

Modulatory Effects of Dexamethasone upon Virulence Attributes of *Pseudomonas Aeruginosa* ATCC® 27853™

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a versatile and opportunistic human pathogen whose virulence derives from several factors as extracellular proteases, pyocyanin and pyoverdine and biofilm formation, among others. Dexamethasone is a glucocorticoid widely used as anti-inflammatory. Despite its immunosuppressive role, the interplay between dexamethasone and bacterial virulence remains uncovered. **Objectives:** In this study, the dexamethasone modulatory effects on planktonic growth, surface adhesion, cell surface hydrophobicity, biofilm formation and pyocyanin and protease secretion by *P. aeruginosa* ATCC®27853™ was evaluated. **Results and Conclusion:** The results showed that dexamethasone decreases planktonic cell growth rate and biofilm development. In other hand, the medicine increases proportional pyocyanin secretion and, in high concentrations, collaborates with the bacterial adherence.

Key words: *Pseudomonas aeruginosa*, dexamethasone, biofilm formation, pyocyanin, protease

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INTRODUCTION

Pseudomonas aeruginosa is a versatile human opportunist pathogen that can survive in many different environmental conditions. It is associated with many cases of infection in different parts of human body, especially when there are pre-existent injuries as skin burns and other types of wound^{1,2}. It is also the most important lung pathogen in patients with cystic fibrosis³.

The pathogenicity of *P. aeruginosa* is mainly related with its capacity to degrade host's tissues by the action of proteases and exotoxins and also to escape from antibiotic attacks when in biofilm phenotype^{4,5}. The genome of *P. aeruginosa* encodes a wide arsenal of virulence factors which includes the production of hemolytic and non hemolytic phospholipase, rhamnolipid, pyocyanin, pyoverdine and alginate; also, hemolytic and non hemolytic phospholipase,

rhamnolipid, pyocyanin, pyoverdine and alginate; also, some adhesins and lectins that mediate the bacterial adhesion to biotic and abiotic surfaces. The manifestation of virulence factors, in fact, secondary metabolites is controlled by a quorum sensing system that comes from the communication between cells by N-Acyl homoserine lactones. The expression of virulence factors still directly depends on the physical environment conditions or nutrient availability; these are responsible to determine the stimulation or repression of the growing rate and metabolic activity of bacterial cells^{6,7}.

The dexamethasone is a glucocorticoid ca. twenty five times more powerful than cortisone. It is from synthetic origin and presents fast therapeutic response. This molecule is used as anti-inflammatory and anti-allergic drug, usually prescribed on the treatment of cystic fibrosis. However, available data about the interplay between dexamethasone and *P. aeruginosa* infection shows that high doses of glucocorticoid may impair pulmonary host defense by increasing bacterial density⁸.

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By other side, it is practically impossible to avoid the exposition of a pathogen as *P. aeruginosa* after the xenobiotic intake⁹. It is known that glucocorticoids may induce phenotypical alterations that result in deferent effects on the growing and antimicrobial susceptibility¹⁰. Surprisingly, the dexamethasone influences on the expression of virulence factors of *P. aeruginosa* still remain unknown.

The present study evaluated the dexamethasone effects on planktonic and biofilm growths, adhesion to surfaces, proteolytic activity and pyocyanin secretion of *P. aeruginosa* ATCC®27853™.

MATERIALS AND METHODS

Strain and culture media: The strain here used was *P. aeruginosa* ATCC®27853™. Culture media used were (i) Luria Bertani enriched (enrLBB) with 150 mM (NH₄)₂SO₄, 100 mM KH₂PO₄, 34 mM sodium citrate, 1 mM MgSO₄ and 0.1% glucose (pH 7) and (ii) 0.25% trypticasein soy broth plus 5% peptone (PTSB). The dexamethasone phosphate (Ducto Pharmaceuticals Co.) was added to the culture broths until 0.001, 0.01, 0.1, 1 and 10 μM which is in conformity with the plasmatic concentration range previously determined¹¹.

Adaptation inocula: In order to adapt the bacterium to the presence of xenobiotic at different concentrations, cells were grown twice in planktonic phase. Aliquots of 100 μL with 1×10⁸ CFU mL⁻¹ were added to 1 mL of PTSB or enrLBB with dexamethasone (0.001-10 μM). The cultures were carried out at 37°C, 150 rpm and normoxia for 24 h.

