Formation of biofilm on various implant abutment materials

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The characteristics of prosthetic implant components, such as the type, material, and surface roughness of abutments, can affect biofilm formation. Since an ideal abutment surface for the reduction of bacterial adhesion has yet to be found, this in vitro study aimed to quantify biofilm formation on laser-treated titanium, zirconia, and titanium surfaces. Sterile titanium, zirconia, and laser-treated titanium discs were placed in sterile 48-well plates. Biofilm formation was induced by adding sterilized, unstimulated human saliva and suspensions of *Porphyromonas gingivalis (Pg)*, *Aggregatibacter actinomycetemcomitans (Aa)*, and *Prevotella intermedia (Pi)* to the wells. Viable bacteria in the biofilm were quantified with real-time polymerase chain reaction in conjunction with propidium monoazide. The disc material, the type of bacteria, and their interactions had significant effects on the bacterial counts. On all surfaces, the *Pg* count was significantly higher than both the *Pi* and *Aa* counts (P = 0.0001). The highest count of periodontal pathogens was found on laser-treated surfaces. The second highest and the lowest counts were found on zirconia and titanium surfaces, respectively.

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An oral biofilm is typically composed of complex and dynamic microbial populations in a polymer matrix that originates from bacteria and saliva.[1] Evidence shows that that the processes of bacterial colonization and biofilm formation and maturation are the same on natural teeth and dental implants.[2] Bacterial adhesion and biofilm formation on dental implants result in pocket formation and loss of supporting bone, which can lead to implant failure.[3-5]

Despite numerous attempts by researchers to enhance osseointegration by changing the surface topography of dental implants, previous studies have confirmed that the surface properties of dental implants tend to increase bacterial colonization.[2-11] Therefore, it is necessary to fabricate surfaces that not only improve osseointegration but also decrease bacterial colonization.[2] Prosthetic implant components (such as abutments) significantly affect microbial adhesion. Surface roughness and abutment material affect microbial adhesion and biofilm formation.[6] The formation of biofilm on the abutment surface may compromise soft tissue integration, allow biofilm migration into peri-implant sulcus, and cause the subsequent development of mucositis and peri-implantitis.[7]

Although surface roughness has been found to influence bacterial adhesion, studies on the nature of these effects have shown contradictory results. Bollen et al showed that a surface roughness less than 0.2 μm did not cause a significant change in the number of adhered bacterial colonies or their pathogenesis.[8] Quirynen & Van Assche found no significant difference in subgingival plaque on surfaces with moderate and low surface roughness.[9] Grössner-Schreiber et al reported that bacterial adhesion and plaque formation on modified surfaces were significantly less than on polished surfaces.[10] However, Wennerberg et al found no clinically significant differences among titanium abutments with different surface roughness values.[11]

In addition to surface roughness, abutment materials can also affect biofilm formation.[6] Titanium remains the standard material for fabrication of abutments due to its optimal physical properties, biocompatibility, and high corrosion resistance. Zirconia has been suggested as a substitute for titanium, due to its optimal esthetic properties.[12,13] However, the clinical evidence supporting the superiority of zirconia abutments to titanium is controversial, and the biological advantages of zirconia are a matter of debate.[14] Previous studies have yielded controversial results on bacterial adhesion to both titanium and zirconia abutments.[15,16] Scarano et al found less bacterial adhesion to zirconia discs than to titanium discs.[15] In contrast, de Oliveira et al found no significant difference in the adhesion of bacteria to zirconia and titanium abutment surfaces.[16] Recently, titanium abutments with a laser-treated collar (Laser-Lok, BioHorizons) were introduced to the market; however, studies on the microbial biofilm accumulation on these surfaces are scarce.[6]

