**Potassium loss from chlorhexidine-treated bacterial pathogens is time- and concentration-dependent and variable between species**

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**Abstract**

Chlorhexidine is a membrane-active antimicrobial agent with bactericidal activity, and is used extensively in infection prophylaxis and treatment. Whilst known to induce membrane damage that results in loss of internal solutes from bacteria, the present study sought to determine the rate and extent of cytoplasmic potassium loss, and whether any species-specific differences exist. Direct measurement of potassium was achieved using flame emission spectrophotometry. Exposure of selected test species to minimum inhibitory or minimum bactericidal concentration (MIC; MBC) resulted in solute loss that was both concentration and time-dependent. Within 5 minutes treatment with MIC levels, losses of 3% from *P. aeruginosa*, 9% from *E. coli*, and 15% from *S. aureus* were recorded. Release was greater at MBC levels, while at 5% wt/vol chlorhexidine an elevated loss of 20, 28 and 41% occurred within 5 minutes from *P. aeruginosa, E. coli* or *S. aureus*, respectively. A non-linear release pattern was evident from all three species when treated with 5% chlorhexidine over a 60 minute period. After this contact time, potassium loss from *E. coli* and *S. aureus* rose to 93 or 90%, respectively; in contrast *P. aeruginosa* retained 62% intracellular potassium. Results confirm lethal concentrations of chlorhexidine induce rapid and substantial loss of cytoplasmic potassium from common pathogenic bacteria. However, bacterial responses vary between species and this should be borne in mind when considering the mechanism of action.

**Highlights**

- Chlorhexidine induces concentration dependent bacterial cytoplasmic potassium loss

- Loss is influenced by exposure time with non-linear release rates at high conc.
- Species responses vary; Gram negative's, particularly *P. aeruginosa*, less sensitive
- Loss of membrane integrity does not appear to occur at MBC levels

**Keywords**

Chlorhexidine, antimicrobial, potassium loss, membrane damage

## Introduction

Chlorhexidine is a widely used medical antiseptic possessing broad-spectrum antimicrobial action [1]. The cationic nature of this biguanide is believed to cause an interaction with negatively charged groups on bacterial surfaces, as evidenced by increased surface hydrophobicity following on from electrostatic attraction and attachment of chlorhexidine [2]. Displaying an affinity for surface attachment that is believed to facilitate activity, its consequent usage includes topical applications for skin disinfection [3, 4], wound and burn antisepsis, and as the most common mouthwash for periodontal disease treatment or prophylaxis [5]; hence the agent is extensively employed within healthcare settings. Whilst certain Gram negative clinical isolates display innate resistance to chlorhexidine [6], and potential for resistance development has been shown experimentally [7], this agent is none the less a very effective bactericide in almost all settings. Indeed, with such effective topical activity this biguanide has recently been used in attempts to develop antimicrobial wafers to treat infected wounds; these wafers have proven to be very effective during initial *in vitro* investigations [8, 9].

When *Providencia stuartii* was exposed to this membrane-active agent the treatment resulted in loss of intracellular solutes including potassium [10]. Both scanning and transmission electron microscopy demonstrated that *Pseudomonas stutzeri* exposed to chlorhexidine displayed visible damage to the cell envelope structure, including the cytoplasmic membrane, resulting in gross morphological and structural alterations [11]. In regard to potassium loss as the marker of initial membrane damage [12] the extent, rate and whether species variability exists remains unknown. The present study sought to close this knowledge gap examining the impact of growth inhibitory and lethal concentrations of chlorhexidine upon populations of the commonly encountered pathogenic bacteria *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

## 2. Materials and Methods

### 2.1 Bacterial strains, growth media and reagents

Bacterial strains *E. coli* NCTC 4174, *S. aureus* NCTC 6571, and *P. aeruginosa* NCTC 6750 were obtained from the National Collection of Type Cultures (NCTC, Health Protection Agency Culture Collections, Porton Down, Salisbury, UK.). These strains were stored in Protect Bacterial Preservers (Technical Service Consultants Ltd., Lancashire, UK.) at –80$^{o}$C. The stock cultures maintained on nutrient agar plates were stored at 4$^{o}$C and sub-cultured weekly. A single colony from the stock plate was used to prepare nutrient broth cultures daily, with flasks incubated at 37$^{o}$C for 24 hours in an orbital incubator set at 100 rpm. Nutrient broth and nutrient agar were supplied by Oxoid Ltd. (Basingstoke, UK.) and all media autoclaved at 121$^{o}$C for 15 minutes. Chlorhexidine digluconate (20% wt/vol) and potassium chloride were obtained from Fisher Scientific UK Ltd.

