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ORIGINAL ARTICLE

Evaluation of chlorhexidine substantivity on salivary flora by epifluorescence microscopy

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OBJECTIVE: To evaluate the *in vivo* antimicrobial activity of chlorhexidine (CHX) in saliva 7 h after its application using an epifluorescence microscopy technique.

SUBJECTS AND METHODS: Fifteen volunteers performed a single mouthrinse with sterile water (SM-water) and with 0.2% CHX (SM-0.2% CHX). Saliva samples were taken at 30 s and 1, 3, 5 and 7 h after each application. The bacterial suspension was mixed with the SYTO 9/propidium iodide staining and observed using an Olympus BX51 microscope. The mean percentage of viable bacteria was calculated for each sample.

RESULTS: In comparison with baseline values, the frequency of viable bacteria decreased significantly at 30 s after the SM-0.2% CHX ($P < 0.001$) and presented significant antibacterial activity up to 7 h after the mouthrinse ($P < 0.001$). In comparison with SM-water, the prevalence of viable bacteria was significantly lower at 30 s after the SM-0.2% CHX ($P < 0.001$) and showed a significant antibacterial effect up to 7 h after the mouthrinse ($P < 0.001$).

CONCLUSIONS: Epifluorescence microscopy permits evaluating the antimicrobial activity of CHX on the salivary flora in real-time. Fluorescence assays could be particularly useful to analyse simultaneously the effect of antimicrobials that alter the cytoplasmic membrane integrity on different oral ecosystems.

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Introduction

The study of *in vivo* antibacterial activity of an antiseptic involves the analysis of its immediate effect and of its substantivity. It has been shown that chlorhexidine (CHX) has a greater *in vivo* immediate antibacterial effect and substantivity than other antiseptics used in the oral cavity (Moran *et al*, 1992; Jenkins *et al*, 1994; Elworthy *et al*, 1996; Balbuena *et al*, 1998).

Since the first results were reported by Schiott *et al* (1970), numerous studies evaluating the substantivity of CHX on the salivary flora have been published (Moran *et al*, 1992; Jenkins *et al*, 1994; Elworthy *et al*, 1996; Balbuena *et al*, 1998). The determination of salivary bacterial counts is a test accepted by the scientific community to investigate the *in vivo* antibacterial effect of CHX (Addy and Moran, 1997; Sekino *et al*, 2003), and is considered to be predictive of its substantivity (Roberts and Addy, 1981; Addy *et al*, 1989; Jenkins *et al*, 1994).

In a majority of published series, the quantification of the antimicrobial activity of CHX in saliva was performed using plate culture microbiological techniques (Moran *et al*, 1992; Jenkins *et al*, 1994; Elworthy *et al*, 1996; Balbuena *et al*, 1998). However, some authors have questioned the reliability of these techniques and, as an alternative, have proposed the application of fluorescent assays that use specific fluorochromes to mark viable and non-viable bacteria (Weiger *et al*, 1998). The LIVE/DEAD® BacLight™ fluorescence solution (Molecular Probes, Leiden, The Netherlands) contains two nucleic acid dyes, SYTO-9 and propidium iodide (PI). The simultaneous application of these two fluorochromes enables bacteria with intact membranes (emitting green fluorescence) to be differentiated from bacteria with damaged membranes (emitting red fluorescence). The SYTO-9/PI dual staining could be used by means of epifluorescence microscopy (Weiger *et al*, 1998; Boulos *et al*, 1999), confocal laser scanning microscopy (Hope and Wilson, 2004; van der Mei *et al*, 2006; Filoche *et al*, 2007), flow cytometry (Lehtinen *et al*, 2004; Berney *et al*, 2007), fluorescence spectroscopy using a fluorescence microplate reader or laser-induced fluorescence capillary electrophoresis (Hoerr *et al*, 2007).

