Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

Chlorhexidine compounds in cosmetic products

Risk assessment of antimicrobial and antibiotic resistance development in microorganisms

Opinion of the Panel on Biological Hazards of the Norwegian Scientific Committee for Food Safety:

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Acknowledgements

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Terminology and definitions

Acquired resistance: Describes development of insusceptibility or a decrease in susceptibility resulting from genetic changes in a microorganism due to mutation or the acquisition of genetic material.

Antibiotics: The term has traditionally referred to natural organic compounds synthesised by microorganisms that kill or inhibit growth of other microorganisms. Many antibacterial agents in clinical use are derived from natural products, but most are then chemically modified (i.e. semi-synthetic) to improve their properties. Some agents are totally synthetic (e.g. sulphonamides, quinolones). Therefore, the terms "antibacterial agent" or "antimicrobial agent" are preferred to "antibiotic" to include both natural and synthetic compounds. However, in the literature the term antibiotic is also often used for semi-synthetic and synthetic compounds.

Antimicrobial agents: A general term for the drugs (antibiotics), chemicals, or other substances that either kill or stop the growth of microbes. The concept of antimicrobial agents applies to disinfectants, preservatives, sanitising agents, and biocidal products in general.

Antimicrobial resistance: The characteristic of a strain of a microorganism that enables it to survive or avoid inhibition by a defined concentration of an antimicrobial agent. While the terminology regarding antimicrobial action and resistance is well-understood, that relating to biocidal resistance is still the subject of debate. A culture is considered resistant to a biocide when it is not inactivated by a common in-use concentration of a biocide, or by a biocide concentration that inactivates other strains of that organism.

Antimicrobial susceptibility: Describes the degree to which a target microorganism is affected by an antimicrobial agent. There are no clear "cut-off" concentrations that are widely accepted to denote sensitivity or resistance of the various bacterial species to disinfecting agents.

Antiseptic agent: A substance applied topically to living tissue that prevents or inhibits the growth of microorganisms.

Biocide/Biocidal products: According to Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market, biocidal products are defined as "Active substances and preparations containing one or more substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert to controlling effect on any harmful organism by chemical or biological means". The word "biocide" is in common use, and means a "biocidal product".

Biofilm: Microbial biofilms are populations of microorganisms that are concentrated at an interface (usually solid/liquid), and typically surrounded by an extracellular polymeric slime matrix. Flocs are suspended aggregates of microorganisms surrounded by an extracellular polymeric slime matrix that is formed in liquid suspension.

Co-resistance: The process in which selection for resistance to one type of antimicrobial also selects for resistance to another antimicrobial agent due to linkage of the resistance genes on the same genetic unit.

Cross-resistance: The process in which resistance to one antimicrobial agent confers resistance to another since the same mechanism of resistance applies to both drugs.

Disinfectant: A substance that is used in the inanimate environment to destroy or eliminate specific species or groups of microorganisms.

Intrinsic resistance: A natural property of an organism resulting in decreased susceptibility to a particular antimicrobial agent.

Minimum Inhibitory Concentration (MIC): The lowest concentration of a given agent that inhibits growth of a microorganism under standard laboratory conditions.

Normal flora: Indigenous microbial flora of human external, and some internal, surfaces like the skin, mouth, gastrointestinal tract, and upper respiratory tract.
The normal flora contains numerous bacterial species, and numerous strains within each species. Although it may contain pathogens, the vast majority are commensals that contribute to general health as well as to resistance to colonization by pathogens. However, some low-virulence bacteria of the normal flora may, under certain circumstances, become opportunistic pathogens.

Selection: A process by which some bacterial species or strains of bacteria in a population are selected for by having a specific advantage over other microorganisms. Antibacterial substances may provide a more resistant sub-population with such an advantage, enabling them to increase their relative prevalence.

Strain: A subset within a bacterial species differing by some minor, but identifiable, differences.

Summary

Chlorhexidine and its salts are reported as being used in cosmetics as an active ingredient to give the desired effect or as a preservative in concentrations of up to 0.3 %. Such products include mouthwashes, hair dying and bleaching formulations, shampoos, anti hair "aging" products and exfoliants, body lotions, eye creams, face cleansers, sun cream, after-sun lotions, eye makeup removers, and facial masks. Within the health sector, chlorhexidine is used in formulations for preoperative skin disinfection, in treatment of wounds and burns, for urinary bladder flushing, for catheter disinfection, and in ophthalmology and gynaecology. The commonly used concentrations in medical products range from 0.05 to 4 %. In cosmetic products, chlorhexidine is commonly used in combination with other agents with antimicrobial activity in order to improve the biocidal effect.

The available information on chlorhexidine consumption is limited, but the total annual use of chlorhexidine in cosmetic products in Norway has been estimated to be 200 kg. In addition, chlorhexidine is applied in medical formulations, and in 2009 a total of 2 254 kg were used in such products (www.whocc.no).

Bacterial resistance to chlorhexidine can be a natural property of the organism (intrinsic) or acquired by mutation and/or mobile genetic elements such as plasmids. The terms "intrinsic" and "acquired" resistance are related to resistance mechanisms, and a distinction should be made between these terms and the term "antimicrobial resistance" that is related to survival after exposure to the antimicrobial agent. Intrinsic resistance mechanisms towards chlorhexidine are particularly characteristic of Gram-negative bacteria, but also of bacterial spores, mycobacteria, and, under certain conditions, staphylococci also display such mechanisms. There are limited published data on acquired chlorhexidine resistance in bacteria, but from those available, acquired resistance towards chlorhexidine has been described from members of the *Streptococcus* spp., *Staphylococcus* spp,. and Enterobacteriaceae. This resistance may result from increased expression of chromosomally located efflux pumps, acquisition of plasmid-encoded efflux pumps, or changes in susceptibility by other presently unknown mechanisms.

Literature on the development of resistance due to chlorhexidine in cosmetics is currently not available. However, it can be speculated that chlorhexidine in such products may add to the selection of microorganisms with increased tolerance to chlorhexidine. Although some reports on the correlation between chlorhexidine and antibiotic resistance are conflicting, some efflux pumps have been shown to mediate export of both chlorhexidine and other antimicrobial agents. Cell wall changes that reduce their permeability may also play a role as a common resistance mechanism between chlorhexidine compounds and other antimicrobial agents, some of which are of clinical importance. Dissemination of plasmids carrying multi-resistance between staphylococci showing co-resistance to chlorhexidine and clinically important antibacterial agents has also been reported. Thus, a contribution by chlorhexidine in cosmetic products to increased occurrence of resistance to clinically important antimicrobial agents cannot be excluded. However, resistance problems are most probably of less importance for chlorhexidine than for antibiotics or for biocides containing quaternary ammonium compounds.

Samandrag

Klorheksidin og salt av klorheksidin vert brukte i kosmetikk enten som aktiv ingrediens som skal gi ynskt effekt eller som konserveringsmiddel i konsentrasjonar på opp til 0,3 %. Slike kosmetiske produkt inkluderer munnskyljevatn, midlar for farging eller bleiking av hår, sjampoar, midlar mot "håraldring", hudskrubbepodukt, hudlotionar, kosmetiske augekremar, anletsrensarar, solkremar, ettersolingskremar, augesminkefjernarar og ansiktsmasker. Innan helsesektoren vert klorheksidin nytta i legemidlar for preoperativ huddesinfeksjon, ved handsaming av mekaniske sår og brannsår, ved urinblæreskylling og i kateterdesinfeksjon. Dessutan er klorheksidin brukt innan oftalmologi og gynekologi. Vanlege konsentrasjonar i medisinske produkt er frå 0,05 to 4 %. I kosmetiske produkt vert klorheksidin ofte nytta saman med andre antimikrobielle stoff for å gi auka antimikrobiell effekt.

Tilgjengeleg informasjon om bruk av klorheksidin er sparsam, men den totale årlege bruken i kosmetiske produkt i Noreg er estimert til 200 kg. I tillegg vert klorheksidin brukt i medisinske preparat, og i 2009 var den totale bruken i slike preparat på 2254 kg (www.whocc.no).

Reistens mot klorheksidin hos bakteriar kan skuldast ein naturleg eigenskap hos organismen (ibuande resistens), etter den kan vera tileigna gjennom mutasjonar og/eller mobile genetiske element som plasmidar. Omgrepa "ibuande" og "tileigna" resistens er knytt til mekanismar for resistens, og må skiljast frå omgrepet "antimikrobiell resistens" som er knytt til overleving etter eksponering for eit antimikrobielt stoff. Det å ha ibuande resistensmekanismar mot klorheksidin er særleg karakteristisk for Gram-negative bakteriar, men dette gjeld og for bakteriesporar, mykobakteriar og i nokre tilfelle også for stafylokokkar. Mengda publiserte data på tileigna klorheksidinresistens hos bakteriar er lita. Frå dei publikasjonane som er tigjengelege, vert tileigna reistens mot klorheksidin påvist hos medlemar av *Streptococcus* spp., *Staphylococcus* spp. og Enterobacteriaceae. Denne resistensen kan ha sitt opphav i auka genetisk uttrykk av kromosomalt bundne efflukspumper, tileigning av plasmidkoda efflukspumper eller som fylje av andre og så langt ikkje kjende mekanismar.

