Health Council of the Netherlands

Resistance due to disinfectants

Background report to the advisory report *Careful use of disinfectants*

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to:

the Minister of Health, Welfare and Sport

the Minister for the Environment

No. A16/03E, The Hague, December 21, 2016

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Chapter 1

Introduction

On 4 February 2015, the Minister of Health, Welfare and Sport (VWS) and the Minister for the Environment (IenM) asked the Health Council to give an opinion on disinfectants. Among other things, they want to know whether there are indications of increased microbial resistance to disinfectants due to the rising use of these substances, and whether this can lead to health damage. They also asked whether resistance to antibiotics is increasing due to the (rising) use of disinfectants.

To limit the amount of literature to be reviewed, the ad-hoc committee charged with answering these questions focused primarily on a detailed analysis of resistance development in five (groups of) disinfectants: triclosan, quaternary ammonium compounds, chlorhexidine, silver compounds and reactive chlorine compounds. These case studies have been included in this background document, which belongs with the advisory report drafted by the Committee, entitled *Careful use of disinfectants.* Together, the substances listed adequately reflect the wide variety of chemical compounds used for disinfection. Furthermore, this selection does justice to use in key sectors of society. Finally, a relatively large amount of scientific literature is available on the groups listed. The Committee is of the opinion that applying these limits in no way prevents the questions asked by the minister and secretary of state from being answered.

Chapter 2

Triclosan

2.1 Basic information

Triclosan belongs to the phenol group, substances released from coal tar during the coal distillation process. These substances do not occur naturally. Phenol is the basic water soluble product in this group, and was introduced as a disinfectant nebulising fluid for use during surgery by the surgeon Lister in 1865. The higher the boiling point of coal tar phenols (such as cresol, xylenol), the more toxic they become to micro-organisms (but also to host tissues), and the less water-soluble they become. Most phenols are now produced synthetically. Triclosan and hexachlorophene are members of the bisphenol group, the antimicrobial properties of which were already being studied in 1906 by Ehrlich (Figure 1).

Bisphenols are poorly soluble in water, which limits their applicability. They are easily soluble in organic solvents such as ethanol.[1] Hexachlorophene and Triclosan were marketed in 1948 and 1964, respectively. Hexachlorophene was taken off the market in 1972 due to life-threatening neurotoxic side effects (40 French babies died after talcum powder to which 6% hexachlorophene was added had been applied to the skin). The substances are highly chemically stable; they degrade very slowly in the environment. Triclosan is commonly used as an ‘antimicrobial’ or preservative agent in substances such as pesticides, soaps, detergents, paper products, plastics and building materials, toothpastes and mouthwashes, deodorants and other cosmetics, bedding, underwear and socks,

sponges and in medical implants.[2] Often, the extent to which these products actually protect consumers from damage due to micro-organisms has not been studied. For example, the disinfecting effects of soap containing triclosan were found to be no better than that of soap without triclosan under experimental conditions.[3,4] Triclosan is commonly found in surface water and sediments in nature, likely due to its widespread use and limited degradability. It is estimated that in the U.S., several hundred tonnes of triclosan are released into the environment by water purification companies each year.[5] In Germany, concentrations between 0.01 and 0.6 micrograms/L were measured in water purification plant effluents.[6] In water purification plants and surface waters, triclosan is partially converted into other chlorine-containing derivatives that are even less degradable and potentially more toxic (such as polychlorodibenzo-dioxin). In surface water and soil, triclosan has a half-life of a few weeks; however, this is much longer under anaerobic conditions. The probability of a randomly selected river containing detectable amounts of triclosan in the U.S. is estimated at 60-80%.[7] Surface water in other countries such as England, Germany, Romania, and South Korea was also found to contain triclosan (mean [range] level: 50 [<0.1-2300] ng/L).[8] Sediments contain higher concentrations of triclosan, in part due to its lipophilic character and limited water solubility. Triclosan can be found in nature, particularly near cities. In a national, representative population study conducted in 2003-2004, triclosan was detected in the urine of 75% of the U.S. population.[9] Urine concentrations of triclosan correlated with the use of mouthwashes and sunscreen products that contained the disinfectant.[10] Breast milk[2] and human nasal mucus[11] also often contain triclosan. There are indications that triclosan has side effects, including allergic reactions and disruption of the (thyroid) hormone balance (see Chapter 4 of the advisory report for other harmful side effects of disinfectants). Therefore, in 2016, the European Committee decided not to allow triclosan on the market as a class PT01 disinfectant under the new Biocidal Products Regulation.[12]

2.2 Biocidal action

Triclosan is a broad-spectrum disinfectant: it acts against both Gram-positive and Gram-negative bacteria and some species of mycobacteria and fungi. Viruses with an envelope, *Plasmodium* spp. and *Toxoplasma gondii* are also susceptible to triclosan, but *Pseudomonas aeruginosa* and bacterial spores are not. *Serratia marcescens* and *Morganella morgagii* are inherently minimally susceptible to triclosan.[13] Notably, chlorocresol is able to destroy the contagiousness of

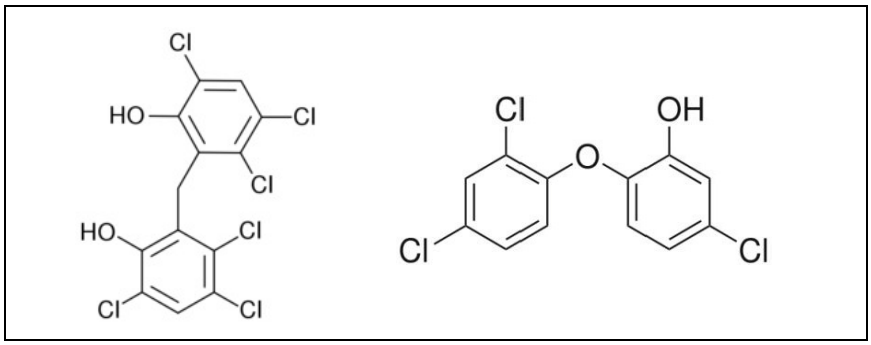

Figure 1 Chemical structures of hexachlorophene (l) and triclosan (r).

prions.[14] This property has not been investigated for triclosan, and in practice, phenols are not used to disinfect surfaces or tissues contaminated with prions.

Ecological MIC/MBC cut-off values for triclosan were recently published for a limited number of human microbial pathogens (see Table 1).[15]

A notable finding of this population study was the appearance of less susceptible subpopulations of *Escherichia coli, Enterobacter* and *Staphylococcus aureus* species, indicating the development of resistance to triclosan.

The bactericidal effect of phenols is based on bonding with amino acids and displacing water molecules. This results in denaturing of cell membrane and cytoplasmic proteins. The effect depends on concentration; at low concentrations, triclosan specifically inhibits the enzyme enoyl reductase, which plays a role in bacterial fatty acid synthesis, resulting in a primarily bacteriostatic effect. At higher triclosan concentrations, all kinds of proteins are denatured, and its effects are bactericidal. Four isotopes of enoyl reductases have been described in prokaryotes, which are found in a variety of micro-organisms. They are

Table 1 Ecological cut-off values for triclosan.

	Triclosan	
	Ecological cut-off value (mg/L)	
Type of micro-organism	MIC_{ecol}	MBC_{ecol}
Salmonella spp.	8	128
Escherichia coli	2	16
Klebsiella pneumoniae	2	8
Enterobacter spp.	1	4
Staphylococcus aureus	0.5	2
Enterococcus faecium	32	64
Enterococcus faecalis	16	32
Candida albicans	16	16

encoded by the genes *fabI, fabK, fabL* and *fabV.* Some species of bacteria, such as *Pseudomonas aeruginosa* and *E. faecalis,* have two types of reductases (*fabI* and *fabK*). Triclosan only inhibits *fabI* encoded enoyl reductase, which is found in species such as *Escherichia coli, Staphylococcus aureus* and *Haemophilus influenzae,* but also in *Mycobacterium tuberculosis*, a finding that has led to research into the use of triclosan-derived compounds as a new class of tuberculostatic agents.[16]

2.3 Is there intrinsic resistance to triclosan?

Answer: yes.

Triclosan is naturally less effective against non-enveloped viruses, while the growth of mycobacteria is slowed, but they are not killed by triclosan. Bacterial spores are entirely resistant to triclosan. Some species of bacteria are intrinsically practically resistant to triclosan, particularly *Pseudomonas aeruginosa*, *Serratia marcescens* and *Morganella morgagni.*[17]

2.4 What mechanisms play a role in intrinsic resistance?

Intrinsic resistance is largely due to reduced interaction of the cell wall of these micro-organisms with triclosan, and/or reduced cell wall permeability or the presence of various efflux pumps.[18] The layered structure of bacterial spore walls makes them resistant to many disinfectants, including triclosan. The presence of a triclosan resistant enoyl reductase enzyme (*fabL, fabK* or *fabV* coded) is also associated with reduced sensitivity or even complete resistance to triclosan, for example in *Pseudomonas aeruginosa*[19, 20] and *Aeromonas salmonicida*[21].

Finally, enzymes that can break down triclosan have been identified in strains of *Pseudomonas putida* and *Achromobacter xylosoxidans subspecies denitrificans* isolated from the environment.[22]

2.5 Does acquired resistance occur?

Answer: yes.

It appears to be fairly easy to select strains of certain bacteria with stable resistance to triclosan in the laboratory by adding triclosan to the medium.[18] This phenomenon has been observed in *Escherichia coli*[23], *Klebsiella oxytoca*[24], *Staphylococcus aureus* [25,26], *Salmonella enterica*[27], *Proteus mirabilis*[17] and

Pseudomonas aeruginosa[28]. Addition of triclosan to simulated riverbeds appears to result in an increase in triclosan-resistant bacteria in these artificial riverbeds; in the field, the higher the level of triclosan contamination in rivers, the larger the proportion of triclosan-resistant bacteria in the sediment.[5,29]

Resistance to triclosan has been observed to a limited extent in daily practice[3], but microbial resistance to triclosan is not monitored systematically. An international study conducted over 10 years ago found that 68 out of 1388 clinical isolates of *Staphylococcus aureus* examined were less susceptible.[30] This is consistent with the 5% of less susceptible MRSA isolates identified in the U.S.[31] *Klebsiella pneumoniae* isolates from the pre-antibiotic era, i.e. prior to 1949 (the so-called Murray collection), have been found to be significantly more susceptible to triclosan (and chlorhexidine and Qacs) than a similar, contemporary collection of *Klebsiella* isolates. The median triclosan MIC at the time was 0.06 mg/L, and is now 0.5 mg/L. The cause of the triclosan MIC 'creep' was not investigated. The old strains did not contain any *qac* genes, but only a minority of modern *Klebsiella* isolates were found to be carrying *qac* genes.[32] Danish research has shown that *Staphyloccoccus epidermidis* isolates from 2010 and 2011 are often less susceptible to triclosan, in contrast to isolates from 1965 and 1966. This appears to be related to, among other things, the presence of a mutation in the *fabI* gene, which codes for the enoyl reductase enzyme in this species.[33] In the U.S., 22% of studied clinical isolates showed decreased susceptibility.[31] Reduced susceptibility has also been observed in *Salmonella* (16 of 428 human and animal isolates[34]), *Acinetobacter baumannii* (20 of 732 clinical isolates[35]) and *Enterococcus faecalis* and *E. faecium* (5 of 272 isolates[36]). Reduced susceptibility to triclosan has sporadically been reported for other bacterial species.[3] Resistance has been observed in *Enterobacter gergoviae*, a species commonly found in contaminated cosmetics. Phenotypic changes in the outer membrane and an increased capacity for biofilm formation play a role.[37]

Cookson et al. identified a genetically transferable form of triclosan resistance in MRSA isolates of patients who washed their hands with triclosan-containing soap on a daily basis.[38] Triclosan resistance was associated with resistance to the antibiotic mupirocin, and was found to be coded by plasmids.

2.6 What mechanisms play a role in the development of triclosan resistance?

Resistance is the result of adaptation, genetic mutation or the acquisition of resistance genes via horizontal gene transfer. Several concurrent mechanisms are

often involved in the development of triclosan resistance, as is clearly shown by genome, transcriptome and proteome analyses.[39, 40] During the above-mentioned in-vitro selection of mutant *Salmonella enterica* strains with reduced susceptibility, resistance was found to be due to increased expression of efflux pump AcrAB-TolC, combined with significantly reduced expression of outer membrane proteins and shortening of the LPS chains.[41] Other authors found that *FabI* mutation combined with mutations in Sigma factors *rpoS* or *rpoD* resulted in high resistance to triclosan. Such strains were also less susceptible to cotrimoxazole and fluoroquinolones.[42] Triclosan belongs to the category of substrates that can be transported by multidrug efflux pumps found in many species of bacteria. Increased expression of such efflux pumps after exposure to triclosan has been described for *Escherichia coli*[43], *Pseudomonas aeruginosa*[28,44], *S. maltophilia*[45] and *A. baumannii*[46]. In *S. maltophilia,* increased expression of the multidrug efflux pump SmeDEF was shown to be the result of triclosan binding to repressor protein SmeT and subsequent increased expression of SmeDEF.[47] In a collection of 31 clinical *Staphylococcus aureus* isolates, all seven triclosan resistant strains showed increased expression of *FabI*-encoded enoyl reductase, and that the six strains with the highest MICs also had a mutation in *FabI* that contributed to the degree of resistance development against triclosan.[48] The increased expression of FabI is likely the result of mutations in the promotor region of the *FabI* gene.[49] Mutations in *Fab* genes have also been found in triclosan resistant *Escherichia coli*[50] and *A. baumannii*[35].

