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Associated of *E. coli* intestinal bacteria with contamination of milk and its products in the city of Hilla, Iraq

Sura I. A. Jabuk*, Zahraa M. Altaee, Anmar M. K. Al-Maamori, Raflaa S.Hussien

Biology Department, College of Science, University of Babylon, Babylon, Iraq

*Correspondence email: suaraihsan@yahoo.com

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ABSTRACT

Food-borne diseases generally correlate with pathogens, such as bacteria, more than 200 food-borne infections caused by food cause significant public health problems worldwide. A total of 100 samples of milk and milk products were gathered randomly from different supermarkets in AL-Hilla City, Iraq, from November 2022 to February 2023 to detect bacteria contamination in these samples. To study the susceptibility of antibiotic 10 antibiotics from different classes. To test the capacity of Enteropathogenic *Escherichia coli* to produce biofilms congo red agar was used. All EPEC isolates were tested for the presence of genes encoded to adhesion *fimH* and *iha* genes. The results showed that the percentage of positive samples was 72(72%), including 19(19%), 30(30%), 14(14%), and 9(9%) isolated from milk, cheese, Yoghurts and cream, respectively. More types of bacteria isolates were *E. coli* (32) isolates than *Staphylococcus aureus* (24), *Staphylococcus epidermidis* (22), *Candida albicans* (21), *Pseudomonas aurogenosa* (18), *Bacillus cereus* (17), *Proteus mirabilis* (16), *Klebsiella pneumonia* (10) and, *Enterobacter aeruginosa* (6). The prevalence of EPEC was 12(37.4%) including 5(15.6), 3(9.3), and 4(12.5) for Polyvalent 2, Polyvalent 3, and Polyvalent 4, respectively. The antibiotic susceptibility to Enteropathogenic *E.coli* observed lower resistance against Imipenem and Meropenem in prevalence 12(100%) and 11(91.6%) from 12 isolates, While the study indicated that there is a higher resistance against Ampicillin in prevalence11 (91.6%). There are three types of colonies: strong, intermediate, weak, and no biofilm in prevalence. The result of PCR showed the prevalence of *fimH* and *iha* genes was 10(83.3%), and 8(66.6%) respectively.

ارتباط بكتيريا الإشريكية القولونية المعوية بتلوث الحليب ومنتجاته في مدينة الحلة بالعراق

سرى احسان عبد جابك[1] ، زهراء محمد الطائي[2]، انمار مهدي كاظم[3] ، رفلاء سابق حسين[4]
جامعة بابل/ كلية العلوم/قسم علوم الحياة

الخلاصة :

ترتبط الأمراض التي تنتقل عن طريق الأغذية بشكل عام بالعديد من مسببات الأمراض، مثل البكتيريا والفيروسات والفطريات والطفيليات، وأكثر من 200 حالة من العدوى التي تنتقل عن طريق الأغذية تسبب مشاكل صحية عامة كبيرة في جميع أنحاء العالم. جمعت عينات الحليب ومنتجات الألبان (100 عينة) بشكل عشوائي من محلات السوبر ماركت في مدينة الحلة، العراق، بين تشرين الثاني (2022) وشباط (2023) للكشف عن التلوث البكتيري لهذه العينات. ، تم تشخيص بكتريا الإشريكية القولونية باستخدام الاختبارات البيوكيميائية ومصليًا باستخدام اختبار تراص الشرائح. وثم دراسة الحساسية للمضادات الحيوية باستخدام 10 مضادات حيوية من مجاميع مختلفة. لاختبار قدرة بكتيريا الإشريكية القولونية المعوية على إنتاج الأغشية الحيوية تم استخدام طريقة احمر الكونكو أجار. تم اختبار جميع عزلات الايشيريكيا القولون الممرضة للامعاء للتأكد من وجود الجين المشفر لالتصاق جينات *fimH* و*ihA* . أظهرت النتائج أن نسبة العينات الإيجابية بلغت 72(72%)، منها 19(19%)، و30(30%)، و14(14%)، و9(9%) معزولة من الحليب والجبن والزبادي والقشدة على التوالى. أكثر أنواع العزلات البكتيرية كانت *E. coli* (32) عزلة ثم *Staphylococcus aureus* (24)، *Staphylococcus epidermidis* (22)، *Candida albicans* (21)، *Pseudomonas aurogenosa* (18)، *Bacillus cereus* (17)، *Proteus mirabilis* (16). والالتهاب الرئوي كليبسيلا (10) والأمعائية الزنجارية (6). كان معدل انتشار EPEC 12 (37.4٪ (بما في ذلك 5 (15.6)، 3 (9.3)، 4 (12.5) لـ متعدد التكافؤ 2، متعدد التكافؤ 3، متعدد التكافؤ 4، على التوالي. وجدت حساسية المضادات الحيوية للإشريكية القولونية المعوية أقل مقاومة ضد الإيميبينيم والميروبينيم في الانتشار 12 (100%) و11 (91.6%) من 12 عزلة، بينما كانت المقاومة الأعلى ضد الأمبيسيلين في الانتشار 11 (91.6%). هناك أربعة أنواع من المستعمرات: قوية، متوسطة، ضعيفة، ولا يوجد بها غشاء حيوي في الانتشار، 4 (33.3%)، 6 (50%)، 1 (8.3%)، 1 (8.3%) على التوالي. أظهرت نتيجة PCR أن معدل انتشار الجين fimH و iha كان 10 (83.3٪) و 8 (66.6٪) على التوالي.

