Quantification of Biofilms formed by *Candida* spp. on Two Types of Plastic Materials used in Pediatric Dentistry and Orthodontics

ALEXANDRU SIMION OGODESCU[1], ALEXANDRU ATTILA MORVAY[2], MAGDA MIHAELA LUCA[1*], ANA EMILIA OGODESCU[1], IRINA ZETU[3]

[1] "Victor Babeş" University of Medicine and Pharmacy, Faculty of Dental Medicine, 2nd Department, Discipline of Paedodontics-Orthodontics, 9 Revolutiei 1989 Blv., 300070, Timisoara, Romania

[2] National Institute of Research – Development in the Pathology Domain and Biomedical Sciences Victor Babes, 99-101 Splaiul Independenei, 050096, Bucharest, Romania

[3] „Gr.T.Popa" University of Medicine and Pharmacy, Faculty of Dental Medicine, Discipline of Orthodontics, 16 Universitatii Str., 700115, Iasi, Romania

Acrylic resins are widely used today both in pediatric dentistry and orthodontics for the manufacturing of space maintainers, interceptive devices or removable orthodontic appliances. All removable orthodontic appliances are composed of porous materials in which microorganisms (fungi, bacteria and viruses) infiltrate, attach and can form biofilms. For this study, two types of materials were chosen in order to test their influence on biofilm formation. For biofilm quantification a strain of Candida spp. was selected among 12 strains isolated from 23 pediatric patients, 7 to 12 years of age, wearing space maintainers or removable orthodontic appliances for at least 4 months at the time of harvest. The materials used in this experiment were the cold-cure acrylic Palapress®vario (Heraeus-Kulzer GmbH, Hanau, Germany) and the visible light acrylic polymerizable system Eclipse (Dentsply DeTrey, Konstanz, Germany). Images of the biofilm formed were obtained using a confocal laser scanning microscope. The images were analyzed using Comstat 2 software. The results showed that in both materials used, the average maximum thickness of the biofilm was above 10 μm. No significant differences were observed in biomass development of the biofilm between the two types of materials tested. Differences were observed in average thickness and maximum thickness of the biofilm. The thickest biofilm was formed on Palapress material. The biofilm formed on Eclipse material surfaces covers 66% of the total aria, while on Palapress material the biofilm covers only 40% of the total aria. These results explain why there are no differences in biomass between the biofilms formed on both materials despite the thickness and average colony size.

Keywords: acrylic resins, biofilms, space maintainers, removable orthodontic appliances

The development of resins represented a great step forward in dental technique, the first thermopolymerisable acrylic resins being developed in 1936 [1].

According to EN ISO 1567 there are four types of resins: thermo polymerized resins (upper 65°C) (either bi-component, or monocomponent), self polymerized (lower 65°C), thermoplastic resins in powder form, photo-polymerized and microwave polymerized resins [2].

Different acrylic resins are widely used today both in pediatric dentistry and orthodontics. They are used for the manufacturing of space maintainers, interceptive devices or removable orthodontic appliances (R.O.A.).

Uncontrolled biofilm formation is a major concern in individuals receiving medical devices such as implants, removable appliances, intubation tubes and catheters. Microorganisms embedded in a hydrated polymeric matrix of biofilms are much more tolerant to antimicrobial agents than are planktonic microorganisms [3].

Although seemingly that only the surface of the R.O.A. is contaminated, the microorganisms that form biofilms also penetrate in the pores and cracks of the acrylic resin generating a real reservoir of bacteria.

Acrylic baseplates of removable orthodontic appliances worn by children were contaminated by mutans streptococci (MS) colonies/biofilms in all cases after 1 week [4].

Biofilms on removable orthodontic appliances act as reservoir of microorganisms, capable of modifying the environmental condition of oral cavity and are difficult to be removed with routine hygiene measures [5].

A contaminated acrylic space maintainer can induce local infections (oral mucosa stomatitis) as well as systemically ones (of upper airways, lungs, kidneys). Dentists, orthodontists, dental-lab technicians dealing with infected R.O.A. have also a high risk through possible cross-contamination.

All R.O.A. are composed of porous materials in which microorganisms (fungi, bacteria and viruses) infiltrate, attach and form biofilms, a 3-dimensional protective matrix in which they buid-up. Especially *Candida albicans* penetrates deeply the pores and cracks of the polymer [6].