The result is that this fluorescence solution detects viable and non-viable bacteria based on the integrity of their cytoplasmic membrane, and it has therefore been

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considered particularly useful in the analysis of the antimicrobial activity of CHX (Hope and Wilson, 2004; Filoche *et al*, 2007). To date, the SYTO-9/PI dual staining has only been used in the study of the antibacterial action of CHX on the oral biofilm (Hope and Wilson, 2004; van der Mei *et al*, 2006; Filoche *et al*, 2007); we have found no references to its use in other oral ecosystems, such as the salivary flora.

The objective of this study was to evaluate the *in vivo* antimicrobial activity of a CHX digluconate mouthrinse on the salivary flora up to 7 h after its application, using an epifluorescence microscopy technique with the LIVE/DEAD® BacLight™ solution.

Material and methods

Selection of the study group

The study group comprised 15 adult volunteers between 20 and 45 years of age, with a good oral health status: minimum of 24 evaluable permanent teeth with no evidence of gingivitis or periodontitis (Community Periodontal Index score = 0) (World Health Organization, 1997) and an absence of caries. The exclusion criteria were: smoking, any type of dental prosthesis or orthodontic device, antibiotic treatment or the routine use of oral antiseptics during the previous 3 months and the presence of any systemic disease that could lead to an alteration in the production and/or composition of the saliva.

Non-stimulated saliva samples (1 ml) were collected from each participant under basal conditions and at 30 s and 1, 3, 5 and 7 h after performing the following mouthrinse under supervision:

1 A single, 30-s mouthrinse with 10 ml of sterile water (negative control) (SM-water).
2 A single, 30-s mouthrinse with 10 ml of 0.2% CHX (Oraldine Perio®; Johnson and Johnson, Madrid, Spain) (SM-0.2% CHX).

The volunteers were not allowed to practise any oral hygiene technique from previous midnight. In the experiment day, the time of sample collection ranged from 11:50 AM (baseline sample) to 7 PM (last sample collected 7 h after ending mouthrinse) for each participant. The volunteers were not allowed to smoke, eat or drink anything for 1 h prior to the collection and during the course of the experiment. The non-stimulated saliva samples were collected using the spitting method (Navazesh and Christensen, 1982). Using a system of balanced randomization, all volunteers performed the two mouthrinses with a washout period of 2 weeks between each test. The project was approved by the Ethics Committee of the Faculty of Medicine and Dentistry of Santiago de Compostela University. Written informed consent was obtained from all participants in the study.

Processing of the saliva samples

The LIVE/DEAD® BacLight™ fluorescence solution was prepared following the manufacturer's recommendations in 5 ml of sterile filtered water using a 0.22 μm Millipore membrane filter (Millipore Ibérica S.A., Madrid, Spain), with a 1:1 ratio of both fluorochromes and was stored at −20°C. The saliva samples were centrifuged at 358 **g** for 6 min. The supernatant was discarded and the pellet obtained was resuspended in 100 μl of sterile water. After homogenizing the bacterial suspension by shaking, it was mixed with 100 μl of the fluorescence solution and was stored in darkness at room temperature for 15 min. Observation was performed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) fitted with an Olympus DP70 camera and a set of filters for fluorescein and Texas Red. The count of viable and non-viable bacteria was performed at high magnification (×100 objective) on 20 microscope fields that presented a minimum of 100 bacteria on epithelial cells, excluding bacterial aggregates (Figure 1). The mean percentage of viable bacteria was calculated for each saliva sample and the difference in the percentage of viable bacteria between two saliva samples was called the 'viability reduction' (VR).

