REVIEW ON THE DEVELOPMENT OF (CROSS-)RESISTANCES TO ANTIMICROBIALS FOLLOWING THE USE OF BIOCIDAL PRODUCTS

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The emergence of resistance after the use of biocidal products

This booklet is a detailed summary of the main literature review on the development of resistance after the use of antimicrobials. It is made of parts of the reports that have been conveniently assembled for an easy access to essential information. You will find here a short summary of the report, the methodology that was used, an introduction on all the biocides that were analyzed and the concluding remarks for each of them. The main conclusions, gaps in knowledge and recommendations are reported here, with little modification.

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Summary of the report

Resistance to antimicrobials is a growing worldwide issue that may spiral out of control if no action is taken to prevent its spread. An effective solution to control microorganisms is to prevent colonization on surfaces by using disinfectants and other biocidal products. But while focus has mainly been on the development of resistance following the use of antibiotics, much less is known about how microorganisms develop resistance following the use of biocidal products. Biocidal products are those that are intended to destroy, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means. Microorganisms are usually considered resistant when they survive exposure to a product which would normally kill them or stop their growth. One major concern is that microorganisms which survive exposure to a biocidal product develop resistance mechanisms that also provide protection against antibiotics used to treat human infection.

This report aims to review the literature pertaining to the resistance and cross-resistance of microorganisms to biocidal products belonging mostly to categories PT1 and PT2. Due to the plethora of biocidal products that are available, this report focuses on the risk associated with the development of resistance and cross-resistance following the use of the following active substances: alcohols, aldehyde-based compounds, hydrogen peroxide, peracetic acid, chlorhexidine, quaternary ammonium compounds, chlorine releasing compounds and weak organic acids. Although not authorized in the EU, triclosan was also included as it constitutes an interesting case study. This report focuses on bacteria, as they are the subject of the vast majority of studies on resistance to biocides. Available data on mycobacteria, yeasts and molds were included when available. Since viruses only replicate (and mutate) within the host, resistance following disinfection with a biocidal product is highly unlikely to happen. Accordingly, no data on this subject was found in the literature.

After analyzing the relevant literature, we found that there is a large amount of data that supports a role for biocidal products in the emergence of resistance to antimicrobials, but the importance of this role largely depends on the type of biocidal product used, the microorganism affected and the method and setting in which the biocidal product was used.

Concerning the risk of development of resistance following the use of biocidal substances, we found that alcohols, hydrogen peroxide, peracetic acid and weak organic acids constitute a highly unlikely risk, aldehyde-based products and chlorine releasing agents constitute an unlikely risk, chlorhexidine and quaternary ammonium compounds constitute a likely risk and triclosan constitutes a highly likely risk. The microorganisms affected are diverse: Gram-positive and Gram-negative bacteria, mycobacteria and yeasts, although most of the data available relates to bacteria. The use of chlorhexidine, quaternary ammonium compounds and triclosan mainly is strongly associated with the development of cross-resistance to other antimicrobials, including antibiotics such as tetracycline, vancomycin, chloramphenicol, ciprofloxacin, imipenem and colistin.

This report also references many gaps of knowledge on the subject, including the lack of standardized biocide testing protocols and comprehensive studies for understanding resistance to biocidal products in practice, not just in the lab. We also recommend implementing a surveillance program and to restrict, whenever possible, the use of biocidal products such as chlorhexidine, quaternary ammonium compounds and triclosan that are associated with the development of resistance and cross resistance.

Methodology

To carry out this review, we have used a pyramidal approach. This approach seems to us to be particularly adapted because it allows us to cover the scope of the field in question (the base of the pyramid) while highlighting the most important elements and proposing a concrete synthesis (the tip of the pyramid). Here are the different steps:

1. A very broad search in the databases (see below) was executed in order to define the framework of work, taking into account the objectives detailed in the previous section.
2. Existing literature reviews and government reports identified in the first step were used to establish an exhaustive list of research articles.
3. The list of reference articles was then expanded: recent publication and the data they present as well as key articles whose importance has been underestimated or even ignored by previous reviews and reports were uncovered. These data were analyzed and integrated into the body of knowledge.
4. The data set from the list of research articles were analyzed in detail and depth. Data concerning the emergence of resistance following the use of biocidal products was extracted and reported in the main body of this review.
5. The most relevant data was discussed and put into perspective; concrete action points, missing data and research needs were identified.

The primary database that was used for the establishment of the primary literature was PubMed. Google scholar was also used for specific searches. High-quality reviews identified through this primary search and additional reports found through the Google search engine completed the primary literature. This primary literature contained research articles and review articles from many different journals. The quality of the articles was assessed on a “one by one” basis.

For this review we decided to target the research on the different biocidal products that are in the scope of the review. This approach allowed us to be more comprehensive, more specific and more able to address the different key point of analysis that were required for this review. These key points are the following:

- The biocidal active substances (PT1 and PT2) that induce the development of resistance to antimicrobials;
- The microorganisms that become resistant to antimicrobials as a result of the use of these active substances;
- The substances (antibiotics and other antimicrobials) against which resistance (cross-resistance or not) occurs as a result of the use of biocidal active substances;
- The uses that lead to the development of antimicrobial resistance in the hospital setting;
- The mechanisms that lead to the development of microbial resistance, following use of antimicrobial products.