Acrylic resin with persistent antimicrobial activities represents a promising method for preventing bacteria- and fungus-induced stomatitis, an infectious disease commonly associated with the wearing of removable orthodontic appliances [7].

Oral biofilms are functionally and structurally organized polymicrobial communities embedded in a self produced extracellular matrix of exopolymers on mucosal, dental and/or oral device surfaces [8]. The oral cavity is a unique environment in the human body, characterized by near-constant presence of liquid water (in saliva), short term extreme temperature fluctuation, externally exposed hard surfaces and by wide variation in carbon and nitrogen input, including a basal component (saliva) that is complex but contains only limited bacterial energy source [9]. The oral

* *email: gheorghescu_magda@yahoo.com; Tel.: 0725724706*

Fig.1. Removable orthodontic appliances with poor hygiene and biofilms under the secondary orthodontic springs

cavity is colonized by a complex microbiota that grows and lives as diverse biofilms on all mucosal and dental surfaces. This microbiota includes protozoa, yeasts, mycoplasmas, *Archaea*, and bacteria. Bacteria are the most numerous and diverse group from which only about half of are culturable [8]. There are over 700 different bacterial species in the oral microflora which colonize the teeth, tongue, oral mucosa, hard palate, carious lesions, periodontal pocket and other surfaces or synthetic materials [10].

The properties of the oral environment determine which organisms colonize, grow and predominate, and result in biofilms with distinct species composition in various habitats of the oral cavity. As a result of the dynamic balance imposed by numerous microbial interactions, component species at a colonization site can remain relatively stable over time but environmental changes may lead to rearrangement in community structure and composition, which can predispose the host site to disease [11].

Candida species are commensal fungi that are found in 30-50% of human oral cavities. Under certain conditions from commensal fungi can transform into opportunistic pathogens that can lead to superficial mucosal and systemic infections. Moreover, *Candida* species are considered the primary causative agents of denture stomatitis, an oral pathology in denture-bearing patients, particularly under the maxillary prosthesis [12].

Experimental part

For this study, two types of materials were chosen in order to test their influence on biofilm formation. For biofilm quantification a strain of *Candida* spp. was selected among 12 strains isolated from 23 healthy pediatric patients, 7 to 12 years of age, wearing space maintainers or removable orthodontic appliances for at least 4 months at the time of harvest**.** All patients have different types of malocclusions or early loss of temporary teeth. The hygiene of their acrylic appliances was poor despite our explanations and effort to convince them to clean their acrylic devices (fig.1).

Isolation of *Candida* strains was done using SDA (Sabouraud Dextrose Agar) culture media. The selection of *Candida* spp. strain was made based on a microtiter plate assay with crystal violet staining, according to [13]. Briefly, the *Candida* strains isolated were cultured on SDA and incubated at 37°C for 48 h. From these cultures an inoculum that matches 0.5 McFarland's standard was made in saline solution (0.9%). This solution was then diluted 1:30 in SDB (Sabouraud Dextrose Broth) growth medium. After these several dilutions, from each strain 150 μL was added to each well, in 8 wells per strain. The microtiter plates were incubated at 37°C, 48 h. After the incubation time the broth was removed from each well, the wells were washed twice with 160 μL 0.9% saline solution, to remove all the planktonic cells. The staining was performed adding 160μL crystal violet 0.1% solution per well and incubating the plates for 10 min at room temperature, then the stain was removed and the wells were washed twice with 170 μL of saline solution 0.9%. Ethanol 96% was added (170 μL) to each well for distaining, for 30 min, and then the OD was measured at 540 nm using an ELISA reader.

The materials used in this experiment were as follows: the cold-cure acrylic Palapress®vario (Heraeus-Kulzer GmbH, Hanau, Germany) and the visible light acrylic polymerizable system Eclipse (Dentsply DeTrey, Konstanz, Germany).

Palapress®vario is a pourable, cold-curing powder and liquid denture base material. The mixing ratio that we used was: 10 g of methylmethacrylate copolymer powder to 7 mL acrylic liquid.

