Molecular characterization of extended-spectrum β-lactamase-producing Escherichia coli and Klebsiella spp. isolates in Mongolia

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antibiotic resistance; CTX-M; Enterobacteriaceae; Extended-spectrum β-lactamase; PFGE

Abstract  Background/purpose: The aim of this study was to determine the molecular characteristics of β-lactamase genes in extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae isolates from Mongolia.

Methods: Fifty-six ESBL-producing Enterobacteriaceae isolates were collected, of which 46 were Escherichia coli, seven were Klebsiella pneumoniae, and three were K. oxytoca. Minimum inhibitory concentrations for selected antibiotics were tested using the agar dilution method, and the β-lactamase genes were determined using polymerase chain reaction combined with sequencing. Pulsed-field gel electrophoresis (PFGE) was used for genotyping all isolates, and phylogenetic grouping was performed on ESBL-producing E. coli isolates.

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Conjugation tests combined with plasmid digestion assays were used to determine whether there was a horizontal spread in Mongolia.

**Results:** Among the 56 ESBL-producing isolates, 43 isolates (76.8%) were resistant to fluoroquinolones, but all isolates were susceptible to carbapenems and amikacin. The polymerase chain reaction sequencing results showed that the dominant CTX-M genotype was CTX-M-15 (19/46, 41.3%) in the ESBL-producing E. coli isolates. By contrast, CTX-M-14 and CTX-M-3 were the major genotypes found in Klebsiella spp. Phylogenetic analysis revealed that 21 ESBL-producing E. coli isolates belonged to group D (21/46, 45.6%), followed by group A (13/46, 28.3%), group B2 (11/46, 23.9%), and group B1 (1/46, 2.2%). Only four E. coli isolates (4/46, 8.7%) belonged to the ST131 clone. PFGE showed that the ESBL-producing Enterobacteriaceae were genetically unrelated. The conjugation assay showed that two plasmids harboring CTX-M-15 in E. coli isolates were genetic unrelated, whereas seven plasmids harboring CTX-M-14 (5/7 and 2/7) and four plasmids harboring CTX-M-55 (4/4) showed genetic relatedness, indicating the dissemination of resistance plasmids in this area.

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### Introduction

Extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (EPE) are increasing rapidly all over the world. At present, EPE are a growing threat to public health, leading to serious infections and raising key therapeutic problems. ESBLs are among the Ambler Class A, resistant to β-lactam antibiotics except cephamycins and carbapenems, and are inhibited by clavulanic acid. In addition, ESBLs are often located on plasmids harboring resistance genes to other antimicrobial classes, resulting in multidrug-resistant isolates and thus also facilitating their transfer to different hosts.

The first ESBLs have evolved from native β-lactamases TEM and SHV by genetic mutation. However, since the mid 2000s, a novel type of ESBL called CTX-M, from environmental *Kluyvera* spp., has emerged worldwide. There are currently >150 different CTX-M-type ESBLs recognized (http://www.lahey.org/Studies/) and grouped into six major subgroups based on their amino acid identities, named the CTX-M-1, -2, -8, -9, -25, and KLUC.

CTX-M has been identified in several members of the Enterobacteriaceae family, and especially in *Escherichia coli*, which is the principal ESBL-producing member of the Enterobacteriaceae. At present, CTX-M-15, derived from CTX-M-3 by a substitution of Asp-240-Gly, which increases its catalytic efficiency against ceftazidime, is recognized as the most widely distributed CTX-M enzyme.

In addition, the current pandemic spread of ESBL-producing *E. coli* has been greatly facilitated by high-risk clones, mainly the clonal group O25B:H4-B2-ST131. ST131 contains the CTX-M-15 enzyme, with high potential virulence, and represents a major public health problem.

Many reports have documented the emergence of EPE. In our previous study, 18.3% of the clinical *E. coli* isolated from patients in Mongolia are ESBL producers. However, the distribution of ESBL genotypes and plasmid characteristics in EPE in Mongolia is still unclear. The aim of this study was to investigate the molecular epidemiology and genetic characteristics of clinical EPE isolates obtained from two Mongolian hospitals.

