Novel Technology to Study Co-Evolution of Humans and *Staphylococcus aureus*: Consequences for Interpreting the Biology of Colonisation and Infection

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Abstract Human nasal carriage of *Staphylococcus aureus* is a textbook example of an apparently neutral interaction between humans and a bacterial species that can still lead to (severe) opportunistic infections. The co-evolutionary aspects of this interaction are slowly surfacing, facilitated by the emergence of a diversity of diagnostic, epidemiological and molecular research tools. Basic microbiology has helped define persistent vs. non-carriage and the genotype of both host and guest has been explored in search of genetic markers for bacterial persistence. This chapter summarises the current state of affairs relating to artificial human colonisation studies with *S. aureus*, large-scale human cohort studies and innovative assessment of the humoral immune status of (non-)nasal carriers. These experimental approaches have recently assisted in identifying bacterial and human determinants and risk factors for staphylococcal carriage. Further refinement of the model by describing the interactions between *S. aureus* and its human host in molecular detail is important since it may pinpoint novel anti-infectious strategies.

1 Introduction

Staphylococcus aureus is a bacterial species that is capable of colonising a number of mammalian hosts [1, 2]. Among these are several companion animals and animals of veterinary importance [3, 4]. These animals and their human keepers can be colonised by *S. aureus* in an innocent, bystander-type way. However, at least in humans such colonisation can develop into active (auto-)infection [5, 6]. This, obviously, poses an important complication. Little is known on the transition from the colonised into the infected state. Questions on the nature of the most relevant pathogen- and host-defined determinants still remain unanswered in many cases. In

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order to provide answers on these and other questions relating to the nature and consequences of nasal carriage in humans, more detailed studies into the precise co-evolutionary (and molecular) interactions between host and visitor need to be undertaken [7, 8]. This chapter describes three recent large studies that may shed some additional light on the important topic of host–pathogen co-evolution: artificial *S. aureus* inoculation studies in human volunteers; longitudinal follow-up of cohorts of individuals with well-defined bacterial carriage status and assessments of the serological response in people carrying or infected with *S. aureus*. However, first this chapter highlights several of the more basic bacterial and host characteristics that are important in establishing a stable colonised state in a susceptible host.

2 The Microorganism

2.1 Detection and Identification

S. aureus is a coccal-shaped bacterial species that lives in small colonies. These "bunch of grape"-like communities are tight clusters of between five and a few dozen individual cells. Precise quantification of colony forming units of *S. aureus* is hampered by their sticky behaviour and it seems highly likely that there must be some ecological advantage to sticking together [9]. How this works, however, is currently ill defined.

Laboratory diagnosis of *S. aureus* is quite straightforward: Gram staining provides a good clue. Selective culture is possible: *S. aureus* is quite tolerant to table salt, it will even grow in solutions of up to 1 M sodium chloride [10]. In combination with an indicator dye, simple broth media can be used for sensitive and specific enrichment cultures. In addition, a few simple agglutination tests will usually provide definite answers as to species' nature. Finally, molecular testing with ribosomal sequence motifs as targets will provide a watertight diagnostic result [11]. In addition to these clinical diagnostics, suited for the laboratory-based identification of *S. aureus,* there also is a new generation of tests that are suited for the direct detection of *S. aureus* in clinical material. These assays still vary from "old-fashioned" in-house laboratory PCR tests, that sometimes still rely on gel electrophoresis for the visualisation of the amplified product [12], to more innovative tests that employ real-time PCR without subsequent steps. These tests are good, although not all laboratories perform them equally well [12]. Most importantly, these tests can be used to reveal within a working day whether or not a patient is a nasal carrier or not. These data can provide a basis to interfere with carriage (by mupirocin and chlorhexidine treatment, for instance) and in that way generate a blockade to auto-infection.

2.2 Habitat and Genome Complexity

S. aureus has a great capacity to survive and it can do that in both animate and inanimate environments [13]. It will survive for weeks in dry environments but it has also been encountered in and on obligate seawater-dwelling mammals [14].

