Phenotypic and genotypic characterization of multidrug-resistant Acinetobacter baumannii isolated in Algerian hospitals

Nabila Benamrouche¹, Ourida Lafer¹, Lahcen Benmahdi², Akila Benslimani³, Wahiba Amhis⁴, Houria Ammari⁵, Farida Assaous¹, Amina Azzam⁶, Kheira Rahal¹, Hassiba Tali Maamar¹

¹ Medical Bacteriology Laboratory, Institut Pasteur of Algeria, Algiers, Algeria
² University Regional Military Hospital of Oran, Oran, Algeria
³ Central Laboratory, Specialized Hospital Establishment Maouche Mohand Amokrane, Algiers, Algeria
⁴ Central Laboratory, Public Hospital Establishment Ibn Ziri of Bologhine, Algiers, Algeria
⁵ Central Laboratory, University Hospital of Beni Messous, Algiers, Algeria
⁶ Microbiology and Parasitology Laboratory, University Hospital of Tizi Ouzou, Tizi Ouzou, Algeria

Abstract

Introduction: the aim of this study was to investigate the drug-resistance and the molecular characterization of carbapenemases, ESBL, and aminoglycoside-modifying enzymes among Acinetobacter baumannii clinical isolates in Algerian hospitals. Methodology: a total of 92 A. baumannii isolates were collected between 2012 and 2016. Antimicrobial susceptibility testings were performed for β-lactams, aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole, rifampicin and colistin. The phenotypic characterization of β-lactamases was investigated. For 30 randomly targeted strains, the carriage of the carbapenemases, ESBL and aminoglycoside-modifying enzymes-encoding genes was determined by PCR. Sequencing was carried out for carbapenemases and ESBL genes.

Results: most of the 92 isolates studied were recovered from hospitalized patients (93.5%) and were mainly from intensive care units (51.1%) and orthopedics (19.6%). The strains were collected primarily from low respiratory tract (33.7%), wounds (23.9%) and urine (16.3%). Multidrug-resistant A. baumannii strains were prevalent (96.7%). High rates of resistance were observed for almost all antibiotics tested (>70%) excluding rifampicin (7.6%) and colistin (5.4%). For the five colistin-resistant strains, MICs ranged between 4 and 128 µg/mL. Positive MBL (83.7%) and ESBL (23.9%) strains were identified. Regarding β-lactams, the blaNDM and both blaSHV and blaCTX-M genes were detected in five and two strains respectively. Sequencing of the genes revealed the presence of blaNDM-1, blaCTX-M-15, and blaSHV-33. For aminoglycosides, aac(6′)-Ib, ant(2″)-I and aph(3′)-VI genes were detected in three, seven and six strains respectively.

Conclusions: here, we report the first co-occurrence of extended-spectrum β-lactamases SHV-33 and CTX-M-15, the carbapenemase NDM-1 and the emergence of colistin-resistant A. baumannii in Algerian hospitals.

Key words: Multidrug-resistant Acinetobacter baumannii; blaCTX-M-15; blaSHV-33; blaNDM-1; colistin-resistance; Algeria.

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Introduction

Acinetobacter baumannii is a Gram-negative opportunistic nosocomial pathogen causing clinical infections and outbreaks in healthcare settings especially in intensive care units (ICUs). This species account for almost 90% of all reported Acinetobacter infections, including respiratory tract infections, bacteremia, meningitis, wound infections and urinary tract infections [1-2].

A. baumannii is able to easily acquire resistance to different groups of antimicrobials and to survive in hospital environment, leading to its persistence and transmission in healthcare settings [1,3]. A. baumannii is a member of the ESKAPE group (E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa and Enterobacter species) of six highly resistant pathogens that are a major cause of antibiotic-resistant infections [4,5].

Multidrug-resistant A. baumannii has become life threatening and is increasingly reported worldwide, including Europe, America, Asia and Africa [1,2]. Over the last decade, the emergence of A. baumannii resistant to carbapenems, a last-line group of β-lactams for treatment of patients infected with multidrug-resistant bacteria, was reported [6-9]. In this context, the World Health Organization recognize carbapenem-resistant A. baumannii (CRAB) as the first critical priority in its list of 12 resistant-bacteria that pose the greatest threat to human health [10].