Alcohols

There are different types of alcohol that are used as biocides, but the more widely used are ethanol and the two isomers of propanol, 1-propanol and 2-propanol, also known as isopropanol. They have rapid and broad-spectrum activity against a large range of microorganisms including bacteria and mycobacteria, fungi and viruses, although they have low activity against spores (1). They are used widely for the disinfection of skin (in hand disinfectants and skin antiseptics for instance) and decontamination of hard surfaces. The antimicrobial activity of alcohol is considered to be significantly lower at a concentration below 50% and is optimal between 60 and 90%. The specific mode of action of alcohols as antimicrobials is still blurry, but since it is more potent when mixed with water, it is generally believed that it causes extreme membrane damage that, in addition with rapid denaturation of proteins, leads to perturbed metabolism and in the end, cell lysis (1).

The COVID19 pandemic has led to more than 120 000 000 contaminations worldwide and more than 2 700 000 death as of the time of writing. To fight against the propagation of the virus, one of the top recommendation made by the WHO is hand washing, both using soap and water if the hands are dirty and with an alcohol-based hand rub if the hands are visibly clean (2). Demand for hand sanitizers has grown tremendously in 2020 and is visibly leading to a widespread adoption and use of the product that has yet to be fully documented. While alcohol-based sanitizers have previously been considered as safe concerning the emergence of resistance, such increase in global use may bring unforeseen consequences for human health.

In this review we found that disinfection with alcohol-based products remains an extremely effective way of killing microorganisms. To the best of our knowledge, and despite the many years of use of alcohols as disinfectants, there have been **no reports on the emergence of resistance** when using appropriate concentrations of product, although biofilms have increase resistance towards disinfection by alcohol. A small increase in resistance was observed for *Saccharomyces cerevisiae, Listeria monocytogenes, Escherichia coli, Sphingobacterium mizutae, Corynebacterium striatum,* and *Acinetobacter baumannii.* **No cross-resistance** with other biocidal products or antibiotics has been reported yet. That being said, the emergence of clinically-relevant *Enterococcus* strains that are resistant to increasing concentrations of alcohol (up to 23%) highlights the need for vigilance. Alcohol-based disinfection efficacy remains very much defined by physical constrains (size of the area to be disinfected, presence of organic matter, …) and **great care should be taken to ensure that correct disinfection procedures are followed, with focus on the volume of disinfectant used and appropriate disinfection timing so that the effective concentration of alcohol reaching the microorganism is attained.** Since the Covid-19 crisis, use of hand rubs containing alcohol has exploded, and increased use leads to more opportunity for misuse. For instance, clinically relevant strains could acclimate to low doses of alcohol and be disseminated through people that use low quality hand rubs, low quantity of hand rubs, or that do not rub for the recommended amount of time, although we consider that scenario unlikely.

Aldehydes

Formaldehyde is a mono-aldehyde. Its clinical use is generally as a disinfectant and sterilant in liquid or in combination with low-temperature steam. Formaldehyde is bactericidal, sporicidal, and virucidal, but it works more slowly than glutaraldehyde. Formaldehyde is an extremely reactive chemical that interacts with proteins, DNA, and RNA *in vitro* (1).

Glutaraldehyde is a dialdehyde that is used as a disinfectant and sterilant for low-temperature surface disinfection and sterilization of endoscopes and surgical equipment. It is also used in the veterinary field, in poultry and pig farms, and for machinery and food processing surface disinfection (1, 3). Glutaraldehyde has a broad spectrum of activity against bacteria and their spores, fungi and viruses, although the mechanism involved for killing seems to be different for each organism (1).

Ortho-phtalaldehyde is a newer type of aldehyde disinfectant that has potent bactericidal and sporicidal activity and has been suggested as a replacement for glutaraldehyde in endoscope disinfection. Ortho-phtalaldehyde is an aromatic compound with two aldehyde groups. The mechanism of action of this biocide seems to be similar to that of glutaraldehyde (1).

In this review we found that increased resistance or tolerance to aldehyde-based disinfectants has been described in various bacterial species, including *E. coli, Pseudomonas aeruginosa* and *Pseudomonas fluorescens, Helicobacter pilori,* spores of *Bacillus* and *Clostridium.* However, most of these reports seem to emerge from lab experiments or a very isolated instrument contamination in the clinical setting, usually blamed on the emergence of biofilm on old medical decontamination devices. Indeed, biofilms of many bacterial species are much more resistant to disinfection with aldehyde-based products. **More concerning is the emergence of resistance amongst the *Mycobacterium* genus, which seem to be causing more and more outbreaks around the globe after colonization of decontaminating devices**. Cross-resistance to other aldehyde-based compounds has been observed, but there are no reports yet of cross-resistance against other biocidal products or antibiotics. The resistance mechanism is not elucidated yet, but may involve reduced efflux systems and differential porin expression, which may in turn lead to increased resistance to some antibiotics, suggesting that the development of cross-resistance in these strains is a possibility. **Since some of these isolates seem to be resistant to formaldehyde, glutaraldehyde and ortho-phtalaldehyde, but all are usually still sensitive to oxidizing agents (such as peracetic acid), it may be wise to generalize the use of such oxidizing agent for decontamination of medical apparatus instead of aldehyde-based disinfectants in the future**.

Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a widely used biocide for disinfection, sterilization, and antisepsis. It is considered to be environmentally friendly because it degrades rapidly into harmless water and oxygen. It is used for the disinfection of human skin, hospital items such as endoscopes, and hard surfaces in healthcare and veterinary institutions (1, 4).

H_2O_2 demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores (1, 4). H_2O_2 acts as an oxidant by producing hydroxyl free radicals (•OH) via the Fenton reaction; hydroxyl radicals attack essential cell components, including lipids, proteins, and DNA. It has been proposed that exposed sulfhydryl groups and double bonds are particularly targeted (1, 4).

