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Genetic Diversity Study of Locally Isolated *Bacillus thuringiensis* Strains from Kuwait Using Random Amplified Polymorphic DNA Analysis

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Authors' contributions

This work was carried out in collaboration between all authors. Author JAQ designed the study, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors EMAA, SAAM and YAAS managed the sample collection, analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: *Bacillus thuringiensis* (Bt) is significant bacteria in the field of insect biological control due to their insecticidal properties and its importance in agriculture.

Aim: The objective of this study was to analyze genetic variation of 15 native isolates of *Bacillus thuringiensis* and four reference strains (total 19) using the random amplified polymorphic DNA (RAPD) technique, this allowed the analysis the genetic diversity of this species in the microbial populations from different soil samples of Kuwait.

Methodology: We isolated 109 *Bacillus thuringiensis* strains, out of which, 15 strains were subspecies *thuringiensis*, using culture and serological method. A rapid typing method of *Bacillus thuringiensis* local isolates from Kuwait soil was established using the RAPD technique. A single

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decamer primer was used to study and characterize *Bacillus* Spp, *Bacillus thuringiensis* var. *thuringiensis*, to differentiate the isolated species. Based on RAPD pattern, data were subjected to cluster analysis using Alpha Ease software.
Results: We found three groups each, with two strains that had a similar pattern of DNA and one group that had three subspecies that have a similar DNA pattern. The rest of the eight isolates each had a unique pattern of DNA. These isolates were classified according to the same chemical and physical characterization, but were different genetically.
Conclusion: In conclusion, using molecular methods for comparison of genomic DNA between different bacterial species of the same genus is a good measurement of genetic relations between different species, which could lead to discovery of new species unique to the local environment.

Keywords: PCR; RAPD; Bacillus; thuringiensis; genetic diversity; Kuwait.

1. INTRODUCTION

Bacillus thuringiensis constitutes the most commonly used biological insecticide and, as such, enjoys general public acceptance [1]. The greatest achievements in microbial pesticides have come from the use of commercial preparations of *Bacillus thuringiensis* (*Bt*). These have been the most effective biological pest control products worldwide [2]. The commercial interest in biological control of insects motivated intensive screening programs to search for new strains in different countries permitting the discovery of new serovares bestowing a different spectrum of entomopathogenic activity [3].

Currently, strains of *B. thuringiensis* are identified and grouped on the basis of their flagellar (H) antigens, a technique that has proven to be useful for species differentiation among the bacilli [4]. However, it has been shown that *B. cereus* can cross-react serologically with some of the *B. thuringiensis* flagella antigen [5] at a frequency close to 30%. Therefore, the distinction between a crystalliferous *B. thuringiensis* strain and *B. cerus* is tenuous, at best, when current techniques are used.

Environment screening for a new and vastly potent strains of *Bacillus thuringiensis* (*Bt*) has become as one of the conceivable approaches for insect resistance controlling [6].

Serotyping is the most widely accepted subspecific classification technique for varieties of Bt, even if strains from the same serovar do not necessarily share the biochemical, genetic, or toxicological attributes. Even though serotyping is a dependable and straight forward technique, it is done only in a few laboratories around the world. Consequently, alternative techniques, particularly molecular techniques, are being established to try to overcome those restrictions [7].

With the development of the molecular techniques the variability, taxonomic and evolutionary studies of microorganisms have increased. Random amplified polymorphic DNA (RAPD) is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence [8].

Utilization of RAPD technique to characterize individuals among the same species is supported by several recent studies [9], to conduct phylogenetic relationships [10], to detect genetic variability among closely related species [11], to reveal genetic markers for certain trait [12]. Several studies on *Bacillus thuringensis*strains displayed different genetic diversity, according to the region where they were isolated [13-17]. The RAPD technique provides a new means for characterization of bacteria. It has been used for epidemiological subtyping of *Listeria*, Campylobacter, *Brucella*, *Legionella* and *Candida* [18-21].