Cell Surface Hydrophobicity (CSH): A protocol involving ammonium sulfate agglutination was used for CSH measurements¹². Briefly, 24 h old dexamethasone-adapted cells were centrifuged (10,000 g for 5 min) and suspended till 5×10⁹ CFU mL⁻¹ in sterile double distilled water. Immediately, 20 μL of each suspension were transferred to central areas of ether-cleansed microscopy slides. Aliquots of 20 μL of ammonium sulfate at different concentrations (1-2.5 M with 50 mM steps, pH 6.8) were gently mixed with the suspensions. The presence of agglutination was verified after 10 min on a black card using a stereomicroscope with 10X magnification.

Adhesion to conditioned surface in presence of dexamethasone: Sterile 96-well polystyrene microtitration plates were conditioned with 100 μL of filter-sterile 0.1% L-asparagine per 2 h at 37°C. Plates were washed twice with sterile 145 mM NaCl and the

wells received 100 μL of enrLBB-dexamethasone (0.001-10 μM) broths. After one hour of incubation at 37°C, wells were washed twice with sterile water. Aliquots of 100 μL of 24 h old dexamethasone-adapted cells containing ca. 1×10⁸ CFU mL⁻¹ were transferred to wells and the plates were incubated for 2 h, at 37°C and 150 rpm. The wells were drained by aspiration and gently washed once with sterile water. Adhered cells were fixed to surfaces with 150 μL of 99% methanol, for 15 min at bench temperature. Supernatants were drained by aspiration and plates were air-dried using a hair fan that remained 40 cm away. Aliquots of 200 μL of aqueous 0.5% Crystal Violet (CV) were added to wells. After 20 min, the excesses of CV were removed by aspiration and the plates were immersed several times in a tank with clean water in order to remove the non-staining CV. The cell-adhered CV was released with 33% acetic acid (150 μL) and the OD₅₉₀ nm were determined in a TP-Reader™ (ThermoPlate Co.) plate reader.

Planktonic growth: One hundred microliter aliquots of 24 h-old dexamethasone-adapted cells containing ca. 1×10⁸ CFU mL⁻¹ were inoculated in 4.9 mL of enrLBB-dexamethasone (0.001-10 μM). The tubes were incubated at 150 rpm, 37°C and normoxia. Culture growths were monitored measuring the OD₆₂₀ nm with 1 h intervals till the achievement of stationary phase in an Ultrospec®1100pro (Amersham Biosciences Co.).

Biofilm growth: Sterile 96-well polystyrene microtitration plates were conditioned with 200 μL of filter-sterile 0.1% L-asparagine per 2 h at 37°C. Plates were washed twice with sterile 145 mM NaCl and the wells received 200 μL of PTSB-dexamethasone (0.001-10 μM) broths. After one hour of incubation at 37°C, wells were washed twice with sterile water. Aliquots of 200 μL of 24 h old dexamethasone-adapted cells containing ca. 1×10⁸ CFU mL⁻¹ were transferred to wells and the plates were incubated for 2 h, at 37°C and 150 rpm (adhesion phase). After the adhesion period, the supernatants were aspirated and the wells were washed five times with sterile 145 mM NaCl. Aliquots (200 μL) of PTSB-dexamethasone (0.001-10 μM) were added to wells and plates were incubated for 24 h, 48 h and 72 h, at normoxia at 37°C. PTSB-dexamethasone were replaced after each 12 h.

At the end of incubation times, biofilm contents were estimated by the XTT reduction assay¹³. Briefly, biofilms were washed three times with 200 μL of sterile 145 mM NaCl and then, 100 μL of sterile 145 mM NaCl and 100 μL of XTT-menadione (Sigma-Aldrich Co.) solution were added on each well. The plates were

statically incubated on the dark for 5 h, at 37°C. After incubation, the contents were transferred to microfuge tubes and centrifuged at 13,000 g, for 5 min. The OD₄₉₂ nm were determined in a TP-Reader (ThermoPlate Co) plate reader.

