Dissemination of carbapenem-resistant *Acinetobacter baumannii* strains carrying the *bla*$_{GES}$, *bla*$_{NDM}$ and *bla*$_{OXA23}$ in Morocco

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Abstract

Background ***Acinetobacter baumannii*** **is a microorganism which has been classified by the World Health Organization in the list of the bacterial strains that pose the biggest danger to human health. This study was performed to determine the susceptibility profile to carbapenems and to detect carbapenemases production in 111 *A. baumannii* isolates. Among these 30 are environmental isolates and 81 are from the three major hospitals in Morocco.**

Methods All strains of *A. baumannii* were tested against diverse antimicrobial agents (13 antibiotic drugs) by the agar diffusion test. Minimum inhibitory concentration (MIC) of imipenem on carbapenem-resistant strains (CRAB) was determined by the E-test technique. Simple phenotypic tests were used to detect carbapenemases and metallo-β-lactamases (MBLs) production including the modified Hodge test, EDTA test, and the cloxacillin test. The presence of carbapenemases-encoding resistance genes of CRAB strains was examined using polymerase chain reaction (PCR).

Results Carbapenem resistance was observed in 23 clinical *Acinetobacter* isolates showing dissemination of the multiresistance profile. Molecular biology techniques indicated that all these strains encoded the naturally occurring *bla*$_{OXA-51-like}$ gene and were proved as *A. baumannii.* The *bla*$_{OXA-23}$ gene was detected in 16 strains (69.6%). The metallo-β-lactamase *bla*$_{NDM}$ gene was detected in five isolates (21.7%). GES-type carbapenemases were found in 15 strains, the existence of three classes of carbapenemases (*bla*$_{GES}$, *bla*$_{NDM}$, and *bla*$_{OXA-23}$) was detected in three strains, while none of the CRAB isolates contained the *bla*$_{OXA-58}$, *bla*$_{OXA-24}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$ or *bla*$_{KPC}$ encoding genes.

Conclusions This study established baseline proof of three classes of carbapenemases producing *A. baumannii* in Morocco, showing the important role of surveillance in controlling their spread.

Keywords ***Acinetobacter baumannii*****, OXA genes, metallo-beta-lactamases, GES genes, Morocco.**

Introduction

Strains belonging to *Acinetobacter baumannii* are now considered the most problematic pathogens causing significant nosocomial 1infections in the world.[1] The ability of this germ to acquire and to disseminate rapidly multidrug resistance makes these infections hard to cure and it is generally combined with an important risk of death.[2] Most often, carbapenems are the best medicines for *A. baumannii* infections

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treatment. However, overconsumption of these medications has caused the occurrence and global propagation of carbapenem-resistant strains, which have become a principal danger in healthcare institutions worldwide.[3] Diverse mechanisms of resistance to carbapenem in *A. baumannii* were reported, including loss of outer membrane protein, change of penicillin-binding proteins (PBPs), over-production of the efflux pump and finally, the best-known of these mechanisms, the production of carbapenemases.[2] The production of oxacillinase (OXA) type carbapenemases, pertaining to class D, remains the main reason of resistance in *A. baumannii*.[3] However, the acquisition of the metallo-beta-lactamase (MBL) enzymes of classes A and B, hydrolyzing carbapenems, may also be among the mechanisms of carbapenem resistance in this germ.[4]

Different classes of MBLs have been represented worldwide in *A. baumannii* isolates belonging to class B such as: imipenemase (IMP), Verona integrin-encoded metallo-beta-lactamase (VIM), Seoul imipenemase (SIM), German imipenemase (GIM), and New Delhi metallo-beta-lactamase (NDM). Recently, carbapenemases of class A, including Guyana extended spectrum beta-lactamase (GES) and *Klebsiella pneumoniae* carbapenemase (KPC), have also been detected.[1,5] The carbapenemases of type bla_{GES} have been identified in *A. baumannii* in many countries around the world such as Turkey,[6] France,[1] Lebanon,[7] and Belgium.[8]

In North Africa, and more specifically in Morocco, the emergence of carbapenemases genes, such as bla_{OXA-24}, bla_{OXA-23}, bla_{OXA-58} and bla_{NDM} in *A. baumannii*, has been detected previously. However, there is presently only little data on the spread of these enzymes into the hospital environment in Morocco. In a recent study, Natoubi et al.[9] have described the characterization of an *A. baumannii* clinical strain harboring the bla_{OXA-58} gene, obtained from a clinical environment in Settat (Morocco). Another report was published by Uwingabiye[10] on the production of carbapenemase in *A. baumannii* isolates, collected from different patients and different places of a Moroccan hospital.

