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A Simple Method for Direct Detection and Discrimination of *A. baumannii* in Tracheal Aspirates without Culture Isolation

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Abstract

Currently, available phenotyping and commercial methods report *A. baumannii* only as *Acinetobacter calcoaceticus-baumannii* complex (ACB) and do not identify individual members of the complex. This is a single blind study aimed to evaluate certain commonly used species-specific genetic markers namely, Intergenic Transcribed Spacer region in 16S rRNA of *A. baumannii* (Ab-ITS) and *gyrB*, for identification of ACB members. These molecular targets were first validated on clinical isolates (n = 200) and subsequently on uncultured tracheal aspirates (n = 172). Among the clinical isolates, 183/200 (91.5%) were positive for Ab-ITS. The clinical isolates 17 (17/200) which are failed to amplify in Ab-ITS PCR were subsequently diagnosed by *gyrB* PCR as *A. calcoaceticus* (n = 2), *A. pitti* (n = 6) and *A. nosocomialis* (n = 9) but not *A. baumannii*. Among the tracheal aspirates, 62 samples were reported as sterile in Advanced Expert System of VITEK-2, among the remaining 110 samples, 68.1% (75/110) samples contained Ab-ITS target. Twenty-five of the sterile samples (25/62) were found to contain Ab-ITS target sequence. Since, our sample processing method enabled identification of all the species of ACB complex by PCR even in uncultured tracheal aspirates, adaptation of our protocol would enable same day (6 - 8 h) reporting and help the clinician make evidence based therapeutic decision quickly.

1. Introduction

Acinetobacter baumannii causes a vast array of infections including ventilator associated pneumonia (VAP), secondary meningitis, urinary tract infections, septicemia and other severe infectious complications in the critical care (CCU) patients [1] [2]. Clinical microbiology laboratories usually report *A. baumannii* as "ACB complex". This includes six individual species namely *A. baumannii*, *A. calcoaceticus*, *A. pitti*, *and A. nosocomialis*, *A. seifertii*, *A. lactucae* [3]. These members have epidemiological and clinically relevant differences. They exhibit different drug susceptibility profiles though they are genetically and phenotypically very similar. In particular, *A. baumannii* is responsible for a large proportion of nosocomial infections. There has been a tremendous increase in antibiotic resistance among *A. baumannii* isolates in the last two decades [4]. Members of ACB complex pose limitations in phenotyping and conventional microbiological identification [5] [6] [7] [8]. Therefore, it would be desirable to apply molecular methods to identify the individual species of the ACB complex [7] [9]. Also, it would be ideal to be able to detect and report the species directly rapidly from the clinical sample without waiting for culture isolation and characterization.

Molecular methods which are used for identification of *Acinetobacter* spp. include ribotyping, polymerase chain reaction (PCR) hybridization with species-specific probes, methods based on the 16S - 23S rRNA intergenic spacer region, tRNA spacer fingerprinting, Pulse Field Gel Electrophoresis (PFGE), methods based on *gyrB* and *rpoB* sequences, PCR-RFLP and AFLP analysis, Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Random Amplified Polymorphic DNA (RAPD) typing [10] [11]. Amplified Ribosomal DNA Restriction Analysis (ARDRA) is rapid and more practical for species identification [12]. The main disadvantage of these typing methods is low reproducibility, especially in terms of global molecular epidemiology [7]. Sequence-based typing, such as single or multi locus or whole-genome sequencing, provides more reliable data for comparison from a geographical point of view or globally. However, the high cost of sequence-based typing prevents implementation by developing countries [6] [13]. Failure to identify the *A. baumannii* in ACB complex may adversely impact both diagnosis and infection control. Thus, there is an unmet need for a rapid and affordable diagnostic tool to identify *A. baumannii* in clinical isolates in general and particularly from uncultured clinical specimens. A promising and robust molecular target for identification of *Acinetobacter* spp. would be the polymorphisms within the rRNA intergenic spacer (ITS) sequences. These rRNA genes are present in the same order (*i.e.* 16S, 23S, and 5S) in their transcriptional

units and well conserved throughout most bacterial species [14]. Among the available genetic targets, intergenic spacer in *A. baumannii* (Ab-ITS) holds-out as a good genetic marker for the species identification as it offers low intra species variation and high degrees of interspecies divergence [1] [9].