الكلمات المفتاحية: الإشريكية القولونية المعوية، الحساسية للمضادات الحيوية، العدوى المنقولة بالغذاء

INTRODUCTION

Foodborne infections are the most broadly distributed health hazard in our country. Toxins in food produce foodborne intoxication or food poisoning. Toxins are generated by microorganisms when they proliferate in meals or after passing through the intestines. Poisonous substances that might be purposefully or accidentally added to food during production, processing, transportation, or storage (Mahdi & Thalij, 2012; Jessberger *et al* ., 2020). The existence of residual amounts of milk pollutants and pathogens in cow dairy farms indicates the quality of the milk (Bauman *et al* ., 2018). *E. coli* is a member of Gram-negative rod bacteria belonging to the Enterobacteriaceae family, which are facultatively anaerobic. *E. coli* has three types of antigens (O lipopolysaccharide antigens on their cell wall, flagella, H antigens, and capsular polysaccharide (K) antigens (Hebbelstrup *et al* ., 2014; Adkins *et al* ., 2018; Hassan & Kassem , 2018). Enteropathogenic *E.coli* is an important pathogroup related to children's diarrhea in the developing world (Alikhani *et al* ., 2013).

The importance of antibiotics has become clear in treating various bacterial infections. The increased wrong use of most antibiotics was accompanied by the emergence of strains

characterized by their high resistance, which extended to include a wide range of antibiotics (Hoffman *et al* ., 2015; Boszczowski *et al* ., 2019)**.** Antibiotic-resistant bacteria have developed into an increasing zoonotic public health issue. Despite extensive research, many individuals suffer from animal-related diseases (Hachemi *et al*., 2019)**.**

Biofilm formation provides several survival benefits to bacterial pathogenicity, such as Increased dispersal capacity, beneficial cell-cell interactions, increased persistence and proliferation in biotic and abiotic environments and external stress protection, enabling survival under severe environmental conditions, enhancing the potential dissemination of antibiotic-resistant genes, enhancing resistance to widely used antibiotics, and increasing morbidity and mortality (Ohadian *et al*., 2014; Soto, 2014; Hu et al ., 2018; Bakar *et al*.,2018). The current study intends to investigate the antibiotic susceptibility of *Enteropathogenic E.coli* contaminated milk and milk products sold under market conditions at Al-Hilla City, Iraq.

MATERIAL AND METHODS

Collection of Samples and Identification of Bacteria

Between November 2022 and February 2023, A total of 100 samples of milk and milk-related products were randomly selected from several neighborhood stores in Babylon province, Iraq. Five grams of milk products were ground up and suspended in 10 ml of brain heart infusion broth, where they were incubated for 24 hours at 37 °C under aerobic conditions. On the Nutrient agar, a loop of milk products was streaking and incubated for 24 hours at 37 °C. Along with confirming diagnostic techniques, positive culture samples were re-cultured in additional selective medium, including Mannitol Salt Agar, Eosin Methylene Blue Agar, MacConkey Agar, and Blood Agar. A gram stain was also performed to distinguish between gram-positive and gram-negative microorganisms (Brown and Smith, 2017; Majid & Saleh, 2022).