### Materials and methods

#### Sampling and isolation of Enterobacteriaceae

The study protocols were approved by the National Ethics Committee of Mongolia (20120925/6). Fifty-six nonconsecutive, nonduplicate clinical isolates were obtained from inpatients hospitalized at National Central Hospital or National Center for Maternal and Child Health in Mongolia. These isolates were collected over a period of 5 months, between February 2013 and June 2013. Presumptive Enterobacteriaceae isolates were identified using standard microbiological methods. The isolates were stored at –80°C in Luria–Bertani (LB) broth containing 20% glycerol (v/v) until used.

#### Antimicrobial susceptibility testing and ESBL confirmation

Antimicrobial susceptibilities were determined by the disk diffusion method on Mueller–Hinton agar (Bio-Rad, Marne la Coquette, France) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. ESBL-producing isolates were screened using the double-disk synergy test in accordance with CLSI guidelines. All ESBL-producing isolates were tested for minimum inhibitory concentration (MIC) of selected antimicrobial agents (from Sigma-Aldrich, St. Louis, MO, USA; amikacin, cefepime, ceftazidime, cefotaxime, ciprofloxacin, ertapenem, levofloxacin; from USP Standards, Rockville, MD, USA: cefoxitin, imipenem, meropenem) using the agar dilution method in accordance with CLSI guidelines. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control strains.

#### Molecular characterization of β-lactamases

The primers used in this study are described in Table 1. Strains with a positive double-disk synergy test were further studied using specific polymerase chain reaction (PCR) amplification and sequencing of ESBL genes. DNA was
extracted with the boiling method, and the ESBL-encoding genes were identified using specific primers for the bla-TEM, blaSHV, and blaCTX-M genes, previously described, and followed by DNA sequencing. The DNA sequences and deduced amino acid sequences were compared with genes in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) or the β-lactamase classification system (http://www.lahey.org/studies/) to confirm the subtypes of β-lactamase genes.

ESBL-producing *E. coli* phylogenetic grouping and ST131 screening

ESBL-producing *E. coli* isolates were assigned to one of the four main phylogenetic groups (A, B1, B2, and D), using a combination of three DNA gene markers (*chuA*, *yjaA*, and TspE4C2). To identify the ST131 clones, PCR for the specific *mdhA* and *gyrB* genotype was used to screen for ST131. Multilocus sequence typing (MLST) was further carried out on suspected ST131 isolates according to the protocol and primers described previously (http://mlst.ucc.ie/).

### Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) of XbaI-digested genomic DNA samples of all isolates were carried out with a CHEF Mapper XA apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the instruction manual. Electrophoresis was performed for 27 hours at 14°C with pulse times ranging from 5 seconds to 35 seconds at 6 V/cm. PFGE profiles were analyzed and compared using the GelCompar II software, version 2.0 (Unimed Healthcare Inc., Houston, TX, USA).

### Conjugation experiments and plasmid analysis

The liquid mating-out assay was carried out to transfer CTX-M genes (CTX-M-14, CTX-M-15, and CTX-M-55) from ESBL-
producing *E. coli* isolates to rifampicin-resistant *E. coli* C600 as described previously. All testing isolates were sensitive to rifampicin at the concentration of 256 μg/mL except isolate 35. As a result, transconjugants were selected on LB plates containing 256 μg/mL rifampicin (Sigma-Aldrich) and 2 μg/mL cefotaxime. The plasmids were extracted as described previously, followed by electrophoresis in a 0.6% agarose gel at 50 V for 3 hours and compared by coelectrophoresis with plasmids of known sizes from *Salmonella* OU7526 to determine the plasmid sizes.30 Plasmid DNA samples extracted from transconjugants were digested with the endonuclease EcoRI (New England Biolabs, Inc., Ipswich, MA, USA). The resulting fragments were electrophoretically separated in 0.8% agarose gels.

