



Effectiveness of hand-cleansing formulations containing tea tree oil assessed ex vivo on human skin and in vivo with volunteers using European standard EN 1499

S. Messenger^a, K.A. Hammer^a, C.F. Carson^a, T.V. Riley^{a,b,*}

^a*Discipline of Microbiology, School of Biomedical and Chemical Sciences, The University of Western Australia, 35 Stirling Highway, Crawley WA 6009, Australia*

^b*Division of Microbiology and Infectious Diseases, Western Australian Centre for Pathology and Medical Research, Hospital Avenue, Nedlands WA 6009, Australia*

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Summary The efficacy of formulations containing tea tree oil (TTO) has been assessed in vitro in previous studies. Products that passed the European suspension test guidelines were investigated further in this study, in vivo with volunteers using the European handwashing method (EN 1499) and ex vivo using freshly excised human skin samples. The activity of 5% TTO in 0.001% Tween 80, in a hygienic skin wash (HSW) and in an alcoholic hygienic skin wash (AHSW) was investigated and compared with that of a non-medicated soft soap (SS, control). These formulations were assessed against *Escherichia coli* K12 as recommended by the European standard. In-vivo results showed that 5% TTO in Tween 80 and the AHSW were significantly more active than the SS after 1 min of handwashing. When assessed ex vivo, these two products were also significantly more active than the reference soap after 1 min of rubbing. Both methods showed that 5% TTO in Tween 80 was generally, although not always, more active than a handwash formulation, and that the AHSW was generally more active than the HSW, although this difference was not significant. The formulations tested, as well as the SS, were more active when assessed in vivo than ex-vivo against *E. coli*, although only the SS and the HSW were significantly more active in vivo. There appeared to be a pattern in the comparison between ex vivo and in vivo results. The antiseptics tested were, on average, 1.28 ± 0.06 times more active when assessed in-vivo than when assessed ex vivo.

* Corresponding author. Address: Department of Microbiology, Queen Elizabeth II Medical Centre, Nedlands 6009, WA, Australia. Tel.: +61-8-9346-3690; fax: +61-8-9346-2912.

E-mail address: triley@cyllene.uwa.edu.au

Nevertheless, the main outcome of the European handwashing method is for the formulation tested to be significantly more active than the SS; both 5% TTO in Tween 80 and the AHSW achieved this both in-vivo and ex-vivo. TTO in Tween 80 and in formulations met the European in-vivo method requirements.

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Introduction

Although it is difficult to demonstrate the effects of handwashing in terms of a decrease in mortality,¹ there is convincing evidence of the relationship between hand hygiene and reduced transmission of infections.²⁻⁴ However, healthcare personnel seldom wash their hands enough; average compliance is usually below 50%.^{5,6} Furthermore, handwashing technique has been described as poor, duration of washing is often shorter than that recommended^{2,5,7} and, although many attempts have been made to change behaviour,⁷⁻⁹ they have not always been effective.^{10,11} Most antiseptic agents can damage skin, leading to a change in microbial flora, an increase in bacterial shedding and thus an increased risk of transmission of micro-organisms.^{3,4} Instead of fighting micro-organisms at any cost (in most cases, at the cost of skin's health), skin integrity as well as its resident flora should be protected and considered an ally against infectious diseases.¹²

Some reports suggest that repeated use of formulations containing tea tree oil (TTO) does not lead to dermatological problems,¹³ and this could be used to encourage better healthcare staff compliance with handwashing. In addition, Hammer *et al.*¹⁴ showed that transient skin organisms were more susceptible to TTO than commensals. This has led to an increased interest in TTO-containing handwash products. The antibacterial activity of TTO has been well established in vitro.¹⁵⁻¹⁹ Recent work²⁰ showed that some TTO-containing formulations, as well as different concentrations of TTO in Tween 80, achieved a $\geq 10^5$ -fold reduction in bacterial cell numbers after 1 and/or 5 min of contact using the European standard suspension test EN 1276²¹ and a $\geq 10^4$ -fold reduction in bacterial cell numbers after 1 and/or 5 min of contact using the European draft standard suspension test prEN 12054.²²

