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Diabetic Foot Ulcer Infections and *Pseudomonas aeruginosa* Biofilm Production During the COVID-19 Pandemic

Marwa A. Yakout* and Ibrahim A. Abdelwahab

Department of Microbiology and immunology, Faculty of Pharmacy, Pharos University in Alexandria, Egypt.

Abstract

During the different waves of the coronavirus (COVID-19) pandemic, there has been an increased incidence of diabetes mellitus and diabetic foot infections. Among gram-negative bacteria, *Pseudomonas aeruginos*a is the predominant causative agent for diabetic foot ulcer infections in low-resource countries. *P. aeruginosa* possesses a variety of virulence factors, including biofilm formation. Biofilm formation is an important benchmark characteristic in the pathophysiology of diabetic foot ulceration. The main objective of the current study was to identify the most commonly isolated organisms and their antibiotic susceptibility patterns in diabetic foot patients during the COVID-19 pandemic. We also determined the genes associated with bacterial persistence and biofilm formation in the predominantly isolated organism. Accordingly, 100 wound swab samples were collected from diabetic foot patients from different hospitals in Alexandria, Egypt. Through phenotypic detection of biofilm formation, 93% (40) of the 43 *P. aeruginosa* isolates examined were categorized as biofilm producers. Molecular detection of the biofilm-encoding genes among the 43 *P. aeruginosa* isolates was as follows: algD (100%), *pelF* (88%) and *pslD* (49.7%), and this highlights a need for biofilm formation inhibitors to prevent the persistence of bacterial pathogens, and thus achieve better clinical outcomes in diabetic foot ulcer infections.

Keyword: Diabetic foot ulcer infections, *Pseudomonas aeruginosa,* biofilm, COVID-19

***Correspondence:** marwa.atef@pua.edu.eg; +201224296850

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INTRODUCTION

According to the International Diabetes Federation, there has been an increased incidence of diabetes mellitus (DM) during the different waves of the coronavirus (COVID-19) pandemic.[1-4] A study in Wuhan reported that diabetic patients constituted 2–20% of all positive cases, and accounted for 7.1% of intensive care unit admissions.[5,6] Another study in New York reported that 33.8% of COVID-19 positive patients were diabetic. Accordingly, several reports have attempted to determine the reason for the correlation between COVID-19 and DM. One hypothesis is that the angiotensin-converting enzyme 2 (ACE2) receptor necessary for the entry of SARS-CoV-2 is overexpressed in diabetic patients taking antidiabetic medications.[5,7-9] In addition, the use of corticosteroids in COVID-19 patients increases blood glucose levels in both diabetic and non-diabetic individuals.[5,10] Additionally, diabetic patients are more vulnerable to viral and bacterial infections.[5, 11] The COVID-19 pandemic has posed many challenges for the diabetic community, such as lack of sufficient resources, overworked health care workers, and scarcity of proper care for diabetic patients to avoid the development of infections.[12, 13]

Diabetic foot ulcer (DFU) is a common complication of DM, with an increasing prevalence worldwide.[14] Thirty-three percent of all diabetic patients are expected to be diagnosed with DFUs at least once during their lifetime. Untreated DFUs can result in leg amputations, permanent disability, and increased mortality rates in DM patients.[13] DFUs are most commonly associated with clinical infections with different etiological agents.[15-22] Microbiological studies have shown that diabetic foot ulcers generally have polymicrobial etiologies. The etiological agent may differ from one individual to another, and from country to country. Aerobic gram-positive bacteria that are frequently isolated in DFUs include *Staphylococcus* spp. beta-hemolytic *Streptococcus* and *Enterococcus* spp. *Escherichia coli, Proteus mirabilis,* and *Klebsiella pneumoniae* are among the gram-negative bacteria that are most commonly isolated in DFUs.[23] *P. aeruginosa* is the predominant causative agent for DFUs in low-resource countries.[15-22] *Pseudomonas* spp. are generally encountered in immunocompromised patients due to their high pathogenicity and variety of virulence factors, including biofilm formation.[24] Virulence factors and biofilm formation are the benchmark characteristics in the pathophysiology of DFUs.[25] The formation of biofilms by bacteria is considered to be the cornerstone that provides bacteria protection against several environmental factors, mediates persistence in medical devices, facilitates immune system evasion, and contributes to the development of antimicrobial resistance.[15,21,22] Antibiotic resistance is a global public health concern, especially in patients with diabetic foot infections (DFIs). Multidrug resistance results in poor clinical outcomes, financial burden, and increased morbidity and mortality in DFU patients.[23]

