained unchanged overtime. The occurrence of genes encoding SEH, SEL, SEP, SER, SET and SE/U increased significantly from 2002 to 2011. Conversely, three enterotoxin genes became significantly less common over time (*seg, sei, sek*) (Table 24).

Overall, of the classical enterotoxin genes, *sea* and *see* were the most common. All the newly described SE/ SEl genes were detected in isolates collected in all three periods. Of these, the *egc* associated genes, *seg, sei, sem, sen, seo* and *selu,* were the most frequently detected (Figure 11).

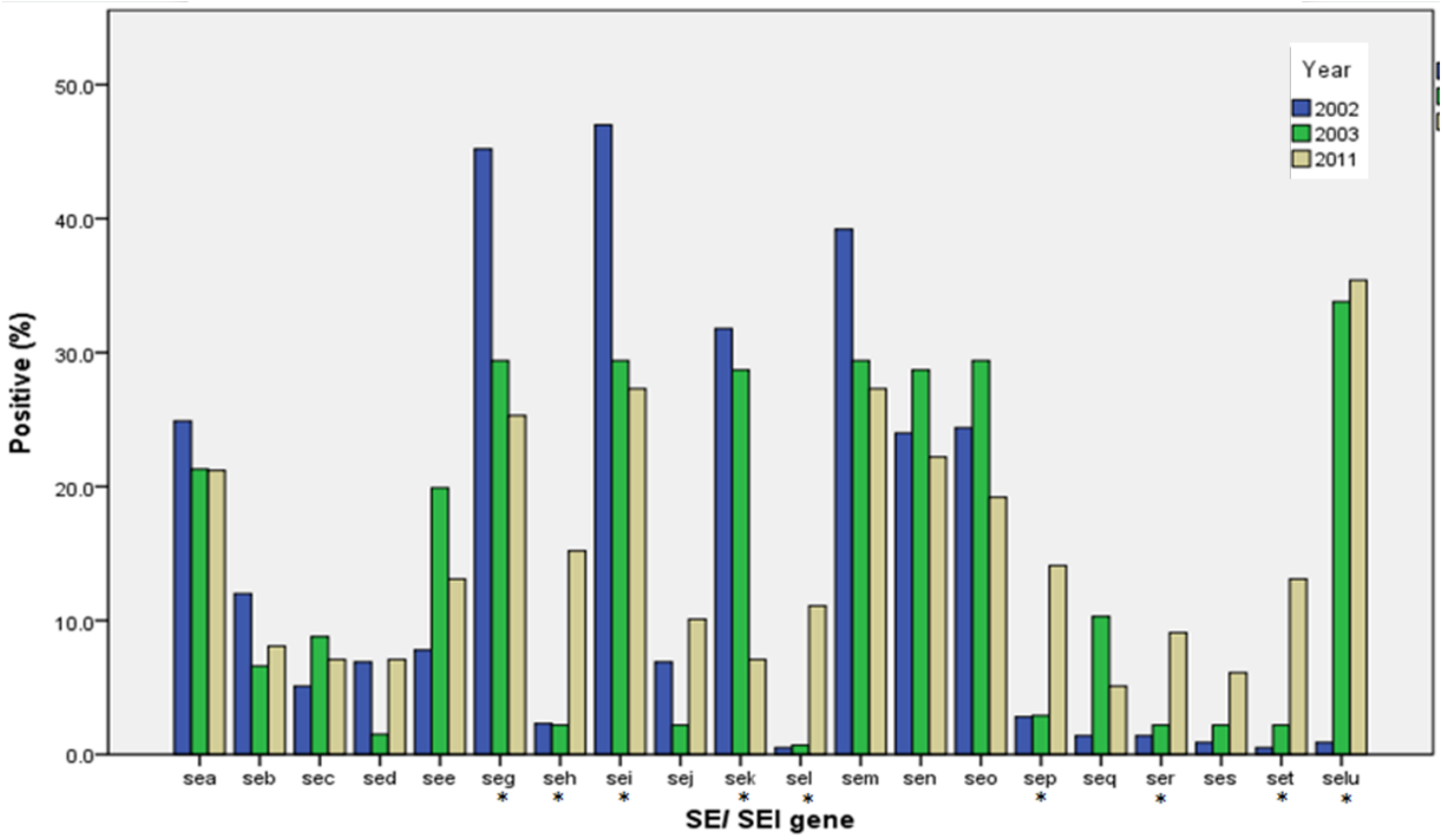
Of the 217 nasal isolates in 2002, 180 (82.9%) harbored at least one SE/ SEl gene. However, the types of SE/SEl genes present varied considerably between isolates with 82 genotypic combinations observed. The most common genotype was *seg-sei-sem* which occurred in ten isolates followed by *sea, sek* and *seg-i-m-n-o* (*egc*).

Of the 136 nasal isolates in 2003, 112 (82.4%) harbored at least one SE/ SEl gene with a total of 52 genotypic combinations of SE/SEls. The most common genotypes were *sek* or *sea* alone with nine isolates each, followed by *seg-i-m-n-o-u* (*egc*).

Of the 99 nasal isolates in 2011, 79 (79.8%) harbored at least one SE/ SEl gene with 51 genotypic combinations of SE/SEls. The most common genotypes were *selu* or *sep* alone comprising seven and six isolates, respectively. The four t1081 MRSA isolates, all harboured *seg-sej-sem-sen-seo-ser-set*.

Although no risk factors investigated in 2002 and 2011 were significantly associated with carriage of SE/SEl genes in *S. aureus* nasal isolates recovered from food handlers, these genes were more frequently detected in food workers who handled cooked meat in the 2002 collection, approaching statistical significance (p= 0.059) (Table 25, 26).

Figure 11. Temporal change of prevalence of SE/ SEl genes among *S. aureus* nasal isolates in 2002, 2003 and 2011.



*Asterisk denotes statistical significance by Chi-squared test for trend comparing prevalence over three periods

Table 24. Prevalence of genes encoding SE/SEls.

Gene	Number of positive (%)			*p* value[a]
	2002 (N=217)	2003 (N=136)	2011 (N=99)	
sea	54 (24.9)	29 (21.3)	21 (21.2)	0.41
seb	26 (12.0)	9 (6.6)	8 (8.1)	0.17
sec	11 (5.1)	12 (8.8)	7 (7.1)	0.36
sed	15 (6.9)	2 (1.5)	7 (7.1)	0.12
see	17 (7.8)	27 (19.9)	13 (13.1)	0.05
seg	98 (45.2)	40 (29.4)	25 (25.3)	**0.002**
seh	5 (2.3)	3 (2.2)	15 (15.2)	**<0.001**
sei	102 (47.0)	40 (29.4)	27 (27.3)	**0.002**
sej	15 (6.9)	3 (2.2)	10 (10.1)	0.57
sek	69 (31.8)	39 (28.7)	7 (7.1)	**<0.001**
sel	1 (0.5)	1 (0.7)	11 (11.1)	**<0.001**
sem	85 (39.2)	40 (29.4)	27 (27.3)	0.02
sen	52 (24.0)	39 (28.7)	22 (22.2)	0.95
seo	53 (24.4)	40 (29.4)	19 (19.2)	0.51
sep	6 (2.8)	4 (2.9)	14 (14.1)	**0.002**
seq	3 (1.4)	14 (10.3)	5 (5.1)	0.03
ser	3 (1.4)	3 (2.2)	9 (9.1)	**0.001**

Table 24. Continued.

Gene	Number of positive (%)			
	2002 (N=217)	2003 (N=136)	2011 (N=99)	*p* value[a]
ses	2 (0.9)	3 (2.2)	6 (6.1)	0.008
set	1 (0.5)	3 (2.2)	13 (13.1)	**<0.001**
selu	2 (0.9)	46 (33.8)	35 (35.4)	**<0.001**

[a]Comparison of prevalence between 2002, 2003 and 2011 by Chi-squared test for trend. Bold face indicates statistical significance ($p < 0.003$).

Table 25. Risk factors for carriage of SE/SEl genes in *S. aureus* nasal isolates in 2002.

Variable	Presence of SE/SEl genes (%)		*p* value	OR (95% CI)
	Positive	Negative		
Gender				
Male	80 (85.1)	14 (14.9)	0.356[a]	1.4 (0.7 – 2.9)
Female	89 (80.2)	22 (19.8)		
Smoking				
Yes	14 (77.8)	4 (22.2)	1.000[b]	1.2 (0.4 – 3.2)
No	61 (74.4)	21 (25.6)		
Age				
≤ 40	91 (85.8)	15 (14.2)	0.267[a]	1.5 (0.7 – 3.1)
> 40	89 (80.2)	22 (19.8)		
Years of experience as a food handler				
≤ 5 years	94 (82.5)	20 (17.5)	0.839[a]	0.9 (0.5 – 1.9)
> 6 years	86 (83.5)	17 (16.5)		
Handled raw meat regularly at work				
Yes	92 (80.7)	22 (19.3)	0.464[a]	0.9 (0.7 – 1.2)
No	77 (84.6)	14 (15.4)		
Handled cooked meat				
Yes	104 (86.7)	16 (13.3)	0.059[a]	2.0 (0.9 – 4.1)
No	65 (76.5)	20 (23.5)		
Handled vegetables				
Yes	96 (88.1)	13 (11.9)	0.244[a]	1.7 (0.7 – 3.9)
No	78 (81.3)	23 (18.7)		

Table 25. Continued.

Variable	Presence of SE/SEl genes (%)		*p* value	OR (95% CI)
	Positive	Negative		
Handled dairy or creamy desserts				
Yes	32 (78.0)	9 (22.0)	0.409[a]	0.7 (0.3 – 1.6)
No	137 (83.5)	27 (16.5)		
Handled bakery				
Yes	51 (78.5)	14 (21.5)	0.308[a]	0.7 (0.3 – 1.4)
No	118 (84.3)	22 (15.7)		
Handled rice, congee, or noodles				
Yes	70 (87.5)	10 (12.5)	0.128[a]	1.8 (0.8 – 4.1)
No	99 (79.2)	26 (20.8)		
Handled salad				
Yes	27 (77.1)	8 (22.9)	0.366[a]	0.7 (0.3 – 1.6)
No	142 (83.5)	28 (16.5)		

[a] Pearson Chi-squared test.

[b] Fisher's exact test.

Table 26. Risk factors for carriage of SE/SEl genes in *S. aureus* nasal isolates in 2011.

Variable	Presence of SE/SEl genes (%)		*p* value	OR (95% CI)
	Positive	Negative		
Gender				
Male	61 (80.3)	15 (19.7)	1.000[b]	1.0 (0.8 – 1.4)
Female	18 (78.3)	5 (21.7)		
Smoking				
Yes	21 (77.8)	6 (22.2)	0.759[a]	0.8 (0.3 – 2.5)
No	58 (80.6)	14 (19.4)		
Age				
≤ 40	17 (73.9)	6 (26.1)	0.422[a]	0.6 (0.2 – 1.9)
> 40	62 (81.6)	14 (18.4)		
Years of experience as a food handler				
≤ 5 years	15 (75.0)	5 (25.0)	0.544[b]	0.7 (0.2 – 2.2)
> 6 years	64 (81.0)	15 (19.0)		
Handling raw meat regularly at work				
Yes	59 (79.7)	15 (20.3)	0.977[a]	1.0 (0.3 – 3.1)
No	20 (80.0)	5 (20.0)		
Daily working hour				
≤ 8 hour	22 (78.6)	6 (21.4)	0.849[a]	0.9 (0.3 – 2.6)
≥ 9 hour	57 (80.3)	14 (19.7)		
Use of gloves at work				
Yes	74 (80.4)	18 (18.6)	0.627[b]	1.6 (0.3 – 9.2)
No	5 (71.4)	2 (28.6)		

Table 26. Continued.

Variable	Presence of SE/SEl genes (%)		*p* value	OR (95% CI)
	Positive	Negative		
Acne				
Yes	10 (71.4)	4 (28.6)	0.473[b]	0.6 (0.2 – 1.8)
No	69 (81.2)	16 (18.9)		
Allergies				
Yes	7 (77.8)	2 (22.2)	1.000[b]	0.9 (0.2 – 4.6)
No	72 (80.0)	18 (20.0)		
Dermatitis				
Yes	2 (100)	0 (0)	1.000[b]	1.0 (0.9 – 1.0)
No	77 (79.4)	20 (20.6)		
Rhinitis				
Yes	18 (85.7)	3 (14.3)	0.553[b]	1.7 (0.4 – 6.4)
No	61 (78.2)	17 (21.8)		
Other chronic diseases				
Yes	4 (100)	0 (0)	0.580[b]	0.9 (0.9 – 1.0)
No	75 (78.9)	20 (21.1)		
Antibiotic use in the last three months				
Yes	16 (80.0)	4 (20.0)	1.000[b]	1.0 (0.3 – 3.5)
No	63 (79.7)	16 (20.3)		
Hospitalization in 12 months				
Yes	3 (60.0)	2 (40.0)	0.265[b]	0.4 (0.1 – 2.3)
No	76 (80.9)	18 (19.1)		

Table 26. Continued.

Factor	Presence of SE/SEl genes (%)		*p* value	OR (95% CI)
	Positive	Negative		
Antibiotic use in the last three months				
Yes	16 (80.0)	4 (20.0)	1.000[b]	1.0 (0.3 – 3.5)
No	63 (79.7)	16 (20.3)		
Hospitalization in 12 months				
Yes	3 (60.0)	2 (40.0)	0.265[b]	0.4 (0.1 – 2.3)
No	76 (80.9)	18 (19.1)		
Pet ownership				
Yes	5 (83.3)	1 (16.7)	1.000[b]	1.3 (0.1 – 11.6)
No	74 (79.6)	19 (20.4)		
Living with a family member who is a health care worker				
Yes	5 (83.3)	1 (16.7)	1.000[b]	1.3 (0.1 – 11.6)
No	74 (79.6)	19 (20.4)		

[a] Pearson Chi-square test.

[b] Fisher's exact test.

4.5 Prevalence and distribution of *qac* genes in nasal *S. aureus* isolates.

There was a slight increase in *qacA/B* positive isolates over the period, from 2.3% to 5.1 % (2003) and 7.1% (2011). However, this change did not reach statistical significance (χ^2= 3.15, df= 1, P_{trend} = 0.076). The *smr* gene was present in 3.7% (2002) and 1.0% (2011) isolates. No *smr* gene was detected in the 2003 collection. There was no significant change of prevalence of *smr* gene over the period (χ^2= 1.19, df= 1, P_{trend} = 0.276) (Table 27).

Co-existence of *qac*A/B and *smr* was observed in two isolates, one each in 2002 and 2011, respectively. These belonged to *spa* types t034 (2002) and t127 (2011) respectively. Both isolates were sensitive to methicillin and to more than two classes of antibiotics. All isolates investigated were negative for *qacG, qacH* and *qacJ.*

Nasal isolates from the three sample periods were pooled for determination of association between the presence of *qac* genes and SE/SEl genes. The presence of *qacA/B* gene was significantly associated with carriage of the *ser* gene (OR= 6.6, 95% CI 1.7 – 25.6, *p*= 0.021) prior to Bonferroni correction for multiple comparison. No association was observed between *qac* genes and other SE/ SEl genes (Table 28).

The *qac* gene positive *S. aureus* isolates were selected for antiseptic susceptibility testing. Isolates harbouring *qacA* had considerably higher MIC and MBC to BC than those habouring *qacB.* The MBC to BC and CHG was significantly higher for *qac*–positive strains in comparison to –negative strains. However, only MIC to CHG

reached statistical significance. No association was found between MIC to BC and the presence of *qac* genes (Table 29).

To determine if there is an association between the type of disinfectant used and the presence of *qac* genes, the type of disinfectant used at each establishment was obtained from managers upon visits. Three establishments (A, B, C) used QAC-containing agents. While the prevalence of nasal colonisation rate did not differ significantly between establishments, the presence of *qacA/B* (7 isolates) and *smr* (1) genes was restricted to isolates from workers at establishments using QAC-based sanitizers. There was a reduced risk for presence of *qac* in operatives employed at establishments using non-QAC products (OR 0.91; 95% CI 0.847-0.976) (Table 30).

The *qac* genes appeared to be more frequently detected in experienced food handlers in both 2002 and 2011, approaching statistical significance (Table 31, 32). Univariate analyses of risk factors for carriage of *qac* genes in the 2011 cross sectional survey revealed that working hours exceeding nine hours a day increased the likelihood for harbouring *qac* positive strains in *S. aureus* colonised workers by twenty fold as compared to workers working less than eight hours a day (p=0.002) (Table 32). However, this variable was not investigated in the 2002 – 2003 study.

Table 27. Prevalence of *qacA/B* and *smr* harbouring *S. aureus* in three samples.

Year	N	Percentage of isolates positive for *qacA/B* or *smr* (95% CI)	
		qacA/B	*smr*
2002	217	2.3 (1.0 – 5.3)	3.7 (1.9 – 7.1)
2003	136[a]	5.1 (2.5 – 10.3)	-
2011	99	7.1 (3.5 – 13.9)	1.0 (0.2 – 5.5)
		P_{trend} = 0.076[b]	P_{trend} = 0.276[b]

[a]Of the 137 isolates collected in 2003,one isolate was not viable.

