Microflora of Laboratory-Customized Dental Implant Abutments

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Abstract

Objective: This study aimed to detect and categorize the microorganisms of customized abutments returned from laboratories due to probable contamination and their role in peri-implantitis.

Methods: The samples included 202 abutments from 10 laboratories. Ten µL aliquots obtained from the microbial suspension of each abutment were inoculated on sheep blood agar (as non-selective enriched media), MacConkey agar (selective media for Gram-negative bacteria), Columbia agar with colistin and nalidixic acid agar (selective media for Gram-positive bacteria) and Sabouraud Dextrose Agar with antibiotics (selective media for fungi). They were then incubated in an aerobic atmosphere at 37°C. The molecular methods based on 16S rRNA gene sequence analysis were used in the identification of isolates that did not fit with any recognized biochemical profiles. The distribution of qualitative variables was presented numerically and according to frequency. Complex sample analysis with a 95% confidential interval was used for analysis.

Results: From a total of 49 detected microorganisms, 44 were aerobic bacteria and 5 were fungi. *Micrococcus luteus*, *Bacillus subtilis*, *Staphylococcus epidermis* and *Candida* (the dominant genus among fungi) were the dominant species observed in samples from all 10 laboratories, and on average presented on 13.7, 6.2, 5.0 and 4.1 abutments, respectively. The most abundant Gram-positive and Gram-negative bacteria were *M. luteus* and *Acinetobacter baumannii*, respectively. *B. subtilis* was the most common bacillus, and *M. luteus* was the most common coccus.

Conclusions: Certain decontamination protocols and regulations must be defined to eliminate bacteria and fungi, as both were present in samples from all of the laboratories.

Key words: Microorganisms, customized abutments, contamination, bacteria, fungi, dental implants

Introduction

Nowadays, using implants in dentistry has become a common procedure because of their similarity to natural teeth, stability and longevity (Adell *et al.*, 1990; Olsson *et al.*, 1995; Arvidson *et al.*, 1998; Buser *et al.*, 1999). To obtain these features, accuracy and precision are necessary during all steps of surgery and the prosthetic procedure. Two significant challenges must be dealt with in order to achieve these goals: Osseointegration and an appropriate mucosal seal (Buser *et al.*, 1992). Although previous research and clinical studies have mainly focused on the role of osseointegration and its importance, recent studies have emphasized the role of soft tissue attachment. Many authors have considered its texture and function to be the priorities; this approach leads to long-term aesthetically pleasing prognoses (Vezeau *et al.*, 1996).

Detachment of hard and soft tissue from implants is a primary reason for failure. It is assumed that the mucosal tissue surrounding the implants and the free gingiva of teeth have a histological and clinical similarity

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(Berglundh *et al*., 1991). Like teeth in the periodontium, healthy and intact mucosa surrounding implants can form a biological seal that ensures appropriate osseointegration. Hence, the biological seal can play a significant role in preventing peri-implantitis and bacterial invasion (Listgarten *et al*., 1991). Although 1.5 mm of marginal bone loss during the first year after surgery, followed by 0.1 mm by the second year, does not support the theory that the primary reason for detachment and bone loss is bacterial, but bacterial-induced loss is suggested to be one of the main reasons behind bone loss and may play both causative and accelerative roles, as it is reported in patients with poor oral hygiene that the rate of bone loss is higher (Misch, 1999).

Lehkolm *et al*. (1986) showed that supragingival and subgingival bacterial microorganisms on teeth and implants are similar regardless of the site and type of abutment. Non-motile rods constituted the majority, and spirochaetes were negligible in both groups. These results demonstrate that the efficacy of peri-implant mucosa is equivalent to free gingiva and as a result maintaining its consistency is important. The quality of this transmucosal attachment can be affected by the abutment material, connection type, the compatibility of the materials, and controlling saliva and other contaminants (Gratton *et al*., 2001).

Based on previous studies by Botero *et al*. (2005), Leonhardt *et al*. (1999), and Listgarten and Lai (1999), who used culture as the method of identification of microorganisms, Salcetti *et al*. (1997) and Shibli *et al*. (2008), who used DNA probe analysis, and Kumar *et al*. (2012), who employed pyrosequencing for detecting microorganisms at healthy and failed implant sites, there is a transition from predominantly Gram-positive, non-motile, aerobic and facultative anaerobic composition towards colonization with a greater proportion of Gram-negative, motile, anaerobic bacteria.

