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Molecular characterization and 16S rRNA sequence analysis of probiotic *lactobacillus acidophilus* isolated from indigenous Dahi (Yoghurt)

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Abstract

Indigenous yoghurt is being the most popular fermented milk product in Pakistan, produced by heterogenous lactic acid bacteria (LAB). Nowadays LAB has drawn particular interest in food and nutrition science because of functional and probiotic attributes. Among various genera of LAB, *Lactobacillus acidophilus* is considered to be a potential candidate. Present study was designed by keeping in view the probiotic attributes of *L. acidophilus*. Isolation was conducted on MRS media supplemented with 0.7% bile salts to screen out bile tolerant isolates. Out of sixty lactobacilli, eighteen were identified as *L. acidophilus* by performing gram staining, catalase, carbohydrate fermentation test and growth at different temperatures. Only six had shown tolerance of pH 2 up to 50% and only three had shown a wide range of antimicrobial activity against tested organisms. *S. epidermis* found to be more sensitive with a maximum zone of inhibition (18 mm) and *A. baumanii* and *S. aureus* were least sensitive with a smallest zone of inhibition (11.5 mm and 12 mm respectively). 16S rRNA gene amplification, sequencing and phylogenetic tree construction of the obtained sequences with the most closely related lactobacillus spp. was performed. Sequences are available in GeneBank and NCBI with the accession numbers KU877440 (WFA1), KU877441 (WFA2) and KU877442 (WFA3). Presence of probiotic *L. acidophilus* in locally fermented product (dahi) has proved its potential along with bile salts supplementation in MRS media is helpful in initial screening of probiotic isolates.

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Introduction

Indigenous yoghurt is a semi-solid fermented milk product that has originated centuries ago. *L. acidophilus* along with other Lactic acid bacteria's (LAB) is its natural inhabitant (Raquib *et al.,* 2003). Some strains of *L. acidophilus* have been reported to have probiotic characteristics (Ljungh and Wadstrom, 2006). Probiotics are the live microorganisms which when administered in adequate amount confer health benefits on the host. Due to their probiotic characters, they improve the health of the host hence are included in the category of nutraceuticals (Reid *et al*., 2003) by producing bio active proteins and maintaining intestinal microbial balance thus reducing the incidence of intestinal infection (Catanzaro and Green, 1997; Hoque *et al.,* 2010).

Proper identification and characterization of lactobacilli includes not only phenotypic but also molecular studies (Donelli *et al.,* 2013). Molecular study focuses on Deoxyribonucleic acid (DNA) sequencing and sequence analysis of evolutionarily stable genes to study bacterial phylogeny and diversity (Tringe and Hugenholtz, 2008). For this purpose genes that code for the 5S, the 16S, the 23S ribosomal ribonucleic acid (rRNA) along with the spaces between these genes are supposed to be potential candidates and among these 16S rRNA gene is the most common part of the DNA used for taxonomic purposes (Palys *et al.,* 1997; Tortoli, 2003; Harmsen and Karch, 2004) due to its highly conserved nature in *Bacillus* spp (Clarridge, 2004). Major application of 16S sequence analysis is identification, classification and estimation of bacterial diversity of isolated pure cultures along with environmental samples without culturing through metagenomic approaches (Rajendhran and Gunasekaran, 2011).

Soomro and Masud (2012) conducted the study on probiotic potential of *lactobacillus spp*. isolated from dahi (Traditional milk product) and reported that among all *L. acidophilus* showed maximum acid and bile tolerance along with a wide range of antimicrobial properties.

Keeping in view the previous study, this study was design to explore probiotic potential of *L. acidophilus* from indigenous yoghurt up to molecular level characterization. In Pakistan, this study will help to provide a more valuable functional food at a cheap cost. The main objectives of the study includes isolation, phenotypic and molecular confirmation of probiotic *L. acidophilus* including sequence analysis and phylogenetic studies of screened isolates by utilizing 16S rRNA gene.

Materials and methods

Sample collection

This study was conducted at Department of Food Technology, University of Arid Agriculture Rawalpindi, Pakistan. 105 Samples of dahi were randomly collected in sterilized test tubes from the local market of district Rawalpindi to explore the potential of this area regarding probiotics covering the area of Sadiqabad, Dhoke kala khan, Iqbal town, Satellite town, Muslim town, Sadar, Shamsabad, Afandi colony, Liayaqat bagh, college road, Pir wadhae, Gulzare quaid, Judicial colony, Khana pull, Shakrial and Chaklala.