The main objective of the current study was to identify the commonly isolated organisms and their antibiotic susceptibility patterns in DFU patients during the COVID-19 pandemic. In addition to phenotypic detection of biofilms, we also determined genes encoding biofilm formation in the predominantly isolated organism.

MATERIALS AND METHODS

One hundred wound swab samples were collected from DFU patients at the Vascular Surgery and Diabetic Foot Unit of Abou Hommos Central Hospital, Alexandria Main University Hospital, Mowasat Hospital, Abou Qir Central Hospital, and Medical Research Institute, in Alexandria, Egypt, between January 2020 and January 2021. The study was approved by the Ethics Committee in Pharos University in Alexandria, and all procedures were performed according to Helsinki ethical standards. Specimens were subjected to Gram staining, and were cultured in mannitol salt agar, MacConkey agar, blood agar, and Sabouraud dextrose agar (Oxoid, Cambridge, UK). Isolates were identified using standard biochemical methods,[26] and *P. aeruginosa* isolates were confirmed with MALDI-TOF/MS (Bruker, Billerica, MA, USA). The identified stock cultures were preserved at −80 °C in 15% glycerol.

Antimicrobial susceptibility test

Antibiotic susceptibility tests were done by disc diffusion method on Mueller–Hinton agar plates (Oxoid) according to Clinical Laboratory Standards Institute (CLSI) 2019 guidelines.[26] The antibiotic discs include: Ceftazidime (CAZ, 30 µg),

Cefepime (FEP, 30 µg), Piperacillin-tazobactam (TZP, 100 µg/ 10 µg), Aztreonam (ATM, 30 µg), Levofloxacin (LEV, 5 µg), Amikacin (AMK, 30 µg), Azithromycin (AZM, 15 µg), Erythromycin (E, 15 µg), Gentamycin (CN, 10 µg), Tetracycline (TE, 30 µg), Cefoxitin (FOX, 30 µg), Chloramphenicol (C, 30 µg), Ampicillin (AMP, 10 µg), Imipenem (IPM, 10 µg), Linezolid (LZD, 30 µg), Amoxicillin-clavulanate (AMC, 20 µg/10 µg), and Methicillin (MET, 5 µg).

Quantification of Biofilm formation by *P. aeruginosa*

200 µL of overnight broth subculture of the tested isolates in sterile trypticase soy broth (TSB) (Oxoid), equivalent to 1.5×10^8 CFU/mL, was added to each well of a 96-well flat-bottomed microtiter plate, in triplicates. The plates were then incubated at 35 °C for 24 h. The next day, the medium was discarded, and the wells were washed with phosphate-buffered saline (PBS, pH 7.2) (Sigma-Aldrich, Milan, Italy). Biofilm fixation was performed by incubating the 96-well plates at 60 °C for 1 h. 0.1% (w/v) Crystal violet was used for staining, and was extracted with 99.5% ethanol.[27] The optical density (OD) value of each well was measured at 620 nm on a microtitre plate ELISA reader (STATFAX2100, Fisher Bioblock scientific, France). The isolates were characterized according to their biofilm-forming ability as strong, moderate, weak, or non-biofilm producers, as previously described by Stepanovic et al.[28]

Molecular detection of biofilm encoding genes in *P. aeruginosa*

DNA was extracted from the *P. aeruginosa* isolates using the boiling method.[29] DNA amplification was performed using Master Mix (iNtRON biotechnology, Seongnam, South Korea). The primers30 (SBS GeneTech, Beijing, China) and the annealing temperatures used are listed in Table 1. PCR program was as follows: initial denaturation at 95 °C for 5 min, followed by denaturation at 95 °C for 1 min, 30 cycles at 58 °C for *algD, pelF* and 56 °C for *pslD* gene for 40 seconds, then 72 °C for 45 seconds, and a final elongation at 72 °C for 5 min.