While there are mechanisms in place in microorganisms to resist oxidative stress, it is unclear how these mechanisms play a role in resisting disinfection with oxidative agents, as the literature on this subject is sparse.

In this review we found that microorganisms in biofilms are much more resistant to decontamination by hydrogen peroxide but that there are a few reports on the emergence of higher resistance towards hydrogen peroxide following exposure to the biocide in *S. cerevisiae*, *E. coli, Mycobacterium tuberculosis* and *Campylobacter jejuni.* Low level cross-resistance to 0.2% hypochlorous acid has been observed in *E. coli* and to 20% ethanol in *S. cerevisiae*. **Nevertheless, these reports are few, and none of them are clinically relevant yet.** The mechanisms of resistance are not fully elucidated yet, but may involve specific enzymes such as catalases, superoxide dismutases, glutathione peroxidases and peroxiredoxins that target ROS and their toxic byproducts, as well as a pleitropic response that is mediated by global regulators such as OxyR or SlyA.

Hydrogen peroxide is an efficient biocide with a non-specific mode of action that readily decompose into non-toxic product in the environment. All **these features make peroxygen compounds such as hydrogen peroxide very attractive disinfectants.**

Peracetic Acid

Peracetic acid is an organic peroxide and a colorless liquid with a characteristic acrid odor reminiscent of acetic acid. Peracetic acid is obtained by reacting hydrogen peroxide with acetic acid in an aqueous solution. In this process, peracetic acid is not obtained as a pure substance but in the form of an aqueous solution containing peracetic acid, acetic acid, hydrogen peroxide and water (5). Peracetic acid is usually considered a more potent biocide than hydrogen peroxide, being sporicidal, bactericidal, virucidal, and fungicidal even at low concentrations (0.3%). Dry-fogging a mixture of hydrogen peroxide and peracetic acid is an efficient way to inactivate non-enveloped and enveloped viruses (including SARS-CoV-2), mycobacteria and bacterial spores. However, some species are more resistant than others (notably *Mycobacterium senegalense*) (6).

As is the case with hydrogen peroxide, peracetic acid is considered to be environmentally friendly as it decomposes to safe by-products (acetic acid and oxygen). Compared to hydrogen peroxide, peracetic acid has the added advantages of being free from decomposition by peroxidases and remaining active in the presence of organic loads. Its acts in a similar manner as H_2O_2, probably by denaturing proteins and enzymes and increasing cell wall permeability by oxidizing sulfhydryl bonds. Its main application is as a low-temperature liquid sterilant for medical devices, flexible scopes, and hemodialyzers, but it is also used as an environmental surface sterilant (1).

In this review we found that microorganisms in biofilms are much more resistant to decontamination by peracetic acid but that reports on the emergence of higher resistance towards peracetic acid following exposure to the biocide are extremely rare. *Salmonella* Typhimurium LT2 cells were shown to resist disinfection by the biocide by remaining in a viable but nonculturable state. **No clinically relevant resistance has been identified yet.** The mechanisms of resistance are mostly unexplored.

Peracetic acid is an efficient biocide with a non-specific mode of action that readily decompose into non-toxic product in the environment. All **these features make peroxygen compounds such as peracetic acid very attractive disinfectants.**

Chlorhexidine

Chlorhexidine is a cationic biguanide, mainly used in the form of its salts, namely chlorhexidine digluconate or chlorhexidine diacetate. Chlorhexidine is used in washing and cleaning products, disinfectants, perfumes and fragrances, cosmetics and personal care products, polishes, waxes and pharmaceuticals. Professional workers in the healthcare field also use it as a hand scrub, a disinfectant for surgical sites, a disinfectant for mucous membranes and wounds, a surface disinfectant, and a disinfectant for instruments. It can also be used for the disinfection of burns and as a non-volatile active ingredient in alcohol-based hand wipes (7).

The first target of chlorhexidine are the cytoplasmic membrane and membrane-bound enzymes, while secondary effects (at higher concentrations) are cytoplasmic leakage and, ultimately, the coagulation and precipitation of intracellular constituents such as proteins and nucleic acids (8).

Chlorhexidine is not sporicidal, although it prevents the development of spores. It has poor mycobactericidal activity and has low activity against most viruses, although lipid-enveloped viruses are more sensitive. It is generally active against other non-sporulating bacteria and yeasts (1). Nevertheless, extremely high MIC values (above 0.1%) have been described for many bacterial isolates, including isolates from *Enterococcus faecalis, Klebsiella pneumoniae, Proteus* spp*., Bacillus subtilis, P. aeruginosa, L. monocytogenes, Enterococcus faecium, Staphylococcus aureus, Streptococcus* spp*., Serratia marcescens, Acinetobacter* spp*., Citrobacter* spp. and *Enterobacter* spp. The maximum epidemiological cutoff was proposed at 0.0064%. It is thus safe to say that amongst these species (and possibly others), clinically resistant isolates have already been discovered (7). Concerning bactericidal activity, 4% chlorhexidine has sufficient bactericidal activity (above 5-$\log_{10}$ reduction) against almost all non-sporulating bacterial species within 3-5 min except *Enterococcus* spp. 2% chlorhexidine is still bactericidal but ineffective against some isolates of *E. faecium*, MRSA and *Staphylococcus epidermidis*. At lower concentrations, bactericidal activity is variable (7).

Although chlorhexidine is popular in consumer products, there is mounting evidence that microorganisms can become resistant to this biocide, and there may be hints of cross-resistance to clinically important antibiotics as well. Here we describe studies which report such cases of increased resistance or cross-resistance to chlorhexidine, as well as the associated resistance mechanism. If mentioned in the study, we will report the form of chlorhexidine that was used in the experiment (digluconate or diacetate), but bear in mind that salts are dissociated in water and the effect of the anion on resistance is likely to be extremely minor compared to the chlorhexidine cation.