A simple sample processing protocol and PCR were optimized for detection of *A. baumannii* in uncultured tracheal aspirates. The internal transcribed spacer region of the ribosomal RNA of *A. baumannii*, called Ab-ITS, was used for identification of *A. baumannii* species. Results of this Ab-ITS-PCR were evaluated against conventional clinical microbiology phenotyping methods and VITEK 2 system. We also present in this article our experience of using *gyrB* based target sequences for differentiation of individual members of ACB complex.

2. Materials and Methods

This is a prospective study conducted from November 2014 to March 2015, at Gleneagles Global Hospitals.

2.1. Specimen Collection

Samples were collected from two tertiary care health centers in Hyderabad for this study.

Category 1: This group comprised of characterized archival clinical isolates which were reported as ACB complex. These isolates were collected from the department of Clinical Microbiology of Gleneagles Global hospital, Hyderabad (n = 200).

Category 2: The leftover tracheal aspirate specimens (n = 172) from the department of clinical microbiology of Nizam's Institute of Medical Sciences (NIMS), Hyderabad were collected, and stored at 2˚C - 8˚C (not more than 48 h) till sample processing was performed for genotyping. No criteria were imposed on inclusion of these samples except that all tracheal aspirates were collected from patients in the intensive care units suffering from pneumonia. The results of clinical microbiology characterization of these tracheal aspirate samples were blinded to our laboratory and were decoded only after the Ab-ITS genotyping was completed.

Four reference strains—*A. baumannii* (ATCC 17978), *A. pitti* (MCC 2077-T), *A. calcoaceticus* (MCC 2329), *A. nosocomialis* (ATCC 17903) belonging to different genome species of ACB complex were obtained from culture collections (ATCC and National Center for Microbial Resource, India), for quality assurance. *Staphylococcus aureus* (ATCC 43300, ATCC 25923) was used as negative control.

2.2. Genomic DNA Preparation

Cell free DNA lysates were prepared from isolates of Category 1 and tracheal aspirates of Category 2, by Tris-EDTA-Triton-X (TEX) buffer protocol (10 mM Tris-HCl pH 8.5, 1 mM EDTA, 1% (w/v) Triton X-100) [15]. Single colonies

isolated on the MHA were inoculated into MH broth and allowed to grow at 37°C for 24 h and processed just like tracheal aspirates. The concentration of DNA (ng/μl) was determined by absorbance (260/280) in Nanodrop ND2000. The sample processing is presented schematically in Figure 1.

2.3. Genotyping of *A. baumannii* by Ab-ITS PCR

PCR assay was performed with the genomic DNA in 20 μl total reaction volume. The PCR reaction mixture contained 20 pmol of each primer, 200 μmol of dNTPs, 1 U of Taq DNA polymerase (KAPA Taq DNA Polymerase, KAPA Biosystems Inc), 2 μl 10X buffer, 1.65 mM MgCl$_2$ and 100 ng of genomic DNA lysate. The primers and PCR conditions used in this study are given in Table 1. The amplicons of ITS region and *gyrB* were resolved and analyzed on 2% agarose gel after ethidium bromide staining and documented in a gel documentation system (UVITEC, UK). The tracheal aspirate's phenotyping results were de-coded after the genotyping was completed.

3. Results

3.1. Phenotyping and AST

The 200 ACB isolates which belonged to Category 1 were identified in the clinical microbiology laboratory using both manual (biochemical) and automated methods and results interpreted as per BSAC guidelines (2015) [16].
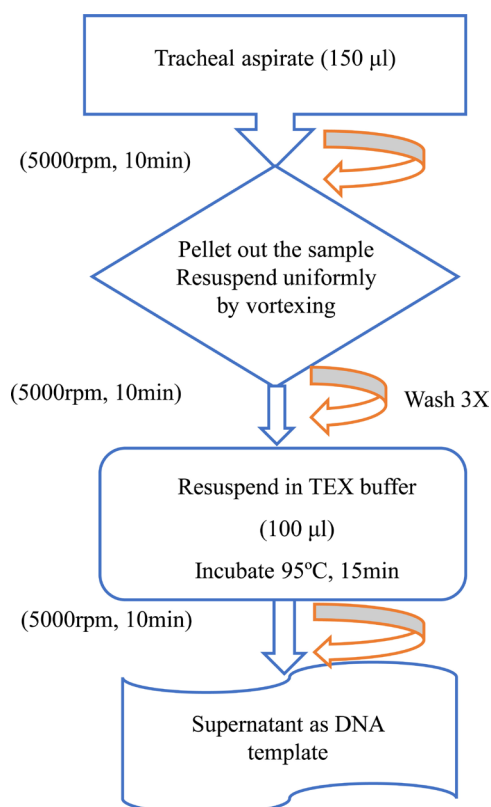

