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Original Research Article

Emergence of Antimicrobial Resistance in *Escherichia coli* Isolates from Gut of Healthy and Diarrheal Paediatric Population

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Keywords

AmpC ß-lactamases, Metallo-ß-lactamases, extended spectrum ß-lactamases, Antibiotic resistance, Multidrug-resistant organisms, Co-existence, Phenotypic detection

A B S T R A C T

Extended-spectrum beta-lactamase (ESBLs), AmpC-type β-lactamases (AmpC) and metallo-ß- lactamases (MBL's) have emerged as the most troublesome resistance mechanism to *E. coli.* The aim of this study was to explore the Beta-lactamases resistance in *E. coli* isolates using various phenotypic methods. During the study period, 40 fecal isolates was collected from each outpatient, inpatient and healthy children attending the Guru Teg Bahadur Hospital in East Delhi, India. Biochemically confirmed *E. coli* isolates were examined for the presence of various beta-lactamase production using standard CLSI methods. Isolates were also subjected to antimicrobial testing using the disc diffusion method. Among 120 isolates, ß-lactamase production was observed in 101(84.16%) isolates. 77 (64.16%) isolates were ESBL producers, 29 (24.16%) were MBL producers and 20(16.66%) were AmpC producers. Co-existence of ESBL with MBL was observed in 13 (10.83%) isolates MBL with AmpC in 8(6.66%) and ESBL with AmpC was observed in 5(4.16%) isolates. The high prevalence of the ß- lactamases production in *E. coli* isolates highlights the importance of various phenotypic methods in routine microbiology laboratory, need of strict guidelines for the antibiotic therapy and a continuous observation to detect the resistant strains, and the implementation of infection control measures to reduce the increasing burden of antibiotic resistance.

Introduction

β-lactam antibiotics being less toxic and higher efficacy are most preferred antibiotics in the world. Selective pressure generated due to the improper and indiscriminate use of the these antibiotics in the hospital settings has led to the emergence of ESBLs, AmpC β-lactamases and metallo-β-lactamases which appears as the most challenging resistance mechanism in the health care settings Deshmukh *et al.* (2011). Multidrug-resistant *E. coli* by virtue of production of various "newer β-

lactamases" is capable of hydrolyzing a wide range of β-lactam antibiotics and confers resistance to many classes of antibiotics (Livermore, 2003; Khan *et al.,* 2008). The production of ESBL's and AmpC β-lactamases occurs simultaneously and AmpC β-lactamases may be co-transferred with the plasmids, thus accelerating the fluoroquinolone and the aminoglycoside resistance (Livermore, 2009). Because of the ability of Metallo beta-lactamases to hydrolyze almost all drugs including carbapenems along with aminoglycosides and fluoroquinolones. They are important and possess an ability to rapidly disseminate as they are plasmid mediated (Walsh *et al.,* 2005). β-lactamases are of significant concern because they confine therapeutic preferences, cause treatment failures and are increasing worldwide (Khan *et al.,* 2008). The detection of the AmpC beta-lactamases mediated resistance in the clinical microbiology laboratory poses a problem, because their phenotypic tests are not standardized. Several phenotypic methods for AmpC detection have been described (Upadhyay, 2010; Polsfuss, 2011; Pasteran, 2010). The present study assisted in determining the prevalence rates of the multidrug resistant *E. coli* expressing β-lactamase enzymes, as residents of gut flora in paediatric patients suffering from diarrhea and in healthy children.

Material and Methods

Clinical specimens: The study population included three groups. Group 1 comprised of 40 children with acute diarrhea (less than 72 hour duration) attending the Out Patient Department services of a tertiary care hospital and not receiving any antibiotic. Group 2 comprised of 40 hospitalized children and receiving antibiotics (oral or I/v for 72 hr or more) for reasons other than diarrhea. Children in this group were receiving antibiotics like monocef, ampicillin, chloromycin, amikacin, vancomycin, ampicillin + chloromycin for complaints like pneumonia, enteric fever, meningitis, respiratory tract infection, malaria etc. Group 3 represented the healthy controls of 40 children below 5 years of age who are not suffering from diarrhea or any other diseases. Informed consent form was collected from all the participants. The study protocol was approved by institutional ethical committee.