Isolation and Characterization of probiotic Lactobacillus

Isolation of lactic acid bacteria was done by streaking loop full of dahi on MRS ((de Man, Rogosa and Sharpe), Oxoid, Basingstoke, UK) media supplemented with 0.7% bile salts (Oxoid). The identification of strains was performed according to their morphological, cultural and biochemical properties based on key characteristics as described in Bergey's manual (Buchanan and Gibbons, 1974). Phenotypic characterization of isolates includes gram staining, catalase test and carbohydrates fermentation test by using analytical profile index (API) kits method. Culture was preserved in 20% glycerol at -20°C.

Acid tolerance of isolates

Acid tolerance of bacterial isolates was conducted by the method described by Hassanzadazar *et al.* (2012) and Singhal *et al.* (2010) with some modifications.

Overnight grown culture of *L. acidophilus* in MRS broth (Oxoid, Basingstoke, UK) at 37°C was inoculated (1%) to MRS broth with adjusted pH of 2, 3 and 4 with 1M HCl. The broths were incubated at 37°C for 5 h and bacterial cell tolerance was determined by measuring absorbance with a spectrophotometer at 600 nm.

The percent difference between the variation of optical density (OD) at pH 7.0 and OD at pH 2, 3 and 4 would give an index of isolates surviving that can be expressed as follows:

$$Surviving\ (\%) = \frac{\text{OD (pH7)} - \text{OD (pH2, 3 or 4)}}{\text{OD (pH7)}} * 100$$

Antimicrobial activity

The antagonistic effect of partially purified bacteriocins of isolated culture was conducted against indicator organisms including *Escherichia coli, Salmonella paratyphi, Staphylococcus epidermis, Acinobacter baumanii* and *Staphylococcus aureus* were tested by the paper disc method (Ohmomo *et al.*, 2000; Soomro and Masud, 2012). For cell free supernatant, firstly pH of the overnight grown culture was adjusted to 5.5 with 1M NAOH than centrifugation was performed at 10,000 rpm for 15 minutes, cell free supernatant was collected and passed through 0.2 um syringe filter to remove bacterial cell.

The concentration of the overnight grown culture of test organisms was adjusted according to 0.5 McFarland. Sterile filter paper discs measuring 6 mm diameter, thin type with an adsorbed aliquot of 25 ul of cell free supernatant, was placed on nutrient agar plates containing a target strain and left overnight at room temp. After that incubation was done for 24 hrs at 37°C the inhibitory activity was evaluated, based on the formation of a clear zone around the paper disk.

Molecular Characterization

Confirmation of lactobacilli isolates by Colony PCR

Molecular characterization of bacterial isolates was done by polymerase chain reaction (PCR) using universal primers (1510R and 9F) (Table 1).

Universal primers	
1510 R	5'-GGCTACCTTGTTACGA-3'
9F	5'-GAGTTTGATCCTGGCTCAG-3'

DNA Template formation

DNA (Deoxyribonucleic acid) template was prepared by the method described by Plourde-Owobi *et al.* (2005) with some modifications. Freshly grown bacterial colonies were picked and transfer to 10ul Tris-EDTA (ethylene diamine tetra acetic acid) buffer (10 ml of 1M Tris-HCl buffer (pH 7.5) and 2 ml of 0.5M EDTA solution (pH 8) make the volume up to 1L) by using sterilized tooth picks and incubate at 80°C for 15 minutes, followed by centrifugation at 12000 rpm for 15-20 minutes to settle the cell debris at bottom and supernatant was used in PCR assay.

PCR assay conditions

DNA template was amplified by PCR, the reaction mixture (10 µl) was comprised of DNA template (2 µl), reaction buffer (1 µl), magnesium chloride (0.6 µl), forward and reverse each primer (0.5 µl), *Taq DNA polymerase* (0.4 µl) mix dNTPs (0.2 µl) final volume was attained by adding nano pure water (4.8 µl). PCR mixture was initially heated at 94°C for 2 minutes, followed by 33 cycles of denaturation at 94°C for 1 minutes, annealing 56°C for 1 minutes and extension 72°C for 90 seconds. A final extension was performed at 72°C for 20 minutes.

