New Culture Media for the Isolation of *Streptococcus mutans* and *Lactobacillus* in the Saliva of Head- and Neck-Irradiated Patients

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The reduction in salivary flow in patients subjected to head and neck irradiation induces changes in the oral microflora and increases the risk of oral mucosal infections. The frequent presence of fungi, particularly *Candida*, in the oral environment of these patients complicates identification of the most important cariogenic bacteria with the commercial CRT Bacteria® (Ivoclar Vivadent) culture media. Such identification is important for the application of chemical measures to control cariogenic bacteria in these patients, since it has been shown that simple fluoride application is unable to control caries in this population.

Objective: The aim of this study was to obtain a simple medium that inhibits *Candida* spp. growth and allows the specific growth of *Streptococcus mutans* and *Lactobacillus* spp. Thus, reliable counts of cariogenic species can be achieved.

Materials and Methods: Stimulated saliva samples from 30 head- and neck-radiotherapy patients were seeded in commercial CRT Bacteria® culture medium and in two different media designed by our group: mitis salivarius bacitracin agar (MSBA), containing 5% potassium tellurite and fluconazole 64 µg/ml (MSBTPF) for the isolation of *Streptococcus*; and Man, Rogosa and Sharpe (MRS) agar, containing bacitracin 0.2 U/ml and fluconazole 32 µg/ml (MRSBF) for the isolation of *Lactobacillus* spp.

Results: *Candida* growth was inhibited 100% in the media developed in this study. In all the samples seeded, growing of colonies in MRSBF was identified as *Lactobacillus*, while in CRT Bacteria® for *Lactobacillus* spp. this species was only isolated in 48.1% of the samples. *S. mutans* was identified in 71.4% of the colonies that grown in MSBTPF medium, while in CRT Bacteria® for *S. mutans*, this species was only identified in 35% of the colonies obtained.

Conclusion: The culture medium developed in the present study was able to inhibit the 100% of *Candida* spp. growth. These new media permit reliable counts of cariogenic bacteria in irradiated patients.

Key words: *Candida*, caries risk, culture medium, head- and neck-irradiated patients, *Lactobacillus* spp., *Streptococcus mutans*

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The drastic reduction in salivary flow produced by radiotherapy leads to a series of oral disorders that in turn imply the frequent appearance of oropharyngeal candidiasis and a high incidence of caries in locations that are unusual among individuals with normal salivation (cusps and smooth surfaces) (Brown et al, 1975; Shannon et al, 1977; Epstein and Scully, 1992).

Head and neck irradiation induces a series of changes in the oral microflora (Almstahl and Wikström, 1999), with an increased presence of *Streptococcus mitis*, *Streptococcus salivarius* and *Lactobacillus* spp. (LB), and a reduction in the levels of *Streptococcus sanguis*. LB are potent acidogenic and aciduric germs that can grow and produce acids under initial conditions of pH 4.5. Likewise, *S. mitis* and *S. salivarius* are strongly acidogenic bacteria, while *S. sanguis* is more sensitive to acid action, and is therefore less abundant

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after radiotherapy. Some authors consider that dental plaque is primarily colonised by these acidogenic bacteria, followed at a later stage by *Streptococcus mutans* (SM) (Sansone et al, 1993).

Although SM has been the organism most directly implicated in the development of caries, a number of studies have reported a low presence of these bacteria in white stain lesions. In this context, it has been postulated that some non-mutans streptococci may cause these initial lesions in patients with an important dietary carbohydrate component (Van Ruyven et al, 2000).

Other species of *Streptococcus*, such as *S. oralis*, *S. parasanguis*, *S. anginosus*, *S. constellatum* and *S. gordonni*, are not affected in terms of either frequency of isolation or amount (Tong et al, 2003).

Approximately 91% of irradiated patients present oral candidiasis during and after head and neck radiotherapy, as a consequence of xerostomia and immune changes. The species involved are particularly *Candida albicans*, together with an increase in SM, LB and a reduction in *S. sanguis*, *Neisseria* spp. and *Fusobacterium* spp. (Brown et al, 1978; Spolidorio et al, 2001).