In the host, physical interactions define the likelihood of developing a successful interaction between man and bacteria. In addition, a variety of bacterial anchors play an important role. The so-called microbial surface components recognising adhesive matrix molecules (MSCRAMMs) form an important category protein facilitating the colonisation of alien niches [15]. MSCRAMMs may recognise host molecules as diverse as collagen, von Willebrand factor and vitronectin. These proteins may also show antigenic variability; simple size variation in regions of repetitive DNA (and, hence, peptides) is also frequently recorded. Overall, *S. aureus* is a bacterial species that is very well equipped for fulfilling its life cycle in a potentially quite hostile *Homo sapiens* environment.

Bacterial versatility is the word that comes to mind and that is also what the staphylococcal genome reflects. Overall, *S. aureus* genomes range between 2 and 3 megabases in length [16]. The genome consists of three major categories of DNA regions. First there is the core genome that is quite well conserved between isolates and encodes all of the requirements needed to fulfil the bacterial life cycle. Second there is core variable DNA. These regions, spread across the entire genome, encode factors that may show genetic variability to a limited degree. This material provides a certain genetic flexibility. Important factors may, for instance, evade host immunity through the Darwinian selection of non-immune-recognised epitopes that are generated through random mutagenesis processes. Last but not least, there are the real variable elements: the so-called mobile genetic elements (MGEs) [17]. These may include transposons, integrated bacteriophages and islands consisting of virulence factors. Such genomic islands play an important role in staphylococcal biology and may be related to host specificity and antimicrobial resistance. The staphylococcal cassette chromosome *mec* (SCC*mec*) is an example of the latter category of elements [18]. On the whole, however, *S. aureus* is a clonal microorganism [19, 20]. This implies that the genome is quite well conserved and that there are significant similarities between many of the *S. aureus* isolates collected in clinical laboratories. However, despite this conservation and the associated genetic flexibility, essentially all strains of *S. aureus* can become invasive to their host given the appropriate conditions [19, 21]. These usually exist in the form of ineffective or waning immunity of the host.

3 The Host

3.1 Human Niches

When living in or on a mammalian host, *S. aureus* interacts with a variety of different molecules and physical conditions. First and foremost, there are the physical constraints. Temperature, salinity, pH, availability of oxygen and hydratation are important determinants of staphylococcal survival. In addition, there are the biological features. In a host, bacteria have to compete with other bacterial species that may be attracted to the same niche [22]. The bacteria have to be able to withstand the innate and acquired immune responses and features such as adherence to extracellular mucus or certain receptor molecules are ultimately of importance

in developing a stable colonised state. Hosts either do or do not provide such conditions. *S. aureus* thrives on human skin, the gastrointestinal tract, the pharyngeal region and, most prominently, the vestibulum nasi of the human nose [1, 23]. The latter ecological niche is considered by far the most important and it is suggested that all colonisation of other anatomical locations derive from nasal carriage.

3.2 Patterns of and Susceptibility to Carriage

Humans can be divided, on the basis of serial cultures, into three phenotypic categories of (non-)carriers: persistent, intermittent and non-colonised individuals [24]. Several humans will be nasal culture positive in over 90% of all culture attempts, others may be positive in only 10–80% of cultures whereas the remaining individuals will be repetitively culture negative. The persistent carriers comprise in the order of 30% of individuals. Interestingly, the intermittent carriers are characterised by a more extensive genotypic diversity among colonising strains, indicating that these individuals occasionally pick up a strain, carry it for a relatively short time and then either clear the strain or become re-colonised again [25, 26]. In this group of carriers the number of viable cells that can be collected from the nose is usually less (up to 1,000-fold) than the number collected from persistent carriers [24]. Several host factors define the differential host susceptibilities and simple ones are gender and age [8]. It has been found that males are more often persistent carriers and it has also been shown that with increasing age the fraction of persistent carriers declines. These are unexplained differences. Another unexplained but very intriguing difference is the influence of fasting glucose level. There is a linear association between the molarity of blood glucose and *S. aureus* nasal carriage: the higher the glucose level, the more likely an individual is to be a persistent carrier. Last but not least: there is a genetic basis for the predisposition to become a carrier. Certain HLA variants have since long been associated with *S. aureus* carriage [27–32], but modern genome-wide testing for genetic variation has recently facilitated more in-depth studies. Single nucleotide polymorphisms in several immune genes, the serine protease inhibitor and the glucocorticoid receptor gene have been shown to be associated with carriage in a statistically significant fashion [33–36].