The European suspension tests (phase 2/step 1) determine whether a preparation possesses adequate antimicrobial properties for a defined application. If so, the formulation should be tested to see whether it possesses antimicrobial properties in

practice (phase 2/step 2), mimicking, in this case, a handwashing test. Therefore, the efficacy of a hygienic skin wash (HSW) and an alcoholic hygienic skin wash (AHSW) that both contained 5% TTO, as well as 5% TTO in Tween 80, was investigated in vivo according to the European handwashing test.²³ An ex-vivo test, which has been shown to be a reproducible potential substitute for in-vivo testing,²⁴⁻²⁶ was adapted to imitate the handwashing technique and also used to assess the activity of TTO. Ex-vivo and in vivo data were compared.

Materials and methods

Micro-organism

Escherichia coli K12 (NCTC 10538) stock cultures were kept on tryptone soya agar (TSA; Oxoid, Basingstoke, UK) plates, stored at 4 °C, and renewed once a week.

Antiseptics

The activity of a HSW containing 5% TTO (100% HSW; Novasel Australia Pty Ltd, Mudgeeraba, Queensland, Australia), an AHSW containing 5% TTO and 10% alcohol (100% AHSW; Novasel), and 5% (v/v) TTO in sterile distilled water (SDW) with 0.001% (v/v) Tween 80 (Sigma, Chemical Co., St Louis, MO, USA) was investigated. TTO was kindly supplied by Australian Plantations Pty Ltd, Wyrallah, NSW, Australia. Batch WUEU04 complied with the international standard for TTO,²⁷ and contained 38.6% terpinen-4-ol, 20.3% γ -terpinene, 8.7% α -terpinene, 3.2% terpinolene, 3.0% α -terpineol, 2.6% α -pinene, 3.6% 1,8 cineole and 3.1% *p*-cymene. Levels of components were assessed by gas chromatography/mass spectrometry, performed by the Wollongbar Agricultural Institute, Wollongbar, NSW, Australia.

Neutralizer

The neutralizing solution used to quench the activity of antiseptics was based on the European standard EN 1276²¹ and contained: 30 g/L Tween 80

(Sigma), 3 g/L lecithin (Sigma), 1 g/L histidine (Sigma), 5 g/L sodium thiosulphate (BDH, AnalaR, Merck Pty Ltd, Victoria, Australia) and 34 g/L potassium dihydrogen phosphate (BDH) in tryptone soya broth (TSB; Oxoid). The efficacy of this neutralizer in quenching the activity of TTO, as well as its absence of toxicity against *E. coli* K12, has been reported previously.²⁰

Control

To compensate for extraneous influences, the results obtained with the products tested were compared with those obtained with a control handwash [soft soap (SS)] as recommended by EN 1499.²³ Experiments using SS were performed under comparable environmental conditions. SS was made of linseed oil (100 g/L; Sigma), potassium hydroxide (19 g/L; Sigma) and ethanol (14 g/L; Fisher Chemicals, Fairlawn, NJ, USA), with SDW as the balance.

In vivo handwashing test EN 1499²³

The number of test organisms released from artificially contaminated hands was assessed before and after hygienic handwashing. The prevalue is the number of colony forming units (cfu) sampled from the skin before treatment, and the postvalue is the number after treatment. The reduction factor (RF) in bacterial cell concentration was calculated as follows:

$\text{Log}_{10} \text{ RF} = \text{Log}_{10} \text{ prevalue} - \text{Log}_{10} \text{ postvalue}$
where RF is a measure of the antimicrobial activity of the disinfectant tested.

Due to volunteer availability, the handwashing studies were carried out on two separate occasions. These will be referred to as Studies 1 and 2.

Subjects

The test was repeated on 13 subjects (eight females and five males) in Study 1, and 14 subjects (eight females and six males) in Study 2. Ages ranged from 22 to 52 years and from 19 to 53 years in Studies 1 and 2, respectively. All subjects had short fingernails and intact skin, and were asked not to use any antibacterial soap or toiletries on their hands for 24 h before the test. All volunteers were informed about the procedure and signed a consent form prior to beginning the study. In each study, the products under investigation were tested on each subject.

