Correlation Between Cell Surface Adhesins Genes and Biofilm Formation in Streptococcus Mutans Bacteria Isolated from Dental Caries

Ahmed Turki Abdul Hassan[1], Farah Tareq Al-Alaq[2]
[1, 2] Department of Biology, College of Science, University of Babylon, Babylon, Hilla City, Iraq
Email: ahmedalnadway34@gmail.com

Abstract
Objectives: Streptococcus mutans (S. mutans) has significant virulence factors associated with the etiology and pathophysiology of dental plaque and caries through adherence to the tooth surface and biofilm formation. The purpose of this study was to investigate correlation between cell surface adhesins genes and biofilm formation in Streptococcus mutans bacteria isolated from dental caries.Materials and Methods: Selective cultivation on mitis-salivarius-bacitracin (MSB) agar, biochemical tests and VITEK 2 system were carried out to isolate and identify S. mutans bacteria from 100 patients with dental caries. Identification of S. mutans isolates was confirmed by conventional PCR using specific pairs of PCR primers targeting Sm479 gene of S. mutans. PCR also were carried out to detect gbpA, gbpB, and spaP genes. Biofilm formation by S. mutans isolates was estimated using the microtiter plate crystal violet method. Results: A total of 42 S. mutans isolates were recovered from 100 clinical samples collected in the current study. There was a statistically significant difference in the biofilm formation ability among the isolates of S. mutans based ($p < 0.05$). Percentages of biofilm formation grade were 14.28% (6/42) strong biofilm producer, 35.71% (15/42) moderate biofilm producer, 28.57% (12/42) weak biofilm producer and 21.42% (9/42) non biofilm producer. Molecular detection of gbpA, gbpB and spaP genes was done for all S. mutans. The results showed that 80.95% (34/42), 85.71% (36/42) and 76.19% (32/42) of S. mutans isolates gave positive results for gbpA, gbpB and spaP and genes, respectively. The gbpA gene encodes glucan-binding protein (Gbp)A, the gbpB gene encodes GbpB, and the spaP gene encodes cell surface antigen, SpaP. Conclusion: It can be concluded that in the absence of only one of (gbpA, gbpB, spaP) genes, the biofilm will be weak, but in the absence of two or more of these genes, the biofilm will not form. Thus, each of these genes has own role in biofilm formation and specific relation to other genes responsible for biofilm formation of the bacterial isolates.
Keywords: biofilm formation, cell surface adhesins, *Streptococcus mutans*, mitis-salivarius-bacitracin agar

1. Introduction

Dental caries, the most common infection affecting the oral cavity and one of the most prevalent infectious diseases worldwide, is characterized by the enamel demineralization of the tooth (Souissi *et al.*, 2021).
Oral microbiota, one of the most complex microbial communities in the human body, contains about 700 kinds of microorganisms that inhabit the human mouth (Lu *et al.*, 2019). Oral Streptococci are primarily members of the viridans group Streptococci, a group of 20 species that are commensal occupants of the oropharyngeal cavity, as well as the gastrointestinal and genital tracts of mammals, and are thought to be the principal cause of dental caries in mammals (Abo Bakr *et al.*, 2021).
Mutans Streptococci (MS) species that have been implicated in dental caries are *Streptococcus mutans* (serotypes c, e, f) and *Streptococcus sobrinus* (serotypes d, g), and are facultative anaerobes, non-motile and catalase-negative gram-positive cocci (Nomura *et al.*, 2020). *S. mutans* is considered the most important etiological microbe of dental caries (Zhang *et al.*, 2020). *S. mutans* bacteria are facultatively anaerobic Gram-positive cocci that belong to the lactic acid-producing bacteria (Chavan, 2015).
The formation of biofilms on tooth surfaces is a predominant factor in the etiology of dental caries (Valen and Scheie, 2018). *S. mutans* is a key contributor to the formation of the pathogenic dental biofilms, mainly due to its ability to synthesize extracellular polysaccharides such as water insoluble glucans or fructans by the action of glucosyltransferases (GTFs) and fructosyltransferase (FTF) (Aqawi *et al.*, 2021).
The glucans are the primary keys that comprise the matrix in cariogenic biofilms (Swedan *et al.*, 2018). Non enzymatic glucan-binding proteins (Gbps) can bind to glucan and are assumed to take part in the sucrose dependent adhesion and the cohesive nature of the dental plaque biofilm (Wang *et al.*, 2020).
Cell surface adhesins of *S. mutans*, such as Gbps and SpaP, are important for bacterial adhesion to tooth surface. *S. mutans* carry *gbp*s genes (*gbpA*, *gbpB*, and *gbpC*) related with the adhesion. The *gbpA*, *gbpB*, and *gbpC* genes encode GbpA, B, and C,