In another form of adaptation, exposure to sub-MIC triclosan was found to result in increased biofilm production[51], greatly reducing susceptibility to triclosan, a phenomenon that has also been observed for other groups of disinfectants and antibiotics. The induction of so-called small colony variants of *Staphylococcus aureus* by triclosan also results in phenotypic resistance to triclosan and to penicillins and aminoglycosides.[52]

2.7 Is triclosan resistance also transferable?

Answer: yes.

Ciusa et al. identified *FabI* genes in clinical *Staphylococcus aureus* that originated from *S. haemolyticus*.[30] Cookson et al. isolated MRSA with reduced susceptibility to triclosan in patients treated with triclosan-containing soap for 2 weeks to combat MRSA carriage.[38] They were able to transfer triclosan resistance to triclosan susceptible *Staphylococcus aureus* in mixed cultures and

on membrane filters, suggesting a plasmid localised gene coding for triclosan resistance.

2.8 Has co-resistance and/or cross-resistance to antibiotics or other disinfectants been observed?

Answer: yes.

Resistance to triclosan together with resistance to one or more classes of antibiotics has been observed regularly, but whether this is coincidental, without any common basis or co-resistance (concurrent presence and transferability of various resistance mechanisms) or of cross-resistance based on the same resistance mechanism often goes unexamined. An interesting observation in this context is that triclosan resistance development in *Escherichia coli* and *Klebsiella pneumoniae* can be selected not only by exposure to triclosan, but also by exposure to benzalkonium chloride or ciprofloxacin.[53] This is due to the increased expression of efflux pumps, which is regulated by so-called global regulators (e.g. MarA, RamA, SoxR) that also regulate the expression of outer membrane proteins, and thus the permeability of the outer membrane in these species. The above-mentioned resistance to triclosan among clinical isolates of *A. baumannii* was found to be associated with increased MICs for imipenem, levofloxacin, amikacin and tetracycline.[35] The reason for this association was not studied, however. In an in-vitro selected, triclosan resistant strain of *A. baumannii* – MIC 256 mg/L – susceptibility to various betalactam antibiotics, fluoroquinolones and doxycyclin was found to be 4 to 8 times lower due to increased expression of a multidrug efflux pump from the RND class.[46] In *S. maltophilia,* increased expression of efflux pump SmeDEF has been shown to be the result of triclosan binding to repressor protein SmeT, which prevents transcription of the *SmeD* gene; this binding lifts the repression, resulting in expression of the efflux pump and thus reduced susceptibility to triclosan and to quinolone-class antibiotics.[47]

In a collection of 400 human and animal *Salmonella enterica* isolates, triclosan resistance was observed in 4% and associated with multiple resistance to a variety of other antibiotics in 56% (this was significantly less common in triclosan-susceptible strains). Elevated expression of efflux pumps was the only explanation found in this group of resistance strains.[34] In one study, in-vitro exposure of *L. monocytogenes* to sub-lethal concentrations of triclosan was found to substantially increase the MICs for gentamicin and other aminoglycosides without selecting for triclosan resistance.[54] The molecular

mechanism for this phenomenon was not identified; there were no mutations to the *16S rRNA* gene. An analogous finding was the selection of so-called pin-point colonies of *Listeria monocytogenes* due to triclosan exposure, a result of a mutation in a haem protein gene. An additional consequence was resistance to aminoglycosides. The researchers suspected that active transport of aminoglycosides no longer took place.[55]

90% of the Triclosan resistant coliform bacteria found in the effluent of a water purification plant in New Jersey, U.S. – all *Citrobacter freundii* – were resistant to four classes of antibiotics. The same study found that the effluent from another site almost always contained triclosan resistance coliform bacteria, and that triclosan resistance correlated with resistance to at least three classes of antibiotics, including chloramphenicol and nitrofurantoin. However, the mechanism of cross-resistance or co-resistance was not determined.[56] The finding that induction of small colony variants of *Staphylococcus aureus* by triclosan also reduces susceptibility to aminoglycosides and penicillins was already mentioned in Section 2.6. However, other studies found no relationship between triclosan resistance and antibiotic resistance in staphylococci.[57, 58] Epidemiological studies also show no signs that triclosan use has caused resistance to methicillin or other antibiotics in *Staphylococcus aureus*.[59] Conversely, an association has been described between methicillin resistance and triclosan resistance in coagulase-negative staphylococci.[31] Additionally, there are published data that show membrane changes in *Staphylococcus aureus* can lead to both triclosan and ciprofloxacin resistance.[60]

In an international, randomised, multi-centre study, no relationship was found between use of triclosan (and other biocide) containing products in the household and the presence of antimicrobial resistance in humans and the environment around said households.[61]

2.9 Has resistance to triclosan been clinically relevant?

Answer: yes.

Contaminated triclosan solutions have resulted in various clinical outbreaks. A recent outbreak of a life-threatening *Pseudomonas aeruginosa* infection in haemato-oncological patients was traced to the use of a contaminated triclosan-containing hand disinfectant. The strain was highly resistant to triclosan (MIC: 2.1 g/L versus 0.5 g/L for wild-type strains) with cross-resistance to six antibiotics typically recognized by efflux pumps. Efflux pump inhibitors were found to be able to reverse the cross-resistance.[62] An earlier conjunctivitis

outbreak among newborns with triclosan-resistant *Serratia marcescens* was caused by a soap solution containing 0.5% triclosan that was contaminated (intrinsically) during the manufacturing process.[63]

2.10 Is co-resistance and/or cross-resistance of triclosan with antibiotics clinically relevant?

Answer: unknown.

It is not known whether cross-resistance with antibiotics induced specifically by triclosan has been clinically relevant. However, it is assumed that reduced susceptibility to antibiotics due to increased expression of efflux pumps, with or without reduced permeability of the cell wall in bacterial pathogens is clinically relevant.[64-68] The use of efflux pump inhibitors in combination with antibiotics has been identified as one of the new treatment strategies to combat this.[69]

Chapter 3

Quaternary ammonium compounds

3.1 Basic information

Although these substances were synthesized and described earlier, the disinfectant properties of certain quaternary ammonium compounds (QACs) were brought to public attention in a publication by Gerhard Domagk in 1935[70] and have since been exploited within healthcare and in other fields. Cationic QACs were found to have particularly strong bactericidal properties. The list of cationic QACs is long, and mixtures of QACs are also used (e.g. benzalkonium chloride, cetrimide). QACs are also in widespread use outside of healthcare, sometimes in combination with other disinfectants such as chlorhexidine. In part due to their surfactant activity, QACs are widely used as microbiocide detergents (cleaning and disinfection) and, at lower concentrations, as preservatives. Common QAC disinfectants are benazalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides), cetrimide (a mixture of various trimethylammoniumbromide compounds), didecyldimethylammonium chloride and cetylpyridinium chloride.

3.2 Chemistry and biocidal action

Cationic QAC disinfectants are organically substituted ammonium compounds, in which a nitrogen atom is bound to 4 carbon side chains of varying lengths. The total number of carbon atoms is >10, and at least one side chain has a length of

between C_8 to C_{18} (see Figure 2). Chain length determines activity. For example, benzalkonium – which has a chain length of C_{12} – is more active than benzalkonium variants with other chain lengths.[71] Benzalkonium with chain lengths of C_{12}-C_{14} are the most active against Gram-positive bacteria, while Gram-negative bacteria are most sensitive to chain lengths of C_{14}-C_{16}.[72] Therefore, mixtures of QACs with various chain lengths are commonly used. Chlorine or bromine anions are located opposite the cationic ammonium group. Such QACs are amphipathic, with both hydrophobic and hydrophilic properties. QACs lower surface tension and, above certain concentrations, critical micelle concentrations (which is different for every QAC solution) QACs form micelles that contribute to their detergent action.

The biocidal action of cationic QACs covers a broad range of micro-organisms, namely Gram-positive bacteria, Gram-negative bacteria, fungi, and certain viruses. QACs are not effective against spores. Hydrophilic viruses (without an envelope) are also poorly susceptible to QACs. The minimum inhibitory concentration (MIC) for benzalkonium chloride for Gram-positive bacteria and yeast species is 4-16 mg/L, while MICs for Gram-negative bacteria are higher (32-128 mg/L), and the minimum bactericidal concentrations (MBC) are 16-32 mg/L and 32- >128 mg/L, respectively.[15] *Salmonella* and *Pseudomonas* species are sometimes resistant to concentrations of 1,000 mg/L. Even higher concentrations are required for swift in-vitro biocidal action (> 5 log reductions in colony forming units (CFU) within 5 minutes). Thus, the user concentration of QACs is important. In practice, this varies between 0.04% (0.4 g/L) and 8% (80 g/L). However, the high concentration in the in-use solution is not the same as the final concentration at the application site, where it is lower due to bonding of the disinfectant with all kinds of (organic) materials at the site of application.

The biocide activity of QACs is based on interaction of the product with the cytoplasmic membranes (and with the outer membrane for Gram-negative bacteria), which disrupts the structure and integrity of the membrane and causes leakage. After being absorbed into the cell, QACs also cause denaturation of

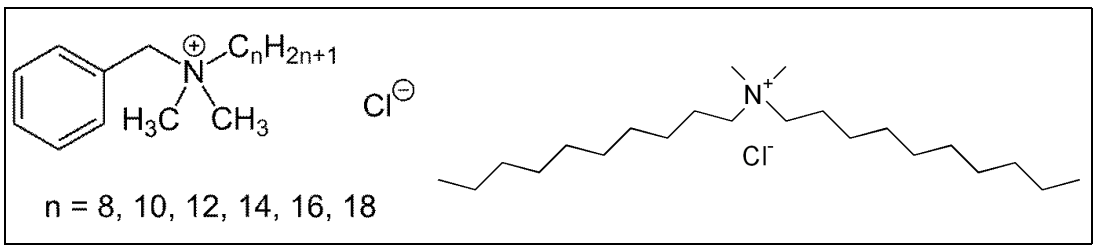

Figure 2 Chemical structure of benzalkonium chloride (l) and didecyldimethylammonium chloride (r).

cytoplasmic proteins. QACs also bind to DNA.[1] The surfactant activity of QACs, expressed as thermodynamic quantities, correlates well with antibacterial properties. A low acidity (pH) in the environment and the presence of anions (including hard water, soaps, carbonates), cotton, talcum powder, phospholipids (serum) and other organic materials reduces the efficacy of QACs.

3.3 Does intrinsic resistance to QACs occur?

Answer: yes.

QACs are naturally less effective against non-enveloped viruses, while the growth of mycobacteria is slowed, but they are not killed by QACs. Bacterial spores and prions are entirely resistant to QACs.[1] Fungal spores and parasitic cysts (excluding *Giardia* cysts) are also less susceptible to QACs. Some Gram-negative bacteria are intrinsically less susceptible / have higher MICs (>100 mg/L), such as *Salmonella* spp and in particular *Pseudomonas aeruginosa*.

3.4 What mechanisms play a role in intrinsic resistance?

Intrinsic resistance is largely due to reduced interaction of the cell wall of these micro-organisms with QACs, and/or reduced cell wall permeability. Gram-negative bacteria in particular are capable of regulating the permeability of their cell walls, for example by varying the number and nature of their porines. The composition and electrical charge of the cell wall may also be different than usual, for example lipid-rich cell walls in mycobacteria (mycoylacylara-binogalactan) or more dense and less negatively charged LPS in the outer membrane in *Pseudomonas aeruginosa.* The lack of a lipid-containing envelope around certain viruses means QACs have nothing to bind to. Prions are proteins, which QACs do not bind to. The complex structure of bacterial spore walls also makes them resistant to QACs.

In bacteria, commonly occurring efflux pumps play a role in the degree of intrinsic susceptibility to QACs. There are 5 classes of efflux systems in bacteria which play a role in bacterial homeostasis.[73,74] They are capable of removing all manner of substances, including certain antibiotics and disinfectants, from the cytoplasm or periplasmatic space (in Gram-negative bacteria). There is one group of ATP-dependent transport systems (ABC class efflux pumps) and 4 groups of proton pump antiporters (MF, SMR, MATE and RND class efflux pumps). The antiporters utilize the electrochemical gradient across the cell membrane (proton motive force), and the product to be expelled is exchanged for

the influx of positively charged hydrogen or sodium ions. The antiporter efflux pumps in particular play a role in susceptibility to QACs. Genes that code for these efflux pumps, including a series of *qac* genes, are present in Gram-negative and Gram-positive bacterial species, and may be found on (conjugative transmissible) plasmids as part of an integron. Various efflux pumps may co-exist in a bacterium. For example, the more efflux pump genes present in *Klebsiella pneumoniae,* the less susceptible they are to QACs.[75] The same is true for *Staphylococcus aureus*.[76]

Interestingly, *Pseudomonas aeruginosa, Pseudomonas putida, Stenotrophomonas* spp. and *Achromobacter* spp. are able to enzymatically break down QACs under aerobic conditions, which may contribute to their natural resistance to QACs.[77] Such strains are currently used for the bioremediation of waste water.[78]

3.5 Does acquired resistance occur?

Answer: yes.