In the present study, we studied whether the RAPD technique can be used for differentiation and subtyping 15 locally isolated of *B. thuringiensis* [22] in order to verify if there is a relation between genetic variability of isolates and the site of isolation.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

The origin of the 15 bacillus strains used for the investigation is presented in Table 1. *Bacillus thuringiensis* indigenous strains were isolated bythe method of Travers et al. [23] with some modification. The samples were collected from various soil samples and grain dusts (on infected date palm trees). Each sample was added to 10 ml of Luria Bertani (LB) broth (1.0% trypton,

0.5% yeast extract, 0.5% Nacl) buffered with 0.25 M sodium acetate in a 125 ml flask and then cultured at 30ºC for 4 h with agitation on a rotary shaker. Afterwards, one ml volume of the culture was platted on LB agar, and incubated for 24 h at 30ºC. The isolates were tested by gram stain, spore staining and biochemical tests. The *B. thuringiensis* isolates were confirmed on the basis of morphology, gram staining and production of protein crystals. Smears were examined under a light microscope to observe the proposal crystal protein as inclusion bodies in the bacterial cell. The crystal-forming colonies were selected and subcultures for future use [23].

2.2 Growth Conditions and Biochemical Tests of Isolated Strains

B. thuringiensis var israelensis ATCC 10792, *Bacillus thuringiensis* ATCC 13366, *Bacillus thuringiensis* ATCC 33679 and *Bacillus thuringiensis* ATCC 13367 were obtained from the American Type Culture Collection, Manassas, Virginia, United States. The local isolates were from different locations in Kuwait. Different methods were combined to reach and develop the best isolation method [22,23].

The biochemical identification of the *Bacillus* strains was carried out in accordance with Bergey's Manual of Systematic Bacteriology [24]. The gram-staining characteristics, the production of catalase and oxidase, hydrolysis of D-glucose, L-arabinose, D-xylose, D-mannitol, gelatin, casein and starch, the reaction in the indole and Voges-Proskauer test, the urease and the lecithinase activities and the growth at pH 6.8, pH 5.7 and 7% NaCl was tested. In addition, all strains were identified by means of API-20 test strips (BioMerieux, Marcy-l'Etoile, France). The sensitivity to penicillin strains was determined by the disk diffusion susceptibility test [25].

2.3 Preparation of DNA Templates for PCR Analysis

Total *B. thuringiensis* DNA was isolated using Promega, USA, DNA genomic isolation kit. Cultures were grown in LB broth (10 g trypton, 5 g yeast extract, 10 g NaCl) to an optical density at 600 nm of 0.8. The cells were harvested by centrifugation and washed once in 0.5 ml of TES (10 mMTris-HCl, pH 8.0, 1 mM EDA, 100 mMNaCl).

Ten milliliters (10 ml) of Bt cells were grown in LB medium. After overnight incubation, 1 ml was centrifuged for 2 min at 14,000 rpm. The supernatant was removed and the pellet was suspended in 480 μl of 50 mm EDTA + 60 ml of 10 mg/ml lysozyme, incubated at 30ºC for 1 h, then centrifuged at 14000 rpm for 2 min and the supernatant was removed and the pellet was suspended in 600 μl of nuclei lysis solution. It was then mixed by pipetting up and down, and incubated at 80ºC for 10 min; 3 ml of RNase solution was added and mixed by inverting for 25 min, incubated at 37ºC for 1 h and then cooled at room temperature. Two hundred milliliters (200 ml) of protein precipitation solution were added and set on ice for 5 min then centrifuged at 14000 for 3 min. The supernatant was transferred to a new tube and 600 ml isopropanol was added, mixed by inverting, centrifuged at 14000 for min, washed with 70% ethanol, and centrifuged for 2 min, and the pellet was dried under vacuum. The dry DNA was re-suspended in 50 ml TE, and stored at 4ºC.

The concentration of DNA and its relative purity was determined using UV spectrophotometer based on absorption 260 and 280 mm, respectively.

2.4 RAPD Primers

Total genomic DNA from each isolate and four reference strains were amplified using five different decamer oligonucleotide primers that were tested by RAPD analysis. Oligonucleotide primers OPB-01 5'- GTTTCGCTCC-3`, OPJ-05 5'-CTCCATGGGG-3', OPJ-06 5'-CGTTCCGCA-3', OPJ-12 5'-ACTCCTGCGA-3' and OPN-02 5'-ACCAGGGGCA-3' all the primers were 60-70% G-C content with no internal repeats they were synthesized by Operon Technologies, Alameda, CA, USA [26].