Så langt er det ikkje tilgjengeleg littaratur om utvikling av resistens som fylje av klorheksidinbruk i kosmetiske produkt. Det er likevel mogeleg at klorheksidin i slike produkt kan vera medverkande til seleksjonen av mikroorganismar som har auka toleranse mot klorheksidin. Sjølv om det ser ut til å vera motstridande informasjon i den vitskapelege litteraturen om samvariasjonen mellom resistens mot klorheksidin og antibiotika, er nokre efflukspumper viste å stå for transport av både klorheksidin og andre antimikrobielle stoff ved ein og same pumpemekanisme. Endringar i celleveggen som fører til redusert permeabilitet kan også spele ei rolle som ein sams resistensmekanisme for klorheksidin og andre antimikrobielle stoff som er klinisk viktige. Spreiing av multiresistensplasmid mellom stafylokokkar som viser ko-resistens mot klorheksidin og klinisk viktige antibakterielle stoff er også rapportert. Ein kan derfor ikkje sjå bort frå at klorheksidin i kosmetiske produkt kan medverka til auka førekomst av resistens mot klinisk viktige antimikrobielle stoff. Det er likevel høgst samsynleg at resistensproblema for klorheksidin er mindre enn det ein ser for antibiotika eller biosidar som inneheld kvartære ammoniumssambindingar.

1 Background

In 2009, the Norwegian Scientific Committee for Food Safety (VKM), Panel on Biological Hazards, received a request from the Norwegian Food Safety Authority to develop a risk assessment regarding development of resistance in microorganisms resulting from the use of chlorhexidine in cosmetic products. In response, an *ad hoc* Working Group of experts was appointed with the mandate to draft an assessment regarding this issue.

2 Definition of cosmetic products

According to the EU Cosmetics Directive (76/768/EEC), "a *cosmetic product* shall mean any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition." Furthermore, Norwegian regulations define *cosmetic products* as products that come into contact with the human body surface (skin, hair, nails, lips, and external genitals), teeth or mucous membranes of the oral cavity (Kosmetikklova, 2005*).*

3 Terms of reference

The Norwegian Food Safety Authority commissioned the Norwegian Scientific Committee for Food Safety to undertake a risk assessment on the application of chlorhexidine in cosmetics with special emphasis on the following topics[1]:

1- Could the use of chlorhexidine in cosmetic products facilitate the development of resistance or reduced susceptibility (tolerance) towards chlorhexidine in microorganisms?
2- Could the efficacy of clinically important antimicrobials be reduced by resistance development due to use of chlorhexidine in cosmetic products? If so, which classes of agents might be affected?
3- Could use of chlorhexidine in cosmetic products alter the microflora of the skin or the oral cavity, or influence the virulence of these microorganisms?

4 Hazard identification

Hazard identification is implicit in the title of this risk assessment and in the terms of reference.

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[1] **Oppdrag**

Mattilsynet ba VKM om en risikovurdering i forhold til bruk av klorheksidin i kosmetiske produkter. Følgende spørsmål ble spesielt ønsket besvart:

1- Kan bruk av klorhexidin i kosmetiske produkter føre til resistens/nedsatt klorhexidin følsomhet hos mikroorganismer?
2- Kan man få redusert effekt av klinisk viktige antimikrobiell midler som følge av mulig resistensutvikling ved bruk av klorhexidin i kosmetiske produkter? I tilfelle hvilke klasser av antimikrobielle midler kan bli berørt?
3- Kan bruk av klorhexidin i kosmetiske produkter gi endringer i hudens og munnhulens mikrobiota og dens virulensegenskaper?

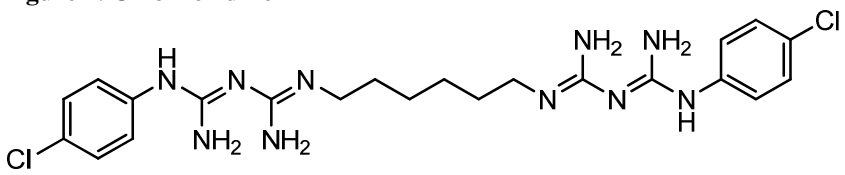
5 Hazard characterization

5.1 Characteristics

5.1.1 Chemical structure and properties

Chlorhexidine is a bisbiguanide compound with antimicrobial activity against bacteria, viruses, and fungi (O'Neil 2006). The structural formula is provided in Figure 1. Chlorhexidine has been given the IUPAC name *N*,*N*,'-hexane-1,6-diylbis[*N*-(4-chlorophenyl) (imidodicarbonimidic diamide)], has a molecular formula of $C_{22}H_{30}Cl_2N_{10}$, and a molecular weight of 505.4 g/mol.

Figure 1. Chlorhexidine



Chlorhexidine is a white to pale yellow, odourless powder. It is only slightly soluble in water and most organic solvents. Since chlorhexidine itself is practically insoluble in water, the commonly used form is a salt of glucuronic acid, chlorhexidine gluconate. Chlorhexidine gluconate is readily soluble in water and alcohol. Concentrated chlorhexidine gluconate is a colourless to pale yellow solution that is odourless and has a strong bitter taste. In aqueous solutions, chlorhexidine salts display maximum biological activity and chemical stability within a pH range of 5-8.

Chlorhexidine gluconate was first synthesised in England by ICI Pharmaceuticals in the 1950s (DAVIES *et al.,* 1954). It was reported to have a high antimicrobial activity, an affinity to skin and mucous membranes, and relatively low toxicity to human cells. Thus, it soon became popular as a topical antimicrobial agent (Paulson 2003).

The antimicrobial activity of chlorhexidine gluconate is pH dependent, being optimal in the range 5.5 to 7.0 (Paulson 2003). This span corresponds with the pH normally found on human tissue where chlorhexidine gluconate may be commonly applied.

5.1.2 Stability

Diluted aqueous solutions of chlorhexidine in concentrations under 1 % are quite heat stable and may be sterilized by autoclaving at 123°C for 15 minutes. Autoclaving of solutions greater than 1.0 % can result in the formation of insoluble residues and is therefore unsuitable. According to producers of commercially available chlorhexidine, aqueous solutions may be stored at room temperature for at least one year, provided that the packaging is adequate (www.sigmaaldrich.com). Prolonged exposure to high temperature or light should be avoided as this affects the stability of chlorhexidine solutions. Aqueous solutions of chlorhexidine are most stable within the pH range of 5 to 8. Above pH 8.0, chlorhexidine base is precipitated and in more acidic conditions a gradual degradation of, and reduction in, the antibacterial activity can be observed (Block 1991).

Several authors report on the microbial degradation of chlorhexidine. Ogase *et al.* (1992) examined the degradation of chlorhexidine by two strains of *Achromobacter xylosoxidans* originally isolated from the reservoir in a hand-washing machine. These two strains were shown to be resistant to chlorhexidine, with MIC (Minimum inhibitory concentrations) values of 1000 µg/ml. The authors found pyrogallol, phenol, and *p*-chlorophenol to be important degradation products. Several intermediate compounds were predicted to be part of the metabolic degradation of chlorhexidine by microorganisms, including p-chloroaniline and phenol. The experiments of Ogase *et al.* (1992) demonstrated the ability of microorganisms to degrade chlorhexidine. Indeed, Tanaka *et al.* (2005) found that an unidentified strain of *Pseudomonas* isolated from activated sludge degraded chlorhexidine to p-chloroaniline, as well as to two metabolites designated CHDI-B and CHDI-C. These two metabolites showed a tenfold reduction in the antibacterial activity towards several test strains, including *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Serratia marcescens.*

5.1.3 Mode of action

Most studies on the mode of action of chlorhexidine have been on bacteria. The positive charge of chlorhexidine attracts negatively charged proteins on the bacterial surfaces, resulting in physical disruption of the membranes, dissipation of the proton motive forces, and inhibition of membrane-associated enzymes. It is assumed that interactions with bacteria occur via cationic binding to phosphate groups of cell wall teichoic acid in Gram-positive bacteria (Albert 1942) and to phosphate groups in the cell walls and membranes of Gram-negative bacteria (Heptinstall *et al.,* 1970).

At bacteriostatic concentrations, chlorhexidine inhibits membrane enzymes and disrupts the interactions between lipids and proteins in the membranes, leading to permeabilization and leakage of cellular components. At higher concentrations, cytoplasmic proteins coagulate, presumably through denaturation, and the cells die. It is believed that the mode of action on fungi and protozoa is similar to that with bacteria, both through disruption of the cell membranes resulting in leakage of cellular components and penetration of the membranes leading to coagulation of cytoplasmic proteins. Chlorhexidine interacts with the envelope of enveloped viruses resulting in release of the viral capsid at high concentration. At lower concentrations, chlorhexidine may interact with envelope proteins or tail structures, resulting in inhibition of transduction (Denton 1991; Lambert 2004; Maillard 2004).

