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Phenotypic and genotypic biosystematics of *E. coli* strains isolated from clinical and environmental samples in the Karbala Governorate

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ABSTRACT

Eight distinct phylogenetic groups of *Escherichia coli* strains have been identified through a novel technique called quadruplex PCR. The exact evolutionary relationships among these bacterial groups remain ambiguous. The Clermont phylotyping technique has been employed in this study to elucidate the phylogenetic groups of *E. coli*. This study aimed to establish the connections between isolates derived from clinical and environmental samples, isolate them, diagnose them, and subsequently classify *E. coli* bacteria into phylogroups and strains. Five hundred samples were collected from Karbala, subsequently cultured, isolated, and diagnosed biochemically, with confirmation via PCR and according to the Clermont classification. In the 201 positive isolates, two pathotype groups (DEC/EXPEC) and their associated subpathotype groups (APEC, UPEC, SEPEC, MPEC) were identified, alongside seven phyllocrops (A, B1, B2, C, D, E, and F), as well as the Unknown group and clade (I, II, V). Commensal *E. coli* was additionally identified. For this investigation, a novel strain classification was established, and these strains were initially catalogued in the NCBI gene library under the designation "Hetero-hybrid pathogenic," which is the plural of "EXPEC + DEC + DEC." An extension number was obtained upon the initial registration of ten new *E. coli* isolates with the NCBI. A novel category was established specifically for this study; *E. coli* bacteria were identified utilising specific diagnostic primers, and pathogenic strains initially discovered in Iraq were diagnosed.

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Introduction

Escherichia coli is one of the most researched bacteria in the world. The digestive system is home to these rod-shaped, facultative anaerobic, Gram-negative bacteria, which usually inhabit warm-blooded animals, particularly mammals, but also birds, reptiles, and fish (Al Jader & Ibrahem 2022; AlJudaibi et al. 2023; Odaa & Rasheed 2025).

Escherichia coli is classified as a significant part of the gut microbiota and a member of the Enterobacteriaceae family. Commensal interactions occur when two species live together without clearly benefiting from one another but also without endangering one another. Numerous commensal microorganisms, mostly bacteria but also archaea and eukaryotes, live in the gastrointestinal system of healthy people (Zhu et al., 2023).

The overall number of cells in the microbial community, which includes more than 500 bacterial species, is 10 times more than the total number of cells in the body. Bacteria in the distal small intestine of the large intestine (colon) are more numerous, reaching

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10^{11}–10^{12} colony-forming units per milliliter. With a 100:1 ratio of anaerobic to aerobic bacteria, the great majority of bacteria in the large intestine are anaerobic species. Despite forming symbiotic relationships, *E. coli* can also have a significant negative impact on its hosts, particularly when it comes to human health, due to its metabolic adaptability, which allows it to live in environments other than those of its hosts, such as soil, water, plants, and food materials (Weiland-Bräuer 2021).

This study aimed to investigate the phylogenetic diversity and pathotypic classification of *E. coli* strains isolated from clinical and environmental sources in Karbala, Iraq. Using the Clermont phylotyping method and quadruplex PCR, the research sought to identify and characterize *E. coli* phylogroups, distinguish between commensal and pathogenic strains, and introduce novel strain classifications, including newly identified hetero-hybrid pathogenic types, with registration in the NCBI gene library.

Materials and Methods

Collection of specimens

A total of 500 specimens—50 of each type (meat, fish, poultry, raw milk, cream, cheese, tap water, river water, stool, and urine)—were collected from Karbala between November 2023 and May 2024, comprising both environmental and clinical samples.

Tap water samples were obtained from residential areas across different districts, while river water samples were collected from various locations along local rivers. Clinical samples were collected and cultured at Hospital City.

Isolation and identification of E. coli

Glossy pink colonies observed on MacConkey agar following cultivation were indicative of *E. coli*. These colonies were further isolated and cultured on Eosin Methylene Blue (EMB) agar, a selective medium used to differentiate *E. coli* strains. On EMB agar, colonies exhibiting a metallic green sheen confirmed the presence of *E. coli*.

