Isolation, Identification, and Antimicrobial Susceptibility Pattern of Shiga toxin-producing *Escherichia Coli* O157:H7 from Food of Bovine Origin in Mekelle, Tigray, Ethiopia

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Research Article

Keywords: Antimicrobial, Bacteriological, Bovine, E. coli O157:H7, Food, Mekelle, Molecular

Posted Date: July 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-692904/v1>

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Abstract

Background: *Escherichia coli* O157:H7 is an emerging and major zoonotic foodborne pathogen with an increasing concern for the emergence and spread of antimicrobial-resistant strains which may results in sporadic cases to serious outbreaks in the whole world. Cattle have been identified as a major reservoir of the pathogen. This study aimed to isolate and characterize Shiga toxin-producing *E. coli* O157:H7 from raw milk, yogurt, and meat of bovine origin and determine their antimicrobial susceptibility pattern.

Methods: A cross-sectional study was conducted from December 2014-June 2015 and a total of 284 milk and meat were collected from different sources in Mekelle. The collected samples were analyzed for the presence of *E. coli* and Shiga toxin-producing *E. coli* O157:H7 and determination of their antimicrobial susceptibility pattern following the standard bacteriological and molecular techniques and procedures, and antimicrobial sensitivity test.

Results: Out of the total 284 samples, 70(24.64%) were positive bacteriologically to *E. coli* and 14.29% were found to be Shiga toxin-producing *E. coli* O157:H7. All (100%) *E. coli* isolates carried the *pal* and 41.67% *eae* gene (EHEC). Of these EHEC isolates 40% and 60% were positive for *stx1* and *stx2*, respectively. *E. coli* isolates were showed the highest level of sensitivity for Gentamycin (91.7%) but the highest level of resistance to Amoxicillin (95.8%). Of the tested isolates, 18(75%) of *E. coli* showed multiple antimicrobial resistance.

Conclusions: The current study revealed the occurrence of Shiga toxin-producing *E. coli* O157:H7 in foods of bovine origin in the study area. So, there is a chance of acquiring infection via the consumption of raw or undercooked food of bovine origin. Thus, awareness creation should be made on foodborne disease caused by Shiga toxin-producing *E. coli* O157:H7 with due consideration on the safe handling and consumption of food of animal origin.

Background

In Ethiopia, both food shortage and lack of appropriate food safety assurance systems are problems that have become obstacles to the country's economic development and public health safety [1, 2]. Animal products are generally regarded as high-risk commodities in respect of pathogen contents, natural toxins, and other possible contaminants and adulterants [3]. Foodborne microorganisms are major pathogens affecting food safety and cause human illness worldwide as a result of the consumption of foodstuff, mainly animal products contaminated with vegetative pathogens or their toxins. Most of these microbes have zoonotic importance resulting in a significant impact on both public health and economic sectors [4]. Moreover, the emergence of multidrug-resistant pathogens presents a global challenge for treating and preventing disease spread through zoonotic transmission [5].

Data regarding foodborne diseases are extremely scarce at a national level and a few studies conducted in different parts of the country showed the poor sanitary conditions of catering establishments and the

presence of pathogenic organisms like *Campylobacter*, *Salmonella*, *Staphylococcus aureus*, *Bacillus cereus*, and *Escherichia coli* [6, 7, 8, 9, 10].

E. coli found in humans can be categorized on basis of genetic and clinical criteria into three main groups: commensal, pathogenic (enteric or diarrheagenic), and extraintestinal pathogenic *E. coli* (ExPEC) [11]. The typical diarrheagenic strains include: enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC) and enteroaggregative (EAEC) *E. coli* [12, 13]. The *E. coli* that cause enteric disease have been divided into pathotypes, based on their virulence factors and mechanisms by which they cause disease [11, 14]. One of these pathotypes, called Shiga toxin-producing *E. coli* (STEC), refers to those strains of *E. coli* that produce at least 1 member of a class of potent cytotoxins called Shiga toxin. The STEC are also called verotoxin-producing *E. coli* (VTEC). The names Shiga toxin (Stx), derived from similarity to a cytotoxin produced by *Shigella dysenteriae* serotype 1 [15], and verotoxin (VT), based on cytotoxicity for Vero cells [16] are used interchangeably. Those STEC that cause hemorrhagic colitis and hemolytic uremic syndrome are called enterohemorrhagic *E. coli* (EHEC) [14, 17]. The Shiga toxin-producing *E. coli* O157 is synonymous with *E. coli* O157:H7 [18, 19]. Pathogenicity of *E. coli* O157:H7 is encoded by a variety of plasmid, bacteriophage, and chromosomal genes [20]. The key virulence factor for the subset of EHEC is the Shiga toxin (*Stx*) family which contains two subgroups: *Stx1* and *Stx2* that share approximately 55% amino acid homology [11]. The ability to produce Shiga toxin was acquired from a bacteriophage presumably directly or indirectly from *Shigella* [20].