Antiseptics

The following antiseptics were investigated: 5% (v/v) TTO in 0.001% (v/v) Tween 80 and 100% HSW in Study 1, and 100% AHSW in Study 2. The prevalues

Table 1 In vivo Studies 1 and 2: Latin square arrangement of the treatments and soft soap control

Testing order	1	2	3
Study 1			
Group 1	Soft soap	5% TTO in Tween 80	100% HSW
Group 2	5% TTO in Tween 80	100% HSW	Soft soap
Group 3	100% HSW	Soft soap	5% TTO in Tween 80
Study 2			
Group 1	Soft soap	100% AHSW	
Group 2	100% AHSW	Soft soap	

TTO, tea tree oil; HSW, hygienic skin wash; AHSW, alcoholic hygienic skin wash. Study 1-Group 1: volunteers 1-4; Group 2: volunteers 5-8; Group 3: volunteers 9-13. Study 2-Group 1: volunteers 1-7; Group 2: volunteers 8-14.

and postvalues were determined for each antiseptic and the SS. Volunteers in each study were separated into groups and the order of testing in both studies was determined by a Latin square design (Table 1).

Contamination fluid

The suspension used for the in vivo test was prepared as described in European standard EN 1499.²³ *E. coli* K12 was grown overnight in two tubes containing 5 mL of TSB at 37 °C, that were then inoculated into two bottles containing 1 L of TSB and incubated for a further 18-24 h at 37 °C. These cultures were then combined and a viable count was performed using the drop counting method (DCM).²⁴ The viable count as well as contamination was then monitored after every four volunteers by the DCM.

Application of the contaminating fluid

The subjects' hands were washed for 1 min with the SS to remove natural transient micro-organisms and then dried on paper towels. The contamination fluid was poured into a container, and the hands were immersed up to the mid metacarpal for 5 s, with fingers spread apart. The surplus was allowed to drain back into the container. Finally, the hands were air dried for 3 min, holding them in the horizontal position with fingers spread out and rotating the fingers to avoid the formation of droplets. The contamination batch was not used for more than 3 h after the first subject's hands had been contaminated.

Prevalues. Immediately after drying, the fingertips and thumb tips were rubbed for 1 min on the base of a Petri dish containing 10 mL of TSB without

neutralizer, with the aim of assessing the release of the test organism before treatment of the hands (one Petri dish per hand). Serial dilutions of the sampling fluid were prepared in phosphate buffered saline [8 g/L NaCl (BDH), 0.2 g/L KCl (BDH), 1.44 g/L Na₂HPO₄ (BDH) and 0.24 g/L KH₂PO₄ (BDH)], and the number of bacteria released was counted using the DCM.

Hygienic handwash procedure. Immediately after prevalue sampling and without recontamination of the hands, the group washed their hands with either the SS or the test product. Five millilitres of SS or antiseptic product were poured into cupped hands that had been premoistened with tap water, and the volunteers performed the six steps of the standard handwashing procedure. As much warm water as necessary to produce lather was added and the handwash procedure was continued for 60 s. Finally, hands were rinsed under tap water for 15 s from distal to proximal with fingertips upright. To avoid recontamination of the sampling area, the fingers had to remain pointing upwards until postvalue sampling. Wrists and lower arms were dried with paper towels by a helper.

Postvalues. Immediately after drying the wrists, the fingertips and thumb tips were rubbed on the base of a Petri dish containing 10 mL of neutralizer for 1 min (one Petri dish per hand). These sampling fluids were serially diluted in neutralizer, and viable counts were performed using the DCM. After tests with the SS and the antiseptics, the subjects were asked to decontaminate their hands using a hospital-grade soap cleanser and then rubbing with 70% ethanol. Colonies were counted after incubation of plates at 37 °C for 24 h followed by a further 24 h incubation to detect any slow-growing colonies.

Ex vivo testing to match in vivo handwashing test EN 1499²³

The ex vivo model has been described elsewhere.^{24,25} In essence, skin samples were taken with consent from patients undergoing plastic surgery, such as breast and abdominal reduction. Samples of about 2 cm² were placed onto diffusion cells containing 1 mL of SDW to keep the dermis moist.