In short, there is an enormous diversity in both the host and the bacterial factors that are important in establishing colonisation. These factors interact in a complex manner and it is this interaction that ultimately defines whether or not a host is susceptible to (long-term) colonisation. A number of essential colonisation factors have been identified through experimental approaches. Unfortunately, most of these experiments have been performed in animal models. Definite proof of the relevance of certain factors requires a human model of colonisation. Results to date from experiments in a human volunteer nasal colonisation model will be succinctly described below.

4 Artificial Colonisation Studies

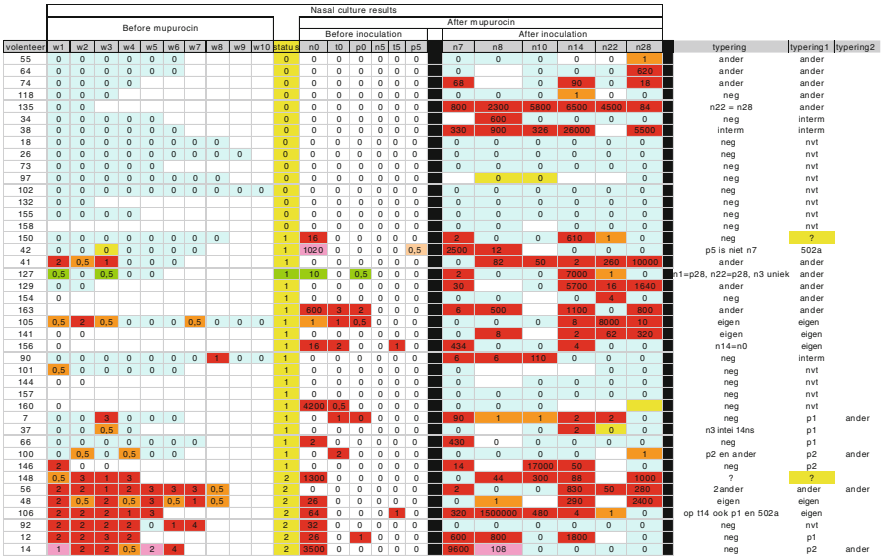
4.1 Setting Up a Nasal Colonisation Study

Artificial human colonisation with an opportunistic pathogen such as *S. aureus* needs careful consideration and preparation [37]. Individuals with certain risk factors have to be excluded from such experiments. These include, among others, diabetes, recurrent skin infections and other skin-related problems, pregnancy, frequent contact with susceptible patients (either work related or in the personal arena) and allergy to certain antibiotic or chlorhexidine [38, 39]. In addition, the inoculum strain(s) should be devoid of known virulence genes and resistance traits (SCC*mec*, superantigens and toxins such as Panton–Valentine leukocidin and others). An "intended release" genetically modified organisms (GMO) protocol with appropriate permissions is required for experiments involving the inoculation of genetic mutants of *S. aureus* [40]. Once ethical and GMO-related hurdles have been taken, colonisation studies are relatively simple to develop. Our studies include at least 16 people when comparing two parameters to provide data with sufficient statistical power. Only routine laboratory equipment found in a good clinical diagnostic microbiology facility is needed.

Our studies are usually staged: we start with assessing the nasal *S. aureus* carriage status of our volunteers. This means that participants are followed for 5–10 weeks and that at least five nasal cultures are taken. This identifies carriers, intermittent carriers and non-carriers [24, 26]. Once the cohort has been established and characterised, the elimination treatment can commence. This consists of nasal mupirocin ointment for 5 days and a 1 week supply of chlorhexidine soap and shampoo. After 6 weeks the colonisation state is re-assessed and individuals without carriage in the rectum, throat and nose can be included in the inoculation experiment.

Volunteers are then nasally challenged with 5×10^9 cells per strain to be used. The maximum number of strains combined to date is 5 which results in an inoculum of 2.5×10^{10}. To date, none of our volunteers has experienced any serious adverse events as a result of the inoculation. Participants have, however, remarked on a "fungus-like" smell of the bacterial inoculum. Volunteers are followed up closely, with an infectious disease specialist on call for the duration of the experiment. Nasal cultures are performed every day during the first week after inoculation and once weekly thereafter. The experiment continues for 3 months after which those individuals who are still colonised by a strain from the mixture are offered a new decolonisation course. The colonisation study protocol is shown in Fig. 1.