STEC is recognized as an important cause of food-borne disease in humans and causes large outbreaks worldwide [21]. *E. coli* O157:H7 is the leading cause of hemorrhagic colitis, hemolytic-uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) in man. These illnesses may lead to death due to improper absorption of nutrients and destruction of certain tissues in the target organs [22, 23]. People of all ages are susceptible to infection with STEC. However, the young and the elderly are more susceptible and are more likely to develop more serious symptoms [24]. Domestic and wild animals are sources of EHEC O157:H7 but the major animal carriers are healthy domesticated ruminants, primarily cattle and to a lesser extent, sheep, and possibly goats. Fresh meat and raw milk are, nevertheless, considered as common vehicles for *E. coli*, particularly for the EHEC (O157:H7) strain. Contamination of meat usually occurs during animal slaughter, as a result of poor slaughter practices, abattoir hygiene, and animal handling practices [20, 25].

Foods of bovine origin are frequently implicated in human outbreaks of Shiga toxin-producing *Escherichia coli* (STEC) O157 [26, 27]. Shiga-toxigenic *E. coli* is transmitted by the fecal-oral route by either consumption of contaminated food or water, from direct contact with infected animals, or via person-to-person contact. Moreover, series of studies on the resistance of *E. coli* which were isolated from animals and humans have strongly suggested that those bacteria which are resistant to antimicrobials used in animals would also be resistant to antimicrobials used in humans [28, 29, 30]. In general, antimicrobial resistance is a global public health problem, and growing scientific evidence indicates that it is negatively impacted by both human and animal antimicrobial usages [31]. Although antimicrobial

therapy is not the primary tool for treating infections caused by STEC O157:H7, multidrug-resistant (MDR) STEC O157:H7 is a public health issue as those strains participate in a reservoir of resistance genes that could be easily exchanged between Enterobacteriaceae in the host and the environment [32]. But little is currently known about the molecular basis of multidrug resistance in STEC O157:H7 isolates of food origin [33]. So, it is of paramount importance to systematically investigate and characterized this recurring food-borne disease. Thus, the current study was conducted to isolate and characterize Shiga toxin-producing *E. coli* O157:H7 from raw milk, yogurt, and meat of bovine origin from different sources in the study area and to determine the antimicrobial susceptibility pattern of the isolates.

Materials And Methods

Study area

The study was conducted from December 2014 to June 2015 in Mekelle city. Mekelle is the capital city of Tigray Regional State, situated about 783 km North of Addis Ababa at 38.5° East longitude and 13.5° North latitude at an altitude of 2300 above sea level. The city has a total population of 215,546 [34], 308 cafeterias, 292 restaurants, 258 supermarkets, and an active urban-rural exchange of goods which has 30000 micro and small enterprises [35]. The weather condition is hot and dry. The mean annual rainfall of the area is 628.8 mm and an annual average temperature of 21°C [36].

Study design and study population

A cross-sectional study on Shiga toxin-producing *E. coli* O157:H7 was conducted from December 2014 to June 2015 on raw milk, yogurt, and meat samples collected from different sources and parts of Mekelle City, Tigray, Ethiopia. The study populations comprised of purposively selected milking dairy cows and slaughtered cattle found in Mekelle City.