Bacterial inoculum

The inocula used for the ex vivo method were prepared as described in European standard EN 1276.²¹ Loopfuls of cells from TSA plates were

transferred into 15 mL of diluent containing 1 g/L tryptone pancreatic digest of casein (Difco, Becton, Dickinson and Co., Sparks, MD, USA) and 8.5 g/L NaCl (BDH) at pH 7, and placed in a 100-mL flask with 5 g of glass beads. The flask was shaken for 3 min using a mechanical shaker.

Application of the contaminated fluid

A volume of 20 µL of bacterial inoculum was placed onto the stratum corneum and left to dry for approximately 3-5 min in a laminar flow cabinet at room temperature.

Prevalue

A volume of 980 µL of TSB was added on to the dried inoculum and it was resuspended by flushing the liquid in and out of the pipette tip for 1 min. Viable counts were assessed using the DCM.

Handwash technique mimicking

Following the prevalue sampling and without recontamination, 40 µL of antiseptic or non-medicated SS solution was added to the bacterial inoculum and rubbed onto the skin for 1 min using a sterile glass rod.

Postvalue

Immediately after rubbing, 940 µL of neutralizer was added to the skin sample, the surviving bacterial cells were resuspended by flushing the liquid in and out of the pipette tip for 1 min, and viable counts were performed using the DCM. Plates were counted after incubation at 37 °C for 24 h and re-incubated for a further 24 h to detect any slow-growing colonies.

Statistical analysis

Studies 1 and 2 were performed on 13 and 14 volunteers, respectively. The ex vivo experiments were repeated at least seven times, and depended upon the availability of skin samples. Student's *t*-tests and ANOVA tests were conducted (Excel[®], Microsoft Corporation, Redmond, WA, USA, Graphpad Prism[®], Graphpad Software Inc., San Diego, CA, USA) for results obtained in Study 1 and in the ex-vivo study. Statistical analysis of Study 2 was done according to European standard EN 1499 and is described further below. In all cases, *P* values of <0.01 were considered to be significant when the antiseptics were compared with the SS, as recommended by the European standard method.

Results

In vivo studies

Both the requirements for acceptance in the EN 1499 were achieved; all results from the 13 subjects in Study 1 and 14 subjects in Study 2 were evaluable, and the overall mean of the \log_{10} prevalues for control and test procedures was at least 6. In both studies, the reduction in bacterial cell numbers obtained with the antiseptic formulations and the SS was not dependent upon the hand tested ($P > 0.05$; Student's t -test). When comparing male and female volunteers, no statistically significant difference was observed between the reduction in bacterial numbers after handwashing with SS, HSW or AHSW. However, 5% TTO in Tween 80 appeared to be significantly more active in females than males (\log_{10} reductions of 4.404 ± 0.709 and 3.268 ± 0.025 , respectively). European standard EN 1499 makes no statement regarding the influence of the volunteers' sex upon the test results because each subject acts as its own control, performing the test with the non-medicated SS as well as with the antiseptic soap under similar environmental conditions.²³

Original bacterial inoculum

The original inocula contained, on average, \log_{10} counts 8.99 ± 0.32 and 8.93 ± 0.15 cfu/mL of *E. coli* cells in Studies 1 and 2, respectively.

Prevalue

In Studies 1 and 2, the number of bacterial cells recovered from hands during prevalue samplings was not significantly ($P > 0.05$; ANOVA) dependent upon the testing day, nor was it dependent upon the product (antiseptic or control) that was tested after prevalue sampling.

Comparison of prevalue and postvalue

The number of bacterial cells recovered after treatment (postvalue) with the non-medicated SS or the TTO-containing products was always significantly ($P < 0.05$; Student's t -test) lower than that in the prevalue (prior treatment). The activity of the SS, 5% TTO in Tween 80 and the AHSW was not significantly influenced by the testing day ($P > 0.05$). However, HSW, assessed in Study 1, achieved a slightly higher ($P = 0.049$) reduction in bacterial cell numbers with Group 2 than with Groups 1 and 3 (Table I).