Simultaneously, to compare the results obtained with epifluorescence microscopy *vs* plate culture, non-stimulated saliva samples (2 ml) were collected from five

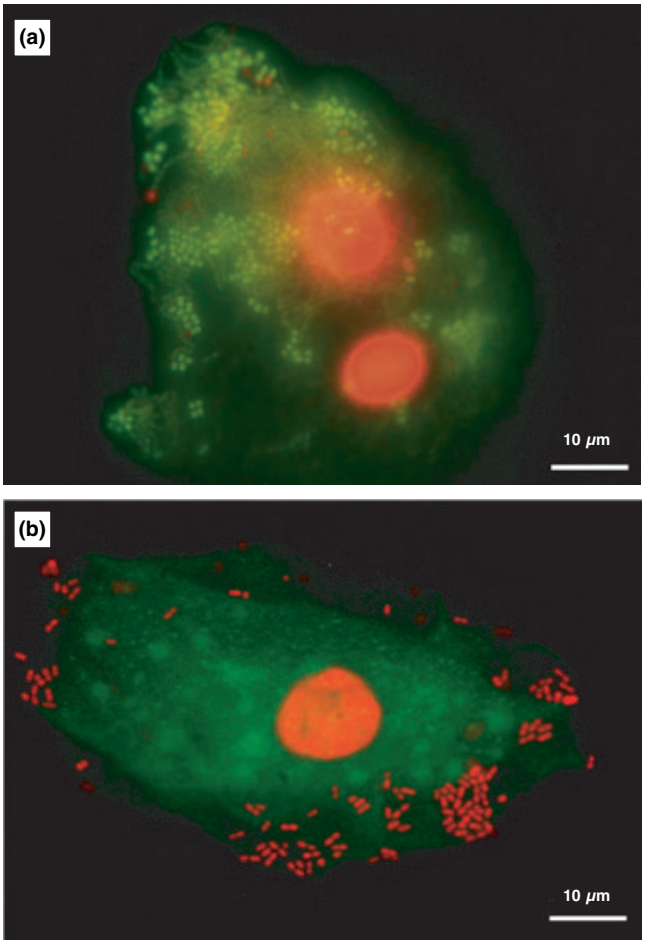

Figure 1 (**a**, **b**) Salivary bacteria with intact membranes (emitting green fluorescence) and with damaged membranes (emitting red fluorescence) on epithelial cells (×100 objective)

participants. Samples were collected under basal conditions and at 30 s and 1, 3, 5, and 7 h after performing the SM-0.2% CHX. Serial dilutions were performed and the resulting samples were cultured and subcultured on conventional culture media for aerobes/facultative anaerobes and obligate anaerobes as previously described (Tomás *et al*, 2008). The number of colony-forming units (CFU ml^{-1}) was determined and the results expressed on a decimal log scale (log_{10} CFU ml^{-1}).

Statistical analysis

The results were analysed using the SPSS version 15.0 statistical package for Windows (SPSS Inc., Chicago, IL, USA). The choice of a parametric or non-parametric test depended on whether or not the values of the quantitative variable analysed presented a normal distribution; this was determined using the Kolmogorov–Smirnov test. All the variables analysed presented a normal distribution. The repeated-measures ANOVA test and simple comparisons were used for intra-mouthrinse and inter-mouthrinse comparisons between two saliva samples. Statistical significance was taken as a *P*-value less than 0.05.

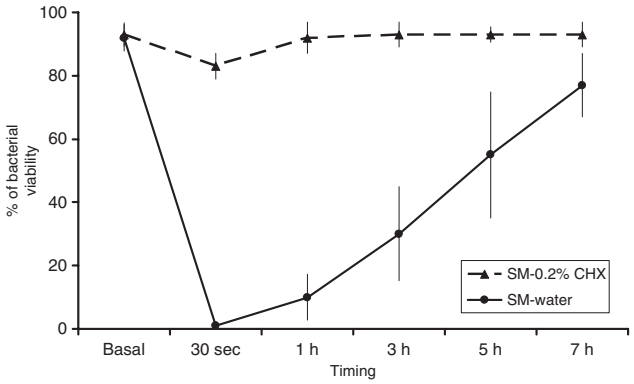

Figure 2 Percentages of bacterial viability in saliva under basal conditions and at 30 s and 1, 3, 5 and 7 h after the application of a single mouthrinse of sterile water and 0.2% chlorhexidine

Results

Figure 2 shows the mean percentages of bacterial viability in saliva under basal conditions and at 30 s and 1, 3, 5 and 7 h after the mouthrinses with SM-water and SM-0.2% CHX.