Sampling technique and sample collection

A total of 284 samples of bovine origin, comprised of raw milk (n=145), yogurt (n=48), and meat (n=91), were collected using a purposive random sampling technique. These samples were collected based on the willingness of the owners until the required sample size was achieved. Raw milk samples were aseptically collected directly from the teats of lactating cows (n=100), whole-sellers (n=17), cafeterias (n=28), and similarly the yogurt samples were collected from dairy farms (n=26) and cafeterias (n=22) using a sterile universal bottle. However, the raw meat samples were collected from abattoirs (n=55), butchery shops (n=16), and restaurants (n=20) during slaughtering and selling. Then, the sections of meat samples were aseptically removed and placed in separate sterile plastic bags to prevent spilling and cross-contamination. Both samples were transported to the Veterinary Microbiology Laboratory of College of Veterinary Sciences, Mekelle University using an icebox and stored at +4°C until the laboratory work was conducted.

Isolation and identification of *E. coli* and Shiga toxin-producing *E. coli* O157:H7.

Microbiological procedures

Isolation of *E. coli* was attempted according to [37] with slight modification. A part of each sample (10 ml or 10 g) was enriched in peptone water (HiMedia, India) (90 ml) and was incubated at 37°C for 24 h. Enriched samples were inoculated on MacConkey Agar (MCA) (HiMedia, India) by four flame technique, and plates were incubated at 37°C for 24 h. Pink-colored colonies were considered presumptive of *E. coli*. Gram staining was performed as per procedures described by [38] to determine the size, shape, and arrangement of bacteria. The organisms revealed gram-negative, pink-colored with rod-shaped appearance, and arranged in single or in pair were suspected as *E. coli*.

A single well-isolated colony was picked from MCA and streaked on Eosin Methylene Blue Agar (EMB) (HiMedia, India) and incubated at 37°C for 24 h. The characteristic green metallic sheen growth of colonies is a presumptive identification for *E. coli*. Colony morphology and colors on MCA and EMB agar plates together with the Gram stain procedure were used as an initial identification of *E. coli* colonies [39]. Such colonies were taken from EMB into nutrient broth and agar for further biochemical examination. Standard biochemical tests (Catalase test, Indole, Methyl red, Voges-Proskauer test, Nitrate reduction, Citrate utilization, and Urease production) were used as confirmation of identification [40, 41, 42, 43, 44, 45, 46]. Triple Sugar Iron test was performed according to [47]. Carbohydrate fermentation tests of the isolates were performed according to the method of [43]. Colonies, that were confirmed as *E. coli*, were further be subcultured onto MacConkey Agar with Sorbitol. A bacterial strain that was used as quality control organisms in this study was a standard strain of *E. coli* ATCC 25922.

PCR amplification of the *pal*, *eaeA* *stx1*, and *sxt2* virulence genes of *E. coli*

E. coli genomic DNA extraction and purification were performed as per the protocol given by PureLink® Genomic DNA Purification Kit, USA, for Gram-negative organisms, and the total genomic DNA was checked by running on 1.0% agarose gel.

All the presumptive isolates' DNA were subjected to multiplex PCR for analyzing for the presence of the *pal* [48], *eae* [49], *stx1*, and *stx2* [50] genes, and further modified by [51]. The amplification was done according to the protocol reported by [52] and [53] using the following specific primers: ECPAL-F-5'GGC AAT TGC GGC ATG TTC TTC C3' and ECPAL-R-5'CCG CGT GAC CTT CTA CGG TGA3' for *E. coli* (280 bp); EHEC-F-5'TGG TAC GGG TAA TGA AAA3' and EHEC-R-5'AAT AGC CTG GTA GTC TTG T3' for *eae* gene of EHEC (360 bp); *Stx1*-F-5'ATA AAT CGC CAT TCG TTG ACT AC3' and *Stx1*-R-5'AGA ACG CCC ACT GAG ATC ATC3' for *stx1* (180 bp); and *Stx2*-F-5'GGC ACT GTC TGA AAC TGC TCC3' and *Stx2*-R-5'TCG CCA GTT