Table II In vivo Studies 1 and 2: \log_{10} reduction in *Escherichia coli* K12 concentration (cfu/mL) after volunteers's handwashing with soft soap or antiseptic, mean(SD)

Log ₁₀ reduction in cfu/mL, mean(SD)	
Study 1	
Soft soap	2.927(0.338)
5% TTO	3.967(0.868)
100% HSW	3.187(0.501)
Study 2	
Soft soap	2.619(0.447)
100% AHSW	2.929(0.258)

TTO, tea tree oil; HSW, hygienic skin wash; AHSW, alcoholic hygienic skin wash.

Comparison of each antiseptic product with the non-medicated SS

The mean \log_{10} RF of each antiseptic was larger than that obtained with the SS (Table II). The difference in RF was therefore tested for statistical significance.

In Study 1, more than one antiseptic was tested in parallel with the SS. Hence one-way ANOVA analysis was used to compare all groups together, followed by Dunnett's post-test to compare each group with the control soap (Prism). TTO (5%) in 0.001% Tween 80 was significantly ($P < 0.01$) more active than the SS. The HSW appeared to be slightly more active than the SS, but this difference was not statistically significant ($P > 0.05$). Finally, 5% TTO in Tween 80 was significantly ($P < 0.01$, Student's t -test) more active than the HSW.

In Study 2, only one formulation was assessed in parallel with the SS, and therefore the difference in reduction in bacterial cells achieved by the AHSW compared with the SS was assessed using the Wilcoxon matched-pairs signed-rank test (Table III).²³ The smaller sum of ranks (positive or negative) was compared with tabulated values from the Wilcoxon table for 14 volunteers. A P value of 0.01 was considered to be significant. The smaller calculated sum of ranks was 10 (which is < 15), and hence the AHSW was significantly more effective than the SS.

Ex-vivo method

Original bacterial inoculum

Original inocula contained, on average, \log_{10} counts of 8.57 ± 0.29 cfu/mL of *E. coli*.

Comparison of prevalue and postvalue

The number of bacterial cells recovered after treatment (postvalue) with the control SS or the

Table III In-vivo Study 2: statistical comparison of values obtained for soft soap and 100% alcoholic hygienic skin wash (AHSW) (Wilcoxon matched-pairs signed-rank test)

Subject	Log reduction factor derived from		Difference	Rank of difference	
	Soft soap	100% AHSW		Without sign	With sign
1	2.394202	3.021678	-0.62748	11	-11
2	2.709113	3.563315	-0.8542	13	-13
3	2.410648	2.837909	-0.42726	6	-6
4	2.453193	2.901844	-0.44865	7	-7
5	3.415063	2.851779	0.563284	2	2
6	3.591754	2.724959	0.866795	1	1
7	2.165978	2.670102	-0.50412	9	-9
8	3.018867	3.235469	-0.2166	5	-5
9	2.559089	2.54519	0.013899	4	4
10	2.426082	2.890294	-0.46421	8	-8
11	2.282982	3.092535	-0.80955	12	-12
12	2.340363	2.849485	-0.50912	10	-10
13	2.108633	3.074552	-0.96592	14	-14
14	2.785049	2.751726	0.033323	3	3

Sum of ranks (+): 10; sum of ranks (-): 95.

TTO-containing products was always significantly ($P < 0.05$; Student's *t*-test) lower than the prevalence (prior to treatment).

Comparison of each antiseptic product with the SS

All antiseptic-containing products were compared with each other as a group, and against the SS. TTO (5%) in Tween 80 and the AHSW were significantly more active ($P < 0.01$) than the SS against *E. coli* (Table IV). The HSW was more active than the SS, although this difference was not significant ($P > 0.05$) (Table IV). TTO (5%) in Tween 80 was significantly more active ($P < 0.01$) than the HSW, and more active (not significant, $P > 0.05$) than the AHSW. Finally, the AHSW was more active than the HSW, although this difference was not statistically significant ($P > 0.05$) (Table IV).