Gel electrophoresis

Gel electrophoresis was performed after PCR amplification in which 1% agarose gel (electrophoresis grade) was prepared and run in TAE (Tris-Acetate EDTA) buffer, whole process was carried out in gel electrophoresis chamber provided with positive and negative charge supply. Gel was run for about 20-30 minutes on 70 volt and 110 mA then stained in ethidium bromide solution and visualized in UV transilluminator.

Sequencing, sequence analysis and phylogenetic tree

The amplified products of screened isolates were sent to macrogen (Korea) for sequencing in sense and antisense directions.

The obtained sequences were analyzed by using BioEdit sequence alignment software and BLAST tool of National Centre for Biotechnology Information (NCBI) (www.blast.ncbi.nlm.nih.gov/Blast.cgi). Final sequences were submitted in public data base of NCBI under the accession # KU877440, KU877441 and KU877442. Phylogenetic tree was constructed by using ClustalW2 phylogeny for which first sequences of closely related lactobacilli were aligned by using Multiple Sequence Alignment (MSA) tool namely MUltiple Sequence Comparison by **L**og- Expectation (MUSCLE).

Closely related sequences (99-100% genetic homologies) of lactobacilli after BLAST along with accession numbers has been picked from NCBI for phylogenetic tree construction, including *L. acidophilus* KP 942831, KT 070880 LN 869545, *L. casei* KC456367, *L. plantarum* AB 362750, AB 362749, AB 362652, KT 725396, KT 852452, KT 852450, KU 644577 and *Lactobacillus* spp JQ 046407 and FJ 532368.

Results

This study was conducted to screen out probiotic *L. acidophilus* from indigenous fermented milk product of Pakistan namely dahi. Samples were streaked on MRS (Oxoid, Basingstoke, UK) agar supplementing 0.7% of bile salts. Eighteen of them were found to be of *L. acidophilus*. Others were *L. plantarum, L. fermentum, L. paraplantarum* and *L. pentosus*. All the isolates were gram positive, catalase negative with no motility. Carbohydrates fermentation test and growth at different temperature of isolates has been shown in Table 2.

Table 1. Oligonucleotide sequences for PCR amplification of conserved region in 16S rDNA gene.

L. acidophilus			
Morphology	Bacilli	Melibiose	-
Gram Staining	+	Mannose	+
Catalase	-	Raffinose	-
Mobility	-	Rhamnose	-
Fructose	+	Gluconate	-
Glucose	+	Amygdaline	+
Galactose	-	Arabinose	-
Lactose	+	Cellobiose	+
Sorbitol	-	Esculine	+
Maltose	+	Nitrate reductase	-
Mannitol	-	CO_2 production from glucose	-
Sucrose	+	Growth at 15°C	-
Ribose	-	30°C	+
Xylose	-	45°C	+
Trehalose	-		
Melizitose	-		

Acid tolerance of L. acidophilus isolates

All the isolates showed variability in acid tolerance or pH tolerance. Only 6 among all were able to tolerate pH 2 with about 50% survival for 5 hrs. The percentage tolerance of all the isolates has been represented graphically (graph 1). The maximum was found to be 51.25% at pH 2 and about all the isolates were survived or tolerated pH 3 and 4 with maximum percentage of 60.25% and 80.95% respectively.

Isolates showing acid tolerance at pH 2 were selected (2, 5, 7, 8, 9 and 11) for antimicrobial testing.

Antimicrobial Activity

From above selected six acid and bile tolerant isolates, only three (5, 8 and 9) showed a wide range of activity against all test organisms. *S. epidermis* was found to be the most sensitive as maximum number of isolates showed zones against it with maximum zone of inhibition which was 18 mm, shown by isolate #8.

Table 2. Phenotypic characterization of *L. acidophilus* including gram staining, catalase test, mobility, sugar fermentation test and growth at different temperatures.