5.1.4 Antimicrobial activity

Chlorhexidine has a broad antimicrobial activity against bacteria, fungi, enveloped viruses, and protozoa (Denton 2009; Maillard 2004).

Bacterial growth is inhibited at concentrations between 0.5 and 10 mg/L, and bactericidal activity is found at concentrations over 5 mg/L, depending on species and strain. Chlorhexidine is not lethal to bacterial spores or acid-fast bacteria. Fungi are more resistant to chlorhexidine than bacteria, with MIC of 10-200 mg/L and Minimum bactericidal concentration (MBC) of 20 (yeast) to over 200 mg/L (moulds). Chlorhexidine is lethal to protozoa at concentrations over 50-150 mg/L depending on species (see 5.3.1). The susceptibilities of viruses are highly variable and reported virucidal concentrations are between 10 and 2500 mg/L. Enveloped viruses are susceptible to chlorhexidine (Kawana *et al.,* 1997).

5.2 Susceptibility testing

The wide range of areas of application and target organisms makes it difficult to establish relevant and standardised tests for microbial susceptibility. For chlorhexidine compounds, both the ability to inhibit growth and to kill microorganisms may be of importance, depending on the area of use. The test conditions have a considerable influence on results and means that comparisons between investigations and extrapolation from laboratory to practical conditions are difficult. Due to the synergistic and antagonistic effects between different ingredients in cosmetics and chlorhexidine, the concentrations needed to inhibit growth and kill microorganisms may be higher or lower than reported in the literature. Chlorhexidine produces insoluble products with chloride, sulphate, phosphate, and citrate. The efficacy is reduced in the presence of organic materials such as serum, blood, and pus. Soaps and other anionic compounds also neutralize its activity (Moore & Payne 2004). Non-ionic surfactants may have synergistic and antagonistic effects with chlorhexidine depending on compound and concentration ratio (Schmolka 1973). A combination of several biocides/preservatives in the same cosmetic product is often used to increase the total antimicrobial activity, to extend the spectrum of activity, or to ensure antimicrobial action in both the water and oil phase. The effects of chlorhexidine can be enhanced by other biocides such as alcohols and QACs (McDonnell 2007). A combination of preservatives active in the oil phase (such as parabens) and the water phase (chlorhexidine) may be used in cosmetic emulsions (Hiom 2004).

In conclusion, antimicrobial activity in cosmetic products containing several ingredients may be lower or higher than the antibacterial activity in laboratory model tests. Susceptibility tests in the laboratory can therefore be used to compare strains/species or in mechanistic studies (for example to test the effects of mutations), but are not necessarily useful for predicting survival in conditions of practical use.

***In vitro* tests**

In tests for general inhibitory activity, microorganisms are exposed to chlorhexidine compounds in nutrient suspension or nutrient agar, and growth is determined after incubation for a specific time. The main advantages of the MIC method are that it is easy to perform and many strains or chlorhexidine compounds can be tested in the same experiment. There are no standard methods for determining the MIC of chlorhexidine compounds, and therefore various approaches have been described in the literature.

In biocidal tests, microorganisms in suspension or on a surface are exposed to chlorhexidine compounds for a specific period of time, followed by neutralisation and determination of the number of viable microbes. For determining biocidal efficacy for usage in hand-washing or mouthwash preparations, the efficacy of products and/active components can be tested using European standardised tests for bactericidal, sporicidal, virucidal, and fungicidal activity (CEN 1997a; CEN 1997b; CEN 1997c; CEN 1997d; CEN 1998; CEN 2002a; CEN 2002b; CEN 2005). These test methods may be used for measuring strain susceptibility.

In the tables presented by Denton (1991) there is generally little correlation between the tolerance level for chlorhexidine in the MIC test and the biocidal test. In another study, *Pseudomonas aeruginosa* strains with raised MIC to chlorhexidine were no less sensitive than the parent strain to chlorhexidine and benzalkonium chloride in bactericidal investigations (Thomas 2005). It is therefore difficult to draw conclusions about resistance to the biocidal effect of chlorhexidine based on results of MIC tests.

Testing susceptibility under conditions of practical use

Standardised tests have been developed to test the efficacy of preservatives in cosmetic products (British Pharmacopoeia Comission 2000; United States Pharmacopeia 2002). These methods could potentially be adapted to test susceptibility of microorganisms under conditions of practical use. The challenge tests are based on inoculation of the pharmaceutical/cosmetic product with bacteria, followed by incubation and sampling for survivors during the storage period (Russell 2003).

For assessing efficacy of hygienic hand-wash, a European standard *in vivo* test (EN1499) has been developed. The method is based on contamination of hands with a test strain, treating with the preparation and measuring the number of viable test organisms before and after treatment. The method can potentially be used to measure susceptibility of microorganisms to chlorhexidine. Standardised tests for measuring the resistance of microorganisms in conditions relevant to mouthwash are not available, and mouthwash preparations are often tested using *in vitro* test methods. Since the resistance of microorganisms is dependent on a range of environmental factors, results from these experiments are not readily extrapolated to practical conditions.

5.3 Resistance mechanisms

The terms "intrinsic" and "acquired" resistance are related to resistance mechanisms and should be distinguished from the term "antimicrobial resistance" that is related to survival after exposure to the antimicrobial agent. Bacterial resistance to chlorhexidine can be a natural property of the organism (intrinsic) or acquired by mutation and/or mobile genetic elements such as plasmids or transposons.

5.3.1 Intrinsic resistance

Intrinsic resistance to chlorhexidine is generally low level resistance/tolerance (see section 5.1.4 and below for inhibiting concentrations of chlorhexidine) and thus for most organisms, with the exception of some viruses and *A. xylosoxidans,* well below the commonly used concentrations in both cosmetic (≤ 3 g/L) and medical products (≥ 0.5 g/L).

There is an overall tendency for Gram-negative bacteria to be more resistant than Gram-positive bacteria to chlorhexidine. Thus, intrinsic resistance to chlorhexidine is particularly demonstrated by Gram-negative bacteria (especially *P. aeruginosa, Proteus* spp., and *Burkholderia cepacia*), but also by bacterial spores, mycobacteria, and, under certain conditions, staphylococci. Spore coats and cortexes are responsible for the high tolerance of bacterial spores to chlorhexidine, while impermeability, due to outer membrane composition and decreased porin expression, contributes to the intrinsic resistance of vegetative cells (McDonnell & Russell 1999; Russel & Day 1993). In mycobacteria, the waxy cell walls and, specifically, the cell wall component arabinogalactan prevent adequate chlorhexidine entry (Broadley *et al.,* 1995; McDonnell & Russell 1999). In *P. aeruginosa,* the outer membrane is responsible for its high tolerance, due to the high cation content that aids in the formation of strong lipopolysaccharide (LPS)-LPS links, and the presence of small-sized porins that do not permit general diffusion (Broadley *et al.,* 1995; Brown 1975; McDonnell & Russell 1999). A less acidic outer membrane LPS, partly due to the high content of phosphate-linked arabinose in the LPS limiting its cation-binding capacity, probably contributes to the high intrinsic resistance to chlorhexidine of *B. cepacia* and some *Proteus* strains (Cox & Wilkinson 1991; McDonnell & Russell 1999). In *S. aureus*, the mucoidal slime layer seems to protect these Gram-positive cells against chlorhexidine (Kolawole 1984; McDonnell & Russell 1999). Certain Gram-negative bacteria produce vesicles by extrusion of their outer cell membranes that attach to bacteria and oral surfaces. Vesicles released by *Porphyromonas gingivalis*

probably promote tolerance to chlorhexidine by binding of chlorhexidine by the vesicle LPS. Vesicles released by *P. gingivalis* have been demonstrated to protect both *P. gingivalis* and other selected oral bacterial species against chlorhexidine concentrations that are 3 times that of their normal MIC (Grenier *et al.,* 1995).

Growth rate and growth-limiting nutrients affect the physiological states of cells and the presence of biocides, such as chlorhexidine, is likely to modify the degree of thickness and cross-linking of peptidoglycan. These factors might explain the modified response to biocides in Gram-positive bacteria such as *Bacillus megaterium* (Gilbert & Brown 1980; McDonnell & Russell 1999).

Formation of biofilms prolongs survival of *S. marcescens* (Marrie & Costerton 1981), *B. cepacia* (Hugo *et al.,* 1986), *Enterococcus faecalis* (Abdullah *et al.,* 2005), and methicillin-resistant *S. aureus* (MRSA) (Oie *et al.,* 1996) on exposure to chlorhexidine. *Haemophilus influenzae*, *P. aeruginosa*, MRSA, and *Streptococcus mutans* exposed to chlorhexidine showed a markedly increased percentage survival when growing in biofilms compared with planktonic cells (Izano *et al.,* 2009; Kreth *et al.,* 2008; Smith & Hunter 2008).