To detect Shiga toxin-producing *Escherichia coli* (STEC), fecal samples were also cultured on CHROMagar STEC and incubated at 37 °C, following the protocol of Al-Saadi et al. (2018).

Biochemical tests

Biochemical identification of *E. coli* isolates was conducted using standard tests as described by MacFaddin (2011).

Molecular confirmation

Polymerase Chain Reaction (PCR) was performed using nine specific primers. The primers and their annealing conditions are listed in Table 1.

The amplification conditions were as follows: an initial denaturation at 94–95 °C for 5 minutes (one cycle), followed by 30 cycles of denaturation at 94 °C for 30–60 seconds, annealing at 50–63 °C for 30–60 seconds, and extension at 72 °C for 30 seconds. A final extension was carried out at 72 °C for 10 minutes.

PCR products were visualized by electrophoresis on 1% agarose gel stained with ethidium bromide, according to the method of Al-Omari and Al-Ammar (2021).

Results and Discussion

Prevalence of E. coli

The prevalence of *E. coli* observed in our study was higher in diarrheal samples compared to the findings of Hashim (2016), although our results for river water and diarrhea samples were consistent with the study conducted by Alabassi et al. (2020), which reported a 75% prevalence. Additionally, our results aligned with the data presented in Table 2 (supplementary) regarding the presence of *E. coli* in tap water. In contrast, local cheese samples collected from Baghdad showed an exceptionally high prevalence of *E. coli*, reaching 94%, which was significantly higher than the values observed in our study.

The lipase test revealed positive results in several samples, particularly in isolates obtained from cream (qaymar), followed by isolates from tilapia fish and river water. These findings support the hypothesis that lipase-producing *E. coli* may originate from contaminated aquatic and dairy environments. This aligns with previous reports suggesting that river pollution due to direct sewage discharge, especially in rural areas (Pulido et al., 2020), plays a role in the spread of these strains. To further enhance enzyme expression, lipase and lipase-specific foldase genes were subcloned into two distinct expression vectors.

Biochemical tests

Biochemical tests were conducted on all *E. coli* isolates, with the results presented in Table 2. The outcomes of tests such as urease, catalase, citrate utilization, and indole production were generally consistent with the findings of Mueller and Tainter (2023). Except for minor discrepancies in indole, catalase, citrate, and urease tests, our findings largely concurred with previous research on biochemical assay profiles of *E. coli*. These consistent patterns support the reliability of these markers in identifying *E. coli* strains across various environmental and clinical samples.

Phenotypic expression of virulence factors

The phenotypic investigation of virulence factors, including biofilm formation, hemolysis, and capsule production, is detailed in Table 2. The results from milk-derived isolates regarding biofilm formation were consistent with the study by Ahmed and Nsaif (2021). Furthermore, findings related to raw milk, ghee, and cheese were similar to those reported by Nadia (2003), who found a 51.6% prevalence of biofilm formation. However, not all dairy samples demonstrated biofilm production, likely due to the plasmid-mediated nature of these traits and environmental influences.

Table 1 Primers and PCR conditions used for gene amplification in this study

Genes	Sequence 5-3	Size	References
chuA	F ATGGTACCGGACGAACCAAC	288	Clermont *et al*. (2013)
	R TGCCGCCAGTACCAAAGACA		
yjaA	F CAAACGTGAAGTGTCAGGAG	211	Clermont *et al*. (2013)
	R AATGCGTTCCTCAACCTGTG		
TspE4C2	F CACTATTCGTAAGGTCATCC	152	Clermont *et al*. (2013)
	R AGTTTATCGCTGCGGGTCGC		
arpA	F AACGCTATTCGCCAGCTTGC	400	Clermont *et al*. (2013)
	R TCTCCCCATACCGTACGCTA		
ArpAgpE.	F ATTCCATCTTGTCAAAATATGCC	301	Lescat *et al*. (2012)
	R GAAAAGAAAAAGAATTCCCAAGAG		
f17A	F CAGAAAATTCAATTTATCCTTGG	537	Bertin, *et al. (*1996)
	R TGATAAGCGATGGTGTAATTAAC		
hlyA	F AACAAGGATAAGCACTGTTCT GGCT	1177	Yamamoto, *et al.* (1995)
	R ACC ATATAAGCGGTCATTCCCGTCA		
aggR	F CTA ATT GTA CAA TCG ATG TA	457	Czeczulin, *et al*. (1999)
	R AGA GTC CAT CTC TTT GAT AAG		
16srRNA	F GACCTCGGTTTAGTTCACAG	585	Wang et al. (1996)
	R CACACGCTGACGCTGACCA		