In this review we found that adaptation of bacterial strains to this biocide has been shown countless times, especially for Gram-negative bacteria which can reach clinically relevant levels of resistance. Bacteria (as well as yeasts) in biofilms are especially resistant.

The microorganism to monitor because of their proven high development of resistance, high level of resistance, or potential to develop cross-resistance are *E. faecalis, K. pneumoniae, Proteus* spp*., B. subtilis, P. aeruginosa, L. monocytogenes, E. faecium, S. aureus, Streptococcus* spp*., S. marcescens, Acinetobacter* spp*., Citrobacter* spp., *Enterobacter* spp., *Salmonella* spp., and *Burkholderia cepacia.* More information on the emergence of resistance for these species and others can be found in the main body of the report.

There are many reports of different bacterial isolates developing cross-resistance to other biocidal products or antibiotics after exposure to chlorhexidine. Table 1 is a summary of the

data that was reported in this literature review. More information on cross-resistance following the use of chlorhexidine can be found in the main body of this report.

Organism	Antimicrobial
Salmonella	Tetracycline, triclosan, benzalkonium chloride, chloramphenicol, nalidixic acid.
Escherichia	Triclosan
Klebsiella	Colistin
Burkholderia	Ciprofloxacin, tobramycin, ceftazidime, imipenem, meropenem
Staphylococcus	Ciprofloxacin, tetracycline, gentamycin, amikacin, cefepime, meropenem, vancomycin
Enterococcus	Daptomycin
Organic food isolates	Different levels of increased resistance to benzalkonium chloride, hexachlorophene, triclosan, didecyldimethylammonium bromide, hexadecylpyridinium chloride, cetrimide, imipenem, ceftazidime, sulfamethoxazol, tetracycline, cefotaxime

Table 1 - Reports of cross-resistance following exposure to chlorhexidine

The mechanism of resistance to chlorhexidine is not completely elucidated and is different for different bacterial species but seems to mainly involve modification of the bacterial membrane and the expression of efflux pumps such as the MexCD-OprJ, KpnEF, EfrEF and Qac efflux pumps. Other pleiotropic effect through more global regulators may also be involved. These mechanisms are mostly nonspecific and the high prevalence of efflux pump determinants in chlorhexidine-resistant strains is indicative of the high number of cross-resistance to other antimicrobials (including antibiotics) that are reported in association with chlorhexidine resistance.

That being said, chlorhexidine has been used for more than 50 years, and in real-world applications, while the use of chlorhexidine is linked with the emergence of resistance in bacteria, it does not seem to be a major source of outbreaks, although some occurrences have been described. Nevertheless, the use of chlorhexidine should be restrained to the applications where its greater efficacy has been proven compared to other biocides that are less associated with bacterial resistance. When used, good practice should be followed such that microorganisms are not needlessly exposed to sublethal concentrations of the biocide, a situation which breeds development of resistance.

Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) are surface-active agents that may act as disinfectants and antiseptics (1). Benzalkonium chloride is a widely used QAC and as such has been used as an example in the report. Benzalkonium chloride is a mixture of alkyl benzyl dimethyl ammonium chlorides, in which the alkyl group has various even-numbered alkyl chain lengths. Benzalkonium chloride mixtures comprise of 24 compounds that are structurally similar QACs characterized by having a positively charged nitrogen covalently bonded to three alkyl group substituents and a benzyl substituent (9).

QACs are used for a variety of clinical purposes such as hand scrubbing, preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of noncritical surfaces. Outside of hospitals, QACs are also used as surface disinfectants in household and foodservice settings, comprising the active ingredient of many commercially-available cleaning sprays and wipes (1, 9, 10). QACs are extensively used in SARS-CoV-2-related sanitization in clinical and household settings, which highlights the need to study the potential for the emergence of biocide and antibiotic resistances related to increased use of these compounds (10).

QACs are membrane- active agents effective against non-sporulating bacteria, with a target site predominantly at the cytoplasmic (inner) membrane in bacteria or the plasma membrane in yeasts (1). QACs are sporostatic: they inhibit the outgrowth of spores but not the germination processes. QACs are not mycobactericidal but have a mycobacteriostatic action, although the actual effects on mycobacteria have been poorly studied. QACs have an effect on lipid enveloped viruses (including SARS-CoV-2, human immunodeficiency virus and HBV), but not nonenveloped viruses (1, 11).

Concerning bacteria, the highest MIC values for benzalkonium chloride were described with *Aeromonas hydrophila* (up to 3.1%), *Bacillus cereus* and *Elizabethkingia meningoseptica* (up to 0.78%), *P. aeruginosa* (up to 0.5%), *L. monocytogenes, Enterobacter cloacae* (up to 0.05%), *Achromobacter xylosoxidans*, *B. cepacia* (up to 0.05%) and *Proteus mirabilis* (up to 0.04%). These value are largely higher than the proposed epidemiological cutoff (below 0.0064% for most species) (9), indicating that some level of resistance is already widespread. Of note, with benzalkonium chloride the result of MIC testing depends to some extent on the media composition and plate material showing the need to standardize biocide susceptibility testing (9).

In this review, we found that adaptation of bacterial strains to higher concentrations of QACs has been demonstrated many times, and some strains reach levels of resistance that are clinically relevant.