The oral cavity can include up to 700 bacterial species, only a few of which have been recognized as periodontal pathogens.[6] *Porphyromonas gingivalis* (*Pg*), *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* (*Aa*), and *Prevotella intermedia* (*Pi*) have been reported as periodontal pathogens responsible for periodontal and peri-implant inflammatory conditions.[17,18] Considering the significant association of periodontal pathogens with the occurrence and progression of periodontitis and peri-implantitis, the detection and isolation of these pathogens have been the objective of many studies.[19] The most commonly used techniques for counting microorganisms in previous studies include microbial culture, checkerboard DNA-DNA hybridization, and real-time polymerase chain reaction (PCR), which is also known as *quantitative PCR*. However, these techniques have some limitations. Checkerboard DNA-DNA hybridization has low sensitivity and detects all cells available in the medium, including viable and dead cells.[20,21] The culture technique has low sensitivity as well and only allows the counting of viable cells. Its accuracy highly depends on the technical skills of the operator.[22] Real-time PCR is highly sensitive and detects all the viable and dead cells present in the medium.[20]

In recent years, use of a DNA-binding dye along with real-time PCR has been suggested so that viable cells can be detected more easily. This technique is based on using a propidium monoazide (PMA) dye, which is derived from propidium iodide (PI). The PMA dye penetrates the membrane of dead cells, which have lost their integrity, and bonds to their DNA. When the DNA-PI is stimulated and excited by a certain wavelength, it emits colored light, identifying dead cells. Viable cells can be detected and counted by excluding the cells that PI fluorescence has identified as dead.[23] In contrast to PI, PMA is applicable in PCR and real-time PCR assays. PMA is more selective and less cytotoxic than PI, and some organisms may not stain if PI is used.[24] The dye-PCR methods are superior to the culture method for counting anaerobic oral bacteria, which have a slow growth, because the latter method requires a long period of time and a specific culture medium for the proliferation of anaerobes.[20]

In the current study, real-time PCR was combined with PMA to increase the sensitivity of the viable bacteria count compared to the culture method. To the best of the authors' knowledge, the combined PMA and real-time PCR method has not been used for assessment of the adhesion of periodontal pathogens to different abutment materials. Thus, because the role that various abutment surfaces and materials play on microbial biofilm accumulation requires clarification, this in vitro study combined real-time PCR with PMA in an attempt to assess biofilm formation on laser-treated titanium, zirconia, and titanium surfaces.[25-27]

Materials and methods

This study was conducted on 15 sterile discs (BioHorizons) that were 9 mm in diameter and 1 mm thick: 5 laser-treated titanium (with similar microgrooves at 8-μm intervals), 5 zirconia, and 5 titanium discs.

Saliva collection

Unstimulated saliva was obtained and collected in a sterile container from a candidate after his verbal informed consent was obtained. The candidate was asked to refrain from eating, drinking, using mouthwash, and brushing with toothpaste for a minimum of 2 hours prior to saliva collection. The collected saliva was sterilized via filtration using a filter with 0.22-μm pores.

Bacterial strains and culture conditions

The bacterial strains *Aa* (ATCC 33384), *Pi* (ATCC 25611), and *Pg* (ATCC 33277) were used. After inoculation in brain-heart infusion broth (Merck), the strains were supplemented with 1.0 g/L of yeast extract (Oxoid), 0.1 g/L of cysteine (Sigma-Aldrich), 2.0 g/L of sodium bicarbonate (Merck), 5.0 mg/mL of hemin (Sigma-Aldrich), 1.0 mg/mL of menadione (Merck), and 0.25% (vol/vol) glutamic acid (Sigma-Aldrich). The strains were incubated in a shaking incubator (150 rpm) at 37°C under anaerobic conditions to reach logarithmic growth phase with a count of 10^8 bacteria. (The optical density of each microbial suspension was adjusted to 0.4-0.5 at 650 nm.) Afterward, 1 mL of each microbial suspension was used to prepare a mixed suspension of bacteria for biofilm formation.

Biofilm formation

Biofilm formation was induced as suggested by Sánchez et al.[19] In each group, discs were separately transferred to separate wells of a sterile 48-well plate. Pellicle formation was induced by adding 1 mL of the saliva to each disc-containing well at 37°C. After 4 hours, the discs were removed from the saliva and transferred to the wells of a new sterile plate, and 1 mL of the mixed bacterial suspension was added to each disc-containing well. After 72 hours of incubation in anaerobic conditions, the discs were removed from the wells. To eliminate planktonic and poorly adhered bacteria, the discs were rinsed twice with phosphate-buffered saline.

Quantification of viable bacteria

The viable bacteria in the biofilm formed on the disc surfaces were quantified with PMA–real-time PCR. For this purpose, the bacteria that formed the biofilm on disc surfaces were detached from the disc surface by sonication.

