MOLECULAR DETECTION OF MULTIDRUG RESISTANT *Escherichia coli* ISOLATED FROM BUFFALO MEAT AND OFFAL

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ABSTRACT

Water buffalo (*Bubalus bubalis*) is a significant source of high-quality milk and meat in Egypt. The goal of this research was to investigate the serotypes, virulence genes, and antibiotic resistance profiles of *Escherichia coli* (*E. coli*) detected in buffalo meat and offal. *E. coli* was detected at 26.67%, 56.67%, 60%, 13.33%, and 23.33% of buffalo meat, minced meat, liver, heart, and kidney, respectively. The detected *E. coli* was serologically identified as enteropathogenic *E. coli* (EPEC) O78, O2:H6, O1:H7, O153:H2 and O153:H11. Enterohemorrhagic *E. coli* (EHEC) O91: H21 and O26: H11 in addition to enterotoxigenic *E. coli* (ETEC) O127:H6, O126:H11, and O128:H2. All of the isolated *E. coli* was resistant to penicillin. Furthermore, 85%, 77.5%, 72.5%, 60%, 55%, and 52.5% of the isolates were resistant to erythromycin, ampicillin, cephalothin, nalidixic acid, kanamycin, and oxacillin, respectively. Thirty-one out of forty examined *E. coli* (77.5%) were resistant to at least three antibiotic classes and thus were considered as multidrug resistant (MDR) *E. coli*. Ten MDR isolates were submitted for molecular determination of virulence genes including iron uptake (*iut*A) gene, attaching and effacing (*eae)* gene, and increased serum survival (*iss)* gene, with detection rates of 80%, 30%, and 100%, respectively. In conclusion, buffalo meat and offal can be considered as potential sources of multidrug resistant *E. coli*.

Keywords: *Bubalus bubalis*, buffaloes, buffalo meat, offal, *E. coli*, multidrug resistance, virulence genes

INTRODUCTION

Water buffalo is a potential source of meat that has grown in popularity in Egypt in recent years. Buffalo converts low-quality feed into muscle growth. Increased buffalo meat production would have a significant impact on both human nutrition and the economy. Meat quality is the most important factor in consumer acceptance, and it is determined by its chemical composition, physical characteristics, texture, and microbial profiles **(**Ziauddin *et al*., 1994**)**. Contamination of

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buffalo meat and offal with *E. coli* usually results from bowel rupture during evisceration, indirect contamination with tainted water, and finished product handling and packaging **(**Elabbasy *et al*.,2021**)**. *Escherichia coli* is ubiquitous in human gastrointestinal tract; they are frequently exist in this environment without endangering host health. *E. coli*, on the other hand, is capable of expressing virulence traits that is responsible for a variety of diarrheal disease syndromes that are common around the world. It is estimated that 200 million people are affected by diarrhea on any given day (Bélanger *et al*., 2011). Based on distinct virulence properties, different interactions with intestinal mucosa, distinct clinical syndromes, differences in epidemiology, and distinct O:H serogroups, food-borne *E. coli* is classified as follows: Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), Enterohaemorrhagic *E. coli* (EHEC), and Verocyto-toxigenic *E. coli* (VTEC) (FAO and WHO, 1991). Antibiotic consumption has increased by approximately 40% in a decade because of the emergence of bacteria, but resistance has become a global threat. Aside from clinical settings, antibiotics are widely used in agriculture, food processing, animal husbandry, and aquaculture **(**Van Boeckel *et al*., 2015**)**. The use of broad-spectrum antibiotics places a selective pressure on the bacterial flora, resulting in the emergence of new antibiotic-resistant bacteria **(**Schjørring and Krogfelt, 2011). Resistance to antibiotics in bacterial pathogens is higher in poultry, pigs than other meat animals. As a result, antibiotic-resistant strains from the gut may contaminate meat during slaughtering, and resistant bacteria may infect consumers through the meat. Several countries have reported the transmission of resistant bacteria from meat to humans (Bantawa *et al*., 2019). The current study, therefore, intended to investigate the prevalence of *E. coli* in buffalo meat and offal. Furthermore, determination of the resistance level to antimicrobials and virulence genes in the isolated *E. coli*.

MATERIALS AND METHODS

Samples collection and identification of *E. coli*

A total of 150 random samples of buffalo meat, minced meat, liver, kidney, and heart (n = 30, each) were collected from different butcher shops of different sanitation levels at Zagazig city- Sharkia province, Egypt. For the isolation of *E. coli* from buffalo meat and offal, the standard protocol of APHA (2001) technique was used, which included adding 25 g of each sample to 225 mL of buffered peptone water. This mixture was incubated at 37 ºC for 18 to 24 h, then loop full were streaked on Eosin Methylene Blue (EMB) (Oxoid), 37ºC for18 to 24 h, typical *E. coli* colonies were picked and inoculated in nutrient agar for purification. The isolates were identified by cultural characteristics, Gram staining, and biochemical tests as described by Bergey's Manual of Determinative Bacteriology (Garrity *et al*., 2005). Serological identification of *E. coli* was done by using rapid diagnostic antisera sets (DENKASEIKEN Co., Japan) according to Kok *et al*. (1996).