Based on the review article by Pye *et al*. (2009), anaerobic Gram-negative bacilli such as *Porphyromonas gingivalis* and *Prevotella intermedia*, anaerobic Gram-negative cocci such as *Veillonella* and spirochaetes such as *Treponema denticola* are found in dental peri-implantitis, which resembles chronic periodontitis. The role of *Staphylococcus aureus* and other coagulase-negative staphylococci that are typically found in orthopaedic infections must be discussed further, as they may play a role when isolated from infected sites. The role of coliform bacteria and *Candida* species are debatable as well. Like orthopaedic implants, there is not any effective treatment for infected dental implants and they should be removed (Pye *et al*., 2009).

Another factor affecting the stability and success of implants is the optimum preload. Lower preload causes higher micro-motion that destroys biological width and compromises the health of soft and hard tissues. Proper preparation, surface cleanliness and quality of all of the parts, particularly the fixtures and abutments, are necessary to achieve the optimum preload. Given the increased use of abutments customized in laboratories and the lack of standard protocols for cleaning, polishing, and sterilization in the prosthesis step, achieving this goal can be a challenge.

Micro-particles are believed to be another source of contamination. After laboratory procedures, microparticles such as titanium, carbon, aluminum, which are the result of preparation of the surface of customized abutments, remain even after regular cleaning procedures. It is assumed that micro-particles can affect the fixture-abutment attachment and increase mechanical stress, which leads to leakage and bacterial penetration that may result in biological problems and failure (Micarelli *et al*., 2015).

Canullo *et al*. (2014) confirmed that both argon plasma and ultrasonication can be used as standard cleaning procedures after laboratory procedures. In another study by Micarelli and colleagues (2013), the reverse-torque method was used to measure the preload of implants before and after different cleaning procedures. These authors concluded that eliminating surface contaminates after laboratory procedures significantly improved the reverse-torque values; they found that argon plasma had better results than steam devices (Micarelli *et al*., 2013).

There is a little information available on bacterial microorganisms existing on customized abutments after laboratory procedures and the appropriate protocols for cleaning and sterilization (Canullo *et al*., 2014). Although sterilization protocols are often followed stringently during implant insertion (Adell *et al*., 1984), these precautions are not followed precisely during the second step (abutment attachment and prosthetic procedure). The resulting bacterial contamination can interfere with osseointegration and can negatively affect guided bone regeneration (Esposito *et al*., 1998; Quirynen *et al*., 2005; Quirynen *et al*., 2006).

Laboratories are places in which different microorganisms from different clinics accumulate, so disinfection and sterilization of these microorganisms are necessary. A few studies have been conducted to detect the microorganisms in dental laboratories: the results indicated the presence of *Streptococcus*, *Staphylococcus*, *Candida, Pseudomonas*, *Escherichia coli*, *Klebsiella* and *Enterobacter* (Katberg, 1974; Powell *et al*., 1990; Sofou *et al*., 2002). However, complete spectrometry of dental implant components has not yet been accomplished on the species level (e.g., *Staphylococcus epidermis, S. aureus, etc.)* to categorize these microorganisms. The aim of this study was to detect and categorize the microflora of customized abutments returned from 10 laboratories.

Materials and methods

A total of 202 customized abutments were gathered from 10 laboratories over the course of one year (March 2014 through March 2015). Each abutment belonged to one cast of a particular patient in order to increase the chance of finding more varieties of microorganisms. The abutments were clean when their packs were opened. The impressions used to make the casts and the casts themselves on which laboratory processes took place were disinfected prior to any procedures; therefore, any microbial contamination at the end of the abutment customization, framework fabrication and layering the porcelain could be attributed to laboratories. The aim of the study was not revealed to laboratories' personnel in order to ensure a blind evaluation.