Mitis salivarius is the most selective medium for growth of the different staphylococci, and has also been used for the identification of SM, based on the morphology of the resulting bacterial colonies (Gold et al, 1973; Svanger and Krasse, 1990). However, serial sample dilutions are required to maximally reduce the growth of other streptococci, since both the latter and enterococci interfere with the growth of SM (Schaeken et al, 1986). This medium has been subjected to different modifications, with the incorporation of glucose, saccharose, potassium tellurite, bacitracin, tryptone, etc., in order to secure the greatest possible specificity and selectivity in the identification of SM (Emilson and Brathall, 1976; Tanzer et al, 1984; Kimmel and Tinanoff, 1991; Wan et al, 2002).

Regarding the different species of LB, over 20 of them can be identified in the oral cavity. In this context, the homofermentative bacteria are those with the greatest acid-producing capacity, particularly *L. acidophilus* (Brailsford et al, 2001; Kleinberg, 2002).

In vitro studies have shown that the minimum inhibitory concentration (MIC) of fluconazole for *Candida albicans* isolated within the oral cavity is situated between 1 and 4 μg/ml. Other studies have reported a 100% of growth inhibition with 16 μg/ml fluconazole (Tortorano et al, 1998; Tapia et al, 2003).

Commercial culture media (CRT Bacteria®, Ivoclar Vivadent, Schaan, Liechtenstein) are available, allowing the simple semi-quantitative determination of the presence of SM and LB. These media constitute an efficient tool in defining caries management protocols among these patients. However, such media are not able to inhibit the growth of other species that mask the cariogenic bacteria counts. This is particularly the case of *Candida*, which is rather profuse in the oral cavity of head- and neck-irradiated patients.

The present study describes a culture medium for reliable counting of SM and LB in the saliva of head- and neck-radiotherapy patients, by means of the inhibition of *Candida* growth.

MATERIALS AND METHODS

A cross-sectional study was made involving a series of 30 head- and neck-radiotherapy patients (15 females and 15 males, with a mean age of 40.3 ± 15.9 and 54.2 ± 9 years, respectively). The mean radiation dose delivered was 51.45 ± 12.13 Gy. All patients had completed radiotherapy between 3 and 6 months before sampling, and were programmed for the start of preventive management in a public dental centre of the Valencian Community (Spain). The mean DMFS (decayed, missing and filled dental surfaces) was 34.68 ± 12.7, with a mean stimulated salivary flow of 0.48 ± 0.02 ml/min, and salivary pH values < 6.0 in 81.8% of cases.

A mechanically stimulated (5 minutes chewing on a paraffin tablet) saliva sample was collected. All samples were collected between 8 a.m. and 10 a.m., in a millimeter-graded tube, cold stored (4°C) and transported to the laboratory within 2–3 hours. A sample was seeded in CRT Bacteria®. One of the surfaces of the sample tray contained mitis salivarius bacitracin agar, while the other contained rogosa agar. A sodium monocarbonate tablet was added to the tube to accelerate the growth of LB. The samples were then incubated at 37°C for 48 hours. Another sample was seeded in two media designed by our group, and which contained: mitis salivarius bacitracin agar, 5% potassium tellurite, and 64 μg/ml fluconazole (MSBTPF) for the isolation of SM in one case; and 0.2 U/ml man rogosa sharp medium bacitracin and 32 μg/ml fluconazole (MRSBF) for the isolation of LB in the other. The samples were incubated at 37°C for 72 hours.

