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Article

Association of Colony Morphotype Diversity, Curli Production, and Antibiotic Resistance in Escherichia coli Isolated from clinical sample

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Abstract: This study aimed to examine the correlation of heterogeneity of biofilm with curli production and antibiotic resistance in clinical isolates of Escherichia coli.. 100 clinical isolates collected from urine, wound, and blood samples. The biofilm formation assay results showed 72 isolates (72%) produced biofilms to some degree. Of these, 35 isolates (35%) showed strong biofilm formation., 22 isolates (22%) with moderate biofilm formation, and 15 isolates (15%) with weak biofilm formation. The results achieved on Congo Red Agar medium had a range pattern of colonies with 44 isolates (61.1%) of biofilm-producing isolates showing a high heterogeneity pattern, while 28 isolates (38.9%) showed a low heterogeneity pattern. Curli production was more prevalent in isolates with greater heterogeneity. Of the isolates assessed, 38 of 44 (86.4%) curli-positive isolates had a high corresponding heterogeneity, as opposed to 13 of 28 (46.4%) isolates in the low heterogeneity group ($P < 0.001$). Antibiotic resistance was noted to be high among isolates with high variability, and resistance to ciprofloxacin among these isolates was noted to be at 79.5% as compared to cefotaxime at 46.4% resistance, and resistance to cefotaxime was noted to be at 84.1% among these with variability as compared to 53.6% to ciprofloxacin and to trimethoprim/sulfamethoxazole 72.7% compared to 42.9% in low heterogeneity isolates, with statistically significant differences ($P < 0.05$). Statistical analysis indicated a strong positive correlation between heterogeneity and biofilm density ($r = 0.74$) and between heterogeneity and curli production ($r = 0.69$).

Keywords: Escherichia coli; Biofilm heterogeneity; Biofilm formation; Curli production; Antibiotic resistance; Clinical isolates

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1. Introduction

E. coli is a common bacteria in humans and is part of the intestinal flora. Some strains have virulence factors and are capable of causing several infections., as urinary tract infections, bacteremia, wound infections, and gastrointestinal infections. E. coli is a most cause of community-acquired and hospital-acquired disease , and its causing resistance to antibiotics [1]. The success of E. coli in causing and sustaining infections is due to its ability to form biofilms, which are degrade as microbial communities that help in adhere to living and non-living places and are surrounded by an extracellular matrix composed of polysaccharides, proteins, and extracellular nucleic acids [2]. Because of this structure, a bacterial cells protection is against environmental conditions, immune components, antibiotics, and becomes more difficult to treat in effect causing chronic and recurrent

infections [3]. In recent decades there has been a shift in scientific interest from studying biofilms as homogenous structures to studying biofilm heterogeneity. Biofilm heterogeneity deals with the physiological, and structural differences among the cells within the same biofilm. Different levels of oxygen and conditions of stress in the environment give rise to sub-populations of cells differentiated by their growth and metabolic rates and their response to antibiotics [4]. Heterogeneity is a key characteristic that gives biofilms their ability to survive and adapt. Within a biofilm, populations of cells with high antibiotic tolerance can emerge compared to other cells, contributing to persistent infection and treatment failure [5]. Numerous studies have shown that structural and physiological heterogeneity within biofilms is associated with increased colonization capacity, pathogenicity, and the development of drug resistance. Among the important components associated with biofilm formation in E. coli are curly fimbriae, amyloid protein fibers involved in bacterial adhesion to surfaces and host cells [6]. They also contribute to intercellular cohesion and the formation of the biofilm's three-dimensional structure. Curly fimbriae are a key factor in the development of coarse, dry colonies on specialized media such as Congo Red Agar, and can be used as a phenotypic indicator to assess certain biofilm characteristics. Studies have shown that curl production is associated with increased biofilm formation and enhanced survival in various environments by E [7]. coli, as well as its role in increasing bacterial cell resistance to antimicrobial agents. Furthermore, variations in curl production levels among isolates may reflect a degree of phenotypic heterogeneity that can influence bacterial behavior and pathogenicity [8]. In addition, antibiotic resistance is one of the most serious problems associated with clinical E. coli isolates. Recent years have seen a significant increase in resistance rates to many clinically used antibiotics, including fluoroquinolones, cephalosporins, and sulfonamides. Research indicates that biofilm formation along with its heterogeneity leads to an increase in resistance to antibiotics which poses a challenge to the eradication of such infections [9].

