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Characteristics of 32 *Acinetobacter baumanni* isolates co-harboring OXA-Carbapenemases and 16S rRNA methylase gene

Padde John Roberts, Jama Suleiman Hassan, Xiaohan Li and Mingcheng Li*

Department of Clinical Microbiology, School of Laboratory Medicine, Beihua University, Jilin, 132013, PRC, China.

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Multiple drug-resistant strains of Acinetobacter have become common in hospitals worldwide. The problem becomes more acute with existence of hidden Carbapanemase-resistance genes that co-exist with other resistance determinants. Based on this, this research aims to determine the occurrence and co-existence of OXA-type carbapenemases and 16S rRNA methylase gene among clinical isolates of *A. baumannii* in a Jilin hospital, China. Between January 2012 to December 2013, 32 *A. baumanii* non-repetitive strains were isolated from nosocomial clinical specimens. Susceptibility to antibiotics was determined by the standard microdilution method and phenotypic testing was used to detect the presence of carbapenemases. PCR was performed using specific primers for detection of class D carbapenemase genes and 16S rRNA methylase gene. Results revealed that all imipinem resistant *A. baumannii* isolates were resistant to all antibiotics tested except polymycin E. The isolates harbored different Oxacillin Type Carbapenemases, that is, 62.5% of the isolates harbored OXA-51, 37.5% OXA-23, 18.8% OXA-58, 3.1% OXA-24 and 59.4% harbored 16S rRNA methylase gene. Of the 19 isolates harboring 16S rRNA methylase gene, 47.4% coexisted with at least one oxacillinase gene while 40.6% of the 32 *A. baumanii* isolates harbored more than one different oxacillinase gene. At least one oxacillinase gene was detected in 28.1% of the isolates that were phenotypically negative for carbapenemase production. The current study therefore shows co-existence of resistance determinant genes and existence of hidden genes responsible for resistance. Multiple mechanisms are likely to work in synergism to produce this phenotype.

Key words: *A. baumannii*, metallo-β-lactamases, nosocomial infection.

INTRODUCTION

Acinetobacter baumannii is a glucose non-fermentative Gram-negative bacillus classified as an opportunistic pathogen and is usually involved in infectious outbreaks originating in intensive care units (Peleg et al., 2008). The infections caused by *A. baumannii* include bloodstream infections and ventilator-associated pneumonia as well as urinary tract infection. The extensive dissemination of carbapeneme-resistant *A. baumannii* clonal strains

*Corresponding author. E-mail: limingcheng1964@163.com Tel: (+86) 0432-64608560. Fax: (+86)-0432-64608115.

causing episodes of bacteremia and/or sepsis mostly result of transmission via multiple contaminated surfaces and objects and transiently colonized health care workers' hands (Perez et al., 2007).

The beta-lactam antibiotics are an important group of antibiotics that have been used to treat infections caused by various microorganisms, including *A. baumannii*, (Livermore and Woodford, 2006) due to their efficacy and safety and because their activity can be increased by chemical modification. This was until 1970s' where some isolates of *A. baumannii* were found to be resistant to a wide range of antibiotics including broad spectrum beta-lactams, aminoglycosides and flouroquinolones (Renu et al., 2010). However, decreased susceptibility to carbapenems has been reported worldwide (Uvizl et al., 2011; Trecarichi et al., 2011). This fact is associated with high mortality and morbidity rates, prolonged hospital stays and increased treatment-related costs.

The emergence of carbapenem resistance in *A. baumannii* has become a global concern since these β-lactams are often the only effective treatment left against many multidrug-resistant strains. The major mechanism of carbapenem resistance in *A. baumannii* is the production of OXA-type genes (OXA-23,OXA-24,OXA-51 and OXA-58) and/or metallo-β-lactamase (VIM,IMP,NDM) (MBL) enzymes belonging to Ambler classes D carbapenemases or Class B Metalo-β-lactamase (MBL) respectively (Poirel et al., 2008). A recent development has been the discovery of a novel group of narrow-spectrum OXA β-lactamases in carbapenem-resistant strains, some of which have acquired the ability to hydrolyse the carbapenems (Opazo et al., 2012). These enzymes belong to three unrelated groups of clavulanic acid-resistant □-lactamases, represented by OXA-23, OXA-24 and OXA-58, that can be either plasmid- or chromosomally-encoded. *A. baumannii* also possesses an intrinsic carbapenem-hydrolysing oxacillinase, the expression of which may vary and may play a role in carbapenem resistance (Lee et al., 2006).