Protease secretion: Supernatants of planktonic or biofilm growths on PTSB-dexamethasone (0.001–10 μM) broths, were centrifuged for 5 min at 10,000 g. Aliquots of 100 μL were combined with 500 μL of 0.8% azocasein (Sigma-Aldrich Co.) diluted in 50 mM K₂HPO₄ (pH 7). After 3 h of incubation at 37°C, the reaction was stopped with 500 μL of 1.5 M HCl. The tubes were placed on ice for 30 min and then centrifuged at 16,000 g for 3 min. Supernatants were combined with equal volumes of 1 M NaOH and the OD₄₄₀ nm were measured. One enzymatic activity unit was arbitrarily defined as the amount of enzyme that increments OD₄₄₀ nm in 0.001 unit of absorbance per min of digestion¹⁵. The proportional enzymatic activities were estimated as the enzymatic unities per reduced XTT absorbances¹⁴.

Pyocyanin secretion: Supernatants of planktonic or biofilm growths on PTSB-dexamethasone (0.001–10 μM) broths, were centrifuged for 5 min at 10,000 g. Secreted pyocyanin was extracted with 1 mL of analytical grade chloroform. The chloroform was transferred to clean tubes where aliquots of 1 mL of 1 M HCl were slowly and gently added under shaking in order to change pyocyanin the aqueous phase. The OD₅₂₀ nm of pyocyanin extracts were measured and the concentrations were determined by the multiplication of the values per 17.07. The proportional secretions of pyocyanin were estimated as corrected absorbances of pyocyanin per reduced XTT absorbances¹⁶.

Statistical analysis: All the tests described above were carried out in triplicate, in three different situations. Variance analyses were conducted using the Kruskal-Wallis (Student-Newman-Keuls) test followed by multiple comparison test of Tukey, in the BioEstat@ 5.0 statistics package (SC Mamirauá/MCT/CNPq). A p threshold of 0.05 was considered for difference statements.

RESULTS

Cell Surface Hydrophobicity (CSH): It was only observed a slight alteration on bacterial agglutination due to the glucocorticoid interference (Table 1). Cultures grown in presence of 10 μM, 1 μM and 0.1 μM dexamethasone were slightly more hydrophobic after 24 and 48 h of exposure. All other treatments did not differ

Table 1: Lowest molarities of ammonium sulfate that promoted cell aggregation

Concentration of dexamethasone (μM)	Incubation time (h)		
	24	48	72
Control	1.70 M	1.70 M	1.80 M
10	1.60 M	1.65 M	1.70 M
1	1.60 M	1.60 M	1.70 M
0.1	1.60 M	1.65 M	1.70 M
0.01	1.70 M	1.70 M	1.80 M
0.001	1.70 M	1.70 M	1.80 M

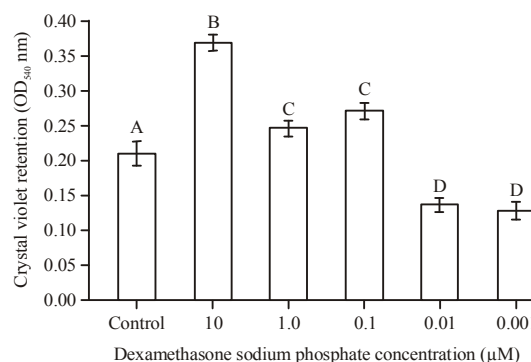


Fig. 1: Dexamethasone-mediated adhesion of *Pseudomonas aeruginosa* ATCC®27853™ to L-asparagine conditioned polystyrene surfaces. Dissimilar letters over the bar errors denote statistically supported differences (p<0.05)

from the control (no dexamethasone), showing that lower dexamethasone concentrations seem not to interfere on the bacterial hydrophobic behavior.

Additionally, with the prolongation of time, cells tended to become less hydrophobic for all treatments, once higher concentrations of salt were required for the agglutination of cells.

Adhesion to conditioned surface: The presence of dexamethasone altered the bacterial adhesion ability on L-asparagine conditioned acrylic surfaces (Fig. 1). Higher concentrations of dexamethasone (≥ 0.1 μM) showed better adhesion rates, when compared to the control. However, in 0.01 and 0.001 μM treatments, an opposite effect that negatively interfered on adhesion has happened.