Since the introduction of QACs in the market after 1950, the development of resistance to QACs has been reported. For example, benzalkonium chloride-resistant strains of *Pseudomonas* (MIC ± 1,000 mg/L) already caused contamination of disinfectant solutions and hospital infections during the first decades of use.[79] Exposure to QACs in vitro appears to result in less susceptible strains, a phenomenon observed in *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella* Typhimurium and *Serratia marcescens*.[80-85]

In the food industry, *Serratia marcescens* strains have been isolated from foot baths with disinfectants that can no longer be killed adequately (< 5 log reduction in 5 minutes) when exposed to ready-to-use concentrations (2,000 mg/L) of benzalkonium chloride.[86] Such highly benzalkonium-resistant strains of *Serratia marcescens* were also found during an outbreak of septic arthritis related to use of contaminated disinfectant solution in medical practice.[87] Strains of *Listeria monocytogenes* with reduced susceptibility to QACs are found with some regularity in the food industry.[88] Some resistant *L. monocytogenes* clones persist and spread throughout the food chain.[89] Strains less susceptible to QACs are also found in veterinary practice, for example, *Staphylococcus* species.[90]

In human medicine, *Staphylococcus aureus* is showing signs of a so-called MIC creep, meaning the average MIC for QACs in this bacterial species – or at least among clinical isolates – is slowly but surely on the rise, from < 8 mg/L to > 16 mg/L.[59,91] For example, Zmantar recently found that 20% of a clinical

collection of staphylococci had a MIC for benzalkonium chloride of 16-32 mg/L[92], but that this percentage was lower (9%) in a study performed in other parts of the world.[93]

It is still difficult to predict whether and when mutants will evolve in in-vitro experiments with QACs, and under what conditions they will appear in daily practice. There is no systematic monitoring for QAC resistance in relevant bacteria.

3.6 What mechanisms play a role in the development of QAC resistance?

Resistance is the result of adaptation, genetic mutation or the acquisition of resistance genes via horizontal gene transfer. Exposure to QACs may result in increased biofilm production, greatly reducing susceptibility to QACs[94], a phenomenon that has also been observed for other groups of disinfectants and antibiotics. Biofilms consisting of multiple bacterial species are particularly resistant to QACs.[95] Adaptation to QACs can also manifest as changes to the cell membrane, the outer membrane in Gram-negative bacterial species, the density and structure of porines, or increased expression of efflux pumps. Exposure to sub-lethal QAC concentrations also causes stress and an SOS response in the exposed micro-organisms, increasing mutation rates and the chance of gene transfer.[96] A schematic overview of possible micro-organisms responses to exposure to sub-lethal concentrations of QACs may be found in the article by Tezel and Pavlostathis.[77]

Various mutations lead to changes in cellular membrane composition, reducing permeability to QACs.[97] *Pseudomonas aeruginosa* and *Campylobacter jejuni* lower permeability by changing the number of porines and protein composition of their outer membrane.[98,99] Elevated expression and horizontal transfer and dissemination of genes that code for efflux pumps in the SMR class are likely the most important explanations for acquired resistance to QACs. *EmrE, SugE*, *qacE*, *qacEΔ1*, *qacG, qacH, qacI, qacJ, qacZ* and *smr* genes in particular code for efflux pumps that can remove QACs from the cell. *EmrE, smr* and *SugE* are non-QAC-specific multidrug efflux pumps, while the other efflux systems are more specific to QACs. These genes are particularly common on mobile genetic elements such as transposons, plasmids and integrative-conjugative elements (ICE) on chromosomes. For example, *qacE* and *qacEΔ1,* often located on class 1 integrons, are widely spread via plasmids among Gram-negative bacteria and *qacEΔ1* also among Gram-positive bacteria.[100] SMR class genes have also been found on chromosomes, however, for example in

Enterobacter cloacae.[101] The presence of *qacEΔ1* in food pathogens such as *Salmonella* correlates strongly with high MICs (≥ 512 mg/L) for QACs.[102] Among Gram-negative bacteria such as *Escherichia coli, Salmonella* Typhimurium, *Serratia marcescens, Pseudomonas aeruginosa, Burkholderia pseudomallei* and *Acinetobacter baumannii,* RND class efflux pumps (AcrB, AcrF, sdeAB, AdeABC) also contribute to impaired susceptibility to QACs.[73,82,84] In *Staphylococcus aureus,* the presence of *qacA* and *qacB* genes from the MFS class of efflux pumps is also important for QAC susceptibility.[73,103] According to Jennings, the prevalence of the latter class of genes appears to be increasing in MRSA (see Figure 3).[103]

Smr was also more common in clinical *Staphylococcus aureus* isolates the more often patients had suffered from skin infections with staphylococci.[104] Exposure of MRSA to QACs induces the expression of *qacA/B* genes in vitro. In a recent study, 70% of isolated MRSA strains were found to have *qacA/B* genes, while only 2% of methicillin susceptible *Staphylococcus aureus* isolates possessed such genes.[105] On average, clinical *Staphylococcus aureus* isolates with *qac* genes were found to have a MIC for QACs that was four times higher.

In a multi-centre study in Asia, over 50% of MRSA isolates from 1998/1999 were already *qacA/B* or *smr* positive.[91] In Denmark, no *S. epidermidis* strains

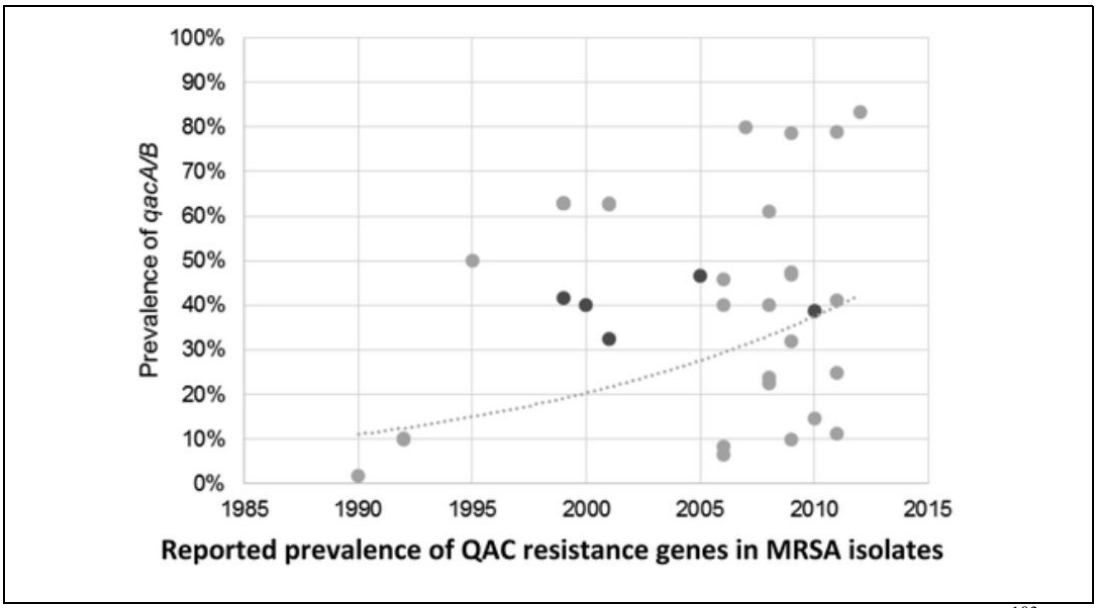

Figure 3 Reported prevalence of QAC resistance genes in MRSA isolates (taken with permission from Jennings et al.[103] Copyright 2015 American Chemical Society).

isolated from blood prior to the introduction of chlorhexidine had *qac* genes, while over half of current *S. epidermidis* blood isolates in that country carry *qac* genes.[106] Chlorhexidine use in the hospital since the 1960s has been associated with the selection and spread of *qac* genes within this species.[107] Furthermore, there appear to be unexplained major differences in the prevalence of *qac* genes in clinical *Staphylococcus aureus* isolates worldwide.[108]

Other resistance mechanisms may also be at play. QAC resistance in *L. monocytogenes* was not correlated with the presence of specific efflux pumps, but with a change in cell wall composition, resulting in strains that were no longer susceptible to bacteriophages as well.[88] In staphylococci, not all QAC-resistant isolates were in possession of one or more of the known *qac* genes.[92] In vitro selected *Escherichia coli* mutants with reduced susceptibility to benzalkonium chloride appeared to have stably higher numbers of RND class efflux pumps of type AcrAB, alongside a change in the porine composition in their outer membrane.[81]

3.7 Is resistance also transferable?

Answer: yes.

The first gene coding for an SMR-class efflux pump, *smr*, was found in both non-conjugative and conjugative plasmids in clinical isolates of *Staphylococcus aureus* and other staphylococci.[100] In the laboratory, with the help of recombinant plasmids, resistance to QACs in *Staphylococcus aureus* has successfully been transferred to *Escherichia coli* and to *Streptococcus sanguis* via transformation.[109,110] *Qac* genes in clinical isolates of various other bacterial species (including *Escherichia coli, Listeria* spp. and *Pseudomonas aeruginosa*) have also been found in various transferable genetic elements such as plasmids, which also contain genes that code for resistance to antibiotics.[111] There are also direct observations that suggest plasmid-mediated transfer of QAC resistance in practice.[112, 113]

3.8 Has co-resistance with resistance to antibiotics been observed?

Answer: yes.

As mentioned above, genes that code for the major QAC efflux pumps are found on mobile genetic elements. Such elements often – but not always – contain

genes that code for various classes of antimicrobial agents. Many articles note the existence of combined resistance to QACs and antibiotics. Recently:

- Sidhu et al.[112] identified a plasmid (pST6) containing the *qacB* gene alongside an (incomplete) β-lactamase gene containing transposon Tn552 in an *S. epidermidis* strain isolated in the food industry. In a follow-up study, they found plasmids in 19 of 78 (24%) benzalkonium-resistant *Staphylococcus* isolates that hybridised with both *qacA/B* and *blaZ* probes[111]
- Jeong et al.[114] identified a class 1 integron containing *qacF* in addition to $bla_{\text{VIM-2}}$ and $bla_{\text{OXA-3}}$, *aacA4* and *aadAI* (aminoglycoside R genes) and *catB3* (chloramphenicol resistance) in a carbapenemase-producing *Pseudomonas aeruginosa* isolate. The integron was located on a plasmid and could be transferred to *Escherichia coli.*
- Johnson et al.[115] found a class 1 integron on a plasmid containing resistance genes for QACs, silver, tetracyline, aminoglycosides, trimethoprim and β-lactam antibiotics in a bird pathogenic *Escherichia coli* strain. The plasmid was transferable to various *Escherichia coli* strains and to *Salmonella enterica* via conjugation.
- In a review of all publicly available full bacterial genome sequences, Pal et al.[116] found that *qacEΔ1* was commonly located on plasmids together with antibiotic resistance genes and that in general, bacteria with biocide resistance genes also had antibiotic resistance genes more frequently than bacteria without biocide resistance genes. Although environmental isolates only rarely displayed the combination of biocide resistance and antibiotic resistance in plasmids (<0.7%), this was more common in human and animal isolates (5-7%). Plasmids with combined resistances were also more often conjunctive.
- Sun et al.[117] found megaplasmids in a *Pseudomonas aeruginosa* (282-kb) and a *Pseudomonas putida* (409-kb) strain containing several transposons with integrons containing genes that code for resistance to QACs, mercury, β-lactam antibiotics, quinolones, aminoglycosides, chloramphenicol, sulphonamide and trimethoprim, and several other classes of antibiotics.
- Buffet-Bataillon et al.[118] found an epidemiological relationship between reduced susceptibilities to QACs and cotrimoxazol among 153 bacteremia isolates of *Escherichia coli.*
- He[119] found that cloning an SMR efflux pump (*emmdR* gene) from *E. cloacae* to *Escherichia coli* not only made the receptor strain less susceptible to benzalkonium chloride, but also to quinolones and trimethoprim.

- Soumet et al.[80] found that *Escherichia coli* strains were not only less susceptible to QACs, but also to β-lactams, chloramphenicol and quinolones after adaptation to sub-MIC QACs.
- Maseda et al.[82] found that an in vitro selected, QAC-resistant *Serratia marcescens* mutant was also less susceptible to fluoroquinolones, tetracyclines and chloramphenicol.

In a collection of over 1600 clinical *Staphylococcus aureus* strains, statistically significant correlations between their MICs for benzalkonium chloride and multiple types of quinolones and the glycopeptide antibiotic vancomycin were observed.[58] The presence of *qac* genes was not examined in this study.