Determined Enteropathogenic *E.coli* by Antisera

According to kit instructions, the *E. coli* positive cultures and biochemical tests were serologically analyzed using the slide agglutination test (Remel) *E.coli* Agglutinating Sera consists of Polyvalent O antisera for EPEC

Polyvalent2:O111:K58(B4)/O55:K59(B5)/O26:K60(B6)/O126:K71(B16)/ O119:K69 (B14),
Polyvalent 3: O128:K67(B12)/ O86:K61(B7)/ O127:K63(B8)/ O114: K90(B) /O125:K70(B15),
Polyvalent4: O124: K86(B)/O44:K74(L)/ O124:K72(B17)/ O112: K66 (B11).

Biofilm Formation

Detection of the ability of biofilm formation by Enteropathogenic *E.coli* was carried out by Congo Red Agar methods according to Arciola *et al*., 2005.

Susceptibility Test of Antibiotics

To study the antibiotic susceptibility of Enteropathogenic *E.coli* isolates against 10 antibiotics from different classes [Ampicillin (AMP 10 µg), Cefotaxime (CTX 30 µg), Imipenem (IPM 10 µg), Meropenem (MEM 10 µg), Gentamicin (GEN 10 µg), Amikacin (AMK

30 µg), Tetracycline TET 30 µg), Ciprofloxacin (CIP 5 µg) and, Chloramphenicol (CHL 30 µg). By using the Kuarby and Baur method. The inhibitory zones' diameters were measured and compared to those established by (CLSI, 2023).

DNA Extraction

The boiling technique was used to extract the DNA as previously stated by (Boroumand *et al*., 2019).

Determine Biofilm formation genes

All EPEC isolates were tested for the presence of adhesion gene *fimH, ihA* encoded to type 1 fimbriae, and Iron-regulated, respectively. The primers used and PCR conditions are shown in Table (1). Then the PCR products showed by electrophoresis by using 1.5 % agarose gel staining with safe stain dye.

Table (1) primers sequence and PCR Condition

Gene	Direction	Sequences (5→3)	Product size (bp)	References
FimH	F	GTTGTTCTGTCGGCTCTGTC	400	Rahdar *et al* ., (2015)
	R	TAAATGTCGCACCATCCAG		
iha	F	CTGGCGGAGGCTCTGAGATCA	827	Lee *et al* ., (2016)
	R	TCCTTAAGCTCCCGCGGCTGA		

Conditions of PCR

initial denaturation	94°C for 5 min	
denaturation	94°C for one min	30 cycles
annealing	55 for 20 s	
extension	72°C for one min	
final extension	72°C for 10 min	

RESULTS AND DISCUSSION

One hundred milk and milk products samples, including cheese 35(35%), milk 29(29%), yoghurt 21(21%), and cream 15(15%). Some of them were local, and the others were imported. These samples were chosen randomly from a variety of neighboring supermarkets Figure (1).