For the identification of the different species, some rapid identification system galleries comprising microwells with different culture media was used: API® 20 Strep (Biomérieux, Marcy l'Etoile, France) for *Streptococcus* spp., API® Candida (Biomérieux) for *Candida* spp., and API® 50 CH (Biomérieux) for *Lactobacillus* spp. Regarding gram-positive cocci and gram-negative

Table 1 Number of cases in which each species was identified in the different culture media

	CRT Bacteria® SM	MSBTPF	CRT Bacteria® LB	MRSBF
S. mutans	7 (35%)	20 (71.4%)	-	-
Candida	9 (45%)	0 (0%)	14 (51.9%)	0 (0%)
Others	4 (20%)	8 (28.6%)	0 (0%)	0 (0%)
Lactobacillus	-	-	13 (48.1%)	30 (100%)
Total	**20**	**28**	**27**	**30**

CRT Bacteria® SM: commercial medium for the identification of *S. mutans* (Vivadent)
MSBTPF: medium developed in the present study for the identification of *S. mutans*
CRT Bacteria® LB: commercial medium for the identification of *Lactobacillus* spp. (Vivadent)
MRSBF: Medium developed in the present study for the identification of *Lactobacillus* spp.

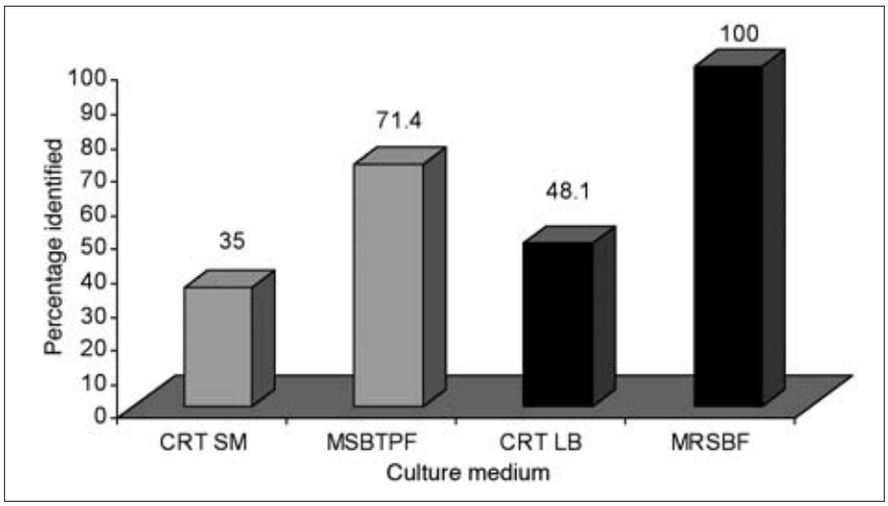

Fig 1 Percentages of *S. mutans* and *Lactobacillus* spp. identified with each culture medium. CRT SM: percentage of *S. mutans* identified with the CRT Bacteria® medium for *S. mutans*. MSBTPF: percentage of *S. mutans* identified with the medium designed in the present study. CRT LB: percentage of *Lactobacillus* spp. identified with the CRT Bacteria® medium for *Lactobacillus* spp. MRSBF: percentage of *Lactobacillus* spp. identified with the medium designed in the present study.

bacilli, API® Staph and API® 20 E (Biomérieux) systems were used, respectively.

RESULTS

In CRT Bacteria® medium for SM, SM growth was observed in seven cases, whereas *Candida* and gram-positive catalase-positive cocci growth were recorded in nine and four samples, respectively. In the medium developed in the present study, *Candida* growth was completely inhibited, and the growth of SM was observed in 20 of all the samples studied (Table 1). From the eight bacterial species that were not identified as SM, two of them were identified as *S. uberis*, two as *Enterococcus faecalis*, two as *Lactococcus lactis*, one as *Leuconostoc* and one as *S. agalactiae*.

When medium MRSBF was used, a 100% of LB growth was recorded in all the plaques where colonies were seen. On the contrary, when CRT Bacteria® medium for LB was used, only 48.1% of colonies were identified as LB. *Candida* spp. were isolated in 14 CRT Bacteria® medium for LB, whereas *Candida* growth was completely inhibited (Table 1).

Fig 1 shows the percentages of SM and LB identified by means of the commercial culture medium com-

pared to MRSBF and MSBTPF media. It can be observed that in all cases, growing colonies in MRSBF were identified as LB, while in CRT Bacteria® for LB, these species were only isolated in 48.18% of samples. SM was identified in 71.42% of the colonies that were grown in MSBTPF medium, whereas in CRT Bacteria® for SM, this species was only identified in 35% of the colonies obtained.

