Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*

Short running title: Antimicrobial activity of chlorhexidine and essential oils

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Synopsis:

Objectives: Effective skin antisepsis and disinfection of medical devices are key factors in preventing many healthcare acquired infections associated with skin microorganisms, particularly, *Staphylococcus epidermidis*. The aim of this study was to investigate the antimicrobial efficacy of chlorhexidine digluconate (CHG), a widely used antiseptic in clinical practice, alone and in combination with tea tree oil (TTO), eucalyptus oil (EO) and thymol against planktonic and biofilm cultures of *S. epidermidis*.

Methods: Antimicrobial susceptibility assays against *S.epidermidis* in a suspension and in a biofilm mode of growth were performed with broth microdilution and ATP bioluminescence methods respectively. Synergy of antimicrobial agents was evaluated with checkerboard assay.

Results: Chlorhexidine exhibited antimicrobial activity against *S. epidermidis* in both suspension and biofilm [Minimum inhibitory concentration (MIC) 2–8 mg/ L]. Of the essential oils thymol exhibited the greatest antimicrobial efficacy (MIC 0.5–4 g/ L) against *S. epidermidis* in suspension and biofilm followed by TTO (MIC 2–16 g/ L) and EO (4–64 g/ L). Minimum inhibitory concentrations of CHG and EO were reduced against *S. epidermidis* biofilm when in combination (MIC 8 reduced to 0.25-1 mg/ L and 32-64 reduced to 4 g/L for CHG and EO respectively). Furthermore, the combination of EO with CHG demonstrated synergistic activity against *S. epidermidis* biofilm with a fractional inhibitory concentration index (FICI) of <0.5.

Conclusion: The results from this study suggest there may be a role for essential oils, in particular EO, for improved skin antisepsis when combined with CHG.

Introduction: Incision of human skin is a common practice in the clinical setting, for example during surgery, when taking blood or inserting intravascular devices. Adequate skin antisepsis is therefore essential in avoidance of healthcare associated infections (HAI) which may occur post incision, commonly from resident microorganisms located within the skin, particularly *S. epidermidis.*[1, 2] *Staphylococcus epidermidis* is common on both the surface of human skin and also within the deeper layers where it may exist as microcolonies, which, like bacterial biofilms, exhibit increased resistance to antimicrobials including antiseptics.[3] Unfortunately, HAI do arise following incision of skin[4,5] and are likely to be associated with increased prevalence of microbial resistance to antibiotics and antiseptics and inadequate skin antisepsis which encompasses both the contact time between skin and antiseptic prior to incision and permeation of the antiseptic within the skin.[6-10] Chlorhexidine is one of the most widely used antimicrobials within clinical practice for skin antisepsis and is currently recommended within the Evidence-Based Practice in Infection control (EPIC)[11] and Healthcare Infection Control Practices Advisory Committee (HICPAC)[12] guidelines. However, its antimicrobial efficacy is significantly reduced by factors including pH and organic matter.[13] Therefore additional strategies for skin antisepsis or improvement of existing methods need to be considered.

The antimicrobial efficacy of essential oils has been known for several years and many studies have demonstrated activity against bacteria, fungi and viruses.[14] More recently, in the light of increased antimicrobial resistance within the clinical setting, the potential of essential oils for the prevention and treatment of infection has been researched in several studies.[15-19] Indeed, TTO has recently been shown to be more effective than CHG at clearing superficial skin sites and lesions colonised with

63 methicillin resistant *S. aureus*.[17] In addition, preliminary research within our laboratory
64 has demonstrated that several essential oils (EO, TTO, thymol, lavender, juniper and
65 citronella) have antimicrobial efficacy against a wide range of microorganisms associated
66 with HAI.[20] Many of the essential oils are thought to disrupt cell membranes by the
67 lipophilic compounds contained within the essential oil, such as terpenes. However,
68 essential oils are mixtures of many heterogeneous compounds, and the main components
69 responsible for their antimicrobial activity and the mode of their activity are not well
70 understood.[14] Furthermore, essential oils have been shown to act as effective penetration
71 enhancers, increasing permeation and improving retention of drugs within the skin.[21,22]
72 The aim of this study was to investigate the antimicrobial efficacy of aqueous
73 CHG alone and in combination with TTO, EO and thymol against planktonic and biofilm
74 cultures of *S. epidermidis*.
75