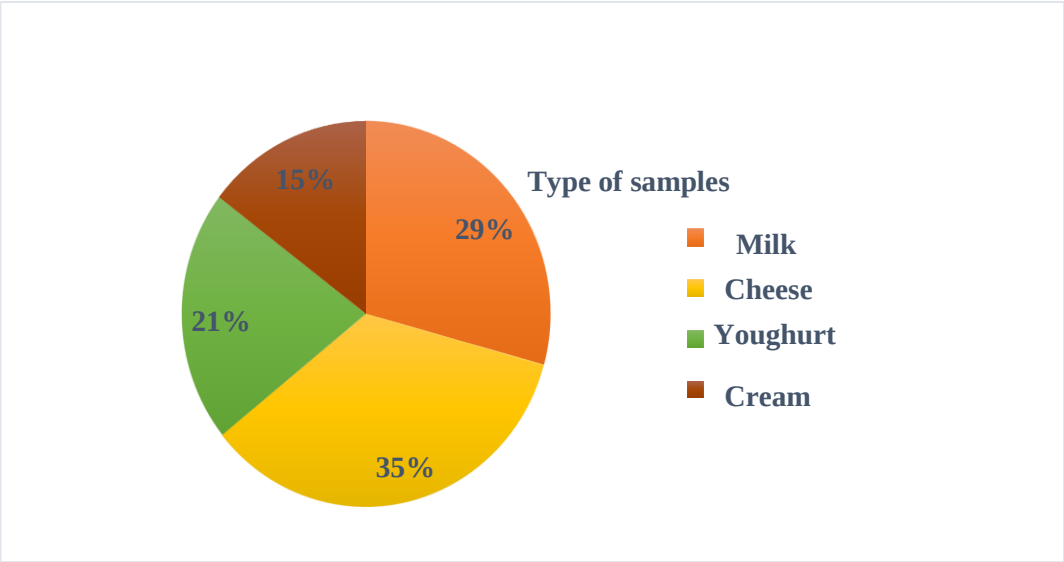

Figure (1): Distribution of milk and milk products samples

Isolation and primary identification were performed according to the standard microbiological procedures, including culture characteristics, bacterial cells' arrangements in Gram's stain, biochemical reaction (Catalase, Coagulase, Oxidase, Indole, Methyl red, Citrate, and Voges Proskauer) test (Cappuccino and Welsh, 2020). The results showed the percentage of positive samples was 72(72%), including 19(19%), 30(30%), 14(14%), and 9(9%) isolated from milk, cheese, yogurts and cream, respectively Table(1).

Table (1): Prevalence of positive and negative samples according to the type of milk samples

Type of samples	Positive samples NO. (%)	Negative samples NO. (%)	Total NO. (%)
Milk	19(19%)	10(10%)	29(29%)
Cheese	30(30%)	5(5%)	35(35%)
Yogurt	14(14%)	7(7%)	21(21%)
Cream	9(9%)	6(6%)	15(15%)
Total	72(72%)	28(28%)	100(100%)

The results showed the more prevalent type of bacteria isolates was *Escherichia coli* (32) , then *Staphylococcus aureus* (24), *Staphylococcus epidermidis* (22), *Candida albicans* (21), *Pseudomonas aurogenosa* (18), *Bacillus cereus* (17), *Proteus mirabilis* (16), *Klebsiella pneumonia* (10) and, *Enterobacter aeruginosa* (6) Table (2).

Table (2): Bacteria isolated from milk and milk products (n=72**)**

Type of bacteria	Milk	Cheese	Yogurt	Cream	Total
Escherichia coli	9	12	5	8	32
Staphylococcus aureus	6	10	5	3	24
Staphylococcus epidermidis	4	11	3	4	22
Candida albicans	4	10	6	1	21
Pseudomonas aurogenosa	8	5	3	2	18
Bacillus cereus	7	6	4	-	17
Proteus mirabilis	8	5	1	2	16
Klebsiella pneumonia	5	4	-	1	10
Enterobacter aeruginosa	2	4	-	-	6

Other researchers culture milk samples found the percentage of *E. coli*, *K.pneumoniae* and *S. aureus* was (21.3%), (14.6%) and (11.4%) (Berhe et al ., 2020). Thaker *et al* ., (2013) found the prevalence of *S. aureus* was 10 (6.25 %). Ombarak *et al* ., (2016) also isolated *E. coli* from raw milk samples. Out of 47 raw milk samples, the percentage of *S. aureus* was 12 (25.53%) samples depending on cultural and biochemical features (Jahan *et al* ., 2015). Another researcher found 60 (80%) of the 150 samples revealed positive for *E. coli* infection (Walaa and Alaa, 2021).

The potential of milk products occurs during handling, processing, and distribution. As a result, it may be used to encourage personal health care. As a consequence, tight control and monitoring procedures were proposed to reduce the danger of spreading animal-associated pathogens to individuals. In comparison with canned food, commercially canned foods are considered healthy when manufactured under carefully regulated conditions. When canned food shows signs of spoilage, leakage spurt, de-smell, or mold, do not use it (Walaa and Alaa, 2021; Ali & Jarjees , 2022).

By utilizing an antiserum *E. coli* slide agglutination test, the EPEC serogroups were identified. The result showed the prevalence of EPEC was 12(37.4%) including 5(15.6), 3(9.3), and 4(12.5) for Polyvalent 2, Polyvalent 3, Polyvalent 4, respectively Table (3).