DISCUSSION

Diminished salivary flow induces an increase in bacterial plaque, a reduction in sugar clearance and saliva buffering capacity, a rise in the presence of anaerobes and acidogenic and aciduric bacteria, as well as an increase in species associated with opportunistic infections (e.g. viruses and fungi) (Almstahl and Wikström, 1999).

Several studies have analysed the characteristics of the oral microflora in head- and neck-irradiated patients. In this context, there is general agreement that total streptococci counts increase after radiotherapy. However, Llory et al (1972) reported a decrease in the presence of *S. salivarius* and *S. sanguis*, and an elevation in *S. mitis*, while Tong et al (2003) observed an increase in both *S. salivarius* and *S. mitis*, in association with a reduction in *S. sanguis*. All these authors agree that LB counts increase after irradiation (Llory, 1971; Joyston-Bechal et al, 1992; Keen et al, 1994; Tong et al, 2003).

The commercial CRT Bacteria® media do not yield reliable count information on the presence of cariogenic bacteria (SM and LB) in the saliva of these patients, since *Candida* contamination complicates identification of the target bacterial species.

Regarding the selective medium for SM identification, different authors reported the addition of glucose, saccharose, potassium tellurite, mannitol or bacitracin in different proportions to mitis salivarius agar. In the present study, addition of potassium tellurite MSBA was performed. The medium commonly used for the identification of LB is MRS agar, which contains polysorbate, manganese and magnesium acetate. These components are nutrients that favour the growth of LB. Bacitracin was also added to this medium in the present study. Fluconazole was added to both media, since it exerts effective antifungal action against *C. albicans*, the microorganism most commonly found in the oral cavity of head- and neck-irradiated patients, and in general in all immunesuppressed patients (Tapia et al, 2003).

The high percentage (100%) of LB growth in MRSF medium is consistent with the studies carried out by other authors who reported the presence of LB in high concentrations in most head- and neck-irradiated patients (Brown et al, 1978; Joyston-Bechal et al, 1992). Nevertheless, the commercial CRT Bacteria® medium yielded *Candida* growth in 46.6% of cases. Such growth was so abundant in most cases that it was impossible to identify LB because of the overgrowth of *Candida* spp. However, when fluconazole was added to the medium, *Candida* growth was completely inhibited.

In the medium MSBTPF, no growth was recorded in 12.5% of the samples that were grown in the commercial medium. SM growth was observed in 71.4% of the colonies that were identified in the MSBTPF medium; in the rest of the colonies that were grown in MSBTPF medium, *S. uberis*, *E. faecalis*, *Lactococcus lactis*, *Leuconostoc* spp. and *S. agalactiae* were identified. However, we have found no references in the literature reporting these species in the saliva of head- and neck-irradiated patients. In the commercial medium for the isolation of SM, these bacteria were specifically identified in only 35% of the patients; in 45% of cases *Candida* growth was recorded, and in 20% of the commercial medium, gram-positive catalase-positive cocci were identified. The incorporation of fluconazole to the culture media allowed the complete inhibition of *Candida* spp.

The development of a reliable selective medium to count cariogenic bacteria in the saliva and bacterial plaque of head- and neck-irradiated patients is necessary. The reasons to obtain reliable counts of cariogenic bacteria are the need to individualise antiseptic treatment (particularly regarding high-concentration fluorides and chlorhexidine).

It has been shown that the usual treatment regimens administered to patients with normal saliva flow are not always effective. Such individuals show a strongly diminished natural protection (Katz, 1982; Makkone et al, 1986; Rozir, 2001). In contrast, the daily application of high-concentration fluoride, with chlorhexidine varnish on a three-monthly basis, is capable of reducing the risk of caries in such patients in the same way that has been observed in the general population (Llena and Bagan, 2004).

In conclusion, the addition of fluconazole to selective media for the growth of SM and LB is able to inhibit the growth of *Candida*. Some modifications in the medium used in this study are thus required for the growth of *Streptococcus*, in order to avoid the growth of other organisms such as those described in our study.

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