REVIEW

Common therapeutic approaches for the control of oral biofilms: microbiological safety and efficacy

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ABSTRACT

Triclosan is widely employed in many consumer and healthcare products. The increasing employment of triclosan in a range of consumer products where there is no proven benefit for hygiene has been severely criticised. Laboratory studies demonstrate theoretical risks that the wide-scale use of triclosan might compromise its efficacy as well as the activity of third-party antibiotics. The precautionary principle would dictate against the use of triclosan, at least in those products where there was no demonstrable health benefit. The theoretical risks, however, are not supported by either field or clinical studies, or by laboratory studies using bacterial microcosms. Numerous clinical studies, as well as historical data, demonstrate the clinical benefits of hygiene adjuncts such as triclosan and triclosan/co-polymer in oral care products where these compensate for deficiencies in mechanical hygiene (brushing and flossing). The balance of risk and benefit is firmly in favour of the continued use of dentifrices (toothpastes) and mouthwashes containing active agents such as triclosan.

Keywords Cross-resistance, enoyl reductase, periodontitis, resistance, triclosan

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INTRODUCTION

The primary factor in good oral health is the routine control of dental plaque, a natural biofilm, formed on tooth surfaces and compacted at gingival margins. Such plaque is associated with caries, and gum inflammation (gingivitis) or degradation (periodontitis) of the gums and proximal bone. Root canal infection and chronic periodontitis lead to continual challenge of the systemic circulation by a plethora of oral microbes, either singly or as 'septic infarcts' originating from the gingival pocket or tooth-pulp [1]. The articles in this issue present overviews of the potential of such chronic inflammation, and overt infection, to predispose individuals to cardiovascular disease either directly or indirectly [1]. In this context, oral health is not only important from an aesthetic standpoint, but is also central to the general wellbeing of individuals and potentially to both the quality and length of life. The single most important route to oral health, in an otherwise healthy individual, is regular and appropriate hygiene. However, over 90% of adults in Western Europe must resort to reparative dental work and over 50% have some form of periodontal disease [2,3], while the majority claim to brush their teeth at least twice-daily [4]. Although many factors, such as genetics, smoking and diabetes, influence the susceptibility of an individual to periodontitis, the process of tissue destruction is initiated by plaque bacteria and can be controlled by regular brushing. Active ingredients in oral care products, such as dentifrices and mouthwashes, have the potential to

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compensate for shortfalls by having significant effects on the vitality of dental biofilm, but they do not substitute for appropriate mechanical cleansing. The efficacy of such products, particularly those employing triclosan/copolymer, is supported by numerous clinical studies [4], where the evidence in favour of triclosan use in oral hygiene is overwhelming. In spite of this proven efficacy and a greater understanding of the possible links between oral health and systemic infection, routine use of such products has been criticised on the basis of theoretical risks of antibiotic resistance development.

Over three decades, with the exception of sporadic reports of triclosan insensitivity, there has been no general reduction in the effectiveness of this agent against its target bacteria. Recent developments in home and household products have, however, dramatically increased the environmental exposure to triclosan. Such novel, market-driven applications have been subject to severe criticism in view of the absence of proven benefit for hygiene to offset the theoretical potential for resistance development. Laboratory studies demonstrating such potential have concentrated on enteric bacteria or pseudomonads, even though triclosan is not noted for its activity against either. Long-term, sub-lethal exposure of *Escherichia coli* to triclosan can lead to the selection of mutant clones with significantly reduced susceptibility, either mutated in an enoyl-reductase enzyme (FabI) or overexpressing multidrug efflux pumps. Initial concerns that mutations in FabI might be capable of horizontal transfer between environmental bacteria and nosocomial pathogens, or that parallel processes might occur directly in Gram-positive pathogens, have subsided. Similarly, concern about possible selection of resistance towards third-party agents (antibiotics) that might share the FabI gene target have proven unfounded. Although efflux mutants have been demonstrated in the laboratory, they have not been observed in retrospective analyses of isolates from the hospital or domestic environment, where agents such as triclosan have been widely employed, suggesting that efflux-on and FabI mutants are unable to compete in natural microbial communities.

This review will consider the evidence associated with triclosan-associated changes in susceptibility of organisms in both laboratory and field studies.

BACKGROUND

Triclosan is the most commonly used and most potent example of the chlorinated diphenyl ether class of antibacterial compounds [5]. Since its introduction in the 1960s, it has been widely used as an antiseptic in clinics and hospitals [6,7], within medicated soaps and hand-washes and as therapeutic baths for methicillin-resistant *Staphylococcus aureus*-infected patients [8,9]. The efficacy of triclosan in the control of methicillin-resistant *S. aureus* skin infections is proven [10,11] and is its main benefit in antiseptic hand-washes for laboratory and hospital surgical/medical units [7]. Triclosan has also been employed widely in a variety of personal products, including shampoos, toothpastes, and deodorants [12–14].