In fish samples, the presence of biofilm-forming *E. coli* mirrored results obtained by Machado et al. (2023), where the prevalence reached 100%. Similarly, the findings from Najaf chicken samples, which showed a 77.4% prevalence of biofilm production, aligned closely with the study of Trang et al. (2023). Urine isolates yielded a 60.7% prevalence, consistent with the findings of Crecencio et al. (2020). *E. coli* was also detected in tap water samples in varying concentrations, and the prevalence of biofilm formation among these samples was similar to that reported by Sherif et al. (2024), at approximately 55%.

The entry of *E. coli* into drinking water sources is attributed to cracks or breaks in the water distribution network, corroborating the results of Gaihre et al. (2024), who reported biofilm formation within polyethylene water pipes. Our study also confirmed hemolysin production, consistent with the findings of Abberton et al. (2016), demonstrating *E. coli*'s ability to cause red blood cell lysis. Capsule production was identified in isolates from various sources, in agreement with the findings of Al-Mousawey and Abed (2023), as well as with capsule-producing strains identified in poultry samples from the study by Hong et al. (2023).

Correlation between isolates

To determine the proportion of similarity between the biochemical test parameters and the virulence factors, numbers were assigned to the cluster groups based on the arrangement of all 201 Karbala samples in the cluster diagram (Figure 1). The diagram was divided into two halves for better visibility and the samples were coded to facilitate tracking on the chart. In the diagram, we observe that Cluster (1) includes samples from urine, feces, and a separate fish isolate, while Cluster (2) includes a large group of isolates representing almost all types of study samples (fish, meat, urine, milk, cheese,

cream, river water, a single feces isolate, and a single tap water isolate).

Cluster (3) includes single isolates from urine, feces, and milk, and Cluster (10) connects the three clusters (1, 2, and 3). Cluster (4) includes a group consisting of chicken, meat, urine, river water, tap water, cheese, milk, and a single stool isolate, and it is connected to Cluster (10) by Cluster (11).

Cluster (5) includes meat, urine, and chicken; Cluster (6) includes river water, urine, chicken, milk, and stool. Cluster (12) connects Cluster (5) to Cluster (11), and Cluster (13) connects Cluster (6) to Cluster (12). Cluster (7) includes river water, milk, and cream, and is connected to Cluster (13) by Cluster (14).

Clusters (8, 9, 17, and 18) all contain stool isolates, and the final Cluster (19) contains a chicken isolate and connects to Cluster (18).