With the exception of the two above-mentioned studies, we are not aware of other research on the prevalence of genes coding for carbapenemase in *A. baumannii* strains in Morocco.

The objectives of the present research were to assess the propagation of carbapenem-resistant *A. baumannii* in both clinical and environmental isolates from Morocco and to detect the types of carbapenemases (oxacillinase, MBL, and extended spectrum beta-lactamase – ESBL) involved in this resistance.

Methods

Origin of samples

The samples studied were collected from environmental and clinical samples in a period of sixteen months, from April 2015 to July 2016. The environmental strains were selected according to biotope which can possibly contain *Acinetobacter* spp., and we opted for soil sampling isolated in diverse sites located in Meknes city, Morocco. The clinical strains are from different hospital departments, which admitted patients with symptoms suggestive for *A. baumannii* infection. Those isolates were collected from three major hospitals in Morocco and are designated in this study as Hop 1, Hop 2 and Hop 3, which correspond respectively to the Military Hospital, Regional Hospital of Meknes (North-East of Morocco) and University Hospital Center of Casablanca (Central Western part of Morocco).

Isolation and identification of *A. baumannii*

The preparation of the environmental samples was done by suspending soil in sterile distilled water at a rate of 10%. After decantation for thirty minutes, one drop of the supernatant was inoculated on nutrient agar (Biokar Diagnostics, Allonne, France). The clinical stains were directly cultured on nutrient or Mueller-Hinton agar plates and cultivated at a temperature of 37°C for 18 h to 24 h. Bacterial colonies suspected as *Acinetobacter* spp. were identified firstly by classical techniques of

bacteriology: morphology, Gram, presence of catalase and growth at 44°C, and then by biochemical tests API 20NE (bioMérieux, Marcy-l'Étoile, France). PCR amplification of the bla_{OXA-51} gene was also performed to confirm the phenotypical identification of the *A. baumannii* strains.[11]

Antibiotic susceptibility testing

The susceptibility of our strains was assessed on Mueller-Hinton agar, using the standard disk diffusion method in accordance with the recommendations of the CA-SFM 2016.[12] Each strain was tested against 13 antibiotics including ticarcillin (75 µg), piperacillin (100 µg), ticarcillin/clavulanic acid (75/10 µg), piperacillin/tazobactam (100/10 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), tobramycin (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), and ceftriaxone (30 µg). The interpretation of the susceptibility results was realized in conformity with the CA-SFM breakpoints.

All strains showing reduced susceptibility to one of the carbapenems, imipenem (IMP) and/or meropenem (MEM), were defined as carbapenem-resistant *A. baumannii* (CRAB).

Multidrug-resistant strains (MDR) were marked as isolates presenting resistance to at least three antibiotics belonging to different families.[13]

Etest® strip (AB BIODISK, Solna, Sweden) was used to determine the minimum inhibitory concentrations (MICs) for all CRAB strains, and the results were interpreted according to the breakpoints recommended by CA-SFM 2016.[12]

Quality control was realized using *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (CLSI 2016).[14]

Phenotypic diagnosis of carbapenemases

The determination of a possible production of carbapenemase among CRAB was carried out by the modified Hodge test as described by Bakour et al. 2015.[15] This test was performed to demonstrate a synergy of enzymatic activity between strains producing carbapenemases (strain tested) and reference strains (*Escherichia coli* ATCC 25922, *Enterobacter cloacae* U2A2242 producing KPC-3 carbapenemase).