The main resistance mechanism among A. baumannii isolates is the production of β-lactamases enzymes which include extended spectrum-β-
lactamases (ESBLs) as TEM-92, SHV-5, CTX-M-2 and CTX-M-15, metallo-β-lactamases (MBLs) as IMP-1, VIM-1 and NDM-1 and carbapenem-hydrolyzing class D β-lactamases (CHDLs), also called oxacillinases (OXAs) as OXA-23, OXA-24 and OXA-58 [11,12].

Resistance to aminoglycosides is prevalent worldwide. It is mainly mediated by aminoglycoside-modifying enzymes (AMEs) including acetyltransferases (AACs) as AAC(6’)-I enzymes, phosphotransferases (APHs) as APH(3’)-VI enzymes and nucleotidyltransferases (ANTs) as ANT(2’’)-I [12]. Recently, 16S rRNA methylases have been reported worldwide among Gram negative bacilli, including Acinetobacter spp. [13].

The purpose of this study was to evaluate the antimicrobial resistance rates and molecular mechanisms of ESBLs, carbapenemases and AMEs to multidrug A. baumannii isolated in Algerian hospitals during a 5-year period starting from January 2012. Here, we report the first co-occurrence of extended-spectrum β-lactamases SHV-33 and CTX-M-15, the carbapenemase NDM-1 and the emergence of colistin-resistant A. baumannii in Algerian hospitals.

Methodology

Bacterial strains

Ninety two non-repetitive A. baumannii clinical isolates were collected from several hospitals from Algiers (n = 64), Blida (n = 6), Tipaza (n = 3), Setif (n = 2), Boumerdes (n = 1), Bejaia (n = 1), Oran, n = 13), Tiaret (n = 1) and Tamanrasset (n = 1) between January 2012 and December 2016.

All clinical isolates were identified by standard microbiological methods using Api 20NE identification system (bioMérieux, Marcy l’Etoile, France).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disc diffusion method according to the CLSI guidelines [14]. The antibiotics tested, based on national drug nomenclature, were ticarcillin (75 µg), piperacillin (100 µg), ticarcillin-clavulanic acid (75/10 µg), ceftazidime (30 µg), imipenem (10 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), rifampicin (30 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg).

Phenotypic detection of production of Extended-spectrum β-lactamase (ESBL) and metallo-β-lactamase (MBL)

Detection of ESBL and MBL production were performed by phenotypic confirmatory disc diffusion test (PCDDT) and combined disc test (CDT) as previously described [17].

PCR assays

Thirty A. baumannii isolates were randomly targeted, using the “alea” function of the Excel 2013 software, among the 92 strains for the detection of the carbapenemase, ESBL and AME-encoding genes according to phenotypic test results. Bacterial DNA was extracted by boiling. Conventional PCR simplex assay was performed in a 50 µL reaction mixture containing the final concentrations of 0.5 µM primer, 2.5 U/µL Taq polymerase, 2 mM MgCl₂, 0.2 mM dNTPs, 10 µL

Table 1. Primers sequence of the targeted genes.

| Gene | Forward | Primer sequence (5’-3’) | Reverse | Size (bp) |
|------|---------|-------------------------|---------|-----------|
| blatem | ATAAAATCTTGTGAGACGAAA | GACAGTTACAATGCTTAATCA | 1080 |
| blaSHV-5 | TTATCCCTGACTTTGACACC | GATTTCGTGTTTGCTGCGG | 796 |
| blactxM-1 | GGTGAAAAATATCAGCTGATC | TGGGTGACGATTCCATCGCGC | 864 |
| blactxM-2 | ATGATGACTCAGACGTCC | TGGGTTACGATTCCATCGCGC | 867 |
| blandm | AATTGAATTGGCCCATATATTGC | TCGAGCGAGCTGGCTGCGC | 621 |
| blavim | GGTGTTTTGGTGCATATCGCACC | TGGCTGCKGACGAAKTCTGACCGC | 390 |
| blaqmp | GCAGAATATCAGTGCTTAAY | GGTTCATAAAYAACAACCACCC | 232 |
| blaoXAS-58-like | ATGAAATTATAAAAATTTG | ATAAATATGAAAAACACCCCA | 841 |
| aac(6’)-Ib | ATGACTGACCATGACCTTAG | AACCACCTACACGGCTCGG | 476 |
| aph(3’)-VI | CCGAAACACGGTAAAAAGT | TTCCCTTTTGTCAGGTTC | 716 |
| ant(2’’)-I | GACACAACCGGAGGCACAT | CGCATACGCCGACTGAAAGC | 524 |
5×buffer, 3 μL DNA template and nuclease-free water. Amplification conditions consisted of denaturation at 94 °C for 7 minutes and 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. PCR products were detected in 2% agarose gel. All PCR primers targeting resistance genes used in this study are listed in Table 1.