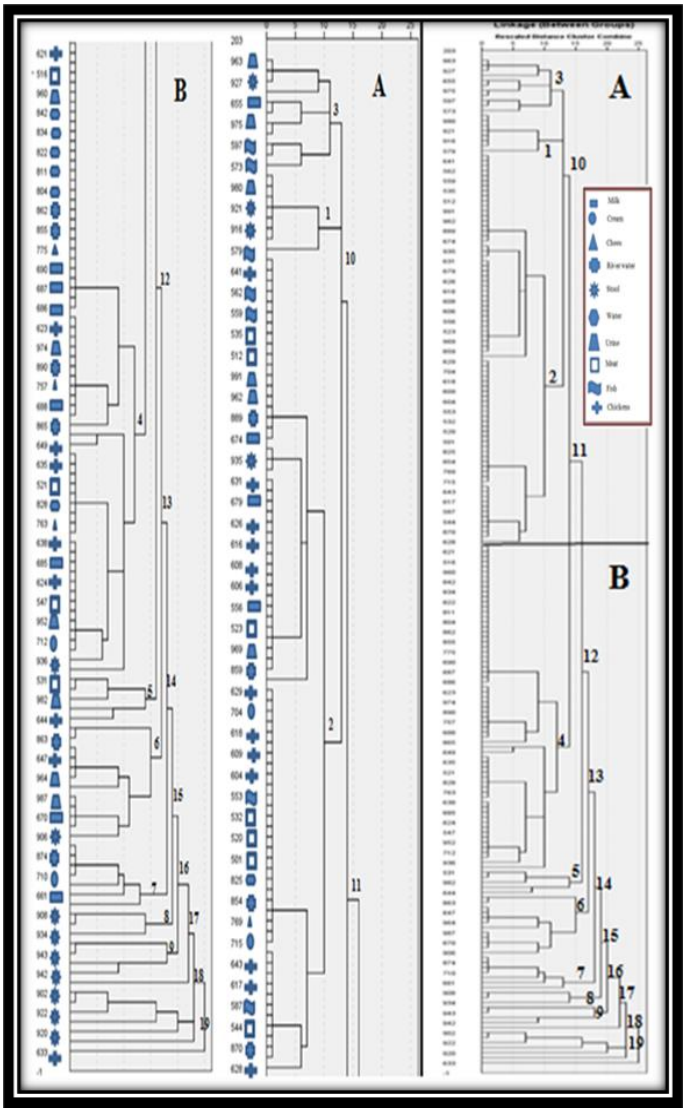

Fig 1. Dendrogram showing the relationship among *E. coli* isolates.

Molecular identification of E. coli

PCR analysis confirmed the presence of the 16S rRNA gene in all 201 isolates. Urinary isolates matched the findings of Mohammed et al. (2022), while diarrheal isolates showed consistency with the studies of Ibrahim (2016) and Jubran et al. (2019). Isolates from other environmental and clinical sources were in line with the findings of Zaki et al. (2021). However, since the 16S rRNA gene is also present in related species such as E. *fergusonii* and *E. albertii*, it is not sufficient on its own for definitive identification of *E. coli* (Clermont et al., 2013).

Phylogenetic grouping of E. coli isolates

Conventional PCR was performed in a quadruplex pattern on 201 *E. coli* isolates. The gel plate images (for illustration purposes only) show the distribution of the four genes (chuA, yjaA, TspE4C2, arpA) across the samples and specimens. Table (3, supplementary) presents the distribution pattern used to determine the pathotypes of the studied *E. coli* isolates.

Figure (2) shows the PCR-positive products classified as follows:

Group A (+, –, –, + / +, –, –, –) includes isolates (1, 3);

Unknown group (+, –, +, + / +, +, +, +) includes isolates (2, 19, 30 / 5, 13, 18, 20, 24, 29);

Group D, E (+, +, –, +) includes isolate (4);

Group B2 (–, +, +, + / –, +, +, –) includes isolates (7, 8, 9, 12, 15, 23, 26 / 6, 11, 22);

Group E, clade I (+, +, +, –) includes isolates (10, 14, 17, 21, 25, 28, 31);

Group F (–, +, –, –) includes isolates (16, 27).

Phylotyping of the Babylon meat samples indicated the presence of groups A, Unknown, and Clade I, represented by isolates (1, 3), (2, 19, 30 / 5, 13, 18, 20, 24, 29), and (10, 14, 17, 21, 25, 28, 31), respectively. These findings are consistent with Joshua et al. (2017). Additionally, the result for group B2 (10%) agrees with Clermont et al. (2013).

The results shown in Figure (3) revealed the following:

Group C, A includes {(BF=94, 96), (KF=112), (NF=124)};

Group E, clade I includes {(BF=95, 101), (KF=107, 108, 114), (NF=116, 117, 118, 119, 121)};

Group A also includes (KF=111);

A separate group includes {(KF=113), (NF=123)}.

The Unknown group includes (BF=98, 102), (BC=103, 104), (KF=106, 110), (NF=115, 122)}.