Materials and methods

Materials

Congo red agar for demonstrating slime production in the test strain *S.epidermidis* was prepared by mixing 0.4 g of Congo red (Hopkins and Williams Ltd, Essex, UK), 25 g sucrose (Fisher Scientific, Leics, UK) and 5 g of agar No.1 (Oxoid, Basingstoke, UK) with 490 mL of brain heart infusion (BHI) (Oxoid) and sterilised according to the manufacturers' recommendations. Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) (Oxoid) were also prepared and sterilised in line with the manufacturers' recommendations. Phosphate buffered saline (PBS), aqueous CHG (20% in water), TTO (40.2% terpinen-4-ol and 3.5% cineole), EO (82.9% cineole), thymol (>99.5%) and dimethylsulphoxide (DMSO) were purchased from Sigma-Aldrich (Dorset, UK) and glucose from Fisher Scientific. White walled, clear bottom, tissue culture treated 96-well microtitre plate were from Corning Incorporated (NY, USA) and clear, round bottom 96-well plates from Barloworld Scientific (Staffordshire, UK).

Microorganisms

Staphylococcus epidermidis RP62A [23] and a clinical isolate of *S.epidermidis*, TK1, (University Hospital Birmingham NHS Trust, Birmingham, UK) were stored on MicroBank beads (Pro-Lab Diagnostics, Cheshire, UK) at -70˚C until required.

Preparation of antimicrobial agents

Aqueous CHG was diluted with MHB to obtain a stock solution of 512 mg/ L. Thymol, TTO and EO were diluted with MHB to obtain stock solutions of 512 g/ L. Five percent (v/v) DMSO was added to the essential oil stock solutions to enhance the solubility of the oils in solution.

Preparation of S.epidermidis inoculum for suspension assay

Suspensions of *S.epidermidis* for the suspension assays were prepared by inoculating 10 identical colonies of overnight cultures of *S.epidermidis* from MHA into sterile PBS. The bacterial concentration was adjusted to 1 x 10^8 cfu/ mL by diluting the culture with sterile PBS and measuring the optical density at 570 nm. The suspensions were further diluted with MHB to obtain inocula containing 1x10^6 cfu/ mL.

Determination of MIC and MBC of CHG, TTO, EO and thymol against S.epidermidis in suspension

Minimum inhibitory concentration of aqueous CHG and TTO, EO and thymol were determined using a broth microdilution assay in line with CLSI (formerly NCCLS) guidelines.[24] The antimicrobial activity of 5 % (v/v) DMSO was also studied on a separate microtitre plate alongside the assay. Each well containing 100 µL of antimicrobial agent was inoculated with 100 µL of *S.epidermidis* suspension containing 1x10^6 cfu/ mL. Following 24 h incubation in air at 37˚C, the wells were inspected for microbial growth and the MIC was defined as the lowest concentration which did not show visual growth. Controls containing antimicrobial agent in broth and broth with

inocula were also included. Minimum bactericidal concentrations were determined by removing the total volume (200 µL) from each of the clear wells and mixing with 20 mL of cooled molten MHA, which was then allowed to set. Plates were incubated in air at 37˚ C for 24 h. Minimum bactericidal concentration was defined as the first plate yielding no growth. The assay was performed in triplicate.

Checkerboard assay to assess antimicrobial activity of CHG in combination with TTO, EO and thymol against S.epidermidis in suspension

The antimicrobial activity of aqueous CHG in combination with TTO, EO and thymol was assessed in a suspension assay by the checkerboard method.[25] In brief, serial double-dilutions of the antimicrobial compounds were prepared (256 to 1 g/L for EO and TTO, 64 to 0.25 g/ L for thymol and 64 to 0.5 mg/L for CHG). Fifty microlitres of each CHG solution was added to the rows of a 96-well microtitre plate in diminishing concentrations and 50 µL of the essential oil to the columns in diminishing concentrations. The wells were then inoculated with 100 µL of *S.epidermidis* suspension containing $1x10^5$ cfu (the final concentrations of EO and TTO ranged from 64 to 0.25 g/L, thymol 16 to 0.06 g/L and CHG 16 to 0.125 mg/L). Columns 10, 11 and 12 served as controls containing MHB and inoculum alone, and antimicrobial compounds separately with the inoculum. The microtitre plates were incubated in air at 37˚ C for 24 h and MIC for both antimicrobial compounds in combination was determined. To assess synergistic or antagonistic activity of antimicrobial combinations, the fractional inhibitory concentration (FIC) and FIC index (FICI) were determined using the following formulae:

$$FIC = \frac{\text{MIC of CHG or natural compound in combination}}{\text{MIC of CHG or natural compound alone}}$$

$$FICI = \text{FIC of oil} + \text{FIC of CHG}$$

FICI $\leq$ 0.5 were regarded as synergistic effect, values between 0.5 to 4.0 as indifferent and over 4.0 as antagonistic activity. The assay was performed in duplicate microtitre plates.