In addition to outer membrane impermeability, intrinsic resistance can be associated with the activity of basal levels of efflux by pumps actively removing chlorhexidine from the membrane core. Efflux pumps contributing to intrinsic resistance of chlorhexidine include MepA in *S. aureus* (chlorhexidine MIC 0.04-1.25 mg/L) (Huet *et al.,* 2008; Kaatz *et al.,* 2005), MexCD-OprJ pump in *P. aeruginosa* (chlorhexidine MIC 10 mg/L) (Fraud *et al.,* 2008), AcrAB-TolC in *E. coli* (Levy 2002), CepA in *Klebsiella pneumoniae* (chlorhexidine MIC 16-32 mg/L) (Fang *et al.,* 2002), and to a lesser extent SdeXY in *S. marcescens* (chlorhexidine MIC 2.5 mg/L in *E. coli*) (Chen *et al.,* 2003). Chromosomal efflux pumps can be induced, so that an apparently susceptible strain can overproduce a pump to become tolerant. It has been claimed that the activity of bisbiguanides, such as chlorhexidine, is unaffected by hyperexpression of efflux presumably because they do not become solubilised within the membrane core (Gilbert & Moore 2005). However, chlorhexidine can induce the MexCD-OprJ pump (chlorhexidine MIC >50 mg/L after serial passage in increasing concentrations of chlorhexidine) and hyperexpression of *mexCD-oprJ* in a mutant strain also enhanced chlorhexidine tolerance (chlorhexidine MIC 10 mg/L increased to 20 mg/L) (Fraud *et al.,* 2008). It has recently been shown by global transcriptomic analyses of *P. Aeruginosa*, that chlorhexidine at sub-MIC concentrations (8 µM (4 mg/L)) up-regulated *mex*C and *mex*D, 14 and 6 times respectively, after 10 minutes exposure time (Nde *et al.,* 2009). The clinical consequence is, however, uncertain.

Rather than preventing the drug from reaching its target, a cell can also inactivate the drug to become resistant. A chlorhexidine-degrading enzyme has been discovered in *A. xylosoxidans* (MIC chlorhexidine >125-500 mg/L) (Nagai & Ogase 1990; Ogase *et al.,* 1992).

The intrinsic resistance of non-enveloped viruses towards chlorhexidine is probably due to a reversible adsorption to the viral capsid. This adsorption does not lead to penetration inside the phage particles, but may result in inhibition in the transduction ability of the virus (Maillard & Beggs 2009).

Studies on chlorhexidine resistance in fungi have mainly been conducted on the yeast *Saccharomyces cerevisiae.* The glucan composition of the cell wall may play a role in limiting entry of chlorhexidine into *S. cerevisiae*. Furthermore, uptake of chlorhexidine is

reduced in older cultures in which *S. cerevisiae* cells show increased cell wall thickness and reduced porosity (Hiom *et al.,* 1995; Hiom *et al.,* 1996). The yeast *Candida albicans* is less sensitive to chlorhexidine than *S. cerevisiae* due to lower uptake (Hiom *et al.,* 1995). Also *C. albicans* biofilms produce multidrug-tolerant subpopulations of persister cells that display resistance to chlorhexidine (growth in 100 mg/L chlorhexidine) that is not influenced by efflux transporters. Such persister formation is not dependent on formation of a complex biofilm structure, but rather on the ability to attach to a surface (Lafleur *et al.,* 2006). For moulds that are generally more tolerant to chlorhexidine than yeasts, no studies are available that indicate the mechanism involved in their high intrinsic resistance. However, McDonnell and Russell (1999) speculate that cell wall composition is involved.

For protozoa, the cyst forms are invariably the most tolerant to chemical disinfectants such as chlorhexidine (Khunkitti *et al.,* 1998b; McDonnel & Russel 1999). The cellulose cyst wall appear to act as a barrier for uptake of chlorhexidine in *Acanthamoeba castellanii* thereby contributing to its high intrinsic resistance (Khunkitti *et al.,* 1998a). The effect of biofilm formation on chlorhexidine tolerance by *Acanthamoebae* (Gray *et al.,* 1995) has not been explored.

5.3.2 Acquired resistance

The resulting tolerance level due to the presence of acquired resistance mechanisms, including acquisition of the *qacA* gene described below, is far less than the commonly used chlorhexidine concentrations of up to 3 g/L in cosmetic products and between 0.5 and 40 g/L in products for medical use.

Increased chlorhexidine efflux can be achieved by over-expression of chromosomal multidrug efflux protein MepA in *S. aureus* due to mutations resulting in premature termination or amino acid substitutions in the regulatory protein MepR or substitution in MepA (chlorhexidine MIC 0.04-1.25 mg/L increased 2-32 fold in mutants) (Huet *et al.,* 2008; Kaatz *et al.,* 2005). Mutational up-regulation of multidrug efflux pump SdeAB in *S. marcescens* also resulted in increased tolerance to chlorhexidine (chlorhexidine MIC 25 mg/L increased to 100 mg/L in mutant) (Maseda H *et al.,* 2009).

Acquisition of plasmid-encoded efflux pumps results in increased tolerance to chlorhexidine in staphylococci. QacA plasmid-encoded efflux pumps providing tolerance to chlorhexidine (MIC 2-4 mg/L) are common in clinical strains of *S. aureus* and other staphylococci (Leelaporn *et al.,* 1994; Littlejohn *et al.,* 1992; Paulsen *et al.,* 1996; Poole 2002). The clinical significance of *qacA* mediated chlorhexidine tolerance has not been determined (Milstone *et al.,* 2008). Furthermore, no studies have been published testing how much QacA actually contributes to the raised chlorhexidine MIC levels in staphylococci. Studies comparing MIC for chlorhexidine in cells expressing QacA wild-type with cells expressing QacA mutants/no QacA have only been conducted in *E. coli* (Hassan *et al.,* 2006; Hassan *et al.,* 2008; Wu *et al.,* 2008*)*. An unnamed antiseptic resistance protein, differing from QacA in size and providing tolerance to chlorhexidine (chlorhexidine MIC 6.25 mg/L), was found encoded on a transferable 50-kb plasmid in MRSA (Yamamoto *et al.,* 1988).

Finally, changes in susceptibility may include other currently unknown mechanisms. DNA from *Streptococcus sanguis* strains that had developed stable tolerance to chlorhexidine (chlorhexidine MIC 64-128 mg/L) was used to transform susceptible, competent *S. sanguis* to increased chlorhexidine tolerance (chlorhexidine MIC 64 mg/L) (Westergren & Emilson 1980) proving that this tolerance was an inheritable trait. In *Pseudomonas stutzeri,* stable

tolerance to chlorhexidine (chlorhexidine MIC 50-100 mg/L) was probably developed by a mutation that resulted in nonspecific alteration of the cell envelope (Tattawasart *et al.,* 1999), and stable adaptive tolerance to chlorhexidine was readily achieved in both *Salmonella enterica* serovar Virchow and *E. coli* O157 (Braoudaki & Hilton 2004), probably due to outer membrane modifications. In *S. mutans,* Clp serine protease, involved in the general stress response, assists *S. mutans* in resistance against chlorhexidine through adaptation (Deng *et al.,* 2007).

To date, limited data have been published on acquired chlorhexidine resistance in bacteria. Most studies exploring substrate specificities of novel resistance mechanisms usually test resistance to quaternary ammonium compounds, rather than chlorhexidine, if a biocide is included in the test panel.

5.4 Resistance among microbes in the normal flora of humans and in the environment

The microflora of the skin

The skin is generally an unfavourable place for microbial growth. It is subject to periodic drying and the pH ranges from 3 to 5, which is non-optimal for most bacterial species. However, certain species are able to establish themselves under such conditions, and these constitute the normal flora of the skin. The skin may be considered as a single organ, but its flora varies at different locations of skin surfaces. Bacterial populations in warm humid places, like the axillae, umbilicus, and interdigital spaces, are rich and numerous, in contrast with the microflora on dryer parts of the skin. Hair follicles, sebaceous glands, and sweat glands provide attractive habitats for microorganisms, where a variety of bacteria and fungi reside (Høiby 1993; Linton 1982; Madigan *et al.,* 1997; Tortora *et al.,* 1989; Tortora *et al.,* 1998). Many factors may influence the composition of microflora of an individual's skin, including temperature, humidity, age, sex, race, and occupation (Roth & James 1988).

The skin flora consists primarily of Gram-positive bacteria. Cultivation-based studies have shown that these include *Staphylococcus* spp., *Micrococcus* spp., *Corynebacterium* spp., *Streptococcus* spp., *Propionibacterium* spp., and yeasts belonging to the genus *Pityrosporum* (Cogen *et al.,* 2008; Gao *et al.,* 2007; Høiby 1993; Madigan *et al.,* 1997; Tortora *et al.,* 1989). However, little is known about the presence of non-cultivable or rare species in the microbiota of the skin, and a more complex microflora may be present (Gao *et al.,* 2007). With the exception of *Acinetobacter* spp., Gram-negative bacteria are almost always minor constituents of the normal skin flora, (Cogen *et al.,* 2008; Gao *et al.,* 2007; Madigan *et al.,* 1997; Martro *et al.,* 2003).