PMA treatment

Treatment with PMA was performed as described by Sánchez et al.[19] In a microtube, 100 μM of PMA was added to 250 μL of bacterial suspension (prepared by use of the biofilm separated from the disc surfaces), and then the microtube was incubated at 4°C on a rotator without light for 10 minutes. Next, the uncapped microtube was placed on ice, subjected to a 600-W halogen light irradiation from a 20-cm distance for 5 minutes, and then centrifuged at 12,000 rpm for 3 minutes. The cell sediment was dissolved in 100 μL of RNase/DNase–free water. DNA extraction was then performed. To evaluate the effect of each experimental step on the viability of bacteria, all the aforementioned steps were repeated without addition of PMA in a negative control group consisting of a suspension from each abutment surface and each bacteria. (This group was not included in statistical analyses.)

DNA extraction

A DNG-Plus DNA extraction kit (SinaClon BioScience) was used to extract bacterial DNA. First, 200 μL of the DNG-Plus

suspension was added to 100 μL of the cell suspension treated with PMA (previous step), and the microtube was vortexed for 15 minutes. Then 300 μL of isopropanol was added to the microtube, which was vortexed for 10-15 seconds. The microtube was then centrifuged at 12,000 rpm for 15 minutes. Next, the supernatant was removed, and 1 mL of 75% ethanol was added to the microtube, which was centrifuged at 12,000 rpm for 5 minutes. The resulting supernatant was discarded, and an additional 1 mL of 75% ethanol was added to the microtube, which was centrifuged again at 12,000 rpm for 5 minutes. The supernatant was discarded.

The microtube was placed in Thermoblock (Eppendorf) at 65°C until the microtube content was completely dried. Next, 50 μL of deionized distilled water was added to the microtube and placed in Thermoblock at 65°C for 5 minutes. The microtube was then vortexed, and 50 μL of DNA was extracted.

Real-time PCR
Viable bacteria present in the biofilm on the discs were quantified by PMA–real-time PCR using standard curves. To draw standard curves, PMA was used as described earlier to treat 1 mL of the suspensions of viable *Pg*, *Pi*, and *Aa*, which included 10^6 colony-forming units (CFUs) per 1 mL and had an optical density of 550 nm. The concentration of DNA was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific). Serial dilutions of each type of DNA were prepared using PCR-grade sterile water (Thermo Fisher Scientific) to obtain 10^1-10^6 CFUs/mL, and then real-time PCR was carried out with a specific primer for detection and quantification of bacterial DNA (Bioer Technology).[22,28]

Real-time PCR was performed in a total volume of 120 μL of reaction mixture (SYBR Premix Ex Taq II, Takara) as follows: amplification with 35 cycles at 95°C for 5 minutes; denaturation at 95°C for 15 seconds; annealing at the temperatures of 50°C, 56°C, and 52°C for *Pg*, *Aa*, and *Pi*, respectively, for 30 seconds; and extension at 72°C for 30 seconds.

The characteristics of primers were assessed using melt curves. Standard curves were drawn based on quantitation cycle values obtained from real-time PCR of *Pi*, *Pg*, and *Aa* and total concentration of bacteria (in log CFUs/mL). The standard curves were used to quantify viable bacteria by real-time PCR. For this purpose, bacterial suspension was prepared using bacteria isolated from the biofilm. Treatment with PMA and DNA extraction were performed as described earlier. All tests were performed in triplicate.

Statistical analysis
The results were expressed as mean values and standard deviations. Statistical analyses were performed via the Kolmogorov-Smirnov test, 2-way analysis of variance (ANOVA), and the Bonferroni multiple comparisons test. The Kolmogorov-Smirnov test revealed that the log counts of bacteria in all groups based on the type of disc (laser-treated, zirconia, and titanium) and type of bacteria (*Pi*, *Pg*, *Aa*) had normal distributions. Assumption of the homogeneity of variances was met for all groups with regard to the log count of bacteria. Thus, these data were analyzed using the 2-way ANOVA. The level of statistical significance was set at α = 0.05 for overall and α = 0.016 (α = 0.05/3 = 0.016) for individual pairwise comparisons.