In comparison with the baseline values, the frequency of viable bacteria decreased significantly at 30 s after the SM-water (VR = 10.13 ± 0.51, $P < 0.001$) and SM-0.2% CHX (VR = 91.35 ± 4.37, $P < 0.001$). In comparison with the baseline values, 0.2% CHX presented significant antibacterial activity up to 7 h after the mouthrinse (VR = 14.14 ± 11.56, $P < 0.001$). In comparison with the values obtained 30 s after the SM-0.2% CHX, a significant recovery of the bacterial population was observed in the later saliva samples taken after its application, with the VR ranging from −9.53 ± 6.24 at 1 h ($P < 0.001$) to −76.06 ± 11.72 at 7 h ($P < 0.001$) (Table 1).

Under basal conditions, the frequency of viable bacteria was similar in the premouthrinse sample of SM-water and SM-0.2% CHX. In comparison with SM-water, the prevalence of viable bacteria was significantly lower at 30 s after the mouthrinse with 0.2% CHX (VR = 81.93 ± 2.46, $P < 0.001$). In comparison with SM-water, 0.12% CHX showed a significant antibacterial effect up to 7 h after the mouthrinse (VR = 16.26 ± 11.72, $P < 0.001$) (Table 1).

Applying plate culture techniques, the salivary bacterial concentration at baseline was 8.146 ± 0.300 log_{10} CFU ml^{-1}. After the mouthrinse with SM-0.2% CHX, the salivary bacterial concentrations detected were: at 30 s 5.481 ± 0.652 log_{10} CFU ml^{-1}, at 1 h

Table 1 Intra-mouthrinse and inter-mouthrinse comparisons of the percentages of viable bacteria in saliva under basal conditions and in the samples collected at 30 s and 1, 3, 5 and 7 h after the application of a single mouthrinse of sterile water and 0.2% CHX

Intra-mouthrinse analysis (SM-water and SM-0.2% CHX); mean difference ± standard deviation (%)

		BASAL vs *1 h*	*BASAL* vs *3 h*	*BASAL* vs *5 h*	*BASAL* vs *7 h*
	BASAL vs *30 s*	*30 s* vs *1 h*	*30 s* vs *3 h*	*30 s* vs *5 h*	*30 s* vs *7 h*
SM-water	10.13 ± 0.51**	0.60 ± 1.29	0.26 ± 1.48	−0.40 ± 1.68	−0.26 ± 0.79
		−9.53 ± 1.18**	−9.86 ± 1.45**	−10.53 ± 1.76**	−10.40 ± 0.91**
SM-0.2% CHX	91.35 ± 4.37**	81.92 ± 8.66**	61.35 ± 15.09**	35.50 ± 18.77**	14.14 ± 11.56**
		−9.53 ± 6.24**	−28.93 ± 14.10**	−54.33 ± 19.21**	−76.06 ± 11.72**

Inter-mouthrinse analysis (SM-water vs SM-0.2% CHX); mean difference ± standard deviation (%)

Water vs *CHX BASAL*	*Water* vs *CHX 30 s*	*Water* vs *CHX 1 h*	*Water* vs *CHX 3 h*	*Water* vs *CHX 5 h*	*Water* vs *CHX 7 h*
0.85 ± 5.49	81.93 ± 2.46**	81.93 ± 7.95**	62.86 ± 15.32**	38.13 ± 19.80**	16.26 ± 11.72**

BASAL, saliva sample collected under basal conditions; 30 s, saliva sample collected at 30 s after the application of the different mouthrinses; 1 h, saliva sample collected 1 h after the application of the different mouthrinses; 3 h, saliva sample collected 3 h after the application of the different mouthrinses; 5 h, saliva sample collected 5 h after the application of the different mouthrinses; 7 h, saliva sample collected 7 h after the application of the different mouthrinses; SM, single mouthrinse; CHX, chlorhexidine.
*$P < 0.05$; **$P < 0.001$.