The staphylococcal group can be divided into two subdivisions: coagulase-positive and coagulase-negative. Coagulase-positive staphylococci are regarded as the most virulent, and are more often associated with disease than coagulase-negative staphylococci. *S. aureus* is the most common coagulase-positive *Staphylococcus* species in man. *S. aureus* can sometimes be found on the skin of individuals who are nasal carriers, as the skin can be contaminated with bacteria from the mucosal linings of the nose. Several species of coagulase-negative staphylococci can be found on human skin, *Staphylococcus epidermidis* being the most common (Cogen *et al.,* 2008). *S. epidermidis* and other coagulase-negative staphylococci are increasingly recognized as cause of nosocomial infections, and also play important roles in implant-related infections. *S. aureus* is of special concern since this is a leading human

pathogen. There has been a dramatic increase in *S. aureus* strains that are resistant to antimicrobial agents, including MRSA. MRSA are resistant to all beta-lactam antibiotics and are considered a major threat in human medicine.

Staphylococci harbouring *qac* genes can be resistant to other antimicrobial agents (described under section 5.5). Several studies have shown the occurrence of *qac* genes in MRSA isolates (Gillespie *et al.,* 1986; Mayer *et al.,* 2001; Noguchi *et al.,* 1999; Noguchi *et al.,* 2005). An early investigation showed that among MRSA strains isolated in Japan in 1992, 10.2 % contained the *qacA/B* gene (Noguchi *et al.,* 1999). Another study reported that among MRSA strains from Europe isolated during the period 1997 to 1999, 63 % contained the *qacA/B* gene (Mayer *et al.,* 2001). Numerous successful MRSA clones have been described and characterized, and many have a worldwide distribution (Deurenberg *et al.,* 2007). The presence of *qac* genes among MRSA clones is currently unknown. Selection of *qac* containing staphylococci may therefore contribute to increased occurrence of strains resistant to different antimicrobial agents. Although QacA may pump chlorhexidine, resulting in increased tolerance, the practical consequence is unknown. As the conferred resistance level is much lower than the concentrations used in cosmetics, it may be of negligible importance (Milstone *et al.,* 2008). Thus, whether the presence of the *qacA* gene will be an advantage for staphylococci during exposure to chlorhexidine cannot be concluded with today's knowledge.

Acinetobacter spp. can be a part of the normal skin flora. *Acinetobacter baumannii* is an important nosocomial pathogen of increasing importance. Of special significance is the increasing frequency of resistance to a variety of antimicrobial agents. Unfortunately, little is known about the susceptibility of *Acinetobacter* spp. to disinfecting agents (Martro *et al.,* 2003; Wisplinghoff *et al.,* 2007).

We have not found any reports about reduced susceptibility to disinfectants among other microorganisms considered to be part of the normal flora of the skin.

The microbiological flora of the skin can be exposed to chlorhexidine via various products like skin cleansing products, hand disinfectants, preoperative bathing formulations, and cosmetic products (Denton 1991; Milstone *et al.,* 2008). Hand cleansing with chlorhexidine has been shown to reduce the number of microbes on skin by between 86 and 92 % (Askgaard 1975; Lowbury & Lilly 1973). In general, skin cleansing with chlorhexidine significantly reduces normal skin flora, Gram-negative organisms, and *S. aureus* (Denton 1991). Whole body washing with chlorhexidine is reported to reduce skin colonisation (Brandberg 1989; Davies *et al.,* 1997; Kaiser *et al.,* 1988). Little knowledge is available on potential selection of certain types of bacteria after such exposure. In a study of bathing in a 4 % chlorhexidine solution, counts of *S. aureus* and Gram-negative bacteria after treatment were no different to those from before treatment (Davies *et al.,* 1997). In another study, it was found that whole body washing with 4 % chlorhexidine reduced MRSA at one sample site, but no effect was detected at other sample sites (Wendt *et al.,* 2007).

To our knowledge, no data exist on how cosmetics containing chlorhexidine will affect the skin flora, and the selection pressure on the normal flora of the skin from chlorhexidine in cosmetic products is not easily predicted.

Oral microflora

The oral cavity is colonized by a wide range of mircoorganisms in complex ecosystems that change throughout life. The oral microbiota is one of the most complex mixtures of bacteria

known. A recent report using pyrosequencing analyses estimated the number of species-level phylotypes to be greater than 19000 (Keijser *et al.,* 2008), with 97 % of the sequences occurring in 45 genera. The most prevalent species belonged to the *Streptococcus*, *Veillonella*, *Corynebacterium*, *Actinomyces*, *Fusobacterium*, *Prevotella*, *Neisseria*, *Porphyromonas,* and *Haemophilus* genera. Less than 50 % of the flora were cultivable. Commensal streptococci constitute a major part of the oral bacterial flora that gradually becomes more complex with time. Among the oral streptococci, *Streptococcus mitis* is the most prevalent (Aas *et al.,* 2005). The streptococci form the basis for adhesion of other colonizing bacteria, such as *Actinomyces* and *Veillonella* species. The composition varies at various sites in the oral cavity, depending on nutrient availability, salivary flow rate, and oxygen tension, as well as on the host's dental and gingival health conditions, diet, and age. The commensal oral bacteria exist mainly as biofilms on mucosal and tooth surfaces. The flora also protects against colonization by potential pathogenic bacteria like *S. aureus, E. faecalis, Streptococcus pneumoniae, Streptococcus pyogenes, Neisseria* species, members of the Enterobacteriaceae family, *H. influenza,* and actinomycetes (Sweeney *et al.,* 2004). Therefore, disruption of the ecological balance by antimicrobial agents may have an impact on the health of the individual (Avila *et al.,* 2009).

Mouthwashes containing chlorhexidine are mainly advertised as anti-halitosis agents. Chlorhexidine mouthwash has been studied also for its effect on oral biofilms and dental diseases, and even for prevention of ventilator-associated pneumonia (Hutchins *et al.,* 2009). The use of chlorhexidine as a mouthrinse is related to its ability to adsorb onto a variety of surfaces and to its broad spectrum of antimicrobial activity over a wide pH range. Being cationic, the molecules can bind to carboxyl-, phosphate- and hydroxyl-groups of negatively charged surfaces. This allows the agent to bind to oral mucosal surfaces and to remain in the oral cavity for prolonged periods. This so-called substantivity is considered important for the oral antibiofilm effects, although chlorhexidine loses some of its activity upon adsorption to surfaces (Baker *et al.,* 1978; Moran & Addy 1984)

Numerous studies confirm the preventive effect of chlorhexidine mouthrinses on dental biofilm formation and gingivitis development. Chlorhexidine is regarded as the most efficacious agent in reducing accretion of biofilm on teeth, and chlorhexidine mouthrinse has been included in numerous studies as the "gold standard" against which the efficacy of other agents may be compared (Petersen & Scheie 1998). A single mouthrinse with 0.2 % chlorhexidine reduces oral flora by between 80 and 95 % (Schiott 1973). The control of dental biofilms is central for oral health maintenance, and therefore chlorhexidine mouthrinsing represents a useful adjunct for subjects unable to perform effective conventional mechanical tooth cleaning.

There has been little focus on the possibility of chlorhexidine resistance development or development of co- or cross-resistance with antibiotics among members of the oral flora. In an early study, it was concluded that two years of daily mouthrinses with chlorhexidine resulted in a slight change in distribution towards bacteria that were less sensitive to chlorhexidine (Schiott *et al.,* 1976a). In 1980, chlorhexidine-sensitive *S. sanguis* ATCC10558 (now *Streptococcus gordonii)* was shown to develop tolerance to chlorhexidine upon growth in continuous culture under exposure to chlorhexidine (see section 5.3.2) (Westergren & Emilson 1980).

A total of 424 clinical *S. mutans* isolates were studied in Finland for chlorhexidine susceptibility, along with susceptibility to amoxicillin, penicillin, cefuroxime, erythromycin,

tetracycline and sulphamethoxazole-trimethoprim (Järvinen *et al.,* 1993). Chlorhexidine was highly effective against all the *S. mutans* isolates and the MIC did not exceed 1 mg/L. The strains were also sensitive to the antibiotics tested. The authors concluded that although there is an increasing and continuous selection pressure on the oral microbiota from chlorhexidine mouthrinses, oral *S. mutans* has remained susceptible in Finland (Järvinen *et al*., 1993). The mouthrinsing habits of the test subjects were, however, unknown, and therefore their actual chlorhexidine exposure is uncertain.