The microbiology aspects of the experiments are also quite simple. Samples are taken and diluted in physiological salt, part of which is plated on blood agar. The rest of the material is immersed in an enrichment broth. If the agar cultures show growth within 4 days, putative *S. aureus* colonies (all different colony morphologies included to a maximum number of 20) are selected and re-cultured. These are speciated by standard lab technology and up to 16 strains are frozen at –80°C for use at a later stage. These follow-up studies may involve overall genotyping, assessment of gene absence or more detailed microbiological analyses. When there is growth in



	Nasal culture results																												
	Before mupirocin											After mupirocin																	
												Before inoculation						After inoculation											
volenteer	w1	w2	w3	w4	w5	w6	w7	w8	w9	w10	status	n0	t0	p0	n5	t5	p5	n7	n8	n10	n14	n22	n28	typering	typering1	typering2
55	0	0	0	0	0	0					0	0	0	0	0	0	0	0	0	0	0	0	1	ander	ander	
64	0	0	0	0	0	0					0	0	0	0	0	0	0	0		0	0	0	620	ander	ander	
74	0	0	0	0							0	0	0	0	0	0	0	68		0	90	0	16	ander	ander	
118	0	0	0								0	0	0	0	0	0	0	0	0	0	1	0	0	neg	ander	
135	0	0									0	0	0	0	0	0	0	800	2300	5800	6500	4500	84	n22 = n28	ander	
34	0	0	0	0	0						0	0	0	0	0	0	0		600	0	0	0	0	neg	interm	
38	0	0	0	0	0	0					0	0	0	0	0	0	0	330	900	326	26000		5500	interm	interm	
18	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	neg	nvt	
26	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	neg	nvt	
73	0	0	0	0	0	0					0	0	0	0	0	0	0	0	0	0	0	0	0	neg	nvt	
97	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0		0	0			0	neg	nvt	
102	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	neg	nvt	
132	0	0									0	0	0	0	0	0	0	0	0	0	0	0	0	neg	nvt	
155	0	0	0	0							0	0	0	0	0	0	0	0	0	0	0	0	0	neg	nvt	
158											0	0	0	0	0	0	0	0	0		0	0	0	neg	nvt	
150	0	0	0	0	0	0	0	0			1	16	0	0	0	0	0	2	0	0	610	1	0	neg	?	
42	0	0	0	0	0	0	0				1	1020	0	0	0	0	0,5	2500	12		0	0	0	p5 is niet n7	502a	
41	2	0,5	1	0	0	0					1	0	0	0	0	0	0	0	82	50	2	260	10000	ander	ander	
127	0,5	0	0,5	0	0						1	10	0	0,5	0	0	0	2	0	0	7000	1	0	n1=p28, n22=p28, n3 uniek	ander	
129	0	0									1	0	0	0	0	0	0	30		0	5700	16	1640	ander	ander	
154	0										1	0	0	0	0	0	0	0	0		0	4	0	neg	ander	
163											1	600	3	2	0	0	0	6	500		1100	0	800	ander	ander	
105	0,5	2	0,5	0	0	0	0,5	0	0	0	1	1	1	0,5	0	0	0	0	0	0	6	8000	10	eigen	eigen	
141	0	0									1	0	0	0	0	0	0	0	1		2	62	320	eigen	eigen	
156	0										1	16	2	0	0	1	0	434	0	0	4	0	0	n14=n0	eigen	
90	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	6	6	110	0	0	0	neg	interm	
101	0,5	0	0	0	0	0					1	0	0	0	0	0	0	0				0	0	neg	nvt	
144	0	0									1	0	0	0	0	0	0	0		0	0	0	0	neg	nvt	
157											1	0	0	0	0	0	0	0	0	0	0	0	0	neg	nvt	
160	0										1	4200	0,5	0	0	0	0	0	0	0				neg	nvt	
7	0	0	3	0	0	0					1	0	1	0	0	0	0	92	1	1	2	2	0	neg	p1	ander
37	0	0	0,5	0							1	0	0	0	0	0	0	0		0	2	0	0	n3 intel 14ns	p1	
66	0	0	0	0	0	0	0				1	2	0	0	0	0	0	430	0	0	0	0	0	neg	p1	
100	0	0,5	0	0,5	0	0					1	0	2	0	0	0	0	0	0	0	0		1	p2 en ander	p2	ander
146	2	0	0								1	0	0	0	0	0	0	14		17000	50		0	neg	p2	
148	0,5	3	1	3							2	1300	0	0	0	0	0	0	44	300	88		1000	?	?	
56	2	2	1	2	3	3	3	0,5			2	0	0	0	0	0	0	2	0	0	830	50	280	2 ander	ander	ander
48	2	0,5	2	0,5	3	0,5	1	0,5			2	26	0	0	0	0	0	0	1		290		2400	eigen	eigen	
106	2	2	2	1	3						2	64	0	0	0	1	0	320	1500000	480	4	1	0	op t14 ook p1 en 502a	eigen	
92	2	2	2	2	0	1	4				2	32	0	0	0	0	0	0	0	0	0	0	0	neg	nvt	
12	2	2	3	2							2	26	0	1	0	0	0	600	800	0	1800		0	neg	p1	
14	1	2	2	0,5	2	4					2	3500	0	0	0	0	0	9600	108	0	0	0	0	neg	p2	ander