Figure 1. Schematic of tracheal samples processing through TEX method.

Table 1. List of primers and PCR conditions used in this study.

Target gene	Primer name	Sequence (5'-3')	Amplicon size (bp)	PCR conditions	Reference
Ab-ITS	Ab-ITS F	CATTATCACGGTAATTAGTG	208	94 ℃ × 5 min 94 ℃ × 25 s 53 ℃ × 40 s ⎫ 35 cycles 72 ℃ × 50 s 72 ℃ × 6 min	[9] [10]
	Ab-ITS R	AGAGCACTGTGCACTTAAG			
gyrB	D14	GACAACAGTTATAAGGTTTCAGGTG	428	94 ℃ × 5 min 94 ℃ × 1 min 60 ℃ × 30 s ⎫ 25 cycles 72 ℃ × 1 min 72 ℃ × 10 min	[18]
	D19	CCGCTATCTGTATCCGCAGTA			
	D16	GATAACAGCTATAAAGTTTCAGGTGGT	193		
	D8	CAAAAACGTACAGTTGTACCACTGC			
	Sp4F	CACGCCGTAAGAGTGCATTA	294		
	Sp4R	AACGGAGCTTGTCAGGGTTA			

The microbial profiles of all tracheal aspirates were determined using VITEK-2 (BioMérieux, Marcy l'Etoile France) in the clinical microbiology department, as per Clinical & Laboratory Standards Institute (CLSI, 2015) guidelines (Wayne 2017) [17].

3.2. Ab-ITS PCR Results

Category 1 (n = 200) ACB isolates

183 (91.5%) isolates were positive for Ab-ITS and a typical pattern of amplicons in agarose gel electrophoresis is represented in Figure 2. Results of identification and drug sensitivity profiles of ACB isolates are listed in Table 2.

3.3. Characterization of the Tracheal Aspirates

After phenotyping, 110 tracheal aspirates were reported as: *A. baumannii* (n = 43), Polymicrobial growth (n = 23), Colonizers (n = 20), *K. pneumonia* (n = 8), *P. aeruginosa* (n = 7), *E. coli* (n = 2), MRSA (n = 3), Diphtheroids (n = 2) and others (n = 2). Sixty-two tracheal aspirates (36%, 62/172) were reported as sterile by VITEK-2. Among these 172 tracheal aspirates, 90 (52.3%) were positive for *A. baumannii* specific marker (Ab-ITS). Among the 62 samples which were reported as sterile by Advanced Expert System of VITEK-2, twenty-five (25/62) were positive for Ab-ITS. Decoded phenotypic data of tracheal aspirates in comparison to our PCR are presented in Table 3.

3.4. Genotyping of ACB Complex by *gyrB* PCR

Several isolates (17/200) of Category 1 tested negative for Ab-ITS PCR. Since *gyrB* PCR has been successfully applied to discriminate and identify members of ACB complex [9] [10] [18]. We first optimized the amplification conditions for the four members of ACB complex: they were *A. calcoaceticus*, *A. pitti* and *A. nosocomialis* in addition to *A. baumannii* and interestingly all the seventeen of them were identified as *A. calcoaceticus* (n = 2), *A. pitti* (n = 6) and *A. nosocomialis*

Lane L- Low range ladder (50bp to 1500bp), Lane PC - Positive Control (ATCC 19606), Lane 1-2-Clinical ACB isolates, Lane 3-4- Tracheal aspirates, Lane NC – No target control

Figure 2. Agarose gel electrophoresis of Ab-ITS PCR.

Table 2. Performance of genotyping and phenotyping approaches used in the identification of ABC isolates.

