Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

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Original Paper

The potential contribution of food contact surfaces to the incidence of *Escherichia coli* in Egyptian meat outlets, as well as their biofilm and pathogenicity characteristics.

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ARTICLE INFO

Keywords

Biofilm traits
Equipment surfaces.
Escherichia coli
Meat contact
Virulence

Received 04/08/2023
Accepted 16/08/2023
Available On-Line 01/10/2023

ABSTRACT

The study aimed to determine *Escherichia coli* prevalence, and their virulence and biofilm formation traits in meat-contact and equipment surfaces at butcher shops and supermarkets in Al-Menofia governorate, Egypt. One hundred swabs were collected from ten butcher shops and ten supermarkets (50 of each). The isolation and identification of targeted pathogens were conducted using standard culturing methods, biochemically using the VITEK2 compact system, and PCR techniques. PCR was used to investigate the genetic components that promote virulence and biofilm formation traits. *Escherichia coli* was confirmed in 20% of swabbed samples. *E. coli* detection rates were slightly higher from butchers than from supermarkets (24% vs. 16%) ($P > 0.05$). Eight serotypes and four pathotypes of *E. coli* were identified with enterohaemorrhagic accounting for 55%. Six of the eight *E. coli* isolates co-expressed *Crl* in all *CsgA* biofilm genes, with four, EHEC, also expressing *Stx*1. Strong virulence and a high prevalence of biofilm-producing components in isolated E. coli, together with popular Egyptian purchasing behavior, raise concerns about the potential contamination of meat products and, eventually, humans.

1. INTRODUCTION

Escherichia coli was rated at the top of several priority pathogens by food safety agencies due to the frequency and severity of illness they cause (Bintsis, 2017). According to the Egyptian Ministry of Health and Population's surveillance system, bloody diarrhea (dysentery) had the highest incidences over 8 years between 2006 and 2013, with 13.7 cases/100.000 (Abdel-Razik et al., 2017). Menoufia had the 7th highest risk index score for bloody diarrhea (dysentery) (Abdel-Razik et al., 2017).

The third-most often zoonotic disease in humans, Shiga toxin-producing *Escherichia coli* (STEC) infection, increased from 2015 to 2019 (EFSA and ECDC, 2021). *Escherichia coli*, including STEC, resulted in a total of 52 foodborne outbreaks, 59 hospitalizations, and one fatality (EFSA and ECDC, 2021).

E. coli is commonly found in the intestines of wild animals as well as the majority of domesticated animals. *E. coli* illnesses can spread directly when people consume animal products or water contaminated with human or animal feces. Animal products are commonly contaminated during the slaughtering and processing of animals (García et al., 2010). Also, these illnesses can indeed be directly spread to people as well as indirectly through contaminated surfaces used in the handling and processing of meat (Sabeq et al., 2022).

Unhygienic cleaning and disinfection of contaminated surfaces used in handling and processing meat makes it possible for these foodborne pathogens to generate a barrier of protection against stressors (Capozzi et al., 2009, Ripolles-Avila et al., 2022). Microorganisms are embedded in biostructures called biofilms for this purpose, which are largely composed of hydrated extracellular polymeric substances (Flemming and Wingender, 2010). Most bacteria in the natural environment survive by way of a biofilm to adapt to the harsh environment, with only a small portion of bacteria living planktonically or suspended in liquid food (Zhao et al., 2017). One of the virulence factors that make foodborne pathogens more hazardous is the presence of biofilm-forming genes. *Escherichia coli* has been found to persist for hours or days after initial contact on hands, clothes, utensils, and surfaces of food-processing facilities (Park et al., 2020).

The majority of research done in Egypt concentrated on determining the frequency of *Escherichia coli* in food sold from retail stores, especially supermarkets. However, very few studies, particularly those conducted in butcheries, assessed the prevalence of these pathogens on surfaces used for food contact or processing. Rarely have these isolates from surfaces used in food contact or processing been investigated for genetic determinants responsible for biofilm formation capacity. Therefore, the current study aimed to determine the prevalence and possible public health significance of *Escherichia coli* on surfaces and equipment used in meat contact and -processing at retail stores, specifically butcher shops, and supermarkets in Egypt's governorate of Al-Menofia. Also, the isolated pathogens were assessed for genetic variables affecting their potential to produce biofilms.