DNA sequencing of carbapenemases and ESBL genes detected was performed using Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed using an ABI Prism 3100 genetic analyser (Applied Biosystems, Foster City, CA, USA).

**Results**

The strains were collected from various clinical specimens, including lower respiratory tract (33.7%), wounds (23.9%), urine (16.3%), blood (9.8%), cerebrospinal fluid (4.3%), upper respiratory tract (3.3%), catheter and drain (2.2%) respectively, pericardial liquid, rectal swab, tracheostomy cannula and urinary catheter (1.1%) respectively. These samples were from intensive care units (ICUs) (51.1%), orthopedics (19.6%), medicine (13.0%), surgery (9.8%) and 6 outpatients (6.5%).

**Antimicrobial susceptibility testing**

The 92 isolates investigated showed high resistance rates to β-lactams: ticarcillin (95.6%), piperacillin (98.9%), ticarcillin-clavulanic acid (90.2%), ceftazidime (96.7%) and imipenem (83.7%), to aminoglycosides: gentamicin (86.9%), amikacin (79.3%), tobramycin (83.7%) and netilmicin (61.9%), to fluoroquinolones: ciprofloxacin (91.3%) and levofloxacin (84.8%) and to trimethoprim-sulfamethoxazole (82.6%). However, resistance rates to rifampicin (7.6%) and colistin (5.4%) were low (Figure 1).

Eighty nine (96.7%) *A. baumannii* strains showed multidrug-resistance. Combined resistance to carbapenems, aminoglycosides and fluoroquinolones was observed in 74 (80.4%) cases and accounted for 37/74 (50.0%) of the invasive isolates.

Regarding aminoglycosides, eleven resistance profiles including resistance to one to four aminoglycosides were defined among MDR *A. baumannii* isolates. The resistance to all aminoglycosides was prevalent (n = 54; 58.7%) strains, followed by the resistance to gentamicin and tobramycin (n = 13; 14.1%) strains (Table 2). MICs to netilmicin were ranged between 0.016 µg/mL to 256 µg/mL with MIC₅₀ and MIC₉₀ both at 256 µg/mL. MICs to colistin were ranged between 0.064 µg/mL and 128 µg/mL with MIC₅₀ at 0.5 µg/mL and MIC₉₀ at 1 µg/mL. For the five colistin-resistant strains, the MICs were 4 µg/mL for four strains and 128 µg/mL for one strain.

**Phenotypic detection of β-lactamases (ESBL and MBL)**

Among the 92 strains screened for ESBL and MBL production, 22 (23.9%) were PCDDT positive and 77 (83.7%) were CDT positive, indicating the probable production of ESBL or MBL.

**Molecular detection of resistance genes**

**Carbapenemase and extended-spectrum β-lactamase-encoding genes**

Out of the eight resistance genes investigated, five genes (*blaTEM*, *blaCTXM-2*, *blaIMP*, *blaVIM* and *blaOXA-58-like*) were not detected in any of the tested strains.