Results
The mean (SD) count of *Aa* was 11.3163 (0.0869), 10.7641 (0.1276), and 9.6941 (0.1658) log CFUs/mL in the laser, zirconia, and titanium groups, respectively (Chart). The mean (SD) count of *Pi* was 11.3437 (0.1972), 10.2500 (0.1692), and 10.0831 (0.2245) log CFUs/mL in the laser, zirconia, and titanium groups, respectively. The mean (SD) count of *Pg* was 12.1176 (0.1972), 10.425 (0.2507), and 10.1213 (0.1843) log CFUs/mL in the laser, zirconia, and titanium groups, respectively. The overall mean (SD) bacterial count was 11.59 (0.42), 10.48 (0.28), and 9.97 (0.27) log CFUs/mL in the laser, zirconia, and titanium groups, respectively. A significant difference existed in the overall log counts of bacteria among the 3 groups (*P* = 0.0001).

According to the Bonferroni test, the log count of bacteria in the laser-treated group was significantly greater (*P* = 0.0001) than those in the zirconia and titanium groups (Table). In addition, the log count of bacteria in the zirconia group was significantly greater than that in the titanium group (*P* = 0.0001). The log count of *Pi* was lower than that of *Aa*, but this difference was not statistically significant (*P* = 1.0000). The log count of *Pi* was significantly lower than that of *Pg* (*P* = 0.0001). The log count of *Aa* was also significantly lower than that of *Pg* (*P* = 0.0001).

Discussion
In the present study, the numbers of biofilm-forming bacteria were significantly greater on laser-treated surfaces than on the other 2 tested surfaces. Also, the bacterial count was significantly higher on the zirconia surface than on the titanium surface. The overall count of *Pg* on all 3 surfaces was higher than the count of other bacteria.

Biofilm formation on dental implant surfaces is among the major causes of implant failure.[29] The best solution to prevent microbial infections of implants is to decrease colonization of oral bacteria on these surfaces.[30] Evidence shows that the characteristics of prosthetic implant components, such as the material and surface roughness of abutments, affect microbial adhesion and biofilm formation.[6]

The surface of prosthetic components must be designed to either minimize bacterial colonization or exert antimicrobial effects without adversely affecting the adjacent tissues.[31] A study on the design of dental implants showed that micron- or submicron-scale structural properties enhance osteoblastic differentiation and subsequent bone formation around dental implants; however, the effect of these surface modifications on the adhesion and proliferation of oral microorganisms has yet to be elucidated.[32]

The results of the current study on bacterial adhesion to different abutment surfaces were in line with those reported by Scarano et al and Nascimento et al.[15,21] Similar to the present study, both studies reported significantly different bacterial counts in the titanium and zirconia groups. However, Nascimento et al reported the greatest bacterial adhesion to cast titanium, followed by machined titanium and zirconia, and Scarano et al showed that bacterial adhesion to zirconia surfaces was less than that to titanium surfaces, findings that differed from the results of the present study.[15,21] These disparities may be due to differences in study designs between their in vivo studies and the present in vitro study or in the methods of
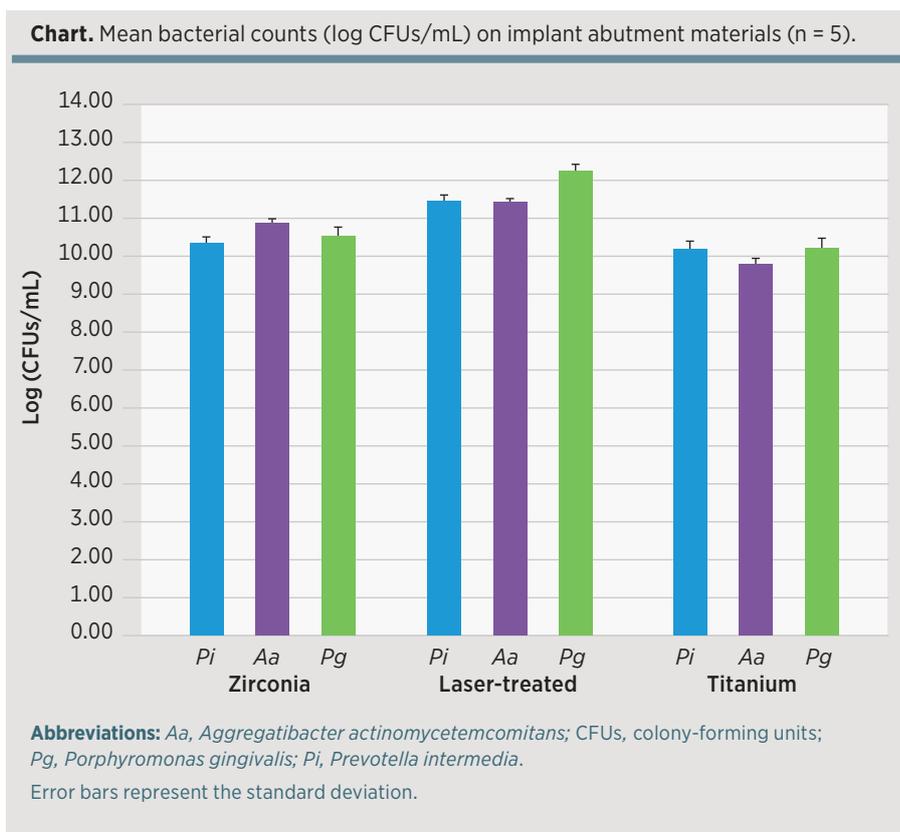
Chart. Mean bacterial counts (log CFUs/mL) on implant abutment materials (n = 5).