### 2.2 Determination of antimicrobial sensitivity

Minimum inhibitory concentrations (MIC) were determined using a 2-fold macro broth dilution assay with 10 ml volumes of nutrient broth. This dilution series was inoculated so as to contain 1 x $10^6$ cfu/ml of test organism and the presence or absence of growth was recorded over 24 hours aerobic incubation at 37$^o$C by measuring optical density at 650 nm in a spectrophotometer (Thermo Spectronic Helios *E*). Minimum bactericidal concentrations (MBC) were established by a replica plating procedure with five 20 μl volumes from each culture transferred to the surface of a 135 mm nutrient agar plate, with presence or absence of growth recorded after 24 hours aerobic incubation at 37$^o$C. Bacterial viability was measured by using a standard drop plate counting procedure with 5 x 20μl volumes of a 10-fold dilution series from each sample plated onto the surface of a 95 mm nutrient agar plate. After 24 hours aerobic incubation at 37$^o$C suitable dilutions were counted and viability calculated. All assays were conducted in triplicate on three separate occasions.

### 2.3 Assessment of loss of intracellular potassium

Assessment of potassium released by bacterial cells was undertaken using a flame atomic absorption spectrophotometer (Model AA3110, Perkin Elmer Las UK, Beaconsfield, UK.) with the method used described elsewhere [13]. Briefly, the instrument was set and calibrated with suitable potassium standards; washed bacterial suspensions of ~1 x $10^6$ cfu/ml (range 5 x $10^5$ – 5 x $10^6$ cfu/ml) in ultrapure deionised water in polymethylpentene flasks were exposed to either MIC, MBC, or 5% wt/vol chlorhexidine digluconate. Loss of cytoplasmic potassium was measured after selected exposure periods up to 24 hours. All assays were conducted in triplicate on three separate occasions.

## 3. Results

### 3.1 Evaluation of antibacterial activity of chlorhexidine

Initially the MICs and MBCs for chlorhexidine against the test bacterial strains were determined (Table 1a) and from the results it is apparent that the Gram positive *S. aureus* is considerably more susceptible than the Gram negative organisms. Further testing confirmed that viability was lost at MBC levels as no living cells were detectable from an initial population of $6 \times 10^6$ cfu/ml at 24 hours (Table 1b). However, the response was time-dependent as 60 minutes with MBC was insufficient to cause complete loss of viability of the Gram negative organisms; no living cells were present after 60 minutes exposure to 5% wt/vol chlorhexidine (Table 1b).

### 3.2 Examination of cytoplasmic potassium loss from bacterial populations exposed to chlorhexidine

Experimental results gathered confirm treatment with chlorhexidine causes cytoplasmic potassium release from all exposed bacterial populations (Figures 1-3). Upon scrutiny it is apparent that time and concentration parameters have an impact upon both the rate and extent of potassium expulsion (Table 2). Individual species responses were also noted, with *S. aureus* displaying the greatest sensitivity (Figure 2; Table 2). This Gram positive organism lost 0.42 mg/L (15%) intracellular potassium within 5 minutes exposure to MIC (0.2 μg/ml) in comparison to *P. aeruginosa* which had lost 0.11 mg/L (3%) upon exposure to MIC (4.0 μg/ml), with *E. coli* voiding 0.29 mg/L (9%) under such conditions. Over 24 hours contact with MIC levels, the quantity of potassium expelled after the loss observed during the initial 5 minute exposure did not increase to an appreciable degree in any of the test species.

After 5 minutes treatment with 5% wt/vol chlorhexidine, substantial losses were recorded as 28% for *E. coli*, 41% for *S. aureus*, and 20% for *P. aeruginosa*. Furthermore, at this high concentration an interesting feature was noted in regard to the rate of potassium loss. The release rate of potassium with respect to time was found to vary, the highest rate recorded during the initial period of treatment (Table 2). Regardless of quantity lost, a substantially reduced rate was noted between 5 to 30 minutes of treatment for all three of the test species, and thereafter rates were observed to increase (Table 2). This pattern was displayed by all three species suggesting a common element of response to the biguanide.