Group B2 includes {(KF=105, 109), (NF=120), (BF=97, 98)}. These results are consistent with those reported by Ortega-Enríquez et al. (2024).

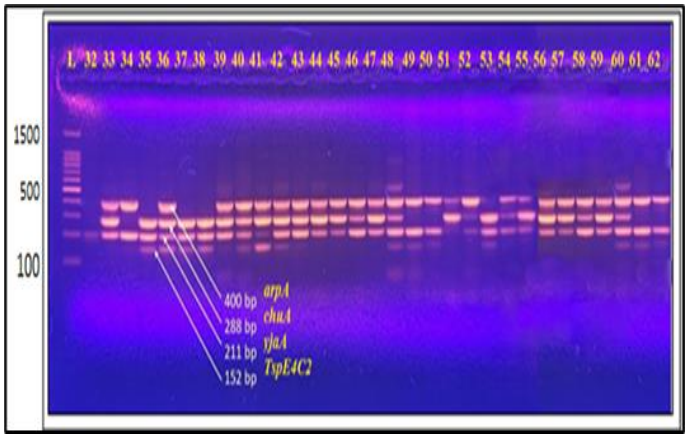

Fig 2. Quadruplex PCR profiles using the new Clermont phylo-typing method. Agarose gel electrophoresis was performed for 60 minutes at 70 volts to target the arpA, chuA, yjaA, and TspE4C2 gene sequences.

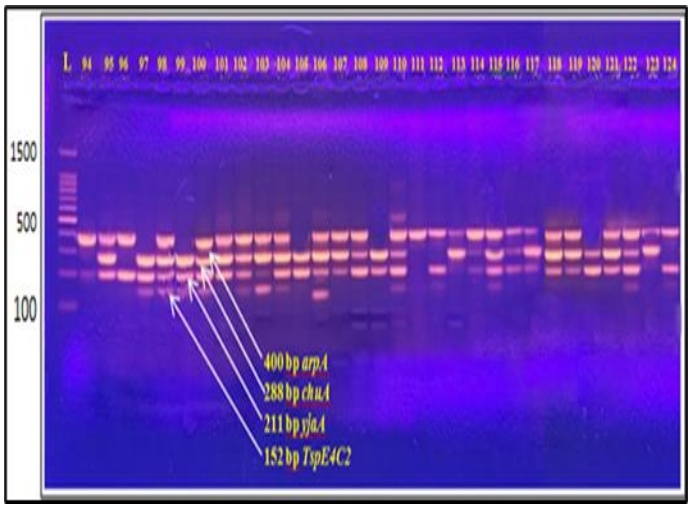

Fig 3. Quadruplex PCR profiles for phylo-typing**.** Agarose gel electrophoresis was conducted for 60 minutes at 70 volts to target gene sequences including *TspE4C2*, *chuA*, *yjaA*, and *arpA*.

Analysis of the distribution of samples according to quadruplex patterns

The current study assessed the distribution of phylogroups in 201 isolates using the customized PCR method described by Clermont and colleagues. Table (3) shows the distribution of isolates according to the Quadruplex patterns. The highest percentage was observed in the Unknown group, which accounted for 57 isolates (28.3%). This was followed by group B2 with 54 isolates (26.8%), and group E, clade I with 50 isolates (24.8%).

The result for group F (3.1%) matched the findings reported by Ortega-Enríquez et al. (2024) and Clermont et al. (2013). However, our results did not align with a recent study conducted in Baghdad, where the reported percentages for groups A, B2, C, D, and F were 4%, 2%, 24%, 12%, and 6%, respectively.

Molecular detection of virulence genes

Multiplex and conventional PCR analyses were used to detect a panel of virulence genes across the isolates. These included hlyA, f17A, aggR, eae, and ipah genes. The aggR gene (457 bp), ArpAgpE (301 bp), and eae gene (881 bp) were amplified successfully. Among these, aggR was detected in 134 isolates (66.6%). The ipah gene showed the broadest distribution, with particularly high prevalence in cream (58.3%), stool (57.6%), and milk (55%) samples, as shown in Table 4 (supplementary). These findings were in agreement with previous studies by Al-Huchaimi et al. (2017, 2018).