Over the past 10 years, the broad-spectrum antimicrobial activity of triclosan has led to its incorporation in an extended range of product formulations intended for home use. For example, a recent survey of liquid soaps in the USA revealed that 45% contained antibacterial agents, most of which included triclosan [15]. A recent, market-driven development has been the introduction of hardware products composed of plastics that have triclosan incorporated within them. Such products, marketed in the name of hygiene, claim to confer a degree of colonisation resistance to a wide variety of objects, including chopping boards, children's toys, carpets, and food storage containers. The hygienic gains associated with this new and increasingly widespread use in the home are largely undemonstrated.

Triclosan is a broad-spectrum antimicrobial agent that is especially active against Gram-positive species such as *S. aureus*, but it is ineffective against the Pseudomonadaceae [5]. The bias away from Gram-negative species is responsible for its widespread use in topical healthcare applications and in oral hygiene. Triclosan differs from more conventional agents of this class, e.g., strong oxidisers, bisbiguanides and aldehydes, in that the majority of environmental species, including many yeasts, are not susceptible.

When first introduced, triclosan was thought to act in a fashion similar to other halogenated phenols such as tricarbamilide, pentachlorophenol, and trichlorocarbanilide [16], by interacting with cell membranes to cause non-specific cytoplasmic losses [16]. Lack of susceptibility in the

Pseudomonadaceae was attributed to exclusion, by the outer-membrane, from the more vulnerable cytoplasmic membrane [17]. Mechanistic studies failed to distinguish between an entire class of membranotropic antimicrobial agents that included bisbiguanides, quaternary ammonium compounds [18], surfactants, phenylethers, and phenoxyethers. Many of these molecules were noted as uncouplers of oxidative phosphorylation from respiration; this has recently been confirmed for triclosan [19]. Uncoupling depletes the ATP pool [18] and disrupts ATP biosynthesis, active transport and osmoregulation, leading to growth inhibition through a multiplicity of targets [17,18]. Such a multiplicity of targets underpins our confidence that resistance will not develop. Many environmental organisms, including many of the Pseudomonadaceae, Actinomycetes and spore-forming bacilli, do not succumb to these and can actively degrade triclosan.

The discovery that chronic, sub-lethal exposure of *E. coli* to triclosan can lead to the selection of mutant clones that possess a significantly reduced MIC [20–24] has led to an upsurge of interest in its pharmacology. Triclosan has proved to be a potent inhibitor of the enoyl acyl carrier protein (ACP) reductase (FabI) of *E. coli* [20,21,24], an essential enzyme in fatty acid biosynthesis for many bacterial species, including Gram-positives [20,22,23]. The enzyme is conserved among bacterial species, with homologous target enzymes (InhA) being functionally important in various mycobacteria, bacilli and staphylococci [25]. Naturally occurring triclosan-resistant ACPs (InhK) have been documented. *Pseudomonas aeruginosa* possesses both triclosan-sensitive and triclosan-resistant FabI homologues. In species such as *E. coli*, triclosan can select for FabI mutants that, for this relatively insensitive organism, are tolerant of the agent at high concentration [21,24]. If this was replicated in Gram-positive bacteria, and if ACP was the sole target, then such mutations would significantly decrease triclosan's efficacy. Importantly, if ACPs were a target for other therapeutic agents and the ACP of *Mycobacterium tuberculosis* is the target for isoniazid, then their activity might also be compromised [26]. McMurry *et al*. demonstrated that partial isoniazid resistance in *Mycobacterium smegmatis* could be conferred by mutations in InhA, the gene homologous to FabI [25], but isoniazid-selected *M. tuberculosis* mutants remain susceptible to triclosan, suggesting separate interactive sites [27,28]. Selection of mutant ACPs by triclosan would be problematic if antibiotics used for treating staphylococcal and enterococcal infections shared this target. This is, however, not the case; cross-resistance has not been encountered for staphylococci [29]. Rather, the major implication of ongoing triclosan use is the possible selection of triclosan-tolerant strains.

If the ACP enzymes were highly conserved and equally important in all clinically relevant organisms, there would be a remote possibility of horizontal transmission of tolerance from environmental to clinically important organisms. Thus, it can be argued that triclosan should be confined to those applications where there are proven gains in hygiene. Recent publications suggest, however, that, not only is the ACP function conferred by a variety of isoenzymes, but even those with a high degree of homology to the FabI of *E. coli* possess significant functional differences.