S. No.	Sample Source	Ab-ITS* (n)	*gyrB*[#] (n)	ACB members(n)
1	Respiratory secretions (73)	69	4	*A. baumannii* (n = 69), *A. nosocomialis* (n = 2), *A. pitti* (n = 2)
2	Blood (50)	47	3	*A. baumannii* (n = 47), *A. nosocomialis* (n = 1), *A. pitti* (n = 2)
3	Wound swab (38)	33	5	*A. baumannii* (n = 33), *A. nosocomialis* (n = 3), *A. pitti* (n = 2)
4	Sputum (33)	29	4	*A. baumannii* (n = 29), *A. nosocomialis* (n = 3), *A. calcoaceticus* (n = 1)
5	Body fluids (6)	5	1	*A. baumannii* (n = 5), *A. calcoaceticus* (n = 1)
	Total (200)	183	17	

*Species specific marker (Ab-ITS); [#]gyrase B gene (DNA topoisomerase); n = Number of positives.

Table 3. Decoded phenotypic data of tracheal aspirates in comparison to our PCR genotyping.

| Microbial ID from Tracheal aspirates	VITEK-2 Results (n)	Ab-ITS * Results (n)
A. baumannii	43	39
E. Coli	2	0
Klebsiella pneumonia	8	0
Pseudomonas aeruginosa	7	0
Diphtheroids	2	0
Serratiamarcescens + *A. baumannii*	1	1
MRSA	3	0
[a]Polymicrobial growth	21	11
[b]Polymicrobial growth with *Acinetobacter spp*	2	2
[c]Colonizers with *A. baumannii*	2	2
[d]Colonizers without *A. baumannii*	18	9
[e]No growth	62 (Sterile)	25
TOTAL	172	90[#]

*—Ab-ITS-16S-23S rRNA gene intergenic spacer region, [a,b,c,d,e]—Results as obtained from Advanced Expert System of VITEK-2, [#]—Total number of Ab-ITS positive samples (including sterile samples).

(n = 9). The results are documented in **Figure 3**.

Four of the tracheal isolates were reported as ACB complex but when we screened them by Ab-ITS PCR, they did not contain the Ab-ITS target sequence (n = 4). These tracheal aspirates were processed as shown in scheme **Figure 1** and subjected to *gyrB*-PCR with the primer sets for the above mentioned four species of ACB complex and analyzed by agarose gel electrophoresis to identify the species (**Figure 4**). Two of them were identified as *A. nosocomialis* (n = 2) and we could not confirm the *Acinetobacter* species of the other two samples.

3.5. Statistical Analysis of the Performance of PCR Assays

MedCalc software (MedCalc Statistical Software version 15.6.1, MedCalc Software,
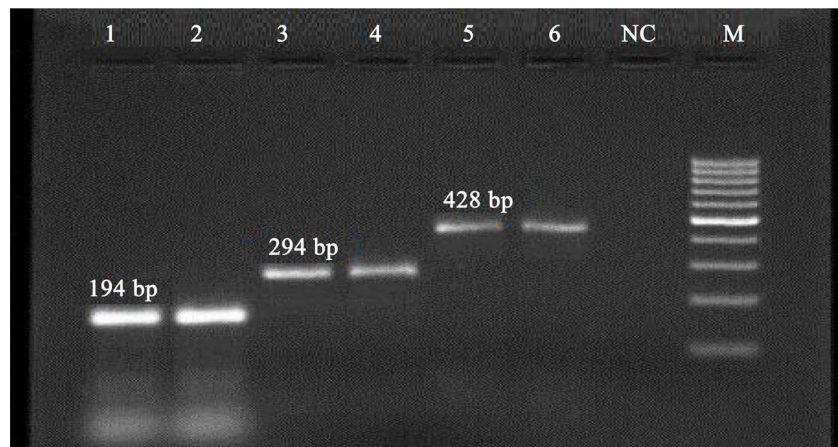
Lane M- Molecular marker (100bp to 1000bp), Lane 1- Positive Control (*A. pitti* MCC 2397), Lane 2, 4 & 6 - ACB clinical isolates, Lane 3 - Positive Control (*A. nosocomialis* ATCC 17903), Lane 5 -Positive Control (*A. calcoaceticus* MCC 2077(T), Lane NC - No target control