	Zones of inhibition				
Isolates	*E. coli*	*S. aureus*	*S. epidermis*	*A. baumanii*	*S. paratyphae*
2	±	++	++	+	-
5	+++	++	++++	++	++
7	+	+	+++	+	-
8	++	++	++++	+++	+++
9	++	++	++++	++	+++
11	±	±	+++	±	±

Mixed responses were seen in case of *E. coli* and *S. paratyphi* with a maximum zone of inhibition was about 13.5 mm (isolate # 5) and 14 mm (isolate # 8). *S. aureus* and *A. baumanii* showed least sensitivity with minimum zones of inhibition of about 10.5 mm (isolate # 9) and 12.5 mm (isolate # 8) respectively.

Molecular confirmation of L. acidophilus

In present study primers 9F and 1510R were used for the amplification of conserved region of 16S rRNA, resulted in a product of 1.5 kb (1500bps) fragments confirming that the isolated bacteria were from genus lactobacillus (Figure 1).

Sequencing and Phylogenetic tree analysis

On the basis of acid, bile tolerance and antimicrobial studies only three isolates 5 (WFA1), 8 (WFA2) and 9 (WFA3) of *L. acidophilus* out of eighteen were selected for DNA sequencing and also for further studies (which are not part of this paper). Opology of the phylogenetic tree showed all the three isolates are in one clad of genus Lactobacillus. All the three were formed sister clad with *L. acidophilus* and have been shown in Fig 2, sequence similarity percentage of about 99% with already submitted sequences of *L. acidophilus* with slight mutation or difference in sequences.

Table 3. Antimicrobial activity of isolates against five different pathogens (*E. coli, S. aureus, S. epidermis, A. baumanii*) by using disc diffusion method. Zone of inhibition: (-) = no activity, 0-4mm (±), 4-8mm (+), 8-12mm (++), 12-16 mm (+++) and 16-20 mm (++++).

	Zones of inhibition				
Isolates	*E. coli*	*S. aureus*	*S. epidermis*	*A. baumanii*	*S. paratyphae*
2	±	++	++	+	-
5	+++	++	++++	++	++
7	+	+	+++	+	-
8	++	++	++++	+++	+++
9	++	++	++++	++	+++
11	±	±	+++	±	±

Discussion

The concentration of bile salts in human intestine is almost about 0.7% along with water being the major component and cholesterol being a minor component. Keeping in view the above concentration of bile, in present study 0.7% concentration was selected for the initial screening of bile salt tolerant as well as viable isolates. Bile salts breakdown lipid bilayer and proteins of bacterial cell by disrupting cellular homeostasis thus cause cell death (Mandal, 2006). Bile tolerant isolates of *L. acidophilus* was found to be 30% among all gram positive LAB.

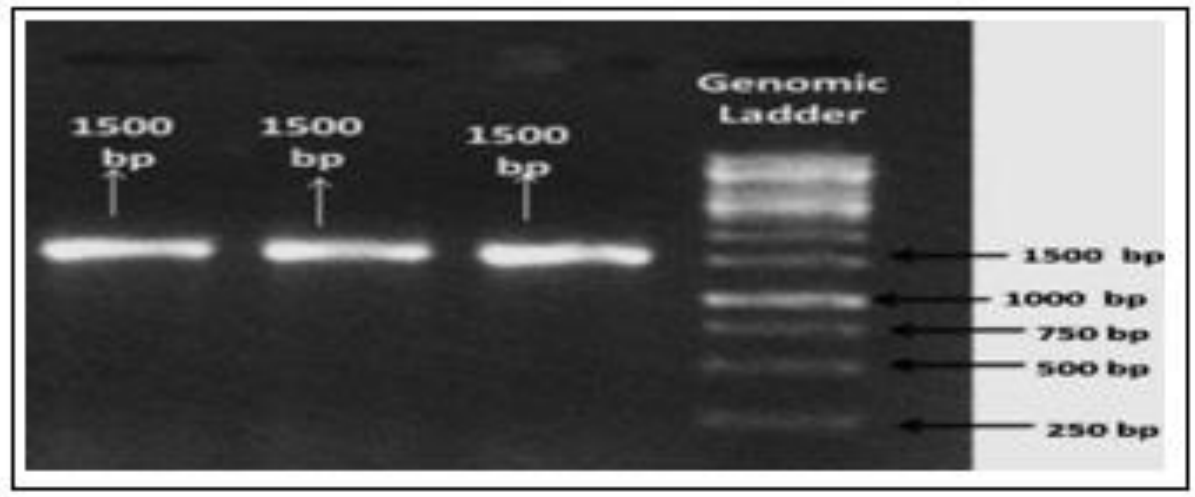


Fig. 1. PCR amplification of DNA of probiotic isolates of *L. acidophilus* shows band of 1500 bps using primers 1510R and 9F at 56 °C annealing temperature. The genomic ladder / DNA marker of 1Kb was used to locate the PCR product.