Heath *et al*. conducted molecular studies concerning the interaction of triclosan with Gram-positive bacteria [22,23]. They identified the FabI component of *S. aureus* (saFabI) and found that the homologues from *E. coli* had similar specific activities, and also that *S. aureus* FabI expression complemented that of *E. coli* FabI (Ts). While staphylococcal FabI was interchangeable with the *E. coli* FabI enzyme, the latter was specific for NADH, whereas the staphylococcal enzyme exhibited specific and positive cooperative binding of NADPH. Triclosan inhibited both enzymes, but triclosan-resistant forms were hexachlorophene-sensitive and hexachlorophene-resistant, respectively. By contrast, Heath *et al*. identified considerable homology between the *E. coli* ACP and that of *Bacillus subtilis*, but noted an equal preference for NADH or NADPH as a cofactor. *B. subtilis* was noted to possess a second ACP homologue, but it was substantially less susceptible to triclosan and could functionally replace the FabI homologue.

Research concentrating on the interaction of triclosan with ACP enzymes loses sight of the multi-targeted mechanisms of this and structurally related biocides [16,30,31]. While triclosan is different from other chlorinated phenolics, because its inhibitory action centres on one enzyme, bactericidal activity involves a plethora of non-specific perturbations. Villalain *et al*. [30] showed

that triclosan disrupts the membrane integrity of typical oral bacteria, which are actively lysed at 'use' concentrations, reinforcing the view that triclosan is primarily a membranotrophic agent. Differences in ACP enzymes between Gram-positive and Gram-negative bacteria are insufficient to explain their marked differences in susceptibility.

Early work attributed the 'intrinsic' resistance of Gram-negative bacteria to the presence of an impenetrable outer-membrane [32]. There is now very strong evidence that this barrier is augmented by the expression of efflux pumps, capable of actively removing many inimical agents from the cell [33]. Efflux pumps such as acrAB can be up-regulated through the multiple antibiotic resistance (*mar*) operon in response to sub-lethal stress due to certain inducer substances (tetracycline and salicylates [34], pine-oil [35], and quaternary ammonium compounds [36]). Induction of efflux is sufficient to confer clinical resistance to many therapeutic agents, but is generally insufficient to influence the outcome of strong biocidal treatments [37]. Efflux is a highly evolved defence mechanism against a wide range of environmental toxins. Mutants that constitutively express efflux pumps suffer a heavy fitness-cost and will generally not be competitive in nature without a favourable selection pressure. In Gram-negative bacteria, sub-lethal exposure to any efflux inducer changes susceptibility to triclosan, which is a substrate. Triclosan cannot induce efflux [21]; therefore, sub-bactericidal exposure of Gram-negative bacteria may select for spontaneous hyper-expressing efflux mutants. Triclosan may thus select for highly resistant clones of *P. aeruginosa* [38], for which the MICs of several other drugs, including ciprofloxacin, were increased up to 1–500-fold.

RESISTANCE DEVELOPMENT AND ENVIRONMENTAL IMPACT

Pure culture studies

Much of the published work concerning triclosan resistance has utilised pure cultures, which are acknowledged to be unrepresentative of the 'real world'. Repeated passage of ex-vivo isolates causes them to lose non-essential traits. Thus, triclosan-insensitive *E. coli* strains were selected during routine and repeated passage against triclosan in monoculture [21,25], identifying ACP as a major target of this biocide; such stable reductions in triclosan susceptibility have been repeated by other groups using *E. coli* [39–41]. In contrast, replication of these selection/training protocols, using over 40 fresh isolates from the mouth, skin and domestic drain, together with representative laboratory strains of typical oral flora (*Fusobacterium nucleatum*, *Lactobacillus rhamnosus*, *Neisseria subflava*, *Porphyromonas gingivalis*, *Actinomyces naeslundii*, *Prevotella nigrescens*, *Streptococcus oralis*, *Streptococcus sanguis*, *Streptococcus mutans* and *Veillonella dispar*), showed that only the enteric species *E. coli* and *Klebsiella oxytoca* undergo selectable decreases in triclosan susceptibility [39,42]. Their susceptibilities to third-party agents were not significantly decreased. None of the remaining test isolates, including other enteric species such as salmonellae, were affected in terms of their susceptibility to triclosan or to any representatives of the test panel [39,42], which showed that the ability to select for triclosan resistance is not universal and might even be restricted to *E. coli*. The multiplicity of ACP in many bacterial genera and differences in susceptibility to triclosan mean that, in other species, there might be more susceptible targets than FabI which may dictate triclosan activity. Alternatively, susceptibility could be dependent on non-specific action at the level of the cytoplasmic membrane [19,43]. In both instances, the presence of multidrug efflux pumps will greatly decrease bioavailability at the pharmacological target. In this respect, high-level triclosan-resistant mutants of *E. coli* are not only affected in their ACP enzymes, but also hyper-express multidrug efflux pumps such as acrAB. *P. aeruginosa*, an organism inherently insensitive to triclosan, requires deletion of the major efflux pumps (mexA, mexB) for triclosan sensitisation. Interestingly, when *P. aeruginosa* is sensitised to triclosan by removal of these pumps, triclosan training selects for strains with the otherwise unexpressed pumps (mexC, mexD) [38]. Triclosan is unusual in that it is a substrate for many different efflux pumps, but does not regulate them. Pump-mediated resistance to triclosan requires cells to be up-regulated, through an unrelated third-party inducer, or constitutive expression. Sub-lethal triclosan exposure of *E. coli* may select for pre-existing constitutive mutants that hyper-express efflux pumps. It has been