Sample processing: Fresh stool samples were collected and inoculated on media as per standard laboratory methods and *E. coli* was identified based on phenotypic characterization (Koneman *et al.,* 1997; Colle *et al.,* 1996).

Antibiotic susceptibility test: The antimicrobial susceptibility testing of the isolates was performed by the Kirby Bauer disc diffusion method (CLSI, 2013). The following antibiotics were tested: norfloxacin (10ug), cefotaxime (30ug), imipenem (10ug), meropenem (10ug), ceftazidime (30ug), azetronam (30ug), nalidixic acid (30ug), amoxicillin (20/10ug), gentamycin (10ug), ciprofloxacin (5ug), ampicillin (10ug), amikacin (30ug), polymixin B (300u), cefotaxime + clavulanic acid (30/10ug), ceftriaxone (30ug) and Piperacillin+tazobactam (100/10ug). Phenotypic methods for Beta lactamase detection were followed as per standard guidelines for screening tests and confirmatory tests. Phenotypic methods for screening and confirmation of β-lactamase detection were followed as per standard guidelines (Samaha Kfoury and Araj, 2003).

Phenotypic methods for screening and confirmation of Beta lactamases

ESBL - Phenotypic screening for ESBL production was performed on *E. coli*

isolates. Recordings of the zone diameters of ceftazidime/cefotaxime/ceftriaxone/aztreonam were performed by disc diffusion test on Mueller Hinton Agar plates using CLSI guidelines (CLSI, 2006; CLSI, 2013). Any isolate showing a reduced (resistant) zone diameter was included for confirmatory testing.

Confirmation - Double Disk Synergy Test (DDST) was performed on isolates resistant to ceftazidime/ cefotaxime/ ceftriaxone/ aztreonam by placing disks of ceftazidime (30 µg) or cefotaxime (30 µg) discs at a distance of 20 mm from the ceftazidime+clavunate (20/10 µg) disc; a enhanced zone of inhibition > or =5mm towards ceftazidime/clavunate (20/10 µg) disc was considered as positive for ESBL production (M'Zali *et al.,* 2000).

AmpC - For AmpC screening a 30ug cefoxitin disk was placed on Mueller Hinton agar plate with lawn culture of *E. coli,* zone diameters less than 18mm was selected for confirmation of AmpC production.

Confirmation - AmpC disk test: Tris-EDTA disk was rehydrated with 20 µl of saline and 4–5 colonies of the test isolate were inoculated on the disk. The inoculated disk was then placed beside a cefoxitin disk on a Mueller Hinton agar plate inoculated with a lawn of *E. coli* ATCC 25922 and incubated overnight. A positive test appears as a flattening or indentation of the cefoxitin inhibition zone near the test disk while a negative test has an undistorted zone (Black *et al.,* 2005).

Boronic acid disk test method: a 30ug cefoxitin disk was supplemented with 300ug phenyl boronic acid. An organism that demonstrated a defined increase(>/=5mm) in zone diameter around the antibiotic disk with added inhibitor compound compared to that with antibiotic containing disk alone was considered to be AmpC producer (Thomson, 2010).

Disk approximation method - Use of disk approximation technique to detect inducible AmpC activity was tested using ertapenem (10ug), cefoxitin (30ug) and amoxicillin-clavulanic acid disks (20/10ug) as the inducing substrates and ceftazidime (30ug) disk as the reporter substrate. Disks were placed at a distance of 20mm, and any obvious blunting or flattening of the zone of inhibition between ceftazidime disk and the inducing substrates was interpreted as a positive result for AmpC (Thomson, 2010; Jacoby, 2009).

MBL-screening was performed in all isolates for imipenem/ ertapenem/ meropenem (10ug) resistance by disc diffusion method.

Confirmation-Combined disc diffusion test: Two 10 µg ertapenem (ERT) discs were placed at a distance of 20mm on a lawn culture of the isolate and 10 µl of 0.5 M EDTA solution was added to one ertapenem disc. A plain EDTA disc can also be placed separately on the same plate to detect its inhibitory effect on test isolates. The zone of inhibition around ERT discs alone and those with EDTA will be compared after 16-18 hours. An increase in zone size of at least 5 mm /> around the ERT-EDTA disc as compared to ERT disc alone was recorded as a positive result (Marchiaro *et al.,* 2005).