ATC TGA CAT TCT G3' for *stx2* (255 bp). Each reaction mixture (50µl) consisted of 5µl of 10x reaction buffer (500mM KCl, 15mM MgCl₂, 100mM Tris HCl pH 8.3, 0.1%w/v gelatin), 5µl of template DNA, 1µl of each primer (the primers were used at a final concentration of 100 M), 3µl of 10mM dNTP mixture (at a concentration of 100µl each), and 1µl of Taq polymerase (3U/l). The remaining volume of the reaction mixture was nuclease-free water. The amplification was carried using a Tianlong PCR Thermocycler with thermal cycling conditions of an initial denaturation at 94°C for 6 min, followed by 35 cycles of denaturation at 94°C for 45s, annealing at 55°C for 30s, extension at 72°C for 45s, and with a final extension at 72°C for 6 min. Finally, PCR products were separated in a horizontal equipment system by running on a 1.5% (w/v) agarose gel containing 0.5g/ml ethidium bromide for 55 min at 110 V using 1XTAE buffer (40 mM Tris, 1 mM EDTA, and 20 mM glacial acetic acid, pH 8.0). The amplicons were visualized under UV-light gel doc and their molecular weight was estimated by comparing them with 100 bp DNA molecular weight marker (Solis BioDyne, Tartu, Estonia) [54].

Antibiotic sensitivity testing

The isolates of *E. coli* were screened for *in vitro* antimicrobial susceptibility using the agar disk diffusion method described by [55]. For this, the following seven different antibiotic discs (Oxoid Ltd., Basingstoke, Hampshire, England) with their concentrations given in parentheses were used in the antibiograms: Erythromycin (E) (15µg), Gentamicin (CN) (10µg), Amoxicillin (Amx) (2µg), Doxycycline (Doxa) (30µg), Tetracycline (TE) (30µg), Penicillin (P) (10µg), and Sulfamethoxazole (SXT) (20µg). Four to five well-isolated colonies from nutrient agar plates were transferred into tubes containing 5 ml of a normal saline solution until it achieved the 0.5 McFarland turbidity standards, and then a sterile cotton swab was dipped into the adjusted suspension and then spread evenly over the entire surface of the plate of Mueller-Hinton agar (Oxoid Ltd., Basingstoke, Hampshire, England) to obtain uniform inoculums. The plates were then allowed to dry for 3 to 5 min. Antibiotic impregnated discs were then applied to the surface of the inoculated plates with sterile forceps. After 18 to 24 h of incubation at 37°C, the plates were examined and the clear zone (inhibition zones of bacterial growth around the antibiotic disc including the disc) diameter for individual antimicrobial agents was measured using a digital caliper and then translated into Sensitive (S), Intermediate (I), and Resistant (R) categories according to the interpretation table of the Clinical and Laboratory Standard Institute [56].

Data management and analysis

All collected data were entered into Microsoft Excel Sheet and analyzed through the SPSS Version 16. Accordingly, descriptive statistics such as percentages and frequency distribution were used to determine the prevalence in the food items, and Chi-square (χ^2) test was applied to assess the association.

Results

The prevalence of *E. coli* and Shiga toxin-producing *E. coli* O157:H7

Out of the total 284 samples collected from the different sample sources, 70 (24.64%) were found to be positive for *E. coli*. The sample-based prevalences of meat, raw milk, and yogurt samples were 41 (45.05%), 18 (12.41%), and 11 (22.91%), respectively. There was a significant difference ($p < 0.05$) among the different sample types in the prevalence of *E. coli* (Table 1). Of the total isolated *E. coli* isolates, 14.29% were found to be Shiga toxin-producing *E. coli* O157:H7.

Table 1
Prevalence of *E. coli* among the different sample types

Sample types	No. of examined	No. (%) of positive samples	χ^2	<i>P</i> -value
Meat	91	41 (45.05%)	32.1	0.000
Raw milk	145	18 (12.41%)		
Yogurt	48	11 (22.91%)		
Total	284	70 (24.64%)		

Prevalence rates of 56.25%, 40%, and 50% were recorded in meat samples collected from butchery, abattoir, and restaurant, respectively. The prevalence of *E. coli* for raw milk samples collected from farms, whole-sellers, and cafeterias were 10%, 17.64%, and 17.85%, respectively. Whereas yogurt samples collected from farms and cafeterias were found to be 23.07% and 22.72%, respectively. The chi-square analysis result revealed that all sample collection sources showed a significant difference ($p < 0.05$) in *E. coli* positivity (Table 2).