5.508 ± 0.432 $\log_{10}$ CFU ml^{-1}, at 3 h 5.779 ± 0.558 $\log_{10}$ CFU ml^{-1}, at 5 h 7.001 ± 0.310 $\log_{10}$ CFU ml^{-1} and 7 h 7.042 ± 0.491 $\log_{10}$ CFU ml^{-1}.

Discussion

The determination of bacterial counts by plate culture is the methodology used in the majority of studies published on the *in vivo* substantivity of CHX on the salivary flora (Moran *et al*, 1992; Jenkins *et al*, 1994; Elworthy *et al*, 1996; Balbuena *et al*, 1998). However, some authors have questioned the reliability of this methodology, referring to numerous difficulties such as: it constitutes a retrospective analysis of bacterial viability (Berney *et al*, 2007); the count is based on measurement of the number of CFU ml^{-1} and not on the number of individual bacteria (Weiger *et al*, 1998); the difficulty in guaranteeing the physiological and metabolic requirements of a polymicrobial population (as found in saliva) for their *in vitro* reproduction (Nadkarni *et al*, 2002; Biggerstaff *et al*, 2006); the existence of factors such as transport and culture media, temperature, atmosphere and incubation period, which affect reproducibility (Boulos *et al*, 1999; Lehtinen *et al*, 2004). These problems could lead to an underestimation of bacterial viability (Boulos *et al*, 1999) or even an overestimation for some genera of bacteria. Furthermore, the heterogeneous nature of the methodologies applied (use of CHX neutralizing agents in the transport and/or culture media, number of serial dilutions, type of bacterial population cultured and the application of different criteria for interpretation of the findings) (Addy *et al*, 1991; Buckner *et al*, 1994; Jenkins *et al*, 1994; Pitten and Kramer, 1999; Sreenivasan and Gittins, 2004) makes comparison of the results between different series difficult.

Some authors have proposed the use of epifluorescence microscopy with specific fluorochromes, such as the LIVE/DEAD® BacLight™ solution, as an alternative method for the quantification of bacterial populations (Boulos *et al*, 1999; Nadkarni *et al*, 2002). The most important advantages of this system include: the rapidity and simplicity of the technique, which quantifies bacterial viability in real-time (Boulos *et al*, 1999); the SYTO-9/PI dual staining allows viable and non-viable bacteria to be counted simultaneously (Boulos *et al*, 1999); and the possibility to detect bacteria that cannot be cultured using plate culture techniques (Joux and Lebaron, 2000; Berney *et al*, 2007).

Some authors studied the correlation between the two techniques (plate culture *vs* epifluorescence microscopy with the SYTO-9/PI dual staining) for the quantification of different bacterial populations and detected that the plate counting and SYTO-9/PI solution counting provided conflicting information on bacterial viability (Boulos *et al*, 1999; Lahtinen *et al*, 2006). In our opinion, the immediate antibacterial effect could be similarly interpreted with both microbiological techniques. However, in accordance with previous authors (Boulos *et al*, 1999; Ihalin *et al*, 2003), we observed that the plate culture technique could overestimate the *in vivo* CHX substantivity, as a significant and progressive increase in the bacterial viability in the different post-mouthrinse saliva samples was detected applying the epifluorescence microscopy with the SYTO-9/PI dual staining. The absence of correlation between fluorescence and plate bacterial count data is likely to be associated with the different characteristics described for each microbiological technique. Moreover, some authors have suggested that: 'The cell membrane has a vital function in bacterial survival, but this does not necessarily imply that a intact cell membrane is viable or culturable' (Joux and Lebaron, 2000; Konings *et al*, 2002; Lahtinen *et al*, 2006) and 'It is unclear whether the observed difference is due to bacteria that have adopted an active but non-culturable state' (Hoefel *et al*, 2003).