Using sterilized gloves, forceps and implant wrenches for each implant, the abutments were removed in coping or porcelain steps before returning the casts from the laboratories to the dental clinics. Each abutment was placed in 1 mL of phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 2 mM NaH_2PO_4, 2.7 mM KCl and 137 mM NaCl at pH 7.4) solution. Adherent microorganisms on the abutments were dislodged in PBS via ultrasonication (5 min) in a 150-W ultrasonic bath (Branson Ultrasonics Co., Shanghai, China) operating at a frequency of 50 Hz. Ultrasonication was followed by rapid vortex mixing (Scientific Industries, Bohemia, NY, USA) at maximum power for 1 min to remove microorganisms that had adhered to the abutments. The abutments were then placed back into their casts. This step was repeated for each abutment separately.

Microbial identification

The microbial suspension was centrifuged for 15 min at 1500 x *g*. The resulting cell sediment was dissolved into 100 µL of PBS. Ten µL aliquots of the microbial suspension were inoculated on sheep blood agar (as non-selective enriched media), MacConkey agar (as selective media for Gram-negative bacteria), Columbia agar with colistin and nalidixic acid agar (as a selective for Gram-positive bacteria) and Sabouraud Dextrose Agar with antibiotics (as selective media for fungi) and were incubated in an aerobic atmosphere at 37°C. If colonies grew, then a smear was prepared and Gram staining followed. The microorganisms were identified according to standard methods **(**Nagano *et al.*, 2008).

Molecular methods based on 16S rRNA gene sequence analysis were used in the identification of isolates that did not fit with any recognized biochemical profiles (Janda and Abbott, 2007). DNA amplification and sequencing of the 16S rRNA gene using a previously described method was carried out (Harada *et al.*, 2008). 16S rRNA-specific amplicons using an AccuPrep® PCR Purification Kit (Bioneer, Daejeon, Korea) and directly sequenced using an ABI3730 automatic sequencer (Applied Biosystems, CA, USA) were purified; the sequences were analyzed using a BLAST algorithm against the NCBI GenBank database.

Data analysis

The distribution of qualitative variables was presented numerically and by frequency. A complex sample analysis was used with a 95% confidential interval for our analysis.

Results

Out of a total of 202 abutments gathered from 10 laboratories (20 samples from each laboratory, except for laboratories number 2 and 3 with 21 samples), 49 different species and genera of microorganisms, for a grand total number of 577 microorganisms, were detected. From these, *Candida*, *Aspergillus*, *Penicillium, Cladosporium* and *Fusarium* were fungi, and the remainder were bacteria. There was no abutment without any microorganisms; the greatest number of species (9) was observed in only one abutment (0.5%), and abutments with three different microorganisms were the most frequent (49%; *Table 1*).

M. luteus, *B. subtilis*, *S. epidermis*, and *Candida* were observed in samples from all 10 laboratories, with a mean number of 13.7, 6.2, 5.0 and 4.1 for each laboratory, respectively (*Table 2*). *M. luteus* was the predominant species with a total number of 137 microorganisms over all of the samples and a 23.7 ± 1.6% relative frequency. On the other hand, 22 microorganisms were seen in only one abutment with a relative frequency below 1% and a cumulative frequency of 4.4% (*Table 3*).

The most abundant Gram-positive and Gram-negative bacteria were *M. luteus* and *A. baumannii*, respectively. *B. subtilis* was the most common bacillus, and *M. luteus* was the most common coccus. Laboratory number 9 was the most contaminated, with 75 microorganisms and 20 different species; the most contaminated abutment, with nine microorganisms, belonged to this laboratory as well (*Table 2*).

Table 1. Pattern of abutment contamination based on number of microorganisms

Number of microorganisms on each abutment	Number of abutments	Relative frequency
1	7	3.5%
2	63	31.2%
3	99	49.0%
4	22	10.9%
5	10	5.0%
9	1	0.5%

Table 2. Number (N) and relative frequency (RF) of microorganisms in abutments from each laboratory