The microorganisms to monitor because of their proven high development of resistance, high level of resistance, or potential to develop cross-resistance include *A. hydrophila*, *B. cereus, E. meningoseptica*, *Pseudomonas* spp., *L. monocytogenes, E. cloacae*, *A. xylosoxidans*, *B. cepacian*, *P. mirabilis, Staphylococcus* spp., *E. coli, Salmonella* spp., *Enterobacter* spp., *Pantoea* spp., *Lactobacillus pentosus*. More information on the emergence of resistance for these species and others can be found in the main body of the report.

There are many reports of different bacterial isolates developing cross-resistance to other biocidal products or antibiotics after exposure to chlorhexidine. Table 2 is a summary of the

data that was reported in this literature review. More information on cross-resistance following the use of chlorhexidine can be found in the main body of this report.

Organism	Antimicrobial
Salmonella	Chloramphenicol, ciprofloxacin, nalidixic acid, tetracycline
Escherichia	Amoxicillin, amoxicillin-clavulanic acid, chloramphenicol, imipenem, tetracycline, trimethoprim, ciprofloxacin, ceftiofur, florfenicol, cefotaxime, chloramphenicol
Pseudomonas	Amikacin, ceftazidime, ciprofloxacin, gentamycin, imipenem, chloramphenicol, polymyxin B, Piperacillin-tazobactam, amikacin, meropenem, colistin
Lactobacillus	Ampicillin, chloramphenicol, tetracycline
Burkholderia	Ceftazidime, ciprofloxacin, meropenem, imipenem
Organic food isolates	Chlorhexidine, ampicillin, sulfamethoxazole, cefotaxime
Listeria	Sodium hypochlorite
Cronobacter sakazakii, Yersinia enterocolitica	Ciprofloxacin, cefotaxime, cefoxitin
Staphylococcus	Gentamicin, erythromycin, ciprofloxacin, chloramphenicol, tetracycline

Table 2 - Reports of cross-resistance following exposure to QACs

The mechanism of resistance seems to be centered around the expression of efflux pumps and modification of the bacterial membrane. These mechanisms are mostly nonspecific which is coherent with the numerous reports of cross-resistance to other biocides and antibiotics.

All things considered, while the use of quaternary ammonium compounds has been reported to be associated with the emergence of resistance in bacteria, it has not been linked to major outbreaks yet. **Nevertheless, the use of QACs such as benzalkonium chloride should be restrained to the applications where its greater efficacy has been proven compared to other biocides that are less associated with bacterial resistance**. When used, good practice should be followed such that microorganisms are not needlessly exposed to sublethal concentration of the biocide, a situation which this report shows breeds development of resistance.

Chlorine releasing compounds

Reactive chlorine species are oxidizing agents that can be used to irreversibly damage microorganisms. These species are released in the medium by chlorine releasing agents such as sodium hypochlorite (NaOCl), also known as household bleach, which is the most used chlorine-based disinfectant (1, 12). As such, the report focused on this product.

NaOCl and its active ingredient HOCl are widely used for sanitation and disinfection purposes in industrial, hospital, and household settings. Sodium hypochlorite is mostly sporicidal, mycobactericidal, bactericidal, fungicidal and is active against certain viruses (13). The use of NaOCl as a disinfectant to clean surfaces and medical equipment has increased during the current SARS-CoV-2 pandemic. Enveloped viruses, such as SARS-CoV-2, are inactivated by NaOCl due to its interaction with the viral outer lipid envelope (12).

For bacteria, the highest MIC values have been found in isolates of *E. faecalis*, (3.2%), *E. coli* (1.2%), *Lactobacillus* spp. (0.4%), *L. monocytogenes* (0.78%), *P. aeruginosa* (0.8%), and *S. aureus* (1.6%) (13). These values are higher than the concentration used in some biocidal products, suggesting that some level of resistance might be attained by some bacterial species. For yeasts, the highest MIC recorded is attained by *Candida albicans* at 1.6%, although the majority of MIC values for *Candida* spp. *Aspergillus* spp., *Penicillum* spp., *Mucor* spp., *Rhizopus* spp. and *Trichoderma* spp. is around 0.2% (13).

In this review we found that while chlorine releasing compounds, as exemplified by sodium hypochlorite or bleach, have been used as a disinfectant since the 1820's (14) and no significant reports of microbial resistance have emerged since then. Low level increases in MIC after exposure to low concentrations of the biocide have been documented in *E.coli, Salmonella enterica, L. monocytogenes* and *S. aureus*, but are probably not a worrisome threat for human health. Similarly, no significant outbreaks have been linked to resistance to sodium hypochlorite disinfection. Biofilms constitute a more resistant reservoir that can lead to dissemination of microorganisms after disinfection procedures, but sodium hypochlorite has the added advantage compared to other biocides of actually dissolving the exopolysaccharide matrix of the biofilm, thereby helping to prevent regrowth of the biofilm.

There are a few reports of low-level cross-resistance to other antimicrobials after exposure to low concentrations of sodium hypochlorite and some of these reports were considered above the threshold for clinical resistance. The substances concerned by cross-resistance are: sodium nitrate, hydrogen peroxide, nalidixic acid, ampicillin-sulbactam, a quaternary ammonium compound, oxacillin, ceftazidime, chloramphenicol, ampicillin, colistin, meropenem and ceftazidim. Again, these reports are few considering the wide usage and the length of time that sodium hypochlorite has been used as a biocide, and there was no report of a link between cross-resistance to antibiotics and actual clinical hazard. The mode of action of sodium hypochlorite is largely non-specific. The mechanism of resistance to the biocide involves several transcriptional regulators and no single gene has been linked to sodium hypochlorite resistance, although an efflux pump that is expressed in presence of sodium hypochlorite has been linked both to increased resistance to the biocide and low-level cross-resistance to some antibiotics.