Modified Hodge test: An IMP/ERT disc (10 µg) was placed at the centre of the lawn of an overnight culture suspension of Escherichia *coli* ATCC 25922, adjusted to one-tenth turbidity of the 0.5 McFarland standards. The test isolates was streaked heavily from the edge of the disc to the plate periphery. The presence of a clover-leaf shaped zone of inhibition was interpreted as production of MBL (Lee *et al.,* 2001).

Out of 120 *E. coli* isolates, 106 (39, 40 and 27) were found as diarrheagenic *E. coli* by multiplex PCR in three groups respectively (data not shown).

Statistical analysis

All outcome data were analyzed using Statistical Package for Social Sciences (SPSS; Version 20.0). The differences between resistance patterns of *E. coli* isolates were determined; chi square test was used to determine the proportion. All P-values were based on 2-tailed tests of significance where $P<0.05$ was considered statistically significant.

Results and Discussion

Demographic profiles of all independent variable factors (breast feeding, dehydration, socio economic status, clinical features and duration of diarrhea) was done for all the participants enrolled in the study as shown in table 1. P value was found to be highly significant for dehydration levels and clinical status between three groups.

Table 2 depicts the confirmatory test for various beta- lactamases. Out of 120 isolates tested, 101(84.16%) *E. coli* isolates revealed ß-lactamase production in all the three clinical groups. As shown in table 3, a total of 77 (64.16%) isolates were ESBL producers, 29 (24.16%) isolates were MBL producer by combined disk diffusion test and Modified Hodge test each.

AmpC was detected in 20 (16.66%) *E. coli* isolates by both zone indentation and boronic acid disk test method while 17 (14.16%) isolates were positive by disk approximation test. MBL and AmpC production was more frequently detected in healthy group as compared to study subjects. For AmpC detection, boronic acid disk test method was found to be more easy and consistent when repeated.The co-production of ESBL/MBL/ AmpC ß- lactamases together was not observed in any isolate in all the three groups. Co production of ESBL and MBL was maximum in group 2 (20%) followed by 10% and 2.5% in group 3 and 1 respectively, coproduction of MBL and AmpC was maximum in diarrheal group 10% followed by 5% each in group 2 and 3 and the co production of ESBL and AmpC was observed in group 1 and 2 only with 2.5% and 10% respectively.

Antibiotic resistance profile of *E. coli* isolates (Figure 1) showed that most isolates were sensitive to polymixin B($P=1.000$), ceftriaxone ($P=1.000$), amoxicillin ($P=0.772$) and cefotaxime+ clavulanic acid ($P=1.000$). Maximum resistance was shown towards cefotaxime ($P=0.01$) followed by ciprofloxacin ($P=0.740$) and norfloxacin ($P=0.709$) in OPD/IPD isolates as compared to controls. The isolates from control group showed maximum resistance to ciprofloxacin followed by norfloxacin and cefotaxime. Other antibiotics which were found to be statistically significant were ceftazidime($P=0.006$), azetronam ($P=0.046$), nalidixic acid($P=0.01$), amoxicillin, gentamycin ($P=0.001$), amikacin ($P=0.01$), and Piperacillin+tazobactam ($P=0.037$).

This study emphasized on the presence of resistant phenotypes for a range of antimicrobial classes with a focus on beta-lactamase producers. Broad spectrum beta-lactams resistance mediated by extended spectrum β-lactamase (ESBL), AmpC and metallo beta lactamase (MBLs) enzymes are an increasing problem worldwide. β-lactamases are encoded either by the chromosomal or plasmid mediated genes (Mary *et al.,* 2005). The infections caused by multidrug-resistant DEC *E. coli* that produce various β lactamase enzymes have been reported in recent past in developing countries and they are associated with a

significant morbidity and mortality in children (Itokazu *et al.,* 1996).