[b]Chi-square test for trend.

Table 28. Association between the presence of *qac* genes and SE/SEl genes.

Gene		Number of isolates (%)					
		qacA/B		*p*-value[a]	*smr*		*p*-value[a]
		Positive	Negative		Positive	Negative	
sea	Pos	1 (1)	103 (99)	0.090	0 (0)	104 (100)	0.126
	Neg	18 (5.2)	330 (94.8)		9 (2.6)	339 (97.4)	
seb	Pos	3 (7)	40 (93)	0.410	2 (4.7)	41 (95.3)	0.208
	Neg	16 (3.9)	393 (96.1)		7 (1.7)	402 (98.3)	
sec	Pos	2 (6.7)	28 (93.3)	0.364	0 (0)	30 (100)	1.000
	Neg	17 (4)	405 (96)		9 (2.1)	413 (97.9)	
sed	Pos	0 (0)	24 (100)	0.614	0 (0)	24 (100)	1.000
	Neg	19 (4.4)	409 (95.6)		9 (2.1)	419 (97.9)	
see	Pos	1 (1.8)	56 (98.2)	0.491	1 (1.8)	56 (98.2)	1.000
	Neg	18 (4.6)	377 (95.4)		8 (2.0)	387 (98)	
seg	Pos	3 (1.8)	160 (98.2)	0.085	4 (2.5)	159 (97.5)	0.728
	Neg	16 (5.5)	273 (94.5)		5 (1.7)	284 (98.3)	
seh	Pos	1 (4.3)	22 (95.7)	1.000	1 (4.3)	22 (95.7)	0.378
	Neg	18 (4.2)	411 (95.8)		8 (1.9)	421 (98.1)	
sei	Pos	3 (1.8)	166 (98.2)	0.053	4 (2.4)	165 (97.6)	0.733
	Neg	16 (5.7)	267 (94.3)		5 (1.8)	278 (98.2)	
sej	Pos	2 (7.1)	26 (92.9)	0.332	0 (0)	28 (100)	1.000
	Neg	17 (4.0)	407 (96.0)		9 (2.1)	415 (97.9)	

Table 28. Continued.

Gene		Number of isolates (%)					
		qacA/B		*p*-value[a]	*smr*		*p*-value[a]
		Positive	Negative		Positive	Negative	
sek	Pos	3 (2.6)	112 (97.4)	0.426	2 (1.7)	113 (98.3)	1.000
	Neg	16 (4.7)	321 (95.3)		7 (2.1)	330 (97.9)	
sel	Pos	2 (15.4)	11 (84.6)	0.099	0 (0)	13 (100)	1.000
	Neg	17 (3.9)	422 (96.1)		9 (2.1)	430 (97.9)	
sem	Pos	4 (2.7)	145 (97.3)	0.325	2 (1.3)	147 (98.7)	0.724
	Neg	15 (4.7)	288 (95.3)		7 (2.3)	296 (97.7)	
sen	Pos	3 (2.7)	110 (97.3)	0.428	0 (0)	113 (100)	0.120
	Neg	16 (4.7)	323 (95.3)		9 (2.7)	330 (97.3)	
seo	Pos	3 (2.7)	109 (97.3)	0.429	0 (0)	112 (100)	0.121
	Neg	16 (4.7)	324 (95.3)		9 (2.6)	331 (97.4)	
sep	Pos	0 (0)	24 (100)	0.614	0 (0)	24 (100)	1.000
	Neg	19(4.4)	409 (95.6)		9 (2.1)	419 (97.9)	
seq	Pos	2 (9.1)	20 (90.9)	0.235	0 (0)	22 (100)	1.000
	Neg	17 (4.0)	413 (96.0)		9 (2.1)	421 (97.9)	
ser	Pos	3 (20)	12 (80)	0.021	0 (0)	15 (100)	1.000
	Neg	16 (3.7)	421 (96.3)		9 (2.1)	428 (97.9)	
ses	Pos	2 (18.2)	9 (81.8)	0.073	0 (0)	11 (100)	1.000
	Neg	17 (3.9)	424 (96.1)		9 (2.0)	432 (98.0)	

Table 28. Continued.

Gene		Number of isolates (%)					
		qacA/B		*p*-value[a]	*smr*		*p*-value[a]
		Positive	Negative		Positive	Negative	
set	Pos	2 (11.8)	15 (88.2)	0.156	0 (0)	17 (100)	1.000
	Neg	17 (3.9)	418 (96.1)		9 (2.1)	426 (97.9)	
selu	Pos	3 (3.6)	80 (96.4)	1.000	0 (0)	83 (100)	0.376
	Neg	16 (4.3)	353 (95.7)		9 (2.4)	360 (97.6)	

[a] Comparison between the presence of *qacA/B/smr* genes with SE/SEl genes using Fisher's exact test. Statistical significance was set at 0.003 (0.05/20).

Table 29. Comparison of MICs and MBCs of BC and CHG for *S. aureus* nasal isolates.

Genotype	BAC (mg/L)		CHG (mg/L)	
	MIC	MBC	MIC	MBC
qacB + smr	1	2	1	1
qacB	1	2	2	4
qacB	≤ 0.5	1	1	2
qacB	≤ 0.5	1	1	2
qacB	1	2	2	4
qacA	4	8	4	8
qacB	1	2	2	2
qacB	≤ 0.5	1	1	2
qacA	4	8	2	4
qacB	2	2	2	4
qacB	1	2	1	2
Range (*qac* gene positive strains [a])	0.5 – 4	1 – 8	1 – 4	1 – 8
Range (*qac* gene negative strains) [c]	0.5 – 2	0.5 – 2	0.5 – 2	0.5 – 2
p-value[a]	0.171	**0.001**	**0.003**	**< 0.001**

[a] Eight strains were not viable.

[b] Comparison of MIC/MBC between *qac* gene -positive isolates and -negative isoaltes using Mann-Whitney U test. Statistical signifance was set at 0.05 and is shown in bold face.

[c] A total of ten *qac* gene negative strains was tested.

Table 30. Association between use of disinfectant type and carriage of *qac* genes in *S. aureus* nasal isolates in 2011.

Site[a]	Active ingredient used in routine disinfection	Number of positive	
		qacA/B	*smr*
A	Alkyl dimethyl benzyl ammonium chloride 1.29%, Alkyl dimethyl ethyl-benzyl ammonium chloride 1.29%	3	0
B	Alkyl dimethyl benzyl ammonium chloride 10%, Ethanol 1.9%	1	1
C	Chlorhexidine gluconate 15%	3	0
D	Sodium Hypochlorite 3.35%	0	0
E	Hydrogen peroxide 6.9%, Peroxyacetic acid 4.4%, Octanoic acid 3.3%	0	0
F	Sodium Hypochlorite 8.4%	0	0

[a] Disinfectants used at sites A, B and C are QAC compounds.

Table 31. Risk factors for carriage of *qac* genes in *S. aureus* nasal isolates in 2002.

Variable	Presence of *qac* genes (%)		*p* value[a]	OR (95% CI)
	Positive	Negative		
Gender				
Male	7 (7.4)	88 (92.6)	0.553	1.7 (0.5 – 5.4)
Female	5 (4.5)	105 (95.5)		
Smoking				
Yes	1 (5.6)	17 (94.4)	0.332	4.7 (0.3 – 79)
No	1 (1.2)	80 (98.8)		
Age				
≤ 40	4 (17.4)	19 (82.6)	0.382	1.8 (0.5 – 6.6)
> 40	8 (10.5)	68 (89.5)		
Years of experience as a food handler				
> 6 years	5 (25)	15 (75)	0.058	3.4 (1.0 – 12.3)
≤ 5 years	7 (8.9)	72 (91.1)		
Handled raw meat regularly at work				
Yes	7 (6.1)	107 (93.9)	0.845	1.1 (0.3 – 3.7)
No	5 (5.5)	86 (94.5)		
Handled cooked meat				
Yes	5 (4.2)	114 (95.8)	0.247	0.5 (0.2 – 1.6)
No	7 (8.1)	79 (91.9)		
Handled vegetables				
Yes	7 (6.4)	102 (93.6)	0.712	1.2 (0.4 – 4.1)
No	5 (5.2)	91 (94.8)		

Table 31. Continued.

Variable	Presence of *qac* genes (%)		*p* value[a]	OR (95% CI)
	Positive	Negative		
Handled dairy or creamy desserts				
Yes	2 (4.9)	39 (95.1)	1.000	0.8 (0.2 – 3.8)
No	10 (6.1)	154 (93.9)		
Handled bakery				
Yes	4 (6.2)	60 (93.8)	1.000	1.1 (0.3 – 3.8)
No	8 (5.7)	133 (94.3)		
Handled rice, congee, or noodles				
Yes	3 (3.8)	77 (96.2)	0.374	0.5 (0.1 – 1.9)
No	9 (7.2)	116 (92.8)		
Handled salad				
Yes	3 (8.6)	32 (91.4)	0.452	1.7 (0.4 – 6.5)
No	9 (5.3)	161 (94.7)		

[a] Fisher's exact test.

Table 32. Risk factors for carriage of *qac* genes in *S. aureus* nasal isolates in 2011.

Variable	Presence of *qac* genes (%)		*p* value[a]	OR (95% CI)
	Positive	Negative		
Gender				
Male	5 (6.6)	71 (93.4)	0.662	0.7 (0.1 – 4.1)
Female	2 (8.7)	21 (91.3)		
Smoking				
Yes	5 (6.9)	67 (93.1)	1.000	0.9 (0.2 – 5.1)
No	2 (7.4)	25 (92.6)		
Age				
≤ 40	0 (0)	24 (100)	0.351	0.3 (0.1 – 4.6)
> 40	7 (9.3)	92 (90.7)		
Years of experience as a food handler				
> 6 years	3 (15)	17 (85)	0.144	3.9 (0.7 – 16.2)
≤ 5 years	4 (5.1)	75 (94.9)		
Handled raw meat regularly at work				
Yes	7 (9.5)	67 (90.5)	0.241	5.7 (0.3 – 103)
No	0 (0)	25 (100)		
Daily working hour				
≥ 9 hour	6 (21.4)	22 (78.6)	**0.002**	19.1 (2.2 – 167.3)
≤ 8 hour	1 (1.4)	70 (98.6)		
Use of gloves at work				
Yes	0 (0)	7 (100)	1.000	1.1 (1.0 – 1.2)
No	7 (7.6)	85 (92.4)		

Table 32. Continued.

Variable	Presence of *qac* genes (%)		*p* value[a]	OR (95% CI)
	Positive	Negative		
Acne				
Yes	0 (0)	14 (100)	0.589	0.9 (0.8 – 1.0)
No	7 (8.2)	78 (91.8)		
Allergies				
Yes	1 (11.1)	8 (88.9)	0.498	0.6 (0.1 – 5.4)
No	6 (6.7)	84 (93.3)		
Dermatitis				
Yes	0 (0)	2 (100)	1.000	0.9 (0.8 – 1.0)
No	7 (7.2)	90 (92.8)		
Rhinitis				
Yes	0 (0)	21 (100)	0.340	0.9 (0.8 – 1.0)
No	7 (9)	71 (91)		
Other chronic diseases				
Yes	0 (0)	4 (100)	1.000	0.9 (0.8 – 1.0)
No	7 (7.4)	68 (92.6)		
Antibiotic use in the last three months				
Yes	0 (0)	20 (100)	0.339	0.9 (0.8 – 1.0)
No	7 (8.9)	72 (91.1)		
Hospitalization in 12 months				
Yes	0 (0)	5 (100)	1.000	0.9 (0.8 – 1.0)
No	7 (7.4)	87 (92.6)		

Table 32. Continued.

Factor	Presence of *qac* genes (%)		*p* value[a]	OR (95% CI)
	Positive	Negative		
Pet ownership				
Yes	0 (0)	6 (100)	1.000	0.9 (0.8 – 1.0)
No	7 (7.5)	86 (92.5)		
Living with a family member who is a health care worker				
Yes	0 (0)	6 (100)	1.000	0.9 (0.8 – 1.0)
No	7 (7.5)	86 (92.5)		

[a] Fisher's exact test.

4.6 Distribution of *spa* types and their relationship with virulence genes.

Spa typing for 2002 and 2011 nasal isolates revealed 39 *spa* types clustered into 13 *spa* clonal clusters (*spa*-CCs) and 42 *spa* types into 10 *spa*-CCs respectively. Between two and 65 isolates were present in each *spa*-CC. Diversity index indicated that there was a slight increase of *spa* type diversity over the period (D_{2002} = 0.939 vs D_{2011} = 0.948), reflecting the absence in 2011 of 28 *spa* types observed in 2002 and 29 types newly detected in 2011. In particular, the number of *spa*-CC15 associated types reduced significantly from 23% to 11% (p=0.046) whilst *spa*-CC45 associated types increased from 4 to 12% (p=0.004) (Table 33). The ST188 associated *spa* cluster is significantly associated with the presence of SE genes. Other *spa* clusters were not associated with the presence of *qac* and SE/SEl genes (Table 34). CC45 associated *spa* cluster was significantly associated with phenotypic resistance to cefoxitin, ciprofloxacin and tetracycline whilst *spa* cluster C associated strains with erythromycin resistance (p<0.002) (Table 35), other *spa* clusters did not appear to be associated with resistance to antibiotics tested.

Of the 89 persistent carriers identified in 2002 and 2003, eighty of them yielded isolates with identical *spa* types on both occasions. The most common *spa* types were t189 (18.8%), t084 (12.5%), t091 (7.5%), t437 (7.5%) and t034 (6.3%). The remaining nine subjects had either a mutation or a change in their colonizing strains (Table 36). *Spa* types observed in transient carriers were similar to those of

persistent carriers. The mutation rate of the *spa* locus amongst persistent carriers is 1 mutation per 223 months (2 mutations per 89 x 5 persons-months).

Table 33. Comparison of *spa* clonal clusters and types of *S. aureus* nasal carriage strains in 2002 and 2011.

spa cluster (n) [a]	Predicted MLST	*spa* type (no. of isolates) [b] 2002	*spa* type (no. of isolates) [b] 2011	*p*-value[c]
Cluster A (55)	CC15/ST7 /ST568	**t084(15)**, t085 (6), **t091 (13**), t796 (3), t1190, t2932 (3), t2949, t5864, t11518	**t084 (3)**, **t091 (7),** t7568	**0.046**
Cluster B (20)	CC45 /ST291	t026 (3), t050 (3), **t1081 (2)**	t937, **t1081 (8)**, t1857, t4981, t5598,	**0.004**
Cluster C (42)	ST30 /ST239	t012 (4), **t021 (8)**, **t338 (12),** t1239 (3), t12778 (2)	t030, t037 (2), t253, t584, t2868, t8917, **t338 (5), t021**	0.555
Cluster D (9)	CC5	t7738 (2)	t002 (4), t179, t668, t688	0.052
Cluster E (9)	ST6 /ST8	**t701 (2)**	**t701 (4)**, t304 (3)	0.052
Cluster F (16)	CC1	**t127 (7)**	**t127 (8),** t527	0.311
Cluster G (3)	ST88	t786	t2592, t4016	0.232
Cluster H (20)	ST541	**t034 (13)**, t571 (3)	**t034 (3),** t3625	0.189
Cluster I (65)	ST188 /ST12	**t189 (43)**, nt1[d], t8139	**t189 (17**), t213 (2), t888	0.513
Cluster J (7)	ST1619	t364 (2), t616 (5)	none	0.069
Cluster K (3)	ST72	t3092	t148 (2)	0.232
Cluster L (4)	no data	t2459 (3)	nt2[d]	0.628
Cluster M (2)	ST25	t3232	t401	0.529

[a]Defined by visual analysis as described by Ruppitsch et al. (2006).

[b]Bold fonts represent *spa* types which are present in both 2002 and 2011. Number of isolates is shown in bracket, if more than one. The *spa* types t282, t2196, t3625, t4864 were excluded from grouping because of the presence of less than five repeats.

[c] Comparison of number of isolates in the *spa*- CC (Chi-square test).

[d] nt = new type nt1 Repeat succession: 07-23-12-21-17-254; nt2 Repeat succession: 23-34-34-16-34-33-13.

The remaining isolates were singletons not belonging to any of the clusters above, 51 in 2002 and 11 in 2011.