Finally, phenotypic adaptation such as biofilm formation still appears to contribute to reduced susceptibility of the bacteria encased in the biofilm to both biocides and antibiotics, a well-known phenomenon.[77,120] Biofilms consisting of multiple bacterial species are often extra resistant to biocides, including QACs.[121] Reduced cell wall permeability can also reduce the efficacy of both biocides and antibiotics.[122]

3.9 Has cross-resistance with other disinfectants been observed?

Answer: yes.

Cross-resistance between QACs and other groups of disinfectants has been found for various biocide resistance mechanisms. Reduced cell wall permeability, either intrinsic or acquired, makes the bacteria less susceptible to several classes of disinfectants.[123,124] Resistance due to acquisition and/or increased expression of efflux pumps will have such an effect, as efflux pumps are often not particularly specific.[82,125,126] *Pseudomonas aeruginosa* strains made resistant to QACs via in vitro exposure are also less susceptible to chlorhexidine.[127] The susceptibility to QACs such as benzalkonium chloride in larger population studies also correlates well with the susceptibility to chlorhexidine.[58]

3.10 Has cross-resistance with certain antibiotics been observed?

Answer: yes.

As mentioned in Section 3.6, efflux pumps are one of the major mechanisms involved in QAC resistance. Some SMR class efflux pumps partially have a limited substrate profile; they are relatively QAC-specific. Other members of this

class of efflux pumps and other classes of efflux pumps have a substrate profile that is considerably broader than QACs alone, however. Most efflux pumps are also capable of transporting various groups of antibiotics out of the cell, and are called multidrug efflux pumps (see Table 2[77]). The genes that code for these multidrug efflux pumps are usually located on the chromosomes, while genes for the more specific efflux pumps (e.g. *qac* genes) are often found on extra-chromosomal, mobile elements.[128] In particular, aminoglycoside antibiotics, tetracyclines (plasmid bound *Tet* genes coding for tetracycline-specific efflux pumps from the MFS class) and chloramphenicol can be removed from the cell via efflux pumps. Macrolide antibiotics and related groups such as lincosamines and ketolides are also removed by certain efflux pumps. The chromosomally coded multidrug efflux pumps NorA from the MFS class can also export QACs from the cell.

Table 2 Efflux pomps that provide QAC resistance.[77]

Pump family	Efflux proteins that eject QACs	Typical antibiotic substrates
Resistance Nodulation Division (RND)	YhiUV-TolC, AcrAB-TolC, MexAB-OprM, CmeABC, CmeDEF, SdeXY, OqxAB	Aminoglycosides, β-lactams, Chloramphenicol, Erythromycin, Fluoroquinolones, Novobiocines, Rifampine, Tetracyclines, Trimethoprim
Major Facilitator Superfamily (MFS)	QacA, QacB, NorA, NorB, MdeA, EmeA, MdfA	Aminoglycosides, Chloramphenicol, Erythromycin, Fluoroquinolones, Lincosamides, Novobiocin, Rifampin, Tetracyclines
Multidrug And Toxic Compound Extrusion (MATE)	MepA, NorM, PmpM	Aminoglycosides, Fluoroquinolones
Small Multidrug Resistance (SMR)	QacE, QacEΔ1, QacF, QacG, QacH, QacI, QacJ, smr, EmrE, SugE	Aminoglycosides, Chloramphenicol, Erythromycin, Tetracyclines

This also applies to the RND class multidrug efflux pump AcrAB and for the MATE class NorM and MepA pumps. This means there is cross-resistance with antibiotics if expression of such efflux systems increases or if they spread among previously susceptible bacterial species due to horizontal gene transfer.[128] In *Staphylococcus aureus,* cross-resistance between quaternary ammonium compounds and quinolones has been determined.[129,130]

3.11 Has resistance to QACs been clinically relevant?

Answer: yes.

(Pseudo)-outbreaks due to contaminated QAC solutions occur with some regularity, generally due to external contamination of in-use solutions. Most

contaminations involve mycobacteria, *Serratia marcescens* and members of the so-called glucose-non-fermenting Gram-negative rods group, including various *Pseudomonas* and *Burkholderia* species. What these species have in common is that their natural habitats are found in nature – particularly wet niches – their sessile growth (in biofilms) in nutrient-poor environments, their intrinsic resistance to various classes of antibiotics, and their relative resistance to disinfectants.[131] The most recent outbreak of QAC-resistant organisms in healthcare was reported in 2003. However, QAC resistance can also be associated with disease outside of the healthcare sector. For example, a 1998-1999 outbreak of listeriosis in the U.S. – with 108 disease cases, 14 deaths and 4 cases of abortion – was caused by *L. monocytoges* type 4b that was resistant to benzalkonium chloride. The strain had a plasmid with resistance cassette *bcrABC* in a transposon, and was traced back to a factory that manufactured hot dogs.[132] Since then, at least nine outbreaks of this type have been reported.[133] Such QAC-resistant strains are found regularly in the food industry, but little research has been performed on the association with the use of QAC compounds in this industry. In a Spanish sausage factory where QACs were used as surface disinfectants, fourteen strains of *L. monocytogenes* were isolated over a two-year period, belonging to five different PFGE clones. All were benzalkonium chloride resistant. Two of these clones had previously been found in a slaughterhouse belonging to the same company, and four clones were of MLST 121, an ST type that persists in various countries.[89]

3.12 Is co-resistance with antibiotics clinically relevant?

Answer: yes.

Infections and outbreaks of bacteria resistant to multiple classes of antibiotics are increasingly common. The susceptibility of such strains to QACs (or other disinfectants) is often not determined. Incidental findings such as those reported above show that combined use of antibiotics and QACs in human and veterinary sectors is common practice, creating selective pressures due to both groups of antibacterial substances in these sectors. The relative contributions of resistance to both groups to the creation or combating of a clinical problem is not (yet) clear. In a recent Dutch study of the genomes of 96 *L. monocytogenes* isolates from patients with meningitis, mortality was most strongly correlated with the presence of a *qacH* gene on a new plasmid that must have entered the *Listeria* population about 20 years ago. The risk of mortality or permanent damage has since increased significantly (from 27% to 62%). The presence of this *qacH* gene

was strongly associated with resistance to benzalkonium chloride and higher MICs of the strains for amoxicillin and gentamicin.[134]

3.13 Does cross-resistance of QACs with antibiotics have practical consequences?

Answer: unknown.

It is often stated that cross-resistance of QACs with antibiotics has yet to have any practical consequences. It has not been determined scientifically whether, and if so to what degree, use of disinfectants has contributed to the rise of antibiotic resistance over the past decades. Whether and to what degree antibiotic use has contributed to impaired susceptibility of pathogenic bacterial species to QACs is also unknown. In order to determine whether co-resistance is based on a single mechanism, i.e. whether cross-resistance exists, and how the observed resistance patterns are related in specific cases, the mechanisms of resistance to both classes of antimicrobial substances must be elucidated along with the evolutionary backgrounds of the involved genes.

Chapter 4

Chlorhexidine

4.1 Basic information

Chlorhexidine was developed in England by ICI in 1950, has been on the market since 1954, and has become very popular in patient care, particularly over the past twenty years. Chlorhexidine, with or without cetrimide, in aqueous solutions or as a tincture in 70% alcohol, is currently one of the most commonly used disinfectants for skin and mucous membranes of patients and care providers. Chlorhexidine is also widely used outside of healthcare, for example, in hand sanitiser gels, mouthwash and other consumer products. The degree to which chlorhexidine is used as a disinfectant in agriculture and veterinary sectors is unknown.

4.2 Chemistry and biocidal action

Chlorhexidine is a biguanide (see Figure 4). At a neutral pH, it is a bivalent cation that is freely soluble in water as a gluconate.

The biocidal action of chlorhexidine covers a broad range of micro-organisms, including both Gram-positive (MICs [after 24 hours of exposure] are usually ± 1 mg/L) and Gram-negative bacteria (MICs usually ± 2 mg/L), fungi and viruses. Higher concentrations (> 10 mg/L) are required for swift biocidal activity (> 5 log reductions in CFU within 5 minutes) in vitro. Thus, the in-use concentration of the chlorhexidine solution is important. In practice, this varies

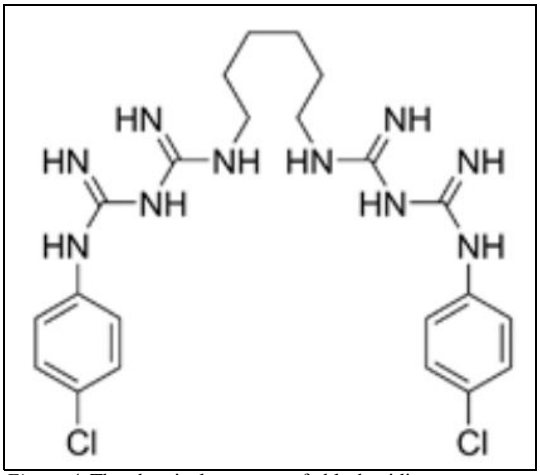

Figure 4 The chemical structure of chlorhexidine.

between 0.1% (1,000 mg/L) and 4% (40,000 mg/L). However, the high concentration in the in-use solution is not the same as the final concentration at the application site. It is lower there due to dilution and bonding of the disinfectant with all kinds of (organic) materials in the area it is used. In one study, after direct application of 2% chlorhexidine to the skin, less than 0.01% (100 mg/L) of the active substance was measurable.[135]

The biocidal activity of chlorhexidine is based on the product binding to the negatively charged cytoplasmic membranes (and to the outer membrane in Gram-negative bacteria) and entry into the cell.[1] At low concentrations, chlorhexidine inhibits enzymes in the membranes and promotes membrane permeability, inhibiting cell growth. At higher concentrations, chlorhexidine coagulates cytoplasmic proteins, nullifies the membrane potential (proton motive force) and shuts down the membrane-bound ATPase enzyme, killing the cell. A low acidity in the environment and the presence of anions (including hard water, soaps, carbonates) and/or phospholipids (serum) reduces the efficacy of chlorhexidine.

4.3 Does intrinsic resistance to chlorhexidine occur?

Answer: yes.

Chlorhexidine is naturally less effective against yeasts and moulds than against bacteria, while mycobacteria are inhibited in their growth but not killed by chlorhexidine, and bacterial spores are entirely resistant.[1]

Some Gram-negative bacteria are intrinsically less susceptible / have higher MICs (10-100 mg/L), such as *Proteus* spp*, Providencia* spp*, Serratia marcescens, Pseudomonas* spp and other glucose-non-fermenting rods such as *Acinetobacter baumannii.*[136]

4.4 What mechanisms play a role in intrinsic resistance?

Intrinsic resistance is largely due to reduced binding to and permeability of the cell wall of these micro-organisms to chlorhexidine. The composition and electrical charge of the cell wall may also be different than usual, for example, lipid-rich cell walls in mycobacteria or more dense and less negatively charged LPS in the outer membrane in *Pseudomonas aeruginosa.*

Efflux pumps also appear to contribute to the intrinsic resistance to chlorhexidine, for example, in *Acinetobacter baumannii* and *Staphylococcus aureus*.

Biofilm growth patterns also lead to significantly lower susceptibility to chlorhexidine, a phenomenon that has been observed in *Serratia marcescens, Burkholderia (Pseudomonas) cepacia, Proteus, Klebsiella* and *Escherichia coli.* Biofilms with such multi-resistant strains are generally difficult to remove from surfaces.[137]

4.5 Does acquired resistance occur?

Answer: yes.

Exposure to chlorhexidine in vitro has been shown to result in less susceptible strains, a phenomenon observed in *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Serratia marcescens.*[138,139]

Exposing *Salmonella* Typhimurium to ready-to-use chlorhexidine consumer products also resulted in strains with (reversible) reduced susceptibility to the disinfectant. Repeated exposure of *Porphyromonas gingivalis* to sub-MIC chlorhexidine also resulted in strains with reduced susceptibility to the disinfectant.[140] Stepwise exposure of *Campylobacter jejuni* and *Campylobacter coli* to increasing concentrations of chlorhexidine also resulted in reduced susceptibility that proved stable and was associated with membrane changes and activation of efflux pumps.[99] However, it is still difficult to predict whether and

when mutant variants will arise in such experiments. Under clinical conditions, chlorhexidine-resistant strains sometimes do and sometimes do not appear.