These phenotypic methods are easy and are able to discriminate between various classes at some extent. The production of beta-lactamase and multidrug resistance was more common in diarrheal and non diarrheal group; this may be due to the improper use of drugs and accumulation of virulent factors which are common in diarrheagenic *E. coli*. It was observed that, children admitted to the hospital receiving antibiotics harboured high Beta lactamase producing *E. coli* similar to children with that of acute diarrhea, suggesting the continued existence of resistant population in the community of this susceptible population. Further, it also indicates the extensive use of antibiotic in pediatric population. High ESBL producing isolates may likely to have emerged due to indiscriminate use of these antibiotics as indicated by high cefotaxime resistance. In our study, the prevalence of various β lactamases in DEC *E. coli* was alarmingly high and these enzymes were also found in healthy isolates. However; On the contrary the low level of resistance in healthy children cannot be ignored which may have developed due to selective pressure from environment and horizontal gene transfer.

The ESBL production was found to almost similar in group 1 and 2 (64.16 percent); and low in healthy controls, among all other β lactamases. The high production of ESBL is a matter of great concern as the effectiveness of third generation cephalosporins substantially decrease. The Study for Monitoring Antimicrobial Resistance Trends (SMART) Program 2007 showed similar levels of ESBL production by *E. coli* isolates but from a different site (intra-abdominal infections) (Hawser *et al.,* 2009). It was mentioned that ESBL levels were highest in India (79 percent). In other studies, ESBL production was found to be very low, this may be due to the masking of ESBLs entirely by the over expression of AmpC b-lactamases, (Thomson, 2001) or by the induction of AmpC b-lactamase by clavulanate used in synergy tests (Sturenburg *et al.,* 2004).

In the present study, AmpC production was seen in 17.5 percent isolates as compared to that in other studies that had reported a high prevalence of the AmpC producer's up to 37.5 percent (Subha *et al.,* 2003). In another Indian study, 3.3 per cent of isolates produced AmpC β lactamases (Ratna *et al.,* 2003). Study at Kochi, India showed 12.5 percent AmpC production. After a first screening procedure, each of the three phenotypic AmpC tests used in this study was capable of confirming the majority of AmpC beta-lactamase-producing *E. coli* strains but the boronic acid disk test method gave consistent result throughout. AmpC type β-lactamases may also be carried on plasmids which can easily spread to other organisms within a hospital or geographic region since they confer resistance to cefoxitin. Boronic acid disc potentiation test has been used by various workers for detection of AmpC (Thomson, 2010; Hawser *et al.,* 2009). The AmpC producing organisms can act as a hidden reservoir for the ESBLs. Also, the high-level expression of the AmpC β-lactamases may mask the recognition of the ESBLs and it may result in a fatal and an inappropriate antimicrobial therapy. In the present study, all isolates resistant to cefoxitin, did not show AmpC production, suggesting that cefoxitin resistant could be due to some other enzymatic mechanism (ESBLs, MBLs) or non-enzymatic mechanism like porin channel mutation (Rawat *et al.,* 2012; Bethel, 2004).

Table.1 Demographic profile of the isolates

S.no.	Variables	OPD (40) N (%)	IPD (40) N (%)	CONTROLS (40) N (%)	P-value
1.	**Age (in years): mean (SD)**	2.84 (7.93)	2.32 (5.71)	1.73(1.67)	0.67
2.	**Age group:**				
	< 1yr	16(40)	20(50)	16(40)	
	1year-3year	14(35)	6(15)	10(25)	0.324
	3year-5year	10(25)	14(35)	14(35)	
3.	**Breast feeding status**				0.481
	Still breast feeding	27(67.5)	30(75)	25(62.5)	
	Breast feeding stopped	13(32.5)	10(25)	15(37.5)	
4.	**Dehydration status**				
	Severe dehydration	33(82.5)	3(7.5)	0	
	mild dehydration	7(17.5)	7(17.5)	2(5)	<**0.001***
	no dehydration	0	30(75)	38(95)	
5.	**Clinical status**				
	no symptoms	0	35(87.5)	40(100)	
	Vomiting only	32(80)	3(7.5)	0	
	Fever only	7(17.5)	2(5)	0	<**0.001***
	Both (vomiting +fever)	1(2.5)	0	0	
6.	**Duration of diarrhea**				0.08
	<2 days	28(70)	9(22.5)	4(10)	
	3-7 days	12(30)	0	0	
7.	**Economic status (income/year)**				
	(< 1 lakh)	31(77.5)	32(80)	20(50)	**0.005***
	(≥1 lakhs)	9(22.5)	8(20)	20(50)	