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2. MATERIAL AND METHODS

Ethics approval

All protocols used in this work were approved by Benha University's Faculty of Veterinary Medicine's Institutional Animal Care and Use Committee Research Ethics number (BUFVTM, 24-6-23).

2.1. Sample collection

In brief, one hundred samples were collected from meat-contact and -processing surfaces and equipment at selling outlets, butcher shops, and supermarkets, in Al-Menofia governorate, Egypt. Surface samples with varying surface areas based on size, exposed structure, and pollution were collected by rubbing with sterile cotton swabs moistened with sterile physiological water (ISO, 2015a, b). Swabs were placed in a screw-capped tube with 10 ml buffered peptone water.

2.2. Isolation and identification of Escherichia coli

2.2.1. Isolation and identification

One ml of the stock sample, containing swabs, was inoculated into MacConkey broth enrichment at 37°C then positives reinoculated and incubated at 44°C, and plating positive enrichment tube on Eosin Methylene Blue agar and Tryptone Bile X glucuronide agar at 37°C and 44°C, respectively, were used for the isolation and differentiation of enteropathogenic *Escherichia coli* following (ISO, 2005). The presumptive *E. coli* was identified using the Gram-negative identification (GNI) card of the automated VITEK2 system (compact model, bioMérieux).

2.2.2. Serological identification

Presumptive positive samples of *E. coli* were subcultured onto nutrient agar slopes and sent to the Animal Health Research Institute Laboratory (Dokki Giza, Egypt) for serotyping. Serotyping of *E. coli* followed a fully described methodology for *Enterobacteriaceae* identification (Ewing, 1986).

2.3. Molecular characterization of Escherichia coli isolates

Isolates were analyzed with PCR to assess eight confirmed *E. coli* isolates for the presence of biofilm and virulence genes including *CsgA* (Knöbl et al., 2012), *Crl* (Knöbl et al., 2012), *CsgD* (Ogasawara et al., 2010), and *Stx1 and Stx2* (Dipineto et al., 2006) under comparable published conditions, as fully outlined in our prior research (Sabeq et al., 2022).

2.4. Statistics

For statistical analysis, SPSS Statistics 20 (SPSS Inc., USA) was conducted. The data collected from various shops was analyzed using descriptive statistics such as frequency, percentage, and/or proportion. Significant differences across shops were evaluated using the student t-test with a P value of <0.05.

3. RESULTS

The prevalence of *Escherichia coli* on swabbed surfaces and equipment from butchers and supermarkets is shown in Table 1 as both tentative and confirmed. Biochemical characterization of isolates swabbed from 100 food contact surfaces and equipment using the VITEK2 compact system indicated presumptive incidences of *Escherichia coli* of 31%, 19 from butchers and 12 from supermarkets. *Escherichia coli* incidence was 20%, with 12 cases from butchers and 8 cases from supermarkets, totaling 20/100, according to serotyping. Butchers had a little higher prevalence of *E. coli* than supermarkets (P > 0.05).