Table 2
Prevalence of *E. coli* among the different sample types and sample sources

Sample type	Site of collection	Total No. of samples examined	Total No. of samples positive	χ^2	<i>P</i> -value
Meat	Abattoir	55	22 (40%)	35.2	0.000
	Butchery	16	9 (56.25%)		
	Restaurant	20	10 (50%)		
	Total	91	41 (45.05%)		
Raw milk	Farm	100	10(10%)	35.2	0.000
	Whole seller	17	3(17.64%)		
	Cafeteria	28	5(17.85%)		
	Total	145	18 (12.41%)		
Yogurt	Farm	26	6(23.07%)	35.2	0.000
	Cafeteria	22	5 (22.72%)		
	Total	48	11 (22.91%)		

PCR amplification results of the virulence genes of *E. coli*

The PCR result indicated that all (100%) of the tested isolates of *E. coli* carried the *pal* gene and 41.67% *eae* gene (EHEC). Among the *eae* gene carried *E. coli* isolates 40% were carried *stx1* gene, and 60% of them were carried *stx2* gene.

Antimicrobial susceptibility profile of *E. coli* isolates

The *E. coli* isolates isolated from meat, raw milk, and yogurt were showed the highest level of sensitivity for Gentamycin (91.7%). However, the highest level of resistance was observed against Amoxicillin (95.8%) (Table 3). Multidrug resistance was detected in 75% of the isolates.

Table 3
In vitro antimicrobial drug sensitivity results of *E. coli* isolates

Antimicrobial agents	Result		
	Sensitive	Intermediate	Resistant
Gentamycin	22(91.7%)	0%	2(8.3%)
Penicillin	1(4.2%)	1(4.2%)	22(91.7)
Amoxicillin	1(4.2%)	0%	23(95.8%)
Doxycycline	10(41.7%)	2(8.3%)	12(50%)
Tetracycline	14(58.3)	0%	10(41.7%)
Sulfamethoxazole	19(79.2%)	0%	5(20.8%)
Erythromycin	7(29.2)	4(16.7%)	13(54.2%)

Discussion

The current study revealed an overall prevalence rate of 24.64% for *E. coli*. This finding was in line with the reports of [57] (20%), [58] (20.2%), [59] (21.6%), [60] (23.4%), [61] (23.7%), [10] (27.3%), and [62] (27.3%). It was higher than the reports of [63] (8.15%), [64] (9.23%), [65] (12.38%), [66] (15.89%), and [67] (19%). However, it was lower than the findings of [68] (33.9%), [69] (38%), [70] (46.26%), [71] (61.7 %), and [72] (63%). The present study also revealed an overall prevalence of 14.29% for Shiga toxin-producing *E. coli* O157:H7 (EHEC). This finding was almost in agreement with reports [73] (10.2%), [61] (10.4%), [74] (10.4%), [75] (10.9%), [76] (11.43%), [77] (17.47%), and [71] (18%). But, it was higher than the findings of [78] (0.54%), [64] (1.92%), [79] (2.1%), [65] (2.4%), [80] (2.4%), [68] (2.9%), [81] (3.38%), [57] (3.5%), [82] (4.2%), [67] (5.2%), [58] (5.4%), [83] (6.3%), [84] (8.8%), [85] (9%), [60] (9.1%), and [86] (9.3%). However, it was lower than the finding of [87] (25%), [88] (36.1%), [89] (41.66%), and [90] (69%). The variation might be due to differences in sample types of food of bovine origin, sources of the samples, methodological approaches, diagnostic techniques, prevalence calculation/interpretation, geographical locations, hygienic condition, handling and transportation of samples, and contamination rates from utensils and personnel. Generally in the present findings prevalence of *E. coli* was higher in the samples collected from butchery, restaurant, cafeteria, and whole seller other than the other sources. This might be due to food establishments in the study area were found to have poor sanitation and were not maintained well, specifically, poor repair condition of kitchens, improperly managed toilet facilities, inappropriate solid waste receptacles, lack of standard dishwashing compartments, and lack of handwashing lavatories in a large number of establishments [8].