The effect of pulsing chlorhexidine on oral bacterial ecosystems, including ten oral bacteria, was studied *in vitro*. The results of this study (McBain *et al.,* 2003) support the previous observation that chlorhexidine alters the susceptibility distribution (Schiott *et al.,* 1976b), but concluded that there were no significant alterations in distribution of sensitivity to chemically unrelated biocides or antibiotics including triclosan, erythromycin, penicillinV, vancomycin, and metronidazole. Notably, the chlorhexidine exposure lasted for only five days. The bacteria studied were laboratory strains of *Actinomyces naeslundii, Fusobacterium nucleatum, Lactobacillus rhamnosus, Neisseria subflava, Prevotella nigrescens, P. gingivalis, S. mutans, S. sanguis, S. oralis,* and *Veillonella dispar*.

Based on the few clinical studies on susceptibility change or resistance development in response to chlorhexidine exposure, Sreenivasan and Gaffar concluded in a review article that chlorhexidine reduces dental biofilm, but without altering the microbial tolerance to either chlorhexidine or to commonly used antibiotics (Sreenivasan & Gaffar 2002).

The oral microflora, including the early tooth colonizers *S. gordonii, S. oralis,* and *S. mitis* constitute a pool of genetic material both from cells inhabiting the oral cavity, and from transiting cells (Hakenbeck *et al.,* 1998; Seppala *et al.,* 2003)). Several factors in the oral cavity, including competence factors for streptococci, could promote horizontal gene transfer among the bacteria. Furthermore, both naked DNA and bacteriophages can survive in human saliva. It has been shown experimentally that oral biofilms represent suitable environments for genetic transfer to potential pathogens (Roberts & Mullany 2006), and it is likely that this could also occur *in vivo*.

The oral bacterium *F. nucleatum* co-aggregates with other oral species and plays a role in oral biofilm formation as a bridge between early and late colonizers (Kolenbrander & London 1993). Exposing *F. nucleatum* to sub-inhibitory concentrations of chlorhexidine resulted in induced bacteriocine production (Okamota *et al.,* 2000). Whether this might affect the oral ecology is, however, unknown.

Environment

As chlorhexidine compounds enter the environment via the sewage system they will inevitably act on environmental microbes. In a study by Lawrence *et al.* (2008), the effects of chlorhexidine on microbial biofilms from river water were examined. The authors observed significant effects of chlorhexidine at a concentration of 100 µg/L on the protozoan, algal, cyanobacterial, and bacterial biomass. At this concentration, a virtual elimination of the protozoan community in the biofilms could be observed, resulting in lowered grazing activity.

Nuñez and Moretton (2007) examined the bacterial resistance patterns to several disinfectants, including chlorhexidine, in hospital sewage effluents in Buenos Aires. Between 10^3 and 10^6 chlorhexidine resistant bacteria/100 mL were isolated from the samples. The bacterial populations resistant to disinfectants were mainly members of the

Enterobacteriaceae family, *Staphylococcus* spp., and *Bacillus* spp. Bacterial isolates were tested for their resistance patterns by an agar dilution method using chlorhexidine in increasing concentrations. The chlorhexidine MIC in the resistant bacteria isolated from the hospital sewage ranged from 50 to 150 mg/L, and included *Shigella dysenteriae, Shigella flexneri, P. vulgaris, Aeromonas hydrophila, Alcaligenes* sp., *Acinetobacter* sp., and *P. aeruginosa.* The authors conclude that hospital effluents are of importance in the bacterial resistance selection process, particularly in the case of disinfectants.

5.5 Resistance link between chlorhexidine compounds and other antimicrobial agents

There are conflicting reports on whether there is a correlation between chlorhexidine tolerance and antibiotic resistance. Koljalg (2002) reported good correlation between chlorhexidine and antibiotic susceptibility in both MIC and MBC among clinical Gram-positive bacteria, and mainly in MBC among clinical Gram-negative bacteria. Resistance to ciprofloxaxin, imipenem, cefotaxime, ceftazidime, gentamicin, and aztreonam appeared to indicate increased chlorhexidine tolerance among Gram-negative bacteria (Koljalg *et al.,* 2002). On the other hand, no link between vancomycin resistance and chlorhexidine tolerance in enterococci has been demonstrated, as vancomycin-resistant enterococci and vancomycin-susceptible enterococci had equivalent susceptibility to chlorhexidine (Anderson *et al.,* 1997). Studies on *E. coli*, *P. aeruginosa, S. marcescens,* and *Proteus mirabilis* have not revealed any increase in chlorhexidine resistance among antibiotic resistant bacteria (Michelbriand *et al.,* 1986; Sykes & Matthew 1976). Some reports indicate a higher MIC to chlorhexidine among MRSA compared with methicillin sensitive *S. aureus* (MSSA) (Brumfitt *et al.,* 1985; Cookson *et al.,* 1991; Mycock 1985). However, bactericidal activity is reported to be similar for MRSA and MSSA (Cookson *et al.,* 1989; Haley *et al.,* 1985). Cookson (2005) discusses the relevance of increased chlorhexidine resistance (QacA mediated) among MRSA. The *qacA*-positive strains were not killed more slowly in an *in vitro* rate of kill test (Cookson *et al.,* 1991). MSSA transcipients with the *qacA* gene transferred to them from MRSA were also killed rapidly *in vitro* and *in vivo.* Chlorhexidine MICs of MRSA isolates with different pulsed field gel electrophoresis genotypes from six geographically disparate hospitals were not further elevated 6 years after the first appearance of the strain. Additional unpublished data would suggest that the *qacA* chlorhexidine resistance gene did not convey a significant advantage to MRSA, in that resistance to gentamicin and chlorhexidine of the isolates referred to the reference laboratory, fell from approximately 90 % in 1984 to approximately 50 % in the late 1980s (Cookson 2005).

Some efflux pumps have been shown to mediate export of both chlorhexidine compounds and other antimicrobial agents by the same pump. The available MIC levels of antimicrobial agents due to these pumps are only listed below if a clinical breakpoint is available for comparison. Clinical breakpoints given are according to the European committee on Antimicrobial Susceptibility testing (EUCAST) (http://www.eucast.org/clinical_breakpoints/) that are only available for those antimicrobial agents which are in clinical use in Europe against the bacteria in question. Among such chromosomally encoded efflux pumps are;

- MepA in *S. aureus* transporting a range of structurally different compounds such as chlorhexidine, benzalkonium chloride, pentamidine, fluoroquinolones (MIC levels of 0.16-1.25 mg/L, clinical breakpoint of >1 mg/L for the ciprofloxacin in staphylococci), and tigecycline (MIC levels of 4-16 mg/L, clinical breakpoint of >0.5 mg/L) (Huet *et al.,* 2008; Kaatz *et al.,* 2005; McAleese *et al.,* 2005).
- *E. coli* AcrAB-TolC pump exporting biocides such as chlorhexidine, quaternary ammonium compounds and triclosan as well as penicillins (MIC levels of 2-32 mg/L,

clinical breakpoints of >8 mg/L in Enterobacteriaceae), cephalosporins, chloramphenicol (MIC level of 3.13 to >160 mg/L, clinical breakpoints of >8 mg/L), fusidic acid, macrolides, fluoroquinolones (MIC level of 3.13-20 mg/L, clinical breakpoints of >16 mg/L fro nalidixic acid), novobiocin, trimethoprim, tetracyclines (MIC level of 0.5 mg/L, clinical breakpoints of >2 mg/L), and rifampicin (Hirata *et al.,* 2004; Levy 2002; Ma *et al.,* 1993; McMurry *et al.,* 1998; Nishino & Yamaguchi 2001; Okusu *et al.,* 1996).

- Pump SdeXY in *S. marcescens* conferring reduced susceptibility to several antimicrobial agents including ampicillin (MIC level of 16 mg/L, clinical breakpoints of >8 mg/L in Enterobacteriaceae), erythromycin, tetracycline (MIC level of 16 mg/L, clinical breakpoints of >2 mg/L for tigecylcine), and ciprofloxacin (MIC level of >8 mg/L, clinical breakpoints of >1 mg/L) in addition to benzalkonium chloride, triclosan and to a lesser extent chlorhexidine (Chen *et al.,* 2003; Hornsey *et al.,* 2010).
- SdeAB in *S. marcescens* resulted in increased tolerance to chlorhexidine as well as to cetylpyridinium chloride, benzalkonium chloride, quinolones (MIC levels of 1.56-25 mg/L, clinical breakpoints of >1 mg/L for norfloxacin and ofloxacin in Enterobacteriaceae), tetracycline, and chloramphenicol (MIC level of 100 mg/L, clinical breakpoints of >8 mg/L) (Maseda H *et al.,* 2009).
- The MexCD-OprJ pump in *P. aeruginosa* induced by chlorhexidine to enhance tolerance to chlorhexidine and also to clinically relevant antibiotics such as quinolones (MIC levels of 2->4096 mg/L, clinical breakpoints of >1 mg/L for ciprofloxacin and >2 mg/L for levofloxacin in *Pseudomonas*), macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, some penicillins (MIC level of 1-4096 mg/L, clinical breakpoints of >16 mg/L), and some cephems (MIC levels of 0.5-256 mg/L, clinical breakpoints of >8 mg/L for cefepime and ceftazidime) (Fraud *et al.,* 2008; Masuda *et al.,* 2000; Morita *et al.,* 2003).