Preparation of S.epidermidis biofilm

The ability of *S.epidermidis* strains to produce slime was confirmed by culturing the bacteria on Congo red agar.[26] The optimal conditions and incubation period for the production of the bacterial biofilms were established in preliminary experiments over a 72 h period (data not shown). Bacterial biofilms were prepared by aliquoting 200 µL of the bacterial suspension containing $1x10^5$ cfu/ mL into the wells of white walled, clear bottom, tissue culture treated 96-well microtitre plates. Four wells in the last column of each plate were left blank to serve as bioluminescence negative control. Suspensions of *S.epidermidis* were prepared in MHB supplemented with 2% (w/v) glucose. Microtitre plates containing *S.epidermidis* suspensions were incubated in air at 37˚ C for 48 h (these conditions demonstrated in preliminary experiments to be optimal conditions for production of a confluent biofilm with approximately $5.5x10^6$ cfu per well - data not shown).

Determination of MIC and MBC of CHG, TTO, EO and thymol against S.epidermidis in biofilm

Microtitre plates containing *S.epidermidis* biofilms were washed once with sterile PBS to remove any unbound bacteria. Antimicrobial agents were diluted with MHB to obtain CHG concentrations ranging from 128 mg/ L to 0.25 mg/ L, thymol 128 to 0.25 g/L and EO and TTO 256 to 0.5 g/L. Two hundred and fifty microlitres of each antimicrobial agent was added to each microtitre plate well. Columns 11 and 12 served as controls containing the biofilm and saline alone and MHB alone without bacterial biofilm. Antimicrobial activity of 5% (v/v) DMSO against the bacterial biofilm was also tested on a separate plate. Following incubation at 37˚C in air for 24 h, the wells were washed once with sterile PBS and the microbial viability was determined using an ATP bioluminescence assay (ViaLight MDA Bioassay kit, Cambrex, Berkshire, UK). In brief, 100 µL of Bactolyse was added with 100 µL of saline into each well and the plates sonicated at 50 Hz for 30 min to release and lyse the cells of the bacterial biofilm. Fifty microlitres of ATP-monitoring reagent was added to each well and luminescence measured (Lucy 1, type 16 850 fluorescence measurer, Rosys Anthos Labtech Instruments). Minimum inhibitory concentration was defined as the minimum concentration of antimicrobial agent that inhibited further growth of the initial biofilm (control well containing biofilm treated with saline) and MBC was defined as the concentration that produced below or equal to the background level of luminescence (empty well). The assay was performed in duplicate microtitre plates.

Checkerboard assay to assess antimicrobial activity of CHG in combination with TTO, EO and thymol against S.epidermidis in biofilm

Microtitre plates containing *S.epidermidis* biofilms were washed once with sterile PBS to remove any unbound bacteria. Antimicrobial agents were diluted with MHB as described previously and 125 µL of each of the antimicrobial dilutions aliquoted into each well in decreasing concentrations. Columns 10 and 11 contained biofilm and antimicrobial compounds alone at various concentrations and column 12 served as control with biofilm with saline and clear wells with saline. The plates were incubated in air at 37˚C for 24 h after which the wells were emptied and the FIC and FICI values determined by ATP bioluminescence as described previously. The assay was performed in duplicate.

Results

Determination of MIC and MBC of CHG, TTO, EO and thymol against S.epidermidis in suspension and in biofilm

Chlorhexidine digluconate, TTO, EO and thymol demonstrated antimicrobial activity against *S.epidermidis* RP62A and *S.epidermidis* TK1 in both suspensions and biofilms (Table 1). Minimum inhibitory concentration and MBC of CHG were 4-fold higher against *S.epidermidis* growing in biofilm compared to cells in suspension (MIC 2 to 8 mg/ L and MBC 4 to 16 mg/ L). Of the three essential oils thymol was the most effective with MIC ranging from 0.5 to 4 g/ L and MBC 2 to 16 g/ L. All antimicrobials tested reduced the luminescence from the bacterial biofilms to below negligible levels with concentrations of 16 mg/ L (CHG), 2-8 g/ L (thymol), 32-64 g/ L (TTO) and 256 g/ L

(EO). Five percent (v/v) DMSO, which was used as co-solvent in oil solutions, did not show antimicrobial activity against *S.epidermidis* in biofilm or suspension.