Table 2 displays the serotypes, rates, and classification of *Escherichia coli* isolated from food contact surfaces and equipment swabbed from butchers and supermarkets. Serotyping of the twenty *Escherichiacoli* isolates revealed that half of the twelve butchers isolates were enterohaemorrhagic *Escherichia coli* (*EHEC*), three were enteropathogenic (*EPEC*), two were enterotoxigenic (*ETEC*), and only one was enteroinvasive *Escherichia* coli (*EIEC*). Enterohaemorrhagic *Escherichia coli* (*EHEC*) was also prevalent in supermarkets, While enteropathogenic (*EPEC*) and enterotoxigenic (*ETEC*) both contributed three isolates, and enteroinvasive *Escherichia coli* (*EIEC*) was not detected. It is worth noting that some butchers and supermarkets recovered multiple *E. coli* serogroups. For example, from swabbed cutting boards, fillers, and/or mincers, eleven isolates were recovered from five butcher shops, yielding either three serogroups (1 shop) or two (4 shops) (data not shown). The estimated occurrences of the four identified pathotypes in this investigation were 55% (11/20) for EHEC, 10% (4/20) for ETEC and EPEC (4/20), and 5% (1/20) for EIEC (Table 2). Only one EPEC serotype, O146:H21, accounted for 20% of all isolates. Four ETEC isolates were identified, representing two serotypes, O127:H6 and O15:H2, and were evenly distributed among butchers and supermarkets.

The virulence and biofilm genes of *Escherichia coli* (*n*=8) isolated from butcher shops and supermarkets are compared in Table 3 and illustrated in Figure (1). The *csgA*, *Crl*, and *stx*1 genes were identified in three butcher's EHEC isolates and one supermarket EHEC isolates. Two isolates, one from *EPEC* and one from ETEC, expressed biofilm-forming genes, *csgA* and *Crl*, whereas two isolates from *ETEC* and *EIEC* did not express biofilm or other virulence genes.

Table 1 Presumptive and confirmed prevalence of *Escherichia coli* on food contact surfaces and equipment swabbed from Butchers and Supermarket (*n*=100)

Pathogen	Butchers Swab (*n*=50)				Supermarket Swabs (*n*=50)				Total confirmed (*n*=100)	
	Presumptive		Confirmed		Presumptive		Confirmed			
	No.	%	No.	%	No.	%	No.	%	No.	%
Escherichia coli[1]	19	38	12	24	12	24	8	16	20	20

[1]*Escherichia coli* isolates were tentatively identified using the VITEK2 compact system and confirmed by serotyping.

Table 2 Serotypes and classification of *Escherichia coli* (*n*=20)isolated from food contact surfaces and equipment swabbed from Butchers and Supermarket.

	Serotypes	Groups	Butcher		Supermarket		Total	
			No.	%	No.	%	No.	%
Escherichia coli	O117:H4	EHEC	2	16.67	1	12.5	3	15
	O121:H7	EHEC	1	8.33	ND	ND	1	5
	O26:H11	EHEC	1	8.33	3	37.5	4	20
	O103:H2	EHEC	2	16.67	1	12.5	3	15
	O124	EIEC	1	8.33	ND	ND	1	5
	O146:H21	EPEC	3	25.00	1	12.5	4	20
	O127:H6	ETEC	1	8.33	1	12.5	2	10
	O15:H2	ETEC	1	8.33	1	12.5	2	10
	Total		12	100	8	100	20	100

ND, not detected; E. coli, Escherichia coli

Table 3 Characterization of virulence and biofilm genes of *Escherichia coli* isolated from food contact surfaces and equipment swabbed from Butchers and Supermarket.

Pathogen (Targeted genes)	Serotypes (ID)	Origin	Genes[1]
Escherichia coli (*n*=8)	O117:H4	Butcher	*Stx*1, *csgA*, *Crl*
	O121:H7	Butcher	*Stx*1, *csgA*, *Crl*
	O26:H11	Butcher	*Stx*1, *csgA*, *Crl*
	O124	Butcher	-
	O146:H21	Supermarket	*csgA*, *Crl*
	O103:H2	Supermarket	*Stx*1, *csgA*, *Crl*
	O127:H6	Supermarket	*csgA*, *Crl*
	O15:H2	Supermarket	-

[1]Five genes were analyzed, including *csgA*, *Crl*, *csgD*, *stx*1, and *stx*2. *E. coli*, *Escherichia coli*