Antimicrobial resistance

Antimicrobial resistance (AMR) was assessed using the disc diffusion method with *E. coli*-ATCC 25,922 as a reference strain for 40 selected isolates (4 from each represent the serotypes O_{78}, O_2:H_6, O_1:H_7, O_{153}:H_2, O_{153}:H_{11}, O_{91}:H_{21}, O_{26}:H_{11}, O_{127}:H_6, O_{126}:H_{11}, O_{128}:H_2). According to Clinical and Laboratory Standards

Institute (CLSI, 2017) recommendations, the disc diffusion method was used with *E. coli*-ATCC 25,922 as a reference strain. Antimicrobial discs were used: erythromycin (E) (15 µg), penicillin (P) (10 UI), oxytetracycline (T) (30 µg), ampicillin (AM) (10 µg), nalidixic acid (NA) (30 µg), sulfamethoxazole (SXT) (23.75 µg), enrofloxacin (EX) (5 µg), cephalothin (CN) (30 µg), oxacillin (OX) (1 µg), neomycin (N) (30 µg), kanamycin (K) (30 µg), ciprofloxacin (CP) (5 µg), chloramphenicol (C) (30 µg), gentamicin (GEN) (10 µg). The relevant drug-impregnated discs were placed on the agar surface after each strain's inoculum was streaked on Mueller-Hinton agar (Himedia, Mumbai, India). The multiple antibiotic resistance index (MARI) was studied. The MARI is a tool for assessing health risks. This indicator is useful for determining the spread of bacterial resistance in a community when the isolate is resistant to more than three drugs (Christopher *et al*., 2013). The MAR index is calculated by dividing the number of antibiotics to which the tested serotypes showed resistance by the total number of antibiotics examined for sensitivity. A MARI score of more than 0.2 implies a high risk of contamination and antibiotic use in the environment.

PCR Assay

Polymerase chain reaction (PCR) monitoring of *iss*, *iut*A genes as described by Yaguchi *et al*. (2007). The *eae*A virulence-determinant gene was carried out as described by Wang *et al*. (2002). The genomic DNA of the examined strains was extracted according to the manufacturer's instructions for the QIAamp DNA Mini Kit (Qiagen, GmbH, Germany/Catalog No.51304). The primer pairs and condition are shown in Table (1)

RESULTS AND DISCUSSIONS

Due to a relative increase in the production and consumption of buffalo meat and offal in Egypt, it was decided to determine the prevalence of *E. coli* in the buffalo meat and offal. The results of this study showed that 8/30 (26.67%), 17/30(56.67%), 18/30(60%), 4/30(13.33%), and 7/30(23.33%) of buffalo meat, minced meat, liver, heart, and kidney, respectively harbored *E. coli* (Table 2). *E. coli* was previously recovered from buffalo meat samples with rates ranged from 19.5% to 32.4% (Biswas *et al*., 2008) and 8.8% (Shekh *et al*., 2013) in India. In addition, *E. coli* was detected in 54% of examined meat samples in Nepal (Bantawa *et al*., 2019). The level of contamination may be due to contamination of hides, unhygienic processing of the carcass during handling that led to raw meat and offal contamination with *E. coli*. Furthermore, surrounding environments have been contaminated through poor disposal contaminated bowel content and feces of animals. Serological identification of *E. coli* reveled presence of enteropathogenic (EPEC) O_{78}, O_2:H_6, O_1:H_7, O_{153}:H_2 and O_{153}:H_{11}. Enterohemorrhagic (EHEC) O_{91}: H_{21} and O_{26}: H_{11} in addition to, enterotoxigenic (ETEC) O_{127}:H_6, O_{126}:H_{11}, and O_{128}:H_2 types (Table 2). Nearly similar *E. coli* O_{26}, O_{45}, O_{103}, O_{111}, O_{121}, O_{145}, and O_{157} in ground beef, beef trim, serotypes were obtained by Wang *et al*. (2012).