Eclipse is a visible light polymerizable system with a substantially reduced manufacture time for removable orthodontic appliances or space maintainers. Eclipse is composed of urethane oligomers, a class of materials which has found wide acceptance in various dental applications and is free of methyl, ethyl, propyl or butyl monomers. From the producer technical data (www.dentsplymea.com) we noticed an extended hydrolytic stability and the fact that plaque growth on devices appears to be equal to or better than that of a typical acrylic appliance. The material was light polymerized in a special developed processing unit from the Department of Dental Technology, Faculty of Dental Medicine, "Victor Babeş" University of Medicine and Pharmacy.

From each type of material, square coupons were made (1.5x1.5 cm). A 200 μm of *Candida* spp. inoculum that matches 0.5 McFarland standards in saline solution (0.9%), diluted 1:30 in SDB growth medium was placed on each coupon. The coupons containing the culture medium and *Candida spp*. were incubated at 37°C for 48h in 100% UR chamber.

After the incubation period the coupons were rinsed with distillated water, stained with acridine orange (AO) in acetate buffer solution (Sigma) for 2 min at room temperature, rinsed in pure water, air dried at room temperature and examined using the Leica DM 2500 microscope's 63x oil immersion objective with 1.3 numerical aperture. Fifteen randomly selected fields (176x132 μm) on the surface of each coupon were analyzed by microscopy and image processing. The fields were scanned at 400 Hz using a 488 nm argon laser at 25% intensity, also using the Leica DM 2500 microscope's 63x oil immersion objective with 1.3 numerical apertures. The confocal images obtained were analyzed using COMSATAT 2, special computer software designed for biofilm quantification [14, 15]. The following parameters were measured and compared: biomass, average thickness and maximum thickness, surface to volume ration, coverage percentage, diffusion distance and average colony size.

Results and discussions

Results of the microtiter plate assay revealed the strain which had the best capacity to develop a denser biofilm (fig. 2). Thus, after the microtiter plate assay the *Candida* spp. number 28 was selected for further experiments regarding the influence of the above mentioned plastic materials on biofilm formation.

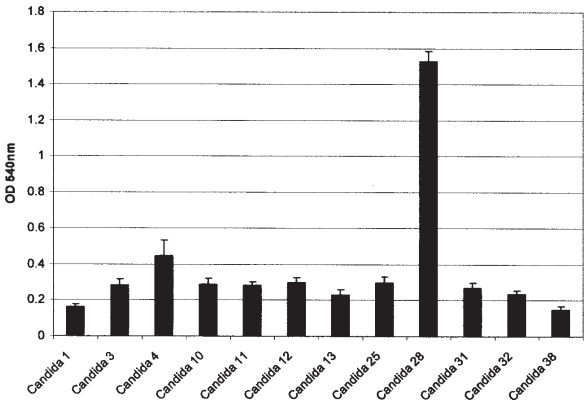

Fig. 2. Optical density of Candida spp. biofilm formed in microtiter plate assay

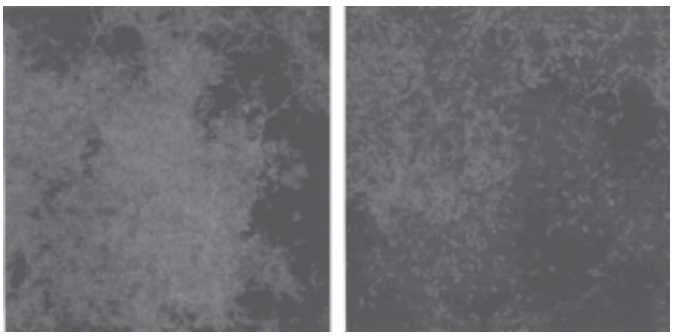

Fig.3. Aspects of the Candida spp. (no. 28) biofilm on PALAPRESS (a) and ECLIPSE (b)

Table 1
ANALYSIS OF CANDIDA SPP. NO. 28 BIOFILM PARAMETERS USING COMSTAT 2

Materials	Biomass $\mu m^3/\mu m^2$	Average thickness μm	Maximum thickness μm	Surface to volume ratio $\mu m^2/\mu m^3$	Average diffusion distance μm	Maximum diffusion distance μm	Average colony size μm^2	Coverage percentage
Palapress	5.04±2.47	10.84±3.23	16.96±0.42	0.18±0.05	0.77±0.64	32.44±21.05	43.05±25.12	40.93±27.81
Eclipse	4.67±2.05	8.75±3.27	13.09±0.64	0.20±0.05	0.86±0.55	34.29±18.90	29.94±7.26	66.00±15.45

All values represent the mean ± standard deviation

The CLSM images showed that the biofilms formed were predominantly composed of blastospore cells on both types of surfaces tested (fig. 3). These findings can be related to the pH value of the culture media or to other components in the culture media (glucose or sucrose), as it was described in other studies [16].