QacA plasmid-encoded efflux pumps are common in clinical strains of *S. aureus* and other staphylococci specifying tolerance to structurally dissimilar cations such as chlorhexidine, benzalkonium chloride, and cetrimide (Littlejohn *et al.,* 1992; Paulsen *et al.,* 1996; Poole 2002). The *qacA* gene is typically found on multi-resistance plasmids that may encode resistance to aminoglycosides, penicillin, tetracycline, and trimethoprim (Archer *et al.,* 1986; Leelaporn *et al.,* 1994; Paulsen *et al.,* 1996; Sidhu *et al.,* 2002; Tennent *et al.,* 1989). A gentamicin resistance plasmid carrying *qacA* was found in an MRSA clone in the United Kingdom (Cookson 2005). *qacA/B* and a gene conferring resistance to β-lactams have proved to co-reside on large plasmids in various staphylococcal species of both clinical and food-processing origin (Anthonisen *et al.,* 2002; Bjorland *et al.,* 2005; Sidhu *et al.,* 2002; Sidhu *et al.,* 2001) and such plasmids can be taken up by plasmid-free *S. aureus,* indicating that the resistance genes have the potential to be transferred to pathogens under selective stress (Sidhu *et al.,* 2001).

An unnamed antiseptic resistance protein, differing from QacA in size and providing tolerance to chlorhexidine and benzalkonium chloride, was found encoded on a transferable 50-kb plasmid in MRSA also harbouring resistance to aminoglycosides (Yamamoto *et al.,* 1988).

Cell wall changes may also play a role as common resistance mechanism between chlorhexidine compounds and other antimicrobial agents by reducing permeability. *P. stutzeri* that developed stable resistance to chlorhexidine also demonstrated a variable increase in resistance to polymyxin B, gentamicin, nalidixic acid, erythromycin, and ampicillin

(Tattawasart *et al.,* 1999). *Salmonella enterica* serovar Virchow that had adapted to chlorhexidine demonstrated cross-resistance to tetracycline and triclosan, whereas *E. coli* O157 demonstrated cross-resistance to triclosan (Braoudaki & Hilton 2004).

In summary, it is mainly intrinsic mechanisms that show cross-resistance and few acquired mechanisms show co-resistance to chlorhexidine and other antimicrobial agents, including clinically relevant antibiotics among some human pathogens, e.g. *S. aureus*. The resulting tolerance levels from both intrinsic mechanisms (5.3.1) and from the presence of the acquired resistance mechanisms, including acquisition of the *qacA* gene (5.3.2), are far less than the commonly used chlorhexidine concentrations of up to 3 g/L in cosmetic products. On the other hand, it may be speculated that exposure of bacteria to residual concentrations of chlorhexidine from cosmetics might favour the spread of resistance towards clinically important antimicrobials. Evidence for a role of chlorhexidine-containing cosmetics in such resistance development is, however, lacking.

6 Link between resistance to chlorhexidine compounds and pathogenicity; genotype and phenotype

The link between resistance to chlorhexidine and pathogenicity has been little studied. Some studies indicate that exposure to low doses of chlorhexidine may render microorganisms less virulent. Galice *et al.* (2006) showed that growth of *Streptococcus agalactiae* in the presence of sub-inhibitory concentrations of chlorhexidine resulted in lower toxin production, but the mechanism behind this was not determined. Similarly, exposure of *C. albicans* to chlorhexidine resulted in reduced phospholipase activity, an important pathogenicity factor (Kadir *et al.,* 2007).

Efflux pumps that confer antimicrobial resistance in microorganisms probably have greater clinical relevance than previously assumed. Certain classes of efflux pumps not only harbour resistance to antimicrobial agents used in therapy, but also have a role in bacterial pathogenicity. Efflux pumps that export antimicrobial agents may also export virulence determinants, such as adhesins, toxins, and other proteins that are important for colonization and survival of bacteria in their hosts (Piddock 2006). Piddock (2006) reviewed several studies that demonstrate that the lack of efflux pump expression by Gram-negative bacteria (like *S. enterica* serovar Typhimurium, *E. coli*, *Erwinia amylovora*, *P. aeruginosa*, *Campylobacter jejuni*, and *Neisseria gonorrhoeae*) has a deleterious effect on the ability of the bacteria to be pathogenic in animal models. Recently, it was reported that lack of AcrAB in *K. pneumoniae* not only resulted in higher susceptibility to several antibiotics, but also to reduced capacity to cause pneumonia in a mouse model. There is, however, a lack of studies on the pathogenicity of mutants over-expressing efflux pumps associated with reduced susceptibility to chlorhexidine.

Most mutations or acquired elements that provide resistance also introduce some biological cost to their host, although genetic adaptations are likely to reduce this fitness burden (Andersson & Levin 1999). Studies on the fitness cost of expressing chlorhexidine resistance genes have not been published, but through their role as exporters of multiple substances it is possible that some of these efflux pumps may express chlorhexidine resistance accompanied by a fitness benefit. Derepression of a multidrug efflux pump, MtrC-MtrD-MtrE in *N. gonorrhoeae,* belonging to the same efflux pump family as the AcrAB-TolC that contributes to chlorhexidine resistance in *E. coli,* increased both resistance to multiple antibiotics and, at

the same time, provided a fitness benefit *in vivo*. This resulted in increased gonococcal survival and infectivity, probably through export of natural immune effectors at the infection site (Warner *et al.,* 2007).

Biofilm formation is considered an important virulence factor for *S. epidermidis* and chlorhexidine is used to prevent biofilm formation in medical applications. A small increase in biofilm formation of *S. epidermidis* on polystyrene when exposed to sub-inhibitory concentrations of chlorhexidine in a laboratory model has been observed (Houari & Di Martino P. 2007). Concentrations of 0.00 2% or higher prevented biofilm formation. No induction of biofilm growth was found for other bacteria tested (*E. coli, K. pneumoniae, P. aeruginosa*). As far as we know, increased biofilm formation of staphylococci during application of chlorhexidine has not been reported.

7 Exposure assessment

Chlorhexidine and its salts may be added to cosmetics, as active ingredients or as preservatives, at concentrations of up to 0.3 %, according to current EU legislation (Council Directive 76/768/EEC). In the EU countries reliable information on products containing chlorhexidine is almost absent. According to the Voluntary Cosmetic Registration Program (VCRP) provided by Food and Drug Administration in USA (http://www.fda.gov/Cosmetics/GuidanceComplianceRegulatoryInformation/VoluntaryCosmeticsRegistrationProgramVCRP/default.htm), chlorhexidine and its salts are used as active ingredients in 43 cosmetic products, and as preservatives in 95 cosmetic products. Furthermore, The Environmental Working Group database in USA (*www.ewg.org*) reports that chlorhexidine or its salts are included in 979 cosmetic products on the American market. These products include hair dying and bleaching formulations, shampoos, anti hair “aging” products and exfoliants, body lotions, eye creams, face cleansers, sun cream, after-sun lotions, eye makeup removers, facial masks, and mouthrinses. Approximately 80 % of the cosmetic products containing chlorhexidine in this database are intended for hair treatments, and therefore not intended for prolonged contact with the body. The Environmental Working Group database reports that, in cosmetic products, chlorhexidine is commonly combined with other agents possessing antimicrobial activity, such as parabens, phenoxyethanol, chlorphenesin, triclosan, hydantoin, benzalkonium chloride, hydroxypropyl trimonium chloride, sorbate, iodine propynyl butylcarbamate, and benetrimonium methosulphate. The combined effect of these agents on microorganisms is not easily assessed.

Creams and lotions containing chlorhexidinemay leave residual concentrations that are below MIC for some bacteria. As for mouthrinses, it has been estimated that by rinsing with 10 ml of a 0.2 % chlorhexidine solution, 67 % will be spat out, 3 % will be swallowed, while 30 % will be bound to mucosal surfaces and subsequently slowly released over a period of time (Gjermo *et al.,* 1975). It is generally accepted that the retention and subsequent slow release of the agent is crucial for the effect on dental biofilms and prevention of gingivitis development. Upon dermal application of 5 ml of 5 % chlorhexidine solution, it was found that more than 60 % of the chlorhexidine present 5 minutes after application remained even after 6 h, while 17 % remained after 24 h (Carret *et al.,* 1997). The binding and slow release of low concentrations exposes the flora to a selective pressure that might favour growth of tolerant bacteria.

In cosmetic products, chlorhexidine is added to mouthwashes with the intention of halitosis reduction and to prevent dental biofilm formation and gingivitis development. As an example, chlorhexidine is reported to be used in the following product types in Norway: Corsodyl Mouthwash, Denivit Original Denivit Mint, Solidox Frisk Pust Munnskyllevann (NFSA).