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**Table 2. Distribution of aminoglycosides resistance profiles among MDR *A. baumannii* isolates**

| Aminoglycosides resistance profiles | Number (%) |
|-----------------------------------|------------|
| Susceptible*                      | 3 (3.3)    |
| Gm                                | 1 (1.1)    |
| Ak                                | 6 (6.5)    |
| Tm                                | 1 (1.1)    |
| Nt                                | 1 (1.1)    |
| Gm-Ak                             | 3 (3.3)    |
| Gm-Tm                             | 13 (14.1)  |
| Gm-Tm-Nt                          | 1 (1.1)    |
| Gm-Ak-Nt                          | 1 (1.1)    |
| Gm-Ak-Tm                          | 8 (8.7)    |
| Gm-Ak-Tm-Nt                       | 54 (58.7)  |

Gm: gentamicin; Ak: amikacin; Tm: tobramycin; Nt: netilmicin. *Strains were susceptible to the four aminoglycosides tested.
Among the 21/30 CDT positive isolates, the \textit{bla}_{\text{NDM}} gene was detected in five strains. The strains were recovered from different hospitals in Algiers, Boumerdes, Blida and Tipaza.

Two strains were positive for both \textit{bla}_{\text{SHV}} and \textit{bla}_{\text{CTX-M-1}} genes among 8/30 PCDDT positive isolates and were collected from an Algiers hospital.

Sequencing of the genes revealed the presence of \textit{bla}_{\text{NDM-1}}, \textit{bla}_{\text{CTX-M15}}, and \textit{bla}_{\text{SHV-33}}. Detailed data are shown in Table 3.

### Aminoglycoside-modifying enzymes-encoding genes

The screening of 26/30 aminoglycosides resistant isolates for resistance genes revealed the presence of the \textit{aac(6')-Ib} (n = 6), \textit{ant(2'')-I} (n = 7), and \textit{aph(3')-VI} (n = 6) genes.

Combined aminoglycoside-modifying enzyme (AME) resistance genes was observed with \textit{ant(2'')-I} and \textit{aph(3')-VI} (n = 2) and association of ESBL, MBL and AME resistance genes was also identified, namely \textit{bla}_{\text{SHV}}-\textit{bla}_{\text{CTX-M-1}}-\textit{aac(6')-Ib} (n = 2), \textit{bla}_{\text{NDM}}-\textit{ant(2'')-I} (n = 1), \textit{bla}_{\text{NDM}}-\textit{ant(2'')-I} (n = 3) and \textit{bla}_{\text{NDM}}-\textit{aph(3')-VI} (n = 1). Detailed data are shown in Table 3.

### Discussion

Multidrug resistant \textit{A. baumannii} represent a major threat in nosocomial infections, mostly in ICUs.

In this study, the most frequently strains were obtained from lower respiratory tract (33.7%), followed by wounds (23.9%) and were mostly from intensive care units (51.1%) and orthopedics (19.6%).

### Antimicrobial susceptibility testing

In our study, most of the isolates showed high antibiotic resistance rates to \(\beta\)-lactams, aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole and low resistance rates to rifampicin and colistin. Our findings are moderately higher than the Algerian Antimicrobial Resistance Network (AARN) data recorded in \textit{Acinetobacter} spp. during the same period (cefazidime 85.6%, imipenem 73.4%, amikacin 65.7%, ciprofloxacin 80.1% and trimethoprim-sulfamethoxazole 72.2%) [18], and studies carried out in Western Algeria in 2013 and Algiers in 2015 [19,20].

In accordance with results reported around the world [11], almost all \textit{A. baumannii} strains in our study were MDR and carbapenem-resistant \textit{A. baumannii} (CRAB) were prevalent (83.7%). The widespread of these strains has described worldwide [11]. The high rate of CRAB isolates is a concern, as carbapenems represent the treatment of choice for infections caused by \textit{A. baumannii}, leading to limited therapeutic options.

In this study, five strains were resistant to colistin. In recent years, the emergence of colistin resistance in \textit{A. baumannii} clinical isolates has been increasingly reported in several countries worldwide, becoming a public health concern as this antibiotic is used for the treatment of infections due to CRAB [21,22].