## 4. Discussion

Collectively the chlorhexidine sensitivities of the bacterial species examined are in agreement with previous findings [10, 11, 14] confirming the biguanide is an effective bactericide at relatively low concentration with a spectrum of activity covering both Gram positive and negative organisms. Whilst it has been reported previously [6] that some Gram negative species display levels of innate resistance, or perhaps more correctly described as reduced susceptibility because they succumb at higher biguanide concentrations, this seems to be a restricted strain-specific phenomenon.

The main purpose for the current study was to examine the impact of chlorhexidine upon the cytoplasmic potassium pool in populations of common pathogenic bacteria. Chlorhexidine is an antibacterial agent known to target the cytoplasmic membrane leading to structural disruption and loss of intracellular contents [10, 14]. Potassium flux is a primary marker for cytoplasmic membrane damage [12] and, given that biguanides are membrane active

compounds, direct detection for loss of this solute was assessed. Data obtained confirm that both sub-lethal and lethal concentrations of chlorhexidine cause potassium depletion in treated bacterial populations (Figures 1-3). The rate and extent of this loss is both time and concentration-dependent (Table 2). Previous research demonstrated that the Enterobacteriacea *Providencia stuartii* voids potassium in a concentration-dependent manner when treated with this agent [11]; however, data presented here confirm that time of exposure substantially influences response (Figures 1-3). Using electron microscopy Tattawasart and colleagues [10] demonstrated that *P. stutzeri* loses structural integrity within 5 minutes treatment with 100 mg/L chlorhexidine. Our study using 5 times this concentration induced between 20-41% losses of potassium in the three test species over this time period, confirming the rapid onset of action. However, after 60 minutes exposure a notable variation between the three organisms was evident: while both *E. coli* and *S. aureus* released 93 and 90% of total potassium, respectively (Figure 1 and 2), loss from *P. aeruginosa* had only risen to 38% (Figure 3). This suggests there were intact but non-viable or non-culturable (Table 1b) *P. aeruginosa* cells retaining the solute.

Recent investigation has shown *Bacillus subtilis* and *E. coli* both lose a substantial proportion of internal phosphorus after 3 hours exposure to 0.75 mg/L chlorhexidine [14]. When proportioned as phospholipid or nucleotide/nucleic acid, these results confirmed the Gram positive organism lost more intracellular constituents, providing evidence the lipopolysaccharide (LPS) layer acts in some way to reduce chlorhexidine activity. Using a similar range of concentrations with *E. coli* (0.6 - 14 μg/ml) and *S. aureus* (0.2 - 0.4 μg/ml) the responses observed during this investigation support the view that the presence of a LPS layer impedes chlorhexidine action, perhaps by sequestering the agent

into the LPS structure. Lacking the LPS layer, the elevated extent of loss in Gram positive species over the initial exposure period probably reflects easier access of chlorhexidine to the cytoplasmic membrane, and supports the view that such bacteria are more susceptible to the biguanide [14]. The additional observations with Gram negative species, that *P. aeruginosa* lost much less potassium than *E. coli* when examined under equivalent conditions, provides clear evidence that species-specific responses occur and probably result from differences in composition and structure of the cell envelope.

When taken together these varied studies provide unequivocal evidence the bacterial cell envelope is damaged by the biguanide. Bearing this in mind, one aspect requiring consideration relates to the amount of potassium voided from a fixed population and whether there is a correlation with loss of viability. The responses from populations treated with MIC and MBC levels of chlorhexidine are clearly different. Whilst having released potassium, cells in the populations exposed to MIC levels have been able to retain viability (Table 1b). This observation suggests that cells are able to release some potassium without losing viability, perhaps through later re-accumulation. Alternatively, differential potassium loss by cells might be occurring with a sub-population retaining higher levels of the cation and in doing so permit such cells to maintain viability.