*Statistically significant

Table.2 Phenotypic confirmatory tests for beta lactamases

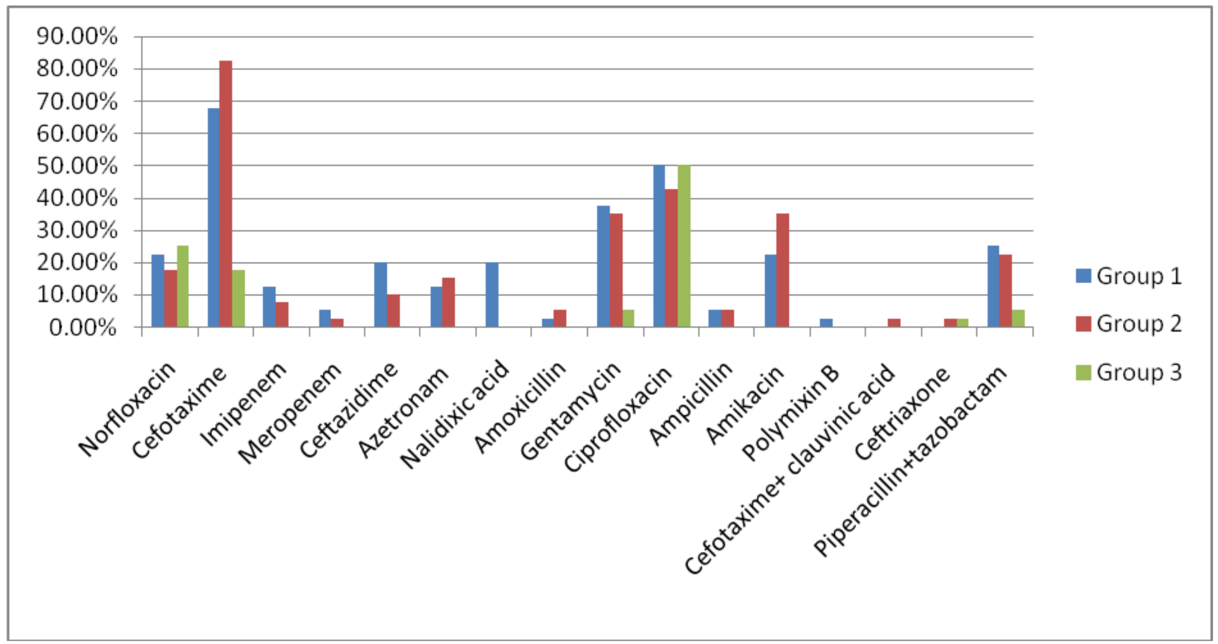
B-lactamases	Confirmatory tests	Figure	Explanation	Quality controls
ESBL	Double Disk Synergy Test (DDST)		Double Disk Synergy Test (DDST) method indicating ESBL producing isolate having increased zone size with ceftazidime +clavulanic acid (> or =5mm) as compared to cefotaxime disk alone.	*E. coli* ATCC 25922 was used as negative control and *K. pneumoniae* ATCC 700603 as positive control.
AmpC	AmpC disk test		AmpC disk test with the upper Tris/EDTA disk inoculated with the test isolate and placed adjacent to a cefoxitin (FOX) disk on a lawn of cefoxitin resistant *E. coli* isolate. The indented zone margin is evidence of hydrolysis of cefoxitin by the AmpC enzymes that diffused out of the permeabilized cells on the Tris/EDTA disk. AmpC disks (filter paper containing tris-EDTA), CX: 30µg cefoxifin disks	*E. coli* ATCC 25922 was used as negative control and *E. cloaceae* BAA-1143 as a positive control.
	Boronic acid disk test		Representing Boronic acid disk test method indicating MBL producing strain having increased zone size with 300µg of phenyl boronic acid+ cefoxitin (≥5mm) as compared to 30 µg cefoxitin disc alone.	
	Disk approximation test		Representation of disk approximation test. No zone flattening of ceftazidime toward ertapenem disk (inducing substrate) showing negative result. ERT: ertapenem (10 µg), CAZ: Ceftazidime (10 µg), AMC: Amoxillin-clavulanate (20/10 µg).	
MBL	Double disk potentiation test		Double disk potentiation test showing increase in zone size with ertapenem disk potentiated with EDTA to test isolate when compared with plain ertapenem disk placed in a lawn of test organism.	*E. coli* ATCC 25922 as negative control and *K. pneumoniae* ATCC/BAA-1705/1706 was used as positive control.
	Modified Hodge test		Modified Hodge test for screening of metallo β-lactamase where the positive strain (*K. pneumoniae* ATCC 1705) is producing indentation in zone of inhibition of imipenem to *E. coli* ATCC 25922.	