In Taiwan, the percentage of *Staphylococcus aureus* strains (all MRSA) with reduced susceptibility to chlorhexidine (MIC ≥4 mg/L) increased from <5% to >40% between 1990 and 1995, and remained stable at that level until the end of the observation period in 2005. *qacA/B* genes were found in 55% of strains with reduced chlorhexidine susceptibility, first in one MRSA clone, but later in seven different clones.[141] In one Scottish centre, no reduced susceptibility to chlorhexidine was seen after introduction of chlorhexidine washcloths in the ICU, and all MRSAs remained free of *qac* genes.[142] In contrast, a recent case study described selection of an MRSA variant with reduced chlorhexidine susceptibility (USA300 type) in a patient during use of chlorhexidine. The bacterium had obtained a plasmid with the *qacA* gene.[143] The same was recently reported for a *Klebsiella oxytoca* strain in a patient with a diabetic foot infection treated with topical chlorhexidine compresses.[144]

In a Swedish study, the intensity of chlorhexidine use per hospital ward was associated with reduced chlorhexidine suseceptibility.[145]

4.6 What mechanisms play a role in acquired resistance?

Genes that code for efflux pumps appear to subject to positive selection due to years of disinfectant use, particularly *qac* genes (*A/B* and *smr*).[91] *Smr* was also more common among clinical *Staphylococcus aureus* isolates the more often patients had suffered skin infections.[104] Exposure of MRSA to chlorhexidine induces the expression of *qacA/B* genes in vitro. On average, clinical MRSA isolates with *qac* genes had higher MICs for chlorhexidine. In *Pseudomonas*, changes in the protein composition of the outer membrane were also found, explaining the reduced susceptibility to chlorhexidine.[138]

In a multi-centre study in Asia, over 50% of MRSA isolates from 1998/1999 were already *qacA/B* or *smr* positive.[91] In Sweden, reduced susceptibility to chlorhexidine and *qac* genes were more common in *S. epidermidis* strains that had caused a nosocomial infection than among isolates of commensal skin flora.[14] In Denmark, no *S. epidermidis* strains isolated from blood prior to the introduction of chlorhexidine had *qac* genes, while over half of current *S. epidermidis* blood isolates in that country carry *qac* genes.[106] Chlorhexidine use in the hospital since the 1960s has been associated with the selection and spread of *qac* genes within this species.[107] Furthermore, there appear to be unexplained major differences in the incidence of *qac* genes in clinical *Staphylococcus aureus* isolates worldwide.

4.7 Is acquired resistance also transferable?

Answer: yes.

Resistance to chlorhexidine with *Staphylococcus aureus* has been transferred to *Escherichia coli* in the laboratory using recombinant plasmids, and to *Streptococcus sanguis* via transformation.[109,110] *Qac* genes have also been found in various transferable plasmids that also carry resistance to antibiotics in clinical isolates. There are also direct clinical observations that suggest plasmid-mediated transfer of resistance to chlorhexidine in hospital practice.[143]

4.8 Has co-resistance with resistance to antibiotics been observed?

Answer: yes.

Strains of *Pseudomonas, Proteus* and *Providencia* with reduced susceptibility to chlorhexidine are usually also resistant to aminoglycosides, polymyxine and other antibiotics.[147,148]

Conversely, MRSA are often less susceptible to chlorhexidine than MSSA. MRSA more often have plasmid-coded *qacA/B* genes than contemporary MSSA strains, which are more susceptible to chlorhexidine.[105,149,150] Multiresistant *Enterococcus* spp. almost all have efflux pumps that can eject biocides.[151]

In a collection of over 1600 clinical *Staphylococcus aureus* strains, significant correlations were found between MICs for chlorhexidine and benzalkonium chloride, quinolones and, to a lesser degree, β-lactam and macrolide antibiotics.[58] In a small (n=52) collection of *Pseudomonas* strains (various species) isolated from slaughterhouses, however, no significant correlations were found between MICs for chlorhexidine and those for antibiotics.[152] In a collection of 53 *Staphylococcus aureus* strains resistant to methicillin in combination with high resistance to mupirocine, susceptibility to chlorhexidine was found to be significantly reduced (MIC_{90}=16 mg/L) and associated with the presence of efflux pumps (*qac* and *nor* coded). In the presence of serum albumin, the MBC_{50} was even 256 mg/L.[153]

The chlorhexidine MICs for independent isolates of the globally successful multiresistant *Klebsiella pneumoniae* clone ST 258 are higher than for other clones of this species.[154] The reduced susceptibility was not associated with the expression of qac genes, and must therefore be based on other mechanisms. In another study, carbapenem-resistant *Klebsiella pneumoniae* were found to often

carry *qac* genes, and carriage of *qac* genes was associated with reduced susceptibility to chlorhexidine.[155]

A multiresistant strain of *Pseudomonas aeruginosa* responsible for an outbreak of infections in a Japanese hospital was chlorhexidine-resistant. The strain was found to have a new integron with resistance genes against β-lactams and aminoglycosides. Unfortunately, the mechanisms of resistance to chlorhexidine were not examined.[148]

Brief exposure of a *Burkholderia lata* strain to chlorhexidine in vitro resulted in increased expression of surface membrane proteins including ABC transporter protein, which was associated with a reduced susceptibility to certain antibiotics.[156]

4.9 Has cross-resistance with other disinfectants been observed?

Answer: yes.

Pseudomonas aeruginosa strains that have been made resistant to QACs via in vitro exposure were also less susceptible to chlorhexidine.[127] Exposure of an *Acinetobacter baylyi* strain to sub-MBC concentrations of chlorhexidine resulted in reduced susceptibility to both chlorhexidine and to oxygen radicals, consistent with bacterial response to oxidative stress.[157] Furthermore, larger populations studies have shown that susceptibility to chlorhexidine correlates well with susceptibility to quaternary ammonium compounds, particularly benzalkonium chloride.[58]

4.10 Has cross-resistance with certain antibiotics been observed?

Answer: yes.

In *E. faecalis* strains resistant to various antibiotics and to chlorhexidine, inhibition of the efflux pump EfrAB using EDTA significantly increases susceptibility to both antibiotics and chlorhexidine.[151] Apparently, EfrAB lowers intracellular concentrations of both types of substances under normal conditions. Clinical strains resistant to multiple antibiotics are often also less susceptible to biocides. This combination of resistance to biocides and antibiotics is rarely to never seen outside of clinical settings.

4.11 Has resistance to chlorhexidine been clinically relevant?

Answer: yes.

Use of chlorhexidine to combat MRSA in an ICU did not work in a *qacA/B* positive MRSA clone (ST 239) that was actually able to spread better after the introduction of daily chlorhexidine treatment of MRSA-positive patients. Other MRSA clones, without *qacA/B* genes, were fought successfully. The MBC for the ST239 was three times higher (78 mg/L) than for the other strains (26 mg/L).[158]

Frequent use of chlorhexidine for perineum disinfection resulted in selection of chlorhexidine-resistant *Providencia, Proteus* and *Pseudomonas* in one study, and subsequently resulted in urinary tract infections with these resistant strains.[159] The strains were also resistant to various antibiotics. The problem was limited to departments where this form of disinfection was practised.

(Pseudo)-outbreaks due to contaminated chlorhexidine solutions occur with some regularity, generally due to external contamination of in-use solutions of chlorhexidine. Most contaminations involve bacteria from the so-called glucose-non-fermenting Gram-negative rods group, such as various species of *Burkholderia, Flavobacterium, Pseudomonas, Ralstonia, Achromobacter* and *Stenotrophomonas*. What these species have in common is that their natural habitats are found in nature – particularly wet niches – their sessile growth (in biofilms) in nutrient-poor environments, their intrinsic resistance to various classes of antibiotics, and their relative resistance to disinfectants.[131] The last outbreak was reported in 2003. In one study from Trinidad, 11/180 (6%) of disinfectant solution samples were found to be contaminated with *Pseudomonas* spp. Chlorhexidine solutions were involved in all cases.[160]

4.12 Is co-resistance with antibiotics clinically relevant?

Answer: yes.

Infections and outbreaks of bacteria resistant to multiple classes of antibiotics are increasingly common. The susceptibility of such strains to chlorhexidine (or other disinfectants) is often not determined. Incidental findings such as those reported above show that combined use of antibiotics and chlorhexidine in patient care is common practice, creating selective pressures due to both groups of antibacterial substances. The relative contributions of resistance to both groups to the creation or combating of a clinical problem is not (yet) clear. In one

case, treatment of MRSA in an ICU stumbled when eradication of a MRSA clone with reduced chlorhexidine susceptibility failed.[158]

4.13 Does cross-resistance of chlorhexidine with antibiotics have practical consequences?

Answer: unknown.

In order to determine whether co-resistance is based on a single mechanism, i.e. whether cross-resistance exists, the mechanisms of resistance to both classes of antimicrobial substances must be elucidated in specific cases.

PS. Warnings about the risk of resistance due to increasing use of chlorhexidine in healthcare have been issued for some time.[161-163]

Chapter 5

Silver

5.1 Basic information

Silver is a (transition) metal that occurs naturally in minerals and ores in the ground. Humans have used it for a broad range of applications throughout history, such as the creation of utensils, jewellery and coins. Its use in photography and electronics is more recent. The estimated annual mining production was 24,000 tonnes in 2012.[164]

The metal has well-documented antimicrobial properties against a broad range of micro-organisms.[164-166] As such, the ancient Greeks already used it to treat sores and wounds and for the preservation of food and water. Prior to the introduction of antibiotics in the 1940s, it was perhaps the most important antimicrobial substance.[164] It is still used in a broad range of medical applications due to its efficacy at low concentrations and relatively low toxicity for human cells. There are a large number of formulations available in the market[166], including silver nitrate solutions and silver-containing ointments that often also contain other antimicrobial substances such as sulphadiazine (a sulphonamide) or chlorhexidine. The treatment of burns and (diabetic) chronic wounds is currently the most important area of application.[164-168] (Nano)silver containing antimicrobial compresses are currently also available for the same indications. Silver compounds are also used in eye care to prevent infections. Medical devices and implants, such as catheters and heart valves, are regularly coated with an antimicrobial compound based on silver to prevent biofilm

formation. Silver is used in hospitals and elsewhere for the disinfection of drinking, bathing and swimming water and to combat *Legionella* in hot water systems. In dentistry, large quantities of silver were used in amalgam until recently, a compound that is about 35% silver.[167] However, this use is unrelated to the antimicrobial properties of the metal.

In recent years, use of antimicrobial silver outside of the medical domain has also grown dramatically. Silver is added to a wide range of products for a variety of purposes (preventing infections, preservation, odour control), including cosmetics, personal hygiene products, textiles, kitchen equipment, household equipment, childrens' toys, construction materials, etc.[166] The silver is often bound to polymers in the form of nanoparticles. Products with 'nanosilver' are currently the largest group of commercial 'nanoproducts''.[164] Advances in manufacturing technology for nanoparticles, impregnation techniques and polymer technology are stimulating this trend and contribute to greater efficacy, longer action and lower toxicity of antimicrobial silver for humans.[166]

5.2 Chemistry and biocidal action

The mechanism of antimicrobial action of silver have been the subject of scientific inquiry for decades. It has yet to be elucidated fully. This is particularly true for the effect of silver nanoparticles. The properties of silver depend on the presence of free Ag^+ ions.[164] They replace the hydrogen in the SH groups of proteins located at the surface of micro-organisms by forming silver-sulphur bonds. This blocks electron transport and respiration. The membrane potential (proton motive force) collapses. Damage to the cell membrane allows silver ions to enter the cytoplasm, where they cause further damage by binding to nucleic acids and inactivating enzymes. They also stimulate the formation of very harmful reactive oxygen species (ROS). The entire process eventually leads to microbial cell death.[164]

The increased activity of silver in its nanoparticle form is likely due to greater release of silver ions, more effective delivery of these ions to smaller surfaces, and greater production of harmful reactive oxygen species.[166] Additionally, the nature of the capping agent used in the production of nanoparticles to prevent them from becoming too large or clumping together also plays a role.[164] Added polymers can further strengthen its action.[166]

The antimicrobial action also depends on circumstances such as temperature, acidity, the presence of halogen ions (chlorine, bromine and iodine) and, in nanoparticles, on the presence of divalent cations. Silver ions quickly bind to proteins and form complexes with free chloride, phosphate and sulphate ions.

Therefore, a slow but steady release of silver ions is required for long-term antimicrobial action.[168]

The actual contribution of silver compounds and silver-containing compresses to the prevention of wound infections and wound healing is a topic of scientific debate. One published randomised controlled trial (RCT) and two Cochrane reviews[169-171] concluded that there is insufficient evidence for such a contribution. An international consensus document was published in response, emphasising the positive aspects of wound treatment with silver-containing compresses.[172,173]

5.3 Does intrinsic resistance to silver occur?

Answer: yes.

There is limited scientific evidence on intrinsic resistance to silver. Silver and nanosilver work against a broad range of Gram-negative and Gram-positive bacteria and viruses[164], but are less effective against bacterial spores, mycobacteria and protozoan cysts[166]. Gram-positive bacteria, such as *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus* spp., are less susceptible than Gram-negative bacteria such as *Escherichia coli*, *Salmonella* typhimurium and *Campylobacter* spp.[174-178] Addition of silver to soil promotes the abundance of Gram-positive species and mycobacteria.[179]

5.4 What mechanisms play a role in intrinsic resistance?

The intrinsic resistance of mycobacteria and bacterial spores to silver is based on limited cell envelope permeability. This also applies to the reduced susceptibility of Gram-positive versus Gram-negative bacteria. The former has a cell wall consisting of a thick layer of peptidoglycans. This prevents silver ions and nanoparticles from reaching the cytoplasmic membrane and penetrating the cytoplasm.[176-178,180,181] Additionally, some bacteria also naturally have efflux pumps that can eject silver ions that have entered the cell. This is the case for wild-type *Escherichia coli*, for example.[182] Some bacteria found in mines, such as *Pseudomonas stutzeri* strain AG259 and *Bacillus megaterium*, are highly resistant to silver. They can accumulate significant quantities of extracellular silver in the form of nanoparticles of metallic silver or silver sulphide.[183,184] This property is hoped to be useful for the industrial production of silver nanoparticles.[184]

5.5 Does acquired resistance occur?

Answer: yes.