2.5 RAPD Amplification

Amplification reactions were performed in volumes of 25 μl. Each reaction mixture contained 200 mM each of dATP, dCTP, dGTP and dTTP, 1.2 mM of primer, 50 ng of template DNA and 1.5 units of UltimaTaq DNA polymerase (Perkin Elmer, USA) in 1 x PCR buffer (10 mM, TrisHCl pH 8.0, 50 mMKCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100). A programmable DNA thermal cycler 2400 (Perkin Elmer Cetus, Norwalk) was used. A denaturing precycle of 5 min at 94ºC was followed by 30 cycles of 1 min at 94ºC, 1 min at 35ºC, 2 min at 72ºC and a final post-cycle of 7 min at 72°C to allow completion

of DNA extension. On completion, samples were cooled to 4ºC until electrophoresis was performed.

To ensure that the amplified DNA bands originated from gnomic DNA and not the primer artifact, negative control was carried out for each primer/strain combination. No amplification was detected in control reactions [27].

2.6 Resolution of the Amplification Product

The RAPD products were resolved by agarose gel electrophoresis and photographed under UV light [28]. Twenty microliter aliquots of the reaction products were mixed with 2 µl of hyperbasic loading solution (30% sucrose, 0.05% bromophenol blue, 30 mM/l EDTA, pH 8) and applied to agarose gel prepared in tris-acetate EDTA buffer (0.05 µl Tris, 0.005 mm sodium acetate. 0.8 mM EDTA, pH 7.9) containing 0.5 µl/mg ethidium bromide. After electrophoresis for 1 h at 125 V, the gels were excited by medium wave ultraviolet light and photographed. A molecular size marker (100 bp ladder, BRL) was included in each gel. RAPD polymorphic products were compared using Pair-wise comparisons and applying the cluster analysis to construct a dendrogram representing the difference and the relationship between the isolates. The computer analysis of RAPD patterns was performed by using the software Alpha Ease stand alone (Alpha Innotech, San Leandro, CA, USA). The resulting similarity matrix was used to construct a dendrogram employing the complete linkage method with arithmetic mean included in the molecular evolutionary genetics analysis software [29].

3. RESULTS

Genomic DNA was isolated successfully from all the soil and the reference strains of *Bacillus thuringiensis var thuringiensis*. Out of five decamer primers tested, one primer OPB-01, which generated good and reproducible bands, was selected to characterize the 15 sub-species of *B. thuringiensis* isolates.

Based on the distinct banding patterns obtained from RAPD-PCR (Fig. 1), multiple banding profiles were detected. The amplified bands had a variation in the size and number of amplified fragments. The size of amplified fragments ranged from approximately 100 bp to approximately 1500 bp. The separation of all the RAPD fragments of the gel produced patterns containing less than five visually detectable bands (Fig. 1). The results are shown as a dendrogram in Fig. 2 and presented in Table 1. The similarity matrixes calculated from the RAPD data were used to generate a dendrogram by using complete linkage (furthest neighbor) cluster method for frequency.

Table 1. RAPD type (Primer OPB-01), growth temperature, source and location of the 15 *Bacillus thuringiensis* var *thuringiensis* strains from Kuwait soil