**Results**

**Description of the bacterial isolates**

During the study period, we collected 223 isolates, of which 56 (25.1%) from hospitalized patients were identified as ESBL producers. Among these, 46 isolates were identified as *E. coli*, seven were *K. pneumoniae*, and three were *K. oxytoca*. Antimicrobial susceptibility analyses showed that 39 ESBL-producing *E. coli* isolates (39/46, 84.8%) were resistant to fluoroquinolones (including ciprofloxacin and levofloxacin). Only one *K. pneumoniae* isolate was resistant to fluoroquinolones (1/7, 14.6%), and all three *K. oxytoca* isolates were resistant to fluoroquinolones. Importantly, all these EPE isolates were susceptible to carbapenems (including imipenem, meropenem, and ertapenem) and the aminoglycoside (amikacin) generally used for EPE treatment.

**Table 2** Phylogenetic group, β-lactamase genes, and MIC ranges among ESBL-producing isolates

<table>
<thead>
<tr>
<th>Phylogenetic group</th>
<th><em>Escherichia coli</em> (n = 46)</th>
<th><em>Klebsiella pneumonia</em> (n = 7)</th>
<th><em>Klebsiella oxytoca</em> (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-3</td>
<td>A 13 (28.3)</td>
<td>2 (9.5)</td>
<td>3 (42.9)</td>
</tr>
<tr>
<td>CTX-M-14</td>
<td>B1 1 (2.2)</td>
<td>2 (9.5)</td>
<td>2 (66.7)</td>
</tr>
<tr>
<td>CTX-M-15</td>
<td>B2 11 (23.9)</td>
<td>1 (4.3)</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td>CTX-M-14/15</td>
<td>D 21 (45.6)</td>
<td>13 (28.3)</td>
<td>3 (42.9)</td>
</tr>
<tr>
<td>CTX-M-22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-14/22</td>
<td></td>
<td>1 (14.2)</td>
<td></td>
</tr>
<tr>
<td>CTX-M-24</td>
<td></td>
<td>3 (14.3)</td>
<td>3 (6.5)</td>
</tr>
<tr>
<td>CTX-M-27</td>
<td></td>
<td>3 (9.1)</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td>CTX-M-55</td>
<td></td>
<td>2 (9.6)</td>
<td>8 (17.4)</td>
</tr>
<tr>
<td>CTX-M-22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-24</td>
<td></td>
<td>1 (9.1)</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td>CTX-M-27</td>
<td></td>
<td>2 (18.2)</td>
<td>8 (17.4)</td>
</tr>
<tr>
<td>CTX-M-55</td>
<td></td>
<td>2 (18.2)</td>
<td>8 (17.4)</td>
</tr>
<tr>
<td>CTX-M-22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other β-lactamase</td>
<td>TEM-1 7 (53.8)</td>
<td>9 (81.8)</td>
<td>6 (85.7)</td>
</tr>
<tr>
<td>SHV-1</td>
<td></td>
<td>29 (63.0)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>MIC range (µg/mL)</td>
<td>CTX 32→256</td>
<td>16→256</td>
<td>16→256</td>
</tr>
<tr>
<td>CAZ</td>
<td>16→128</td>
<td>0.5→16</td>
<td>0.5→128</td>
</tr>
<tr>
<td>FOX</td>
<td>2→32</td>
<td>&lt;2→8</td>
<td>2→64</td>
</tr>
<tr>
<td>FEP</td>
<td>4→64</td>
<td>2→16</td>
<td>1→64</td>
</tr>
<tr>
<td>CIP</td>
<td>4→32</td>
<td>4→32</td>
<td>0.06→32</td>
</tr>
<tr>
<td>LVX</td>
<td>4→64</td>
<td>4→32</td>
<td>0.25→64</td>
</tr>
</tbody>
</table>

* Four isolates belonged to the ST131 clone.

Data are presented as n (%) or range.