All in all, if recommended guidelines for the use of chlorine releasing compounds are followed so as to limit exposure of microorganisms to sublethal dose of biocide, the risk for the development of resistance and cross-resistance should be limited.

Lactic acid and other weak acids

Weak organic acids such as acetic acid (the main ingredient of vinegar) and lactic acid have been used for centuries to preserve food and decontaminate infected environments (15). Nowadays, in addition to their role as food preservative, the use of acetic and lactic acid as disinfectants seems to be mostly in the decontamination of meat carcass in the food industry.

Despite their long-standing widespread use, the antimicrobial mode of action of weak acids is still not fully understood. The inhibitory effect of weak organic acid might be due to membrane perturbations that result from acids interacting with the membrane and accumulation of the weak acid anion inside the cytoplasm, which would lead to osmotic stress and perturbation of certain enzymatic metabolic reactions (16).

Acetic acid and lactic acid mostly have a fungistatic and bacteriostatic effect, inhibiting the outgrowth of these organisms, although depending on the concentration, pH and time of treatment, a bactericidal activity can be observed (17). Interestingly, acetic acid at 6% had a significant bactericidal effect on mycobacteria in solution (18), and sorbic acid also inhibits the outgrowth and germination of bacterial spores (19).

In this review, we found that there are some reports in the literature that some strains of *E. coli*, *S.* Typhimurium*, S. aureus, C. jejuni* and *L. monocytogenes* can become resistant to acidic conditions, and thus more resistant to disinfection by organic acids such as lactic acid. There is one report that antibiotic resistant bacteria may also be more resistant to disinfection by lactic acid, which could potentially result in increased exposure of humans to antibiotic-resistant bacteria. However, these reports are scarce, especially considering the widespread use of organic acid as biocides, and their impact on public health are largely unknown. Because bacteria have been in widespread contact with these substances for such a long time, it is unlikely that they can develop worrisome resistance in the future. The same is true for cross-resistance to antibiotics. There is no actual report of cross-resistance to other antimicrobials following the use of weak organic acid and **there is thus insufficient evidence to suggest that the use of organic acids can lead to antibiotic or biocide resistance**. The precautionary principle should still apply, and care should be taken to ensure that microorganisms are exposed as little as possible to sublethal concentrations of the biocide.

Triclosan

Triclosan is a chlorinated biphenyl antimicrobial agent that is widely used in household products, including cosmetics and antimicrobial soaps (20). At low concentration, it inhibits fatty acid biosynthesis by targeting a highly conserved enoyl-acyl carrier protein reductase (ENR, *fab* genes) (21, 22). There are various mechanisms that are known to confer triclosan resistance in bacteria (23, 24), including the overexpression of ENR (25), the presence of mutated and/or triclosan-resistant ENR (26, 27); and the upregulation of efflux pumps (28-30). Triclosan exhibits particular activity against Gram-positive bacteria but is also effective against Gram-negative bacteria and yeast (1, 20).

In this review we found that there is a wealth of data that shows that bacteria such as *E. coli, P. aeruginosa*, *Staphylococcus* spp., *Salmonella* spp., *Acinetobacter* spp., *Campylobacter* spp., *Enterococcus* spp., and some mycobacteria readily develop resistance to triclosan. More information on the emergence of resistance for these species and others can be found in the main body of the report.

There are many reports of different bacterial isolates developing cross-resistance to other biocidal products or antibiotics after exposure to triclosan. Table 3 is a summary of the data that was reported in this literature review. More information on cross-resistance following the use of triclosan can be found in the main body of this report.

Organism	Antimicrobial
Escherichia	Chloramphenicol, trimethoprim, tetracycline, amoxicillin, amoxicillin/clavulanic acid, trimethoprim, benzalkonium chloride, chlorohexidine, ciprofloxacin, tobramycin, levofloxacin and cefepime
Pseudomonas	Tetracycline, ciprofloxacin, amikacin, levofloxacin, carbenicillin, chloramphenicol
Staphylococcus	Vancomycin, ciprofloxacin, ampicillin
Salmonella	Cefotaxine, ceftazidime, ciprofloxacin, nalidixic acid, aminoglycosides
Acinetobacter	Imipenem, levofloxacin, amikacin, tetracycline, piperacillin, doxycycline
Stenotrophomonas	Tetracyclin, chloramphenicol, ciprofloxacin

Table 3 - Reports of cross-resistance following exposure to triclosan

The mechanisms of resistance are diverse variations of mutations in *fabI, fabI* overexpression and efflux pumps, which is coherent with the vast number of cross-resistance that were reported.

Triclosan might have adverse effects for human health (31) and it has been shown that human absorption of triclosan leads to changes in the microbiome population and size (32, 33), indicating that resistance and cross-resistance could develop in the human body. All things considered the decision to ban triclosan in the EU seems to be well advised.

General conclusions

Does the use of biocidal products in the clinical setting lead to the emergence of resistance to antimicrobials (including antibiotics)?

The short answer is yes. The long answer is that there is a large amount of data that supports a role for biocidal products in the emergence of resistance to antimicrobials, but the importance of this role largely depends on the type of biocidal product used, the microorganism affected and the method and setting in which the biocidal product was used. These specificities are addressed in the following sections by answering questions that cover the objectives of the review.

What are the active biocidal substances that lead to antimicrobial resistance?