Planktonic growth: As a rule of thumb, the bacterial growth was negatively affected by the presence of dexamethasone resulting in growth curves whose stationary phases were reached with lower cell contents (Fig. 2). Surprisingly, this reduction was inversely related to concentration. Despite this fact, all treatments presented on the same time intervals the lag, log and

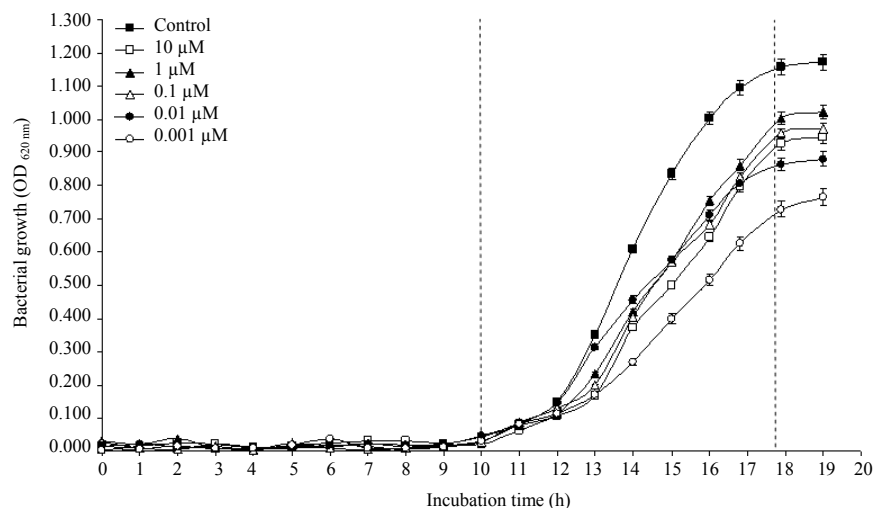


Fig. 2: Dexamethasone-mediated planktonic growth of *Pseudomonas aeruginosa* ATCC®27853™

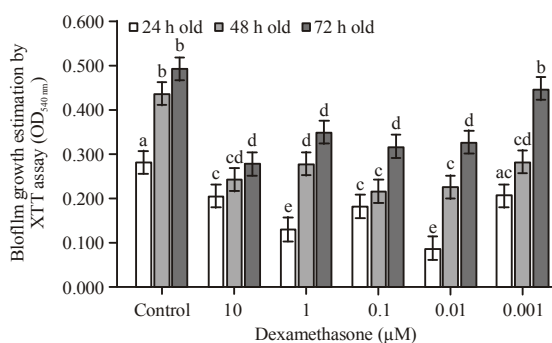


Fig. 3: Dexamethasone-mediated biofilm formation of *Pseudomonas aeruginosa* ATCC®27853™. Dissimilar letters over the bar errors denote statistically supported differences ($p < 0.05$)

stationary phases. Lag phases for all treatments and control lasted for 10 h and log rampings occurred between the 10th and 16th h post-inoculation. This denotes a marked similarity in the growth behavior.

Biofilm formation: Dexamethasone reduced considerably ($p < 0.05$) the cell content in biofilms regardless to time incubation. Exceptions were detected only for the minor dexamethasone concentrations at the challenge (0.001 μM), where cell growths for 24 and 72 h old biofilms did not differ from negative control ($p > 0.05$) (Fig. 3).

Noteworthy, with the decrease in dexamethasone concentrations differences between 48 and 72 h old

biofilms tended to become bigger ($p < 0.05$). This was interpreted as a tendency for delayed prolongation in biofilm formation.

Protease secretion: A time-dependent increase in proportional protease activity was observed for all treatments with dexamethasone in both growth phenotypes, planktonic and biofilm (Fig. 4). In the particular case of biofilms, dexamethasone promoted a similar growth pattern ($p > 0.05$) for all concentrations which diverged from negative control ($p < 0.05$). However, whereas dexamethasone promoted a gradual increment in the proportional activity ($0.03 < p < 0.001$), control displayed an abrupt increment between 24 and 48 h ($p < 0.00001$). Despite this, at the end of biofilm growth (72 h) all groups, including control, presented a similar activity ($p > 0.05$).