PCR products were separated on 2% agarose gel in TBE buffer, stained with 2 µg/mL ethidium bromide, and visualized under ultraviolet transillumination (BIORAD, Italy).[30,31]

Statistical analysis

Statistical analysis of the data was performed using IBM SPSS software version 20.0. (IBM Corp, Armonk, NY, USA). The chi-square test and Fisher's exact test were used. Statistical significance was set at a p-value of 5% or lower.

RESULTS

The present study included swabs from 100 DFU patients (75 male and 25 female) admitted to the Vascular Surgery and Diabetic Foot Unit of Abou Hommos Central Hospital, Alexandria Main University Hospital, Mowasat Hospital, Abou Qir Central Hospital, and Medical Research Institute, Alexandria, Egypt, between January 2020 and January 2021. The ages ranged from 44 to 76 years.

Microbiological Culture Results

The microbiological culture of the 100 DFU swabs yielded monomicrobial bacterial growth in 76 samples (76%), polymicrobial bacterial growth (2–3 microorganisms) in 20 samples (20%), and *Candida albicans* in 4 samples (4%) (Table 2).

The microbial species isolated from the DFU specimens are listed in Table 3. The

Table 1. Primers used for detection of genes involved in Biofilm formation by *Pseudomonas aeruginosa*

Gene	Primers	Annealing Temp.	Band Size
algD	F-CTACATCGAGACCGTCTGCC R-GCATCAACGAACCGAGCATC	58	593
pelF	F-GAGGTCAGCTACATCCGTCG R-TCATGCAATCTCCGTGGCTT	58	789
pslD	F- TGTACACCGTGCTCAACGAC R- CTTCCGGCCCGATCTTCATC	56	369

Table 2. Culture results of 100 diabetic foot ulcers

Growth pattern in culture	No.	%
Bacterial growth	96	96.0
Monomicrobial	76	76.0
Polymicrobial microorganism (2 – 3)	20	20.0
Fungal growth		
Candida albicans	4	4.0
Total	100	100.0

majority of the isolates were gram-negative (88%), and *P. aeruginosa* was the predominant gram-negative bacteria isolated (43%), followed by *K. pneumoniae* (21%), *P. mirabilis* (11%), *E. coli* (9%), and *Serratia marcescens* (4%). The gram-positive bacteria isolated were *S. aureus* (5%) and *Enterococcus* spp. (2%). *C. albicans* was the only fungal species isolated (5%).

Antibiotic Susceptibility Profile

Antibiotic susceptibility in the forty-three *P. aeruginosa* isolates was as follows: Ampicillin (100%), followed by Aztreonam (74%), Amikacin (72%), Levofloxacin, Amoxicillin-clavulanate (69.7%), Cefoxitin (46.5%), Ceftazidime (41%), Imipenem (32.5%), and varied degrees of resistance to other antibiotics (Table 4).

Antibiotic susceptibility profile of the twenty-one *K. pneumoniae* isolates was as follows: Ampicillin (95%), followed by Amoxicillin-clavulanate, Levofloxacin and Cefoxitin (76%), Amikacin (71.4%), Aztreonam (57.1%), and varied degrees of resistance to other antibiotics. The eleven *P. mirabilis* isolates were resistant to Aztreonam, Cefoxitin, Cefazolin and Chloramphenicol (72%), Ampicillin, Piperacillin-tazobactam and Levofloxacin (63%), Amikacin (54%), and varied degrees of resistance to other antibiotics. Of the nine *E. coli* isolates, 77% were resistant to levofloxacin, followed by amoxicillin-clavulanate, gentamycin, amikacin and cefoxitin (66%), and ampicillin (55%). Four isolates of *S. marcescens* were resistant to ampicillin (25%) (Table 4).

Of the five *S. aureus* isolates, 96% were resistant to tetracycline and methicillin and with varying degree of resistance to other antibiotics. Of the two *Enterococcus* spp. isolates, 50% were resistant to cefoxitin, as shown in Table 4.