While many studies have examined biofilm generation and antibiotic resistance in E. coli independently, there is little known about the interplay between biofilm heterogeneity, curl production and antibiotic resistance remains relatively limited, particularly in clinical isolates [10]. Therefore, this study aims to evaluate the relationship between biofilm heterogeneity, curl production, and antibiotic resistance in clinical E. coli isolates to better understand the mechanisms contributing to the successful initiation and persistence of these infections [11].

2. Materials and Methods

Study Design

This cross-sectional laboratory-based study was conducted from [date] in the laboratories of the Department of Life Sciences/College of [college/institution name]. The study aimed to evaluate the relationship between biofilm heterogeneity, curl production, and antibiotic resistance in clinical Escherichia coli isolates.

Collection of Bacterial Samples

One hundred clinical isolates of Escherichia coli were collected from various clinical sources, including:

- Urine samples.
- Wound samples.
- Blood samples.

Samples were collected from patients visiting various hospitals and health centers according to established clinical sample collection procedures.

Bacterial Isolation and Identification

Primary Isolation: Samples were cultured on the following media:MacConkey Agar And Eosin Methylene Blue Agar (EMB).The plates were incubated at 37°C for 24 hours.

Microscopic and Biochemical Identification: Isolations were identified using:Gram staining**,** Oxidase test**,** Indole test**,** Methyl red test **,**Foggs-Brøscauer test and Citrate consumption test**.**

Confirmatory Identification: The identification of the isolates was confirmed using VITEK 2 Compact system .

Biofilm Formation Test

The ability of the isolates to form biofilms was evaluated using the microtiter plate assay, according to the method of Christensen et al. with some modifications.

Procedure

- Isolates were cultured in Tryptic Soy Broth medium containing 1% glucose for 24 hours at 37°C.
- The cultures were diluted 1:100.
- 200µL was added to the wells of a 96-well microplate.
- The cultures were incubated for 24 hours at 37°C.
- The wells were washed three times with PBS.
- The cells were fixed with methanol.
- The biocrystals were stained with 0.1% Crystal Violet.
- Excess stain was removed, and the wells were washed with distilled water.
- The stain was dissolved in 95% ethanol.
- The optical absorbance was measured at 570 nm using an ELISA reader.

Based on the OD value, the isolates were classified into:

- Non-biofilm producer
- Weak biofilm producer
- Moderate biofilm producer
- Strong biofilm producer

Evaluation of Phenotypic Variation and Curl Production

Congo Red Agar (CRA) was used to study colony phenotypes and evaluate curl fiber production.The medium consisted of:Brain Heart Infusion Agar (37 g/L) ,Sucrose (50 g/L) and Congo Red (0.8 g/L).After sterilization, the components were added according to standard instructions.

Procedure

- Isolates were cultured using the surface-striating method on CRA plates.
- The plates were incubated at 28°C for 48–72 hours.
- Colonies were visually inspected, and their morphological patterns were recorded.
- Type Classification

RDAR (Red, Dry, and Rough) → Strongly Curly-Producing.

BDAR (Brown, Dry, and Rough) → Curly-Producing.

PDAR (Pink, Dry, and Rough) → Limited or Absent Curly-Producing.

SAW (Smooth and White) → Non-Curly-Producing.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby–Bauer Disk Diffusion Method according to the guidelines of the Clinical and Laboratory Standards Institute. The antibiotics was used Ciprofloxacin (5 μg) , Cefotaxime (30 μg) ,Gentamicin (10 μg) , Amikacin (30 μg) , Trimethoprim/Sulfamethoxazole (25 μg) , Nitrofurantoin (300 μg) and Imipenem (10 μg).

Statistical Analysis

Data was entered and analyzed using:IBM SPSS Statistics Version 26 Statistical tests included:Frequencies and percentages, Mean ± standard deviation, Chi-square test,Pearson correlation test and One-way ANOVA when needed. The results were considered statistically significant when:$P \leq 0.05$.