The antimicrobial susceptibility test results of *E. coli* isolates isolated from meat, raw milk, and yogurt were showed the highest level of sensitivity for Gentamycin (91.7%). It was almost in agreement with the

reports of [67] (100%), [57] (82.5%), and [91] (81.82%). The 79.2% isolates were susceptible to Sulfamethoxazole which was in agreement with the reports of [91] (78.79%). However, the highest level of resistance was observed against Amoxicillin (95.8%) which was in line with the reports of [60] (100%), and [78] (100%). In addition, 41.7% of the isolates were resistant to Tetracycline which was in agreement with the reports of [59] (47.6%) but was higher than the findings of [92] (4%), [73] (5.1%), [77] (23.08%), [60] (27.7%), and [93] (32%). However, it was lower than the findings of [81] (80.6%), [68] (81.8%), [67] (63.63%), and [91] (60.61%). The present study was also revealed that 20.8% of the isolates were showed resistance against Sulfamethoxazole which lower than the findings of [93] (36%). In general, in this study, 75% of the isolates were developed multidrug resistance which was higher than the reports of [59] (46.0%) and [60] (66.3%) but was lower than the report of [74] (93.2%). Antimicrobial resistance may arise either spontaneously by selective pressure or due to antimicrobial misuse by humans or overuse in feeding or treatment of animals by farmers [94].

Conclusion And Recommendations

The present study revealed a relatively high occurrence of *E. coli* as well as Shiga toxin-producing *E. coli* O157:H7 in food of bovine origin in Mekelle City and isolates developed a high level of multi-drug resistance to the antimicrobials tested. Hence, foods of bovine origin can serve as a potential vehicle for transmitting *E. coli*, in particular, Shiga toxin-producing *E. coli* O157:H7, and its presence indicates a serious public health hazard and give a warning signal for the possible occurrence of a foodborne outbreak in humans through the consumption of raw or undercooked food items. As a result, both its presence and development of a multi-drug resistance should receive particular attention and is an alert for the concerned bodies. Therefore, a coordinated effort is needed to reduce or eliminate the risk posed by these pathogens at various stages in the food chains and on the appropriate use of antimicrobials both in veterinary and human treatment regimes. Moreover, awareness creation should be made on foodborne disease caused by Shiga toxin-producing *E. coli* O157:H7 with due consideration on the safe handling and consumption of food of animal origin and selection and safe use of antimicrobials.

Abbreviations

DNA-Deoxyribonucleic acid; *E. coli* O157-*Escherichia coli* O157; *E. coli*-*Escherichia coli*; EAEC-Enterocaggregative *E. coli*; EHEC-Enterohemorrhagic *E. coli*; EIEC-Enteroinvasive *E. coli*; EPEC - Enteropathogenic *E. coli*; ETEC-Enterotoxigenic *E. coli*; ExPEC-Extraintestinal pathogenic *E. coli*; HUS-Hemolytic-uremic syndrome; MDR-Multidrug-resistant; PCR-Polymerase Chain Reaction; STEC-Shiga toxin-producing *E. coli*; Stx-Shiga toxin; TTP-Thrombotic thrombocytopenic purpura; VT-Verotoxin; VTEC-Verotoxin-producing *E. coli*

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research work was funded by College of Veterinary Sciences, Mekelle University.

Authors' contributions

MW, GG, YT, and NA participated in conception and proposal development, sample collection, laboratory analysis, supervision, validation, data analysis, and write-up of the original draft and reviewing and editing of the final version of the manuscript. AK, MA, NA, HT, and AB were involved in the conception and proposal development, data analysis, validation, and write-up of the original draft and reviewing and editing of the final version of the manuscript. All authors contributed to the final version of the manuscript and approved the submission.

Acknowledgments

The authors would like to acknowledge the College of Veterinary Sciences, Mekelle University for financial support. We would like to extend our acknowledgment to owners, managers, and workers of the different dairy farms, abattoir, butcher shop, restaurant, milk whole seller, and cafeteria of the study site for their keen interest and cooperation during the collection of the samples, and to the College of Veterinary Sciences Staff members who directly or indirectly helped us during the research period.

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