Abbreviations: *Aa, Aggregatibacter actinomycetemcomitans;* CFUs, colony-forming units; *Pg, Porphyromonas gingivalis; Pi, Prevotella intermedia.*

Error bars represent the standard deviation.

Table. Pairwise comparisons of bacterial counts (log CFUs/mL) on implant abutment materials.

Group (I)	Group (J)	Mean diff (I – J)	SE	P	95% CI LB	95% CI UB
Zirconia (n = 5)	Titanium (n = 5)	0.5137ᵃ	0.0675	0.0001	0.3442	0.6833
Laser-treated (n = 5)	Zirconia (n = 5)	1.1127ᵃ	0.0675	0.0001	0.9431	1.2822
	Titanium (n = 5)	1.6264ᵃ	0.0675	0.0001	1.4568	1.7959
Pi (n = 15)	*Aa* (n = 15)	−0.0326	0.0675	1.0000	−0.2021	0.1370
	Pg (n = 15)	−0.3292ᵃ	0.0675	0.0001	−0.4988	−0.1597
Aa (n = 15)	*Pg* (n = 15)	−0.2967ᵃ	0.0675	0.0001	−0.4662	−0.1271

Abbreviations: *Aa, Aggregatibacter actinomycetemcomitans*; CFUs, colony-forming units; diff, difference; LB, lower bound; *Pg, Porphyromonas gingivalis; Pi, Prevotella intermedia*; UB, upper bound.

ᵃStatistically significant difference.

assessment that were used. The present study used PCR, while Scarano et al used electron microscopy and Nascimento et al used a checkerboard DNA-DNA hybridization.[15,21]

Experiments by de Avila et al showed that different abutment surfaces significantly affect biofilm formation and the accumulation of anaerobic biofilm on zirconia surfaces is less than that on titanium and control surfaces.[6] However, the quantitative results in their study were based on counting of CFUs, which differs from the real-time PCR performed in the present study.

Several studies have shown that biofilm formation has a significant association with surface roughness.[6,7,9,15,21] More biofilm is formed on rougher surfaces, but surface roughness values of less than 0.20 μm have an insignificant effect on biofilm formation.[7] It is theorized that greater accumulation and maturation of plaque occur on rougher surfaces because they enhance the primary adhesion of bacteria and thus prevent bacterial elimination.[9]

Inconsistencies in the findings of different studies may be attributed to the difference in the surface roughness of samples.[6,15,21] A recent study showed that the surface roughness of titanium discs is significantly lower than that of zirconia discs, while other previously mentioned studies have found that the

surface roughness values of titanium and zirconia are almost the same.[33] Other factors, such as chemical composition, surface energy, and hydrophobicity, may be responsible for the finding of less bacterial adhesion to zirconia abutments than titanium abutments, but these were not evaluated in the present study.[34,35] Discs evaluated in the present study had similar standards to the abutments found commercially, and the manufacturer provided these discs; in most previous studies, discs were manually fabricated and prepared by the researchers.