Checkerboard assay to assess antimicrobial activity of CHG in combination with TTO, EO and thymol against S.epidermidis in suspension and in biofilm

Combination of CHG with TTO, EO and thymol demonstrated indifferent activity against *S.epidermidis* RP62A and TK1 when grown in suspension (Table 2). In biofilm, CHG in combination with EO demonstrated synergistic activity against both strains of *S.epidermidis*, with FICI of 0.156 and 0.188 for *S.epidermidis* RP62A and TK1 respectively (Table 3).

Discussion:

The aim of this study was to assess the antimicrobial efficacy of aqueous CHG and three essential oils (TTO, EO and thymol) against the common skin microorganism, *S.epidermidis*, and to determine the antimicrobial activity of CHG in combination with the oils.

The results demonstrate that CHG, EO, TTO and thymol exhibit antimicrobial activity against *S.epidermidis* when growing both in suspension and as a biofilm. However, the concentration of essential oils required to achieve the same level of growth inhibition as CHG is several orders of magnitude higher (g/ L for essential oils compared to mg/ L for CHG). Thymol showed increased activity against *S.epidermidis* growing in biofilm compared with planktonic cells. This is unusual as biofilms are considered to be

more resistant to antimicrobial agents compared to planktonic cells.[3] Partitioning of oil, especially pure compound thymol, in oil suspension as well as in extracellular matrix in bacterial biofilm, may alter thymol activity. In a previous study by Nostro *et al*.,[27] only small differences between biofilm and planktonic cultures susceptibility to thymol was demonstrated. Furthermore, in the study by Al-Shuneigat *et al*.,[15] staphylococci in a biofilm mode of growth demonstrated increased susceptibility to an essential oil based formulation compared with planktonic cells, which concurs with our findings in relation to thymol. Thymol is a phenolic compound that has both hydrophilic and hydrophobic properties which may enhance diffusion of this compound in a biofilm and allow its access to bacterial cells where it alters the permeability of plasma membranes.[27]

Combining CHG with TTO, EO and thymol did not improve its antimicrobial activity against *S.epidermidis* TK1 and RP62A strains during their planktonic phase of growth, however, reductions in CHG concentrations required to inhibit growth of both *S.epidermidis* strains in biofilm were observed. Of the three essential oils used in this investigation, EO demonstrated the best potential for combination with CHG. Synergistic activity between EO and CHG was demonstrated against biofilms of both strains of *S. epidermidis* (FICI 0.19 and 0.16 for TK1 and RP62A respectively). To our knowledge, this is the first report of synergism between EO and CHG.

Previous research that has investigated the synergistic activity of an essential oil and an antimicrobial agent has suggested that the synergism may be due to their action on both different[28] or similar targets on the bacterial cell (i.e. cell membranes).[29] Eucalyptus oil and its main component 1,8-cineole are thought to act on the plasma membranes, the same target as CHG. However, TTO (and its main antimicrobial component terpinen-4-

ol) and thymol also have lipophilic properties and target cellular membranes without showing synergy in combination with CHG. Therefore, the interaction of EO and CHG requires further studies to establish the mode of action of the potential synergism. It is possible that not only one component is involved in the synergistic interaction between EO and CHG, but a mixture of several components. Moreover, it has been suggested that cationic CHG diffusion in the biofilm is hindered by the negatively charged extracellular matrix, changing the physicochemical properties of the extracellular matrix and its tertiary structure.[30] Chlorhexidine is likely to remain in the aqueous phase in the oil suspension (Log P of CHG 0.037 [31]). Both EO, which consists of several heterogeneous compounds, and CHG have hydrophilic and hydrophobic properties, and it may be possible that they alter ionic interactions in extracellular matrix of the biofilm, as well as act on the same target on the bacterial cell. However, further studies are needed to establish the mode of action of EO and CHG in combination.

The use of essential oils for the prevention and treatment of infection has been gaining popularity within the research field over the past decade.[19,32-34] Furthermore, the antimicrobial activity of TTO [16,17], thymol [27] and EO [35] has been reported against several important pathogens. However, there has been little research to assess the efficacy of essential oils in combination with CHG against *S. epidermidis,* which is the major microorganism associated with skin related HAI. Chlorhexidine is widely used as a skin antiseptic within the clinical setting and is the recommended antimicrobial within the EPIC and HICPAC guidelines.[11,12] However, infection rates associated with surgical incision of the skin remain high.[4] Thus the current strategies adopted for skin antisepsis need to be considered with a view for improvement.