As far as carbohydrate fermentation test was concerned Boukhemis *et al.* (2009) and Pyar and Peh (2014) reported the same results regarding the fermentation pattern of *L. acidophilus*. Environments having high acidity inhibit the metabolism and reduce the growth and viability of Lactobacilli. In present study strict selection criteria was followed for acid tolerance by keeping in view about pH of stomach before (1.5) and after (3-4) having meal along with the time for which food remained in stomach (4-5 hrs).

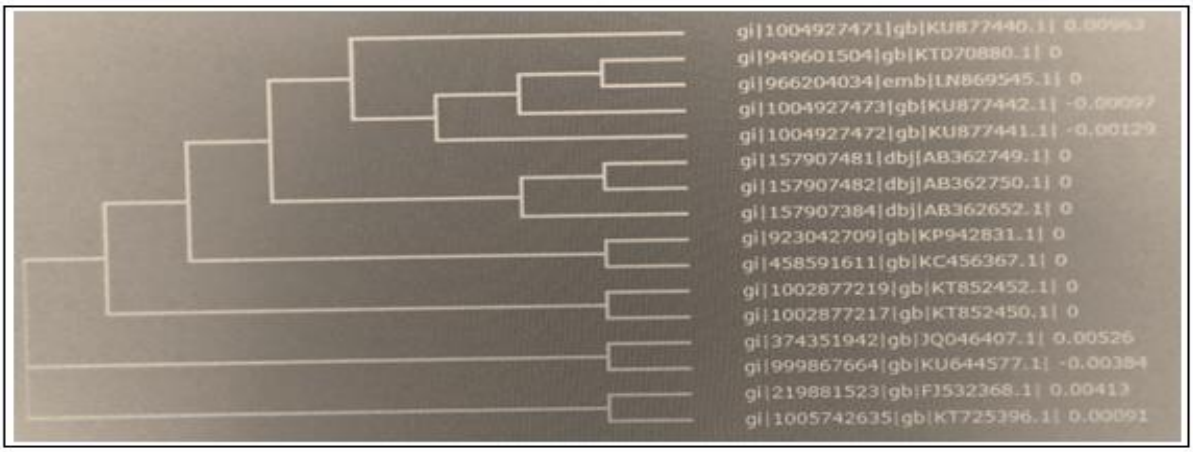
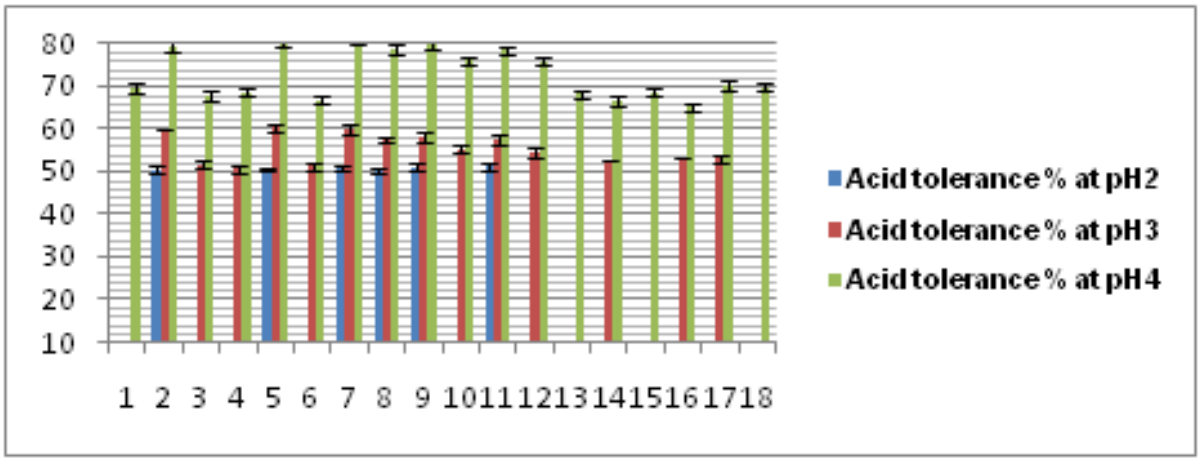