Laboratory number		1	2	3	4	5	6	7	8	9	10	Total
Candida spp	N	9	9	3	3	3	3	5	1	4	1	41
	RF	22	22	7.3	7.3	7.3	7.3	12.2	2.4	9.8	2.4	100
Micrococcus luteus	N	13	15	9	13	15	15	17	12	15	13	137
	RF	9.5	10.9	6.6	9.5	10.9	10.9	12.4	8.8	10.9	9.5	100
Bacillus subtilis	N	10	14	10	3	4	4	6	8	1	2	62
	RF	16.1	22.6	16.1	4.8	6.5	6.5	9.7	12.9	1.6	3.2	100
Staphylococcus epidermis	N	5	5	8	3	4	3	5	8	5	4	50
	RF	10	10	16	6	8	6	10	16	10	8	100
Pseudomonas aeruginosa	N	7	3	7	2	1	0	2	2	0	1	25
	RF	28	12	28	8	4	0	8	8	0	4	100
Streptococcus alpha hemolytic	N	2	1	2	0	0	0	0	1	0	0	6
	RF	33.3	16.7	33.3	0	0	0	0	16.7	0	0	100
Micrococcus lylae	N	1	4	3	1	0	3	1	3	0	1	17
	RF	5.9	23.5	17.6	5.9	0	17.6	5.9	17.6	0	5.9	100
Diphtheroids	N	0	2	7	0	0	2	1	8	1	0	21
	RF	0	9.5	33.3	0	0	9.5	4.8	38.1	4.8	0	100
Aspergillus	N	0	1	0	3	0	3	1	0	5	0	13
	RF	0	7.7	0	23.1	0	23.1	7.7	0	38.5	0	100
Neisseria	N	0	0	4	0	0	1	0	1	0	0	6
	RF	0	0	66.7	0	0	16.7	0	16.7	0	0	100
Acinetobacter baumanni	N	0	0	0	7	3	6	0	0	7	8	31
	RF	0	0	0	22.6	9.7	19.4	0	0.0	22.6	25.8	100
Penicillium	N	0	0	0	4	1	1	0	2	0	0	8
	RF	0	0	0	50	12.5	12.5	0	25.0	0	0	100
Bacillus cereus	N	0	0	0	3	8	7	6	3	9	4	40
	RF	0	0	0	7.5	20	17.5	15	7.5	22.5	10	100
Staphylococcus haemolyticus	N	0	0	0	3	2	3	1	0	2	4	15
	RF	0	0	0	20	13.3	20	6.7	0	13.3	26.7	100
Kocuriarosea	N	0	0	0	7	2	5	3	1	8	9	35
	RF	0	0	0	20	5.7	14.3	8.6	2.9	22.9	25.7	100
Cladosporium	N	0	0	0	0	3	0	0	3	0	0	6
	RF	0	0	0	0	50	0	0	50	0	0	100
Fusarium	N	0	0	0	0	0	2	0	1	3	0	6
	RF	0	0	0	0	0	33.3	0	16.7	50	0	100
Enterococcus faecium	N	0	0	0	0	0	0	0	0	4	4	8
	RF	0	0	0	0	0	0	0	0	50	50	100

Discussion

Besides osseointegration, the long-term success of dental implants relies on the quality of peri-implant mucosa and the appropriate attachment of connective tissue to the supracrestal surface of the implant's components (Buser *et al*., 1992). Therefore, achieving tight and durable soft tissue sealing can help to prevent microorganism invasion and, as a result, infection and crestal bone loss, which leads to implant failure (Comut *et al*., 2001). One of the factors that can compromise this seal is implant level impressions; use of this technique has increased in recent years because of the growing demand for aesthetics and the necessity of laboratory customization. This procedure not only increases the risk of detachment of peri-implant mucosa but also the transmission of contamination, consisting of micro-particles and microorganisms. Because laboratories are places in which different species of microorganisms accumulate, determining the species of microorganisms returned from laboratories to dental clinics is important for selecting the most efficient disinfection and sterilization methods. This information also can be used to establish regulations for laboratories to prevent the spread of microorganisms.

To our knowledge, until now no study has evaluated and categorized the microorganisms of customized abutments returned from laboratories at the species level. The samples were gathered from abutments undergoing the final steps of customization, in which contamination was anticipated to be higher than those in earlier stages. The samples from each laboratory were obtained in one session to prevent disinfection of abutments by laboratory personnel. The samples were stored at 4°C to inhibit reproduction and changes in the microflora ratio.