When considering routes for cytoplasmic potassium release from the bacterial cell, the possibilities are mostly limited to solute specific regulated efflux systems [15] or non-specific, unregulated exit via diffusion across a damaged membrane. Whilst the former may contribute, the rate and extent of loss would seem to suggest that physical disruption of the membrane bilayer is the primary means for solute escape. The recent work by Cheung and colleagues [14] along with a previous study by Russell's research group [11] provide clear visual

evidence that physical breaches to the cell envelope occur when bacterial cells are exposed to chlorhexidine. Data presented here show both rapid and near total loss of the major inorganic intracellular solute occurs from both the *S. aureus* and *E. coli* populations exposed to 5% chlorhexidine. With no viable cells detected after 60 minutes treatment (Table 1 b) this supports the view that total cell destruction has taken place. However, as substantial potassium is retained within bacterial cells (viable or otherwise) treated with either MIC or MBC, our results also suggest that the cytoplasmic membrane does not lose structural integrity at these levels of chlorhexidine even after 24 hours.

Early investigations into the primary means through which chlorhexidine induced antibacterial activity suggested inhibition of ATPases [1, 16], but this has since been disproven [17]. Activity is now agreed to result from general cell envelope damage [18] which in turn leads to loss of the proton motive force required to energise ATPase function [17]. Hence, the earlier studies were only incorrect in identifying loss of ATPase activity as the initial antibacterial event instead of a secondary response occurring during a cascade of unfolding actions. The results reported here shed more light on this cascade of events that follow bacterial treatment with chlorhexidine. The cationic nature of the antibacterial compound probably leads to direct interaction with the negatively charged surface layer of bacterial cells that in turn causes concentration and time-dependent damage to the cell envelope [11, 14]. Such damage results in the release of cytoplasmic solutes including potassium that we observed in this study. At MBC levels the lethal event is probably loss of membrane potential, or similar effect, rather than resulting from physical damage; at very high chlorhexidine concentrations the primary response would seem to result from destruction of the bacterial cells causing massive potassium loss. In summary, it has been shown here that

chlorhexidine induces concentration and time dependent potassium loss from common pathogenic bacteria. This loss is rapid and substantial though, importantly, species-specific responses are evident.

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## References

[1] A.D. Russell, Chlorhexidine: antibacterial action and bacterial resistance, Infection 14 (1986)212-215.

[2] B.M.A. El-Falaha, D.T. Rogers, A.D. Russell, J.R. Furr, Effect of some antibacterial agents on the hydrophobicity of wild-type and envelope mutants of *Escherichia coli*, Curr. Microbiol. 12 (1985) 187-190.

[3] J.L. Faoagali, N. George, J. Fong, J. Davy, M. Dowser, Comparison of the antibacterial efficacy of 4% chlorhexidine gluconate and 1% triclosan hand wash products in an acute clinical ward, Am. J. Infect. Control 27 (1999) 320-326.

[4] L.P. Derde, M.J. Dautzenberg, M.J. Bonten Chlorhexidine body washing to control antimicrobial-resistant bacteria in intensive care units: a systematic review, Intensive Care Med. 38 (2012) 931-939.

[5] K. Lorenz, G. Bruhn, C. Heumann, L. Netuschil, M. Brecx, T. Hoffmann, Effect of two new chlorhexidine mouth rinses on the development of dental plaque, gingivitis, and discolouration. A randomized, investigator-blind, placebo-controlled, 3-week experimental gingivitis study, J. Clin. Periodontol. 33 (2006) 561-567.

[6] S.A. Hammond, J.R. Morgan, A.D. Russell, Comparative susceptibility of hospital isolates of Gram-negative bacteria to antiseptics and disinfectants, J. Hosp. Infect. 9 (1987) 255-264.

[7] U. Tattawasart, J.-Y. Maillard, J.R. Furr, A.D. Russell, Development of resistance to chlorhexidine diacetate and cetylpyridium chloride in *Pseudomonas stutzeri* and changes in antibiotic susceptibility, J. Hosp. Infect. 42 (1999) 219-229.

[8] O. Labovitiadi, A.J. Lamb, K.H. Matthews, *In vitro* efficacy of antimicrobial wafers against methicillin-resistant *Staphylococcus aureus*, Ther. Deliv. 3 (2012) 343-355.

[9] O. Labovitiadi, A.J. Lamb, K.H. Matthews, Lyophilised wafers as vehicles for the topical release of chlorhexidine digluconate – Release kinetics and efficacy against *Pseudomonas aeruginosa*, Int. J. Pharm. 439 (2012) 157-164.

[10] T. El-Moug, J.R. Furr, A.D. Russell, Effect of chlorhexidine on a chlorhexidine-sensitive and a chlorhexidine-resistant strain of *Providencia stuartii*, Lett. App. Microbiol. 1 (1985) 37-40.