Table 3. Frequency of microbial isolates from 100 diabetic foot ulcers

Gram reaction		No.	%
Gram negative	*P. aeruginosa*	43	43.0
	K. pneumoniae	21	21.0
	P. mirabilis	11	11.0
	E. coli	9	9.0
	Serratia marcescens	4	4.0
Gram positive	*S. aureus*	*5*	*5.0*
	Enterococcus spp.	2	2.0
Fungi	C. albicans	5	5.0
Total		100	100.0

Quantification of Biofilm formation by *P. aeruginosa*

Of the forty-three *P. aeruginosa* isolates examined, twenty-three (53.5%) were strong biofilm producers, ten (23.2%) were moderate biofilm producers, seven (16.3%) were weak biofilm producers, and just three isolates (7%) were non-biofilm producers.

Molecular detection of Biofilm encoding genes in *P. aeruginosa*

The following genes encoding biofilm exopolysaccharides were identified in the 43 *P. aeruginosa* isolates: *algD* (100%), *pelF* (88%) and *pslD* (49.7%). The presence of *algD, pslD,* and *pelF* genes was noted in a large proportion of the 43 *P. aeruginosa* isolates. Our findings revealed that 82.6 % of the 23 strong biofilm producers had the genotypic pattern *algD* +/*pslD* +/*pelF* +, while the rest were *algD* −/*pslD* −/*pelF* −. On the other hand, 66.6% of the three non-biofilm producers carried the biofilm encoding genes, as shown in Table 5.

DISCUSSION

DFU is a debilitating consequence of DM with an increasing prevalence worldwide.[14] During the different waves of the COVID-19 pandemic, DM was increasingly diagnosed worldwide. Based on the recommendation of the International Diabetes Federation, increased care should be given to diabetic patients to avoid the devastating complications of DM.[1-4] Among the hypothesized reasons contributing to the increased incidence of DFIs during the COVID-19 pandemic is the increased expression of the ACE2 receptor necessary for the entry of SARS-CoV-2

Table 4. Resistance profile of the tested isolates to different antimicrobial agents

Name of micro-organism	Total No. of isolates	Antibiotics % of Resistance																
		AZM	E	ATM	IPM	FEP	CAZ	AMC	AMP	TZP	LEV	CN	AMK	FOX	MET	C	LZD	TE
Pseudomona aeruginosa	43	–	–	32 (74.0)	14 (32.5)	12 (27.9)	18 (41.0)	30 (69.7)	43 (100.0)	11 (25.5)	30 (69.7)	30 (69.7)	31 (72.0)	20 (46.5)	–	–	–	–
Klebsiella pneumoniae	21	–	–	12 (57.1)	5 (23.0)	7 (33.3)	–9 (42.0)	16 (76.0)	20 (95.2)	8 (38.0)	16 (76.0)	–	15 (71.4)	16 (76.0)	–	9 (42.0)	–	9 (42.0)
Proteus mirabilis	11	–	–	8 (72.0)	2 (18.0)	0 (0.0)	1 (9.09)	2 (18.0)	7 (63.0)	7 (63.0)	7 (63.0)	2 (18.0)	6 (54.0)	8 (72.0)	–	8 (72.0)	–	8 (72.0)
Escherichia coli	9	–	–	3 (33.0)	2 (22.0)	5 (55.0)	–	6 (66.0)	5 (55.0)	2 (22.0)	7 (77.0)	6 (66.0)	6 (66.0)	6 (66.0)	–	3 (33.0)	–	3(33.0)
Serratia marcescens	4	–	–	–	0 (0.0)	0 (0.0)	–	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	–	0 (0.0)	–	–
Staphylococc aureus	5	2 (40.0)	2 (40.0)	–	–	–	–	–	–	–	1 (20.0)	–	–	1 (20.0)	4 (80.0)	1 (20.0)	0 (0.0)	4 (80.0)
Enterococcus spp.	2	0 (0.0)	0 (0.0)	–	–	–	–	–	–	–	0 (0.0)	–	–	1 (50.0)	–	0 (0.0)	0 (0.0)	0 (0.0)

in diabetic patients.[5,7-9] Other reported reasons include the rise in blood glucose levels noted in patients receiving corticosteroids.[5,10] Additionally, diabetic patients are more vulnerable to viral and bacterial infections.[5,11] Other challenges contributing to the increased incidence of DFIs include a lack of sufficient resources, overworked health care workers, and a scarcity of proper care for diabetic patients to avoid the development of infections.[13]