Bacteria that are naturally susceptible to silver may gain resistance mechanisms through mutations or through HGT.[185] This is particularly true for Gram-negative bacteria.[186] The Gram-positive *Staphylococcus aureus* does not appear to be able to acquire resistance mechanisms via mutation or HGT.[187]

5.6 What mechanisms play a role in acquired resistance?

Various researchers have succeeded in making *Escherichia coli* bacteria resistant (MIC>1024 ppm) to silver nitrate in the laboratory via stepwise exposure to increasing concentrations of the substance. The resistant bacteria were found to have chromosomally coded efflux pumps that were overexpressed as a result of mutations in the regulator genes.[182,186,188,189] The resistant bacteria also had reduced membrane permeability due to the lack of certain porines in their outer membrane.[182,186] These are channels composed of protein filled with water that allow hydrophilic compounds such as silver ions to enter the cell via diffusion. Thus, resistance was due to a combination of reduced passive influx of silver ions and increased active efflux.

The efflux pump in *Escherichia coli* is genetically coded by a cluster of genes, the *cus* system.[74,186,188,190,191] It includes genes that code for parts of the pump and associated regulator genes. The system is primarily involved in copper homeostasis, but can also remove silver ions. It is also found in other clinically relevant Gram-negative bacteria (*Citrobacter freundii* and *Shigella sonnei*). However, researchers were unable to induce silver resistance in these bacteria, despite the presence of the *cus* system.[186]

Gram-negative bacteria appear to acquire their resistance genes to silver via HGT more often than via mutations; in many cases, resistance genes appear to be located on mobile genetic elements such as plasmids and ICEs. The first example discovered was the bacterium *Salmonella enterica* serovar Typhimurium. The burn unit of a hospital in Boston had to be closed in the 1970s after a number of patients died due to contamination with this bacterium.[192] The bacterium was found to carry a specific type of plasmid (pMG101) with genes for resistance against silver and certain antibiotics. Resistance to silver was found to be due to a cluster of genes, the so-called *sil* gene cluster, which has a great deal in

common with the chromosomal *cus* cluster of the *E. coli* bacterium.[186] It contains genes that code for two different efflux pumps, one gene that codes for a protein that can bind silver ions in the periplasmic space, and regulator genes that can detect the presence of silver and control the production of efflux pumps and the absorbing protein.[166,167,186,193] The protein may act as a first line of defence in the periplasmic space, binding the silver ions before they can penetrate the cytoplasm.[164,167] The protein is likely also able to transport silver ions to and from the efflux pumps, and thus strengthen the function of the pump.[186]

Plasmids with *sil* genes have also been found in other Gram-negative bacteria, particularly bacteria in the *Enterobacter cloacae* complex[186,194-196], but also in *Klebsiella*[196], *Escherichia coli*[196] and *Pseudomonas aeruginosa*[197].

Other bacteria, such as *Pseudomonas stutzeri* and *Acinetobacter baumannii,* have also been found to have not otherwise specified, plasmid-localised silver-resistance genes.[198-200]

Recently, two extremely resistant strains of *Enterobacter cloacae* complex and *Klebsiella pneumoniae* were found in a US hospital.[96] Microscopic investigations showed that exposure of these strains to silver compounds resulted in the presence of metallic silver in the polymer layer outside of the cell. This suggests that the bacteria have gained the ability to reduce and precipitate ionic silver.[196]

5.7 Is acquired resistance also transferable?

Answer: yes.

Resistance obtained due to mutations in chromosomal DNA can only be transferred vertically, i.e. to descendants. Resistance genes located on plasmids can also be transferred horizontally. Laboratory experiments have also shown that the previously mentioned silver resistant strain of *Salmonella enterica* serovar Typhimurium can transfer its silver resistance to silver susceptible strains of *Salmonella enterica* serovar Typhimurium and *Escherichia coli.* The latter was found to be able to transfer this acquired resistance to another *Escherichia coli* strain.[192] It should be noted that the involved plasmid (pMG101) is a type optimally transferred at temperatures below 25 °C. Transfer is slowed at temperatures above 37 °C. This reduces the risk of spread within and between bacterial species in a patient population.[196] On the other hand, resistance to silver is also found on countless other plasmids.[196,200] The plasmid-bound silver resistance of a strain of *Acinetobacter baumannii* was also transferable to a silver

susceptible strain of *Escherichia coli* in the laboratory.[200] The recipient subsequently displayed more effective silver efflux.[166]

In the past, acquired resistance has been shown to be unstable and easily lost in the absence of silver[200,201], but recent research suggests that resistance provided by the plasmid-bound sil system is likely not quickly lost in the absence of silver. In resistant *Enterobacter cloacae* and *Klebsiella pneumoniae,* possession of this system was associated with minimal fitness costs.[186] A silver resistant *Salmonella enterica* serovar Senftenberg was found to express *sil* genes constitutively, regardless of the absence or presence of silver.[202] The strain was found in a poultry farm where only trace amounts of silver were expected to be present.

5.8 Has co-resistance with resistance to antibiotics been observed?

Answer: yes.

There are a number of examples of plasmids that carry genes for both silver resistance and resistance to one or more antibiotics. The silver resistant *Salmonella enterica* serovar Typhimurium from the burn unit of the Boston hospital contained resistance genes against silver, tellurite, mercury, ampicillin, chloramphenicol, tetracycline, streptomycin and sulphonamides on its pMG101 plasmid [192,193] The resistance profile was found to be transferable in its entirety to a coli bacterium via conjugation.

Recent Portuguese research with various clones of a related serovar of *Salmonella enterica* revealed that *sil* genes are generally present, and are located on the chromosomes together with resistance genes for antibiotics in some clones, and in others are located together with resistance genes for antibiotics on non-transferable plasmids.[203,204]

In the *Enterobacter clocae* complex, the presence or absence of a plasmid was found to make an important distinction between an avirulent strain from a plant and a pathogenic strain that had caused sepsis in three patients. The plasmid was found to contain both functional *sil* genes and resistance genes for various antibiotics.[195]

Two bacteria isolated from a molar with an amalgam filling from the *Enterobacter clocae* complex were also found to contain *sil* genes and be resistant to ampicillin, erythromycin and clindamycin.[194] The *sil* genes were found to be located on a plasmid, but it was not determined whether antibiotic resistance genes were located on the same plasmid.

In a strain of *Acinetobacter baumannii* resistant to ten antibiotics and thirteen metals, the silver resistance factors and antibiotic resistance genes were likely located on different plasmids.[200] However, resistance genes against metals and antibiotics are often found on the same plasmid in this bacterial species.[200]

An outbreak of hospital acquired infections due to ESBL-producing *Klebsiella pneumoniae* occurred in Sweden a few years ago. The bacterium was found to contain a large plasmid that, in addition to resistance genes for various groups of antibiotics, also contained resistance genes against biocides (quaternary ammonium compounds) and heavy metals, including silver.[205] The plasmid could be transferred to *Escherichia coli*, but its presence was not stable there. Recent research in the same country found that *sil* genes are relatively common in ESBL producing *Escherichia coli* bacteria.[174,206,207]

A strain of *Mycobacterium smegmatidis* made resistant to silver in the laboratory displayed increased resistance to isoniazide. However, the mechanism of this resistance was not elucidated.[208]

5.9 Has cross-resistance with other disinfectants been observed?

Answer: yes.

In addition to silver ions, the chromosomal *cus* system in *Escherichia coli* can also pump copper ions out of the cell.[74]

5.10 Has cross-resistance with certain antibiotics been observed?

Answer: yes.

The chromosomal *cus* efflux system in *Escherichia coli* has a high specificity for silver and copper ions.[74,209] However, there are indications that the system may also be able to pump the antibiotics phosphomycin[210] and ethionamide, and substances dinitrophenol and dinitrobenzene[209] out of the bacterial cell.[66]

Various studies have found an association between silver resistance and reduced susceptibility to cephalosporins and/or carbapenes in *Enterobacteriaceae*.[174,182] This is likely associated with a reduction in outer membrane permeability due to a reduction in the number of porines (water-filled protein channels), via which both silver ions and hydrophilic antibiotics can penetrate the bacterial cell. Reducing the number of porines in the outer membrane is a known resistance mechanism against hydrophilic cephalosporins and carbapenems, particularly in ESBL-producing enterobacteria.[211]

5.11 Has resistance been clinically relevant?

Answer: yes.

There have been regular published reports in scientific medical journals of Gram-negative, silver-resistant bacteria since the 1960s.[192,194-197,201,212-220] In the 1970s, three patients in a burn unit of a Boston hospital died due to infections with a *Salmonella* bacteria that was resistant to silver and several antibitoics.[192]

Despite these reports, the significance of silver resistance remains subject to debate. Some scientists point out that silver resistance is a rare occurrence, despite the fact silver compounds have been used in medical practice for decades.[221-226] A few researchers also note that silver resistance is mainly observed in bacteria from the *Enterobacter cloacae* complex, which are rarely involved as primary pathogens in chronic wounds.[222] Furthermore, resistance is such that silver compresses still remain effective.[222,224]

Other scientists believe this scepticism is unfounded.[164,167,227] In one U.S. study, ten of every seventy isolated gut bacteria from patients admitted to a hospital carried resistance genes against silver.[167] In a recent German study, 63% of 164 clinical isolates of *Enterobacter cloacae* complex were found to be carrying silver resistance genes.[195] The presence of a silver resistance gene bearing plasmid was found to be an important differentiator between an avirulent strain found in plants and a virulent strain involved in three cases of sepsis. Additionally, the genes were mostly found in subspecies that frequently cause hospital acquired infections.[195] Very recently, U.S. researchers reported on the discovery of two strains of *Enterobacter cloacae* complex and *Klebsiella pneumoniae* with extremely high silver resistance that cannot be treated using commercially available silver compresses.[196]

Research into the prevalence of silver resistance in bacteria outside of the medical domain is rarely ever conducted. Silver resistant bacteria have been found in the environment in silver mines[198], silver processing industry effluents[355] and in coastal waters[356]. No silver resistance genes were found In *Escherichia coli* bacteria found in wild birds.[206] Addition of silver nanoparticles to a membrane bioreactor with activated sludge for sixty days resulted in a significant increase in silver resistance genes in the bioreactor.[228] The bacterial activity in the sludge and water quality of the reactor effluent did not change, however. In another study with simulated micro-ecosystems, the capacity of silver nanoparticles to increase antibiotic resistance in natural bacterial populations in marine sediments at relevant exposure levels was tested.[229] The

results were negative, contrasting with previous laboratory studies. This suggests that the influence of silver nanoparticles on natural bacterial populations is difficult to predict, and must be examined for each individual environmment.[230] Silver resistance has sporadically been observed in the food chain.[202,231]

Despite differing viewpoints on the clinical significance of silver resistance, there is broad consensus among experts that monitoring the development of silver resistance is necessary.[174,221-225] This is particularly important due to the swiftly growing number of silver nanoparticle containing consumer products that are becoming available.

5.12 Are cross-resistance and co-resistance with antibiotics clinically relevant?

Answer: possibly.