[11] U. Tattawasart, A.C. Hann, J.-Y. Maillard, J.R. Furr, A.D. Russell Cytological changes in chlorhexidine-resistant isolates of *Pseudomonas stutzeri*, J. Antimicrob. Chemother. 45 (2000) 145-152.

[12] P.A. Lambert, S.M.Hammond Potassium fluxes, first indications of membrane damage in micro-organisms, Biochem. Biophys. Res. Comm. 54 (1973) 796-799.

[13] N.H. O'Driscoll, O. Labovitiadi, T.P.T. Cushnie, K.H. Matthews, D.K. Mercer, A.J. Lamb, Production and evaluation of an antimicrobial peptide-containing wafer formulation for topical application, Curr. Microbiol. 66 (2013) 271-278.

[14] H.-Y. Cheung, M.M.-K. Wong, S.H. Cheung, L.Y. Liang, Y.-W. Lam, S.-K. Chiu, Differential actions of chlorhexidine on the cell wall of *Bacillus subtilis* and *Escherichia coli*, PLoS One 7 (2012) e36659.

[15] W. Epstein, The roles and regulation of potassium in bacteria, Prog. Nuc. Acid Res. Mol. Biol. 75 (2003) 293-320.

[16] E.F. Gale, E. Cundliffe, P.E. Reynolds, M.H. Richmond, M.J. Waring, The molecular basis of antibiotic action, (1981) Wiley, London.

[17] T. Kuyyakanond, L.B.Quesnel, The mechanism of action of chlorhexidine, *FEMS Microbiol. Lett.* 100 (1992) 211-216.

[18] K.Barrett-Bee, L. Newboult, S. Edwards, The membrane destabilising action of the antibacterial agent chlorhexidine, FEMS Microbiol. Lett. 119 (1994) 249-254.

## Figure and Table Legends

Table 1 a) Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) b) Loss of viability of bacterial populations treated with MIC, MBC and 5%wt/vol chlorhexidine.

Table 2 Rate of loss of cytoplasmic potassium where $m_1$ is the slope of the gradient between 0 and 5 minutes exposure, $m_2$ the slope between 5 and 30 minutes, and $m_3$ the slope between 30 and 60 minutes.

Figure 1 Assessment of potassium leakage from ~$1x10^6$ cfu/ml *E. coli* treated with MIC (0.6 μg/ml), MBC (14 μg/ml), or 5% wt/vol chlorhexidine. Total potassium was measured using a sonicated suspension yielding 3.2 mg/L. Assays were performed in triplicate on three separate occasions.

Figure 2 Assessment of potassium leakage from ~$1x10^6$ cfu/ml *S. aureus* treated with MIC (0.2 μg/ml), MBC (0.4 μg/ml), or 5% wt/vol chlorhexidine. Total potassium was measured using a sonicated suspension yielding 2.8 mg/L. Assays were performed in triplicate on three separate occasions.

Figure 3 Assessment of potassium leakage from ~$1x10^6$ cfu/ml *P. aeruginosa* treated with MIC (4.0 μg/ml), MBC (20 μg/ml), or 5% wt/vol chlorhexidine. Total potassium was measured using a sonicated suspension yielding 3.2 mg/L. Assays were performed in triplicate on three separate occasions.

Table 1

a)

| Organism | MIC (μg/ml) | MBC (μg/ml) |
|---|---|---|
| *S. aureus* | 0.2 | 0.4 |
| *E. coli* | 0.6 | 14 |
| *P. aeruginosa* | 4.0 | 20 |

b)

| Chlorhexidine concentration | Exposure time (minutes) | Bacterial Viability (cfu/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | *S. aureus* | | *E. coli* | | *P. aeruginosa* | |
| | | Control | Treated | Control | Treated | Control | Treated |
| MIC | 60 | $6.21x10^6$ | $5.47x10^5$ | $7.43x10^5$ | $6.2x10^5$ | $4.33x10^5$ | $4.07x10^5$ |
| | 1440 | $6.08x10^6$ | $2.13x10^2$ | $6.03x10^4$ | $6.0x10^3$ | $4.9x10^5$ | $1.12x10^5$ |
| MBC | 60 | $6.23x10^6$ | ‹ 10 | $7.33x10^5$ | $7.33x10^3$ | $3.93x10^5$ | $4.67x10^2$ |
| | 1440 | $6.22x10^6$ | ‹ 10 | $6.23x10^4$ | ‹ 10 | $4.21x10^5$ | ‹ 10 |
| 5%w/v | 60 | $1.65x10^6$ | ‹ 10 | $4.3x10^5$ | ‹ 10 | $2.37x10^5$ | ‹ 10 |