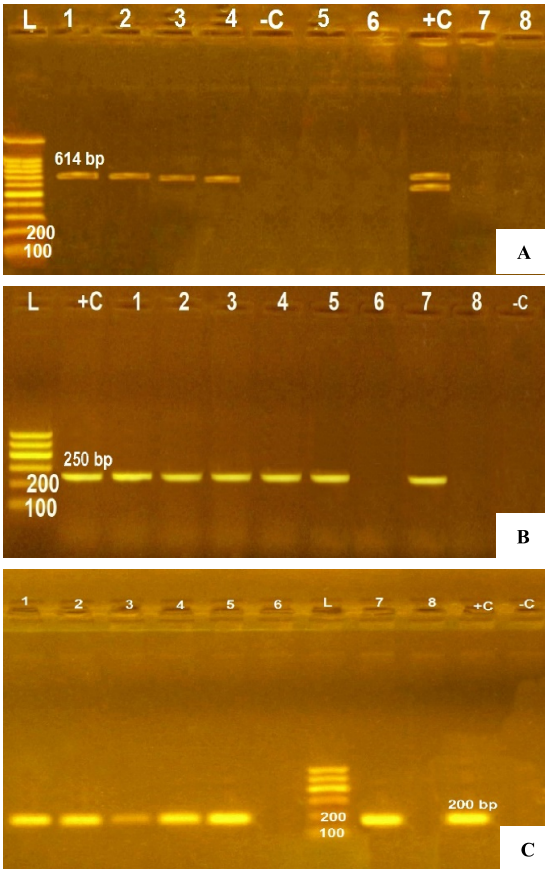

Figure 1 PCR characterization of virulence, and biofilm. Formation traits in *Escherichia coli* (*n*=8), isolated from butcher shops and supermarkets with expected amplicon size. The amplified genes were a: *Stx*1 at 614 bp and *Stx*2 at 779 bp (A); b: *Crl* at 250 bp (B); c: *CsgA* at 200 bp and *CsgD* at 501 bp (C). Lane M: 100 bp DNA ladder; C+: Positive control; C-: Negative control; Isolates of lanes from 1-8 or 1-2 or 1-5 in each gel were recorded for each targeted gene.

4. DISCUSSION

In this study, the estimated incidences of the four identified pathotypes were 55% (11/20) for *EHEC*, 10% (4/20) for *ETEC* and *EPEC* (4/20), and 5% for *EIEC* (1/20) (Table 2). The previously published pathotype frequencies of diarrhoeagenic *Escherichiacoli* (DEC) from a clinical survey in Brazil were 52.6% (359/683) for *EPEC*, 32.5% for *EAEC*, 6.3% for *ETEC*, 4.4% for *EIEC*, and 4.2% for STEC(Ori et al., 2018). *EHEC* O157:H7 is the most commonly reported STEC serotype, but three of the currently detected serogroups—O26, O103, and O121—have also been clinically incriminated in human disease and classified as STEC serotypes and are included in what is known as "the big six," along with serotypes O45, O111, and O145 (Wang et al., 2012). The majority of *EHEC* infections are known to originate from asymptomatic animals, in particular ruminants, although person-to-person spread also occurs in outbreaks. The fact that *EHEC* strains express *Stxs* is what causes serious pathology in humans. The Shiga toxin (Stx) of EHEC disrupts protein synthesis by cleaving ribosomal RNA, killing the poisoned epithelial or endothelial cells(Spears et al., 2006). It has previously been reported that STEC strains that produce Stx1 can also cause HUS (Zhang et al., 2007). To assess the risk of currently isolated *STEC* strains, O26:H11, O103:H2, and O121:H19 belong to Sero-pathotype B, which includes non-O157 serotypes that cause occasional outbreaks but are relatively common in sporadic cases associated with HUS and HC. O117:H7 and O146:H21 are sero-pathotype D associated with diarrhea without severe symptoms but not with outbreaks or sporadic HUS cases (Karmali et al., 2003). The higher prevalence of *EHEC* in swabbed food contact surfaces and premises, particularly O26:H11, in comparison to other pathotypes, as well as the genetic encoding of Shiga toxin (*Stx1*), raises concerns and deserves to be taken seriously by Egypt's food safety authority.