On the other hand, the quantification of bacteria by epifluorescence microscopy may also have some associated problems such as bacterial coaggregation (even after homogenization), the staining of cellular elements or contaminating material (Nadkarni *et al*, 2002). Also, there are authors who considered that the microscopic examination of the staining bacteria is tedious, time consuming and unsuitable for testing large number of samples (Singh, 2006). In this sense, an interesting objective will be to analyse if the sensitivity of bacterial detection on the salivary flora with the SYTO-9/PI dual staining could increase with the use of other fluorescence reader techniques such as the confocal laser scanning microscopy, the flow cytometry or the fluorescence microplate reader. Other problems that may be associated with the SYTO-9/PI dual staining include: the existence of a percentage of people (8–12% of males and almost 1% of females), who show a lowered sensitivity to green light resulting in an inability to distinguish green and red (daltonism) (Hope *et al*, 2002), the influence of the physiological state of the bacteria on the staining properties (exponential-growth phase *vs* stationary phase) (Berney *et al*, 2007), a significantly greater non-specific binding to the matrix and greater background fluorescence than other proposed fluorochromes (Biggerstaff *et al*, 2006) or the detection of intermediate colours (yellow or orange) of 'unknown' interpretation (Berney *et al*, 2007). The presence of these intermediate colours could be a consequence of different degrees of membrane damage (Boulos *et al*, 1999; Berney *et al*, 2007) or due to slow passage of SYTO-9 through the intact cell membrane (Lehtinen *et al*, 2004), specifically for Gram negative bacteria (Berney *et al*, 2007).

Although some authors have used epifluorescence microscopy or flow cytometry techniques for the analysis of salivary flora (Weiger *et al*, 1997, 1998; Ihalin *et al*, 2003), we have not found any studies in which epifluorescence microscopy was used with the SYTO-9/PI dual staining to evaluate the *in vivo* antimicrobial activity of CHX on salivary flora; the comparison of our results with those obtained by other authors using plate culture techniques should therefore be interpreted with caution.

Using plate culture techniques, some authors have demonstrated that the application of SM-0.2% CHX (10 ml min^{-1}) was associated with an immediate anti-

bacterial effect with a reduction of ≥90% (≥1 $\log_{10}$ CFU ml^{-1}) in the bacterial concentration with respect to the baseline values (Jenkins *et al*, 1991; Reynolds *et al*, 1991; Simonsson *et al*, 1991; Moran *et al*, 1992, 1995) and its substantivity persisted for a minimum of up to 7 h after the mouthrinse, with a reduction ≥90% (≥1 $\log_{10}$ CFU ml^{-1}) (Jenkins *et al*, 1991; Reynolds *et al*, 1991; Moran *et al*, 1992, 1995). In the present series, the SM-0.2% CHX resulted in an immediate decrease in the percentage of viable bacteria (≥90%) and this antimicrobial activity was still detectable 7 h after the mouthrinse at which point, the reduction in viability was 14%. In disagreement with previous studies based on plate culture techniques (Jenkins *et al*, 1991; Reynolds *et al*, 1991; Moran *et al*, 1992, 1995), in the present series, a significant recovery in bacterial viability was detected in all the postmouthrinse saliva samples in comparison with the viability at 30 s after the CHX mouthrinse.

In conclusion, epifluorescence microscopy with the SYTO 9/PI dual staining is an efficient method that permits evaluating the antimicrobial activity of CHX on the salivary flora in real-time. Moreover, the application of fluorescence assays could be particularly useful to analyse simultaneously the effect of antimicrobials that alter the cytoplasmic membrane integrity on different oral ecosystems.

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Author contributions

Dr. Tomás designed the study, analyzed the data and drafted the manuscript. Dr. Limeres performed the selection of the study group and supervised all the mouthrinses. Dr. García-Caballero and Dr. Cousido performed the processing of the saliva samples. Dr. Diz and Dr. Álvarez critically reviewed and edited the manuscript.

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