Forty *E. coli* isolates from 150 buffalo samples were examined for antibiotic susceptibility against 14 antibiotics as shown in Table 3. Higher resistance rates were detected for penicillin (100%), erythromycin (85%), ampicillin (77.5%), cephalothin (72.5%), nalidixic acid (60%), kanamycin (55%), oxacillin (52.5%). Moderate resistance was detected against oxytetracycline and sulfamethoxazole (45%), chloramphenicol

(42.5%), neomycin (32.5%). Meanwhile, *E. coli* isolates were susceptible to gentamicin (67.5%), enrofloxacin (85%), and ciprofloxacin (92.5%). It was not surprising to detect the level of antibiotic resistance where incorrect antibiotic use resulted in the presence of antibiotic residues in cattle and buffalo meat and offal (Morshdy *et al*., 2013). Antimicrobial resistance in *E. coli* has been reported worldwide. For instance, in Ghana; *E. coli* isolated from meat showed resistant 44.44% to tetracycline and chloramphenicol, 68.89% to erythromycin, but 95.56% of *E. coli* was susceptible to ciprofloxacin (Adzitey, 2020). Aslam and Service (2006) found that *E. coli* originated from beef sources in Canada resist chloramphenicol (2.45%) and tetracycline (38%). In Bangladesh, antimicrobial resistance in *E. coli* population from beef samples was 85.71% and 71.43% for erythromycin and oxytetracycline, respectively. Meanwhile, 100% of the isolates were sensitive to ciprofloxacin and gentamicin (Rahman *et al*., 2017). In India, *E. coli* isolated from buffalo meat samples showed resistance rates of 52%, 40%, 36%, 24%, 8%, 8%, and 4% to oxacillin, nalidixic acid, tetracycline, erythromycin, ciprofloxacin, kanamycin and chloramphenicol, respectively (Anas and Malik, 2021). The difference in the resistance pattern may be attributed to the antibiotic classes used for treatment and growth promotion in various countries.

In our study, the MARI revealed that 31/40 (77.5%) isolates of *E. coli* strains had very high MARI value (>0.21) (Table 4), meaning that they were resistant to at least three antibiotic classes. Relatively, 50% of isolated *E. coli* from Indian buffalo meat was considered as MDR *E. coli* (Anas and Malik, 2021). Previous studies have documented the emergence, multiplication, accumulation, and maintenance of antimicrobial-resistant pathogenic *E. coli* in both human and veterinary medicine (Molina-López *et al*., 2011). Antibiotics are frequently used to treat infected humans and animals, as well as for prevention and growth promotion in livestock. Many data demonstrate that poor antibiotic selection and overuse may lead to resistance in diverse bacteria, making bacterial infection treatment more challenging (Kolář *et al*., 2001).

Because *E. coli* is the most common Gram-negative bacterium in humans, it is also the most common cause of urinary tract infections, a common cause of both community and hospital-acquired bacteremia, and a cause of diarrhea (Kaper *et al*., 2004). Furthermore, resistant *E. coli* strains can transfer antibiotic resistance genes to other *E. coli* strains in the gastrointestinal tract, as well as acquire resistance from other organisms (Österblad *et al*., 2000). Although *E. coli* serotyping is a standard approach for making appropriate diagnosis and conducting epidemiological investigations during foodborne outbreaks, it cannot be depended on alone to categories a strain of *E. coli* and thus the identification of specific virulence genes is required as well (Barlow *et al*., 1999). In our study, ten isolates were submitted for molecular determination of virulence genes. It was found that the iron uptake gene *iut*A gene was detected in 80% of the tested *E. coli* isolates (Figure, 1A). Furthermore, two serotypes O_1:H_7 (enteropathogenic *E. coli*) and O_{91}: H_{21} (enterohemorrhagic *E. coli*) contained the 3 examined genes as shown in Table 5. The *iut*A gene was previously detected in 50% of *E. coli* isolated from urinary tract infection in Mexico (lopez-banda *et al*., 2014), 35.1% extraintestinal pathogenic *E. coli* (Jørgensen *et al*., 2019), 40% of *E. coli* isolated from meat and meat products from Egypt (Hanan *et al*., 2020). The presence of *iut*A in *E. coli* shows that it can utilize heme,

Table 1. Oligonucleotide primers sequences encoding for amplification of virulence genes.

Gene	Primer sequence (5'-3')	Conditions	PCR Product (bp)
*iut*A	GGCTGGACATGGGAACTGG	94°C 5 minutes, 94°C 30 seconds, 63°C 30 seconds, 72°C 30 seconds, for 35 cycle, 72°C 7 minutes.	300
	CGTCGGGAACGGGTAGAATCG		
*eae*A	ATGCTTAGTGCTGGTTTAGG	94°C 5 minutes, 94°C 30 seconds, 51°C 30 seconds, 72°C 30 seconds, for 35 cycle, 72°C 7 minutes.	248
	GCCTTCATCATTTCGCTTTC		
iss	ATGTTATTTTCTGCCGCTCTG	94°C 5 minutes, 94°C 30 seconds, 54°C 30 seconds, 72°C 30 seconds, for 35 cycle, 72°C 7 minutes.	266
	CTATTGTGAGCAATATACCC		

Table 2. Prevalence and Serotyping of *E. coli* strains isolated from buffalo meat and offal samples (n= 30).