Table 3. Relative frequency and number of each microorganism based on their prevalence

Microorganism	Number of microorganisms	Relative frequency (%)
Micrococcus luteus	137	23.7 ± 1.6
Bacillus subtilis	62	10.7 ± 2.5
Staphylococcus epidermis	50	8.7 ± 1.0
Candida spp	41	7.1 ± 1.5
Bacillus cereus	40	6.9 ± 0.5
Kocuri arosae	35	6.1 ± 0.8
Acinetobacter baumannii	31	5.4 ± 0.4
Pseudomonas aeroginosa	25	4.3 ± 1.6
Diphtheroids	21	3.6 ± 0.3
Micrococcus lylae	17	2.9 ± 0.5
Staphylococcus haemolyticus	15	2.6 ± 0.2
Aspergillus	13	2.3 ± 0.9
Penicillium	8	1.4 ± 0.7
Entrococcus faecium	8	1.4 ± 0.9
Streptococcus alpha hemolytic	6	1.0 ± 0.5
Neisseria	6	1.0 ± 0.7
Cladosporium	6	1.0 ± 0.7
Fusarium	6	1.0 ± 0.6
Staphylococcus caprae	5	0.9 ± 0.7
Staphylococcus aureus	4	0.7 ± 0.4
Enterococcus faecalis	4	0.7 ± 0.3
Nocardia	4	0.7 ± 0.5
Gemella haemolysans	3	0.5 ± 0.3
Non-hemolytic streptococcus	2	0.3 ± 0.2
Bacillus megaterium	2	0.3 ± 0.2
Kocuria kristinae	2	0.3 ± 0.3
Staphylococcus carnosus	2	0.3 ± 0.2
Achromobacter cholinophagum	1	0.2 ± 0.2
Tamlense aeromicrobium	1	0.2 ± 0.2
Aeromona sichthiosmia	1	0.2 ± 0.2
Arthtobacter woluwensi	1	0.2 ± 0.2
Pseudomonas spinose	1	0.2 ± 0.2
Bacillus thuringiensis	1	0.2 ± 0.2
Bacillus firmus	1	0.2 ± 0.2
Brachybacterium faecium	1	0.2 ± 0.2
Aerococcus viridans	1	0.2 ± 0.2
Brevibacterium casei	1	0.2 ± 0.2
Flavobacterium ferrugineum	1	0.2 ± 0.2
Kocuria carniphila	1	0.2 ± 0.2
Deinococcus grandis	1	0.2 ± 0.2
Dermacoccus profundi	1	0.2 ± 0.2
Curtobacterium citreum	1	0.2 ± 0.2
Staphylococcus cohnii	1	0.2 ± 0.2
Staphylococcus lentus	1	0.2 ± 0.2
Kytococcus aerolatus	1	0.2 ± 0.2
Macrococcus carouselicus	1	0.2 ± 0.2
Enterobacter cloacae	1	0.2 ± 0.2
Micrococcus flavus	1	0.2 ± 0.2
Rothia mucilaginosa	1	0.2 ± 0.2

Overall, we obtained samples from 202 customized abutments from 10 laboratories that were used for cultivation. Viruses such as human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV) need a considerable volume of blood to transfer and previous studies have shown negative results of cultivation for viruses. Therefore, viruses were not considered to pose a serious contamination risk and were not accounted for in this study.

As predicted, because of characteristics of the laboratory environments, all 44 detected bacterial species were aerobic; five genera of fungi were found as well. Pye *et al.* (2009) showed that most species that play a role in peri-implantitis are Gram-negative, motile anaerobic organisms. However, *Staphylococcus* and *Candida*, which were detected in this study, existed in 55% of sites with peri-implantitis in some studies (Rams *et al.*, 1990; Leonhardt *et al.*, 1999); their infrequent presence may be due to probable cross-infection (Pye *et al.*, 2009). *S. aureus* is shown to have the ability to adhere to titanium surfaces, which can be crucial for colonization and resulting infection (Harris and Richards, 2004).

Although the species found in this study were aerobic or facultatively anaerobic, and were not among those microorganisms which frequently exist in peri-implantitis, they may facilitate colonization of other species by compromising the peri-mucosal seal and forming micro-gaps. Also, increased osteoclastogenesis may occur due to bacterial activity and endotoxins (Ujiie *et al.*, 2012).