The available information on chlorhexidine use is limited, thus the figures given here are only coarse estimates. The total use of chlorhexidine included in cosmetic products has been estimated by NFSA to be 200 kg. This includes chlorhexidine included in mouthrinse products and hair products. Chlorhexidine found in cosmetics purchased by Norwegians during travel abroad is not included.

In addition to cosmetic products, chlorhexidine is also found in medical formulations. Such formulations may be used for preoperative skin disinfection, in treatment of wounds and burns, for urinary bladder flushing and in catheter disinfection, in ophthalmology and gynaecology. In Norway eight medical products are currently registered. These products contain chlorhexidine in concentrations from 0.05 to 4 %. The yearly consumption of chlorhexidine in these products is estimated to be 2 254 kg (www.whocc.no).

8 Data gaps

There seems to be a lack of reliable data on the use of chlorhexidine in cosmetic products, the food industry, in dental practices, as well as for veterinary and human medical purposes. Therefore the relative importance of cosmetic products containing chlorhexidine for development of resistance is difficult to quantify.

Published information on the pharmacokinetics of chlorhexidine compounds is scarce. The stability of these agents on the skin and mucous membranes is not well described, and the absorption, distribution, metabolism, and excretion in animals and humans are not thoroughly described in the available literature.

Few studies have been conducted that test chlorhexidine susceptibility under conditions of practical use. Moreover, there are few studies investigating acquired chlorhexidine resistance in bacteria. Most studies exploring the substrate specificities of novel resistance mechanisms/genes, test resistance to quaternary ammonium compounds rather than chlorhexidine should a biocide be included in the test panel.

Conclusive information on the development of resistance due to chlorhexidine in general, and in cosmetic products in particular, is currently lacking. The available literature on resistance towards chlorhexidine and co- and cross-resistance to other antimicrobial agents is generally old. The situation may have changed with respect to resistance patterns, and the available literature may not reflect the current situation.

Knowledge about changes in the virulence of microbes of the oral cavity or skin microbiota due to chlorhexidine exposure is scarce.

We have not been able to find any studies that reveal how much QacA contributes to the raised chlorhexidine MIC levels in staphylococci.

9 Risk characterization: Answers to the questions

9.1 Could the use of chlorhexidine in cosmetic products facilitate the development of resistance or reduced susceptibility towards chlorhexidine in microorganisms?

Chlorhexidine has been used for over 50 years, primarily in clinical applications. As far as we know, treatment failure due to acquired resistance has not been reported. Acquisition of resistance in laboratory tests has been reported, but the tolerance levels are relatively low. However, it is not clear whether tolerant mutants could survive in practical applications as the test methods for resistance are often based on growth inhibition, and low correlation with the bactericidal effect has been reported. Little is known about the mechanisms behind acquired resistance to chlorhexidine. The most studied mechanism is the multidrug efflux pump, QacA, which also confers reduced susceptibility to several related antimicrobials. This plasmid-borne determinant is widespread among clinical *S. aureus* strains. The prevalence of *S. aureus* harbouring QacA in domestic products, including cosmetics, is not known. It has never been proven that QacA confers resistance to either medical or cosmetic user-concentrations of chlorhexidine.

The use of chlorhexidine is more limited in cosmetics than in clinical products, and resistance has been less extensively investigated. Based on present knowledge, development of resistance due to the use of chlorhexidine in cosmetics cannot be excluded. However, resistance development is probably less common for chlorhexidine than for antibiotics or biocides containing quaternary ammonium compounds.

9.2 Could the efficacy of clinically important antimicrobials be reduced by resistance development due to application of chlorhexidine in cosmetic products? If so, which classes of agents might be affected?

Some reports have shown that there are resistance links between chlorhexidine and other antimicrobial agents (see section 5.5).

Cross-resistance can be mediated by efflux pumps in both Gram-negative and Gram-positive bacteria that have been shown to export both chlorhexidine and clinically important antimicrobial agents. Such plasmid-encoded efflux pumps (QacA), which so far only have been reported in staphylococci, may confer resistance to other biocides such as quaternary ammonium compounds. Chromosomally encoded efflux pumps may also transport clinically important antibacterial agents in classes including macrolides, β-lactams, quinolones, amphenicols, trimethoprim, rifamycin, fusidic acid, tetracyclines, and lincomycin, the anti-protozoan pentamidin, or biocides such as quaternary ammonium compounds and triclosan. Chromosomally encoded pumps must often be induced to confer resistance and, even after over-expression, the levels of antibiotic resistance are sometimes relatively low and unlikely to compromise therapeutic effectiveness. However, some human pathogens show MIC levels above clinical breakpoints for; i) a novel tetracycline (*S. aureus*), ii) β-lactams, quinolones, amphenicols, and tetracyclines (Enterobacteriacea), and iii) β-lactams and quinolones (*P. aeruginosa*).

Plasmids showing co-resistance to chlorhexidine and to clinically important antibacterial agent classes such as aminoglycosides, β-lactams, tetracyclines, and trimethoprim have been shown to disseminate between staphylococci. Co-localisation of resistance determinants to chlorhexidine and other antibacterial agents, on mobile elements such as plasmids, may also

contribute to transfer of resistance to other bacteria and be selected for in an environment where the chlorhexidine levels are below the MIC levels.

In summary, there is some evidence of co- and cross-resistance between chlorhexidine and a range of unrelated antibacterial agents such as antibiotics and disinfectants. The tolerance level of microorganisms to chlorhexidine is generally low and probably not of clinical importance. However, application of chlorhexidine in cosmetic products may lead to exposure to highly variable concentrations. Exposure to sub-inhibitory concentrations may contribute to increased occurrence of resistance to clinically important antimicrobial agents. However the contribution of chlorhexidine in cosmetic products to such resistance compared with their use in other applications or the use of antibacterial agents in clinical practice is probably of lesser significance.

9.3 Could use of chlorhexidine in cosmetic products alter the microflora of the skin or the oral cavity, or influence the virulence of these microorganisms?

To our knowledge, there are only a few clinical studies on the effect of chlorhexidine on skin microbiota and no studies on the use of cosmetics. For oral microbiota there are only a few studies on the effect of chlorhexidine in mouthrinses. Since Gram-negative bacteria are generally more tolerant to chlorhexidine than Gram-positive bacteria, one could expect there to be selection of Gram-negative flora on skin, but studies do not indicate that such selection occurs. There is a clear need for investigations on the effects after long-term use. The association between tolerance to chlorhexidine and virulence has been little studied, but two publications report a reduction in virulence after exposure to chlorhexidine. The intrinsic resistance mechanisms found in bacteria are also involved in virulence, but increased expression of these mechanisms and the potential effect on virulence has not been investigated. It would be speculative to conclude that chlorhexidine leads to induction of intrinsic mechanisms involved in virulence or increased fitness. In conclusion, there are presently no indications that the use of chlorhexidine alters the microflora of the skin or the oral cavity or that use of chlorhexidine leads to increased virulence of microorganisms.

10 Conclusions

Chlorhexidine is used in a wide range of products, including cosmetic products.

Although some information is available, there seems to be a lack of reliable data on the amounts of chlorhexidine used.

The main conclusions on the questions raised by The Norwegian Food Safety Authority in the Terms of reference are:

- Conclusive information on the development of resistance due to chlorhexidine in cosmetic products is currently lacking.
- Although the literature is not conclusive, it is probable that chlorhexidine in cosmetic products adds to the selection pressure towards more chlorhexidine-tolerant microorganisms among the skin and mouth flora.
- Intrinsic or acquired low level resistance/tolerance towards chlorhexidine is found in a diverse range of microorganisms, and this tolerance is facilitated by several mechanisms. However, the resistance levels are far less than that of the commonly used chlorhexidine concentrations of up to 3 g/L in cosmetic products.
- There are conflicting reports on the correlation between chlorhexidine and antibiotic resistance. However, some efflux pumps have been shown to mediate export of both chlorhexidine (although at a level far below the commonly used chlorhexidine concentrations in cosmetic products) and clinically important antimicrobial agents (β-lactams, quinolones, amphenicols and tetracyclines) at levels above the clinical MIC breakpoints. Furthermore, cell wall changes that reduce the permeability may also play a role as a common resistance mechanism between chlorhexidine and other antimicrobial agents, some of which are of clinical importance. Furthermore, dissemination of multi-resistant plasmids between staphylococci showing co-resistance to chlorhexidine and clinically important antibacterial agent classes such as aminoglycosides, β-lactams, tetracyclines, and trimethoprim has been reported. Although chlorhexidine tolerance levels are far lower than the commonly used concentrations in cosmetic products, the contribution to increased occurrence of resistance to clinically important antimicrobial agents by chlorhexidine in cosmetic products cannot be excluded. However, data exploring the range of chlorhexidine concentrations upon cosmetic use and their influence upon selection of co- or cross-resistance towards clinically important antibiotics are currently lacking.
- At present there are no indications that the use of chlorhexidine alters the microflora of the skin or the oral cavity or that its use leads to increased virulence of microorganisms.

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