Comparison of in vivo and ex vivo results

The in vivo results (data obtained in Studies 1 and 2 for the SS were aggregated) were compared with the ex

Table IV Ex vivo study: \log_{10} reduction in *Escherichia coli* K12 concentration (cfu/mL) after products' challenge, mean(SD)

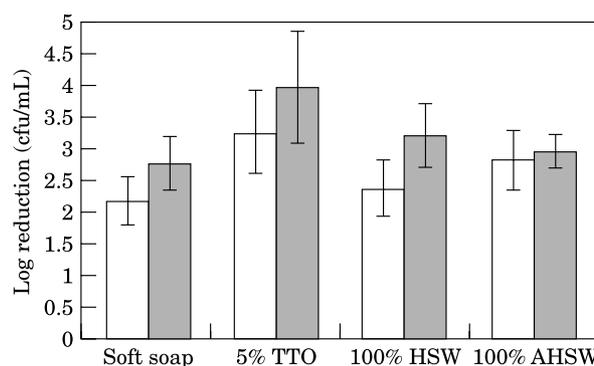
Products	\log_{10} reduction in cfu/mL, mean(SD)
Soft soap	2.126(0.426)
5% TTO	3.257(0.655)
100% HSW	2.365(0.448)
100% AHSW	2.801(0.469)

TTO, tea tree oil; HSW, hygienic skin wash; AHSW, alcoholic hygienic skin wash.

vivo results. The formulations tested, as well as the SS, were more active when assessed in vivo than ex vivo against *E. coli*, although only the SS and the HSW were significantly more active in vivo ($P < 0.05$) (Figure 1). There appeared to be a pattern in the comparison of ex vivo and in vivo results. The antiseptics tested appeared to be, on average, 1.28 ± 0.06 times more active in vivo (from 1.05 times with AHSW to 1.35 times better with HSW) than ex vivo.

Discussion

The activity of TTO and TTO-containing formulations has been assessed previously in suspension²⁰ according to European standards EN 1276²¹ and

**Figure 1** Comparison of the efficacy of the soft soap and tea tree oil (TTO) formulations depending upon the test performed (mean ± SD). Data obtained for the soft soap in vivo (Studies 1 and 2) were aggregated. HSW, hygienic skin wash; AHSW, alcoholic hygienic skin wash; open bars, ex vivo; solid bars, in vivo.

prEN 12054.²² When assessed with suspension test EN 1276, the AHSW achieved a \log_{10} reduction ≥ 5 after a 5-min contact time against *Staphylococcus aureus*, *Acinetobacter baumannii*, *E. coli* K12 and *Pseudomonas aeruginosa*, and its activity was not significantly ($P > 0.05$) influenced by interfering substances, thus fulfilling EN 1276 requirements. TTO (5%) in Tween 80 and the HSW also complied with European guidelines when assessed against *E. coli* K12, and against both *E. coli* K12 and *A. baumannii*, respectively. We also showed that 5% TTO in Tween 80 and the AHSW passed the European standard after a 1-min contact time when tested against *E. coli* K12. With the suspension test prEN 12054, the 55% AHSW and TTO in Tween 80 also passed the European recommendations within a 1-min contact time.²⁰

Our ex vivo and in vivo results showed that 5% TTO in Tween 80 was generally more active than 100% AHSW, which was itself more active than the HSW. These findings are similar to the results obtained in vitro with the European suspension tests described above.²⁰ Both studies showed that it is as important to assess the activity of an active compound as it is to assess the final formulation into which it has been incorporated, as synergy or antagonism can occur between the active component and the other components of the formulation. This was well illustrated by 5% TTO in Tween 80 being significantly more active than 5% TTO incorporated into different formulations. The presence of 10% alcohol in the AHSW was also significant, and it appeared to enhance the activity of TTO in the formulation compared with TTO alone (HSW) when assessed ex vivo and in vitro.²⁰ Other studies have also demonstrated that the combined activity of alcohol with an active compound such as Triclosan²⁵ or chlorhexidine²⁸ was greater than each agent alone.