Combinations of virulence genes and pathotype classification

Strains were classified into different pathotype groups based on their combinations of virulence genes, resulting in three unique groups: hetero-pathogenic strains, which carry virulence genes from multiple DEC pathotypes and comprised 8.9% (18 isolates); hybrid-pathogenic strains, containing a mix of DEC and ExPEC virulence genes, making up 16.4% (33 isolates); and hetero-hybrid pathogenic strains, a novel category introduced in this study that exhibited virulence factors from both ExPEC and multiple DEC types, accounting for the highest proportion—21.8% (44 isolates). This new group has not been previously documented, marking a unique contribution of this study.

Distribution of E. coli by pathogenic group

The pathogenic groups presented in Table 5 (supplementary) include various classifications based on virulence and phylogenetic distribution. DEC strains (EPEC, EHEC, EIEC, EAEC) were observed with respective frequencies of 0.49%, 1.9%, 1.49%, and 4.9%, while the highest frequency, 8 isolates, appeared under the "Unknown" category. ExPEC strains (APEC, UPEC, SEPEC, MPEC) were recorded at rates of 4.4%, 10.9%, 3.4%, and 6.4%, respectively. Commensal *E. coli*, referring to strains that have not undergone mutations or do not carry virulence factors associated with pathogenicity, appeared in 15 isolates (7.4%) distributed across phylogroups B2, E, and Unknown. Hybrid-

pathogenic strains were identified in 33 isolates (16.4%) and were distributed among phylogroups B1, B2, C, D, E, and F, with frequencies of 1, 18, 5, 1, 6, and 2 isolates, respectively. Hetero-pathogenic strains included 18 isolates (8.9%), distributed across phylogroups B2, C, E, F, and Unknown in counts of 6, 2, 6, 1, and 3 isolates, respectively. The hetero-hybrid pathogenic group—newly defined in this study—recorded the highest percentage among all pathotypes, with 44 isolates (21.8%), distributed across phylogroups B2, C, E, F, and Unknown as 12, 1, 15, 1, and 15 isolates, respectively. Additionally, cryptic *Escherichia* clades were identified, including clades I, II, and V, with 10 (4.9%), 11 (5.4%), and 1 (0.49%) isolates, respectively.

The phylogenetic group results from this study differ from those reported by Clermont et al. (2013), where the frequencies of phylogroups A, B1, and B2 were notably higher—18.2%, 18.3%, and 38.1%, respectively—than those observed in our findings. Only the frequency of phylogroup C was comparable, closely matching the 3% reported for French isolates in that study. However, our results align with the findings of Ortega-Enríquez et al. (2024) in identifying the presence of hetero-pathogenic, hybrid-pathogenic, and hybrid strains. The hetero-hybrid pathogenic group, introduced for the first time in this study, represents a novel classification, and thus no prior research exists for direct comparison.

DNA sequencing

Genomic DNA from ten representative isolates was analyzed using BLAST via the NCBI database. All sequences showed high similarity to *E. coli* and were subsequently registered in GenBank with the following accession numbers: PQ326028, PQ326101, PQ326119, PQ326845, PQ326867, PQ326896, PQ326898, PQ327411, PQ327413, and PQ327568.

Conclusion

This study successfully identified and characterized multiple virulent *E. coli* strains using specific diagnostic primers. Notably, the study introduced a new classification—hetero-hybrid pathogenic *E. coli*—which had not been previously reported. These findings contribute significantly to the understanding of *E. coli* diversity and its pathogenic potential in Iraq.

Ethical approval

The authors affirm that the methods used were compliant with the World Medical Association's Code of Ethics (Declaration of Helsinki) and the rules of the applicable clinical research ethics committee. for data confidentiality, the authors affirm that they have complied with their work center's guidelines for the release of patient data. The patients or subjects described in the paper have given their written informed consent, which the writers have acquired in accordance with their right to privacy. This document belongs to the corresponding author.

Availability of data and material

Data are accessible upon request.

Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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