CAZ = ceftazidime; CIP = ciprofloxacin; CTX = cefotaxime; FEP = ceftopime; FOX = cefoxitin; LVX = levofloxacin; ESBL = extended-spectrum β-lactamase; MIC = minimum inhibitory concentration.

Molecular analysis of β-lactamase genes

The nucleotide sequence analysis of the CTX-M, SHV, and TEM genes are shown in Table 2. The dominant genotype was CTX-M-15 (19/46, 41.3%) among the ESBL-producing *E. coli* isolates, and the second most common genotype was CTX-M-14 (14/46, 30.4%) (isolate 69 contained CTX-M-14 and CTX-M-15; Table 2). In addition, CTX-M-55 (8/46, 17.4%), CTX-M-24 (3/46, 6.5%), CTX-M-3 (2/46, 4.3%), and CTX-M-27 (1/46, 2.2%) were also found in *E. coli* isolates. Among *K. pneumoniae*, three isolates had CTX-M-3 (3/7, 42.9%), three had CTX-M-14 (3/7, 42.9%), and one had CTX-M-14/22 (1/7, 14.2%; Table 2). Moreover, two *K. oxytoca* isolates contained CTX-M-3 (2/3, 66.7%) and one contained CTX-M-22 (1/3, 33.3%; Table 2).

Only TEM-1 and SHV-1 were identified in the TEM- and SHV-producing isolates. Twenty-nine *E. coli* isolates contained TEM-1 (29/46, 63.0%), and all *K. oxytoca* isolates contained TEM-1 (3/7, 100%). Among *K. pneumoniae*, six isolates (6/7, 85.7%) had both TEM-1 and SHV-1, and one isolate had only SHV-1 (1/7, 14.3%; Table 2).
Figure 1. Dendrogram of PFGE patterns showing the genetic relatedness of ESBL-producing Enterobacteriaceae isolates. (A) *Escherichia coli*, (B) *Klebsiella pneumoniae*, and (C) *Klebsiella oxytoca*. ESBL = extended-spectrum β-lactamase; PFGE = pulsed-field gel electrophore.
**E. coli phylogenetic groups and ST131 clone**

Phylogenetic analysis of the 46 *E. coli* isolates revealed four main phylogenetic groups (A, B1, B2, and D). Table 2 shows 21 of the ESBL-producing *E. coli* isolates belonged to group D (45.6%). Phylogenetic group A was the second most common, present in 28.3% of the isolates (13/46). Phylogenetic group B2 was represented by 23.9% of the isolates.

![Figure 2](image)

**Figure 2.** Profiles of plasmids harboring CTX-M group genes. (A) Plasmid sizes and numbers in parental isolates and transconjugants. (B) EcoRI restriction digestion profiles of plasmids harboring CTX-M group genes from transconjugants. N.C = *Escherichia coli* C600 is used as negative control; P = parental isolates; P.C = Salmonella OU7526 contain two plasmids with sizes of 50 and 90 kb, respectively, and is used as plasmid size standard control; TC = transconjugants; M = Marker, λ-HindIII.
(11/46), and only one isolate (2.2%) belonged to group B1 (Table 2).

The current pandemic spread of ESBL-producing *E. coli* has been greatly facilitated by high-risk clones, mainly the clonal group O25b-ST131 producing the CTX-M-15 enzyme.16,17 All *E. coli* isolates were subjected to PCR screening for the specific *mdhA* and *gyrB* genotype of ST131, and further confirmed by multilocus sequence typing. The results indicated four *E. coli* isolates belonged to the ST131 clone (4/46, 8.7%). Among them, two isolates contained CTX-M-14 (isolates 14 and 35), one belonged to the ST131 clone (4/46, 8.7%). Among them, although all successful transconjugants showed resistance to cefotaxime compared to the transconjugants harboring CTX-M-15 or CTX-M-55 showed higher level resistance to cefotaxime.

PFGE

PFGE was performed on all confirmed ESBL-producing isolates. Using a > 80% similarity cutoff point, PFGE analysis showed genetic heterogeneity in the CTX-M-producing isolates (Figure 1). Only four pairs of *E. coli* isolates (61 and 66; 19 and 29; 76 and 81; and 2 and 12) and three *K. pneumonia* isolates (44, 47, and 59) showed genetic relatedness (Figure 1).