Table 34. Association between *spa* clusters and the presence of *qac* and SE/SEl genes.

spa cluster	Predicted MLST	Number of isolates (%)								
		qacA/B		*p*-value[a]	*smr*		*p*-value[a]	SE/SEl		*p*-value[b]
		Positive	Negative		Positive	Negative		Positive	Negative	
A	CC15/ST7	2 (3.6)	53 (96.4)	0.652	1 (1.8)	54 (98.2)	0.516	45 (81.8)	10 (18.2)	0.871
B	CC45/ST291	2 (10)	18 (90)	0.172	0 (0)	20 (100)	0.551	17 (85)	3 (15)	0.498
C	ST30/ST239	0 (0)	42 (100)	0.175	1 (2.4)	41 (97.6)	0.659	38 (90.5)	4 (9.5)	0.087
D	CC5	0 (0)	9 (100)	0.703	0 (0)	9 (100)	0.768	8 (88.9)	1 (11.1)	0.495
E	ST6/ST8	0 (0)	9 (100)	0.703	0 (0)	9 (100)	0.768	7 (77.8)	2 (22.2)	0.505
F	CC1	2 (12.5)	14 (87.5)	0.118	1 (6.3)	15 (93.7)	0.378	15 (93.8)	1 (6.3)	0.180
G	ST88	0 (0)	3 (100)	0.891	0 (0)	3 (100)	0.917	2 (66.7)	1 (33.3)	0.451
H	ST541	1 (4.8)	20 (95.2)	0.568	1 (5)	19 (95)	0.449	15 (75)	5 (25)	0.282
I	ST188/ST12	4 (6.2)	61 (93.8)	0.217	1 (1.5)	64 (98.5)	0.416	47 (72.3)	18 (27.7)	**0.037**
J	ST1619	1 (14.3)	6 (85.7)	0.239	0 (0)	7 (100)	0.815	6 (85.7)	1 (14.3)	0.631

Table 34. Continued.

spa cluster	Predicted MLST	Number of isolates (%)								
		qacA/B		*p*-value[a]	*smr*		*p*-value[a]	SE/SEl		*p*-value[b]
		Positive	Negative		Positive	Negative		Positive	Negative	
K	ST72	0 (0)	3 (100)	0.891	0 (0)	3 (100)	0.917	3 (100)	0 (0)	0.549
L	no data	0 (0)	4 (100)	0.856	0 (0)	4 (100)	0.891	4 (100)	0 (0)	0.449
M	ST25	0 (0)	2 (100)	0.925	1 (50)	1 (50)	0.056	2 (100)	0 (0)	0.857
Ungp[c]	n/a	0 (0)	61 (100)	0.073	3 (4.9)	58 (95.1)	0.242	50 (82)	11 (18)	0.854

[a]Comparison of a given *spa* cluster with the remaining *spa* clusters for the presence of *qacA/B* or *smr* by Fisher's exact test. Statistical significance was set at 0.004 (0.05/14).

[b]Comparison of a given *spa* cluster with the remaining *spa* clusters for the presence of SE/SEl by Chi-square test or Fisher's exact test as appropriate. Statistical significance was set at 0.004 (0.05/14).

[c]Ungrouped isolates which did not belong to cluster A to M.

Table 35. Association between *spa* clusters and antibiotic sensitivity.

spa cluster	Predicted MLST	Number of isolates (%)					
		Cefoxitin		*p*-value[a]	Chloramphenicol		*p*-value[a]
		Resistant	Sensitive		Resistant	Sensitive	
A	CC15/ST7	0 (0)	55 (100)	0.213	2 (3.6)	53 (96.4)	0.175
B	CC45/ST291	5 (25)	15 (75)	**<0.001**	1 (5)	19 (95)	0.539
C	ST30/ST239	1 (2.4)	41 (97.6)	0.712	1 (2.4)	41 (97.6)	0.142
D	CC5	0 (0)	9 (100)	0.792	2 (22.2)	7 (77.8)	0.143
E	ST6/ST8	0 (0)	9 (100)	0.792	1 (11.1)	8 (88.9)	0.513
F	CC1	0 (0)	16 (100)	0.657	1 (6.2)	15 (93.8)	0.663
G	ST88	0 (0)	3 (100)	0.926	1 (33.3)	2 (66.7)	0.212
H	ST541	0 (0)	20 (100)	0.589	2 (10)	18 (90)	0.460
I	ST188/ST12	1 (1.6)	63 (98.4)	0.494	7 (10.9)	57 (89.1)	0.189
J	ST1619	0 (0)	7 (100)	0.834	0 (0)	7 (100)	0.572
K	ST72	0 (0)	3 (100)	0.926	0 (0)	3 (100)	0.788
L	no data	0 (0)	4 (100)	0.902	0 (0)	4 (100)	0.728
M	ST25	0 (0)	2 (100)	0.949	0 (0)	2 (100)	0.854
Ungp[c]	n/a	1 (1.6)	61 (98.4)	0.513	6 (9.7)	56 (90.3)	0.323

Table 35. Continued.

spa cluster	Predicted MLST	Number of isolates (%)					
		Ciprofloxacin		*p*-value[a]	Clindamycin		*p*-value[a]
		Resistant	Sensitive		Resistant	Sensitive	
A	CC15/ST7	0 (0)	55 (100)	0.118	2 (3.6)	53 (96.4)	0.451
B	CC45/ST291	5 (25)	15 (75)	**0.002**	1 (5)	19 (95)	0.732
C	ST30/ST239	0 (0)	42 (100)	0.203	3 (7.1)	39 (92.9)	0.359
D	CC5	0 (0)	9 (100)	0.724	1 (11.1)	8 (88.9)	0.377
E	ST6/ST8	0 (0)	9 (100)	0.724	0 (0)	9 (100)	0.623
F	CC1	1 (6.2)	15 (93.8)	0.441	0 (0)	16 (100)	0.426
G	ST88	0 (0)	3 (100)	0.899	1 (33.3)	2 (66.7)	0.145
H	ST541	1 (5)	19 (95)	0.519	2 (10)	18 (90)	0.268
I	ST188/ST12	1 (1.6)	63 (98.4)	0.309	1 (1.6)	63 (98.4)	0.129
J	ST1619	0 (0)	7 (100)	0.778	0 (0)	7 (100)	0.693
K	ST72	1 (33.3)	2 (66.7)	0.101	1 (33.3)	2 (66.7)	0.145
L	no data	1 (25)	3 (75)	0.133	0 (0)	4 (100)	0.812
M	ST25	0 (0)	2 (100)	0.931	0 (0)	2 (100)	0.901
Ungp[c]	n/a	1 (1.6)	61 (98.4)	0.329	4 (6.5)	58 (93.5)	0.386

Table 35. Continued.

spa cluster	Predicted MLST	Number of isolates (%)					
		Erythromycin		*p*-value[a]	Fusidic acid		*p*-value[a]
		Resistant	Sensitive		Resistant	Sensitive	
A	CC15/ST7	6 (10.9)	49 (89.1)	0.226	4 (7.3)	51 (92.7)	0.341
B	CC45/ST291	4 (20)	16 (80)	0.361	2 (10)	18 (90)	0.606
C	ST30/ST239	14 (33.3)	28 (66.7)	**0.001**	3 (7.1)	39 (92.8)	0.385
D	CC5	1 (11.1)	8 (88.9)	0.592	1 (11.1)	8 (88.9)	0.610
E	ST6/ST8	0 (0)	9 (100)	0.223	1 (11.1)	8 (88.9)	0.610
F	CC1	1 (6.2)	15 (93.8)	0.269	2 (12.5)	14 (87.5)	0.479
G	ST88	2 (66.7)	1 (33.3)	0.066	1 (33.3)	2 (66.7)	0.267
H	ST541	2 (10)	18 (90)	0.389	2 (10)	18 (90)	0.606
I	ST188/ST12	6 (9.4)	58 (90.6)	0.101	8 (12.5)	56 (87.5)	0.274
J	ST1619	0 (0)	7 (100)	0.312	1 (14.3)	6 (85.7)	0.518
K	ST72	1 (33.3)	2 (66.7)	0.391	0 (0)	3 (100)	0.733
L	no data	0 (0)	4 (100)	0.516	0 (0)	4 (100)	0.660
M	ST25	0 (0)	2 (100)	0.719	0 (0)	2 (100)	0.813
Ungp[c]	n/a	11 (17.7)	51 (82.3)	0.327	6 (9.7)	56 (90.3)	0.593

Table 35. Continued.

spa cluster	Predicted MLST	Number of isolates (%)					
		Gentamicin		*p*-value[a]	Oxacillin		*p*-value[a]
		Resistant	Sensitive		Resistant	Sensitive	
A	CC15/ST7	0 (0)	55 (100)	0.006	1 (1.8)	54 (98.2)	0.719
B	CC45/ST291	2 (10)	18 (90)	0.567	2 (10)	18 (90)	0.049
C	ST30/ST239	2 (4.8)	40 (95.2)	0.226	1 (2.4)	41 (97.6)	0.578
D	CC5	1 (11.1)	8 (88.9)	0.584	0 (0)	9 (100)	0.839
E	ST6/ST8	1 (11.1)	8 (88.9)	0.584	0 (0)	9 (100)	0.839
F	CC1	4 (25)	12 (75)	0.048	1 (6.2)	15 (93.8)	0.269
G	ST88	0 (0)	3 (100)	0.748	0 (0)	3 (100)	0.944
H	ST541	1 (5)	19 (95)	0.433	0 (0)	20 (100)	0.673
I	ST188/ST12	12 (18.8)	52 (81.2)	0.052	1 (1.6)	63 (98.4)	0.649
J	ST1619	0 (0)	7 (100)	0.506	0 (0)	7 (100)	0.873
K	ST72	0 (0)	3 (100)	0.748	0 (0)	3 (100)	0.944
L	no data	0 (0)	4 (100)	0.679	0 (0)	4 (100)	0.926
M	ST25	0 (0)	2 (100)	0.825	0 (0)	2 (100)	0.962
Ungp[c]	n/a	6 (9.7)	56 (90.3)	0.521	0 (0)	62 (100)	0.267

Table 35. Continued.

spa cluster	Predicted MLST	Number of isolates (%)					
		Penicillin		*p*-value[a]	QD[d]		*p*-value[a]
		Resistant	Sensitive		Resistant	Sensitive	
A	CC15/ST7	41 (74.5)	14 (25.5)	0.228	1 (1.8)	54 (98.2)	0.438
B	CC45/ST291	19 (95)	1 (5)	0.052	0 (0)	20 (100)	0.821
C	ST30/ST239	31 (73.8)	11 (26.2)	0.236	0 (0)	42 (100)	0.651
D	CC5	8 (88.9)	1 (11.1)	0.407	0 (0)	9 (100)	0.917
E	ST6/ST8	6 (66.7)	3 (33.3)	0.284	0 (0)	9 (100)	0.917
F	CC1	14 (87.5)	2 (12.5)	0.314	0 (0)	16 (100)	0.855
G	ST88	2 (79.4)	1 (20.6)	0.506	0 (0)	3 (100)	0.972
H	ST541	14 (70)	6 (30)	0.219	0 (0)	20 (100)	0.821
I	ST188/ST12	48 (73.8)	17 (26.2)	0.141	0 (0)	64 (100)	0.506
J	ST1619	6 (85.7)	1 (14.3)	0.551	0 (0)	7 (100)	0.935
K	ST72	3 (100)	0 (0)	0.494	1 (33)	2 (67)	0.028
L	no data	4 (100)	0 (0)	0.389	0 (0)	4 (100)	0.962
M	ST25	2 (100)	0 (0)	0.625	0 (0)	2 (100)	0.981
Ungp[c]	n/a	53 (85.5)	9 (14.5)	0.113	1 (1.6)	61 (98.4)	0.482

Table 35. Continued.

spa cluster	Predicted MLST	Number of isolates (%)					
		Tetracycline		*p*-value[a]	SXT[e]		*p*-value[a]
		Resistant	Sensitive		Resistant	Sensitive	
A	CC15/ST7	5 (9.1)	50 (90.9)	0.043	2 (3.6)	53 (96.4)	0.419
B	CC45/ST291	10 (50)	10 (50)	**<0.001**	0 (0)	20 (100)	0.589
C	ST30/ST239	5 (11.9)	37 (88.1)	0.203	1 (2.4)	41 (97.6)	0.712
D	CC5	0 (0)	9 (100)	0.169	1 (11.1)	8 (88.9)	0.208
E	ST6/ST8	0 (0)	9 (100)	0.169	0 (0)	9 (100)	0.792
F	CC1	4 (25)	12 (75)	0.309	0 (0)	16 (100)	0.657
G	ST88	2 (66.7)	1 (33.3)	0.082	0 (0)	3 (100)	0.926
H	ST541	4 (20)	16 (80)	0.487	0 (0)	20 (100)	0.589
I	ST188/ST12	12 (18.8)	52 (81.2)	0.467	2 (3.1)	62 (96.9)	0.506
J	ST1619	2 (28.6)	5 (71.4)	0.360	0 (0)	7 (100)	0.834
K	ST72	0 (0)	3 (100)	0.556	1 (33.3)	2 (66.7)	0.074
L	no data	1 (25)	3 (75)	0.544	0 (0)	4 (100)	0.902
M	ST25	0 (0)	2 (100)	0.677	0 (0)	2 (100)	0.949
Ungp[c]	n/a	11 (17.7)	51 (82.3)	0.562	1 (1.6)	61 (98.4)	0.512

[a]Comparison of a given *spa* cluster with the remaining *spa* clusters for the presence of *qacA/B* or *smr* by Fisher's exact test. Statistical significance was set at 0.004 (0.05/14).

[b] Comparison of a given *spa* cluster with the remaining *spa* clusters for the presence of SE/SEl by Chi-square test or Fisher's exact test as appropriate. Statistical significance was set at 0.004 (0.05/14).

[c]Ungrouped isolates which did not belong to cluster A to M.

[d]Quinupristin-dalfopristin

[e]Trimethoprim-sulfamethoxazole

Table 36. Characteristics of altered *spa* types in persistent nasal isolates.

Sample	*spa* type	repeat succession[a]	comments
FL31	NID[c]	23-434-34-254-434-12-23-02-12-23[b]	Change of coloniser
	t571	08-16-02-25-02-25-34-25	strain
FW13	NID	07-23-12-21-17-254	Change of coloniser
	t364	04-34-17-32-17-23-24	strain
JC79	NID	16-02-279-425-25-34-34-25	Change of coloniser
	t021	15-12-16-02-16-02-25-17-24	strain
KO64	NID	23-12-34-34-12-12-12-23-02	Change of coloniser
	t437	04-20-17-20-17-25-34	strain
JC112	t008	11-19-**12**-21-17-34-24-34-22-25	Mutation Glu → Lys
	t12821	11-19-**178**-21-17-34-24-34-22-25	(GAA → AAA)
FL10	t034	08-16-02-25-02-25-34-**24**-25	Deletion of 24bp
	t571	08-16-02-25-02-25-34-25	repeat
FW54	t338	15-21-16-02-25-17-24	Change of coloniser
	t1190	07-23-21-17-34-34-34-34-33-34	strain
SS23	t4864	35-25-17-24	Change of coloniser
	t091	07-23-21-17-34-12-23-02-12-23	strain
KO61	t189	07-23-12-21-17-34	Change of coloniser
	t2196	04-34-22-25	strain

[a]bold face indicates change of *spa* repeats.

[b]upper row denotes *spa* type for the isolate in 2002 and lower row for that in 2003.

[c]not in database.

4.7 Tracking of transmission of *S. aureus* from nasal carriers to hands.

Spa typing results for each establishment was analyzed independently. Subjects with concomitant presence of *S. aureus* with identical *spa* type in their nose and hands were defined as putative sources of spread (index case). Subjects who yielded two positive nasal swab results or were positive only on the second occasion are hereafter referred to as persistent and transient carriers, respectively.

Fourteen establishments sampled in both 2002 and 2003 were selected as case studies for tracking transmission from noses to hands. On the second visit, nasal colonisation was detected in 24% of the subjects. Of these, 16% were persistent and 8% transient carriers. Hand contamination was present in 16.6% of food handlers. Whilst hand contamination was absent in three premises, a considerably higher contamination rate was observed in one site (designated as C). Sampling of this site was repeated after the provision of hand washing training to the staff in the premises. The hand contamination rate dropped from 71% pre-training to 11.1% which was comparable to the rates observed in other establishments (Table 37).

Of the *S. aureus* contaminating hands of food handlers, more than half of the *S. aureus* strains isolated from hands had *spa* types identical to the nasal isolates of other co-workers, including persistent nasal carriers (54.7%) and transient nasal carriers (18.9%). Four subjects (7.5%) simultaneously carried *S. aureus* strains of the same *spa* types in their noses and hands. The remaining 18.9% of hand isolates had

spa types distinct from that of nasal isolates of food handlers in the same workplace (Table 37).

Of note, in establishment C, the first sample revealed three persons who harboured *S. aureus* of the same *spa* types (t189, t338, t127) in their nasal and hand specimens. These subjects were regarded as index cases being plausible primary sources of hand contamination of their co-workers. A person who was nasally colonised and whose strain contaminated the hands of others was defined as an index case. For instance, a person nasally colonised with t189 *S. aureus* led to hand contamination of nine non-nasal carriers and further two persons who were nasally colonised by *S. aureus* of t127 and t2182 respectively (Figure 12). Another index case carrying t338 spread the strain to two other non-nasal carriers. The final index case harbouring t127 spread to four non-nasal carriers and another person who was nasally colonised with t903 (Figure 12). In other establishments, transfer from persistent to others was apparent. However, the low levels of hand carriage rates precluded a clear relationship being seen.

Table 37. Source of hand contamination and *spa* types of *S. aureus* isolated from hands.