Fig. 1 Table showing timing of nasal cultures for assessing the carriage rates among volunteers participating in artificial nasal colonisation studies with *S. aureus.* Studies can be divided into a number of distinct episodes: the before mupirocin/chlorhexidine and after mupirocin/chlorhexidine period and the before and after inoculation episodes. During the first episode the nasal carriage status of the volunteers is determined during a 10-week period. On the basis of the quantitative culture results the volunteers are then stratified into non-carriers, intermittent carriers and the persistent carriers. Before inoculation with *Staphylococci*, the volunteers are treated with mupirocin nasal ointment and chlorhexidine soap and shampoo. The success of this treatment is verified on the basis of nasal, throat and perineal *S. aureus*-specific cultures. Volunteers who are free of *S. aureus* can then proceed to the inoculation phase. Bacteria are instilled in the nose on day one and colonisation is followed up for 28 days. Finally, strains are typed to define colonising isolates

the enrichment broth, medium is plated on blood agar again and cultured bacteria are again speciated following which a single colony is stored. A pre-inoculation and end of study serum sample is also collected, primarily to assess infection parameters, though ultimately these samples will also be used for *S. aureus* antibody serological studies (see below).

4.2 Examples of Previous Colonisation Studies

Three artificial colonisation studies have been published from our laboratory thus far [37, 40, 41]. The first concerned a simple study involving 16 volunteers. Eight persistent and eight non-carriers were included and inoculated with a mixture of four strains. In carriers, their own strain was included in the inoculation mixture. Results clearly showed a human factor in nasal carriage: both the persistent and the non-carriers returned to their pre-inoculation carriage status. Interestingly, carriers tended to re-select their own strain, suggesting the existence of a very intricate

relatedness between host and guest. This was further corroborated by another study which also included intermittent carriers [41]. This study, involving the largest number of volunteer participants to date, showed that intermittent carriers behave in the same way as non-carriers: they eliminate the inoculum in a matter of days and, surprisingly, their anti-staphylococcal antibody profiles are similar to those of non-carriers. So both in humoral responses and nasal elimination kinetics the persistent carriers are a distinct group. This indicates that the paradigm needs changing: there are only two categories of nasal *S. aureus* carriers: those who do and those who do not persistently carry the organism. It is important to note that this implies that the majority of people are non-carriers and that these individuals have "solved" the problem of preventing auto-infection by ensuring that the nose is an inefficient reservoir for *S. aureus* bacteria. The third published inoculation study investigated the elimination kinetics of wild-type *S. aureus* and an isogenic *ClfB* mutant of the same strain [40]. It was shown that the ClfB mutant was eliminated at a statistically significant higher rate than the wild type. This indicates that ClfB, an adhesin belonging to the family of MSCRAMMs and capable of binding both fibrinogen and cytokeratin-10 [42–44], is an essential component in the array of molecules putatively involved in nasal colonisation.