These OXA-type cabarpanemaes (OTC) have been described in different provinces of China. Wang et al. (2011) indicated that OXA-58-like, a carbapenem-resistance determinant located on transferable plasmids and spread by clonal dissemination in most cases, is an emerging threat in China. OXA-51 and OXA-23 in China have been reportedly carried by almost all *A. baumannii* strains isolated due to their chromosomal location (Dijkshoorn et al., 1998).

The 16S rRNA methylase gene is often responsible for the phenotype of Gram-negative pathogens that show high MICs to most aminoglycosides partly due to its enzymatic modification and methylation. 16S rRNA methylase gene has been isolated in *A. baumannii* strains from Korea, Japan, China and other parts of the world with the first gene reported in a strain of *Klebsiella pneumoniae* in France (Wang and Chen, 2005).

The 16S rRNA methylase genes are mostly located on transposons within transferable plasmids, which provide them with the potential to spread horizontally. Some of the *A. baumannii* strains have been found to coproduce extended-spectrum β-lactamases or metallo-β-lactamases, contributing to their multidrug-resistant phenotypes. In addition, studies reveal that *A. baumanii* strains harboring more than one OXA-encoding gene are emerging (Brown and Amyes, unpublished results).

Identification of OTC carrying isolates has been a challenge due to emergency of hidden carbapenem susceptible oxacillinases which may be missed in daily Lab practice. These carbapenem susceptible organisms with hidden oxacillinase genes can spread unnoticed in hospitals. Furthermore, these resistance determinants are located on transferable plasmids consequently easily spread.

The objective of this study was to characterize *A. baumannii* isolates carrying Oxa-type carbapenemase (OTC) from clinical specimens collected from patients in a Jilin hospital in order to clarify the phenotypes and genotypes. The study emphasized on determination of the occurrence and co-existence of OXA-type carbapenemases with 16S rRNA methylase gene.

MATERIALS AND METHODS

Bacteria strains

A total of 32 non-repetitive imipenem-resistant isolates of *A. baumannii* were recovered from clinical respiratory infections in hospitalized patients from January 2012 to December 2013 in a Jilin hospital, China. The identification of strains was performed by standard microbiology procedures including growth on MacConkey agar and simple biochemical tests like Oxidase test, growth on TSI and Eosin Methylene Blue Agar and ability to grow at 44°C. Analytical profile index (API) system procedure was used to biochemically identify the strains.

Kirby Bauer disc diffusion

Antibiotic susceptibility testing was done on all isolates using commercially available discs by the Kirby Bauer disc diffusion method and interpreted as recommended by Clinical Laboratory Standards Unit (CLSI, 2010). The turbidity of the culture suspension was diluted to match 0.5 McFarland standards according to CLSI. The following antimicrobial discs were used: ampicillin (10 µg), piperacillin (160 µg), cephelothin (30 µg), tetracycline (30 µg), aztreaonm (30 µg), chloramphenicol (30 µg), polymycin E (10 µg), cefotaxime (30 µg), ceftaxime (30 µg) and ceftriaxone (30 µg).

Determination of MIC

In all *Acinetobacter spp.* isolates, susceptibility to antibiotics was determined by a standard micro-dilution method according to the Clinical Laboratory Standards Unit (CLSI, 2010).

Phenotypic determination of carbapenemase production

Carbapenemase production in *Acinetobacter spp*. Isolates with a

Table 1. Different genes/enzymes responsible for resistance in *Acinetobacter baumannii* (John Roberts Table).

Gene/Enzymes	Class/family	Antimicrobial class
OXA-23, OXA-24, OXA-51, OXA-58	Class D carbapenemases	Carbapenems
VIM,IMP,NDM	Class B Metalo-β-lactamase (MBL)	Carbapenems
16S rRNA		Aminoglycosides

OXA: Oxacillinase.

MIC for meropenem of >2 mg/L was phenotypically determined by the Modified Hodge Test (MHT) for carbapenemases production (Amjad et al., 2011).