Site	No. Workers (2003))	Nasal carriage status			No. hand positive (%)	Source of hand contamination[a]			
						Non-nasally colonized workers[b]			Nasally colonized[c]
		P	T	N		Persistent carriers	Transient carriers	Others	(Endogenous spread)
A	26	4	4	18	6 (23.1)	t026 (2)	t127, t2932	t3992	t571 (T)
B	18	4	0	14	1(5.5)	t085			
C[d]	54	10	6	38	6 (11.1)	t021, t127, t164, t189, t338	t437		
D	28	6	3	19	5 (17.8)	t189 (2)		t032, t2546	t189 (P)
E	125	22	11	92	9 (7.2)	t084 (2), t189 (3), t616	t091, t5864	t213	
F	66	11	3	52	9 (13.6)	t282, t338, t437 (2), t7738	t012, t3092	t668	t437 (P)
G	43	4	2	37	5 (11.6)	t084 (2), t189	t616	t2919	
H	24	3	3	18	1 (4.2)	t189			
I	58	6	3	49	3 (5.2)	t050, t189	t084		
J	45	6	7	32	5 (11.1)	t189	t571	t035, t183	t189 (P)
K	23	5	2	16	3 (13.0)	t437		t082, t701	
LMN[e]	30	7	0	23	0				
Overall hand carriage (n = 53)						29 (54.7%)	10 (18.9%)	10 (18.9%)	4 (7.5%)
Diversity Index						0.862	1.00	1.00	0.834

[a]Putative source of hand contamination was defined by *spa* typing of hand isolates. Source attributed to presence of a *spa* type of a hand isolate that was identical to that of a nasal isolate from a persistently or transiently colonised co-worker in the same workplace.

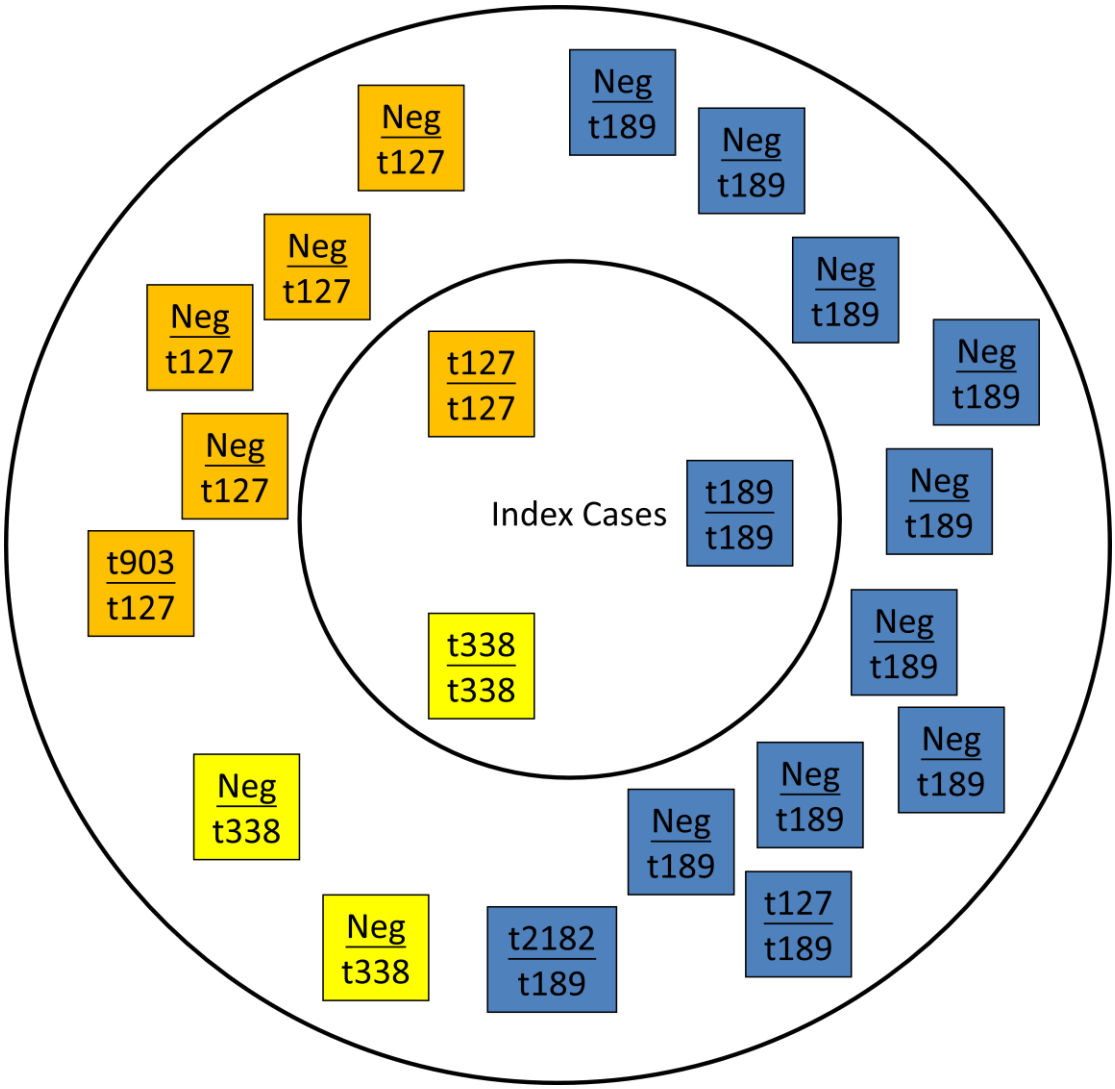
[b]*Spa* type (number of isolates belonging to a given type, shown only if more than one).

[c]*Spa* type and nasal colonization status of hand contaminated subject: P = persistent carrier; T = transient carrier.

[d]Site C: second sample after hand washing training. Eight subjects had left their employment at this time reducing total number to 540.

[e] LMN – three sites where no hand carriage was observed.

Figure 12. Tracking of nose-to-hand spread of *S. aureus* in establishment C.



Each coloured box represents one person. Within each box, upper row indicates the *spa* type of the nasal isolate and that of the lower row belonged to the hand isolate. The same colour indicated the plausible spread from the corresponding index case.

4.8 Antibiotic susceptibility of nasal carriage isolates.

Resistance to ciprofloxacin increased significantly over the period from 1.4% to 7% (p= 0.012). Susceptibility to cefoxitin, chloramphenicol, clindamycin, erythromycin, fusidic acid, gentamicin, oxacillin, penicillin, quinupristin-dalfopristin, tetracycline and trimethoprim-sulfamethoxazole remained largely unchanged. All isolates tested were sensitive to imipenem, linezolid and rifampicin (Table 38).

Methicillin resistance as determined by phenotypic resistance to either oxacillin or cefoxitin in addition to presence of the *mec*A gene in nasal isolates increased over the period from 0.6% (2002) to 0.8% (2003) and 1.2% (2011) (Table 39) but trend analysis did not reach significance. Of the 13 MRSA isolates detected, only five were resistant to more than three classes of antibiotics, and the majority of isolates belonging to SCC*mec* IV or V. One strain isolated in 2002 was typical of HA-MRSA being t189 harbouring SCC*mec* II. However, there was a notable change to predominance of a single spa type, t1081, in 2011.

The presence of SE/SEl genes was not associated with resistance to any antibiotics tested, although MSSA appeared to be more likely to harbour SE genes (82.6%) than MRSA strains (50%) (Table 40). However, resistance to tetracycline (OR= 7.3, 95% CI 2.2 – 23.9, p=0.001) and cefoxitin (OR= 15.2, 95% CI 4.1 – 56.2, p=0.002) was significantly associated with the presence of *qacA/B* gene. Resistance to ciprofloxacin (OR= 7.4, 95% CI 1.4 – 39.4, p=0.050), gentamicin (OR= 4.1, 95% CI 1.7 –

9.8, *p*=0.017) and chloramphenicol (OR= 3.6, 95% CI 1.3 – 10.5, *p*=0.058) also appeared to be associated with *qacA/B* gene carriage, albeit not reaching statistical significance after Bonferroni correction for multiple comparisons. The presence of *smr* gene appeared to be associated with gentamicin resistance prior to Bonferroni's correction but it did not increase the risk for phenotypic resistance to other antibiotics tested (Table 41).

Table 38. Antibiotic susceptibility of *S. aureus* nasal isolates in 2002 and 2011.

Antibiotic[c]	Number of isolates (%), 2002		Number of isolates (%), 2011		*p*-value
	Resistant	Sensitive	Resistant	Sensitive	
CEF	3 (1.4)	214 (98.6)	3 (3.0)	96 (97.0)	0.38[b]
CHL	17 (7.8)	200 (92.2)	7 (7.1)	92 (92.9)	0.81[a]
CIP	3 (1.4)	214 (98.6)	7 (7.1)	92 (92.9)	**0.012**[a]
DA	12 (5.5)	205 (94.5)	4 (4.0)	95 (96.0)	0.78[b]
ERY	32 (14.7)	185 (85.3)	16 (16.2)	83 (83.8)	0.75[a]
FD	25 (11.5)	192 (88.5)	6 (6.1)	93 (93.5)	0.13[a]
GEN	19 (8.8)	198 (91.2)	10 (10.1)	89 (89.9)	0.70[a]
OXA	3 (1.4)	214 (98.6)	5 (5.1)	94 (94.9)	0.11[b]
PEN	169 (78.0)	48 (22.0)	82 (82.8)	17 (17.2)	0.39[a]
QD	3 (1.4)	214 (98.6)	0 (0)	99 (100)	0.56[b]
TET	36 (16.6)	181 (83.4)	20 (20.2)	79 (79.8)	0.44[a]
SXT	6 (2.8)	211 (97.2)	2 (2.0)	97 (98.0)	1.0[b]

[a]Pearson's Chi-squared test. Bold face indicates statistical significance.

[b]Fisher's exact test

[c]CEF, Cefoxitin; CHL, chloramphenicol; CIP, ciprofloxacin; DA, clindamycin; ERY, erythromycin; FD, fusidic acid; GEN, gentamicin; OXA, oxacillin; PEN, penicillin G; QD, quinupristin-dalfopristin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole.

Table 39. Characteristics of MRSA nasal isolates.

Year	Sample	*spa* type	SCC*mec*	Resistance profile[a]
2002	KO02	t189	II	ERY DA SXT GEN CHL TET
n=4 (0.6%)	JC51	t338	IV	ERY TET
	NH38	t437	V	ERY
	LP32	t091	V	TET
2003	FW42	t084	IV	ERY CIP TET
n=4 (0.8%)	FW53	t701	V	ERY DA CIP TET
	FW59	t701	V	ERY DA CIP TET
	HS01	t437	V	ERY DA CIP TET
2011	JC16	t1081	IV	ERY CIP GEN TET
n=5 (1.2%)	JC03	t1081	IV	CIP TET
	JC23	t1081	V	ERY CIP TET
	GM133	t1081	V	FD CIP TET
	JC07	t4981	V	CIP TET

[a] CIP, ciprofloxacin; CHL, chloramphenicol; DA, clindamycin; ERY, erythromycin; FD, fusidic acid; GEN, gentamicin; TET, tetracycline.

Table 40. Association between antibiotic sensitivity and the presence of SE/SEl genes (nasal isolates collected in 2002 and 2011).

Antibiotics[b]		Number of isolates (%)		*p*-value[a]
		SE/SEl		
		Positive	Negative	
Cefoxitin	R	5 (62.5)	3 (37.5)	0.157
	S	254 (82.5)	54 (17.5)	
Chloramphenicol	R	20 (83.3)	4 (16.7)	1.000
	S	239 (81.8)	53 (18.2)	
Ciprofloxacin	R	8 (80)	2 (20)	0.809
	S	251 (82)	55 (18)	
Clindamycin	R	15 (93.8)	1 (6.2)	0.321
	S	244 (81.3)	56 (18.7)	
Erythromycin	R	44 (91.7)	4 (8.3)	0.066
	S	215 (80.2)	53 (19.8)	
Fusidic acid	R	23 (74.2)	8 (25.8)	0.172
	S	236 (82.8)	49 (17.2)	
Gentamicin	R	24 (82.8)	5 (17.2)	0.907
	S	235 (81.9)	52 (18.1)	
Oxacillin	R	3 (50)	3 (50)	0.074
	S	256 (82.6)	54 (17.4)	
Penicillin	R	209 (83.3)	42 (16.7)	0.157
	S	50 (76.9)	15 (23.1)	

Table 40. Continued.

Antibiotics[b]		Number of isolates (%)		*p*-value[a]
		SE/SEl		
		Positive	Negative	
Quinupristin-dalfopristin	R	3 (100)	0 (0)	1.000
	S	256 (81.8)	57 (18.2)	
Tetracycline	R	41 (73.2)	15 (26.8)	0.061
	S	218 (83.8)	42 (16.2)	
Trimethorpim-sulfamethoxazole	R	6 (75)	2 (25)	0.639
	S	253 (82.1)	55 (17.9)	

[a]Comparison of antibiotic susceptibility with the presence of SE/SEl genes by Chi-square test or Fisher's exact test as appropriate. Statistical significance was set at 0.004 (0.05/12).

[b] CEF, Cefoxitin; CHL, chloramphenicol; CIP, ciprofloxacin; DA, clindamycin; ERY, erythromycin; FD, fusidic acid; GEN, gentamicin; OXA, oxacillin; PEN, penicillin G; QD, quinupristin-dalfopristin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole. R, resistant; S, sensitive.

Table 41. Association between antibiotic sensitivity and carriage of *qac* genes.

Antibiotics[b]		Number of isolates (%)					
		qacA/B		*p*-value[a]	*smr*		*p*-value[a]
		Positive	Negative		Positive	Negative	
CEF	R	3 (37.5)	5 (62.5)	**0.002**	0 (0)	8 (100)	1.000
	S	9 (2.9)	299 (97.1)		9 (2.9)	299 (97.1)	
CHL	R	3 (12.5)	21 (87.5)	0.058	2 (8.3)	22 (91.7)	0.143
	S	9 (3.1)	283 (96.9)		7 (2.4)	285 (97.6)	
CIP	R	2 (20)	8 (80)	0.050	0 (0)	10 (100)	1.000
	S	10 (3.3)	296 (96.7)		9 (2.9)	297 (97.1)	
DA	R	1 (6.2)	15 (93.8)	0.470	1 (6.2)	15 (93.8)	0.377
	S	11 (3.7)	289 (96.3)		8 (2.7)	292 (97.3)	
ERY	R	3 (6.2)	45 (93.8)	0.402	1 (2.1)	47 (97.9)	1.000
	S	9 (3.4)	259 (96.6)		8 (3)	260 (97)	
FD	R	3 (9.7)	28 (90.3)	0.102	2 (6.5)	29 (93.5)	0.217
	S	9 (3.2)	276 (96.8)		7 (2.5)	278 (97.5)	
GEN	R	4 (13.8)	25 (86.2)	0.017	4 (13.8)	25 (86.2)	0.005
	S	8 (2.8)	279 (97.2)		5 (1.7)	282 (98.3)	
OXA	R	1 (16.7)	5 (83.3)	0.209	0 (0)	6 (100)	1.000
	S	11 (3.5)	299 (96.5)		9 (2.9)	301 (97.1)	
PEN	R	10 (3.9)	241 (96.1)	0.537	7 (2.8)	244 (97.2)	0.584
	S	2 (3.1)	63 (96.9)		2 (3.1)	63 (96.9)	

Table 41. Continued.

Antibiotics[b]		Number of isolates (%)					
		qacA/B		*p*-value[a]	*smr*		*p*-value[a]
		Positive	Negative		Positive	Negative	
QD	R	0 (0)	3 (100)	1.000	0 (0)	3 (100)	1.000
	S	12 (3.8)	301 (96.2)		9 (2.9)	304 (97.1)	
TET	R	7 (12.5)	49 (87.5)	**0.001**	1 (1.8)	55 (98.2)	1.000
	S	5 (1.9)	255 (98.1)		8 (3.1)	252 (96.9)	
SXT	R	1 (12.5)	7 (87.5)	0.269	0 (0)	8 (100)	1.000
	S	11 (3.6)	297 (96.4)		9 (2.9)	299 (97.1)	

[a]Comparison of antibiotic susceptibility with the presence of *qacA/B* and *smr* genes by Fisher's exact test. Statistical significance was set at 0.004 (0.05/12). Bold face indicates statistical significance.

[b] CEF, Cefoxitin; CHL, chloramphenicol; CIP, ciprofloxacin; DA, clindamycin; ERY, erythromycin; FD, fusidic acid; GEN, gentamicin; OXA, oxacillin; PEN, penicillin G; QD, quinupristin-dalfopristin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole. R, resistant; S, sensitive.

Chapter 5 Summary of Major Findings

5.1 Prevalence of nasal colonisation and hand contamination with *S. aureus*

This work has achieved the aim to compare the nasal colonisation and hand contamination rates amongst food handlers in Hong Kong before, and immediately after, the SARS epidemic with those in 2011. The nasal carriage rates decreased from 35% in 2002 to 23.5% in 2003 and remaining at 22.9% in 2011. In contrast, hand contamination rates continued to decrease over the period: 41.2% (2002), 11.6% (2003) and 3.7% (2011). The sustained reduction in nasal carriage and hand contamination rates over the decade may be attributable to infection control interventions enforced in the community after the SARS epidemic.