Cross-resistance between silver and cephalosporins and carbapenems due to loss of porines in the outer membrane in Gram-negative bacteria is a cause for concern.[174,182] Co-resistance is also frequently observed between silver resistance and β-lactamase production in Gram-negative bacteria.[174,206,207,232] The use of silver, both within the hospital and elsewhere, may therefore contribute to resistance to clinically important antibiotics.[225]

Chapter 6

Chlorine and reactive chlorine compounds

6.1 Basic information

Chlorine was discovered in 1774 by Swedish chemist Carl Wilhelm Scheele. However, he believed it was an oxygen compound. Only in 1810 did British chemist Humphry Davy discover it was a new chemical element. The disinfectant properties of chlorine were discovered in England and France around 1800, and the first proposals for chlorination of drinking water stem from that period. During the London cholera epidemic of 1852, the physician John Snow used chlorine to disinfect the drinking water. Starting in the late nineteenth century, chlorination of drinking water was introduced in a growing number of countries.[233] However, in the 1970s, chlorination of drinking water was discovered to be associated with by-products harmful to health, such as chloroform, due to the reaction between chlorine and organic compounds naturally found in the water.[234] In recent years, drinking water companies have increasingly moved to other methods of drinking water disinfection, including UV radiation or ozone. Drinking water chlorination is no longer used in the Netherlands.[233]

Currently, in addition to elemental chlorine (Cl_2), various reactive chlorine compounds are used for a variety of disinfection purposes (Figure 5). A distinction can be made between substances with an O-Cl bond and substances with an N-Cl bond. The former includes sodium hypochlorite (NaOCl, labelled as bleach when dissolved in water, creating hydrogen hypochlorite

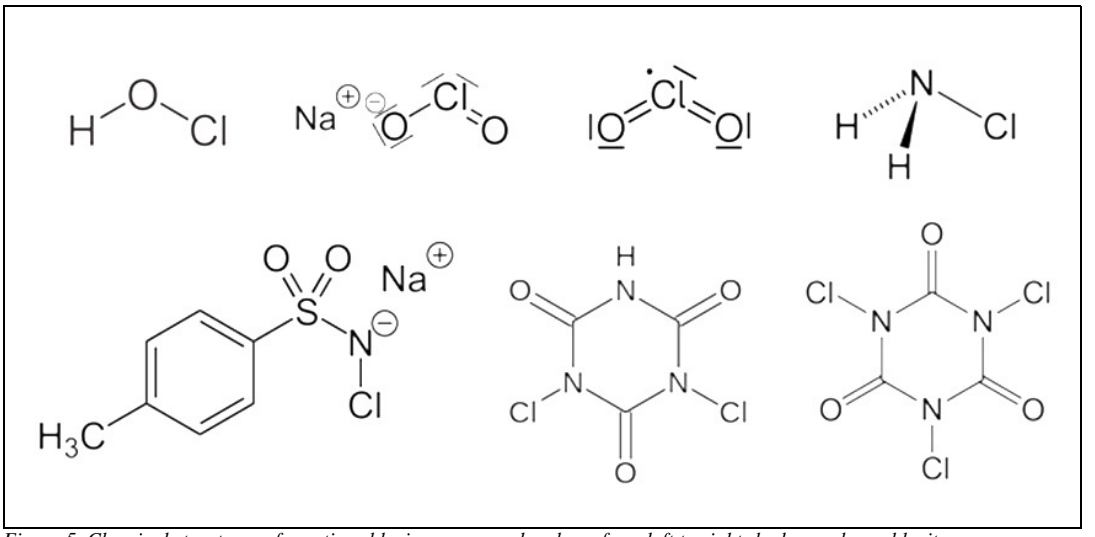

Figure 5 Chemical structures of reactive chlorine compounds: above from left to right: hydrogen hypochlorite, sodium chlorite, chlorine dioxide, monochloramine; below: from left to right: chloramine-T, dichlorisocyanuric acid and trichlorisocyanuric acid

[hypochlorous acid, HOCl]), calcium hypochlorite ($Ca(OCl)_2$), acidified sodium chlorite (ASC, $NaOCl_2$) and chlorine dioxide (ClO_2). The latter includes the chloramines (including monochloramine, NH_2Cl) and the chloramides (dichlorisocyanuric and trichlorisocyanuric acid [$C_3Cl_{2/3}N_3O_3$], Chloramine-T [$C_7H_7ClNO_2SNa$]). Various compounds each have their own advantages and disadvantages, making them more or less suitable for specific applications. For example, the activity of monochloramine and chlorine dioxide is less dependent on pH than that of hydrogen hypochlorite, and the former are also less quickly deactivated by the presence of organic material, so they do not form as many harmful by-products and penetrate better in biofilms when used for drinking water chlorination. Their greater photostability makes dichlorisocyanuric and trichlorisocyanuric acids more suitable for disinfecting swimming water in the open air than hydrogen hypochlorite.

Chlorine and reactive chlorine compounds are among the most commonly used disinfectants in the Netherlands and worldwide. In Belgium, where the use of biocides has been registered for a few years now, sodium hypochlorite is the most used disinfectant, with consumption of 1749 tonnes in 2011. The use of calcium hypochlorite was 30 tonnes, of dichlorisocyanuric and trichloriso-cyanuric acid 207 and 56 tonnes, respectively, and of chlorine dioxide < 2 tonnes.[235] The compounds are used on a large scale for the disinfection of

drinking water, swimming water, cooling water and waste water. They are also very commonly used for the disinfection of hard surfaces in healthcare, the food industry and the private sector. Other applications include the disinfection of endoscopes, antibacterial mouthwashes, root canal disinfection[236], the decontamination of poultry carcasses, fruits and vegetables and combating mastitis in dairy cattle. In addition to use as disinfectants, reactive chlorine compounds are also commonly used as cleaning and bleaching substances in the (paper) industry.

Micro-organisms not only come into contact with reactive chlorine compounds due to conscious human deployment against them. Nature also makes use of these same substances. In the human immune system, neutrophil granulocytes are the first line of defence against bacterial infections. These white blood cells absorb the intruding bacteria via phagocytosis, and subsequently kill them using hydrogen hypochlorite that they form enyzmatically from hydrogen peroxide and chloride ions.[237,238] Reactive chloride compounds have also been found to play important roles in pathogenic and symbiotic interactions between bacteria and their hosts in fruit flies and squid. It is assumed that the production of hydrogen hypochlorite is a common mechanism for controlling bacterial populations on epithelial surfaces in animals.[239] The ability to produce hydrogen hypochlorite ezymatically is also widespread among plants, fungi and bacteria, both terrestrial and aquatic. It is likely that almost all bacteria come into contact with reactive chlorine compounds in their natural environment.[239]

6.2 Chemistry and biocidal action

Reactive chloride compounds are effective against Gram-positive and Gram-negative bacteria, viruses, fungi, algae and protozoa. At higher concentrations, they are also sporicidal. The mechanism of action is not entirely understood, but is based on the powerful oxidative properties of chlorine, which is not fully reduced in any of these compounds. In water, chlorine and various reactive chlorine compounds form hydrogen hypochlorite. This is a powerful disinfectant, even at concentrations below 0.1 mg/L.[240] Between pH 4 and pH 7, the non-dissociated acid (HOCl) is dominant, while above pH 9 the hypochlorite ion is (OCl^-).[241] Both are labelled as ‘free chlorine’. However, the undissociated acid has a much stronger antibacterial effect than the hypochlorite ion. In acidified sodium chlorite, which forms chlorous acid in water, and in chlorine dioxide, which dissolves in water as a gas, chlorine has a higher degree of oxidation. This makes these compounds (even) stronger oxidators than the hypochlorite-forming substances.

Like all disinfectants, reactive chlorine compounds likely kill micro-organisms by concurrently damaging several cellular components. The mechanism of action is not fully understood, but likely varies by type of micro-organism, type of chlorine compound, and exposure conditions.[239,241] Most research data indicate that the cell membrane is the point where lethal damage occurs in vegetative cells. The sulphide groups in sulphur compounds, such as the amino acids cysteine and methionine, are oxidized the most quickly by hydrogen hypochlorite. The nitrogen groups (amines) in proteins can also be oxidised. These changes result in proteins losing their spatial structure, clumping and being broken down. Additionally, hydrogen hypochlorite and chloramines also react - albeit more slowly - with nucleic acids and lipids, which can result in DNA damage and membrane leakage.[239]

The efficacy of disinfection depends on the micro-organism being combated, the type of chlorine compound used (oxidative state of the chlorine, molecular size, charge, lipid solubility) and the conditions under which the substance must perform, such as temperature, light intensity, pH and presence of organic matter.[242]

6.3 Does intrinsic resistance to chlorine occur?

Answer: yes.

Compared with vegetative bacterial cells, bacterial spores are less susceptible to disinfectants. This is also true for chlorine compounds.[243] Mycobacteria are relatively resistant to concentrations of reactive chlorine compounds used in drinking water chlorination.[244] Various *M. avium* strains were found to be 580-2,300 times less susceptible to chlorine and 100-500 times less susceptible to chlorine dioxide than a reference strain of *Escherichia coli*.[244] This explains the presence of mycobacteria in chlorinated drinking water. *Mycobacterium chelonae*, *Bacillus subtilis* (vegetative cells) and *Micrococcus luteus* have been found in endoscope washers despite daily disinfection with chlorine dioxide.[245-247] *Legionella pneumophila*[248-250], *Methylobacterium*[251-253], *Helicobacter pylori*[254] and Sphingomonas species[255] may also be found in chlorinated drinking water, just like mycobacteria. The endemic pathogen *Burkholderia pseudomallei,* common to South-East Asia and Northern Australia*,* is also relatively resistant to chlorine. Viable bacteria of this species can be isolated from water containing 1000 ppm of free chlorine; this is 1000 times the concentration used by Australia to chlorinate drinking water.[256] Some strains of *Pseudomonas aeruginosa* can survive in chlorinated swimming water.[257]

Reduced susceptibility to reactive chlorine compounds (mostly sodium hypochlorite) helps certain strains of *Staphylococcus aureus*[258,259] and *Salmonella*[260] survive disinfections in the poultry industry. In one slaughterhouse, an *Arcobacter butzieri* strain resistant to user concentrations of hypochlorite (200 mg/L of active chlorine) was identified.[261] *Salmonella* Montevideo cannot be removed entirely from tomatoes by immersing them in a 320 ppm chlorine solution made from sodium hypochlorite for two minutes.[262] Some researchers have reported that Gram-negative bacteria are more susceptible to chlorine than Gram-positive bacteria.[263, 264] Others report that it depends on the chlorine compound used.[242]

Treatment of drinking and waste water with reactive chlorine compounds appears to cause shifts in bacterial populations towards species with reduced susceptibility.[265-269]

6.4 What mechanisms play a role in intrinsic and adaptive resistance?

Difficult to penetrate spore and cell walls

The difficult to penetrate wall is the most important explanation for the relatively high resistance of spores to reactive chlorine compounds.[270] Protection of the spore DNA by small acid-soluble proteins and spore core dehydration likely also plays a role.[243]

In vegetative bacteria, the structure of the cell wall also contributes to resistance to reactive chlorine compounds. Mycobacteria have their thick, waxy, mycolic acid containing cell wall to thank for their relatively high chlorine resistance. In chlorine-resistant strains, it is more hydrophobic than in chlorine susceptible strains.[271] The cell wall of Gram-negative bacteria consists of two lipid-containing membranes, while that of Gram-positive bacteria has only one. This may explain why Gram-negative *Escherichia coli* are more susceptible to the lipophilic monochloramine than Gram-positive *Staphylococcus aureus,* but less susceptible to the more hydrophilic chloramine-T.[242]

Slow growth and resting stages

The availability of nutrients in the environment has a major impact on the susceptibility of bacteria to reactive chlorine compounds. Bacteria cultured or living in nutrient-poor environments are more resistant than members of the same species living in nutrient-rich environments. *Mycobacterium avium*, for example, was found to be ten times more resistant to chlorine when cultured in

water rather than in a nutrient medium.[244,271] This likely depends on the much lower growth rate in water. *Enterococcus faecalis* in its stationary phase (the normal phase in water) was 900 times less susceptible to sodium hypochlorite than during its exponential growth phase.[240] Similar findings have been reported for *Escherichia coli*[272], *Klebsiella pneumoniae*[273,274] and *Legionella pneumophila*[275]. This is particularly relevant, because drinking and swimming water are nutrient poor. Therefore, bacteria are more resistant under natural circumstances than in laboratory cultures.[272,275]

Stressful conditions, such as a lack of nutrients, exposure to heat, high salt concentrations or disinfectants, can result in bacteria switching to a resting state with low metabolism. This is labelled a 'viable but non-culturable' state (VBNC state), because the bacteria in this state can no longer be cultured using common culture media, but retain the ability to revive under favourable conditions.[276-279] *Legionella pneumophila* can enter this state under the influence of exposure to reactive chlorine compounds, resulting in a further decrease in susceptibility to these substances.[280-283] Other bacterial species, such as *Burkholderia pseudomallei*[256] and *Helicobacter pylori*[284, 285] can survive in chlorinated drinking water in this state as well.

Sessile lifestyle, biofilm formation and aggregation

Lower chlorine concentrations (<1 mg/L), like those found in chlorinated swimming and drinking water, promote adhesion of planktonic bacteria to a substrate. Higher concentrations inhibit this process.[286] A sessile rather than planktonic lifestyle can make bacteria considerably less susceptible to reactive chlorine compounds. For example, a study of the susceptibility of *Escherichia coli* to chlorine dioxide found that a ten times longer exposure time was required for a 5-log reduction in the number of bacteria if the bacteria were not suspended in water but attached to a steel substrate. On a rough, porous PVC substrate, a 5-log reduction was unachievable.[287] For *Klebsiella pneumoniae*, adhesion to a glass surface was found to reduce susceptibility to chlorine by a factor of 150.[288] The reduced susceptibility may be due to reduced contact between the disinfectant and the bacterial cells. After all, they are no longer surrounded by fluid on all sides.