No	Lab. code	Strain type	Growth temp. (ºC)			RAPD	Source and location
			42	52	55		
1	B7	*Thuringiensis*	+	-	-	3-B	Soil from near Sulaibikat sea shore
2	E1	*Thuringiensis*	+	-	-	3-A	Soil from Ahmadi (hydrocarbon rich)
3	E2	*Thuringiensis*	+	-	-	1-Aa	Soil from Ahmadi (hydrocarbon rich)
4	E3	*Thuringiensis*	+	-	-	2-Aa	Soil from Ahmadi (hydrocarbon rich)
5	E8	*Thuringiensis*	+	-	-	2-B	Soil from Ahmadi (hydrocarbon rich)
6	E16	*Thuringiensis*	+	-	-	1-Ab	Soil from Ahmadi (hydrocarbon rich)
7	E17	*Thuringiensis*	+	-	-	1-B	Soil from Kifan Public Garden
8	E19	*Thuringiensis*	+	-	-	2-Ab	Soil from Messila Beach
9	E20	*Thuringiensis*	+	-	-	2-Ab	Soil from Messila Beach
10	E27	*Thuringiensis*	+	-	-	1-Ab	Soil from Khetan House Garden
11	E28	*Thuringiensis*	+	-	-	1-B	Soil from Khetan House Garden
12	E29	*Thuringiensis*	+	-	-	3-A	Infected Date Palm in KISR Shuwaik
13	E30	*Thuringiensis*	+	+	-	2-B	Infected Date Palm in KISR Shuwaik
14	E31	*Thuringiensis*	+	+	-	3-B	Infected Date Palm in KISR Shuwaik
15	E32	*Thuringiensis*	+	-	-	3-A	Infected Date Palm in KISR Shuwaik

“A, B, Ab, Aa, Ab..” indicate clusters and subclusters according to RAPD similarity pattern

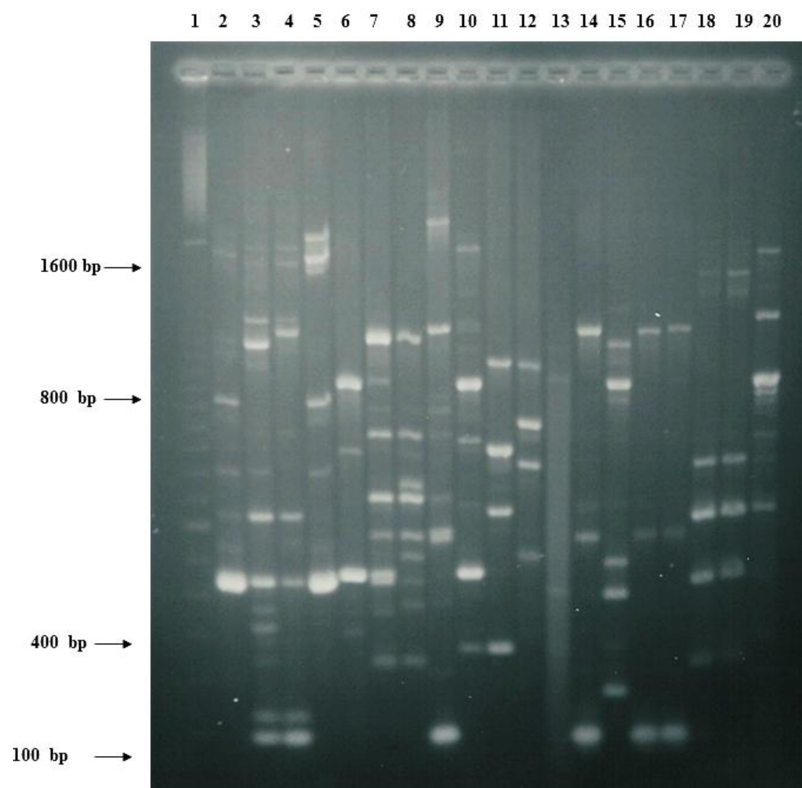

Fig. 1. The RAPD amplified pattern of genomic DNA of soil isolates of *B. thuringiensis* var *thuringiensis*

Lane 1, 100 base pair molecular marker; lanes 2, 18, 19 and 20 are ATCC33679, ATCC10792, ATCC13366, ATCC13367 reference strains respectively. Lanes 3 to 17 are soil isolates B7, E1, E2, E3, E8, E16, E17, E19, E20, E27, E28, E29, E30, E31 and E32 respectively

The dendrogram depicting the relatedness of the isolates is shown in (Fig. 2). Three distinct clusters were apparent *B. Thuringiensis* varieties generate variants, and the results shown in dendrograms of all the isolates may be interpreted as a series of clusters and sub-clusters. Dendrogram analysis showed some regional variation among the isolates between the soil samples from south of Kuwait and samples from central region, but did not indicate a clearly defined habitat location pattern of the DNA polymorphism as some of the samples were from agricultural oil such as samples "E17,E27, E28," and others were from soil with high hydrocarbon content soil such as the samples from Ahmadi city "E1,E2,E3, E8,E16".