Among CRAB strains, the presence of class B carbapenemases (MBL production) was detected using EDTA-disk synergy test.[16]

The cloxacillin test was performed to detect the production of an ESBL with carbapenemase activity (synergy between clavulanic acid and imipenem was interpreted as positive result).[7]

Molecular detection of carbapenemases

Extraction of DNA template

DNA extraction from overnight cultures was performed using the PureLink® Genomic, DNA Mini Kit (Invitrogen, K1820-01, Paisley, UK) in conformity with the manufacturer's instructions.

Detection of carbapenemase-encoding genes

The amplification of the genes coding for the carbapenemases was carried out using the same specific primers described previously by Maroui et al. 2016[17] to identify: bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-58}, bla_{NDM}, bla_{GES}, bla_{KPC} and bla_{VIM} with the difference of two primers bla_{OXA-48} [18] and bla_{OXA-24}.[19]

Amplification reactions were performed in a final volume of 50 µL. Reaction mixtures contained 5 µL of 1× PCR buffer, 2.5 mmol/µL of $MgCL_2$, 100 µm of deoxynucleoside triphosphates (dNTPs), 0.4 µL of each primer, 2U MyTaq DNA polymerase (New England BioLabs Inc., Beverly, MA, USA) and 2 µL of DNA template.

PCR conditions were programmed in this manner: first denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing (57°C for 1 min for NDM and VIM, 60°C for KPC and GES and 52°C for 1 min for OXA-23, OXA-24, OXA-48, OXA-51 and OXA-58) and extension 72°C for 1 min, ending with a final extension at 72°C for 6 min. Amplification products were separated using electrophoresis on 1.5% agarose gel (FMC Bio-product, Rockland, ME, USA).

Reference strains, including *K. pneumoniae* U2A2252 (GES-1), *Enterobacter cloacae* U2A2242 (KPC), *K. pneumoniae* U2A2016 (VIM), *A. baumannii* U2A1863 (OXA58), *A. baumannii*

NCTC13304 (OXA23), *A. baumannii* NCTC13302 (OXA24) and *K. pneumoniae* (NDM)[20] were employed as positive control strains. For each PCR reaction, a negative control was used containing all components except the DNA template, which was replaced by DNase- and RNase-free water (Invitrogen).

Statistical analysis

Statistical analysis was realized using SPSS version 20 software (IBM, Armonk, NY, USA). The comparison between the qualitative variables was performed by the Chi-square and Fisher exact tests. Statistical differences were considered significant for p values less than 0.05.

Results

Bacterial isolates

A total of 111 strains were isolated and identified as *A. baumannii*. Eighty-one of them were isolated from clinical samples and thirty from environment samples. The clinical strains were isolated from males (72.8%) and females (27.2%), and were distributed as follows: 57 were from Military Hospital, 18 from University Hospital Center and 6 from the Regional Hospital. Among these strains, 32 (39.5%) were recovered from urine, 18 (22.2%) from pus, 16 (19.8%) from distal bronchial Levy protected, 6 (7.4%) from bronchial aspirate, 4 (4.9%) from central catheter, 3 (3.7%) from blood cultures, 1 (1.2%) from venipuncture and 1 (1.2%) from pleural liquid. The majority of *A. baumannii* isolates were collected from medical intensive care units (45.7%). The environmental strains were obtained from soil sampling: polluted soil (n=5) exposed to two types of contamination: urban wastewater discharges (n= 3) and solid or liquid household waste (n=2), and an unpolluted soil isolated in gardens near the hospital, which appears uncontaminated (n=25).

Antibiotic susceptibility testing

The antibiotic susceptibility results of the 111 isolates are shown in Table 1. The difference in resistance rates between the clinical isolates and the environmental ones was statistically significant for all antibiotics tested including imipenem (p=0.001) and meropenem (p=0.002). Susceptibility testing showed a high profile of resistance to all antibiotics tested. In addition, among our isolates, twenty-three were carbapenem-resistant and correspond to the clinical isolates. Among these bacteria, twenty-one isolates were resistant to the two carbapenem antibiotics (IPM and MEM) and two isolates were resistant to IPM but sensitive to MEM.