The four predominant species detected in samples from all laboratories were *M. luteus, B. subtilis, S. epidermis and C. albicans. M. luteus* was the predominant species in this study and accounted for 137 out of 577 total microorganisms in 202 abutments. Micrococci are Gram-positive, oxidase-positive and strictly aerobic cocci belonging to the family of *Micrococcaceae*. They are usually non-motile and non-spore-forming and are generally considered to be harmless. However, there have been some reports of their causative role in intracranial abscesses, pneumonia, septic arthritis, endocarditis and meningitis, and they can contaminate the skin, mucosa and oropharynx (Bannerman *et al.*, 2006). According to Wong *et al.* (2001) they can decompose heavy metals such as lead, zinc and nickel, a fact that can destructively affect peri-implant mucosal seals. Micrococci are susceptible to vancomycin, penicillin, gentamicin and clindamycin (Bannerman *et al.*, 2006), but resistant to nitrofurantoin, macrolides (erythromycin), lincomycin and ultraviolet light (Magee *et al.*, 1990; Nakayama *et al.*, 1992; Liebl *et al.*, 2002).

B. subtilis species was the second most prevalent species. It is a Gram-positive, catalase-positive, facultative aerobe that can form a tough, protective endospore that can tolerate extreme environmental conditions of temperature and desiccation. It has the ability to form titanium dioxide (TiO_2) nanoparticles via the biosynthesis of titanium (Kirthi *et al.*, 2011). According to DeQueiroz, sodium hypochlorite accompanied by hydrogen peroxide (Ox-B7) can eliminate *B. subtilis* spores on both porous and non-porous surfaces (DeQueiroz and Day, 2008).

S. epidermis was the third most common species. It is a Gram-positive, catalase-positive, coagulase-negative, facultative anaerobe. According to Rams, the staphylococci in peri-implantitis lesions (15.1%) was considerably higher than in gingivitis (0.06%) or periodontitis (1.2%) lesions; it appears that staphylococci play a role in some implant failures (Rams *et al.*, 1990). It is commonly believed that the most efficient way to deal with *S. epidermis* infections is prevention via disinfection and sterilization of medical equipment, patients and health care personnel (Rogers *et al.*, 2009).

The fourth most prevalent and the predominant microorganisms among the fungi were *Candida*. Their existence on the abutments can be considered worth noting as a result of a recent study that indicated that *Candida* species were predominant in peri-implantitis sites (Schwarz *et al.*, 2015); hence disinfecting and eliminating them after laboratory procedures may lessen their frequency in peri-implantitis. *Candida* species are found mostly in elderly and edentulous patients. Some species, such *C. albicans,* can form chlamydospores in an anaerobic environment and resist severe environmental conditions and some disinfectants (Montazeri and Hedrick, 1984). This fact matters more when *C. albicans* forms subgingival spores in elderly individuals with compromised immune systems. It can also synergize with *S. aureus* and be resistant to vancomycin (Harriott and Noverr, 2009). According to Waltimo *et al.* (1999), *C. albicans* are susceptible to sodium hypochlorite, iodine potassium iodide and chlorhexidine acetate; Trindade showed that *Cymbopogon nardus* essential oil and citronellal can inhibit the adherence of *C. albicans* to dental implants and cover screws (Trindade *et al.*, 2015).

Although there is no standard protocol for the sterilization of implant components, previous studies have shown that argon plasma and ultrasonication yielded acceptable results and eliminated nearly all the microorganisms (Micarelli *et al.*, 2013; Canullo *et al.*, 2014). But these techniques are considered to be costly and not available in every clinic. Therefore, appropriate, alternative disinfectants based on the microorganisms of the abutment are necessary.

Recognition of the type of microbial contamination helps in choosing the most effective disinfectant. Data presented regarding the possible microbial contamination of abutments in dental labs might be more valuable in the future when the possible role of each microorganism has been clarified. The results of this study can be used for further evaluations in selecting disinfectants and also establishing regulations for laboratories. Furthermore, the role and contribution of prevalent species found in this study should be further investigated in peri-implantitis and probable implant failure.

Conclusion

Over all, a total of 49 species of microorganisms were detected, of which 44 were bacteria and five were fungi, with a total frequency of 577 in 202 abutments. The dominant species was *M. luteus*; *Candida* species were the most prevalent among fungi. Both bacterial and fungal decontaminants are necessary for a proper disinfection.

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