5.2 Risk factors for nasal colonisation of food handlers with *S. aureus*

This study has fulfilled the objective to identify risk factors for nasal colonisation with *S. aureus* in food handlers. The recent emergence of LA-MRSA in humans with occupational contact with animals and the high level of raw meat contamination detected in Hong Kong markets led to concern of an increased risk for nasal carriage amongst occupationally exposed workers. Handling of cooked meat (2002) and raw meat (2011) were associated with an increased risk for nasal colonisation with *S. aureus, p*= 0.044 (OR 1.21, 95% CI 1.0 – 1.46) and *p*< 0.001 (OR 2.7, 95% CI 1.7 – 4.5), respectively, although the strains present were not typcal of LA-MRSA. The presence of MRSA carriage was present exclusively in raw meat handlers in both 2002 (Fisher's

exact test, *p*= 0.093) and 2011 (Fisher's exact test, *p*= 0.225) but this did not reach significance. None of the other risk factors investigated were significantly associated with *S. aureus* carriage.

5.3 Prevalence and distribution of genes encoding SE/SEl in nasal *S. aureus* isolates

This study has achieved the aim to determine the prevalence and distribution of genes encoding staphylococcal enterotoxins (SE) and staphylococcal enterotoxin-like proteins (SEl) in all nasal isolates and compare their prevalence between isolates collected at different time periods. Of the three sets of the nasal isolates tested for the presence of SE/SEl genes, 82.9% (n=217), 82.4% (n=136) and 79.8% (n=99) harboured one or more of these genetic determinants, reflecting that the overall prevalence of enterotoxin genes in colonising *S. aureus* strains remained stable over time. While the prevalence of most of the classical enterotoxin genes (*sea, seb, sec, sed*) remained stable over the sampling period, some of the newly described enterotoxin genes increased significantly over the time period.

5.4 Prevalence and distribution of *qac* genes in nasal *S. aureus* isolates

This study has achieved the objective to compare the prevalence and distribution of genes encoding disinfectant tolerance (*qac*) in nasal isolates and to determine the association between the presence of these genes and the type of disinfectant use in sampled establishments. There was a slight increase in *qacA/B* positive isolates over the sampling period, from 2.3% (2002) to 5.1% (2003) and 7.1% (2011), approaching

statistical significance. The *smr* gene was detected in 3.7% in 2002 and rarely present in 2011 (1.0%) of nasal isolates tested and was not detected in 2003. The change of prevalence of *smr* gene between 2002 and 2011 was not significant. Food handlers who worked in the establishments where non-QAC products were used as disinfectants had a reduced risk for carrying *qac* gene positive *S. aureus* (OR 0.91, 95% CI 0.847 – 0.976). The MBC to benzylkonium chloride and chlorhexidine was significantly higher for *qac* gene –positive strains in comparison to –negative strains.

5.5 Distribution of *spa* types and their relationship with virulence genes

This part of the study has completed the aim to determine *spa* types and their relationships with genes encoding enterotoxigenicity and disinfectant tolerance. There was a slight increase of *spa* type diversity from 2002 to 2011. *Spa* typing for 2002 and 2011 nasal isolates revealed 39 *spa* types clustered into 13 *spa* clonal clusters (*spa*-CCs) and 42 *spa* types into 10 *spa*-CCs respectively. The number of *spa*-CC15 associated types reduced significantly from 23% to 11% (p=0.046) whilst *spa*-CC45 associated types increased from 4% to 12% (p=0.004). *Spa* types t189, t213, t888 and t8139 were associated with a lower risk for carrying SE/SEl genes but no other association between *spa* types and presence of virulence genes were observed.The CC45 associated types were significantly associated with resistance to methicillin, ciprofloxacin and tetracycline. The ST30/ ST239 associated types were associated with erythromycin resistance.

5.6 Tracking of transmission of *S. aureus* from nasal carriers to hands

This work has fulfilled the aim to investigate transmission between carriers and non-colonised subjects and to hands. Investigating a subset of the food handlers of whom 9.7% yielded *S. aureus* on hand imprint revealed that 54.7% of these hand isolates were indistinguishable from the nasal isolates of other co-workers who were persistent carriers. Further 18.9% of the hand isolates had *spa* types identical to those of nasal strains from transient carriers. Hand imprint isolates from four workers had the same *spa* types as their own nasally colonised strains. The remaining 18.9% of hand isolates did not match any of the above. This shows the importance of persistant carriers in transmission of *S. aureus* in the workplace.

5.7 Antibiotic susceptibility of nasal carriage isolates

This study has achieved the aim to determine antibiotic susceptibility including methicillin resistance characterization. Resistance to ciprofloxacin increased significantly over the ten year period from 1.4% to 7% (p= 0.012). The level of methicillin resistance increased slowly from 0.6% (2002) to 0.8% (2003) and 1.2% (2011) though not reaching statistical significance. The majority of these MRSA isolates belonged to community associated types. Over the sampling period, there was a change from a more diverse *spa* type to the predominance of a single *spa* type, t1081, in 2011.

Chapter 6 Discussion

This is the first large scale study to investigate the change of *S. aureus* carriage rates among food handlers over time. It investigated nasal carriage and hand contamination, as well as presence of virulence factor and characterisation by *spa* types and antibiotic resistance. Typing allowed for tracking of carriage strains from persistent carriers to hands of food handers. The study commenced before the SARS epidemic thus providing a unique opportunity to investigate nasal carriage and hand contamination rates of food handlers over a decade.

6.1 Prevalence of nasal colonisation and hand contamination with *S. aureus*

No study has investigated the long term changes of prevalence of nasal colonization rates in food handlers. The 23% nasal colonization rate with *S. aureus* in our study is similar to those reported in Ethiopia (Dagnew et al., 2012) and Turkey (Simsek et al., 2009). However, it is lower than the 53.2% carriage rate in food handlers in Kuwait (Udo et al., 2009) and higher than the 15.7% in Brazil (Borges et al., 2010).

Although food handlers were recruited from a wide range of employers, comparison of baseline characteristics revealed that the sample population in 2002 and 2011 was similar. Further stratification of nasal carriage rates by type of workplace, revealed that the workplaces had no effect on prevalence rates, thus confirming that food handlers working in catering establishments in the community did not differ from those in the hospitals or sport facility where an epidemiological link to *S. aureus* or

MRSA spread was previously reported (Oller et al., 2010; McKinnel et al., 2013). This may be explained by the fact that food handlers working for the local hospitals did not have occupational contact with healthcare workers or patients. Porters, who were responsible for transferring foods from the centralized kitchen to wards, were excluded from the study. Food handlers employed in the sports facility had a separate bathroom and changing facility from sports participants and so had no opportunity to share equipment or towels which have been shown to be a risk factor for carriage (McKinnel et al., 2013).

Compared with other occupations in the territory, food handlers are a mobile population with a relatively high turnover rate of staff. Therefore, it would be unlikely to recruit the same individuals after a decade. Despite this, we managed to repeatedly sample 80% of the subjects recruited in the first phase of the study (2002) in 2003. These subjects were included in persistent carriage determination. In the more recent cross sectional survey (2011), only two establishments sampled in the earlier studies were included (comprising 32% of the sample population) as permission to sample at some of the workplaces visited in the first phase of the longitudinal study was not granted. Thus it was important to ensure baseline characteristics of these populations were similar.

The characteristics of the study population in 2002 – 2003 and 2011 were similar except for the proportion of male gender and smokers. These were found to be associated with each other. Neither gender nor smoking was associated with *S.*

aureus colonisation. In addition, the large sample size allowed accurate estimation of carriage rates.

Although nasal colonisation rate may vary temporally, the change does not usually reach statistical significance. In a nine month follow up of *S. aureus* nasal carriers, colonisation rates varied by only two percent (Sakwinska et al., 2010). In one establishment where we sampled twice before the SARS epidemic, the nasal carriage rate did not vary although different individuals were positive on different occasions, neither did the change of hand contamination rate reach statistical significance. These results suggested the presence of an extraneous factor which may lead to the change in carriage rates in our study.

The decrease in *S. aureus* carriage rates suggested that the government enforced hygiene practices implemented in the local food premises during and after the SARS epidemic in response to the outbreak was effective. However, further investigations are needed to confirm the association. The hygiene measures included the mandatory use of gloves and masks during the SARS epidemic. Proper hand washing training was provided to food handlers. From 2005, each food premises was required to employ at least one hygiene supervisor, depending on the size of their premises, to monitor hygiene standards including hand washing practices of their food workers. In addition, inspection of the local food premises became more in-depth and the level of hygiene of each establishment is graded according to Australian and New Zealand criteria and outcomes published and made publicly available (Department of

Health, 2010). Overall, these measures could minimize the contamination of the workplaces and hands of *S. aureus* nasal carriers which in turn would reduce the spread to transient carriers resulting in lower colonisation rates as observed in 2003 and 2011. Transient carriers likely acquired *S. aureus* by touching their noses or face with their contaminated hands. Their hand contamination would have been a result of contact with the food they manipulated or the environmental surfaces. Persistent nasal carriers are known to be more likely to have their hands and surrounding environment contaminated because they harbour a greater load of *S. aureus* compared to transient carriers (Wertheim et al., 2005). Workers who used gloves at work would be less likely to touch their noses and hence reduce opportunities for self-colonisation. Furthermmore, regular disinfection of the food preparation surfaces also decreases microbial load in the working environment. However, the use of disinfectant, particularly QAC compounds, may have selected for disinfectant tolerant strains. This will be discussed further in section 6.4.

Alternatively, epidemiological changes of dietary pattern, choice of clothing and bacterial factors may also accountable for the reduction of carriage rates observed. Intake of yogurt has been shown to reduce nasal colonisation with *S. aureus* (Gluck & Gebbers et al., 2003). Wearing silver coated textile can also minimise colonisation (Gauger et al., 2003). If the circulating *S. aureus* coloniser strains have evolved to be less adhesive over time, less colonised subjects would then be expected. This would

have to be investigated by analysis of adhesion and other genes associated with colonization which is beyond the scope of this study.

Despite the overall decrease of nasal carriage and hand contamination rates between 2002 and 2003, the degree of reduction in supermarkets was significantly less than that in other establishments. In supermarkets, the overall nasal colonisation and hand contamination rates were reduced by only 5.2% and 25%, respectively, as compared to 20% and 56% reduction in other establishments. Consistent with this, the overall hand contamination rates in supermarkets remained high (17%) in 2003 as compared to that in other establishments (3%). However, the overall nasal and hand contamination rates in supermarkets were lower than that in other establishments prior to the SARS outbreak, though not reaching statistical significance. The initial lower carriage rates of food handlers in supermarkets indicated that the establishments were relatively better at implementing hygiene measures before the introduction of hygiene measures in 2003. This might have led to a lower requirement for change in order to comply with the government enforced hygiene measures resulting in a lesser degree of reduction.

Interestingly, a similar drop in the nasal colonisation rate was noted in a national survey of non-institutionalized US citizens from 32.4% in 2001 – 2002 to 28.6% in 2003 – 2004 (Gorwitz et al., 2008). This may be the result of global efforts made during the SARS pandemic to improve hygiene for prevention of coronavirus transmission. A similar effect was also observed in studies of the general public

conducted in Hong Kong. In early 2002, the reported carriage rate among local young adults was 35% (O'Donoghue & Boost, 2004). A recent survey revealed that the carriage rate had fallen to 24% (Zhang et al., 2011). In the months after the epidemic in Hong Kong, telephone surveys revealed a sustainable increase in self-reported hand hygiene practices amongst the general public (Lau et al., 2005). In the survey, over 55% of the respondents had attained only secondary level education or less which was typical for food handlers in the territory.

Since 2003, although the enforced practices of use of gloves and proper hand hygiene has been sustained, we observed that use of masks has become less common amongst food workers, except for those handling sensitive foods such as ready-to-eat cooked meat, and staff displaying respiratory symptoms. Therefore, the sustained low carriage rates highlighted that hand hygiene practices and use of gloves are important in the control of staphylococcal dissemination.

The reduction of nasal colonisation rates was not significantly correlated to that of hand contamination rates, suggesting that there were determinants other than hand contamination contributing to nasal colonization. This is consistent with the multiple determinant nature of *S. aureus* nasal colonisation in humans (Sollid et al., 2014).

In the 2011 cross-sectional survey, the hand carriage rate of food handlers in the centralised kitchen for local hospitals was significantly higher than that of other workplaces. This kitchen is a semi-automatic centralized kitchen shared by 40 workers per shift supplying more than 2000 meals daily. Despite the high degree of

automation, the work load was obviously greater than that in other establishments. The great workload could affect opportunities for personal hygiene and might increase the chance of environmental contamination. Indeed, insufficient time and high workload has been shown to be a barrier to hand washing among food workers in a focus group study conducted by Pragle et al. (2007). More recently, a large variation of hand contamination rates amongst food handlers from different establishments was observed in a study in South Africa (Lambrechts et al., 2014).

6.2 Risk factors for nasal colonisation of food handlers with *S. aureus*

Although numerous risk factors associated with colonisation were investigated, only contact with meat appeared to increase the risk after controlling for confounders by multiple logistic regression. Handling raw meat was found to be a significant risk factor in the 2011 study and approached statistical significance in 2002. The association might have been obscured by the presence of more transient carriers in the first sample. Handling cooked meat was also associated with *S. aureus* nasal colonisation in 2002. It was noted in the longitudinal study that more than half of the meat handlers processed both raw and cooked meat at work, resulting in significant overlap of these exposures. In the cross-sectional survey, 57% of the operatives handled raw meat, predominantly pork, beef and chicken, these workers were at significantly higher risk for nasal colonisation with *S. aureus* (30%) as compared to non-exposed workers (13.4%). This was consistent with a report from the

Netherlands in which greater proportion of meat handlers were found to carry MSSA than the general public (33% versus 24%) (de Jonge et al., 2010).

The increased risk of nasal carriage was confirmed by stratification of nasal colonisation rates of meat handlers by levels of exposure which revealed a significant positive trend. Compared to food handlers who never handle raw meat, subjects that reported "sometimes" handling raw meat at work experienced a two-fold increased risk for nasal colonisation whereas those who reported "always" had a nearly four-fold increased risk.

Studies on association between gender and *S. aureus* nasal colonisation have yielded conflicting results. One study involving more than 4,000 patients prior to surgery, reported that male gender was a risk factor for nasal colonisation (Herwaldt et al., 2004), while in another study, screening of hospital personnel for *S. aureus* carriage failed to establish any statistical association with gender (Eyoh et al., 2012). In our study, gender appeared to be a confounder for nasal colonisation because the majority of meat handlers were male.

Investigation of the effect of smoking on nasal carriage of *S. aureus* found a higher prevalence in smokers (Durmaz et al., 2001). However, smoking was not identified as a risk factor in this study but was found to be associated with male gender. Although the proportion of males in the 2011 study was somewhat higher than in the 2002-2003 sample, the nasal colonization rates remain similar to that of 2003 suggesting that gender is not a risk factor. Colonisation in food handlers reporting either allergic

rhinitis or acne was not increased in contrast to reports elsewhere (Shiomori et al., 2000; Khorvash et al., 2012). Further work is needed to determine if these factors are important in colonization of food handlers. Other previously reported risk factors for colonisation of patients and healthy individuals including recent antibiotic use or hospitalization, and contact with family members who are healthcare workers (Wertheim et al., 2005b; van Nguyen et al., 2014), also did not reach significance. This may be attributable to the small number of food handlers reporting these exposures, in contrast to the subjects of other healthcare based studies.

The prevalence of methicillin resistance among food handlers was comparable to that observed in the general public of this region (Zhang et al., 2011), indicating that food handling may not be a risk factor for MRSA carriage. This was in contrast to our previous findings that butchers handling raw meat exhibited a five-fold increased risk for colonisation with MRSA (Boost et al., 2013b). The risk in butchers may be elevated because they are additionally responsible for primary cut-up of whole pig carcasses, including the snout which has previously been shown to be highly contaminated (Ho et al., 2012). A similar situation was also observed in the Netherlands where workers handling live animals had significantly higher risk of MRSA carriage (Voss et al., 2005) but operatives occupationally exposed to raw meat did not have an increased risk despite 14% of meat samples processed in the production plants being contaminated with MRSA (de Jonge et al., 2010).

In 2011 cross sectional survey, MRSA was exclusively isolated from raw meat handlers and belonged to *spa* type t1081 and a related type, t4981. However, neither t1081 nor t4981 was detected in our previous study on butchers in the territory (Boost et al., 2013a). Interestingly, nasal isolates of live-stock associated t701-MRSA-II colonizing two butchers (Boost et al., 2013a) were detected in nasal MRSA isolates of food handlers as t701-MRSA-V in 2003, indicating that this MRSA strain may have been circulating in the region for some time and possibly associated with meat handling. Due to the small number of MRSA isolates yielded in our study, the association between *spa* types and meat handling cannot be confirmed. The putative animal associated strain t701-MRSA-V has been reported in human clinical isolates (Kinnevey et al., 2014). MRSA t701 belongs to CC6 and has been shown to harbour *sea* but to be negative for PVL (Monecke et al., 2012). One 2003 t701 MRSA and one MSSA isolate also carried *sea* but this enterotoxin gene was absent in the second MRSA t701 isolate.

Contamination of chicken meat by spa types t034 and t002 MRSA appears to be rather common (23%) locally (Boost et al., 2013b). MSSA of t034 and t002 were also detected from meat handlers in this study. Both t034 and t002 MRSA have also been isolated from swine and have been reported in human infections (Kock et al., 2013). It was possible that some food handlers in our study became colonised with these strains because of extended time handling potentially contaminated meat.