In the North African countries, a few cases of colistin resistance among \textit{A. baumannii} has been described in Tunisia and Egypt [23,24]. In Algeria, Bakour et al. in 2014, described one colistin-resistant \textit{A. baumannii} clinical strain that harbored a single mutation in the \textit{pmrB} gene in an Algiers hospital [25].

### Table 3. Detection of ESBL, MBL, aminoglycosides resistance and their related genes among MDR \textit{A. baumannii} isolates.

| Code of isolates | Year of isolation | ESBL test | MBL test | Aminoglycosides resistance | Phenotypic tests | Resistance genes |
|------------------|-------------------|-----------|----------|---------------------------|-----------------|-----------------|
| Aba.1            | 2012              | +         | +        | Ak                        |                 | \textit{bla}_{\text{NDM}}, \textit{aph(3')-VI} |
| Aba.2            | 2012              | -         | +        | Gm-Ak-Tm                  |                 | \textit{bla}_{\text{NDM}}, \textit{ant(2'')-I}, \textit{aph(3')-VI} |
| Aba.3            | 2012              | -         | -        | Ak                        |                 | \textit{aph(3')-VI} |
| Aba.7            | 2014              | +         | -        | Gm-Ak-Tm-Nt                |                 | \textit{bla}_{\text{SHV}}, \textit{bla}_{\text{CTX-M-1}}, \textit{aac(6')-Ib} |
| Aba.8            | 2014              | +         | -        | Gm-Ak-Tm-Nt                |                 | \textit{bla}_{\text{SHV}}, \textit{bla}_{\text{CTX-M-1}}, \textit{aac(6')-Ib} |
| Aba.11           | 2015              | -         | +        | Gm-Tm                      |                 | \textit{bla}_{\text{NDM}}, \textit{ant(2'')-I} |
| Aba.12           | 2015              | +         | +        | Gm-Tm                      |                 | \textit{bla}_{\text{NDM}}, \textit{ant(2'')-I} |
| Aba.15           | 2015              | -         | +        | Gm-Ak-Tm-Nt                |                 | \textit{ant(2'')-I}, \textit{aph(3')-VI} |
| Aba.19           | 2015              | -         | +        | Gm-Ak-Tm-Nt                |                 | \textit{ant(2'')-I} |
| Aba.20           | 2016              | -         | -        | Gm-Tm                      |                 | \textit{apo} |
| Aba.21           | 2016              | +         | -        | Gm-Ak-Tm-Nt                |                 | \textit{apo} |
| Aba.22           | 2016              | -         | +        | Gm-Ak-Tm-Nt                |                 | \textit{apo} |
| Aba.23           | 2016              | +         | -        | Gm-Tm                      |                 | \textit{apo} |
| Aba.25           | 2016              | -         | +        | Gm-Ak-Tm-Nt                |                 | \textit{apo} |

ESBL: extended-spectrum \(\beta\)-lactamase; MBL: metallo-\(\beta\)-lactamase; ICU: intensive care unit; CSF: cerebrospinal fluid; Gm: gentamicin; Ak: amikacin; Tm: tobramycin; Nt: netilmicin.
To delay the emergence of this resistance, combination therapies using tigecycline or rifampicin with colistin were found to be effective [26,27]. In addition, new drugs developed such as cefaracycline and cefiderocol are promising but need to be evaluated in further studies [28].

Seven (7.6%) isolates were resistant to rifampicin. This rate is higher than that (2.8%) reported by Bakour et al. in 2013 in Setif and Tizi-Ouzou [29]. So, this increased resistance rate should prompt attention and be continuously monitored.

Molecular detection of resistance genes
Carbapenemases and extended-spectrum β-lactamases-encoding genes

Five strains harbored the blaNDM gene, identified as blαNDM. The MBL NDM-1, detected originally in Enterobacteriaceae, was later described in Acinetobacter spp. Currently, this enzyme has spread in several countries around the world [30-32].

In Algeria, the first descriptions of NDM-1 in Algerian patient were reported by Boulanger and Bogaerts in 2011 [33,34]. After, this MBL was increasingly reported by other authors in Western: Oran [18], Eastern: Annaba [35,36] and Central Algeria: Tizi-Ouzou (Azzam et al. 34th RICAI 2014, France), Algiers, Bejaia and Setif [19,37-39].