All CRAB strains presented an important level of resistance to cefepime, ciprofloxacin, ceftriaxone, ticarcillin, piperacillin/tazobactam, ceftazidime, and ticarcillin/clavulanic acid (100%). A high level of resistance to aminoglycosides was identified, with 74% resistance to amikacin, 95.7% to tobramycin and 100% to gentamicin (Table 2). The CRAB strains presented resistance to more than 3 drugs in distinct antibiotic classes, indicating the multidrug-resistant (MDR) phenotype. The MICs evaluated for all CRAB isolates (23 strains) showed a high profile of resistance to imipenem (MIC >32 mg/mL)

Phenotypic detection of carbapenemases

Positive MHT test was noted in all CRAB strains suggesting carbapenemase production. Moreover, the inhibition of carbapenemase activity using EDTA test was observed in fifteen *A. baumannii* strains demonstrating the possible production of MBL class B. In addition, five isolates gave positive results with cloxacillin test, suggesting the production of a class A ESBL with carbapenemase activity.

Molecular identification of carbapenemase-encoding genes

PCR detection for carbapenemase genes showed that all the isolates gave positive results for the bla_{OXA-51} gene. Among them, 16 isolates carried bla_{OXA-23} gene, 5 contained bla_{NDM} and 15 contained bla_{GES}. Furthermore, 11 isolates harbored both bla_{OXA-23} and bla_{GES}, indicating that these carbapenemases are the most frequent mechanisms of resistance amongst our strains. The coexistence of bla_{NDM} with bla_{OXA-23} and bla_{NDM} with bla_{GES} was detected in 3 (13.04%)

Table 1. Antibiotic resistance in *A. baumannii* isolates

Antibiotic agents	Resistance rates, n (%)			
	Clinical isolates (n=81)	Environmental isolates (n= 30)	Total (n=111)	p value
Piperacillin	75 (92.6)	16 (53.3)	91 (82)	<0.0001
Ticarcillin	75 (92.6)	13 (43.3)	88 (79.3)	<0.0001
Piperacillin/tazobactam	70 (86.4)	13 (43.3)	83 (75.7)	<0.0001
Ticarcillin/clavulanic acid	74 (91.4)	13 (43.3)	87 (78.4)	<0.0001
Ceftazidime	75 (92.6)	12 (40)	87 (78.4)	<0.0001
Cefepime	72 (88.9)	7 (23.3)	79 (71.2)	<0.0001
Meropenem	21 (25.9)	0	21 (19)	0.002
Imipenem	23 (28.4)	0	23 (21)	0.001
Tobramycin	61 (75.3)	4 (13.3)	65 (58.6)	<0.0001
Amikacin	41 (50.6)	4 (13.3)	45 (40.6)	<0.0001
Ciprofloxacin	64 (79.01)	4 (13.3)	68 (61.3)	<0.0001
Gentamicin	60 (74.1)	4 (13.3)	64 (57.7)	<0.0001
Ceftriaxone	72 (88.9)	13 (43.3)	85 (76.6)	<0.0001

Table 2. Antibiotic resistance of CRAB strains isolated from three hospitals in Morocco

Antimicrobial agent	Resistance rates, % (n)			
	Hop 1 (n=14)	Hop 2 (n=1)	Hop 3 (n=8)	Total (n=23)
Piperacillin	85.7 (12)	100 (1)	50 (4)	74 (17)
Ticarcillin	100 (14)	100 (1)	100 (8)	100 (23)
Piperacillin/tazobactam	100 (14)	100 (1)	100 (8)	100 (23)
Ticarcillin/clavulanic acid	100 (14)	100 (1)	100 (8)	100 (23)
Ceftazidime	100 (14)	100 (1)	100 (8)	100 (23)
Cefepime	100 (14)	100 (1)	100 (8)	100 (23)
Tobramycin	92.9 (13)	100 (1)	100 (8)	95.7 (22)
Amikacin	85.7 (12)	100 (1)	50 (4)	74 (17)
Ciprofloxacin	100 (14)	100 (1)	100 (8)	100 (23)
Gentamicin	100 (14)	100 (1)	100 (8)	100 (23)
Ceftriaxone	100 (14)	100 (1)	100 (8)	100 (23)

Hop 1 – Military Hospital of Meknes; **Hop 2** – Regional Hospital of Meknes; **Hop 3** – University Hospital Center of Casablanca.

and 4 (17.39%) *A. baumannii* isolates, respectively. None of the strains contained bla_{OXA-24}, bla_{OXA-48}, bla_{OXA-58}, bla_{VIM} and bla_{KPC} (Table 3).