Another strategy that bacteria can use to limit their exposure to harmful substances is to envelop themselves with a difficult to penetrate mucous layer of exopolymers. In *Bacillus subtilis,* this is stimulated by exposure to sub-lethal chlorine dioxide concentrations. The genes involved in the formation of the required exopolymers become more active in response to a membrane-bound

kinase that responds to disruption of the membrane potential by chlorine dioxide.[289] Two bacteria isolated from an endoscope washer, one *Bacillus subtilis* and one *Micrococcus luteus*, that were resistant to user concentrations of chlorine dioxide, were characterised by a thick layer of exopolymers.[246,247]

Pseudomonas aeruginosa is also stimulated to produce exopolymers by low concentrations of chlorine dioxide.[289] While common chlorine concentrations (0.1-5 mg/L) can be very effective against 'normal' strains of these bacteria in swimming water, mucous-forming strains can survive in chlorinated swimming or drinking water.[257,290-292] This creates the possibility for chlorination in swimming water to apply selective pressure that promotes the spread of mucous-producing strains with reduced chlorine susceptibility.[292] The production of an extracellular mucous layer associated with lower susceptibility to reactive chlorine compounds has also been described for some strains of *Escherichia coli*[293] and *Staphylococcus aureus*[259].

Combining a sessile lifestyle with the extrusion of exopolymers leads to biofilm formation. It is well-known that bacteria that grow in biofilms (which is increasingly considered the natural lifestyle for bacteria in the environment) are significantly more difficult to combat with disinfectants. *Pseudomonas aeruginosa* is a good biofilm former on any type of surface, including surfaces in swimming pools. There, it can grow in biofilms in the presence of 1-3 mg/L of chlorine, and shock treatments with 10 mg/L of chlorine are required to achieve significant reductions in the number of bacteria in the biofilm.[294] Other researchers have reported that following seven days of exposure to 15 mg/L, live bacteria were still found.[295] Laboratory tests show that *Pseudomonas aeruginosa* bacteria living in biofilms are up to 10,000 less susceptible to chlorine than planktonic cells.[296] When cleaning swimming water with a shock treatment, it is possible that only planktonic bacteria are killed. The more-or-less intact biofilm can act as a reservoir for the bacteria, allowing re-colonisation of the water column between shock treatments.[292] These observations suggest that current swimming water management is inadequate where combating *Pseudomonas aeruginosa* biofilms is concerned.[292]

Pseudomonas species can also form biofilms in drinking water systems. Douterelo et al. found they were mostly involved in the initial stage of adherence to the wall, and that other bacterial species dominated at a later stage.[297] In a laboratory study, drinking water bacteria in a biofilm were 1.6-40 times less susceptible to chlorine than free living bacteria.[298] Bacteria can also find shelter in biofilms (jointly) formed by other bacteria.[299-303] Multispecies biofilms appear even more resistant to reactive chlorine compounds than single-species biofilms.[121,298,304]

Legionella bacteria also form biofilms in drinking water distribution systems.[305] Four to ten times higher concentrations are required to inactivate bacteria in the biofilm compared to planktonic bacteria.[248] Longer exposure is required for the same concentration. This is related to the difficulty the disinfectant has penetrating the biofilm. Monochloramine and chlorine dioxide are better at this than chlorine.[306] Moderate concentrations of reactive chlorine compounds can force *Legionella* bacteria in the biofilm into a VBNC state.[282,307] The biofilms may be grazed by amoebas. The amoebas ingest the *Legionella* bacteria. The bacteria are even more difficult to combat in the amoebas or amoebal cysts. Furthermore, the infected amoebas themselves become more resistant to chlorine compounds.[281] This entire process means the *Legionella* bacteria cannot be treated fully effectively using reactive chlorine compounds (or other disinfection methods).[249,250,308] After every shock treatment with high concentrations of reactive chlorine compounds, recolonisation of the water network occurs from the remaining biofilm fragments, VBNC cells or infected amoebas. Some researchers see indications that frequent use of reactive chlorine compounds results in the selection of less susceptible *Legionella* strains[309], while other do not.[249,306,307] The cooling water of nuclear power plants also cannot entirely be freed from *Legionella*.[310] The bacteria *Burkholderia pseudomallei* uses the same strategies as *Legionella* to survive drinking water chlorination. In the presence of amoebas, effective disinfection requires 100 times more monochloramine than in the absence of these protozoans.[256,311]

Sub-lethal concentrations of sodium hypochlorite, a commonly used disinfectant in the food industry, have been found to induce changes to cell shape and hydrophobicity of the cell surface in *Escherichia coli*, and promote biofilm formation by the bacterium.[312] *Salmonella* bacteria have been shown to form biofilms on food contact materials such as plastic, making them less susceptible to hypochlorite.[313]

In the U.S., hypochlorite is also used for the decontamination of vegetables and sprouting vegetables, among other things to combat *Salmonella.* However, high chlorine concentrations of >320 ppm are inadequate for entirely clearing tomatoes of *Salmonella*.[314] For alfalfa seed, even the recommended treatment with 20,000 ppm of chlorine for 15 minutes cannot entirely remove the risk of infection.[315-317] This is primarily ascribed to the fact that the bacteria hide between the plant tissues, where they find shelter from lethal concentrations. The sprouting plants are also covered by biofilms that may offer shelter to the pathogen.[318] Outbreaks of salmonellosis due to contaminated (sprouting) vegetables are therefore common, despite disinfection with hypochlorite.[315,316,319]

A related strategy that is also based on exopolymer formation is clumping of bacteria in aggregates. This also results in reduced susceptibility to reactive chlorine compounds. Isolates of *Staphylococcus aureus* from recently slaughtered turkeys in a poultry processing company were found to be 100 times more susceptible to 1 mg/L of free chlorine than 'endemic' isolates that had colonised factory equipment further down the processing chain.[258] Some of the endemic isolates were also found to be resistant to 2 mg/L of free chlorine. The higher resistance among endemic isolates was found to be related to formation of an extracellular mucous layer and their ability to clump.[259] In another study, endemic strains were found to be scarce among isolates collected from the incoming birds. However, they were in the majority among isolates of carcasses after plucking. This suggests that chlorine-resistant, clumping strains are selected in the processing chain.[320] The clumping phenotype was correlated with the presence of a specific plasmid.

Klebsiella pneumoniae bacteria cultured under nutrient-poor conditions (such as in drinking water) formed smaller cells than bacteria growing in nutrient-rich conditions.[321] The smaller cells also clumped into aggregates of 10 to >10,000 cells (average 90), which was associated with exopolymer formation. The smaller cells also displayed different lipid composition in their membrane. After exposure to monochloramine, 33% of the SH groups were found to be oxidised in the nutrient-poor cultured cells, compared with 80% in the cells cultured under nutrient-rich conditions.[321] In a study of the related *Klebsiella oxytoca,* aggregate formation was also found to be associated with reduced susceptibility to chlorine.[322] The same was true for *Salmonella*.

Intracellular physiological mechanisms

By definition, bacteria have no defence against lethal doses of biocides. The survival mechanisms described above can prevent the lethal effects of exposure to chlorine to some degree. Furthermore, bacteria have access to other survival mechanisms against reactive chlorine compounds.[239] Bacteria have sensors that can detect early damage and initiate repair mechanisms in response.[239,323,324] The key mechanisms for combating oxidative stress are (increased) production of catalases and perixodases, as well as of methionine sulfoxide reductases that can reduce oxidised SH groups again. Other important mechanisms are increased production of chaperones and proteases that prevent aggregation and accumulation of damaged, irreparable proteins. A third group of mechanisms is focused on maintaining supplies of sulphur and sulphur-containing amino acids in the cell. Whether DNA repair mechanisms are upregulated is unclear. DNA

damage appears to be of secondary importance in the case of reactive chlorine compounds. Standard DNA repair mechanisms are likely sufficient.[239]

Combination of defensive strategies

Bacteria can use multiple defensive strategies at the same time. The resistance of a *Bacillus subtilis* isolate from an endoscope washer to user concentrations of chlorine dioxide was not entirely explained by the production of exopolymers, aggregate formation and increased catalase production. The researchers suspected that additional, unknown intracellular mechanisms also play a role.[247] Some point out that overall resistance of a bacteria is equal to the product of resistance effects of the individual mechanisms.[288]

6.5 Does acquired resistance occur?

Answer: not demonstrated.

In 2008, the EFSA *Panel on Biological Hazards* determined that there are no published data that indicate that (correct) use of four substances for microbial decontamination of poultry carcasses, including chlorine dioxide and acidified sodium chloride, will lead to acquired resistance to these substances.[325] Reduced resistance in certain strains of *Methylobacterium*, *Pseudomonas aeruginosa* and *Escherichia coli* to hypochlorite or dichlorisocyanuric acid could also not be related to the presence of plasmids or the occurrence of mutations in the chromosomal DNA.[251,291,326] Changes in the expression of existing genes may play a dominant role in how bacteria respond to stress due to reactive chlorine compounds.[326]

6.6 What mechanisms play a role in acquired resistance?

Acquired resistance has not been demonstrated.

6.7 Is acquired resistance also transferable?

Answer: not demonstrated.

Acquired resistance has not been demonstrated.

6.8 Has co-resistance and/or cross-resistance with other disinfectants or antibiotics been observed?

Answer: yes.

Cross-resistance between chlorine dioxide, hydrogen peroxide and peracetic acid has been observed in an isolate of *Bacillus subtilis* collected from an endoscope washer where chlorine dioxide was used as a disinfectant.[246] All three substances listed have strong oxidative properties. The mechanisms that contribute to this cross-resistance are unknown, however.[247] Cross-resistance to sodium hypochlorite and sodium nitrite, both of which are commonly used biocides in the food industry, has been observed for *Escherichia coli*[327] and between ASC and trisodium phosphate, citric acid and peracetic acid in *Listeria monocytogenes* and *Salmonella enterica*[328]. Efflux pumps and changes in hydrophobicity of the cell surface are likely the underlying explanation.

In 2008, the EFSA *Biohazard Panel* noted that there were no indications that use of four substances for the microbial decontamination of poultry carcasses, including chlorine dioxide and ASC, contributed to antibiotic resistance.[325] Recent research has shown that, at least in the laboratory, (repeated) exposure to (increasing) sub-lethal concentrations of chlorine, ASC, hypochlorite or chlorine dioxide can result in reduced susceptibility of *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes* to various antibiotics.[312,329-331] Experiments with chicken legs confirmed that application of microbial decontaminants, including ASC, may increase resistance of *Escherichia coli* bacteria found on the meat to certain antibiotics.[332] The induction or activation of efflux pumps and changes to cell wall permeability are mentioned as possible explanations for these observations.[312,329]

There are indications that drinking water chlorination can also contribute to antibiotic resistance.[333] Some researchers are of the opinion that (co-)selection plays a particularly important role in this process. Chlorination of drinking water leads to shifts in bacterial flora, benefiting chlorine resistant species. These bacteria also relatively frequently carry resistance genes for (certain) antibiotics, such as genes that code for RND efflux pumps.[291,334-338] Additionally, reactive chlorine compounds may also induce antibiotic resistance. In a study of *Acinetobacter baumannii,* exposure to chlorine was found to result in an increased expression of antibiotic resistance genes, including genes for efflux pumps.[339] Additionally, there are indications that mutagenic by-products of

drinking water chlorination can cause changes to bacterial DNA that promote the development of antibiotic resistance.[340-342]

Co-selection of antibiotic and chlorine resistance also appears to occur in swimming water. In one study into the presence of *Pseudomonas aeruginosa* on foam and vinyl swimming aids in Dutch public swimming pools (with chlorinated swimming water), the bacterium was detected in 19 of 24 pools and 47 of 175 examined objects. 21% of the isolates showed (intermediate) resistance to one or more of twelve tested, clinically relevant antibiotics, particularly imipenem and aztreonam.[343]

Decades of research into the efficacy of reactive chlorine compounds for the removal of antibiotic resistant bacteria from waste water has yielded inconsistent results.[344,345] Some researchers report that chlorination has significantly increased the proportion of antibiotic resistant bacteria in the remaining bacterial population.[346,347] It remains unclear whether this is due to induction of resistance or selection.[346] Others found that only six of the 125 studied resistance genes show a relative increase in numbers, that absolute numbers drop significantly, and that chlorination thus mostly reduces the presence of antibiotic resistant genes in waste water.[348] Various researchers have reported the presence of chlorine resistant *Bacillus* species in waste water from pig farms treated with chlorine. The bacteria were also resistant to one or more of the antibiotics studied.[349,350] Researchers recently found that low concentrations of chlorine can promote the transfer of antibiotic resistance genes via HGT in *Escherichia coli.*[351] Chloramine was formed in the presence of ammonium, which was identified as the cause for the stimulating effect. High concentrations of chlorine had an inhibitory effect. Other researchers found an inhibitory effect of chlorine on HGT in the absence of ammonium.[352]

6.9 Has resistance been clinically relevant?

Answer: yes.

Reduced bacterial susceptibility to reactive chlorine compounds has undoubtedly contributed to outbreaks of infections due to pathogenic bacteria. In the field of food safety, outbreaks of salmonellosis due to contaminated (sprouting) vegetables and fruits can be named.[314-316] The resistance of biofilm-forming strains of *Pseudomonas aeruginosa* to chlorine contributes to disease burden among swimmers due to swimmer's ear (otitis externa) and folliculitis.[292,343] The disease burden caused by disinfection-resistant and biofilm-forming bacteria in

drinking water, such as *Legionella*, *Mycobacterium avium* and *Pseudomonas aeruginosa*, is substantial.[353,354]

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