This report is not a risk assessment but a literature review that analyzes the available information, which may be lacking in some respects. A more quantitative approach could be undertaken in the future if we address some of the gaps in knowledge and recommendations that we discuss in later sections. Nevertheless, we propose a qualitative ranking (See Table 4 for a summary):

- There are few reports of resistance following the use of **alcohols, hydrogen peroxide, peracetic acid and weak organic acids**. It is unsurprising given that these compounds are abundant in nature (or are a mixture of compounds that are abundant in nature), have been in contact with microorganisms for a long time and do not easily accumulate in the environment (there is thus less chance of developing resistance in environmental settings). We consider that using these active substances constitutes a **highly unlikely risk** for the development of resistance to antimicrobials.

- There are only a few reports on the development of resistance and cross-resistance to antibiotics following the use of reactive chlorine species. These biocidal products are often used in low concentrations for a large amount of time, which may promote the development of resistance and cross-resistance, as well as increase horizontal gene transfer. Aldehyde-based compounds are not associated with cross-resistance, but they are associated with resistance that led to small-scale outbreaks. We thus consider using **reactive chlorine species and aldehyde-based compounds** an **unlikely risk** for the development of resistance and cross-resistance.

- There is a large amount of evidence characterizing the development of resistance and cross-resistance following the use of **quaternary ammonium compounds and chlorhexidine**, including cross-resistance to the last resort antibiotic colistin. The biocidal products have a tendency to accumulate in the environment, and microorganisms in contact with low concentrations of the products have been demonstrated to develop resistance and cross-resistance. These products often lead to the overexpression of efflux pump which may confer resistance to multiple antimicrobials. We consider the use of these biocidal products a **likely risk** for the development of resistance.

- Finally, triclosan, with its single target, has a very large amount of resistance and cross-resistance reported, mostly through the increased expression of efflux pumps and mutations in the *fabI* fatty acid biosynthesis gene. We consider the use of **triclosan, or any potential biocide with a single specific target**, a **highly likely risk** for the development of resistance. It is therefore appropriate that triclosan is not approved for use in the EU.

Alcohols, hydrogen peroxide, peracetic acid and weak organic acids	Highly unlikely
Reactive chlorine species, aldehyde-based biocides	Unlikely
Chlorhexidine, quaternary ammonium compounds	Likely
Triclosan	Highly likely

Table 4 - Qualitative risk ranking

What are the microorganisms that develop antimicrobial resistance following exposure to biocidal products?

A variety of species seems to be affected: Gram-positive and Gram-negative bacteria, sporulating bacteria, mycobacteria and yeasts. Obviously, the type of microorganism affected depends on the biocidal product used. For instance, mycobacteria have developed resistance to disinfection by aldehyde-based products that has led to small outbreaks in the clinical setting. There were many reports on the development of resistance following the use of chlorhexidine in both Gram-positive and Gram-negative bacteria, but Gram-negative were ultimately resistant to much higher concentrations of the substance than Gram-positive bacteria. In general, Gram-negative bacteria seem to have a higher propensity for the development of resistance following the use of biocidal products than other microorganisms.

Which substances (antibiotics and other antimicrobials) are subject to resistance (cross-resistance or not) as a result of the use of biocidal active substances?

As stated previously, all biocidal products analyzed in this report may lead to some level of resistance to themselves, although not all these biocidal substances may lead to clinical resistance that has implications on human health. The biocidal substances that we consider risky are triclosan, chlorhexidine, quaternary ammonium compounds and, to a much lesser extent, reactive chlorine species and aldehyde-based disinfectants.

However, the use of biocidal products sometimes also leads to the emergence of resistance to antibiotics and other antimicrobials. In this report, we found many such occurrences where resistance to one biocidal product led to resistance to a single or multiple antibiotics. Again, in most instances, the resistance was not clinically relevant, but in some cases, it was enough to be potentially detrimental to public health. **Of particular concern, there are reports of colistin (a last resort antibiotic) resistance in some bacterial species following the use of chlorhexidine and quaternary ammonium compounds.** The use of triclosan, chlorhexidine and quaternary ammonium compounds may also lead to the resistance of many other antimicrobials, including antibiotics such as tetracycline, vancomycin, chloramphenicol, ciprofloxacin, imipenem and colistin. More details on all the occurrences of cross-resistance to other biocidal products and to antibiotics are available in the main body of this report.

What are the practices that lead to the emergence of antimicrobial resistance in the hospital setting?

There is a very large number of study reporting that exposure to sub-inhibitory concentrations of a biocidal active substance can lead to the emergence of resistance to the biocidal product and/or other antimicrobials, including antibiotics. Thus, clinical practices where the biocidal product is applied in smaller quantities than recommended (meaning the product will get diluted when applied) or is likely to remain present at low concentration (compared to the recommended concentration for disinfection) or is applied during a too short period of time should be avoided. These recommendations are also valid for the use of biocidal products in household, agricultural or production settings.

There are also reports of stock solutions of chlorhexidine getting contaminated by bacteria and leading to health problems and even death in patients. In some cases, the stock solution was contaminated during production, before it reached the hospital while in others the stock solution or container got contaminated through multiple use, thus contributing to the emergence of resistance.

Further research in the clinical practice (and in other fields) may highlight specific uses of biocidal products that potentiate the development of antimicrobial resistance.

What are the mechanisms that lead to the development of antimicrobial resistance, following the use of biocidal products?