Epidemiological research has shown that the use of synthetic detergents has increased barrier-dependent skin problems.²⁹ Antiseptics remove adhering bacterial contaminants and surface lipids from the skin. After multiple applications, the lipid-dependent efficiency is decreased and the active compounds from the antiseptics penetrate the skin more deeply.¹² Skin lipid removal by antiseptic agents is a big problem associated with hand hygiene. Lipids of plant origin cannot repair a damaged barrier but they are known to improve the feel and overall function of damaged skin.¹² Previous research has suggested that the repeated use of a TTO-containing handwash did not lead to the dermatological problems associated with other formulations.¹³ TTO is active against a wide range of micro-organisms in vitro.^{15,17,18} Moreover, it has

greater activity against transient skin-associated bacterial pathogens than commensal skin flora.¹⁴ Hence, it was anticipated that as a handwash agent, it could be useful to remove potential pathogens while preserving the naturally occurring flora.¹⁴ Our results showed that TTO has significant in vivo activity according to European recommendations, when assessed as an active compound by itself or within a formulation, and therefore could be used in healthcare settings to encourage staff compliance with handwashing.

Ex vivo results in this study were reproducible and, although the antiseptics tested were more active when tested in vivo, both testing techniques appeared to be linked by a constant conversion factor. In a previous study that compared results obtained with an adapted version of the ex vivo test with a rubbing effect with that obtained in vivo with volunteers (EN 1499), Triclosan, *para*-chloro-metaxyleneol, povidone iodine and SS generally performed 1.62 times better in vivo than ex vivo (range 1.13 times better with SS to 2.29 times better with povidone iodine).²⁶ These results are similar to ours which showed the antiseptics to be, on average, 1.28 ± 0.06 times more active in vivo than ex vivo (range 1.05 times better with the AHSW to 1.35 times better with the HSW). Nevertheless, following the European standard method, the important outcome is that the product being tested should achieve a significantly higher reduction than that achieved by the control soap. Thus, the formulation being tested should be significantly more active than the control soap, and 5% TTO in Tween 80 and the AHSW achieved this using both in vivo and ex vivo methods. Although there are no epidemiological data indicating how effective a disinfection procedure has to be in order to prevent hand-transmitted infection,^{2,8,30} even in the controlled environment of a hospital,³¹ these results imply that TTO-based disinfectants may have a role.

Other studies have looked for a substitute to human skin and/or an intermediate test between in vitro and in vivo testing, and included the use of pig skin,^{32,33} monoxenic hairless mice³⁴ and freshly excised samples of euthanized cats and dogs as a substitute to human skin.²⁴ McDonnell *et al.*³³ showed that a 60% isopropanol handwash formulation achieved comparable results when assessed on pig skin samples in vitro and in vivo with volunteers (\log_{10} reductions of 2.4 ± 0.5 and 2.0 ± 0.5 , respectively). The study by Barc *et al.*³⁴ also presented encouraging results, although correlation with in vivo data was dependent upon the formulations tested. Although these studies produced valuable results, it is difficult to rely on

animal models as their skin reacts differently and human skin bacteriology is unique. It is also very difficult to induce infections that would resemble those of humans.³⁵ Although there are major differences between human skin in vivo and ex vivo, such as moisture level, microbial ecology, surface pH and temperature, and the presence of sebum and sweat, the ex vivo model appears to be highly suitable for investigating the efficacy of antiseptic agents directly on skin.²⁴⁻²⁶ The main advantages of ex vivo testing over in vivo testing are that ex vivo tests are easier and cheaper to perform, parameters such as environment temperature and resident flora can be controlled, pathogenic organisms can be assessed, different handwashing conditions can be simulated, and ethical approval is easier to obtain. The present study supports the use of the ex vivo model to investigate the efficacy of antiseptics on skin and may help in predicting the outcomes of in vivo studies.

In conclusion, we have shown that after passing the European in vitro suspension tests,²⁰ 5% TTO in Tween 80 and in formulations also passed the European in vivo handwashing test. The same conclusions were reached when the products were tested with the ex vivo method. The use of antiseptics is critical in healthcare settings for the prevention of transmission of infections.³⁶ However, resistance to commonly used agents has been reported in the laboratory³⁷⁻³⁹ and this has led to the search for new antiseptic agents. Our findings suggest that TTO-containing handwash formulations may help reduce the skin carriage of potentially pathogenic organisms by healthcare staff and thus reduce transmission of nosocomial infections in hospital settings. Finally, we emphasize the fact that the ex vivo test is a suitable method to predict in vivo results. TTO formulations should be investigated further in randomized controlled clinical trials.

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