All the 15 isolates could be grouped in three main clusters (1, 2, 3). Cluster 1 was grouped into two sub-sub-clusters 1-A, 1-B. 1-A included two sub-sub-clusters, we called 1-Aa and 1-Ab; each contained two isolates. The 1-Aa sub-sub-cluster included isolates E2 and the reference strain ATCC 13367; they gave over 65% similarity to each other. The 1-Ab sub-sub-cluster included isolates E27 and E16; they had 60% similarity. The sub-cluster 1-B included two isolates E-28 and E17, and the similarity between them was low at about 30% (Fig. 2).

Cluster 2 was grouped into two sub-clusters, 2-A and 2-B. The sub-cluster 2-A consisted of two sub-sub-clusters called 2-Aa and 2-Ab. This sub-sub-cluster included two reference strains, ATCC 13366 and ATCC 10792, and one sub-isolate E3.

The similarity between E3 and ATCC 10792 was 80% and both showed over 60% similarity to ATCC 13366. The sub-sub-cluster 2-Ab included three isolates and one reference strain. The isolates E20, E19 and the reference strain ATCC 33679 showed over 65% similarity, and the isolates E2 and E19 had 80% similarity. All three showed about 63% similarity with the isolate E8. The sub-cluster 2-B included E30 only and had over 30% similarity with sub-cluster 2-A.

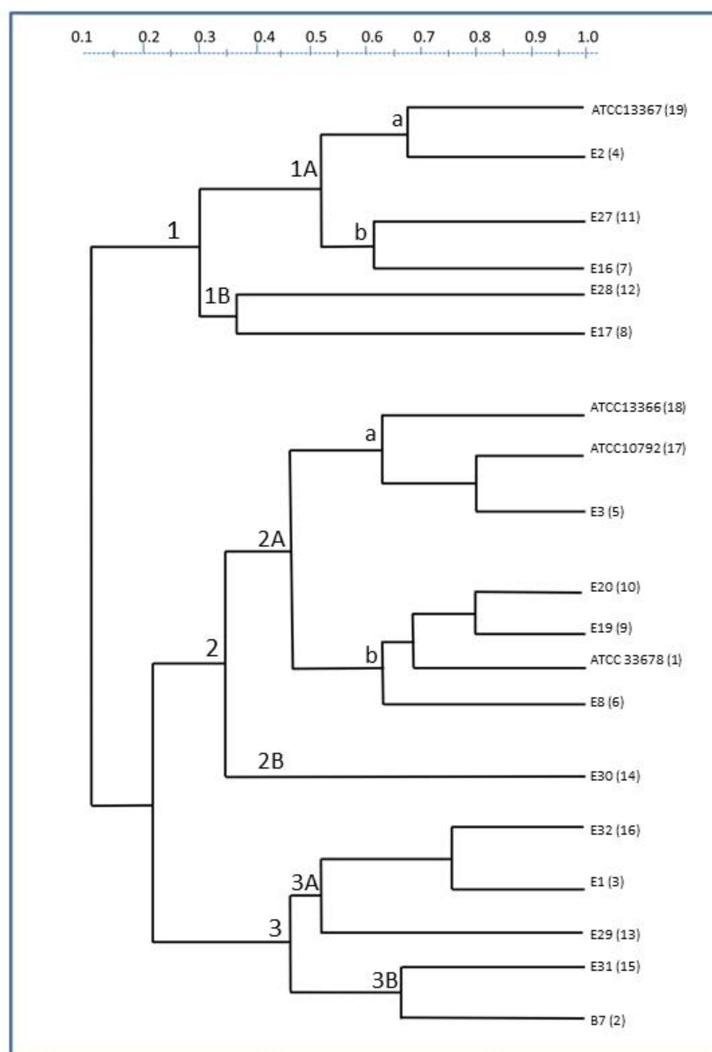

Fig. 2. A dendrogram generated from the similarity coefficient computed from the pattern shown in the agarose RAPD gel experiment using the neighbor joining method

Cluster 3 cluster was grouped into two sub-clusters 3-A and 3-B. The 3-A sub- cluster included three isolates, E32, E1 and E29, with E32 and E1 having 75% similarity. The 3-B sub-cluster included two isolates, E31 and B7, with 68% similarity (Fig. 2).