Figure 3. *gyrB* genotyping of ACB complex by PCR.
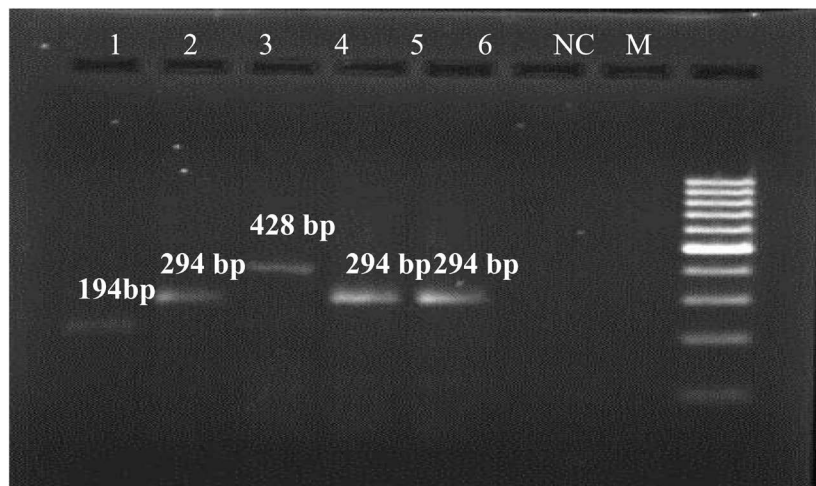
Lane M-Molecular marker (100bp to 1000bp) Lane 1-Positive Control (*A. pitti* MCC 2397) , Lane 2-Positive Control (*A. nosocomialis* ATCC 17903), Lane 3-Positive Control (*A. calcoaceticus* MCC 2077(T), Lane 4 & 5 -Tracheal aspirates positive for *A. nosocomialis,* Lane 6-Tracheal aspirate negative for ACB members. Lane NC-Negative PCR Control.

Figure 4. *gyrB* PCR results of tracheal aspirates.

Ostend, Belgium; https://www.medcalc.org; 2015) was used for statistical analysis of the data.

The Ab-ITS PCR had 92% Sensitivity, 100% Specificity, with a Positive Predictive Value (PPV) of 98.92% and 100% Negative Predictive Value (NPV) (Figure 5).

When we evaluated the performance of the genetic marker Ab-ITS against the reference method VITEK-2, 44(44/110) tracheal aspirates were positive by both PCR and VITEK-2, whereas 31 samples were "false positive" in PCR genotyping. Thus Ab-ITS PCR showed greater sensitivity (100%) and specificity (53.03%) compared to VITEK-2 for identification of *Acinetobacter baumannii* (Figure 6).

4. Discussion

A. baumannii has emerged as one of the most notorious pathogens in health care facilities globally, targeting the critically ill hospitalized patients with its dreadful drug resistance profile. With an MDR rate of 92.1% and no new drug on the horizon, *A. baumannii* infections have become a real challenge for critical care and general physicians [7] [19] [20] [21]. Reporting time for identification of *A. baumannii* in clinical samples directly impacts the outcome of critical care. In this study we have focused on the identification of *A. baumannii* by evaluating a commonly used species-specific genetic marker and subsequently using a simple sample processing protocol for tracheal aspirates coupled to PCR for direct detection

*Conventional phenotyping

		+	-	
Ab- ITS	**+**	181	2	**183**
	-	17	0	
		198	**2**	**200**

Statistical analysis: Sensitivity 92%, Specificity 100%, Positive Predictive Value (PPV) 98.92% and Negative Predictive Value 100% (MedCalc Statistical Software version 15.6.1). *Species identification and characterization done by biochemical tests.

Figure 5. Comparison of species-specific marker (Ab-ITS) vs Phenotypic assay in ACB isolates (n = 200).

VITEK-2

		+	-	
Ab- ITS	**+**	**44**	**31**	75
	-	**0**	**35**	35
		44	**66**	**110**

Statistical analysis: Sensitivity 100%, Specificity 53.03%, Positive Predictive Value (PPV) 58. 67% and Negative Predictive Value (NPV) 100% (MedCalc Statistical Software version 15.6.1). This analysis considered only the clinical samples contained bacterial growth (110/172) and 62 samples were sterile samples.