argued that multidrug efflux pumps, while conferring resistance to clinically useful antibiotics, have evolved to contend with naturally occurring inimical agents [44]. Rickard *et al*. [44] investigated the potential of non-antibacterial consumer products to induce the multiple antibiotic resistance (*mar*) operon of *E. coli*. Of 35 products tested (nine herbs and spices, 19 foods and seven household products), 24 (69%) products were bacteristatic and 22 (63%) induced mar expression. Six products were shown to be powerful *mar* inducers but none were claimed to affect hygiene and none contained triclosan. The authors concluded that *mar* can be induced by exposure to many natural substances, common to the domiciliary setting. Concern that antibacterial agents deliberately added to consumer products might select for *mar*-mediated resistance was short-sighted and failed to recognise the ubiquity of inducers in the environment.

Microcosm studies

Pure cultures maintain organisms within a nutritious environment devoid of competition. Debilitated strains can be perpetuated as pure cultures in the laboratory but are unable to compete in the environment. In the 'real world', microrganisms grow as polymicrobial communities, often in close proximity to one another and attached to surfaces (biofilms). Biofilms are ubiquitous in nature, where phenotypic and genetic diversity confer metabolic capacities that are greater than the sum of the individual community members. Survival within such a community is dependent upon fitness and cross-species interaction. Few environments are colonised by pure cultures. Rather, multiple-species biofilms dominate in locations such as domestic sink drains, the human mouth and gut, sewers and sewage treatment plants. All of these environments will be exposed to varying levels of triclosan, because of its use in domestic cleaning formulations and dentifrices. Accordingly, we have evaluated the effects of chronic, low-level triclosan exposure on such communities.

Domestic drain studies

Stable sink-drain biofilms were established, using constant-depth film fermenters and ex-situ drain biofilm material from a household that had not used triclosan-containing products, other than dentifrices [45]. Microcosms, intermittently fed with artificial dishwater, were maintained at room temperature and constantly moistened with untreated tap water. Cultured microcosms closely modelled the ex-situ material [45] and were used to investigate the long-term effects (6 months) of exposure to a triclosan-containing domestic detergent. Culturable bacteria were archived and antimicrobial susceptibilities were determined. There was a general lack of sensitivity in the evolved biofilm community to triclosan and cationic soaps. Triclosan-tolerant strains of *Aeromonas*, *Pseudomonas*, *Stenotrophomonas* and *Alcaligenes* spp. were detected before and after triclosan exposure. Triclosan products did not select for resistance; rather, they affected community dynamics, causing clonal expansion of pre-existing, less susceptible clones, among which pseudomonads dominated. *Achromobacter xylosoxidans*, an organism later shown to solubilise and degrade triclosan, expanded clonally. There was no change in the triclosan susceptibility of any isolate, and susceptibility to a panel of third-party antibiotics was unchanged.