The majority of *spa* types detected in meat handlers in the 2011 study were absent in the previous survey on butchers (Boost et al., 2013b). Selective medium for MRSA was employed in the butcher study precluding isolation of MSSA colonizing these meat handlers.

Though not reaching significance, it appeared that carriage strains of food handlers exposed to raw meat were more likely to be t189, t127 and t034 (32%) than those of food handlers not exposed at work (16%). In a large scale environmental study of pork production facilities in the United States, MRSA *spa* types t189 and t034 were detected in the workers' shower and changing facilities as well as from the sow site (Larson et al., 2011). Although the isolates in our study were methicillin sensitive, the isolation of these *spa* types indicated that the coloniser strains of food handlers may have been derived from contaminated meat they handled.

Dairy products, chicken and ham have been reported to be an important reservoir for staphylococcal enterotoxins (Chomvarin et al., 2006; Principato et al., 2009; Hwang et al., 2010; Hu et al., 2012). It is therefore reasonable to expect that food handlers in contact with these foods would exhibit an increased risk for colonisation with enterotoxigenic *S. aureus* strains. However, in this study, the presence of SE gene in *S. aureus* colonizing strains of exposed and non-exposed food handlers did not differ significantly.

6.3 Prevalence and distribution of genes encoding SE/SE*l*

Studies on carriage of SE genes in human isolates have largely focused on the classical types. In contrast, this study simultaneously investigated both the classical and novel types of enterotoxins. Of the three samples collected over a decade, more than 70% of *S. aureus* nasal isolates harboured at least one SE gene. This is in agreement with the occurrence of SE determinants as confirmed by PCR reported in 71.2% of *S. aureus* strains colonising food handlers in Kuwait (Udo et al., 2009), 73% from clinical isolates in Germany (Becker et al., 2003) and in 68.4% isolates from milk (Rall et al., 2008) but considerably lower than 95% in food worker associated carriage isolates in Brazil (Rall et al., 2010) and 91.8% in food poisoning outbreak associated *S. aureus* strains in Taiwan (Chiang et al., 2008). All these studies used PCR to detect the presence of classical and novel SE. However, a direct comparison cannot be made because of the inclusion of a different spectrum of SE determinants in each study. Nevertheless, it is evident that the majority of *S. aureus* strains are potentially enterotoxigenic because all SE genes are actively transcribed during cell growth (Derzelle et al., 2009).

Although the overall prevalence of SE genes remained similar over the period from 2002 to 2011, only frequency of *egc* associated determinants and those encoding SEA, SEB and SEC did not change significantly. All these determinants are chromosomally located (Arguidin et al., 2010). This is in contrast to findings in Spain

where rates of SE genes present were shown to have increased between 1997 and 2006 (Argudin et al., 2013). It has been reported that the *egc* associated enterotoxin genes are more commonly found in carriage than invasive isolates and are less immunogenic compared to other enterotoxins (van Belkum et al., 2006). A large scle study in the Netherlands revealed that the egc group was present in a high proportion of carriage strains (63.9%) (van Trijp et al., 2010). It was therefore not surprising to detect a stable proportion of *egc* genes in our strains as all isolates tested originated from asymptomatic nasal carriage in humans.

A significant increase or decrease of prevalence was observed amongst other SE genes. Of note, the SE genes encoding SEJ, SER, SES and SET increased considerably over the period. Initial identification of *ser* led to isolation of numerous plasmid vectors carrying this gene (Omoe et al., 2003), suggesting that a wide range of plasmids could be recipients of *ser*. Further characterisation of the plasmids (pF5) carried by the Fukuoka 5 *S. aureus* strain previously isolated from a SFP outbreak in Japan in 1997 found that *ser* co-existed with *sej*, *ses* and *set* (Ono et al., 2008). The simultaneous increase in incidence of the genotype *sej-r-s-t* indicated that our strains may harbour this plasmid. The selection of this genotype over time may imply the presence of an unknown determinant benefiting survival associated with these genes. Further investigation is needed to confirm this hypothesis.

If the entire SE gene profile is considered, genes encoding novel SE types were more commonly detected than those encoding classical SE types. It was noted that the

majority of our isolates positive for novel SEs did not harbour classical SE determinants. This has also been observed in other studies (Akineden et al., 2001; Blaiotta et al., 2004). However, most public health laboratories include only classical SE types in food poisoning outbreak investigations, indicating the potential of failure to identify an outbreak caused by a novel toxin released by a classical SE strain. Testing for presence of novel SEs should be considered.

Investigation of the production of classical SE in *S. aureus* isolates ranged from 14% in Egypt (El-Shenaway et al., 2014) to 86.6% in Kuwait (Al-Bustan et al., 1996). This discrepancy may be attributable to variation in detection methods. Production of classical enterotoxins was determined by agar diffusion in the 1996 study whereas latex agglutination was used in the later study. A recent study in Botswana investigating the production of SEA through D using latex agglutination found 21.1% isolates positive for these toxins (Loeto et al., 2007).

Of the classical SE types, *sea* was the most prevalent in our isolates over the three samples. The long term circulation of the *sea* gene could be partially explained by its ability to propagate in alternative bacterial hosts other than staphylococci such as *Pseudomonas* (Casas et al., 2010). Type A enterotoxin, being able to retain residual amount of biological activity after exposure to 121^{o}C for 28 minutes, is the most heat resistant amongst all SEs so far identified (Pepe et al., 2006). It is also the most frequent identified agent responsible for SFP outbreaks worldwide (Argudin et al., 2010). The percentage of our isolates positive for *sea* was similar to those reported

in colonizing strains from healthy subjects in Switzerland (Wattinger et al., 2012), but considerably higher than those of medical students in the Czech Republic (Piechowicz et al., 2011).

Of the novel SE types, the most common were SEK and SEI, either alone or in combination with other enterotoxins. These two SE determinants are phylogenetically related to each other and that have been suggested to be associated with atopic dermatitis (Orwin et al*.,* 2001; Na et al., 2012). However, only three percent of our subjects reported suffering from dermatitis. Of the *S. aureus* strains carried by these subjects, *sek* was detected.

Although co-existence of SE genes is common, there is no evidence so far indicating a positive correlation between virulence and the number of SE determinants. Holtfreter et al. (2007) suggested that the presence of SEs strongly influenced invasiveness and was strongly associated with clonal cluster. Van Trijp et al (2010) reported that egc was more common in CC5, CC25, CC30 and CC45 than other carriage isolates. Conversely, interference between SE determinants has been documented by other researchers. In the case of co-existence of SEB and SEP, all $seb^{+}sep^{+}$ strains failed to produce *sep* whereas $seb^{-}sep^{+}$ strains did (Omoe et al., 2005). In *S. aureus* isolates concomitantly carriing *sed* and *ser* genes using ELISA, SED was not detectable, high level of SER was produced (Lis et al., 2012b). Further work is needed to elucidate interactions between the wide array of SE genes.

PCR was chosen to determine the presence of enterotoxin genes rather than immunological methods which detect enterotoxin proteins produced *in vitro* as there are no immunological methods available for detecting novel enterotoxin types. Active transcription of all SEs identified to date have been demonstrated in laboratory controlled conditions (Lee et al., 2007; Ono et al., 2008; Omoe et al., 2013). Although the expression of novel SEs in food is not clear, it is likely to vary according to multiple factors such as pH, water activity and temperature, as is the case for classical toxins (Derzelle et al., 2009). The presence of genes may probably be a good predictor of enterotoxin production. Due to the significant amount of nucleotide sequence homology between subtypes of the determinants, the primers targeting *sec* and *selu* in our study did not differentiate between *sec* subtypes and *selu* variants and it was not possible to differentiate *egc2* from *egc3* and *egc4* (Collery et al., 2009).

Interestingly, the newly reported SE genes were detected in our historical isolates, suggesting long term circulation of these determinants before their recognition and may explain some food poisoning outbreaks. There have been few reports of detection of these novel SEs involving strains isolated before recognition of these toxins. A small study of *S. aureus* isolated from food investigated presence of *sea* – *sep* in current isolates and was able to detect all toxin genes known at that time (Bania et al., 2006a). More recently, an investigation of a small collection of nasal isolates dating from 1997 to 2006 revealed the presence of all SEs except ses and set

over these period (Argudin et al., 2013). Examination of other *S. aureus* isolate collection may reveal earlier presence of these determinants, which are certainly not as novel as initially thought. Their presence in isolates in a wide range of geographical locations (Hwang et al., 2010; Nienaber et al., 2011; Argudin et al., 2013; Xing et al., 2014) indicates spread of these determinants over some time and their detection in older isolates is therefore not surprising as some time would have been needed for this widespread dispersion although genetic recombination resulting in new enterotoxin genes may have occurred (Jarraud et al., 2001).

Currently, Hong Kong public health laboratory detects only enterotoxin type A to D in food poisoning outbreak investigations. If there were other SE responsible for the outbreaks, the link would be missed. Thereby, detection of the complete spectrum of SE present in S. aureus isolates may reveal their high prevalence and necessity to include a wider spectrum of SE for SFP outbreak investigation. As one of the major contributors to SFP is contamination by food handlers, examination of these strains may reflect those involved in local outbreaks. Indeed, colonizer strains of food handlers have been implicated in SFP outbreaks in Hong Kong (CHP, 2011), these can be a reasonable alternative to evaluate the importance of the whole spectrum of SE gene that may be of public health concern in the food industry.

As long as good personal hygiene is maintained and contamination of food with enterotoxigenic *S. aureus* strains minimized, food poisoning outbreaks can be

avoided even if most colonising strains carry SE genes. Proper storage of food is also important in preventing food poisoning outbreaks.

According to a recent review on staphylococcal food poisoning in Hong Kong, SEA is the most common causative toxin responsible for outbreaks (CHP, 2011). However, only enterotoxin A to D were investigated. Strains are currently not typed for presence of other SEs.

6.4 Prevalence and distribution of *qac* genes

This was the first study to determine the prevalence of *qac* genes in *S. aureus* isolates nasally colonising food handlers. In addition, the association between the use of disinfectant types and the occurrence of *qac* genes was also investigated.

In response to the SARS epidemic in 2003, a range of hygiene measures including tightened regulations for controlling food safety in the territory were launched under the scheme of "Team Clean". The enhanced hygiene measures, including regular use of QAC containing disinfectants in the food industry, has led to concerns about the possibility of selecting QAC resistant staphylococci. Of the approved bactericidal agents for use in the local food establishments provided by the Food and Environmental Hygiene Department of the Hong Kong Government, 27% contain QAC. Approximately half of these were approved for use in the food industry following the SARS epidemic. Their increased use may be attributable to the reported superior efficacy of QAC compared to other cleaning agents in food premises (Lalla & Dingle, 2004).

Trend analysis revealed that the increase of *qacA/B* genes over time was approaching statistical significance but the occurrence of the *smr* gene remained stable over time. This suggests that the increased use of QAC may preferentially select *qacA/B* genes. Of note, the smaller numbers of *S. aureus* isolates in 2003 and 2011 samples may have prevented the positive trend reaching significance. To give an estimate with 95% confidence intervals with 5% standard error, a minimum of

139 *S. aureus* isolates would be required in each sample. This is based on a 10% prevalence of *qacA/B* genes previously reported in *S. aureus* colonising the general public in Hong Kong (Zhang et al., 2011).

The prevalence of *qacA/B* and *smr* genes has been comprehensively studied in staphylococci of clinical origin, with emphasis placed on methicillin resistant strains (Leelaporn et al., 1994; Mayer et al., 2001; Alam et al., 2003; Wang et al., 2008; Marchi et al., 2014). Screening of clinical isolates of MSSA from various European countries revealed the presence of *qacA/B* and *smr* in 12% and 5% of isolates, respectively (Mayer et al., 2001). In contrast, our study found a lower prevalence of these antiseptic resistance genes: 7.1% for *qacA/B* and 1% for the *smr* gene. This may indicate geographical differences and distribution.

There has been concern about effect on tolerance to disinfectants following increased use of QAC containing solutions. It is suggested that exposure to low level residue of these agents may lead to selection of strains with *qac* genes. This is particularly important because of the widespread use of chlorhexidine bathing for decolonization of MRSA patients (Batra et al., 2010; Edgeworth, 2011). An MRSA isolate TW (ST239) was shown to be resistant to the chlorhexidine decontamination process and carried *qacA* (Holden et al., 2010).

Presence of *qacA* in blood stream infection isolates was shown to be increased following the introduction of chlorhexidine bathing in a hospital infection control program (Otter et al., 2013b). Higher frequency of *qacB* gene in this study may be

attributable to different exposure of the colonising strains to disinfectants in different settings. This is in consistent with a study of MRSA isolates, revealing that *qacB* was more common than *qacA* (Longtin et al., 2011). Our *qacA* positive isolates displayed considerably higher MIC to benzylkonium chloride and chlorhexidine than strains harbouring *qacB* gene, confirming that *qacA,* but not *qacB,* contributes to a higher resistance level to chlorhexidine (Littlejohn et al., 1992). In our study, between 2002 and 2011, the incidence of *qacB* increased but not *qacA*, despite our *qacA* isolates having higher MICs to disinfectants tested. In agreement with the literature, *qacA* but not *qacB* confers higher level of resistant to disinfectants (Longtin et al., 2011; Otter et al., 2013b).

Of studies performed in the community, *qac* gene positive *S. aureus* has been detected in ATM machines, public trains and healthy people (Zhang et al., 2011; Zhang et al., 2012; Zhou & Wang, 2013).

Although both the MIC and MBC of our isolates to BC and CHX were far below the in-use concentration recommended by the manufacturer, the results reflect only the well-controlled environment in the laboratory where planktonic cells were tested. In real life situations, staphylococci may grow in biofilms which increases their horizontal gene transfer rate by 600-fold and genes present in mobile genetic elements are usually up-regulated when bacteria grow in biofilm (Fux et al., 2005). The ability of *S. aureus* to form biofilm has been demonstrated on different materials including polystyrene, stainless steel, and rubber surfaces (Lee et al., 2014). In

addition, the bacterial population present in the core of a biofilm are physically protected from the biocidal effects of disinfection and likely to be exposed to a sub-lethal concentration of the applied disinfectant. Repeated exposure to low levels of disinfectant up-regulates the expression of efflux pumps mediating for resistance to QACs (Huet et al., 2008). The subsequent propagation of a more resistant clone replacing sensitive strains may further disseminate these bacterial strains or the disinfectant resistance determinants to other bacterial hosts (Dantas et al., 2008). Hospital isolates with higher MICs to chlorhexidine were more lilely to be involved in blood stream infection (Otter et al., 2010). The survival of staphylococci subsequent to disinfection is of particular concern in hospital kitchens in which food may be served to immunocompromised patients. Survivial of such strains which may also harbour SE genes may lead to food poisoning or a rare but fatal form of enterocolitis in susceptible hosts (Kotler et al., 2010).

As the use of QAC containing disinfectants has become more common in the food industry, the prevalence of staphylococci harbouring QAC resistance genes is likely to continue to increase. The isolation of *qacA/B* positive staphylococci carried by food handlers indicated that exposure to disinfectants may increase risk for colonisation with *qac* gene positive strains. This is supported by the presence of *qac* positive *S. aureus* carried only by food handlers working in establishments where QAC disinfectants were used. Food handlers carrying QAC positive staphylococci may act as reservoirs for dissemination of these antiseptic determinants in the community.

Since QAC resistance determinants also co-exist other antibiotic resistance genes, the spread in the community would be a concern. Therefore, continuous monitoring of the occurrence of *qac* gene positive staphylococci colonising food handlers may be necessary.

The association between use of disinfectant type and prevalence of carriage of *qac* gene positive *S. aureus* was consistent with the observation that workers with daily working hours longer than nine were at twenty fold higher risk for carrying *qac* gene positive staphylococci. For colonized workers, their number of years of experience appeared to be positively associated with the likihood for *qac* gene carriage. These findings indicated that prolonged exposure to QAC compounds predisposes individuals to be colonized with strains harbouring genes for disinfectant resistance. In the clinical setting, patients exposed to chlohexidine daily were more likely to suffer from invasive infection caused by organisms with reduced susceptibility to chlorhexidine (Suwantarat et al., 2014).

However, the association has to be interpreted carefully. There are possible confounders which exist because of the presence of QAC compounds in personal products other than disinfectants. QACs are present in many products such as cosmetics, body moisturizers, suncreen applications, fabric conditioners and pain relief topical cream (Hegstad et al., 2010). Exposure to any of these items may increase risks for being colonised with *qac* gene positive staphylococci. However, these exposures were not known for our subjects.

The genes *qacG/H/J* were not detected in any of the isolates in our study. These genes were initially reported in animals (Bjorland et al., 2005). They appear to be rare in human nasal carriage isolates, ranging from 0.26% to 1.03% (Ye et al., 2012). Their low prevalence in human carriage isolates suggests that these animal-associated QAC resistance determinant variants may be host specific. Interestingly, these genes have been reported in clinical isolates of *S. haemolyticus* (Correa et al., 2008).

Although both enterotoxin genes and QAC resistance genes are present in mobile genetic elements, these determinants did not appear to be co-selected. This is consistent with the previous findings that enterotoxin was not produced by QAC positive strains (Heir et al., 1999).