Discussion

The emergence of *A. baumannii* carrying carbapenemases and their worldwide dissemination represent significant public health menaces. Antibiotic resistance in this germ is considered as one of the dangerous nosocomial problems.[1] In this work, we isolated a total of 111 *A. baumannii* from soil samples (n=30) and clinical specimens (n=81).

Statistical analysis demonstrated a significant difference in antimicrobial drugs resistance between environmental and clinical isolates for all antibiotics used ($p<0.05$). The excessive consumption of antimicrobial treatment,

Table 3. Clinical data and carbapenemase types of 23 CRAB

Strains	Clinical data			Carbapenemase types*			
	Specimen	Hospital	Gender	OXA-51	OXA-23	NDM	GES
AB2	Central catheter	Hop 3	F	+	+	-	+
AB4	Central catheter	Hop 3	M	+	-	-	-
AB8	Bronchial aspirate	Hop 3	M	+	-	-	+
AB14	Pus	Hop 1	M	+	-	-	+
AB17	Urine	Hop 1	M	+	-	-	+
AB20	Urine	Hop 1	M	+	+	-	+
AB21	Urine	Hop 1	F	+	-	-	-
AB24	Pus	Hop 1	M	+	+	-	+
AB32	Pus	Hop 2	M	+	+	-	-
AB39	Distal bronchial levy protected	Hop 3	M	+	+	-	-
AB40	Distal bronchial levy protected	Hop 3	M	+	+	-	+
AB41	Central catheter	Hop 3	M	+	+	-	-
AB43	Distal bronchial levy protected	Hop 3	M	+	+	-	+
AB44	Bronchial aspirate	Hop 1	F	+	+	+	+
AB58	Distal bronchial levy protected	Hop 1	M	+	+	-	+
AB68	Urine	Hop 1	F	+	+	+	+
AB71	Urine	Hop 1	F	+	+	-	+
AB72	Urine	Hop 1	F	+	+	+	+
AB76	Distal bronchial levy protected	Hop 1	M	+	+	+	-
AB77	Blood	Hop 1	F	+	+	-	+
AB78	Distal bronchial levy protected	Hop 1	M	+	-	-	-
AB79	Distal bronchial levy protected	Hop 1	M	+	-	+	+
AB80	Distal bronchial levy protected	Hop 1	M	+	+	-	-

*All strains presented negative results with OXA-24, OXA-48, OXA-58, VIM, and KPC.
Hop 1 – Military Hospital of Meknes; **Hop 2** – Regional Hospital of Meknes; **Hop 3** – University Hospital Center of Casablanca.

including carbapenems, in hospitals may explain these results.

Among our clinical strains, the IPM resistance rate was 28.4%. This rate is significantly lower than the one (100%) signaled recently in Morocco by Uwingabiye et al. 2017.[10]

In the current study, we found that the molecular diagnosis of genes, coding for carbapenemase, confirmed the results of the modified Hodge test. However, among CRAB isolates, fifteen strains gave positive results with the EDTA assay (synergy between imipenem and EDTA), while only five strains harbored the bla_{NDM} gene; and all of them were collected from the Military Hospital.

Various research works[21] showed that phenotypic assays prove the MBL production by *A. baumannii* strains and that no MBL encoding genes were identified by molecular methods. These results may be explained by the bactericidal action of EDTA, which can prompt an expanded inhibition zone, giving a false positive that is not related to true MBL production.[22] In contrast, these results may be true positives associated with another MBL gene that has not been screened in this study.

On the other hand, only 5 among 15 GES positive isolates gave a positive result with cloxacillin test (synergy between imipenem and clavulanic acid), showing the importance of

molecular analysis to confirm the existence of GES producing isolates.

As mentioned previously, resistance to carbapenem in *A. baumannii* strains results essentially from the production of class D (oxacillinase) and class B (MBL) carbapenemases. Recently, GES and KPC carbapenemase of class A have also been described in this germ.