Dental plaque studies

The potential effects of a triclosan/copolymer dentifrice on oral microcosms was also studied using constant-depth film fermenters [46] exposed to triclosan at levels equivalent to a normal hygiene regimen [46]. Bacteria exhaustively isolated on various selective media and microcosm plaques were profiled using denaturing gradient gel electrophoresis (DGGE); the isolates were archived and susceptibility to a range of antibiotics was determined. Triclosan/copolymer caused significant reductions in Gram-negative anaerobes and the total anaerobe count. Transient reductions were noted, according to both culture and non-culture methods, in the numbers and diversity of streptococci and actinomycetes. Parallel studies using type cultures of oral bacteria indicated that *N. subflava*, *P. nigrescens* and *P. gingivalis* were highly susceptible to triclosan and that the lactobacilli and streptococci were relatively non-susceptible [46]. Both datasets were compatible with the hypothesis that repeated exposure of oral microcosms to triclosan/copolymer inhibits the most susceptible flora and causes clonal expansion of less susceptible species. This picture is also compatible with *in-vivo* studies. In no instance was there any

change in the susceptibility profile of the isolated species that could be attributed to triclosan.

Environmental surveillance studies

The availability of oral hygiene formulations containing adjuncts that inhibit the formation and development of dental plaque and that are intended to improve other indices of oral health has resulted in guidelines from the American Dental Association and the US Food and Drug Administration to monitor effects on oral microflora. Eight long-term studies (6 months or longer with more than 650 subjects) have examined the microbiological effects of the unsupervised use of triclosan formulations as compared with those of appropriate control dentifrices [47–50]. Qualitative and quantitative evaluations of the oral microflora were made, with particular reference not only to the emergence of opportunistic pathogens, but also to the possible development of microbial resistance, either during the 6-month test period or during a 6-month post-exposure surveillance period. Results from some of these studies were reviewed for both safety and efficacy of triclosan/copolymer by the US Food and Drug Administration and the American Dental Association [47,48]. Significantly, in no instance were there reported changes in antimicrobial susceptibility of the oral microflora, or dysbiosis of a healthy flora during or after exposure. The clinical studies report that a beneficial oral flora was maintained within the dental plaque, with no emergence of periodontal or opportunistic pathogens, including yeasts, among the subjects.

Other long-term studies (3–5 years with more than 500 subjects) examined the effects of brushing with triclosan/copolymer dentifrice upon the severity of periodontitis or the nature of the subgingival microflora [51–54]. Once again, no alterations in antimicrobial susceptibility were reported in any of these studies, and nor were adverse changes in composition of the oral microflora in the subgingival regions noted. Reductions in the progression of periodontal disease were reported among the triclosan/copolymer group [54]. Overall decreases in the subgingival flora, with fewer subjects harbouring periodontal pathogens, i.e., *P. gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans*, were reported [53].

Beyond the immediate effects on oral flora, interest has been focused on environments subject to the greatest exposure to triclosan and other antibacterial agents. Cole *et al*. [55] studied 60 homes in the USA and UK, selected on the basis of antibacterial product use. Analysis of the bacterial species present (1238 isolates) demonstrated a greater proportion of potential pathogens in non-user households. Neither methicillin-resistant *S. aureus* nor strains resistant to oxacillin or vancomycin were recovered, and nor was there evidence of ampicillin- or vancomycin-resistant enterococci. All *E. coli* and *Klebsiella* spp. were susceptible to third-generation cephalosporins. Antibiotic resistance to a single 'preferred' drug agent was not significantly different between user and non-user households. Importantly, the incidence of resistance to antibacterial agents was highest in non-user households. Lear *et al*. [56] investigated industrial environments where heavy biocide use is routine. While triclosan-tolerant isolates were retrieved, the authors concluded that these were naturally tolerant *Pseudomonas* spp., and the data did not predict resistance to normally lethal levels. No evidence linked biocide residues to tolerance in the environments investigated. A similar study evaluating clinical isolates of *P. aeruginosa* and *S. aureus* over a 10-year period [57] revealed evidence of changes in susceptibility to biocides between 1989 and 2000 for *S. aureus*, but failed to support the hypotheses that increased biocide resistance had led to antibiotic resistance. Negative correlations between antibiotic and biocide susceptibility were seen as 'a useful reason for the continued use of biocides in the hospital environment'.

Aiello *et al*. [58] conducted a large (224 households), 12-month study into the potential effects of antibacterial products in the domestic environment. This involved detailed microbial isolation and analysis, and logistic regression analysis of susceptibility data showed that the use of antibacterial products did not generate a significant increase in antimicrobial drug resistance after 1 year and had not influenced overall susceptibility to triclosan.

CONCLUSION

There is currently insufficient evidence to affirm that the uncontrolled use of triclosan in domestic products is totally free of risk. Every intervention

must be evaluated according to the balance between its risk to the consumer and environment and its potential and real benefits. The clinical effectiveness of oral hygiene formulations containing triclosan, including their role in the prophylaxis and treatment of common oral maladies, is unquestionable, and the risk of resistance development following from triclosan use is overstated and does not justify its removal from oral hygienic products.

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