which are known to play an important role in the adhesion of *S. mutans* to glucan molecules, a kind of extracellular polysaccharide of plaque matrix (You, 2019).

GbpA contributes to the development of optimal plaque biofilm, which minimizes stress on the bacterial population. A deficiency of GbpA results in loose binding to the EPS matrix, resulting in a weak non-uniform biofilm structure. Thus, GbpA has important roles as a protein for formation of firm and stable biofilm (Matsumoto-Nakano, 2018).

The *gbpB* gene expresses an adhesion protein named glucan-binding protein B which facilitates the binding of glucan to the physical and biological surfaces (Rouabhia and Semlali, 2021; Wang *et al.*, 2020). It was also found to be homologous with peptidoglycan hydrolases of other Gram-positive microorganisms, while results of a comparative genomic analysis of the *gbpB* region suggested a functional relationship between genes involved in cell shape and cell wall maintenance (Fujita *et al.*, 2007).

S. mutans also carry the *spaP* gene, which encodes the cell surface antigen, SpaP. The SpaP is also known as Ag I/II, PAc, AgB, Pl, Sr, SpaA, PAg, SspA, SspB and SoaA, which adheres to salivary agglutinin glycoprotein (SAG) and proline-rich protein of the acquired pellicle on the tooth surface as a kind of surface fibrillar adhesion (Jeong *et al.*, 2013). PAc is known to be correlated with virulence of the organism for development of dental caries and participates in bacterial adherence to teeth via interaction with the salivary pellicle, which is termed sucrose-independent adhesion (Ancuceanu *et al.*, 2019). The aim of this study was to investigate correlation between cell surface adhesins genes and biofilm formation in *Streptococcus mutans* bacteria isolated from dental caries.

2. Material and Methods

Study Population

The current study included collection of 100 samples of adults with dental caries aged 18-67years. The study samples were reqruited from patients attending the teaching dental clinics at the College of Dentistry of Babylon University. The samples comprised 100 soft caries lesions obtained from the outermost layer of carious dentin and removed with a sharp, sterile excavator (Villhauer *et al.*, 2017).

Sample processing and identification of *S. mutans* bacteria: soft caries samples were placed in Eppendorf tubes containing one ml brain heart infusion broth, then transferred to the microbiology laboratory within 10-15 min. Next, they were added to 10 ml brain heart infusion broth in the laboratory and incubated at 37°C for 24 h. In the next day, the growth in the brain heart infusion broth was inoculated onto the surface of the growth medium MSB agar by streaking [preparation of MSB agar media was following the method of (Abeas *et al.*, 2020). It was prepared by addition of selective agents: bacitracin antibiotic and sucrose, at the optimal levels determined to the mitis-salivarius agar (MSA). MSA is the main components of the prepared media that suppress the growth of most microorganisms but allows the growth of *Streptococcus spp*. This media prepared according to the manufacturer's instruction by dissolving 90.07 g of MSA in 1000 ml purified/distilled water, and heat to dissolve the medium completely. Afterward sterilized by autoclave at 15 lbs pressure (121°C) for 15 minutes and left to cool until 45-50 °C and aseptically add 1 ml of sterile 1% Potassium Tellurite solution. Mix well and pour into sterile petri plates. To inhibit bacteria other than mutans Streptococci; bacitracin and sucrose were added to MSA medium; since a relative resistance of *S. mutans* to high concentration of both bacitracin and sucrose had been reported], which is the most sensitive and selective media for the culture of *S. mutans* bacteria. *S. mutans* bacteria were identified using the traditional microbiological methods, including culture identification based on colony morphology on the selective media (highly convex, raised, light – blue, frosted glass appearance with smooth surface) (Koneman *et al.*, 1997).