6.5 Distribution of *spa* types and their relationship with virulence genes.

In both 2002 and 2011, the most frequently detected *spa* type was t189. Isolation of t189 as the most common *spa* type, representing 19% of the isolates in both years is noteworthy because this spa type is rarely reported as a human nasal commensal. A large scale cross sectional survey of human *S. aureus* nasal carriage involving more than 30,000 participants from nine European countries failed to detect t189 among methicillin resistant strains (den Heijer et al., 2013). However, *spa* typing was not performed on methicillin sensitive isolates which are known to differ from methicillin-resistant counterparts. In Hong Kong, screening of two to five year old children revealed that 12% of their carriage MSSA isolates belonged to t189 (Ho et al., 2012b), indicating that this strain is indeed ommon in Hong Kong.

In a large-scale animal study, cats and dogs appeared to be reservoirs of t189 *S. aureus* (Ho et al., 2012c). However, only three of the 17 food handlers carrying t189 MSSA reported owning a dog or a cat. Recently, genome sequencing of a bacteraemia associated MRSA strain belonging to ST 188, *spa* type t189, revealed that this clone, while negative for PVL, harboured several virulence genes which encode human immune evasion factors responsible for the suppression of chemotaxis, complement activation and phagocytosis (Ip et al., 2014), making it more likely to survive in nasal cavity. It has been suggested that *S. aureus* t189 may acquire methicillin resistance under selective pressure. In China, *S. aureus* ST188-t189 was reported as one of the most common clones responsible for bacteraemia

(Yu et al., 2012), highlighting the pathogenic potential of t189 *S. aureus* in humans. Therefore, food handlers carrying *S. aureus* t189 may have an increased risk for infection.

MSSA t189 was reported as the second most common *spa* type in clinical isolates in mainland China (Chen et al., 2014), predominantly originating from Sichuan and Jiangxi provinces where pig farming is an important commercial activity. These provinces supply 5,000 live pigs daily to Hong Kong for local consumption. Screening of *S. aureus* in a pig production facility found t189 in a sow site and a shower facility for workers (Larson et al., 2011). It would be possible that some of the food handlers were colonised while handling t189 *S. aureus* contaminated raw pork supplied from these Chinese provinces.

In 2011, 42 *spa* types were detected, the three types most frequently observed being t189 (17%), t127 (8%) and t034 (3%). These *spa* types were more frequently isolated from food handlers exposed to raw meat, but this did not reach significance. The *spa* types t189 and t127 belong to CC1 which includes ST1 and ST188 whereas t034 has been reported in both ST1 and ST398 in MRSA (Vanderhaeghen et al., 2010, Franco et al., 2011, Larson et al., 2011). The widely disseminated live-stock associated *S. aureus* ST398-t034 is methicillin resistant. However, all *S. aureus* t034 in our study were sensitive to methicillin. It has been suggested that the clone t034 might have initially originated from humans and lost human immune modulating factors with the acquisition of cassette chromosome *mec* during the human-to-

animal transfer (Price et al., 2012). Nevertheless, the existence of MSSA t034 in live-stock cannot be excluded because most studies have investigated only MRSA so the presence of MSSA could have been missed. A recent Japanese study reported MSSA t034 of swine origin (Asai et al., 2012).

The distribution of *spa* types identified in our study differed from those recently reported in local clinical isolates of MRSA, in which the most prevalent *spa* type was t1081 (50%) (Cheng et al., 2011). This spa type represented only 8% of our isolates, whilst the most common *spa* type, t189, in our study was rare in clinical isolates (Cheng et al., 2011). However, as the survey of clinical isolates only examined MRSA, the *spa* types present are likely to differ from *S. aureus* carriage isolates, which comprised of mainly MSSA.

Five MRSA isolates were detected in the 2011 cross-sectional survey. Four MRSA strains belonged to t1081 and one to a related *spa* type t4981, which is a single repeat deletion variant of t1081. Interestingly, t4981 MRSA has only been previously reported in monkeys (van den Berg et al., 2011). Three of the subjects colonised with t1081 worked in close proximity in the same establishment, indicating the possibility of cross-contamination between these operatives.

A recent study of local pork butchers (Boost et al., 2013) revealed a high colonisation rate with the most common Asian linage of LA-MRSA, ST9. This strain was not isolated possibly due to a wider range of meat types and sources used in the catering establishments than the local wet markets. This is supported by the clonal diversity

observed in food handlers being greater than that in butchers. Once again however the study of butchers focued on MRSA and so did not provide any data on MSSA carriage or *spa* types.

Though the exposures of the patients to livestock was unknown, clinical isolates in Europe yielded t127 (Franco et al., 2011) and both t189 and t127 in Malaysia and China (Ghaznavi-Radl et al., 2010; Yu et al., 2012). Comparison of human and swine t127 isolates indicated that these consisted of two distinct clones (Franco et al., 2011), suggesting that *S. aureus* strains of divergent origins may belong to the same *spa* type. As eight isolates of t1081 *S. aureus* strains were found in meat handlers, it is possible that these were livestock associated if this clone had been introduced into the pork production facilities. Further work to compare these strains with clinical isolates of t1081 with respect to the presence of virulence factors and clonal similarity is needed.

Typing of isolates in our study was restricted to *spa* typing of colonising isolates. Since access to food in the kitchens was not permitted, there was no attempt to match isolates from food handlers to those of the food being prepared. There have been some studies that have demonstrated a relationship between strains in food handlers and srains isolated from foods using *spa* typing (Arguidin et al., 2012). It is suggested that pulsed field gel electrophoresis (PFGE) can distinguish between isolates of the same *spa* type making this a more discriminatory tool. However, its use in the food industry is limited and there appears to be only one published study

which examined staphylococcal isolates from food handlers and dairy products (Tondo et al., 2000). Other studies using PFGE have documented indistinguishable isolates from food involved in food poisoning outbreaks and those colonizing nares or hands of food handlers (Haga et al., 2007; Yan et al., 2012; Gallina et al., 2013).

The distribution of clonal lineages in specific foods is largely unknown, as most published reports did not include this data (De Boer et al., 2009; Di Giannatale et al., 2011; Hu et al., 2013). However, a recent study did report the *spa* types present in food and found these predominantly belonged to CC5, CC30, and CC45 (Arguidin et al., 2012). Clones belonging to these *spa* types were also found in nasal isolates of our study, indicating their potential as food contaminating strains.

There was a considerable change of circulating *spa* types between 2002 and 2011. In particular, it was observed that *spa*-CC15 incidence fell while that of *spa*-CC45 increased significantly over the period. Although only nine *spa* types were detected in both samples, it appeared that some clones have been circulating in the region for more than a decade.

Examination of the rate of mutations in the *spa* locus revealed that there had been only two mutations over the period of 445 sample months which equates to a molecular clock of one per 223 months in the *spa* locus. This mutation rate is considerably slower than those previously reported (Kahl et al., 2005; Sakwinska et al., 2010), indicating that colonising strains in our subjects rarely undergo mutations. A possible explanation for the observed differences in mutation rates may be

variation in selective pressure on the strains in the studies. The subjects in the previous studies were exposed to health-care environments where antimicrobial agents are regularly used. They were either cystic fibrosis patients or health care workers whilst in our study subjects were food handlers working in the community where the selective pressure is lower.

6.6 Tracking of transmission of *S. aureus* from nasal carriers to hands

In this study, *spa* typing was used to investigate the transmission dynamics of *S. aureus* amongst food handlers. This is limited by the fact that certain routes of transmission may be indifferent due to its low discriminatory power compared to more recently introduced teachniques such as whole genome sequencing (WGS). Strains differentiable by WGS may otherwise be indistinguishable using *spa* typing which takes only a portion of the bacterial genome into account. The use of WGS enables the identification of single nucleotide polymorphism (SNP) such as insertions or deletions (Harris et al., 2010). This provides additional information that may increase discriminatory power of strains belonging to the same lineage. This allows the order of transmission events to be characterised.

Subjects in this study were sampled twice in determination of carriage status. Without further sampling points, some transmission events may be undercovered because transient carriers who were negative at the sample collection timespots would have been missed. Collecting serial samples over time may enable carriage status to be thoroughly characterised and hence more transmission routes could be identified.

There are several possible routes of transmission that may occur in the food industry. The organism may initially spread to workers' hands via contaminated food followed by indirectly colonising others' nares via, for instance, formites. Alternatively, food

handlers may self inoculate a strain into their nares through handling contaminated food and subsequent touching of their faces. The use of *spa* typing cannot differentiate these routes of transmission events.

Determination of routes of transmission in the food industry allows proper hygiene measures to be implemented. For instance, effective environmental cleaning can prevent spread via formites whereas proper use of gloves and masks can miminise the contaminaton of food and discourage self-inoculation.

Determination of *spa* types and comparison of types present in persistent and transient nasal carriers with those present on the hands allowed the transmission between food handlers leading to hand contamination to be elucidated. It was found that the the most important source of hand contamination was persistent carriers, accounting for more than half of the contamination. Persistent *S. aureus* nasal carriers are known to harbour higher loads of the organism and tend to contaminate the surrounding environment more often than transient carriers (Wertheim et al., 2005a). This is further supported by the fact that the hand isolates with *spa* types matching with nasal isolates from persistent carriers were more clonal than those originating from transient carriers, indicating that there may be a common source of contamination originating from a few persistent carriers. The most frequently isolated strain was t189 which was also the most prevalent nasal carriage strain isolated from food handlers in 2011. This predominant type has also been reported in human infections (Rijnders et al., 2009; Ip et al., 2014), pork production shower

facilities (Larson et al., 2011) and from roasted meat (Young et al., 2014), indicating the epidemic potential of this strain.

Colonised workers may contaminate their hands and the surrounding environment from which other co-workers may acquire the bacteria. A Japanese study investigating staphylococcal transmission in one establishment using ribotyping demonstrated that spread from contaminated hands of a food handler to cooking equipment and subsequent contamination of another worker (Kishimoto et al., 2004). Up to 90% of nasal carriers have been shown to simultaneously harbour the bacteria in their noses and on their hands (Wertheim et al., 2005). However, in our study, endogenous hand contamination was surprisingly rare which might be attributable to regular hand washing. The overall low level of hand contamination observed in this study was similar to the 9% hand contamination rate reported in a survey of Finnish airline-catering workers (Hatakka et al., 2000) and 8.4% for food handlers in Spanish restaurrants (Sospedra et al., 2012), but lower than the 16.5% reported for food handlers employed in university cafeterias in Ethiopia (Andargie et al., 2008).

Notably, in the establishment C where an initial high rate of hand contamination was observed in the first visit, the contamination rate went down considerably after implementation of a hand washing training programme to staff. The disappearance of staphylococcal strains originated from “other sources” after hand washing training indicated that the working environment may have become cleaner as a result of

reduced opportunity for cross contamination via dirty hands. The introduction of hygiene training to food handlers in hospitals has been shown to significantly reduce the level of contamination of food processing surfaces, clothing and hands of operatives as well as improving overall hand hygiene (Lazarevic et al., 2013).

The large diversity of *spa* types detected among strains originating from transient carriers and other sources suggested that these workers share different sources of contamination. The *spa* types t091, t127, and t701 have been reported from food poisoning outbreaks in China (Yan et al., 2012). The t701 type has also been reported in an SFP outbreak in Italy, while t127 and t084 were involved in SFP incidents in Germany (Wattinger et al., 2009; Fetscch et al., 2014). Another two *spa* types t571 and t3992 have been suggested to be of livestock origin causing human infections and colonisation (Kock et al., 2013).

It has been suggested that food contamination can be of either animal or human origin. Both nasal carriers and contaminated foods are possible sources of *S. aureus* contamination (Moon et al., 2007; Todd et al., 2008). Contamination of meats with HIEC negative *S. aureus* has indicated the possibility of contamination of animal origin (Benito et al., 2014). The isolation of human associated *S. aureus* CC8, CC15, CC30 and CC45 from ready-to-eat food suggested cross contamination by food handlers (Baumgartner et al., 2014). More recently, CC8 and CC30 were isolated from SFP outbreaks in Japan (Suzuki et al., 2014), indicating that the presence of these clones in food may pose a risk for the consumers.

In summary, the majority of hand contamination was attributable to cross-contamination from other persistently colonised co-workers who may contaminate the food preparation surfaces if proper hand washing is not conducted. Whilst it is possible to decontaminate nasal carriers and this is frequently attempted in the hospital (Wertheim et al., 2005c; Ammerlaan et al., 2009; Bode et al., 2010), this is not feasible in the food industry. This highlights the importance of disinfection in the food industry as a complement to hand washing. In addition, it may be preferable to reduce handling of foods frequently associated with SFP by persistent carriers, although this would be difficult as it would require repeated sampling to define status.

6.7 Antibiotic susceptibility of nasal carriage isolates.

While resistance rates to most classes of antimicrobial agents remained stable over the entire sampling period, resistance to ciprofloxacin increased significantly. This may reflect the increased use of this agent in the territory since the SARS epidemic (Yap et al., 2004; Lam et al., 2009). A similar trend of resistance to fluoroquinolones was also observed in the United States (Gorwitz et al., 2008).

Despite the steadily increasing local incidence of community associated MRSA infections, the prevalence of MRSA in catering workers in our study (1.1%) did not differ from rates observed in the general population in 2004 (1.4%) and currently (2%) in Hong Kong (O'Donoghue & Boost, 2004; Zhang et al., 2012). Notably, all

MRSA isolates in our study came from workers exposed to raw meat, further investigation of sources of MRSA in the general population are needed.

With one exception, the MRSA isolates nasally colonizing food handlers are typical of CA-MRSA, harbouring SCC*mec* IV or V. The first case of CA-MRSA was reported in the region in May 2004 (Ho et al., 2004), which was after the collection of MRSA nasal isolates investigated in our study. This suggests that these resistance determinants may have been present in the region long before they were recognized.

In 2002, one HA-MRSA isolate and three CA-MRSA isolates, coming from four different establishments, were detected. The HA-MRSA strain was isolated from the nares of a food handler working in a supermarket and was t189-MRSA-SCC*mec*II. Whether this person had previous exposure to health-care facilities was unknown.

In 2003, four MRSA strains belonging to SCC*mec* IV or V were isolated. The presence of identical t701-MRSA-V strains with the same antibiotic resistance phenotype in two food handlers working in close proximity suggested the possibility of cross contamination. Resistance to non-beta lactam agents was observed for all MRSA isolates over the whole period, demonstrating the ability of these strains to become multi drug resistant.

The apparent increase of MRSA incidence over time in this study may have been confounded by prolonged freezing of 2002 and 2003 specimens prior to detection of *mecA* gene. Freezing of MRSA isolates has been shown to result in loss of *mecA* gene, converting the resistant strains to susceptible (Griethuysen et al., 2005).

Whilst nine of the total 13 MRSA strains were resistant to both erythromycin and clindamycin, four isolates remained resistant to erythromycin only. As confirmed by a simple D test, these four isolates did not display inducible clindamycin resistance phenotypes. This resistance phenotype displaying erythromycin resistance only has been demonstrated to be encoded by *msr* or *mph* (Wendlant et al., 2013). Further investigation of the anitbiotic resistance determinants in these isolates may reveal the presence of these genes. It was interesting to note that there appeared to be a higher liklihood of an MSSA strain harbouring an enterotoxin but a larger sample size would be needed to confirm this association.

Overall, eighty-five percent of penicillin resistant isolates harboured *qacA/B* genes. The co-existence of genes encoding QAC tolerance (*qacA/B*) and that for beta-lactamase on the same plasmid has been demonstrated in staphylococci isolated from food (Sidhu et al., 2001). Phenotypic resistance *of smr* positive staphylococci to erythromycin and tetracycline has also been reported (Heir et al., 1999; Sidhu et al., 2001). In our study, the resistance rates to tetracycline, cefoxitin, gentamicin, ciprofloxacin and chloramphenicol appeared to be more common among *qacA/B* positive staphylococcal isolates. This association may be due to genetic linkage of *qacA/B* and determinants encoding resistance to these antibiotics or the non-specific multidrug efflux outcome of the *qacA/B* encoded protein *per se.*

Although *qacA* isolates in our studies did have an elevated MBC to disinfectants tested, this increase was modest amongst *qacB* positive isolates, suggesting the

association between the presence of these genetic determinants and susceptibility to QAC products may not be as strong as we previously thought. The association between methicillin resistance and the presence of *qacA/B* and/or *smr* was previously reported for *S. aureus* carriage isolates in Hong Kong (Zhang et al., 2011). Although it appears that certain MRSA clones such as CC22 were more likely to acquire QAC gene (Otter et al., 2013), no association between the presence of QAC genes and genetic background was observed in our isolates. However, this may be due to the small number of samples positive for QAC genes in this study.