Table 2

| Organism | Rate of Loss of Cytoplasmic Potassium ($mg/L\ min^{-1}$) | | | | |
|---|---|---|---|---|---|
| | MIC | MBC | 5% wt/vol CHD | | |
| | $m_1$ | $m_1$ | $m_1$ | $m_2$ | $m_3$ |
| *S. aureus* | 0.07 | 0.13 | 0.22 | 0.009 | 0.03 |
| *E. coli* | 0.05 | 0.08 | 0.17 | 0.021 | 0.05 |
| *P. aeruginosa* | 0.01 | 0.02 | 0.12 | 0.003 | 0.016 |

Figure 1

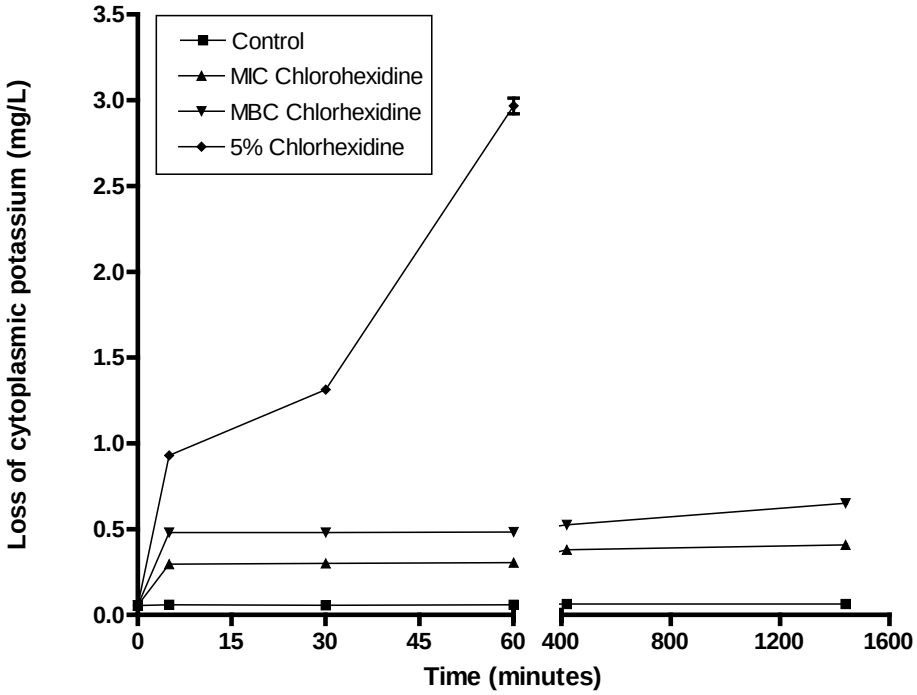
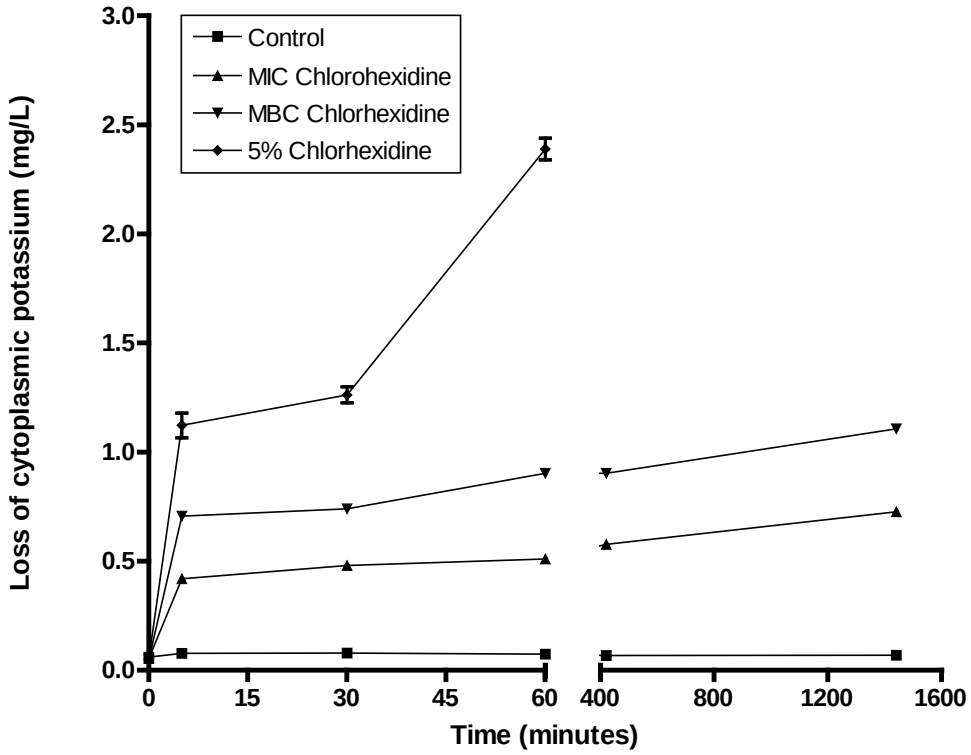