First characterization for identification depends on (Gram staining, microscopic examination and biochemical tests). *S. mutans* isolates were Gram-positive, spherical cells and appear in medium chains, catalase-negative, and their capacity to ferment mannitol, sorbitol, melibiose, raffinose and inulin (Shimomura-Kuroki *et al.*, 2011). Identification of *S. mutans* was confirmed by conventional PCR assay using specific pairs of PCR primers targeting *Sm479* gene of *S. mutans* according to (Chen *et al.*, 2007). *S. mutans* isolates were sub-cultured routinely on brain heart infusion broth and stored as glycerol stock at 20°C for long-term preservation.

Biofilm Formation Assay

Biofilm formation by S. mutans isolates was estimated using the microtiter plate crystal violet method according to (Stepanović et al., 2000; Zhou et al., 2018). Then the isolates of S. mutans from the glycerol stock were cultured on brain heart agar media and incubated at 37°C in a candle jar for 48 h. A sterile plastic loop transferred a loopful of bacterial colonies into a tube containing 5 ml of isotonic saline and adjusted to match the 0.5 McFarland turbidity standard. Then, 100 µl from the standardized saline was transferred into 10 ml brain heart broth and 200 µl of each of the diluted solutions was transferred to a sterile flat-bottom 96-well plate containing 100 µl of fresh media per well (Brain heart infusion broth + 5% sucrose) in triplicates and incubated at 37°C in a candle jar for 24 h. The negative control wells contained all components except the bacteria. Following incubation, the broth was removed, and the wells were gently washed three times with saline. The plates were left to dry, then followed by biofilm quantified using 200 µl of 0.1% crystal violet for 15 min. The excess stain was washed off by saline and inverted on tissues and left to dry, after that

resolubilized by 100 µl of 98% ethanol for 15 min. The optical density (OD) was measured at 570 nm by Stat Fax-2600 microplate reader (Awareness Technology, USA). The results obtained by the microtiter plate were classified according to (Stepanović et al., 2000). the cut-off OD (ODc) of the negative control was defined as: OD ≤ ODc: Non-adherent, ODc < OD ≤ 2 × ODc: Weakly adherent, 2 × ODc < OD ≤ 4 × ODc: Moderately adherent, 4× ODc < OD: Strongly adherent.

PCR-Based Detection of Cell Surface Adhesins Genes

DNA Extraction and PCR Oligonucleotide Primers

The bacterial isolates were cultivated on MSB agar and incubated anaerobically at 37°C for 48 h in a candle jar, then loopful colonies were transferred by a sterile plastic loop into 5 ml of brain heart broth, then incubated at 37°C for 24 h. Genomic DNA was extracted from S. mutans isolates by using Bacterial DNA Extraction Kit (Geneaid, KOREA) following the manufacturer's instructions. PCR oligonucleotide primers used in this study to detect virulence genes, synthesized by (macrogen, Korea), are listed in table (1). The primers used were previously published or designed in this study using the PCR primers designing tool available at NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) using Streptococcus mutans strain NG8 (NZ_CP013237.1) as a DNA template.

For PCR-based molecular identification of S. mutans, the following PCR primers were designed in this study: 5' TCGCGAAAAAGATAAACAAACA -3' (forward primer) and 5'-GCCCCTTCACAGTTGGTTAG -3' (reverse primer). The expected PCR amplicon size is 479 bp. PCR primers were designed to be specific for S. mutans and include a conserved region among all S. mutans Sm479 available sequences in the Gene Bank database. The Primer-Blast analysis indicated the specificity at that time. The lyophilized primer powder was reconstituted using nuclease-free water to achieve a concentration of 100 pmol/µl and then was adjusted to the working solution of 10 pmol/µl.

PCR Reactions

The primers sequences were selected to amplify genes responsible for S. mutans virulence traits are glucan-binding protein A (GbpA), glucan-binding protein B (GbpB), and cell surface antigen and multi-functional adhesion SpaP as shown in table (1). Amplification reactions were carried out using 96 well PCR thermocycler containing 5 µl of template DNA with 1.5 of each forward and reverse primer, 12 µl of Master Mix (Taq DNA Polymerase, optimized green buffer, MgCl2, and dNTPs) and the volume was completed to 25 µl by adding nuclease-free water. Amplification conditions were mentioned in table (2).