PCR detection of OXA- genes and 16S rRNA genes

The boiling method was used to extract DNA from the bacteria as described by Vaneechoutte et al. (1995). Briefly, one colony of a pure culture grown on TSI slanting agar was re-suspended in 50 µl of LB broth and incubated at 37°C in an orbital shaker for 14 h. 50 µl of the growth was centrifuged and resultant pellet re-suspended in 50 µl of distilled water and heated at 100°C for 10 min. After centrifugation in a micro-centrifuge, at 6000 x g for 3 min, the supernatant was kept at -20°C for further use.

PCR was carried out in a 25 µl reaction volumes with 1 µl of extracted DNA, 12.5 µm of the PCR master Mix containing 1.25 U of Taq Polymerase, 1 x PCR buffer containing 0.1 mM $MgCl_2$ and 240 µM of each dNTP. Specific primers used are shown in Table 1. PCR conditions were as follows: 94°C for 5 min and then for 35 cycles, 94°C for 30 s, 57°C for 45 s, 72°C for 30 s and then final extension at 72°C for 10 min. Amplified products from the isolates were analyzed by electrophoresis on 1% (w/v) agarose gel stained with Gel Red.

Competent cells were prepared from protocol given by Joe (2005). The DNA band of interest was excised, purified with a QIAqick PCR purification kit, ligated to pGEM-T Easy vector (Promega), transformed into *E. coli*$_{DH5\alpha}$ competent cells, and the white colonies which were successfully transformed and selected were screened. Nucleotide sequencing was performed directly on cloned fragments using an ABI Prism 377 DNA sequencer as a control and to rule out non-specificity in case of novel gene sequences. Sequence similarity searcher was carried out with the BLAST program available at the website of the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov).

RESULTS

Antimicrobial susceptibility to imipenem determined by Kirby Bauer disc diffusion

Phenotypically, all *A. baumannii* strains were found to be extended-spectrum β-lactamase-producing (ESBL) and all isolates were imipenem resistant (data not shown). In imipenem-resistant *A. baumannii* isolates, phenotyping showed resistance to chloramphenicol (100%), gentamicin (100%), amikacin (100%), ciprofloxacin (100%) and levofloxacin (100%) as well as β-lactams and aztreonam (100%). However, all isolates (100%) were susceptible to polymyxin E.

Minimum inhibitory concentrations (MICs)

The MICs of the 32 *A.baumanii* isolates were determined. All isolates (100%) were resistant to all drugs tested, that is, Cephalosporines (Cefetamet, Ceftazidime, Cefotaxime, Cefuroxime and Ceftriaxone) as their MICs were > 2 mg/L. These isolates were resistant to three or more classes of antibiotics. According to the definition given by A. P Magiorakos et al. (2011), we described these isolates as MDR.

Phenotypic determination of carbapenemase production in *A. baumannii*

Imipenem-resistance by disc diffusion method was found in all 32 isolates of *Acinetobacter spp.* Of the 32 imipenem-resistant *Acinetobacter spp*. 18 (56.3%) were carbapenemase producers when tested by MHT. Whereas, remaining 14 (43.8%) isolates did not show evidence of carbapenemase production.

16SrRNA gene

Thirty two Imipinem resistant *A. baumannii* strains were tested for 16S rRNA methylase genes. Of the 32 *A. baumanii* isolates, 19 (59.4%) harbored 16S rRNA methylase gene of which 11 (61.1%) tested positive for carbapanemase and also 8 (57.1%) out of 14 of the non-carbapenemase producing isolates harbored 16S rRNA methylase gene. We also found that 47.4% of the isolates harboring 16S rRNA methylase gene co-existed at least with one oxacillinase gene (Table 2 and Figure 1).

Carbapenem-resistant genes

Thirty-two imipinem resistant *A. baumannii* strains were

Table 2. Multiplex PCR primers for detecting genes encoding oxa carbapenemase (John Roberts Table).