The present study included swab samples from 100 patients with DFUs (75 male and 25 female). Other studies have also supported the male predominance of foot ulceration and its associated complications.[32,33] Culture results of the 100 specimens showed 76% monomicrobial bacterial growth, 20% polymicrobial bacterial growth, and 4% fungal growth. Hitam et al. also reported a similar percentage (28.8%) of polymicrobial infections in DFI patients.[34] Additionally, culture results showed that the majority of isolates were gram-negative (88%) bacteria, and *P. aeruginosa* was the predominant microorganism isolated (43%), followed by *K. pneumoniae* (21%), *P. mirabilis* (11%), *E. coli* (9%), and *S. marcescens* (4%). Among gram-positive bacteria, *S. aureus* was the most common isolate. *Pseudomonas* spp. was regarded as the main causative agent of DFI by Hitam et al.,[34] Hatipoglu et al.,[35] Hobizal et al.,[36] and Ramakant et al.[37] *P. aeruginosa* is also reported to be the most predominant causative agent for DFIs in low-resource countries.[15-22] *P. aeruginosa* should not be regarded as a normal flora in burn wounds and diabetic foot patients. *P. aeruginosa* can cause extensive tissue damage in diabetic patients and result in sepsis.[24] Additionally, *S. aureus* has been reported to be the most common gram-positive etiological pathogen of DFI.[34-37]

Antibiotic susceptibility testing revealed that 100% of the forty-three *P. aeruginosa* isolates were resistant to Ampicillin, followed by Aztreonam (74%), Amikacin (72%), Levofloxacin, Gentamycin and Amoxicillin-clavulanate (69.7%), Cefoxitin (46.5%), Ceftazidime (41%), Imipenem (32.5%), and varied degrees of resistance to other antibiotics was observed. Multidrug resistance (MDR) was observed in 30 (69.7%) of the *P. aeruginosa* isolates. Sivanmaliappan et al.[24] reported that 55.5% of *P. aeruginosa* were

Table 5. Relationship between phenotypic biofilm characteristic and genotypic biofilm characteristic among *P. aeruginosa* isolates (Manual)

Phenotypic pattern of biofilm, no. (%)	Genotypic pattern of biofilm, no. (%)		χ^2	FEp
	AlgD +/pslD +/pelf +	AlgD -/pslD -/pelf -		
Strong 23 (53.5%)	19 (82.6%)	4 (17.4%)	0.953	0.473
Moderate 10 (23.2%)	7 (70%)	3 (30%)	0.332	0.674
Weak 7 (16.3%)	5 (71.4%)	2 (28.6%)	0.132	0.656
Non 3 (7%)	2 (66.6%)	1 (33.4%)	0.184	0.558
Total	33	10		

χ^2: Chi-square test, FE: Fisher Exact, p: p-value for comparing between the studied groups

multidrug resistant (MDR); 100% were resistant to ampicillin, 83.3% to piperacillin, and 66.6% to ceftazidime, gentamycin and imipenem. However, our results show that ceftazidime, imipenem, and piperacillin/tazobactam combination display higher activity as antipseudomonal agents. Banar et al.[30] also stated that ceftazidime displayed high activity in *P. aeruginosa* isolates. In a study on DFI in Tanzania, resistance was noted for all commonly used antibiotics, except imipenem (100% sensitivity). This can be attributed to the fact that imipenem is expensive in low-resource countries.[38] Previous reports described the increased efficacy of piperacillin-tazobactam against several virulence traits, such as adhesion, biofilm production, and flagellin production.[39] The increased prevalence of MDR has been noted in different studies worldwide.[30,31,40,41] This can be attributed to extensive use of antibiotics, which gives a selective advantage for survival of pathogenic bacterial strains. MDR *P. aeruginosa* guarded by biofilms that are difficult to penetrate can survive and develop more resistance.[41,42]