The analysis of biofilms using Comstat 2 software showed that in both materials used, the average maximum thickness of the biofilm was above 10 μm (table 1). Differences were observed between the average thickness of the biofilm. The thickest *Candida* spp. no. 28 biofilm was formed on Palapress acrylic material with an average thickness of 10.84 μm. Mean biofilm thickness provides a measure of the spatial size of the biofilm and is the most common variable used in biofilm literature. Santana et al. (2013) obtained similar results regarding average thickness, using a *Candida albicans* strain (ATCC 90028) on poly(-methlymethacrylate) (PMMA) acrylic resin coated with saliva and YNB (yeast nitrogen base) culture media supplemented with glucose (1%) or sucrose (1%) [16].

No significant differences were observed in biomass development of the biofilm between the two types of materials tested (table 1). Lower values of biomass were obtained in a study on PMMA resin after 48 h in YNB supplemented with 100 mM glucose [17]. Faot et al. (2014) in a study on efficacy of citric acid denture cleanser and the effects of residual biofilm and recolonization, obtained higher biovolume for the biofilm formed by *Candida albicans* (ATCC 90028) on PMMA in YNB supplemented with glucose 100 mM for 72 h (the medium was changed every 24 h) [18].

Average and maximum diffusion distance indicate the distance over which nutrients and other substrate components have to diffuse from the voids to the microorganism within the biofilm colony [15]. Insignificant differences were observed regarding the average and maximum diffusion distance in the *Candida* spp. no 28 biofilm formed on tested materials.

Differences were observed in average colony size. The area sizes of microcolonies at the substratum provide valuable information about the organization of the biofilm community. The differences recorded could be due to material characteristics. This is consistent with recent studies showing that a significantly higher proliferation of *Candida albicans* was observed on the surface of denture base materials with the highest polar contribution to surface free energy (i.e. Eclipse, Mucopren), and this result supports the assumption that there might be a relationship between the polar contribution to surface free energy of the substratum material and *Candida albicans* proliferation [19]. The biofilm formed on Palapress material surfaces is characterized by tall (10 μm average thickness, 16 μm maximum thickness) and large microcolonies (43 μm^2) compared to the biofilms formed on Eclipse material surfaces which are characterized by low (8 μm average thickness and 13 μm maximum thickness) and small microcolonies (29.9 μm^2).

In regards to the coverage percentage, the biofilm formed on Eclipse material surfaces covers 66% of the total aria, while on Palapress material the biofilm covers only 40% of the total aria. These results explain why there are no differences in biomass between the biofilms formed on both materials despite the thickness and average colony size. While the biofilm formed on Palapress material has microcolonies that are taller and larger, it present less microcolonies on a given surface, compared to the number of microcolonies formed by *Candida* spp. no. 28 on Eclipse material. In a study on biofilm-forming ability and pathogenicity, *Candida* isolates were cultured on two different materials (silicon and acrylic resin). The biofilm formed on silicone surfaces had a significant higher biomass (2.17-6.61 mg) compared to the biofilm formed on acrylic resin surfaces (0.25-1.50 mg) [20]. Estivill et al. (2011) in a study on biofilm formation of 84 strains of *Candida*, three clinical materials (Teflon™, PVC, polyurethane) were tested and concluded that *C. albicans*, *C. parapsilosis* and *C. tropicalis* produced more biofilm on

Teflon™ compared to PVC and polyurethane, whereas *C. glabrata* and *C. krusei* biofilms showed no differences among the three materials [21].

The *Candida albicans* colonies on a new polymer used to create complete dentures were studied in [22].

Conclusions

Candida spp. was able to form biofilm on both plastic materials tested. Differences were observed in average thickness and maximum thickness of the biofilm. The thickest biofilm was formed on Palapress®vario material. The microcolonies of the biofilm formed on Palapress®vario were larger and they occupied a greater aria compared to those formed on Eclipse material. The coverage percentage was higher for the biofilm formed on Eclipse material which explains the insignifican differences in the biomass values between the biofilms formed on the tested materials.

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