Fig. 2. Phylogenetic tree of lactobacilli spp. showing maximum percentage of similarity (99-100%), constructed by using ClustalW2 phylogeny for which first sequences of closely related lactobacilli (*L. acidophilus* KP 942831, KT 070880 LN 869545, *L. casei* KC 456367, *L. plantarum* AB 362750, AB 362749, AB 362652, KT 725396, KT 852452, KT 852450, KU 644577 and *Lactobacillus* spp JQ 046407 and FJ 532368) were aligned by using Multiple Sequence Alignment (MSA) tool namely multiple Sequence Comparison by Log- Expectation (MUSCLE).

Intensive reduction in both bacterial viability (Mishra *et al.,* 2005; Liu *et al.,* 2013) and tolerance (Prasad *et al.,* 1998; Mandal *et al.,* 2006) at pH 2 and 3 has been reported. Similar decrease in viability and tolerance of bacterial culture at low pH was also reported by Hassanzadazar *et al.* (2012).

Liong and Shah (2005) suggested that resistance at pH 3 should be set as standard for acid tolerance of probiotic culture. So if considerable amount of bacterial culture will tolerate pH up to 2 or 3, it can be a potential candidate for probiotic.

Antimicrobial activity of the Lactobacilli against both gram positive and gram negative bacteria is one of their most prominent and widely studied properties (Kaushik *et al.,* 2009). *L. acidophilus* showed variable sizes of inhibition zones reported in different studies. Against *E. coli* maximum zone of inhibition was of 26 mm (Goderska and Czarnecki, 2007). Soomro and Masud (2012) reported zones of *L. acidophilus* against *E. coli* (11.5 mm), *Bacillus cereus* (8.0 mm), *Enterococcus faecalis* (11.5 mm), *S. aureus* (10.5 mm) and *Salmonella. typhi* (8.0 mm), thus revealing the strong antimicrobial activity of *L. acidophilus*.



Graph 1. Acid tolerance percentage of *L. acidophilus* isolates at different pH (2, 3 and 4) after 5hr of incubation. Data based on mean of three readings, error bars representing standard deviation.

PCR amplification by targeting universal gene is the most commonly used molecular technique for spp level identification. Utilization of 16S rRNA, 16S-23S rRNA intergenic spacer region (ISR), or 23S rRNA, for amplification in a universal gene by designing specific primers is the simplest and easiest tool for successful identification of LAB (Kim *et al.,* 2005). Similar set of primer was previously used by Rahayu *et al.* (2009), for amplification of bacterial 16S rRNA gene and reported the PCR product of 1.5 kbs or 1500 bps. As far as sequencing was concerned, results showed slight mutation or difference in genetic material (DNA) which might be due to difference in environmental conditions of the region or area of isolation, served as a determinant of bacterial identification at both genus and species level (Clarridge, 2004). Kusmiyati *et al.* (2014) isolated lactobacilli from naturally fermented milk product, after 16S rRNA gene sequence analysis and phylogenetic study he found that similar isolates formed sister clad with their respective reference isolate. This showed that after BLAST, phylogenetic tree construction was proved to be a vital tool for proper identification and also for studying sequence similarities and differences among different strains and spp.

Conclusion

These are preliminary studies focusing isolation and 16S rRNA sequencing of probiotic *Lactobacillus acidophilus* from indigenous yoghurt (Dahi). Like previous studies present study also supports "indigenous yoghurt (Dahi) as a potential source of probiotics.

As far as isolation of lactobacilli on MRS supplemented with bile is concerned, it is helpful in initial screening for probiotic isolates but other screening tests like acid tolerance and antimicrobial activity are also necessary and cannot be ignored. Along with this molecular study regarding identification of lactobacilli is more authenticated up to specie level characterization of probiotics when compared with phenotypic characterization (Carbohydrate fermentation test or API 50).

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