Table (3): Number of Enteropathogenic *E.coli* isolates

EPEC NO.(%)	Another type of *E.coli* NO.(%)	Total NO.(%)
Polyvalent 2: 5(15.6) Polyvalent 3: 3(9.3) Polyvalent 4 : 4(12.5)	20(62.5)	32(100)
12(37.4)	20(62.5)	

Enteropathogenic *E. coli* (EPEC) strains have been associated with epidemics of infantile diarrhea in infants. Since enteric pathogens with diarrheagenic *E. coli* pathotypes are rarely sought for in clinical laboratories worldwide, most often, especially in endemic areas, their incidence in children under two and their importance in instances of community-acquired diarrhea remain unclear (Estrada-Garcia *et al* ., 2009; Aslani and Alikhani, 2009). Ghali-Mohammed et al ., 2023 found the percentage of of *E. coli* was (58.8%),while Dowidar and Khalifa , 2023 present the prevalence of *E.coli* isolated from milk samples was 23%.

When studying the antibiotic susceptibility to Enteropathogenic *E.coli* found higher sensitivity against Imipenem and Meropenem in prevalence 12(100%) and 11(91.6%) from 12 isolates, While the higher resistance against Ampicillin in prevalence11(91.6%) Table (4) Figure (2).

Table (4) Antibiotic susceptibility test of Enteropathogenic *E.coli* isolates (n=12)

Antibiotic	Resistant NO.(%)	Intermedium NO.(%)	Sensitive NO.(%)
Ampicillin	11(91.6)	-	1(8.3)
Cefotaxime	5(41.6)	2(16.6)	5(41.6)
Imipenem	-	-	12(100)
Meropenem	1(8.3)	-	11(91.6)
Gentamicin	4(33.3)	2(16.6)	6(50)
Amikacin	7(58.3)	1(8.3)	4(33.3)
Tetracycline	8(66.6)	1(8.3)	3(25)
Ciprofloxacine	5(41.6)	-	7(58.3)
Chloramphinicol	9(75)	-	3(25)

The results obtained by Li *et al* ., (2020) showed that 112 *E.coli* isolates were mostly resistant to tetracycline, ampicillin, trimethoprim/ sulfamethoxazole, (52%), amoxicillin followed by ampicillin, compound trimethoprim/ sulfamethoxazole, and nalidixic acid in prevalence 42%, 37%, 33%, and 32% respectively, While all isolates are sensitive to imipenem. While another researcher found the higher resistance of 45 *E. coli* isolates was 50% to amoxicillin, trimethoprim, and tetracycline. then chloramphenicol, imipenem, gentamicin, and ciprofloxacin in percentages 81.3 %, 74.8%, 72.0 %, and 69.5% respectively (Adzitey *et al*., 2021). The antimicrobial susceptibility revealed high resistance to amoxicillin and streptomycin in percentage 85.71% and 80.95%

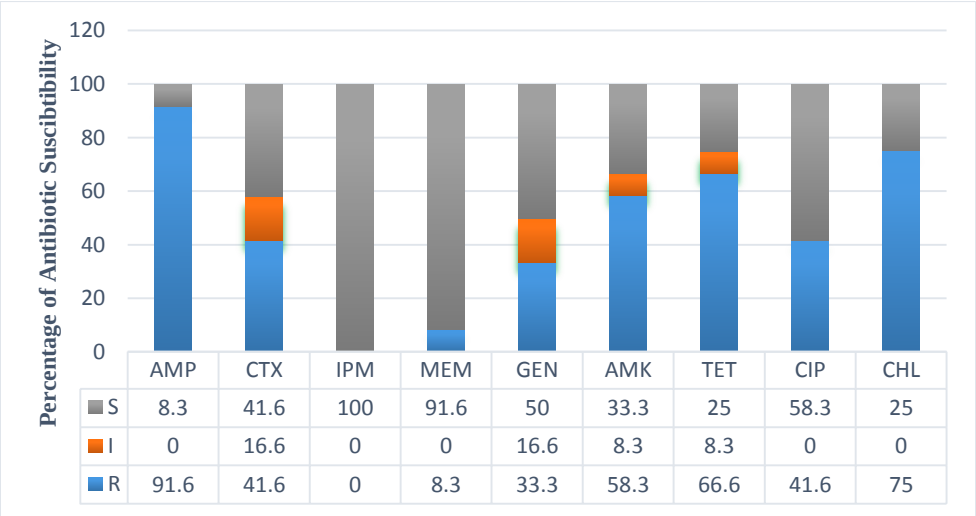

Figure (2) Percentage of antibiotic susceptibility of Enteropathogenic *E.coli* isolates (n=12)