The EPEC strains are not typically found in healthy individuals because they are traditionally linked to diarrhea and high mortality rates of 10% to 40% in young children in developing countries (Chen and Frankel, 2005). Two categories of enteropathogenic *Escherichia coli* (EPEC) exist typical EPEC (tEPEC) and atypical EPEC (aEPEC). The presence of the EPEC attaching and effacing (A/E) factor plasmid encodes bundle-forming pilus (BFP), which is found only in tEPEC, distinguishes these two groups (Hernandes et al., 2009). Humans are the only reservoir for typical EPEC; however, for atypical EPEC, both animals and humans may serve as reservoirs. Although the typical EPEC lacks the genes necessary to produce Shiga toxin (Stx), both tEPEC and aEPEC do contain a chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE), which encodes important virulence proteins like intimin proteins, which are required for the attachment of the EPEC to the epithelial host cells (Ochoa et al., 2008). In the current study, only one *EPEC* serotype, O146:H21, accounting for 20% of all isolates, was retrieved. *STEC* non-O157 strains like O26:H11 or H-, O103:H2, O111:H-, O117:H7, O121:H19, and O146:H21 have been linked to severe illness in humans and have been reported to be more common in animals and as food contaminants (Blanco et al., 2004, Amézquita-López et al., 2016, Söderlund et al., 2016). According to earlier studies, Atypical EPEC is more closely related to *STEC*, frequently has the potential to acquire verotoxin genes, and both strains appear to be emerging pathogens (Söderlund et al., 2016). Fortunately, the currently isolated serotype, O146:H21, is an atypical *EPEC* lacking the Shiga toxin gene.

Enterotoxigenic *E. coli* (ETEC) are water and foodborne pathogens that adhere to the epithelium via colonization factors and are distinguished by the production of diarrheagenic heat-labile (LT) and heat-stable (ST) enterotoxins, estimated to cause 2.5 million cases and 700 000 deaths in children below 5 years of age (Gonzales-Siles and Sjöling, 2016). In the current study, four *ETEC* isolates representing two serotypes, O127:H6 and O15:H2, and evenly distributed among butchers and supermarkets were identified. In addition to producing the enteroaggregative heat-stable toxin, strain O127:H6 had all of the virulence traits of typical *EPEC* strains (Trabulsi et

al., 2002). There are no animal reservoirs for Enteroinvasive *E. coli*, and infected humans appear to be the main source of infection, especially in low-income countries where poor general hygiene promotes their spread and transmission is primarily oral-fecal (Pasqua et al., 2017). In line with recent findings, serogroup O124 is one of the most frequently reported serogroups among EIEC strains (Ori et al., 2018).