Figure 6. Comparison of species-specific marker (Ab-ITS) vs VITEK-2 in tracheal aspirates (n = 110).

and confirmation of *A. baumannii* in uncultured clinical sample. It is well known that tracheal aspirates are rich source of mixed microbes. Our protocol worked remarkably well on these samples and was able to identify *A. baumannii* correctly. Ab-ITS seems to be a more reliable and sensitive genetic marker for identification of *A. baumannii*. Sequences of the ITS regions have been found to have low levels of intra-species variation and high levels of interspecies divergence [9] [10]. We used the primer sequences of *Chen et al.* (2007) and it was interesting to note that these primers proved absolutely specific to *A. baumannii [10]*. Including species specific *gyrB* primers along with Ab-ITS primers would be an ideal way to make differential diagnosis of ACB complex directly from uncultured clinical samples. *Acinetobacter* genus consists of about 33 species and six of them, grouped as ACB, are more frequently associated with human diseases. They are *A. baumannii*, *Acinetobacter* 13TU and *Acinetobacter* genome species 3 were frequently found in several outbreaks [7]. Commercial systems like API 20NE and VITEK-2 are increasingly being used for identification of species but these systems fail to discriminate closely related species [22]. The six members of ACB complex are very closely related and difficult to differentiate by conventional phenotyping or as was shown in this study even by automated systems like VITEK 2 and are missed sometimes even by PCR. Individual members of ACB complex should be identified correctly from *A. baumannii* as their drug sensitivity profiles are different [5]. Ab-ITS PCR is a reliable, accurate method and can be applied directly on uncultured tracheal aspirates for rapid identification of *A. baumannii*. The sample processing protocol used in this study is derived from the original protocol published decades ago for processing a variety of clinical samples for the detection of *M. tuberculosis* and therefore, it should work well with any type of clinical specimen [19]. However, since the microbial population would be different in each type of specimen, our protocol is currently being validated on other types of clinical specimen.

The combination of genetic markers used in this study, provides powerful information not only on identification of *A. baumannii* but also on probable outbreak potential and allows, to some extent, prediction of carbapenem resistance. The superiority of our genotyping approach compared to VITEK-2 is evident from the sensitivity profile and helps significantly in the differential diagnosis of individual members of ACB complex. Further, our protocol can be applied to both clinical isolates and for direct detection from uncultured clinical samples. This is certainly an advantage as results could be intimated to clinician the same day to make quick therapeutic decisions. Sixty-two tracheal aspirates were reported as sterile by VITEK-2 (mainstay in many advanced clinical microbiology laboratories) while twenty-five of them found to harbor Ab-ITS target sequence specific for *A. baumannii*. Availability of multiple copies of 16S-23S rRNA in bacterial cells ensures superior sensitivity of PCR compared to culture. It is possible that these patients apparently had *A. baumannii* infection and the antimicrobial therapy killed them. Such information from the "so called sterile sam-

ples" will be very useful for transmission, antibiotic therapy and molecular epidemiology. Our method has not yet been validated on *A. seifertii*, *A. lactucae* which are also members of ACB complex and further we have demonstrated our method only on clinical isolates and tracheal aspirates. We are hopeful that the sample processing protocol would work equally well with all types of clinical specimen as it was originally reported [15]. The in-house sample processing and PCR methods for clinical specimen can be easily adopted for rapid detection of *A. baumannii* much before culture results are obtained. Implementation of this simple and economical protocol would help the clinician to make evidence based therapeutic decision on the same day.

5. Conclusion

This study showed that the 16S - 23S rRNA intergenic spacer region possesses high discriminatory and better sensitivity to identify *A. baumannii* to the species level among ACB complex compared to conventional microbiology techniques. In this study, a comparison between a well-established species-specific genetic marker, Ab-ITS and a commonly used microbial identification system VITEK 2, was made. A convenient and rapid sample processing method was evaluated, for identification of individual species of ACB complex and their drug resistance profiles. The results are available in about 6 - 8 h, enabling the clinicians in infection control. Implementation of this protocol would help not only in control of hospital acquired infections but also improve the outcome of such infections.

Acknowledgements

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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