The higher resistant *E.coli* isolated to β-Lactam antibiotic (Ampicillin) used in the present study related to the three types of resistant mechanisms including hydrolyzing the cyclic amide bond of the β-lactam ring by producing β -Lactamase enzymes (decreases bactericidal activity), inhibition the ability of antibiotic to bind to Penicillin Binding Proteins (PBPs) by decreased permeability to the antibiotic through the outer cell membrane of the bacteria. In gram-positive bacteria, the peptidoglycan layer is near the bacteria's surface, and there are few barriers for the drug to reach its target and altered PBPs. This explains the resistance of *E.coli* to most commercially available β-lactams (Whalen *et al*., 2019). The current study's findings demonstrated that EPEC isolates may produce biofilm in varying degrees. There are four types of colonies: strong (black colony), intermediate (black to red colony), weak (red colony), and no biofilm (white colony) in prevalence, 4 (33.3%), 6(50%), 1(8.3%) and, 1(8.3%) respectively Table (4), Figure (2).

Table (4): Biofilm formation by Enteropathogenic *E.coli* isolates

	Strong NO.(%)	**Moderate NO.(%)**	**Weak NO.(%)**	**No biofilm NO.(%)**	**Total NO.(%)**
Milk	3(25%)	1(8.3%)	-	-	4(33.3%)
Cheese	-	4(33.3%)	-	1(8.3%)	5(41.6%)
Yogurt	-	1(8.3%)	1(8.3%)	-	2(16.6%)
Cream	1(8.3%)	-	-	-	1(8.3%)
Total	4(33.3%)	6(50%)	1(8.3%)	1(8.3%)	12(100)

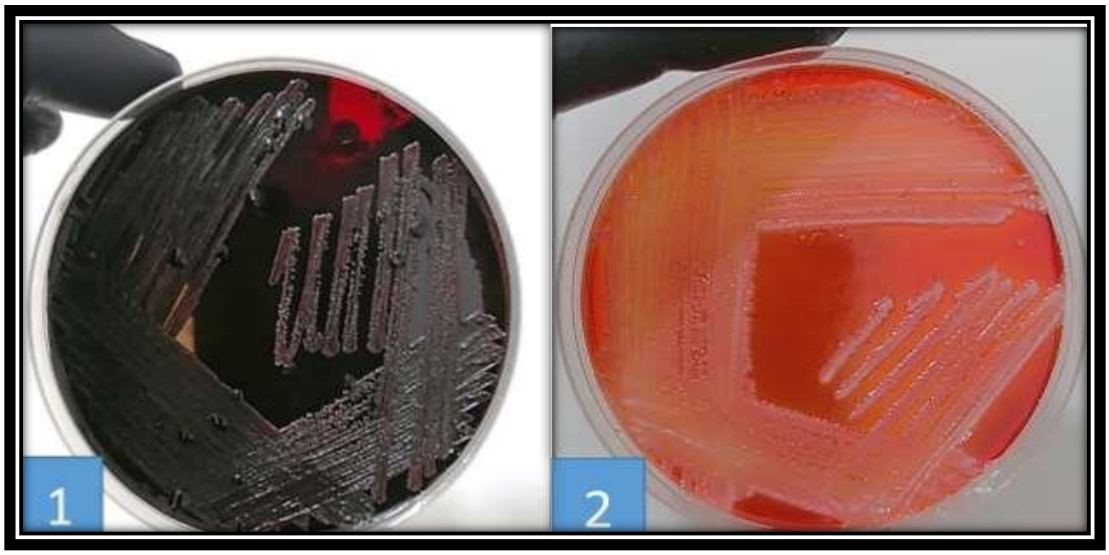

Figure (2) *Escherichia coli* on Congo red agar
1- Biofilm formation 2- Non-biofilm formation

The result of PCR showed the prevalence of *fimH* and *iha* genes was 10(83.3%), and 8(66.6%) respectively. Abd El-Baky *et al* ., (2020) found the percentage of *fimH* was (48/64, 75%). Also, another researcher showed that the most frequent virulence gene among isolated strains was *fimH* (98.0%) (Jama-Kmiecik et al ., 2022). The more prevalent genes were *fimH* 88.37%

(Abdul-Ghaffar and Abu-Risha, 2017). While other researchers found the prevalence of adhesins genes was (*fimH*, 98%, and *iha*, 26%) (Rashki *et al*.,2017). Badi *et al* ., 2018)found the percentage of *fimH* was 96.9%