In Table 1, two strains, E30 and E31, are capable of growth at 52 Celsius. This is unusually high for *Bacillus thuringiensis*, these could have specially evolved to resist the high temperature commonly found in this part of the world (Hot arid zone).

The placement of the fifteen *B. thuringiensis* in three major clusters and ATCC reference strains in the second major cluster indicated the effectiveness of the RAPD - PCR technique as a powerful method to differentiate between bacterial strains from different subspecies.

4. DISCUSSION

The use of molecular methods can provide a measure of genetic relatedness, which will allow distinguishing between unrelated strains, and identifying separate isolates in the same strain; and provide a tool to answer some of the most fundamental questions related to microbial diversity in the soil environment.

Random amplified polymorph DNA (RAPD) analysis is a DNA fingerprinting technique used

to detect genomic polymorphism [26]. RAPD analysis has been widely used in numerous applications, including gene-mapping, detection of strain diversity, population analysis, epidemiology and the demonstration of phylogenetic and taxonomic relationships [30]. Its popularity arises from its ability to quickly detect polymorphism at a number of different loci using nanogram quantities of genomic DNA. In this study, the RAPD method was used to analyze polymorphism for *Bacillus thuringiensis* var *thuringiensis,* and it was found that this sub-species is highly heterologous on the genetic level.

The RAPD analysis of the 15 strains of *B. thuringiensis* revealed 15 different DNA profiles with the primer OPB-01. The DNA profiles attained with the primer OPB-01 are shown in Fig. 2. With this primer, individual strains showed up to four different DNA bands, whereas only two distinct bands became visible with other primers (data shown). The different DNA profiles, which could be determined with the primer OPB, were substituted into strain types (Table 1).

Within the 15 *B. thuringiensis* strains, a total of eight isolates typed could be distinguished. The majority of *B. thuringiensis* deriving from the soil were classified as 1-A and 2-A types. One of the three strains from the infected tree was identified as the strain type 2-B while the rest were identified as 3-A.

The RAPD patterns, which were found in one isolate of *B. thuringiensis,* E2, clearly differed from those of the rest of the isolates. It was striking that the isolate E3 showed large similarities with the ATCC 10792 reference strain; the closest similarities were in the large type 2-A. This could indicate the uniqueness of the isolate to the soil of origin as the references were from the North American agricultural soil.

These results show that the primer OPB-01 is very suitable for characterization of *B. thuringiensis* strains, which will give more differentiated RAPD profiles. It has been observed that random primers with high GC content (60%) resulted in a greater and better reproducible number of strain specific bands. This result is in agreement with the findings of other researchers [31].

Brousseau et al. [30], reported on the feasibility of the RAPD techniques, rapid identification of commercial strains of *B. thuringiensis.* As in the present investigation, the authors revealed discriminating DNA fingerprints using only a single primer. Nevertheless, for epidemiological sub-typing of bacterial strains, simultaneous use of different primers has been recommended [26,32].

The RAPD study presented here indicate that it could provide an alternative to serotyping for *B. thuringiensis*. Serotyping has provided a valuable subspecific classification of *B. thurigiensis* for over four decades, but suffers limitations [32]. Some strains cannot be typed because they lack flagella or agglutinate and specialist antisera are needed. Furthermore, typing based on whole genome patterns of one kind or another has become the norm for pathologically important microorganisms [33].

5. CONCLUSION

In conclusion, RAPD is a simple and reproducible technique, which can be adapted to work reproducibly with soil bacterial colonies rather than purified genomic DNA. This technology allows rapid identification of closely related commercial strains of *B. thuringiensis.* This technique should prove to be a useful tool for assessment and quality control of this important biological insecticide. *Bacillus thuringiensis* isolates can be characterized, identify and differentiate using RAPD obtained data. The generated RAPD specific markers might be used in the tracking of these isolates.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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