Regarding to planktonic growth, dexamethasone induced a continuous increment on the proportional protease activities in function of time for all treatments. Different from biofilms, here controls did not have a pronounced increment after the first 24 h of incubation. Expressive increments in proportional protease activities were observed between the 48th and 72nd h ($p < 0.0001$). Comparisons among treatments after 72 h revealed that, excepting the 1 μM treatment, all the other treatments showed higher activities ($p < 0.05$).

Although the proportional protease activities of biofilms and planktonic growths were not compared in present study, it is markedly that in the first growth phenotype the protease secretion per cell was higher.

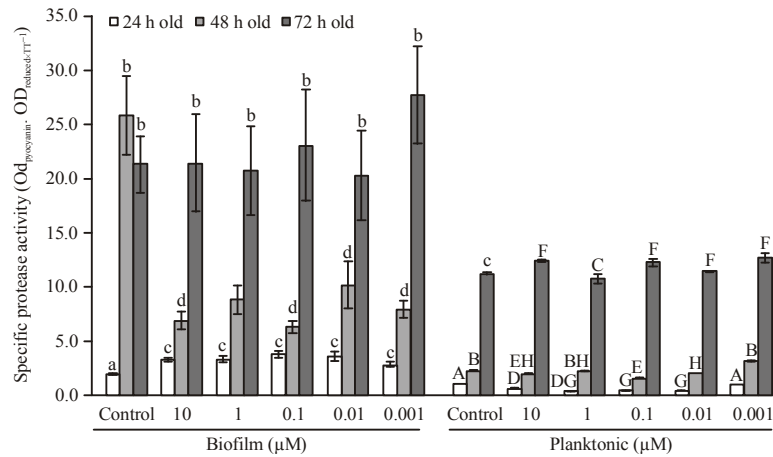


Fig. 4: Dexamethasone-mediated protease secretion by *Pseudomonas aeruginosa* ATCC@27853™. Dissimilar letters over the bar errors denote statistically supported differences ($p < 0.05$). Biofilm vs planktonic protease secretions were not compared

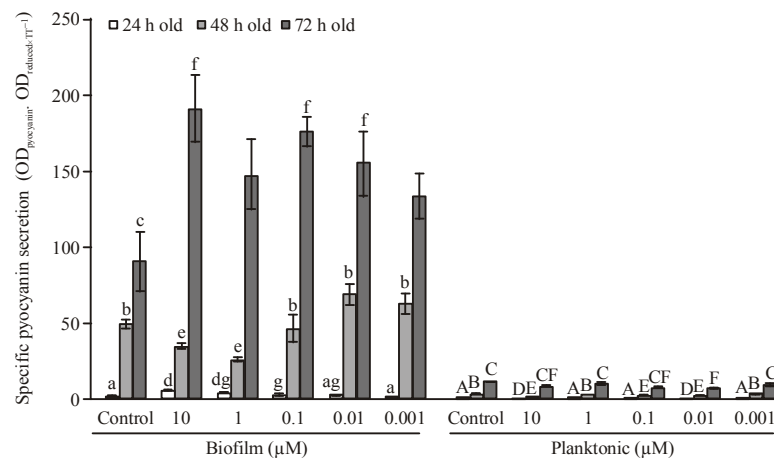


Fig. 5: Dexamethasone-mediated pyocyanin secretion by *Pseudomonas aeruginosa* ATCC@27853™. Dissimilar letters over the bar errors denote statistically supported differences ($p < 0.05$). Biofilm vs planktonic pyocyanin secretions were not compared

Pyocyanin secretion: Continuous time-related increments in proportional pyocyanin secretion were observed for *P. aeruginosa* ATCC@27853™ grown as biofilm or planktonic isolated cells ($p < 0.05$) (Fig. 5). In biofilms, dexamethasone exerted a variable and controversial effect until the 48th h post-inoculation, according to the concentration. However, the corticoid in all concentrations induced a final increment in 72 h old biofilms in relation to controls ($p < 0.005$). Less predictable were the results obtained for planktonic cells which oscillated among dexamethasone concentrations in all biofilm ages. However, as occurred in biofilms, noticeable increments were detected in 72 h old cells ($p < 0.005$).

As it occurred for proportional protease activity, biofilm entrapped cells secreted more pyocyanin than planktonic counterparts (statistics were not carried out).