Table(1): Virulence Genes and Sequences of PCR Oligonucleotide Primers

Virulence gene	Primers sequence (5' - 3')	Expected amplicon size (bp)
gbpA	F: GGTGGTTCTGTGCCTGATGA R: TTGCCAGCCTGATACACGTT	162
gbpB	F: AGCAACAGAAGCACAACCATCA R: CCACCATTACCCCAGTAGTTTCC	150
spaP	F: GACTTTGGTAATGGTTATGCATCAA R:TTTGTATCAGCCGGATCAAGTG	101

Table 2. Programs of PCR Thermocycling Conditions

Gene	Temperature °C/ Time				
	Initial denaturation for 1 Cycle	Cycling condition for 40 Cycle			Final extension for 1 Cycle
		Denaturation	Annealing	Extension	
Sm479	94/3 min	95/30 sec	55/30sec	72/59 sec.	72/5 min
gbpA	94/3 min	95/30 sec	55/30 sec	72/59 sec.	72/5 min
gbpB	94/3 min	95/30 sec	55/30 sec	72/59 sec.	72/5 min
spaP	94/3 min	95/30 sec	51/30 sec	72/59 sec.	72/5 min

Five µl of mixture reaction were analyzed by 1% TBE agarose gel electrophoresis. The PCR products were stained with Safe Red dye (5 mg/ml) in a 1× TBE buffer at 80 volts for 1 hour and visualized by placing on a UV transilluminator and photographed directly. The size of the PCR products was estimated from the electrophoretic migration of products relative to 6 µl of DNA Ladder ranging from 100-10000 bp (Cleaver Scientific, UK).

3. Statistical Analysis

Statistical analyses were performed by using statistical analysis software (Statistical Package for the Social Science) (SPSS) version 20. Chi-square test (χ^2) was employed to assess the association between qualitative variables and the study groups (disease outcome). Correlation coefficient between two quantitative variables was also computed to know the strength of the relationship between them. For all tests, a probability (P) of <0.05 has been considered as significant.

4. Results

A total of 42 S. mutans isolates were recovered from 100 clinical samples collected in the current study. S.mutans colonies appeared pale-blue in color about 1-2 mm in diameter, avoid or spherical in morphology with raised or convex surface adhered well to the medium surface as shown in figure (1). Also S. mutans isolates are characterized by their ability to ferment a number of sugars such as (mannitol, sorbitol, raffinose, melibiose, sucrose), it gave negative result to catalase, oxidase, urease, Simmon citrate, and motility. While it gave positive result to VP test and it has been shown that S.mutans can grow in 4% Nacl but do not grow in 6.5% Nacl concentration.

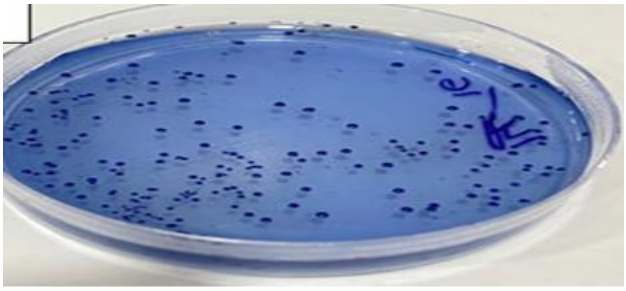

Figure (1): S.mutans colonies on MSB agar

The results of the present study showed that percentages of biofilm formation grade were 14.28% (6/42) strong biofilm producer, 35.71% (15/42) moderate biofilm producer, 28.57% (12/42) weak biofilm producer and 21.42% (9/42) non biofilm producer as shown in figure (2).

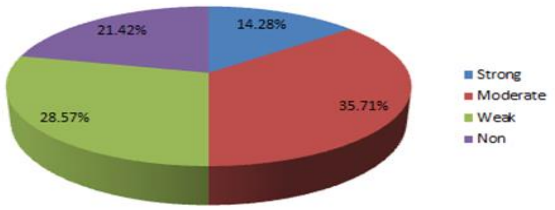

Figure (2): Percentages of biofilm formation grade of S. mutans

The results of this study showed there was a statistically significant difference in the biofilm formation ability among the isolates of *S. mutans* based (p = 0.05).