3. Results

Distribution of Clinical Isolates

The study included 100 clinical isolates of Escherichia coli collected from various clinical sources. The majority of isolates (60%) were from urine samples, followed by wound samples (25%), and then blood samples (15%) as showed in table 1 and [12].

Table 1. Distribution of Clinical *E. coli* Isolates According to Sample Source

Sample Source	No. of Isolates	Percentage (%)
Urine	60	60.0
Wound	25	25.0
Blood	15	15.0
Total	**100**	**100**

Biofilm formation capacity of isolates

Crystal Violet assay results showed that 72 isolates (72%) were capable of biofilm formation to varying degrees, while 28 isolates (28%) showed no significant biofilm formation capacity as showed in table 2 and figure1 [13].

Table 2. Classification of *E. coli* Isolates According to Biofilm Formation

Biofilm Category	No. of Isolates	Percentage (%)
Strong Biofilm Producer	35	35.0
Moderate Biofilm Producer	22	22.0
Weak Biofilm Producer	15	15.0
Non-Biofilm Producer	28	28.0
Total	**100**	**100**

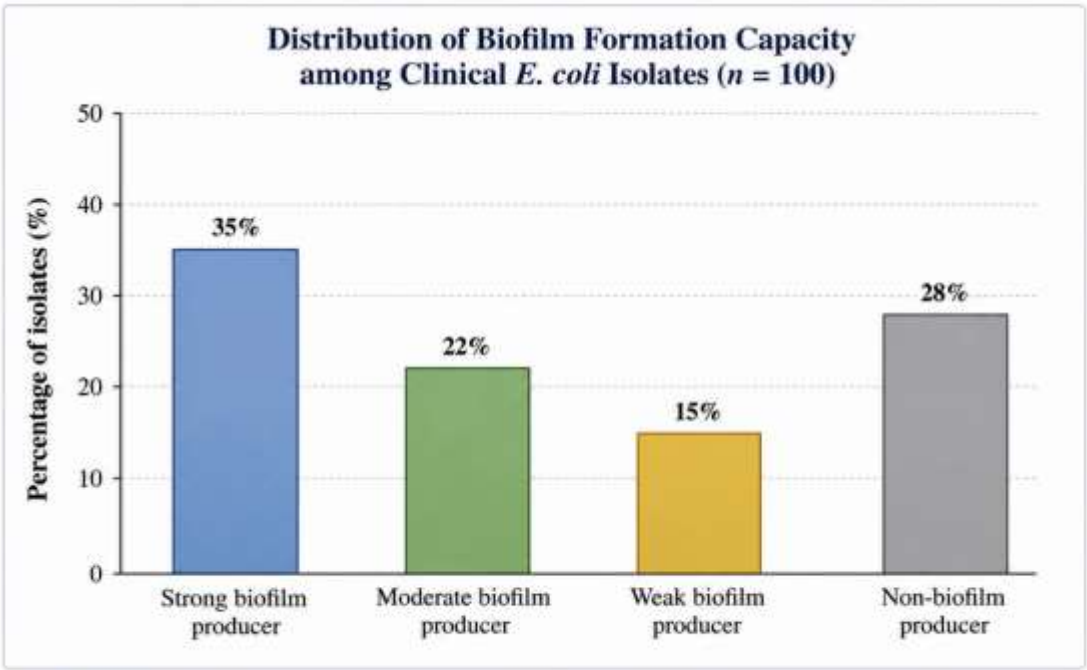

Figure 1. Distribution of biofilm formation capacity among clinical *Escherichia coli* isolates (n = 100).

Figure 1.Distribution of biofilm formation capacity among clinical Escherichia coli isolates (n = 100).

Colony morphology on Congo Red Agar

The biofilm-producing isolates (72 isolates) exhibited four distinct morphological patterns when cultured on Congo Red Agar as showed in table 3 and figure 2and 3 [14].