Primer	Sequences	Expected size (bp)
OXA-23 F	GATGTGTCATAGTATTCGTCGT	501
OXA-23 R	TCACAACAACTAAAAGCACTGT	-
OXA-51 F	TAATGCTTTGATCGGCCTTG	353
OXA-51 R	TCGATTGCACTTCATCTTGG	-
OXA-24 F	ATGAAAAAATTTATACTTCCTATATTCAGC	246
OXA-24 R	TTAAATGATTCCAAGATTTTCTAGC	-
OXA- 58 F	AAGTATTGGGGCTTGTGCTG	599
OXA- 58 R	CCCCTCTGCGCTCTACATAC	-
16SrRNAF	GTCGTAACAAGGTGGTTGC	1450
16SrRNAR	GGGTTTCCCGTTCGGAAAT	-

OXA: Oxacillinase.

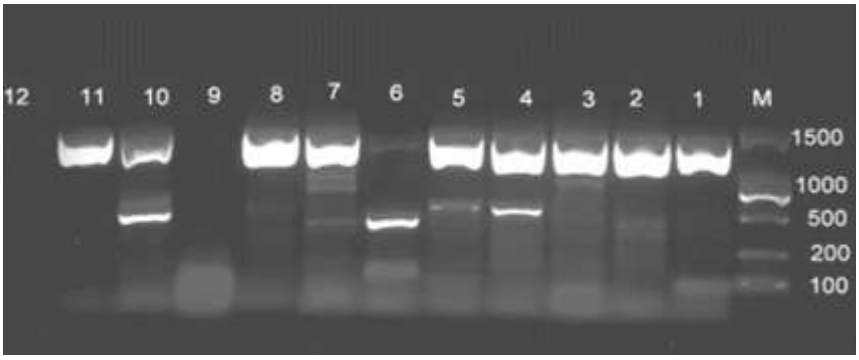

Figure 1. Agarose gel electrophoresis of PCR patterns of genomic DNA from clinical isolates of *A. baumannii* harboring 16srRNA methylase gene. Lane M: 100bp DNA size marker; lane 1~11: are 1450bp positive for 16SrRNA; Lane 6 and 9 are negative for 16SrRNA; Lane 12: negative control.

Table 3. Distribution of 16srRNA metheylase gene among MBL and non MBL producing *Acinetobacter baumannii* (John Roberts Table).

Types	MBL producers	No MBL producers	Total (n =32)
16S rRNA gene	11(61.1%)	7(38.9%)	18(56.25%)
No 16S rRNA gene	8(57.14%)	6(42.86%)	14(43.75%)

MBL: Metallo beta lactamase.

studied to determine presence of oxacillinase genes. Twenty out of 32 (62.5%) *A. baumannii* isolates harbored OXA-51 gene with 75% of these isolates being carpanemase producers and the remaining 25% Non carbapenemase producers. Of the 32 *A. baumannii* strains, 37.5% isolates harbored OXA-23 gene and 75% of them were carbapenemase producers. Six out of 32 (18.6%) *A. baumannii* isolates harbored OXA-58 gene and 50% were non- carbapenemase producers. Only one isolate contained OXA-24 gene.

Co-existence of different bla_{OXA} genes among 32 isolates of *A. baumannii* are indicated in Tables 3 and 4. None of the isolates carrying bla_{OXA-24} associated with bla_{OXA-23} or bla_{OXA-58} genes. A big percentage (72.7%) of *A. baumannii* isolates containing bla_{OXA-51}-like gene coexisted with all other OXA-like genes, because this gene is intrinsic to *A. baumannii* isolates (Figure 2).

Cloning and sequencing confirmed that the PCR products were 100% identical to the OXA-51 genes (GenBank accession nos AY795964 and AY949204).

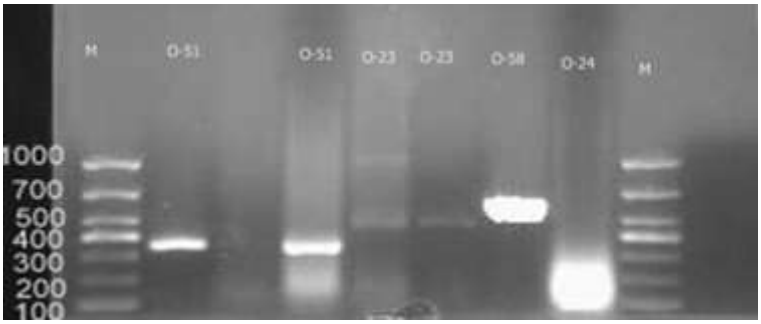

Figure 2. Agarose gel electrophoresis of PCR patterns of genomic DNA from clinical isolates of *A. baumannii* harboring OXA-type Carbapenemase genes. Lane M: 100bp DNA size marker; O-51 is OXA-51, O-23 is OXA-23, O-58 is OXA-58 and O-24 is OXA-24 genes respectively.