Control
MIC Chlorohexidine
MBC Chlorhexidine
5% Chlorhexidine
Loss of cytoplasmic potassium (mg/L)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
15
30
45
60
400
800
1200
1600
Time (minutes)

Figure 2


Control
MIC Chlorohexidine
MBC Chlorhexidine
5% Chlorhexidine
Loss of cytoplasmic potassium (mg/L)
3.0
2.5
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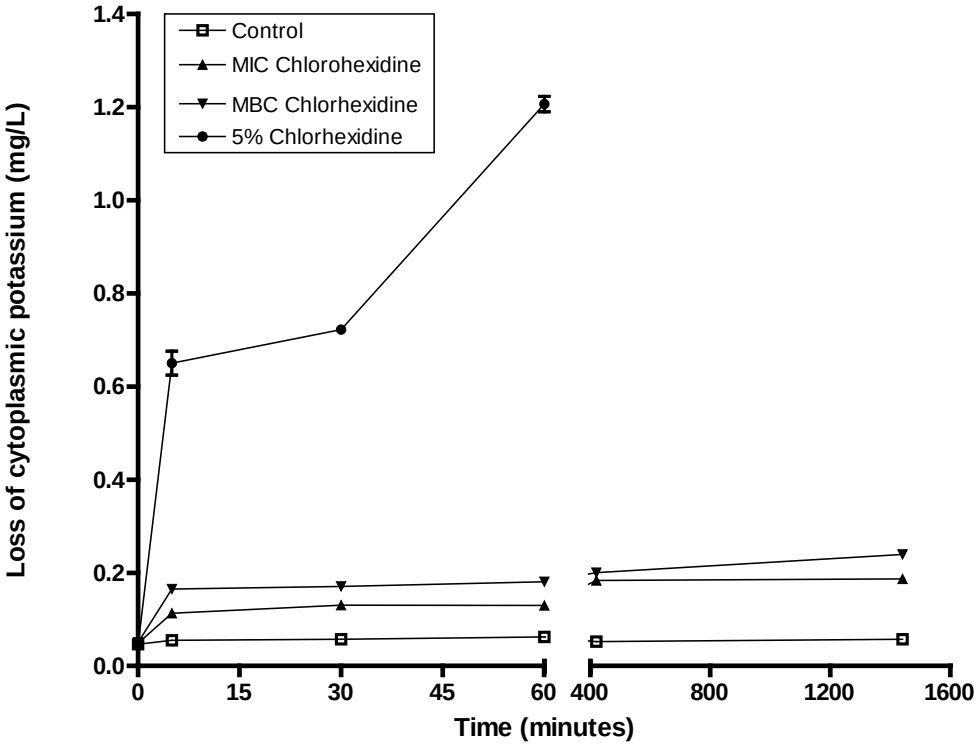
15
30
45
60
400
800
1200
1600
Time (minutes)

Figure 3


Control
MIC Chlorohexidine
MBC Chlorhexidine
5% Chlorhexidine
Loss of cytoplasmic potassium (mg/L)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
15
30
45
60
400
800
1200
1600
Time (minutes)