Table 3. Distribution of Colony Morphotypes on Congo Red Agar

Morphotype	No. of Isolates	Percentage (%)
RDAR	26	36.1
BDAR	18	25.0
PDAR	12	16.7
SAW	16	22.2
Total	**72**	**100**

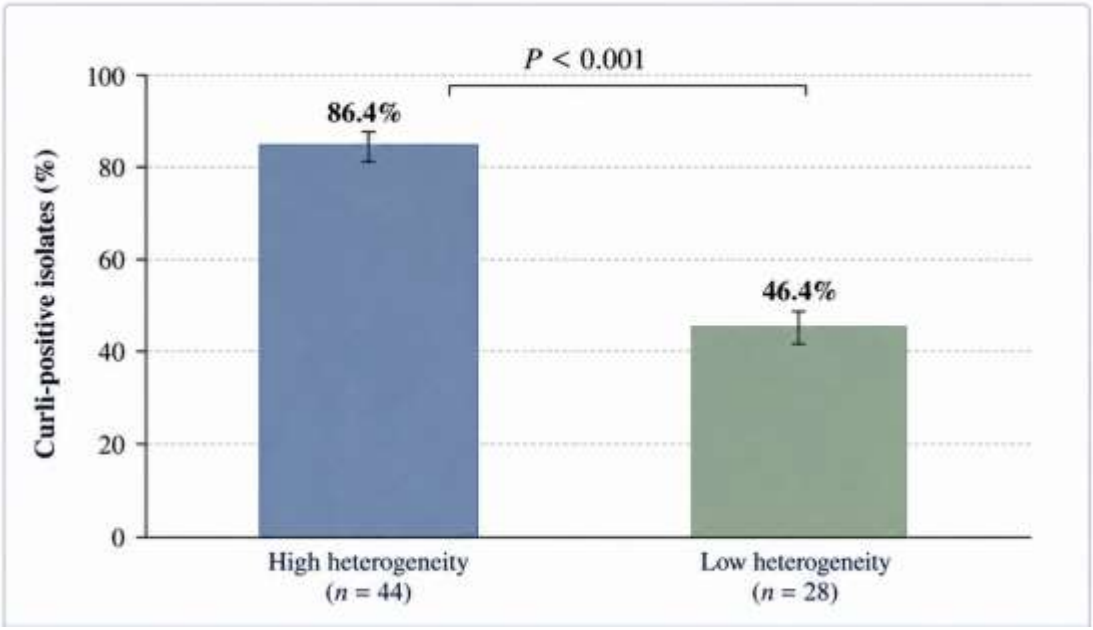

Figure 2. Comparison of Curli Production Between High and Low Phenotypic Heterogeneity Groups of Clinical *Escherichia coli* Isolates.

Figure3: *E. coli* biofilm morphotypes on Congo Red (CR) agar.

The Relationship Between Heterozygosity and Curl Production

Isolates were classified into two groups based on morphological pattern:

- High Heterozygosity (RDAR + BDAR) = 44 isolates.
- Low Heterozygosity (PDAR + SAW) = 28 isolates.

Curl production results showed that 38 out of the 44 high-heterozygosity isolates were positive for curl production (86.4%), while only 13 out of the 28 low-heterozygosity isolates were positive for curl production (46.4%) as showed in table 4 [15].

Table 4. Association Between Phenotypic Heterogeneity and Curli Production

Heterogeneity Group	Curli Positive n (%)	Curli Negative n (%)	$\chi^2 = 12.84$, $P < 0.001$
High Heterogeneity	38 (86.4)	6 (13.6)	
Low Heterogeneity	13 (46.4)	15 (53.6)	

The relationship between heterogeneity and antibiotic resistance

Antibiotic susceptibility testing results showed that isolates with high heterogeneity exhibited higher resistance rates compared to isolates with low heterogeneity as showed in table 5 [16].

Table 5. Antibiotic Resistance Rates Among High and Low Heterogeneity Isolates.

Antibiotic	High Heterogeneity (%)	Low Heterogeneity (%)	P-value
Ciprofloxacin	79.5	46.4	0.004
Cefotaxime	84.1	53.6	0.006
Trimethoprim/ Sulfamethoxazole	72.7	42.9	0.011
Gentamicin	54.5	28.6	0.032
Amikacin	20.5	10.7	0.218
Nitrofurantoin	15.9	7.1	0.191
Imipenem	4.5	0.0	0.372

Multidrug-Resistant (MDR) Isolates

The study showed that 32 isolates (72.7%) from the high heterogeneity group were multidrug-resistant (MDR), compared to only 10 isolates (35.7%) from the low heterogeneity group as showed in table 6 and [17].