Table 4. Distribution and co-existence of different bla_{OXA} genes among clinical isolates of carbapenem resistance *Acinetobacter baumannii* (John Roberts Table).

Isolate	MBL	OXA-51	OXA-23	OXA-24	OXA-58	16S rRNA
1	X	X	X	0	0	X
2	X	X	0	0	0	X
3	X	0	0	0	0	X
4	0	0	0	X	X	X
5	0	0	X	0	0	X
6	0	0	X	0	0	0
7	X	X	0	0	0	X
8	X	X	X	0	0	X
9	X	X	X	0	0	0
10	X	X	0	0	0	X
11	0	0	0	0	X	X
12	0	0	0	0	0	0
13	X	X	X	0	0	0
14	0	0	0	0	0	X
15	X	X	0	0	X	X
16	X	X	0	0	0	X
17	0	X	0	0	0	X
18	0	0	0	0	0	0
19	X	0	0	0	0	0
20	0	0	0	0	X	0
21	X	X	X	0	0	0
22	X	X	0	0	0	0
23	X	X	X	0	0	X
24	0	X	0	0	0	X
25	X	X	0	0	0	X
26	X	0	X	0	X	0
27	0	X	0	0	0	0
28	X	X	X	0	0	0
29	0	X	X	0	X	X
30	0	X	X	0	0	X
31	X	X	0	0	0	0
32	X	X	X	0	0	0
Total	**19**	**20**	**13**	**01**	**06**	**18**

OXA: Oxacillinase; MBL: Metallo beta lactamase. X= Positive, 0 = Negative.

DISCUSSION

Carbapenem resistance in *A. baumannii* has been increasingly detected in Asian countries, which is the same case with China (Zong et al., 2008; Wang and Chen, 2005). In China, national resistance surveillance data from intensive care units (ICU) at 19 teaching hospitals (1996 to 2002) showed that 5% of *Acinetobacter* isolates were resistant to imipenem (Wang and Chen, 2005). However, another national surveillance program involving 10 geographically disparate hospitals found that resistance to carbapenems increased from 4.5% in 2003 to 18.2% in 2004 (Wang and Chen, 2005).

Carbapenems (e.g., imipenem and meropenem) and aminoglycosides are the drugs of choice in the treatment of *Acinetobacter* infections in almost all medical centers, but are being compromised by the emergence of carbapenem-hydrolyzing-lactamase (carbapenemase) of molecular classes D, especially OXA-type and methylasation of 16S rRNA methylase gene respectively. Furthermore, several studies have indicated that imipenem may be hydrolyzed by the extended-spectrum-lactamases of Gram-negative bacteria.

In this study, *A. baumannii* isolates were described as Multi-Drug Resistant (MDR) strains as the isolates were resistant to all antibiotics tested with exception of polymyxin E. This was based on the definition of MDR as given by Magiorakos et al. (2011). These findings also agree with Muthusamy and Boppe's claim that strains of *A. baumannii* are emerging as MRD (Muthusamy and Boppe, 2012). These results agree with the findings of Dalin et al. (2015) who observed that, all the isolates of *A. baumanni* isolated in an affiliated hospital of Jilin, China were resistant to all antibiotics.

By using the modified Hodge test as a screening test for carbapanemase production (Amjad et al., 2011), we found that 19 (56. 25%) of 32 *A. baumannii* isolates were carpanemase producing isolates. This agrees with Irfan et al. (2008) observation suggesting the presence of OTC enzymes since they are considered to be the mechanism of resistance in these organisms. This finding is also consistent with reports from other tertiary care hospitals around the world and even China (Zong et al., 2008; Wang and Chen, 2005). The resistance of *A. baumanni* to antimicrobial agents is mediated by almost all resistance mechanisms found in bacteria. Methylation of 16S rRNA methylase gene has emerged as a mechanism of high-level resistance to aminoglycosides among Gram-negative bacteria. In this study, the 16S rRNA methylase genes were amplified and detected in 19 (59.38%) of the isolates and 61.1% were carbapenemase producers. These findings are consistent with other published reports suggesting that *A. baumannii* carries 16S rRNA methylase gene (Kyungwon et al., 2011). As 16S rRNA methylse gene confer high-level resistance to aminoglycosides, its occurrence in *A. baumannii* limits the clinical use of aminoglycosides.