Conventional PCR was carried out using specific primers targeting the *Sm479* gene, according to the programs listed in table (2). After that gel electrophoresis showed that 42 isolates produced the specific 479 bp DNA fragment when compared with DNA ladder: as shown in figure (3).

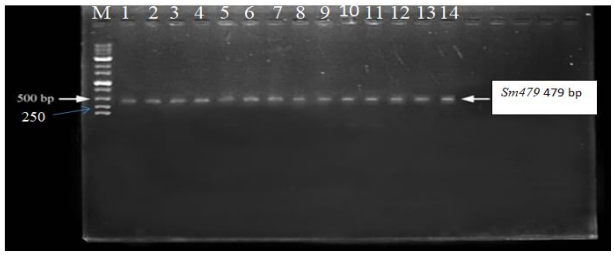

Figure (3): 1% Agarose gel electrophoresis at 80 volt for 1 hour for PCR-based detection of specific (Sm479) gene of S. mutans visualized under U.V light at 280 nm after staining with Safe Red dye. Lane M: DNA marker (100-10000) bp; Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14) isolates give positive result for this gene, the size of product is 479 bp.

Molecular detection of *gbpA*, *gbpB* and *spaP* genes was done for all *S. mutans* using specific primers targeting these genes, according to the sequences and programs listed in table (1) and (2) respectively. After that gel electrophoresis detected that 80.95% (34/42), 85.71% (36/42) and 66.66% (28/42) of *S. mutans* isolates gave positive results for *gbpA*, *gbpB* and *spaP* genes, respectively when compared with DNA ladder; as shown in figure (4 A, B, C).

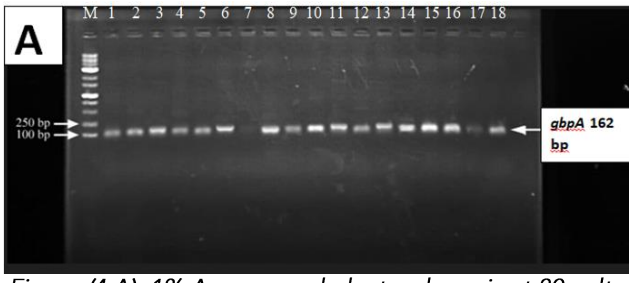

Figure (4 A): 1% Agarose gel electrophoresis at 80 volts for 1 hour to detection of PCR product of gbpA gene (162 bp) in S. mutans visualized under U.V light at 280 nm after staining with Safe Red dye. Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,8,9,10,11,12,13,14,15,16,17,18) isolates give positive result for this gene; Lane (7) isolate give negative result.

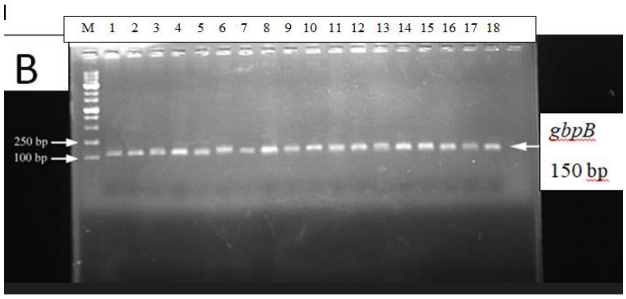

Figure (4 B): 1% Agarose gel electrophoresis at 80 volt for 1 hour to detection of PCR product of gbpB gene (150 bp) in S. mutans visualized under U.V light at 280 nm after staining with (Safe Red dye). Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18) isolates give positive result for this gene.

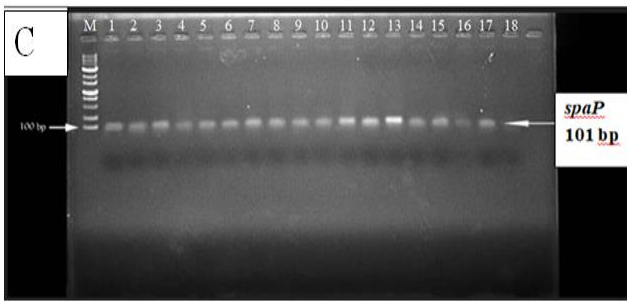

Figure (4 C): 1% Agarose gel electrophoresis at 80 volt for 1 hour to detection of PCR product of spaP gene (101 bp) in S. mutans visualized under U.V light at 280 nm after staining with (Safe Red dye). Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17) isolates give positive result for this gene; Lane (18) isolate give negative result.