Table 6. Prevalence of Multidrug Resistance (MDR) Among the Studied Isolates

Heterogeneity Group	MDR Positive n (%)	MDR Negative n (%)
High Heterogeneity	32 (72.7)	12 (27.3)
Low Heterogeneity	10 (35.7)	18 (64.3)
$\chi^2 = 9.67$, P = 0.002		

Correlation between Biofilm Density, Heterogeneity, and Curl Production

Pearson correlation analysis showed a strong positive correlation between heterogeneity and biofilm density as show in table 7 and figure 4.

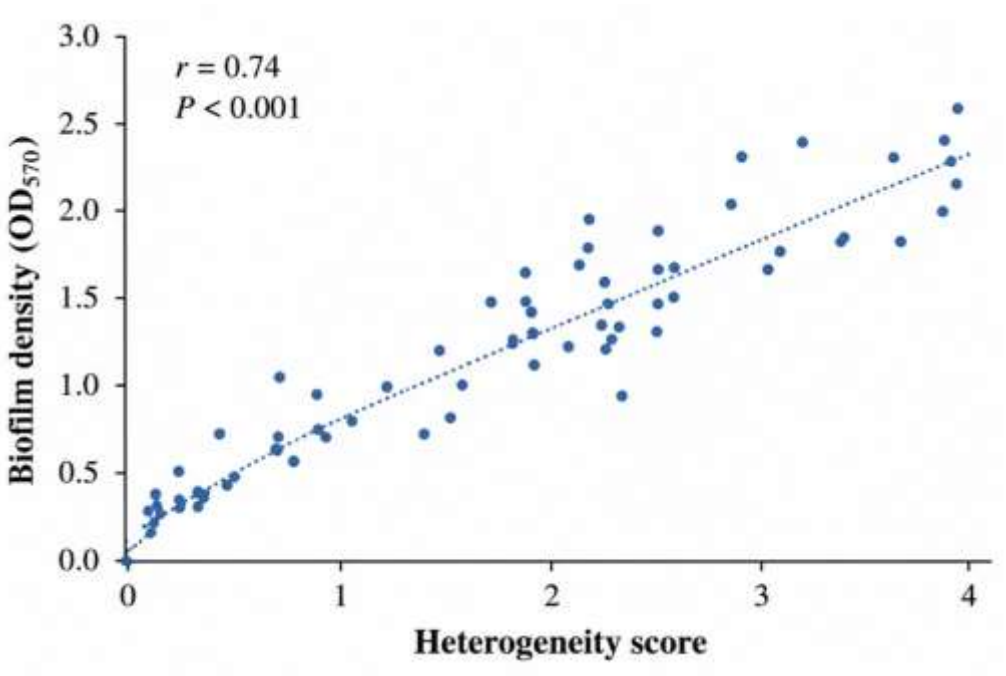

Figure 4. Positive correlation between phenotypic heterogeneity score and biofilm biomass (OD_{570}) in *Escherichia coli* isolates (r = 0.74, P < 0.001).

Table 7. Correlation Between Heterogeneity, Curli Production, and Biofilm Density

Variables	Correlation Coefficient (r)	P-value
Heterogeneity vs. Biofilm Density	0.74	<0.001
Heterogeneity vs. Curli Production	0.69	<0.001
Curli Production vs. Biofilm Density	0.72	<0.001

The study results showed that 72% of E. coli isolates were capable of biofilm formation, and that curly-producing morphological patterns (RDAR and BDAR) were the most common among highly heterogeneous isolates. High heterogeneity was also associated with increased curly production and higher rates of antibiotic resistance and multidrug-resistant isolates, with a strong positive correlation between heterogeneity and biofilm density. These findings suggest that biofilm-related morphological differences may contribute to enhanced survival of clinical isolates [18].

4. Discussion

This study aimed to investigate the relationship between biofilm-related phenotypic heterogeneity, curl fiber production, and antibiotic resistance in clinical Escherichia coli isolates. The results showed that a biofilm was formed by the isolates and clear variation in colony morphology on Congo Red Agar was identified [19]. Additionally, a strong relationship was identified between the production of curls and the resistance to antibiotics. The study results showed 72% of the isolates formed biofilms to some degree. This aligns with other research that shows significant biofilm formation capability of E. coli., particularly clinical isolates from chronic infections and urinary tract infections. This ability is due to the bacteria's many surface structures and adhesion factors that enable colonization and the establishment of organized bacterial structures [20].