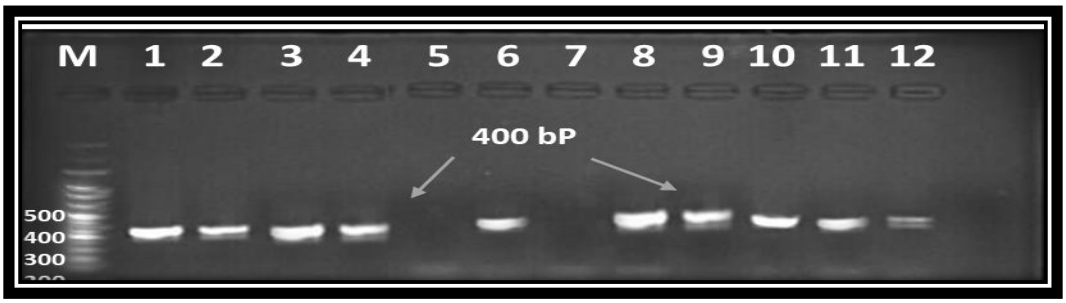

Figure (3) Electrophoresis by 1.5 % agarose gel at 70 volts 60 min after staining with red Safe for *fimH* gene (400 bp) PCR products for Enteropathogenic *E. coli*. Lane (M) molecular size marker for DNA molecules (1500-bp ladder). show positive and negative results.

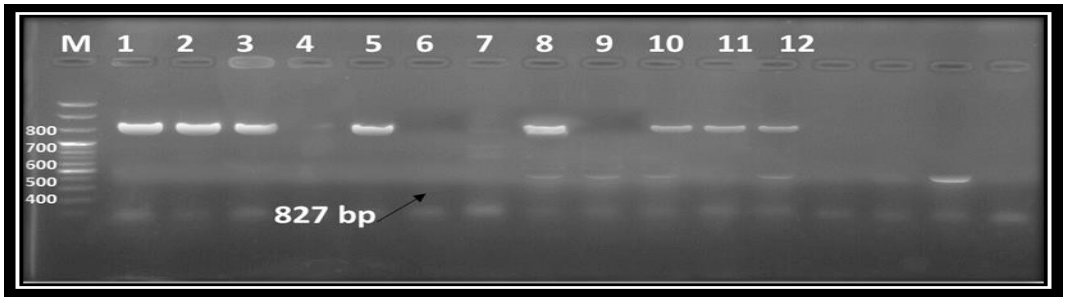

Figure (4) Electrophoresis by 1.5 % agarose gel at 70 volts 60 min after staining with red Safe *ihA* gene (827 bp) PCR products for Enteropathogenic *E. coli*. Lane (M) molecular size marker for DNA molecules (1500-bp ladder). show positive and negative results.

Table (5) Prevalence of biofilm formation gen among Enteropathogenic *Escherichia coli* isolates n=12

Gene	**Positive Samples**	
	Number	%
fimH	10	83.3
ihA	8	66.6

Food-contact surfaces that build biofilms can seriously harm your health. Biofilms reduce the effectiveness of sanitizers, result in economic losses for the industry, contaminate meat, and promote antimicrobial resistance (Mazaheri *et al* ., 2021). In compared to planktonic bacteria, biofilms increase bacteria's tolerance to environmental stress in the food industry, such as washing, disinfection, and inhibition. (van den Brom *et al* ., 2020). The creation of biofilms is a crucial part of the microbial life cycle in nature. Cross-contamination during unclean food preparation processes and raw or undercooked food intake are the primary causes of bacterial

transmission in food processing resources. Foodborne bacteria develop biofilms to survive in a range of unfavorable environments, which are commonly a cause of continuing contamination and outbreaks of foodborne illness (Bai *et al* ., 2021).

CONCLUSION

The results of that study showed the high contamination of Enteropathogenic *E.coli* and the ability to biofilm formation can be attributed to the use of raw milk, the whole process of manual production, and the maintenance of traditional cheeses, especially at the distribution and sale stage at ambient temperature. Food-contact surfaces that build biofilms can seriously harm your health. The higher resistant *E.coli* isolated to β-Lactam antibiotic (Ampicillin) used in the present study related to the three types of resistant mechanisms Given that there are very few and limited studies nationwide in this area, there are no comprehensive statistics and data on this issue, so it is recommended that more detailed studies be carried out in research centers with more oversight by the Ministry of Health on food.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript

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