The current study evaluated *Pg*, *Pi*, and *Aa*, since they are considered to be the main pathogens responsible for both periodontal and peri-implant diseases.[17,18] *Pg* has been isolated from 85% of areas exhibiting chronic periodontitis.[36] This microorganism has several pathogenic mechanisms responsible for inflammation.[37] The pathogenesis of *Pg*-elicited periodontal disease depends on the ability of this microorganism to bond to host cells, the acquired pellicle, and other microorganisms.[38]

In the present study, the total count of *Pg* was significantly greater than that of *Aa* and *Pi*; this finding was in accordance with that of Almaguer-Flores et al.[32] In their study, *Pg* had the highest count in the formed biofilms on all surfaces. The results of the present study were also in agreement with those of Nascimento et al, who isolated *Pg* and *Aa* from 95.84% and 91.67% of their samples, respectively.[21] Quirynen & Van Assche reported *Pg* and *Pi* to be the key periodontal pathogens among 40 bacterial species and confirmed the presence of these bacteria on all surfaces they tested (minimally and moderately rough).[9]

A review of the literature yielded only 1 study, by Di Giulio et al, that compared bacterial biofilm accumulation on laser-modified, sandblasted, and machined surfaces.[29] The authors evaluated the effect of surface topography and implant material on *Pg* biofilm and reported the lowest adhesion of this pathogenic microorganism to laser-treated surfaces. However, they only evaluated 1 bacterial strain, while the present study evaluated 3 bacterial strains.[29] The presence of different bacterial strains and their interactions in the suspension can affect their adhesion and accumulation.[39]

Oral biofilm is composed of complex and dynamic microbial populations in a polymer matrix originating from the bacteria and saliva.[2] When biofilm is formed, bacteria become more resistant to antibiotics and host defense mechanisms.[32] In other words, biofilm provides a safe environment for the bacteria and protects them from the effects of antibiotics and antiseptics.[20] The formation of biofilm is a complex procedure. Biofilm formed in vitro is an actual biofilm only if several bacterial strains are present in it (similar to dental plaque biofilm). The process of biofilm formation and bacterial adhesion are affected by several factors, including the oral environment, bacterial properties, and surface properties. Moreover, the culture medium has a critical role in bacterial adhesion and the amount of biofilm formed in vitro.[40] The main strengths of the present study were an approach that included the use of 3 bacterial strains that are involved in periodontitis and peri-implantitis to induce biofilm formation and the use of saliva as a culture medium to better simulate the oral clinical environment.[32]

Different methods of bacterial counting may be responsible for the different results obtained in various studies. Microbial culture and real-time PCR are commonly used methods for the counting of bacteria. However, these methods have limitations, including overestimation or underestimation of bacterial counts. A culture medium can only be used for the counting of viable bacteria that are capable of forming colonies in the presence of nutrients.[20] Real-time PCR has revolutionized gene assessment.[41] However, this method has its limitations as well, such as the detection of all bacteria present in a sample, including both viable and dead bacteria. The present study used real-time PCR along with PMA; this technique increases the sensitivity of detection of viable cells.[20]

In vitro biofilm formation on surfaces was a limitation of this study. Since biofilm formation in the oral environment is a complex procedure, the results of this in vitro study cannot be transferred to a clinical setting.[42] Several factors, such as wettability, chemical composition, surface energy, hydrophobicity, and surface roughness, affect the bacterial adhesion to surfaces of implant components, and these factors could not be evaluated in this study.[34,35]

Conclusion
Within the limitations of this study, the results showed that the surface properties of dental implant abutments may affect the adhesion of the bacteria responsible for peri-implant inflammatory diseases. The results also showed that the bacterial count was the highest on laser-treated titanium surfaces, second highest on zirconia surfaces, and lowest on titanium surfaces.

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Disclaimer
The authors report no conflicts of interest pertaining to any of the products or companies discussed in this article. The manufacturer (BioHorizons) provided the titanium, zirconia, and laser-treated discs used in this study.

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