The study also revealed Four distinct colony morphologies were found on Congo Red Agar, with RDAR and BDAR being the most common morphologies among biofilm producing isolates. This indicated variability in the production of the extracellular matrix., particularly curly fibers, which play a crucial role in bacterial cell adhesion to each other and to various surfaces. Previous studies have shown that Curly fibers are a critical component of E. coli biofilm, enhancing its structure and increasing its resilience to modification of the environment [21]. The current results cleared that curli production was significantly higher in isolates with high heterogeneity than isolates with low heterogeneity. This can be clarified by the isolates that can produce higher amounts of curl showing a greater capacity to construct complex cell communities that exhibit phenotypic diversity. Furthermore, curl production lead to increased cell adhesion within the biofilm, leading to be coarser and more complex colony patterns on culture media. A key finding of this study is the strong positive correlation of r = 0.74 between heterogeneity and biofilm density suggests that greater phenotypic diversity among the isolates may lead to greater biofilm formation. Stewart and Franklin noted that Heterogeneity is a defining trait in structured communities of bacteria that enables the development of subpopulations with varying physiological properties, that enable bacteria to adapt to various environmental changes. The study also cleared that isolates with high heterogeneity have a higher ability to resistance of antibiotics compared to isolates with low heterogeneity, specially to ciprofloxacin, cefotaxime, and trimethoprim/sulfamethoxazole.

This increased resistance may be due to the defensive role of the biofilm, where the outer cellular material limits the penetration of antibiotics into bacterial cells, in addition

to the decrease in the metabolic activity of some cells within the biofilm, which reduces the activity of many antibiotics. The high heterozygosity group also had a substantially greater amount of multidrug resistant (MDR) isolates. This finding aligns with studies in the last few years that show a strong correlation between the development of multi-drug-resistant strains and biofilm formation. Biofilms offer a suitable environment for the exchange of transposable genetic elements and the maintenance of strains more tolerant to various stresses, including antibiotic exposure. Correlation analysis revealed a strong positive relationship between curl production and biofilm density Correlation analysis indicated that curl production and biofilm density had a strong positive correlation. This confirmed that the fibers had an important structural role in biofilm formation and stability. Multiple studies demonstrate that curl serves an important role in the early adhesion stages of biofilm formation. Moreover, it contributes to the cohesion of the bacterial cells in multifaceted biofilms. In conclusion, the results of the present study suggest that phenotypic heterogeneity associated with curl production may play a significant role in biofilm promotion and antibiotic resistance in clinical E. coli isolates. These findings offer insights into the mechanisms of bacterial persistence and treatment failure and highlight the promise of targeting biofilm components, such as curl fibers, as a strategy for the reduction of biofilm-related infections.

5. Conclusion

The results of this study showed that most of the clinical Escherichia coli isolates have the potential of biofilm formation to different degrees and there are obvious differences in the phenotypic pattern of colonies on Congo Red Agar. Furthermore, isolates with high phenotypic heterogeneity were more capable of producing curl fibers than isolates with low heterogeneity. The study also revealed a significant positive correlation between phenotypic heterogeneity and biofilm density, suggesting that increased phenotypic diversity may contribute to enhanced biofilm stability and development. Moreover, the high heterogeneity of the isolates showed a higher rate of antibiotic resistance and a higher proportion of multidrug-resistant (MDR) isolates, highlighting the important role of biofilm and its structural components in the promotion of bacterial survival and tolerance to different therapeutic agents. The significance of these fibers as one of the primary elements in charge of bacterial cell adherence and cohesiveness within the biostructure is further supported by the results, which also demonstrated a strong correlation between curl generation and biofilm formation strength. Thus, it can be said that in clinical E. coli isolates, phenotypic variability linked to curl generation plays a significant role in fostering biofilm development and raising antibiotic resistance. Researching these traits could lead to the development of treatment approaches that target the biofilm and its constituent parts in order to lessen persistent bacterial infections, as well as a better understanding of the mechanisms of pathogenesis and infection persistence.

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