4.3 Ongoing Experiments and Ideas for Future Colonisation Studies

We have preliminary evidence that *S. aureus* strains that harbour prophages that carry immune evasion clusters (IECs) [45, 46] do worse in colonising adult human volunteers. Counterintuitively, the strains that contain the evasion clusters are eliminated from the nasal cavity at a higher rate. Whether this is due to, for instance, elevated antibody levels against several of the IEC-coded proteins is the subject of current investigations. Our preliminary data seem to suggest that this is not the case and that there is no important role of humoral immunity in this selective phenomenon. It might thus be that the IECs play their most important role in immune-naïve individuals, i.e. children during their first encounters with colonising or even infecting *S. aureus* bacteria.

Multiple additional colonisation studies can be envisaged: each and every (potential) MSCRAMM can be assessed to investigate their importance in colonisation of humans (e.g. [47]). Also, it has been suggested that many strains are restricted to certain mammalian hosts (pigs, cow, horses, sheep, goats and many more) [48]. Inoculation studies could be used to corroborate these suggestions, primarily based on bacterial genotyping studies rather than real in vivo assessments. It is not known up to what extent *S. aureus* colonisation is inhibited by the presence of other bacterial species. The species that are most frequently mentioned in this respect are coagulase-negative staphylococci and Coryneform bacteria [49]. Artificial colonisation could be used to investigate this potentially therapeutic bacterial interference phenomenon [7, 50]. Finally, if voluntary participation is feasible, inoculation studies could be used to define the potential selective advantage of multi-resistant

bacteria including methicillin-resistant *S. aureus* (MRSA) or the effect of toxins on colonisation. Finally, inoculation with random mutant libraries could identify many novel adherence factors in a single large-scale in vivo expression technology (IVET)-like experiment [51].

In conclusion, over the years we have developed an important and useful model for the detailed study of the interaction between humans and *S. aureus* bacteria. In vivo selection of the fittest colonisers is feasible and this may help reveal why some *S. aureus* lineages are more successful (or epidemic) than others.

5 Example Of A Cohort Study

5.1 Relevance of Cohort Studies

Cohorting can be used to generate often unexpected clinically relevant data from complex environments and situations. Embedding a microbiological assessment of bacterial colonisation within a cohort study that focuses on other diseases is extremely productive [52, 53]. In Rotterdam in The Netherlands, a cohort of neonates was initiated in 2004 and primarily focused on determinants of non-infectious diseases. Within this so-called Generation R cohort a variety of features have been monitored [54]. These include host genetics, gestational age, immune parameters, gender and birth weight. In addition, day-care attendance, presence of siblings, parental smoking, socio-economic status and gender have been recorded. This has generated extensive databases enabling extrapolations and correlations between a variety of factors and microbiological culture data.

5.2 Microbiology in Generation R

The bacterial carriage study was embedded in the prospective Generation R cohort study. Overall, 10,000 pregnant women and their children were enrolled. Within the so-called Focus cohort, 1,232 women were eligible to participate. Complete follow-up in the Focus cohort of 1,079 infants was available. Visits to the study centre were scheduled at 1.5, 6, 14 and 24 months of age (with a visit rate of 82%). Questionnaires were done at age 2, 6, 12, 24 months and 2 and 5 years. The main goal of the microbiological research within Generation R was to study bacteriological and immunological determinants of nasopharyngeal bacterial carriage in young children and to assess their association with respiratory tract infections. For this reason, serial nasal and nasopharyngeal swabs were taken over extended periods of time and specifically cultivated for *S. aureus, Moraxella catarrhalis, Streptococcus pneumoniae* and *Haemophilus influenzae*. This is enabling the mapping of microbial colonisation dynamics, bacterial interference, the role of human genetic variability (SNP measurements), the definition of Ig and TCR repertoires and the definition of the intricate relations between colonisation, host immune response and respiratory tract infections [55].