Chapter 7 Limitations and Recommendations

This study revealed a sustainable decrease of both nasal colonisation and hand contamination rates with *S. aureus* in food handlers from 2002 through 2011. However, samples were only collected in 2002, 2003 and 2011. The absence of sampling points between 2003 and 2011 prevented any variation during this period being seen. To elucidate if the decline of SFP incidence in 2003 was a consequence of hygiene or a temporary attention shift to viral respiratory illness, a time series analysis involving multiple time points would be more appropriate. In addition, due to the limited budget, samples were collected on one occasion only in 2011. This prevented differentiation of transient from persistent carriers. Further studies on prevalence changes should include sampling points with a shorter interval and similar designs throughout. The sites sampled in this study were all from large catering establishments in Hong Kong. Food handlers working in these premises may differ from those in small-scale restaurants and food kiosks. The presence of hand skin lesions were not assessed during sampling. Having skin lesions may increase the chance of infection and subsequent dispersal of the bacteria to suroundings.

In the investigation of occupational risk factors, raw meat handling was found to be associated with nasal colonisation with *S. aureus* by comparing the carriage rates between meat handlers and workers who did not handle meat using multiple logistic regression to control for confounders. Further characterisation of the nasal isolates

by *spa* typing found that certain clones were more common in workers who handled meat regularly, indicating that one of the sources of their coloniser strains might be from meat. However, since we were not allowed to collect meat samples in the establishments, the origins of *S. aureus* strains could not be confirmed. If possible, a comparison between *S. aureus* strains isolated from food items handled by workers and their nasally colonised strains using molecular typing methods such as PFGE, MLST or *spa* typing may confirm the transmission of the organism from meat to food handlers, or vice versa. In Hong Kong, t1081 MRSA is frequently isolated from clinical specimens (Cheng et al., 2011). The exclusive presence of this clinical epidemic clone in raw meat handlers who did not report any previous health-care exposure suggested a possibility that this lineage may have been introduced to the food industry from an unknown source. Genetic difference in the same lineages colonizing different hosts has been documented in other lineages including ST1 and ST398 (Price et al., 2012; Franco et al., 2011). Further comparison using microarray of the MRSA isolates associated with meat handlers with strains of the same *spa* types of clinical origin and of healthy carriage without occupational exposure to raw meat may confirm the origin of t1081 MRSA strains in this study. As evidence supporting transfer of live-stock asscociated *S. aureus* to humans is accumulating, personal and environmental hygiene should be strengthened in order to prevent its spread.

In this study, antibiotic susceptibility testing was performed in an attempt to preliminarily type bacterial strains. This may allow representative strains with

different antibiotic susceptibility patterns to be selected for more in-depth molecular characterization, making the best use of time and resources. However, overall ST patterns were inadequate in discriminating between strains and thus *spa* typing was conducted. Mupirocin susceptibility was not performed because decolonisation of food handlers is not recommended due to ethical concern and the fact that virtually all people can be colonized at some point during lifetime.

We investigated the presence of enterotoxin genes and disinfectant resistance genes in nasal isolates. These were detected by simplex PCR using primers previously reported. Specificity of primers was checked in Genebank and amplicons were sequenced to determine the identity of the sequence. However, the prolonged storage of 2002 and 2003 isolates in -80^{o}C freezer may have had an impact on the presence of genes harboured in mobile genetic elements such as plasmids and transposons. Of note, these isolates were stored in commercial cryoprotect beads in vials without the addition of QACs. It may be possible that some of the plasmid borne genes encoding QAC were lost because of the absence of selective pressure. In addition, the expression of these genes was not determined because of limited resources available and the fact that some SE proteins have been shown to be controlled at the level of translation (Derzelle et al., 2009). In this regard, developing an immunoassay capable of distinguishing different SE serotypes is necessary.

In addition, the *S. aureus* strains involved in SFP in Hong Kong were not accessible. Further study comparing the characteristics of strains involved in SFP outbreaks and colonising strains in this study may give interesting findings.

Clustering of *spa* types were performed manually because funding was not sufficient to purchase Ridom software. Although the manual clustering algorithm has been proven to be highly correlated to that sorted by Ridom software (Mellmann et al., 2007), a certain extent of human error may remain.

Comparison of *spa* types of *S. aureus* strains isolated from the nares and hands of workers in the same establishments allowed the origins of the organism, either from self-colonisation or other nasally colonised co-workers, to be determined. This suggested *spa* typing can be an affordable and useful tool applicable to routine hygiene monitoring exercises in the food industry.

Subjects with hand isolates showing *spa* types distinct from those of their own nasal isolates and co-workers were defined as contamination from other sources including food preparation surfaces or foods manipulated. Contamination of the food preparation surfaces with *S. aureus* may lead to formation of biofilms allowing the organism to persist on the food processing surfaces where it may grow and produce enterotoxins. Subsequent manipulation of food particularly ready-to-eat food on these surfaces may result in cross contamination. This highlights the importance of environmental cleaning in addition to effective hand washing. A clean workplace can

minimize the risk for cross-contamination. Since gaining access to sample food preparation surfaces and inanimate objects in contact with staff was not possible, further determination of the “other sources” of hand contamination was precluded. Simultaneous comparison of the genetic background of *S. aureus* strains colonizing co-workers with that isolated from commonly accessible surfaces in the food establishments and that from food being handled in the same workplace can provide further insight into the dynamics of bacterial spread in the food industry.

In conclusion, this is the first study that has revealed a long-term sustainable reduction of nasal and hand carriage with *S. aureus* in food handlers. One of the explanations may be attributable to the reinforced hygiene measures implemented in the region over the last decade. Alternatively, changes in epidemiology may also play a role. Epidemiological analyses concluded that occupational exposure to raw meat is the only independent risk factor for nasal colonization with *S. aureus,* but not LA-MRSA, in this population. This emphasizes the need for maintaining personal and environmental hygiene to prevent bacterial dissemination. Use of gloves may not always be possible, in any circumstances, while handling food. In this regard, proper personal hygiene including hand washing should be delivered to food handlers. The occurence of SE genes underscores the toxigenic potential of these strains. The exclusive presence of *qacA/B* gene in *S. aureus* isolates amongst workers exposed to QAC disinfectants indicated that prolonged exposure to these compounds increases risk for colonization with disinfectant resistant staphylococci which may subsequently spread to close contacts and the community. Therefore, proper environmental cleaning should be reinforced in addition to hand washing and use of gloves in order to reduce bacterial load which may otherwise persist in the environment and cross contaminate non-colonized personnel.

Appendices

Appendix 1. Components of lysis buffer for total genomic DNA extraction.

Item	Working Concentration	Volume (µL)	Final Concentration
Lysostaphin	500U	20	25U
Lysozyme	5000U	20	250U
EDTA	0.5M	8	10µM
Tris-HCl	1M	4	10µM
ddH_2O	-	348	

Ho et al., 2012

Appendix 2. Preparation of reagents for plasmid extraction.

Ingredient	Quantity
TENS solution	
1M Tris-HCl	0.5mL
0.5M EDTA	0.1mL
2M NaOH	2.5mL
10% SDS	2.5mL
dH_2O	44.4mL
3M Potassium acetate (pH 5.2)	
Potassium acetate	147.3gm
Glacial acetic acid	Amount to adjust pH to 5.2
dH_2O	Top up to 500mL
1M Tris-HCl (pH 8)	
Tris base	60.6gm
Conc. HCl	Amount to adjust pH to 8
dH_2O	Top up to 500mL
0.5M EDTA (pH 8)	
EDTA	101.1gm
NaOH pellets	Amount to adjust pH to 8
dH_2O	Top up to 500mL
2M NaOH	
Sodium hydroxide	40gm
dH_2O	Top up to 500mL
10% Sodium dodecyl sulfate	
Sodium docedyl sulfate	10gm
dH_2O	Top up to 100mL

Zhang et al., 2011.

Appendix 3. PCR Conditions for detection of SE/SEl genes.

PCR conditions				
		Temperature (^{o}C) x Time (second)		
Target	No. of cycle	Denaturation	Annealing	Extension
sea	30	95 x 60	57 x 60	72 x 60
seb	30	94 x 120	55 x 120	72 x 60
sec	30	94 x 120	54 x 120	72 x 60
sed	30	94 x 120	54 x 120	72 x 60
see	30	94 x 120	54 x 120	72 x 60
seg	30	94 x 60	55 x 60	72 x 60
seh	30	95 x 60	55 x 45	72 x 60
sei	30	94 x 60	51 x 60	72 x 60
sej	35	94 x 60	59 x 60	72 x 60
sek	35	94 x 30	56 x 30	72 x 30
sel	35	94 x 30	51 x 30	72 x 30
sem	35	94 x 30	56 x 30	72 x 30
sen	35	94 x 30	55 x 30	72 x 30
seo	35	94 x 30	56 x 30	72 x 30
sep	30	94 x 60	55 x 60	72 x 60
seq	35	94 x 30	55 x 30	72 x 60
ser	35	94 x 30	55 x 30	72 x 30
ses	35	95 x 60	59 x 60	72 x 60
set	35	94 x 60	58 x 60	72 x 60
selu	35	94 x 30	51 x 30	72 x 30

All reactions were initiated with 94^{o}C for 5 mins and ended with 72^{o}C for 7 mins

Appendix 4. Consent form in Chinese.

同意書

飲食業從業員帶有毒素性金黃葡萄球菌發生率之研究

本人_____（姓名）同意參與此項飲食業從業員帶有毒素性金黃葡萄球菌發生率之研究。

我已經閱讀和理解提交給我的資料。

本人清楚我有權對是次研究發出任何提問，直至滿意為止。

本人明白我須要提供一次鼻液樣本，手掌表面樣本和完成問卷。

本人明白這項研究結果可能會被公佈，但個人資料將是保密，個人身份亦不會被洩露。

_____
簽名

_____
日期

Appendix 5. Consent form in English.

Consent Form

Re: Occurrence of toxigenic *Staphylococcus aureus* among food handlers

I _____ (name) agree to participate in the study on occurrence of toxigenic *Staphylococcus aureus* among food handlers.

I have read and understood the information presented to me.

I have had an opportunity to ask questions about the study and these have been answered to my satisfaction.

I understand that I have to provide a nose swab and a hand swab on one occasion only and complete a questionnaire.

I understand that the results of this study may be published, but my own results will remain confidential and I will not be identified personally in any published work.

_____
Signature

_____
Date

Appendix 6. Questionnaire in Chinese (2002).

代碼 _____
(員工專用)

問卷

飲食業從業人員帶有毒素性金黃葡萄球菌發生率之研究

1. 年齡
   □ ≤ 18 □ 19 – 30 □ 31 – 40 □ 41 – 50 □ 51 - 65

   □ 男 □ 女

2. 你吸煙嗎?
   □ 從不 □ 每星期一包 □ 每星期 2 – 4 包 □ ≥ 每星期 5 包

3. 你在飲食業工作了多少年?
   □ ≤ 1 年 □ 2 – 3 年 □ 4 – 5 年 □ ≥ 6 年

4. 你工作時要處理生肉嗎?
   □需要 □不要

5. 你工作時要處理熟肉嗎?
   □需要 □不要

6. 你工作時要處理蔬菜嗎?
   □需要 □不要

7. 你工作時要處理甜品嗎?
   □需要 □不要

8. 你工作時要處理麵包品嗎?
   □需要 □不要

9. 你工作時要處理粥麵飯嗎?
   □需要 □不要

Appendix 7. Questionnaire in English (2002).

Code number _____
(Official Use Only)

Questionnaire
Re: Occurrence of toxigenic Staphylococcus aureus among food handlers

1. How old are you?
   □ ≤ 18 □ 19 – 30 □ 31 – 40 □ 41 – 50 □ 51 – 65

   □ Male □ Female

2. Do you smoke?
   □ Never □ 1 pack/ week □ 2-4 pack/ week □ ≥ 5 pack/ week

3. How long have you been working in catering industry?
   □ ≤ 1 year □ 2 – 3 years □ 4 – 5 years □ ≥ 6 years

4. Do you handle raw meat at work?
   □Yes □No

5. Do you handle cooked meat at work?
   □Yes □No

6. Do you handle vegetables at work?
   □Yes □No

7. Do you handle dairy/creamy desserts at work?
   □Yes □No

8. Do you handle bakery at work?
   □Yes □No

9. Do you handle rice, congee, noodles at work?
   □Yes □No

Appendix 8. Questionnaire in Chinese (2011).

代碼 _____
(員工專用)

問卷
飲食業從業人員帶有毒素性金黃葡萄球菌發生率之研究

1. 年齡
□ ≤ 18 □ 19 – 30 □ 31 – 40 □ 41 – 50 □ 51 - 65

□ 男 □ 女

2. 你吸煙嗎?
□ 從不 □ 每星期一包 □ 每星期 2 – 4 包 □ ≥ 每星期 5 包

3. 你在飲食業工作了多少年?
□ ≤ 1 年 □ 2 – 3 年 □ 4 – 5 年 □ ≥ 6 年

4. 你每天工作多少小時?
□ ≤ 4 小時 □ 5 – 8 小時 □ 9 – 12 小時 □ ≥ 13 小時

5. 你工作時要處理生肉嗎?
□經常 □間中 □從不

6. 你工作時戴手套嗎? 如有, 你每天更換多少對?
□ ≥ 5 對 □ 3 – 4 對 □ 1 – 2 對 □從不

7. 你工作時洗手的頻率?

如廁後	□恆常	□經常	□間中	□從不
處理熟食前	□恆常	□經常	□間中	□從不
處理生肉後	□恆常	□經常	□間中	□從不
吸煙後	□恆常	□經常	□間中	□從不
接觸鼻或面部後	□恆常	□經常	□間中	□從不

8. 你有慢性疾病嗎?
□ 有 □ 沒有
如有, 請列明 _____

9. 你在 過去三個月有服用抗生素嗎?
□ 有 □ 沒有

10. 你有以下徵狀嗎?
□ 皮膚發炎 □ 敏感 □ 暗瘡 □ 鼻敏感

11. 你在 過去十二個月有否住院?
□ 有 (請回答第 12 題) □ 沒有 (請回答第 14 題)

12. 你在什麼時候住院?
□ 過去三個月內 □ 過去六個月內 □ 六個月之前

13. 你住院多久?
□ 少於 2 天 □ 3 – 5 天 □ 多於一星期

14. 你有養以下寵物?
□ 狗 □ 貓 □ 鳥 □ 沒有

15. 你有家人是醫護人員嗎?
□ 有 □ 沒有

Appendix 9. Questionnaire in English (2011).

Code number _____
(Official Use Only)

Questionnaire
Re: Occurrence of toxigenic Staphylococcus aureus among food handlers

10. How old are you?
☐ ≤ 18 ☐ 19 – 30 ☐ 31 – 40 ☐ 41 – 50 ☐ 51 – 65

☐ Male ☐ Female

11. Do you smoke?
☐ Never ☐ 1 pack/ week ☐ 2-4 pack/ week ☐ ≥ 5 pack/ week

12. How long have you been working in catering industry?
☐ ≤ 1 year ☐ 2 – 3 years ☐ 4 – 5 years ☐ ≥ 6 years

13. How many hours a day do you work?
☐ ≤ 4 hrs ☐ 5 – 8 hours ☐ 9 – 12 hours ☐ ≥ 13 hours

14. Does your job require you to handle raw meat?
☐ Always ☐ Sometimes ☐ Never

15. Do you normally wear gloves? If yes, how many pairs would you use per day?
☐ ≥ 5 ☐ 3 – 4 ☐ 1 – 2 ☐ Never

16. How often do you wash your hands?

after going to toilet	☐ Always	☐ Usually	☐ Sometimes	☐ Never
before handling food	☐ Always	☐ Usually	☐ Sometimes	☐ Never
after handling meat	☐ Always	☐ Usually	☐ Sometimes	☐ Never
after smoking	☐ Always	☐ Usually	☐ Sometimes	☐ Never
after touching noses / faces	☐ Always	☐ Usually	☐ Sometimes	☐ Never

17. Do you have any chronic illness?
☐ Yes ☐ No
If yes, please specify _____

18. Have you taken antibiotics in the last 3 months?
☐ Yes ☐ No

19. Do you suffer from any of the following?
☐ Dermatitis ☐ Allergies ☐ Acne ☐ Rhinitis

20. Have you been hospitalized in the last 12 months?
☐ Yes (Go to Q12) ☐ No (Go to Q14)

21. When were you hospitalized?
☐ in the last 3 months ☐ last 6 months ☐ ≥ 6 month ago

22. How long did you stay in hospital?
☐ ≤ 2 days ☐ 3 – 5 days ☐ ≥ 1 week

23. Do you own any of the followings?
☐ Dog ☐ Cat ☐ Bird

24. Is any member of your family a healthcare worker?
☐ Yes ☐ No

Appendix 10. Ethics approval.

From: ethics, Hr [HRO]
Sent: Friday, December 17, 2010 3:40 PM
To: hrethics@ ; hsmdonog@
Cc: hscmlee@
Subject: HSEARS Application for Ethics Review Approved

Please note that the following application for ethics review of research / teaching projects involving human subjects has been approved:

Project ID: HSEARS20101126002 (Click here to view the application)
Project Title: Occurrence of toxigenic strains of Staphylococcus aureus among food handlers
Principal Investigator: O'DONOGHUE Margaret May
Approval Period: 17-12-2010 to 25-11-2012

Please note that you are responsible for informing the SN in advance of any changes in research proposal or procedures which may affect the validity of this ethical approval. You will receive separate notification should you be required to obtain fresh approval.

CHIEN Wai Tong (hschien)
Using Human Subjects Ethics Application Review System (HSEARS)

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