Resistance transfer and plasmid analysis

*E. coli* isolates with CTX-M-14, CTX-M-15, and CTX-M-55 were further analyzed with conjugation tests to determine whether there was a horizontal plasmid spread in Mongolia. Transfer of ESBL enzyme by conjugation to *E. coli* C600 was successful for 13 (33.3%; including 2 CTX-M-15, 6 CTX-M-14, 1 CTX-M-14/15, and 4 CTX-M-55 producing isolates) of the 39 selected ESBL isolates (Figure 2). Transconjugants harboring CTX-M-15 or CTX-M-55 showed higher level resistance to cefotaxime compared to the transconjugants harboring CTX-M-14 (Table 3). Moreover, although all successful transconjugants showed resistance to cefotaxime, all were still sensitive to fluoroquinolones (Table 3). Plasmid sizes present in parental isolates and transconjugants were verified by Kado and Liu’s29 methods, and the results revealed that all transconjugants contained single plasmid with sizes ranging between 50 kb and 90 kb (Figure 2A). Four CTX-M-14 transconjugants (22', 56', 103', and 104') contained similar plasmid digestion patterns, and this plasmid pattern was also found in a transconjugant of isolate 69 harboring CTX-M-14/15 (Figure 2B). In addition, CTX-M-14 containing a similar plasmid digestion pattern was found in the transconjugants of isolates 6 and 102. All successful transconjugants with CTX-M-55 (from isolates, 3, 20, 37, and 43) also showed similar plasmid digestion patterns (Figure 2B). Two CTX-M-15 transconjugants were shown to be genetically unrelated by plasmid digestion patterns.

### Discussion

In this study, we present the characteristics of 56 ESBL-producing *E. coli* and *Klebsiella* spp. isolates from Mongolia. The EPE isolates showed high resistance to fluoroquinolones, but not to carbapenems and the aminoglycoside. CTX-M-15 was the dominant genotype in ESBL-producing *E. coli* isolates, and CTX-M-14 and CTX-M-3 were the major genotypes among ESBL-producing *Klebsiella* spp. isolates. In addition, plasmids containing the CTX-M-14 or CTX-M-55 enzymes appear to be disseminated in this area.

The CTX-M-15 genotype is reported to be the most prevalent in all continents.31,32 The rapid emergence of CTX-M-15-producing *E. coli* worldwide is attributed to the dissemination of mobile genetic elements, as well as the spread of specific clones, predominantly the international clone ST131.16,17,33 In this study, the dominant ESBL genotype was CTX-M-15 (19/46, 41.3%) in ESBL-producing *E. coli* isolates (Table 2). The second most common genotype is

| Isolate | CTX-M | Parental isolates | Antibiotics (µg/mL) | Transconjugants<sup>*a*<br>CTX | CAZ | CIP | LVX | CTX | CAZ | CIP | LVX |
|---------|-------|-------------------|---------------------|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|
| 23      | 15    | >256              | 128                 | 32              | 64  | 128 | 32  | 0.03 | 0.06 |
| 90      | 15    | >256              | 32                  | 16              | 32  | 256 | 64  | 0.03 | 0.06 |
| 69      | 14/15 | 128               | 32                  | 32              | 32  | 32  | 2   | 0.03 | 0.06 |
| 6       | 14    | 32                | 2                   | 16              | 16  | 32  | 4   | 0.03 | 0.06 |
| 22      | 14    | 16                | 2                   | 16              | 8   | 16  | 2   | 0.03 | 0.06 |
| 56      | 14    | 16                | 2                   | 32              | 8   | 16  | 1   | 0.03 | 0.06 |
| 102     | 14    | 128               | 2                   | 8               | 16  | 32  | 4   | 0.03 | 0.06 |
| 103     | 14    | 64                | 2                   | >32             | 16  | 8   | 1   | 0.03 | 0.06 |
| 104     | 14    | 32                | 2                   | >32             | 6   | 8   | 1   | 0.06 | 0.12 |
| 3       | 55    | >256              | 64                  | 0.25            | 0.5 | 128 | 16  | 0.03 | 0.06 |
| 20      | 55    | >256              | 128                 | >32             | 32  | 128 | 32  | 0.03 | 0.06 |
| 37      | 55    | 256               | 32                  | 32              | 16  | 64  | 32  | 0.03 | 0.06 |
| 43      | 55    | >256              | 16                  | 8               | 4   | 64  | 16  | 0.03 | 0.06 |