As explained in the report, microorganisms use different strategies to resist biocidal products; they may inactivate the product or modify its target, prevent its entry into the cell or increase its removal from inside the cells. The same mechanisms are used to resist antibacterials and antibiotics; as a result, developing resistance against biocidal products can drive resistance to antibiotics. For instance, bacteria with mutated *fabI* have emerged with high level triclosan resistance (this is an example of modifying the target of the antimicrobial, a strategy that is usually mostly used to resist antibiotics). In addition, bacteria may also express ROS-detoxifying enzymes such as superoxide dismutases (SOD), glutathione peroxidases and peroxiredoxins that may confer low-level resistance to disinfection by hydrogen peroxide or other oxidative disinfectant (this is an example of inactivating the antimicrobial or its toxic by-products); as a result, they may become more tolerant towards antibiotics that kill in part by causing an oxidative stress. Modifying the permeability of the cell envelope through mutations in the LPS pathway or by modifying the expression of porins (this is an example of limiting the entry of antimicrobials inside the cell) is another example of resistance mechanism that is common to the fight against biocides and antibiotics. Finally, the formation of biofilms is another strategy used by bacteria and fungi that is efficient to limit the entry of biocidal products and antibiotics. This mechanism of resistance is a major issue, as the data analyzed in this report indicate that all biocidal products reviewed here may be subject to increased resistance when cells are embedded in a biofilm matrix. Both these mechanisms may also prevent the entry of other antimicrobials, such as antibiotics.

Another prevalent resistant mechanism seems to be the expression of efflux pumps. These pumps utilize energy to drive the transport of molecules, including antimicrobials, from the inside of the cells to the outside environment and thus prevent the accumulation of toxic molecules inside the cell. They may be already present in the cell and expressed upon exposure to a biocidal product, or they may be shared through horizontal gene transfer. This mechanism of resistance is problematic since efflux pumps that are expressed or shared following the use

of biocidal products may also drive the export of other antimicrobials, such as antibiotics that are substrates for efflux pumps, leading to potential health hazard.

Gaps in knowledge and research needs

While a significant amount of research is available on the subject of resistance of microorganisms to biocidal products, there are areas that are severely lacking. In this section we identify the gaps of knowledge and research needs to have a better, fuller understanding of how and how often resistance to biocidal products occur, which microorganisms are affected and whether this resistance is clinically relevant:

- Standardized testing protocols are needed for assessing resistance to biocidal products and cross-resistance to antibiotics, both in test tubes/petri dishes and situations that mimic the uses in practice.

- Standardized testing of commercial biocidal products and research on how different formulation of active substances influence the killing of microorganisms and the development of resistance and cross resistances. Data is scarce, but there is evidence reported in this review that suggests that additional components in the formulation of biocidal products may reduce the risk of emergence of resistance.

- No readily available threshold to establish whether bacterial strains are clinically resistant to biocidal products, which makes it hard to evaluate the risks associated with the use of one biocidal product, even when data on increased resistance is available.

- Majority of data on the emergence of resistance following the use of biocidal products available is about Gram-positive and Gram-negative bacteria. It is unknown whether the general lack of data on yeasts and molds and other microorganisms is because these microorganisms do not readily develop resistance following the use of biocidal product or whether there is a lack of research on the subject.

- The majority of bacteria may be present in biofilms, and this review highlighted the fact that microorganisms in biofilm are much more resistant to biocidal products. There is a lack of data on how this resistance occurs in biofilms, and whether the biocidal products may be used to kill microorganisms in biofilms. Furthermore, current data suggests that biocidal products may either promote or decrease biofilm formation. Additional studies are required to understand the conditions in which biocidal products may promote or decrease biofilm formation.

- To identify the potential risk for biocidal products resistance and cross-resistance, both now and in the future, we need detailed knowledge on the quantity of biocides produced, used and recovered in the environment.

- Comprehensive studies are needed to assess the mechanisms of resistance, the genetic/phenotypic factors involved and the contribution of resistance, tolerance and persistence to the survival of the microorganisms. How biocidal substances influence horizontal gene transfer and how this may increase the spread of AMR determinants should be studied as well.

Recommendations

- Surveillance programs should be developed on a national/European level to monitor resistance and cross-resistance of microorganisms in all areas of biocide usage, in particular the health care setting, veterinary setting, household setting and food industry.

- Communication programs targeting the general public and health sector workers should be developed to increase awareness of resistance and cross resistance related to the use of biocidal products. For example, these could be in the form of a reminder to use a specific quantity of product for a specific duration, as is already done for hands cleaning.

- Good Practices surrounding the use of biocidal substances, especially those that carry a high risk of development of resistance and cross-resistance, should be established in concertation with the health sector and if possible, the manufacturer of the biocidal product. These Good Practices should at least ensure that 1) the in-practice concentration reaches the appropriate level *i.e.,* a sufficient amount is applied on a sufficiently small surface, 2) the appropriate contact time between the biocidal substance and the microorganisms to be decontaminated is respected, 3) after decontamination, the potentially remaining microorganisms are not exposed to sub-lethal levels of the biocidal product for extended periods. For non-volatile products, this may be achieved through rinsing off/wiping.

- There should be adequate protocols for the use of stock solutions of biocidal products and containers of biocidal product to ensure sterility over time.

- Commercially available bioindicators used to assess the efficiency of disinfection are sometimes more susceptible to the action of biocidal products than clinically relevant strains. Bioindicators should ideally not be more susceptible than clinically relevant strains.

- There should be incentives to include the CAS number of chemicals in scientific studies (at least in clinical studies), as this would facilitate interconnections between academic researcher and legislators. The unique formula identification (the UFI) code should also be included to identify biocidal substances in commercial products even if its composition changes.

- The use of biocidal products that carry a high risk for the development of antimicrobial resistance, such as chlorhexidine and QACs, in household products and over-the-counter medication should be reevaluated.

- The use of biocidal products that carry a high risk for the development of antimicrobial resistance, such as chlorhexidine and QACs, should be restricted to applications where these biocides are clearly more adapted or efficient than biocidal products that carry a lower risk for the development of resistance.

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