5.3 Preliminary Results

Serial follow-up of young children with nasopharyngeal culture reveals that different bacterial opportunists show completely different longitudinal colonisation kinetics. For both *M. catarrhalis* and *H. influenzae* there is a continuous rise in prevalence from birth onwards, with peak incidences at the age of approximately 30% at the age of 2 years. At 6 weeks of age the colonisation rate peaks at 10%. For these two species increase in age is accompanied by an increasing colonisation rate until saturation levels are reached. For *Pneumococcus* and *S. aureus* the situation is a little more dynamic. The colonisation rates for the *Pneumococcus* at 1.5, 6, 14 and 24 months are 8, 31, 44 and 37%, respectively. The *S. aureus* rates are 52, 20, 15 and 35% [53]. This inverse relationship between the two species has also previously been demonstrated in large-scale cross-sectional population studies [50, 56]. Clearly, the colonisation of the nose and the nasopharynges by different bacterial species is a dynamic and evolving process. The situation is likely to even more complex: many additional bacterial species inhabit the same niche and further investigations should also include, for example, anaerobic bacterial species.

It is interesting to note that persistent carriage does not seem to exist in young children. Although the colonisation prevalence is quite high, serial cultures revealed that few children were persistently culture positive. In addition, genotyping revealed that even children who cultured positive on more than one occasion were colonised by genotypically distinct bacterial strains. This suggests that intermittent rather than persistent carriage is predominant among young children [53]. It might be that these children are "sensing" the microbiological diversity of staphylococci until they find their match and enter into a long-term relationship with a strain. Conversely, the initial encounters with *S. aureus* may also result in a more permanent non-carriage state. Whether this is imposed by humoral immunity, cellular immunity or another driving factor is currently unknown. Environmental, demographic or social determinants of staphylococcal carriage were not identified. Only male gender was associated with carriage. Features such as gestational age, birth weight, breast feeding habits, mothers educational level, (prenatal) smoking habits, the presence of siblings or day-care attendance were of limited importance. The studies in the paediatric cohort clearly reveal that staphylococcal carriage in children is significantly different to adults. Long-term persistence seems to occur in later life and the incidence of carriage shows a highly significant decrease in the first years of life. Whether this is immune regulated or due to other, physiological phenomena remains to be investigated.

Cohort studies are often designed to identify markers of disease. Within the Generation R cohort we specifically addressed the aetiology of atopic dermatitis (AD). We defined correlations between nasal *S. aureus* carriage and the development of AD in later stages of life [52]. When culture results for nasal swabs taken at 1.5, 6 and 14 months of age were correlated with AD determinants, significant associations were documented. For instance, a first positive *S. aureus* culture at the age of 6 months was associated with AD in both the first and the second year of life.

There was also an association with parentally reported AD severity. In addition, frequent colonisation resulted in a more than fourfold increased risk of moderate to severe AD in the second year of life. Although this is still mechanistically unexplained, it is clear that nasal colonisation with *S. aureus* predisposes to AD. We are currently investigating the correlation between nasal carriage and other relevant childhood diseases (including wheezing and asthma) and novel associations have already been identified. Future studies into these correlations should include detailed immunological and physiological assessments. Elucidating the mechanisms of such correlations may lead to the design of novel prophylactic measures for atopic diseases in general [57].

6 Humoral Immunity and *S. aureus* Carriage and Infection

6.1 Technical Aspects of Multiplex Anti-staphylococcal Antibody Measurements

Antibody detection can be done by a variety of long-established methods including a variety of immunodiffusion tests, membrane-based Western blotting assays and enzyme linked immunosorbent assays (ELISA) [58]. Disadvantage of the commonly used ELISA tests is that relatively large amounts of serum (or other test liquids) are required and that the opportunities for multiplex testing are limited. Innovative bead-based immunofluorescence tests, the so-called Luminex technology, overcome this limitation allowing the simultaneous measurement of a large number of antibodies or other factors [59, 60]. We have developed a 40-plex bead-based FACS test for a variety of staphylococcal antigens [61–63]. This can be used to study the immunogenicity of staphylococcal antigens during colonisation and infection and also to study the longitudinal maturation of the anti-staphylococcal antibody response in the early stages of life.