<sup>*a*</sup> MICs to CTX, CAZ, CIP, and LVX of *E. coli* C600 recipient strain were <0.03 µg/mL, 0.12 µg/mL, <0.03 µg/mL, and 0.06 µg/mL, respectively.

CTX = cefotaxime; CAZ = ceftazidime; CIP = ciprofloxacin; LVX = levofloxacin; MIC = minimum inhibitory concentration.
ESBL-producing Enterobacteriaceae in Mongolia

The prevalence of CTX-M genotypes was similar to that reported in a previous study in Korea, located close to Mongolia. However, the clonal spread of the ST131 epidemic E. coli is not linked to ESBL-producing E. coli in Mongolia. In addition, the results of PFGE showed that the majority of strains were genetically unrelated in Mongolia (Figure 1).

Previous studies showed that the phylogenetic group B2 was the most common in many countries, and group A and group B1 were usually isolated as commensals. In Mongolia, the prevalence of group D (21/46, 45.6%) and group A (13/46, 28.3%) was dramatically higher than in other countries. Whether the characteristics and virulence of phylogenetic groups D and A of E. coli in Mongolia are different from those in other countries is worth evaluating.

The high similarity of plasmids encoding CTX-M-14 or CTX-M-55 isolated from transconjugants strongly suggests that plasmid transmission among clonally unrelated strains of CTX-M-14 and CTX-M-55-producing E. coli might be taking place in Mongolia, whereas the diversity among plasmids encoding CTX-M-15 would rule out a common source of these plasmids (Figure 2B). Importantly, similar RFLP plasmid profiles were observed in the transconjugants of isolate 90 (CTX-M-15 producer) and CTX-M-55 producers (Figure 2B). CTX-M-55 is most closely related to CTX-M-15 with only a single amino acid substitution, Ala-77-Val, raising the possibility that the conversion is between CTX-M-14 and CTX-M-55 on the transferable plasmid. As a result, the characterization and dissemination of similar plasmid containing CTX-M-55 or CTX-M-15 in Mongolia is worth investigating.

Previous studies indicate that E. coli uses different mechanisms to provide resistance to fluoroquinolones; however, mutations in the chromosomal genes (gyrA, gyrB, parC, and parE) for topoisomerases are generally required for higher-level fluoroquinolone resistance. In this study, we found a remarkably high prevalence of fluoroquinolone-resistant ESBL-producing E. coli (39/46, 84.8%; Table 2). The conjugation tests showed that all transconjugants were resistant to cefotaxime but sensitive to fluoroquinolones (Table 3). Moreover, isolates 3, 20, 37, and 43 harbored homogenous CTX-M-55 containing plasmids with different susceptibilities to fluoroquinolones (Figure 2). Taken together, these results raised the possibility that the fluoroquinolone-resistant determinants did not coexist in the CTX-M producing plasmid. However, the mechanisms leading to fluoroquinolone resistance in ESBL-producing E. coli in Mongolia is still unclear and thus worth studying.

In summary, CTX-M-15 is the most frequent ESBL produced by E. coli isolated from hospitalized patients in Mongolia, followed by CTX-M-14 and CTX-M-55. Plasmids encoding the CTX-M-14 or CTX-M-55 enzymes appear to be very closely related, suggesting that they could share a common origin.

Conflicts of interest
All authors declare no conflict of interest.

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