Biofilm production is the benchmark characteristic for the development of DFIs, and provides a balance between colonization and infection.[25] Bacteria within biofilms produce their own matrix of extracellular polymeric substances (EPS). EPS contains glycoproteins and polysaccharides that provide protection against several environmental factors, mediate persistence in medical devices, facilitate immune system evasion, and contribute to the development of antimicrobial resistance.[15,21,22] There are three major exopolysaccharides that significantly contribute to the formation and stabilization of the biofilm matrix of *P. aeruginosa*. The pentasaccharide *Psl* is essential to promote both cell–cell and cell–surface interactions, thereby initiating biofilm formation and providing structural support to the formed biofilm. The PslD protein is encoded by the *pslD* gene, a part of the *psl* operon. The *PslD* protein is located in the periplasm/outer membrane and contributes to the export of essential biofilm exopolysaccharides. Alginate is another important polymer that significantly stabilizes biofilm formation and provides additional protection. The synthesis of alginate protein is mediated by the *algACD* operon. The *algD* gene controls the synthesis of the alginate proteins. The *algD* gene controls the production of the final precursor, GDP-mannuronic acid, one of the two monomers of alginate. The pellicle operon controls the synthesis of the third major exopolysaccharide, the Pel protein, which is responsible for pellicle formation.[30,31]

In this context, biofilm formation was evaluated both phenotypically using the crystal violet assay, in addition to molecular detection of genes responsible for biofilm formation, *algD*, *pslD*, and *pelf* . Phenotypic characterization revealed that 93% (40) of the 43 *P. aeruginosa* isolates examined were biofilm producers; 53.4% (23) of *P. aeruginosa* isolates were strong biofilm producers, 23.3% (10) were moderate biofilm producers, 16.3% (7) were weak biofilm producers, and only 6.9% (3) were non-biofilm producers. Kamali et al. reported that 83.75 % of their *P. aeruginosa* isolates were biofilm producers with variable degrees of biofilm production.[31] Banar et al.[30] reported that out of 57 *P. aeruginosa* isolates tested 55 (96.5%) isolates were biofilm producers

with variable degrees of biofilm production, and only 2 (3.5%) isolates were regarded as non-biofilm producers.

The frequency of genes encoding biofilm exopolysaccharides among the 43 *P. aeruginosa* isolates was as follows: *algD* (100%), *pelF* (88%) and *pslD* (49.7%). The present study showed a high prevalence of all three genes, *algD*, *pslD*, and *pelf*, in a considerable proportion of the *P. aeruginosa* isolates. Approximately 82.6 % of the 23 strong biofilm producers showed a *algD* +/*pslD* +/*pelF* + genotypic pattern, while 17.4 % showed *algD* −/*pslD* −/*pelF* −.

Banar et al.[30] also reported similar frequencies of biofilm genes: *pelF* (93%), *pslD* (54.65%), and *algD* (100%), with *algD* +/*pslD* +/*pelF* + being the predominant genotypic pattern among their isolates. Another study by Kamali et al.31 reported that *algD* +/*pslD* +/*pelF* + genotypic pattern (87.5%) was the predominant pattern among their isolates. Pournajaf et al.[43] reported that the frequency of *pslD* and *pelF* genes was 89.5% and 57.3%, respectively, in their isolates. Ghadaksaz et al.[44] reported a frequency of 83.7% for *pslD* and 45.2% for *pelF* in their isolates. However, to the best of our knowledge, only a few studies have investigated the presence of biofilm-encoding genes, *algD*, *pslD*, and *pelf*.[31]

CONCLUSION

During the different waves of the COVID-19 pandemic, there has been an increased incidence of DM and DFIs. *P. aeruginosa* is the predominant etiological agent for DFIs. In the present study, the majority of *P. aeruginosa* isolates were MDR and biofilm producers. A high prevalence of biofilm-encoding genes were identified in this study, highlighting a need for inhibitors of biofilm formation to prevents the persistence of bacterial pathogens, and thereby achieve better clinical outcomes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Both the authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILTY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

The study was approved by the Unit of Research Ethics Approval Committee [UREAC], Pharos University in Alexandria, Egypt.

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