Characterization of foodborne pathogens' biofilms and virulence involves hundreds of genes and regulators. From eight currently isolated *Escherichia coli*, five biofilms, and virulence genes were analyzed, including *csgA*, *Crl*, *csgD*, *stx*1, and *stx*2. Curli are adhesive organelles formed by enteric bacteria such as *E. coli* and *Salmonella* spp. for irreversible attachment after flagella loss (Prüss et al., 2006). Most strains of *E. coli* have the type I fimbriae and curli (aggregative fimbriae) genes, but not all strains phenotypically produce these structures. The pro-inflammatory response is triggered by these structures and is linked to biofilm formation (Olsen et al., 1993). The csg gene cluster, which consists of two divergently transcribed operons, codes for curli fibers. The genes *csgB*, *csgA*, and *csgC* are encoded by one operon, while *csgD*, *csgE*, csgF, and *csgG* are encoded by the other (Gophna et al., 2001). During the development of the fibers, *CsgA* extracellularly self-assembles the curlin subunit, and this action is reliant on a specific nucleator protein controlled by the *CsgB* gene. The outer membrane lipoprotein *CsgG* is involved in the extracellular stabilization of CsgA and CsgB, and the transcriptional activator *CsgD* is necessary for the expression of the two curli fiber operons(Loferer et al., 1997). Curli fimbriae and exopolysaccharide cellulose production, whether in vivo or in vitro, are undoubtedly the most common contributors to medium to high biofilm formation and invasion by commensal and pathogenic *E. coli* and *Salmonella*spp (Gophna et al., 2001). Genetic characterization revealed that the majority of currently isolated pathotypes had the *csgA* biofilm-associated gene, which is consistent with earlier findings that csgA was prevalent in all eight pathotypes tested (99.5%) (Schiebel et al., 2017). In the present investigation, *CsgA* was expressed by six of the eight analyzed *E. coli* isolates, but fortunately not *CsgD*, a gene that transcriptionally controls the production of curli and cellulose as cells change from a planktonic to a multicellular state (Brombacher et al., 2006). *CsgA*, however, has been demonstrated in a prior study to be a protein that is necessary for the formation of biofilm and cell invasion in *E. coli* O157:H7 (Uhlich et al., 2009). Furthermore, according to earlier research, though constitutive *CsgD* expression encourages the development of biofilms and activates transcription from the *csgBAC* promoter; however, *csgBAC* expression is largely influenced by the growth medium and temperature. Additionally, *Crl* is one of a vast network of regulatory proteins for *Csg* production, along with *OmpR*, *RpoS*, *CsgD*, H-NS, and host factor integration. This may explain the co-expression of *Crl* in all *CsgA* genes found in currently isolated *E. coli*. In summary, *Crl* controls the transcription of the csgAB operon through particular protein-protein interactions with RpoS that encourage the binding of the sigma-holoenzyme to the *csgA* promoter (Lelong et al., 2007). This research supports previous findings that many control elements play a role in the complex expression of curli fiber genes (Mika and Hengge, 2014). The *Crl* gene stimulates the expression of various alternative sigma factor RpoS regulon components either negatively or positively, in particular when RpoS levels in cells are low. Although the alternative sigma factor RpoS and the genes it regulates are not necessary for *Escherichiacoli* growth, they play a crucial role in the organism's ability to adapt to a variety of unfavorable growth conditions by regulating the expression of numerous genes that aid in stress resistance and nutrient scavenging (Schellhorn, 2020). In the exponential phase, *Crl* slightly stimulates RpoS's transcription, and in the stationary phase, it regulates a subset of RpoS-dependent genes (Dudin et al., 2013). The growth of STEC biofilms on the surface of numerous pieces of equipment used in the processing and slaughtering frequently causes the contamination of beef carcasses at various stages of processing in food processing facilities (Rivera-Betancourt et al., 2004).

Unfortunately, the current characterized emerging *E. coli* with Shiga toxin and biofilm production ability may negatively contribute to Egypt's high incidence of bloody diarrhea. These biofilm-contaminated tools could potentially contaminate fresh meat products and unadulterated carcasses (Vogeleer et al., 2014). Popular Egyptian purchasing habits involving fresh meat, particularly from butcheries, and noncompliance with hygiene standards recommended by the Egyptian national food safety authority raise worries about the possible contamination of animal-derived foods and, eventually, humans.

5. CONCLUSION

In conclusion, in 20% of swabbed samples, *E.* coli was confirmed. Butchers had higher rates of targeted pathogen detection than supermarkets (24% vs. 16%) ($P > 0.05$). The eight serotypes of *Escherichia coli* isolates were classified into four pathotypes (55% *EHEC*, 20% *EPEC*, 20% *ETEC*, and 5% *EIEC*. Six of the eight *E. coli* isolates co-expressed *Crl* in all *CsgA* biofilm genes, with four of them, *EHEC*, also expressing *Stx1*. The genetic identification of virulence factors like *Stx1*, genes from pathogens isolated from surfaces that come into contact with meat, especially in butcheries, combined with a high incidence of biofilm-producing genes, is a serious concern that needs to be immediately controlled; otherwise, it could potentially contaminate fresh meat products and carcasses, and eventually people via the food chain.

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