6.2 Application of the Luminex Technology and Microbiological Implications

The first tests involving the staphylococcal Luminex test concerned the identification of anti-staphylococcal antibody profiles in healthy adults who were established persistent or non-carriers of *S. aureus* [62]. This showed a number of biologically interesting features. First and foremost, antibody levels in persistent carriers were, on average, higher than in non-carriers and the profiles were quite unique to an individual. The profiles are unique to an individual and remain consistent over a period of at least 6 months. This suggests that the continuous challenging of the immune system by the presence of bacterial cells stimulates antibody production. Second, we showed that the overall antibody profiles, when comparing the two categories of carriers, differed significantly between the two groups. Despite the fact that the panel of staphylococcal antigens was limited to 20 in these initial studies we found

several antibody levels that differed in a statistically significant manner between the two groups. Antibody levels against toxic shock syndrome toxin (TSST), clumping factor A, clumping factor B and enterotoxin A were elevated among persistent carriers. We were also able to show that at least for the anti-TSST antibodies this elevation led to a more pronounced in vitro inhibition of toxin activity. In other words, the differences in antibody levels were also reflected in differences in antibody neutralising activity. It remains a challenge to define whether or not these quantitative and qualitative differences can be associated with the differences in infectious risk and outcome of infections that have been observed clinically between carriers and non-carriers [21, 64].

A second study involved the investigation of intermittent *S. aureus* carriers. The Luminex assay revealed that the antibody profiles of intermittent and non-carriers were essentially identical [41]. No significant differences could be established which led to the conclusion that intermittent carriers are contaminated non-carriers rather than "accidentally negative" persistent carriers. This fits well with the outcome of the artificial inoculation study and was also in agreement with previous studies in which infection risk in intermittent carriers was shown to be the same as that in non-carriers and not elevated like in persistent carriers.

We also used the Luminex system to investigate differences in antibody profiles between various groups of individuals with distinct staphylococcal infections [61]. These experiments focused on assessment of toxin antibody responses in patients with various diseases. For many of the toxins, clearly elevated levels of antibodies were documented in patients compared to healthy controls. This clearly suggests in vivo expression of these toxins during infection. Adults displayed higher anti-toxin antibody levels than children. If certain toxin genes were absent in the infectious strain, the antibody response was lower than that induced by gene-positive strains. Bacteriaemia was associated with a higher prevalence of enterotoxin A among the infectious isolates, whereas bone and joint infections were caused by strains enriched in Luk-PV. This led to the conclusion that during infection a variety of toxins is actively expressed and recognised by the immune system. In addition, certain toxin genes seem to be important aetiological factors during several types of infections.

Finally, defining the humoral immune kinetics at the early stages of life is important [63]. This could provide clues to the identity of major immunogens and it could also show whether (maternal) antibodies provide protection against colonisation or infection. In the Generation R cohort we studied serial serum samples from over 50 children. This showed that, again, the antibody profiles were extensively different between individuals. IgA and IgM levels clearly increased over the first 2 years of life, whereas maternal antibodies steeply decreased in the first half year of life. The maternal antibody levels did not provide any protection against colonisation of the child. Interestingly, the differences in antibody profiles documented for colonised vs. non-colonised children differed, but to a different extent than was defined for (non-)carrying adults. In children, the chemotaxis inhibiting protein of *S. aureus* (CHIPS), Efb and IsdA IgG levels were elevated among the carriers. Again, this shows that these antigens are expressed in vivo and stimulate

the humoral immune response. Their role in colonisation should still be defined further and further research is clearly warranted.

7 Conclusion

The interaction between *S. aureus* and its human host is complex. Both bacterial and human factors are important in establishing a quite individualised interaction, which may amount to an exclusive interaction between a host and a specific bacterial isolate. Although this interaction may be disrupted occasionally, leading to minor (skin lesions, boils, etc.) or more severe (bacteriaemia, sepsis, pneumonia, etc.) infections, in general the interaction is quite silent. *S. aureus* and the human host generally co-exist well, although it is notable that over 50% of individuals remain healthy without being colonised by *S. aureus* strains. More detailed, molecular insight into the factors associated with human non-colonisation may help identify novel measures that may prevent or cure staphylococcal infections. Our integrated model, comprising artificial inoculation studies, access to clinical materials (serum, DNA, nasal secretes and *S. aureus* strains), availability of human and bacterial transcriptomics facilities and the Luminex antibody-profiling system, provides a valuable set of tools for the future studies.

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