Table.3 Distribution of ESBL, MBL and AmpC either single or in combination in three groups

	ESBL n(%)	MBL n(%)	AmpC n (%)			ESBL+MBL n (%)	MBL+AmpC n (%)	ESBL+AmpC n (%)
Groups			Zone indentation test	Boronic acid disk test method	Disk approximation test			
1(n=40)	27(67.5)	5(10)	6(15)	6(15)	4(10)	1(2.5)	4(10)	1(2.5)
2(n=40)	27(67.5)	13(32.5)	6(15)	6(15)	5(12.5)	8(20)	2(5)	4(10)
3(n=40)	23(57.5)	**11(27.5)**	**8(20)**	**8(20)**	**8(20)**	4(10)	2(5)	0
Total (n=120)	**77(64.16)**	**29(24.16)**	**20(16.66)**	**20(16.66)**	**17(14.16)**	13(10.83)	8(6.66)	5(4.16)
P value	0.408	**0.024***	0.611	0.595	0.708	**0.045***	0.728	0.125

*Statistically significant

Figure.1 Distribution of antibiotics among three groups



In the present study, MBL's were low in group 1 and almost same frequency of occurrence in IPD and healthy group. 25 percent isolates demonstrated MBL activity in IMP/meropenem sensitive strain. A previous study from this area has also reported 33.3 percent MBL production in IMP sensitive gram negative strains. (Rawat, 2011). Carbapenems remain the only β-lactams active against the AmpC and the ESBL co producers however, producers of metallo β-lactamases have increased the resistance to carbapenems.

In the present study, coexistence of ESBL and MBL was reported in 10.83 percent isolates, AmpC and MBL co production was showed by 6.66 percent isolates and the AmpC and the ESBL co production was showed by 4.16 percent isolates in all the

three groups. Higher level of co producers among beta‑lactams suggests that there is an increase in horizontal transfer of resistance gene. This reinforces the importance of continuous surveillance. When resistance was conferred by producing a single enzyme, it was commonly the ESBL but when the multiple enzyme production was observed, AmpC and MBLs were more common.

The high prevalence of multidrug resistance in DEC *E. coli* raises the need for an early detection of the β-lactamase producing organisms by simple screening methods, which can help in providing an appropriate antimicrobial therapy and in avoiding the development and the dissemination of these resistant strains. It is important to develop our own antimicrobial program which should be based on the local epidemiological data and international guidelines, to optimize the antimicrobial use among hospitalized patients, to provide a cost-effective therapy and to reduce the adverse consequences of the antimicrobial use (Laxmi, 2008).

In conclusion for this study Amp-C, ESBL and MBL were the main resistance patterns of the strains evaluated. High prevalence of ESBL and plasmid-mediated AmpC was due to extensive use of third-generation cephalosporins. Detection of beta-lactamase by phenotypic methods was an attempt to identify the gut flora *E. coli* drug resistance profile before administration of drugs. Microbiological excellence is needed more than ever, and it is critical that ESBLs, AmpC beta-lactamases, and carbapenemases be promptly and accurately detected.Multiple resistances to antimicrobial drugs among *E. coli* isolates complicate therapeutic management of infections. Early Detection of beta lactamase production is important in routine laboratories in order to establish the antibiotic therapy and to achieve the favourable clinical outcomes. Implementation of simple phenotypic tests in the laboratories will help us to reduce the spread of various beta-lactamase harboring *E. coli* in diseased as well as in healthy children. Understanding the molecular basis of resistance acquisition and transmission can contribute to the development of new strategies to combat this phenomenon. Identification of DEC is important because our study reveals that self limiting diarrhea are being treated with high end antibiotic by overzealous clinicians leading to the current status of multidrug resistant *E. coli* which eventually here lead to the spread of resistant genes to the commensal *E. coli* of the gut microbiota.

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