The antimicrobial activity of CHG alone *in vivo* is reported as being bacteriostatic[36] and may be one factor which contributes to the survival of *S. epidermidis* within the skin following antisepsis and its association with subsequent infection. Furthermore, sub-inhibitory concentrations of chlorhexidine may increase a biofilm-mode of growth of staphylococci,[37] which may reduce the efficacy of skin antisepsis if inappropriate levels of antiseptic are used. The synergistic action of CHG in combination with EO may therefore be one way forward for enhancing both skin antisepsis and potentially disinfection of hard surfaces. The environment in the healthcare setting contributes to the spread of pathogens and transfer of microorganisms between patients and healthcare workers.[38] Microorganisms may reside on surfaces in aggregates embedded in a biofilm rendering them less susceptible to cleaning and disinfection. Furthermore, many medical devices such as central venous catheter hubs and needleless connectors also become colonised with microorganisms capable of producing a biofilm.[39] At present chlorhexidine-based compounds or isopropyl alcohol are commonly used for disinfecting these medical devices prior to use. The synergistic activity between CHG and EO in combination may therefore be of benefit in the clinical setting, for example, in improved skin antisepsis and the elimination of *S.epidermidis* existing as microcolonies which are likely to exhibit increased resistance to CHG alone, and also potentially hard surface disinfection. However, whilst much of the research data advocates the potential use of essential oils in the clinical setting for preventing and treating infection there is little information regarding safety in relation to their use, which needs to be taken into consideration. Therefore further studies are warranted.

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Transparency declarations

None to declare.

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Table 1. MIC and MBC of aqueous chlorhexidine digluconate, eucalyptus oil, tea tree oil and thymol against clinical TK1 and RP62A strains of *S.epidermidis* in suspension and in biofilm.

Strain	**Compound**	**MIC (g/ L for oils, mg/ L for CHG)**		**MBC (g/ L for oils, mg/ L for CHG)**	
		Suspension	**Biofilm**	**Suspension**	**Biofilm**
RP62A	Eucalyptus	4	32	64	256
	Tea tree oil	2	16	4	64
	Thymol	4	0.5	16	2
	CHG	2	8	4	16
TK1	Eucalyptus	8	64	32	256
	Tea tree oil	16	16	64	32
	Thymol	0.5	0.5	4	8
	CHG	2	8	4	16

Table 2. Antimicrobial activity of aqueous chlorhexidine digluconate, eucalyptus oil, tea tree oil and thymol against clinical TK1 and RP62A strains of *S.epidermidis* growing in suspension.

Strain	**Combination**	**MIC of oil (g/L) in combination/ alone**	**FIC of oil**	**MIC of CHG(mg/L) in combination/ alone**	**FIC of CHG**	**FICI**	**Result**
RP62A	CHG + Eucalyptus	4 / 4	1	2/ 2	1	2	Indifference
	CHG + Tea tree oil	2 / 2	1	2/ 2	1	2	Indifference
	CHG + Thymol	1/ 4	0.25	2/ 2	1	1.25	Indifference
TK1	CHG + Eucalyptus	8 / 8	1	2 / 2	1	2	Indifference
	CHG + Tea tree oil	16 / 16	1	2 / 2	1	2	Indifference
	CHG + Thymol	0.25 / 0.5	0.5	2 / 2	1	1.5	Indifference

Table 3. Antimicrobial activity of aqueous chlorhexidine digluconate, eucalyptus oil, tea tree oil and thymol against clinical TK1 and RP62A strains of *S.epidermidis* growing in biofilm.

Strain	**Combination**	**MIC of oil (g/L) in combination/ alone**	**FIC of oil**	**MIC of CHG(mg/L) in combination/ alone**	**FIC of CHG**	**FICI**	**Result**
RP62A	CHG + Eucalyptus	4 / 32	0.125	0.25/ 8	0.031	0.156	Synergy
	CHG + Tea tree oil	4 / 16	0.25	4/ 8	0.5	0.75	Indifference
	CHG + Thymol	0.5 / 0.5	1	8/ 8	1	2	Indifference
TK1	CHG + Eucalyptus	4 / 64	0.063	1 / 8	0.125	0.188	Synergy
	CHG + Tea tree oil	16 / 16	1	8 / 8	1	2	Indifference
	CHG + Thymol	0.25 / 0.5	0.5	4 / 8	0.5	1	Indifference