Our study reveals that 50% of the of 32 *A. baumannii* isolates studied harboring 16S rRNA methylase gene co-existed with at least one oxacillinase genes. This finding agree with that of Strateva et al. (2012) which showed here that *A. baumannii* isolates producing 16S rRNA methylase gene harbored both $bla_{\text{OXA-23}}$-like and $bla_{\text{OXA-51}}$-like genes. This is because these genes are linked with mobile genetic elements and usually confirmed as located on large conjugative plasmids, allowing potential spread among bacterial populations.

In addition, this association with other genes encoding resistance to clinically relevant antimicrobials such as ß-lactams (bla_{SHV}, $bla_{\text{CTX-M}}$, plasmid-mediated AmpC), carbapenems ($bla_{\text{SPM-1}}$, $bla_{\text{NDM-1}}$, $bla_{\text{KPC-2}}$) and fluoroquinolones (plasmid-mediated *qepA*, *aac(6')-Ib-cr* and *qnr* family), allows potential co-selection and maintenance of resistance by use of other antimicrobial agents.

Beta-lactamases are the most prevalent group of enzymes that are responsible for resistance in *A. baumanniii* and more than 50 different types have been identified so far in this strain. Several reports from around the world indicate a large increase in the rates of carbapenem-resistant *A. baumannii* from 8% in 2003 to 52% and 74% in 2005 and finally to 96% in 2007 (Mendes et al., 2009) due to these group of hydrolytic enzymes.

As shown in our study, 20 (62. 5%), 13 (40. 63%), 6 (18.75%), and 1 (3.13%) of the 32 multidrug-resistant strains studied carried OXA-51, OXA-23, OXA-58 and OXA-24 genes respectively. Moreover, 10 (31. 25%) isolates were positive for both OXA-51, OXA-23genes. Our data support those of other studies that demonstrated that OXA-51 may be used as a marker to identify *A. baumannii* (Qi et al., 2008). The OXA-23 genes have been documented in strains associated with outbreaks of carbapenem resistant *A. baumannii* in Asia, Europe and South America (Merkier and Centrón, 2006). Four strains were OXA-58 positive and only 1 (3.4%) strain was positive for OXA-24. This is consistent with studies from other hospitals in Jilin and entire China which suggests that the most widespread oxacillinase gene in *A. baumannii* is OXA-51, OXA-23, OXA 58 and OXA-24 in the order (Su-ying et al., 2015). This study also reveals that 9 (28.1%) out of 32 *A. baumanni*i isolates carried at least one oxacillinase gene though they were negative for carbapenemase production. This suggests the existence of hidden carbapenemase genes. These hidden genes can easily be missed in daily laboratory practice hence posing as a diagnostic and therapeutic challenge and can easily spread unnoticed in the hospital. We also found that 13 (40.63%) out of 32 isolates carried more than two oxacillinase genes, and were MDR.

The main limitation of this study is that it was confined to a single centre and it would be valuable to extend the origin of the strains. Another limitation is the small sample

size which led to a lack of power to determine the individual effects of each broad spectrum antibiotics.

Conclusion

The emergence of broad-spectrum antibiotic resistance profiles in *A. baumannii* clinical isolates is worrying in the hospital. The current study shows co-existence of resistance determinant genes and also existence of hidden genes responsible for resistance. Multiple mechanisms are likely to work in synergism to produce this phenotype. The co-existence and the multiple mechanisms working in synergy led us to an insight of defining *A. baumannii* strains in this study as extensive drug resistant *A. baumannii* (XDRAB), as there was still hope with the use of the highly toxic polymyxin E. The results here highlight that enhanced surveillance and health policies for the detection and control of these MDR pathogens are urgently needed to avoid the emergence and spreading of such organism.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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