In the current study, we observed the existence of bla_{OXA-23} and bla_{GES} in the majority (69.6% and 65.2% respectively) of CRAB, indicating the predominance of these genes among *A. baumannii* Moroccan strains. The emergence of OXA-23 in Morocco is compatible with the global epidemiology of OXA-23 and with many reports from Mediterranean countries.[23] Several research works have documented the propagation of OXA-23 in *A. baumannii* in many Tunisian regions.[24] It is the same for Algeria where OXA-23 in *A. baumannii* strains occurred in distinct geographical areas.[25] Lately, the production of OXA-23 carbapenemase in *A. baumannii* has been detected in Libyan hospitals.[26]

For OXA-58-producing *Acinetobacter* isolates, a lot of research reported their spread in many countries such as France, Belgium, Italy, Australia, the USA, Tunisia, Turkey, Algeria, Egypt, and Morocco.[9,27-33] Similarly, OXA-24 carbapenemase was identified in many countries such as Spain, Bulgaria, Poland, Saudi Arabia, and Algeria. [25,34,35]

In Morocco, OXA-24 was newly detected in *A. baumannii* isolates.[10] The OXA-58 and OXA-24 oxacillinases, previously reported in Moroccan hospitals, were not detected among our isolates.[9,10]

The GES type of ESBLs has been found in various species, mainly in *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*,[36] and has newly occurred in *A. baumannii* strains.[1,8] To our knowledge, our isolates represent the first report of *A. baumannii* strains carrying bla_{GES} genes in Morocco.

Amongst clinical strains resistant to IMP, 65.2% were carbapenemases producers, particularly of GES type of ESBL. This rate is higher than that reported in Turkey[6] (25.3%), Tunisia[24] (50%) and Lebanon (20.4%).[7]

Although previously reported in Tunisia, Kuwait, and in Lebanon,[24,7] the co-existence of both OXA and GES carbapenemases types in *A. baumannii* is not frequent.

A. baumannii is considered one of the nosocomial pathogens of biggest concern; it is widespread in the hospital environment especially in medical intensive care units. These data are consistent with our results. Among the clinical strains, those isolated from medical intensive care units represent 45.7%.

In this study, three strains carried the bla_{NDM}, bla_{GES} and bla_{OXA-23} genes. This represents the first appearance and dissemination of *A. baumannii* resistant to carbapenem by a combination of three carbapenemases genes in Morocco. These 3 strains were collected from hospitalized patients in the Military Hospital of Meknes. The first strain (AB44) was isolated from a female hospitalized in May 2015. The second and the third isolates (AB68 and AB72) were from two females hospitalized in May and June 2016. Antibiotic susceptibility profiles of these three isolates (AB44, AB68, and AB72), harboring this combination of carbapenemases, presented a higher prevalence of resistance for the majority of antibiotics tested. In addition to their high profile of carbapenems resistance, those strains indicated resistance to aminoglycosides family (amikacin and gentamicin). Furthermore, the three strains had relatively similar resistance profiles, and were resistant to all β-lactams tested, except the AB72 strain, which was sensitive to tobramycin. These results are very disturbing because they reflect the occurrence and spread of carbapenemases (Class A, B, and D) in North Africa.

Conclusions

In summary, we report the position of CRAB strains in Morocco, with the dominance of OXA-23, GES, and NDM. This study does not only show the first nationwide evaluation of carbapenemases in *A. baumannii* strains, but it is also the first detection of GES carbapenemases in Morocco. *A. baumannii* remains among the

most disturbing multidrug resistant pathogens. Moreover, carbapenemases-producing strains illustrate an emerging danger in Morocco and abroad. For this reason, regular monitoring of such strains, involving Morocco and neighboring countries, is necessary to avoid their dissemination.

Authors' contributions statement: HEH performed the experimental part (the microbiological and molecular tests), collected the data and performed the statistical analysis, prepared the manuscript. AB designed the study, supervised the laboratory experiments and helped draft the manuscript. NEH, MS, KZ and IM helped in performing the experimental part of the manuscript. KN conducted the PCR amplification. MT helped in performing and interpreting the molecular part. All authors read and approved the final version of the manuscript.

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