These studies support our results and confirm that NDM-producing A. baumannii isolates are nowadays endemic in Algerian hospitals.

blαOXA-58-like was not detected in any strain tested. In recent years, several studies from Algeria described carbapenem resistance among A. baumannii isolates, mainly due to CHDLs OXA-23 and OXA-24 in several regions of Algeria: Tizi-Ouzou (Azzam et al. 34th RICAI 2014, France), Setif, Tlemcen, Sidi Bel Abbes, Algiers and Bejaia [18,39]. OXA-58 was reported only in two studies from Annaba [40] and Tizi-Ouzou (Azzam et al. 34th RICAI 2014, France).

For 16 CDT positive strains, none of blαVIM, blαIMP, blαNDM and blαOXA-58 genes were detected. Several studies reported false MBL-positive results using this test, particularly in CHDLs strains likely due to chelators as EDTA that may inhibit the activity of oxacillinases [41,42].

In our research, two isolates harbored both blαCTX-M15, and blαSHV-33 genes. Several previous studies reported ESBLs-producing A. baumannii strains [11,12]. In Egypt and Pakistan, TEM, CTX-M or SHV genes were previously reported [43,44]. In Algeria, blαTEM-128 was previously described [29].

To the best of our knowledge, this is the first description of SHV-33 and co-occurrence of extended spectrum β-lactamase SHV-33 and CTX-M-15-producing A. baumannii.

For the six PCDDT positive strains, blαTEM, blαSHV, blαCTX-M-1 and blαCTX-M-2 genes were negative. As previously described by Beceiro et al., the intrinsic susceptibility of some strains of A. baumannii to β-lactamase inhibitor clavulanic acid could yield false ESBL-positive results in A. baumannii [45].

Aminoglycoside-modifying enzymes-encoding genes

Among 26/30 aminoglycosides resistant isolates, the three types of AMEs genes were identified with aac(6’)-Ib (n = 6), ant(2’’)-I (n = 7), and aph(3’)-VI (n = 6) genes, alone or associated. As previously described in several studies [12], these findings are in accordance with three aminoglycosides resistance profiles identified: (amikacin), (gentamicin-tobramycin) and (gentamicin-amikacin-tobramycin).

The most frequent resistance profile (gentamicin-amikacin-tobramycin-netilmicin) is probably due to the 16S rRNA methylase ArmA [12].

In Algeria, several previous studies reported the presence of aminoglycoside encoding genes aac(6’)-Ib, aph(3’)-VI, ant(2’’)-I, aac(3)-Ia and aadA [18,19,25,38].

Combined AME resistance genes was observed with ant(2’’)-I-aph(3’)-VI (n = 2) and association of ESBL, MBL and AME resistance genes was also identified (n = 7). The association of aminoglycosides resistance, MBL and ESBL genes identified, highlight the multiple mechanisms involved leading to multidrug resistant A. baumannii strains.

Conclusions

Our results highlight that (i) the high prevalence of multidrug resistant A. baumannii in Algerian hospitals is alarming, (ii) NDM-1 isolates are actually endemic in Algerian hospitals, (iii) co-occurrence of ESBLs SHV-33 and CTX-M-15 is described for the first time in Algeria, (iv) AMEs, often combined, are mediated by aac(6’)-Ib, aph(3’)-VI and ant(2’’)-I-land (v) colistin-resistant strains have emerged in Algerian hospitals.

These findings report a critical situation in Algerian hospitals that requires rational use of antibiotics, strict compliance with hygiene rules, rapid and accurate screening of inpatients and implementation of early precautions for MDR carriers to decrease nosocomial transmission in hospitals. Further investigations are needed to better understand the molecular
epidemiology of this nosocomial opportunistic pathogen.

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Corresponding author
Doctor Nabila Benamrouche, MD, PhD
Medical Bacteriology Laboratory, Institut Pasteur of Algeria, Route du petit Staoueli, Dely Ibrahim, Algiers
Postal code: 16000
Tel: +213023367348
Fax: +213023367348
Email: n_benamrouche@yahoo.fr

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