DISCUSSION

The aim of this study was to evaluate the modulatory influence of dexamethasone on some putative virulence attributes of *P. aeruginosa*. The results demonstrated that dexamethasone may interfere in some bacterial physiological features. Growth curves revealed that bacterial doubling time tends to increase when in contact with the glucocorticoid, what is in accordance with previously published data¹⁰. At first view, this seems to be a good prognostic once an elongation of

doubling time could facilitate the pathogen recognition and elimination by cells and molecules from immune system. However, it must be remembered that such experimental findings were obtained dealing with planktonic cells at controlled conditions what may not reflect the in vivo reality.

By other side, dexamethasone has also incremented CSH and cell adhesion rate. Cell adhesion to abiotic surfaces is related to the presence of different types of adhesins, as outer membrane proteins, LPS, alginate and type IV fimbriae¹⁷. CSH exerts an important role on the microbial adherence to different surfaces, including macrophage and other mammalian cells¹⁸. Treatments with higher concentrations of dexamethasone which showed better adhesion to surfaces had also induced the phenotype of slightly hydrophobic cells. Such positive interplay between CSH and *P. aeruginosa* affinity to adhesion to polystyrene has already been studied by Fonseca and Sousa,¹⁹ who defend that higher CSH results imply in proportional increments on adhesion rates. The results of present study corroborate with this hypothesis and show that higher doses of dexamethasone increase the adhesion capability, what could has a negative impact on patients health, mainly on those who need dedicate devices for mechanical ventilation. The fact that higher concentrations of dexamethasone increase bacterial adhesion must have some clinical relevance, once adhered cells are less prone to be phagocytized by immune system cells and also act as the start up for biofilm formation²⁰.

However, in spite of the higher adhesion capability induced by the corticoid, the microbial loads in biofilms formed under continuous dexamethasone influence were comparatively inferior to control. Taking in account the experimental limitations, under such perspective, dexamethasone exerts a beneficial role when interferes in a negative manner on the biofilm formation. Obviously, this must be confirmed by further experiments using biotic systems.

Pseudomonas aeruginosa has the ability to form biofilm, well-known recognized as an important bacterial pathogenicity characteristic. Part of biofilm formation is related to the control exerted by quorum-sensing signaling, where cells are organized on layers entrapped in a polysaccharide mucous matrix. The biofilm development confers a reduction on the susceptibility to antibiotics and creates a more severe infection in lungs of patients with cystic fibrosis, being considerably harder to eradicate^{4,21}.

Dexamethasone does not affect the proportional activity of proteases when cells grow under biofilm

phenotype. However, in planktonic phase, the same corticoid enhances the virulence when cells are in contact for 48-72 h. As in infectious processes, cells are concomitantly found under the two growth phenotypes, this increase in proteases' secretion should be considerate and more properly investigated.

Among all virulence factors here investigated, may be the secretion of pyocyanin, an important molecule involved in *P. aeruginosa*'s infection^{9,22-25}, was the more affected by dexamethasone. It was shown that even with a lower number of cells, biofilms under dexamethasone influence secrete higher amounts of this phenazine. Such modulation is worthy of concern, because patients presenting biofilm-like patterns of infection by the bacterium would become more prone to harmful effects. From our knowledge, only few groups are evaluating the interference of xenobiotics on pyocyanin secretion modulation. Shen *et al.*²⁴ expressed their concern on the fact that sub-inhibitory concentrations of various antibiotics cause up-regulation on the expression of genes that codify the pyocyanin synthesis and it could complicate the antibiotic therapy expectatives. Other compounds such as curcumin²⁶ and salicylic acid¹⁶ cause down-regulation on the genes with consequent virulence reduction.

Futures experiments enrolling transcriptome-based techniques may elucidate the pathways and mechanisms involved on this apparent positive modulation induced by dexamethasone.

Concluding, dexamethasone on plasma concentrations showed ambiguous virulence modulation with negative interference on bacterial division and biofilm growth. In opposition, positive modulation was detected on adhesion to abiotic surfaces, on proportional secretions of protease by planktonic cells and on proportional secretion of pyocyanin by biofilms. It is recommended to observe the appearing or the exacerbation of manifestations of bacterial virulence when continuous dexamethasone therapy is stated.

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