The statistical analysis showed a significant correlation between biofilm formation and these genes based on (p = 0.05), as shown in the table (3).

Table (3): Correlation between (*gbpA*, *gbpB*, *spaP*) Genes and Biofilm Formation Grade of *S. mutans*

Gene	Biofilm Formation Grade								P value
	None (9)		Weak (12)		Moderate (15)		Strong (6)		
	No	%	No	%	No	%	No	%	
gbpA	6	66.67	10	83.33	13	86.67	6	100	0.00
gbpB	6	66.67	11	91.67	13	86.67	6	100	0.00
spaP	2	22.22	10	83.33	14	93.33	6	100	0.00
P value	0.011								

5. Discussion

Many studies from the literature revealed that S. mutans bacteria could produce biofilm both in vivo and in vitro conditions (Ahn et al., 2018; Kulshrestha et al., 2016; Senpuku et al., 2019).

The results in this study showed that 78.56 % of S. mutans isolates were able to biofilm forming. This results nearly similar to the results obtained by (Jubair, 2015) who demonstrated (90%) of S. mutans isolates were isolated from dental caries have the ability to biofilm forming.

According to data obtained by this study, the emergence of strong, moderate, weak and non-production of biofilm refer to there was a significant difference in the ability to biofilm formation among the recovered isolates.

The results of this study were in disagreement with the results of (Patidar et al., 2012) who showed that out of 100 clinical isolates (92%) showed strong biofilm forming potential and (8%) clinical isolates showed moderate biofilm formation capability.

These difference in the ability to biofilm formation can be explained by differences in growth condition such as ionic forces, pH and the number of subculture (Grivet et al., 2000).

The results in this study nearly similar to the results obtained by (Hossain et al., 2021) who revealed that gbpA gene was present in all clinical isolates with expected size PCR product.

The results in this study nearly similar to the results obtained by (Abo Bakr et al., 2021) who found that the gbpB gene was detected in all S. mutans isolates and produced the expected PCR product of 150 bp.

The result of this study was in consistence with the results of (Abd Al- Zahra, 2018) who found that spaP gene present in (71.42%) of isolates.

While the results of this study are in disagreement with the results obtained by (Israa and Mahdi, 2015) who showed that the *spaP* gene was present in (50%) of isolates from dental caries infections.

The results of this study proved that in the absence of any one of these genes, biofilm formation often will be affected. This result was consistent with the results of (Lynch *et al.*, 2007) who revealed that the reason for the decrease in biofilm thickness varied based on the particular Gbp lost.

GbpA has important role as a protein for formation of firm and stable biofilm,because its deficiency results in loose binding to the EPS matrix, resulting in a weak non-uniform biofilm structure (Matsumi et al., 2015).

6. Conclusion

S. mutans isolates harbored gbpA, gbpB and spaP genes responsible for the virulence traits including glucan-binding protein A, glucan-binding protein B, and cell surface antigen and multi-functional adhesion, respectively. There was a significant difference in the biofilm formation ability among the recovered isolates of S. mutans.

It can be concluded that in the absence of only one of (*gbpA*, *gbpB*, *spaP*) genes, the biofilm will be weak, but in the absence of two or more of these genes, the biofilm will not form. Thus, each of these genes has own role in biofilm formation and specific relation to other genes responsible for biofilm formation of the bacterial isolates. Each cell surface adhesin makes a unique contribution to the development of a mature and optimal dental biofilm, which minimizes stress on the bacterial population through its important role in cellular adherence to tooth surfaces, binding proteins and exopolysaccharides for construction of biofilm and maintenance of a balanced environment. Therefore, any loss of these genes would alter biofilm architecture. Thus, *gbpA*, *gbpB* and *spaP* genes play important role in formation of firm and stable dental biofilm.

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