Serotypes		Meat		Minced meat		Liver		Heart		Kidney	
		No	%	No	%	No	%	No	%	No	%
E. coli		8	26.67	17	56.67	18	60.00	4	13.33	7	23.33
EPEC	O78	1	3.33	2	6.66	2	6.66	-	-	1	3.33
	O2:H6	1	3.33	1	3.33	3	10	1	3.33	2	6.66
	O1:H7	2	6.66	2	6.66	1	3.33	1	3.33	1	3.33
	O153:H2	-	-	2	6.66	2	-	-	-	-	-
	O153:H11	-	-	1	3.33	1	3.33	-	-	-	-
EHEC	O91:H21	1	3.33	2	6.66	2	6.66	-	-	-	-
	O26:H11	1	3.33	2	6.66	2	6.66	1	3.33	1	3.33
ETEC	O127:H6	-	-	2	6.66	2	6.66	-	-	1	3.33
	O126:H11	2	6.66	2	6.66	1	3.33	1	3.33	-	-
	O128:H2	-	-	1	1	2	6.66	-	-	1	3.33

EPEC: enteropathogenic *E. coli*; EHEC: enterohemorrhagic *E. coli*. ETEC: enterotoxigenic *E. coli*.

Table 3. Antimicrobial resistance pattern of the isolated *E. coli* strains from buffalo meat and offal (n = 40).

Antimicrobial Agent	Sensitive	Intermediate	Resistant
penicillin (P)	0	0	40 (100%)
erythromycin (E)	2 (5%)	4 (10%)	34 (85%)
ampicillin (AM)	6 (15%)	3 (7.5%)	31 (77.5%)
cephalothin (CN)	7 (17.5%)	4 (10%)	29 (72.5%)
nalidixic acid (NA)	9 (22.5%)	7 (17.5%)	24 (60%)
kanamycin (K)	10 (25%)	8 (20%)	22 (55%)
oxacillin (OX)	12 (30%)	7 (17.5%)	21 (52.5%)
oxytetracycline (T)	14 (35%)	8 (20%)	18 (45%)
sulfamethoxazole (SXT)	18 (45%)	4 (10%)	18 (45%)
chloramphenicol (C)	20 (50%)	3 (7.5%)	17 (42.5%)
neomycin (N)	23 (57.5%)	4 (10%)	13 (32.5%)
gentamicin (GEN)	27 (67.5%)	3 (7.5%)	10 (25%)
enrofloxacin (EX)	34 (85%)	2 (5%)	4 (10%)
ciprofloxacin (CP)	37 (92.5%)	1 (2.5%)	2 (5%)

Table 4. Multiple antibiotic resistance (MAR) index and antimicrobial resistance profile of the isolated *E. coli* strains from buffalo meat and offal (n = 40).

Resistance pattern	Resistance profile	Number of isolates	Number of antibiotics	MARI
1	P, E, AM, CN, NA, K, OX, T, SXT, C, N, GEN, EX, CP	2	14	1
2	P, E, AM, CN, NA, K, OX, T, SXT, C, N, GEN, EX	2	13	0.92
3	P, E, AM, CN, NA, K, OX, T, SXT, C, N, GEN	6	12	0.85
4	P, E, AM, CN, NA, K, OX, T, SXT, C, N	3	11	0.78
5	P, E, AM, CN, NA, K, OX, T, SXT, C	4	10	0.714
6	P, E, AM, CN, NA, K, OX, T, SXT	1	9	0.642
7	P, E, AM, CN, NA, K, OX	3	7	0.5
8	P, E, AM, CN, NA, K	1	6	0.428
9	P, E, AM, CN, NA	2	5	0.357
11	P, E, AM, CN	5	4	0.285
12	P, E, AM	2	3	0.21
13	P, E	3	2	0.142
14	P	6	1	0.071

MAR Index = a/b, where 'a' equal the number of antimicrobials to which the isolates were resistant and 'b' the total number of antimicrobials to which an *E. coli* isolate was tested.

Table 5. Distribution of virulence genes among *E. coli* serotypes.

No	Serotype	*IutA*	*eaeA*	*Iss*
1	O78	-ve	-ve	+ve
2	O2:H6	-ve	+ve	+ve
3	O1:H7	+ve	+ve	+ve
4	O153:H2	+ve	-ve	+ve
5	O153:H11	+ve	-ve	+ve
6	O91:H21	+ve	+ve	+ve
7	O26:H11	+ve	-ve	+ve
8	O127:H6	+ve	-ve	+ve
9	O126:H11	+ve	-ve	+ve
10	O128:H2	+ve	-ve	+ve

No: isolate number -ve: negative; +ve: positive.

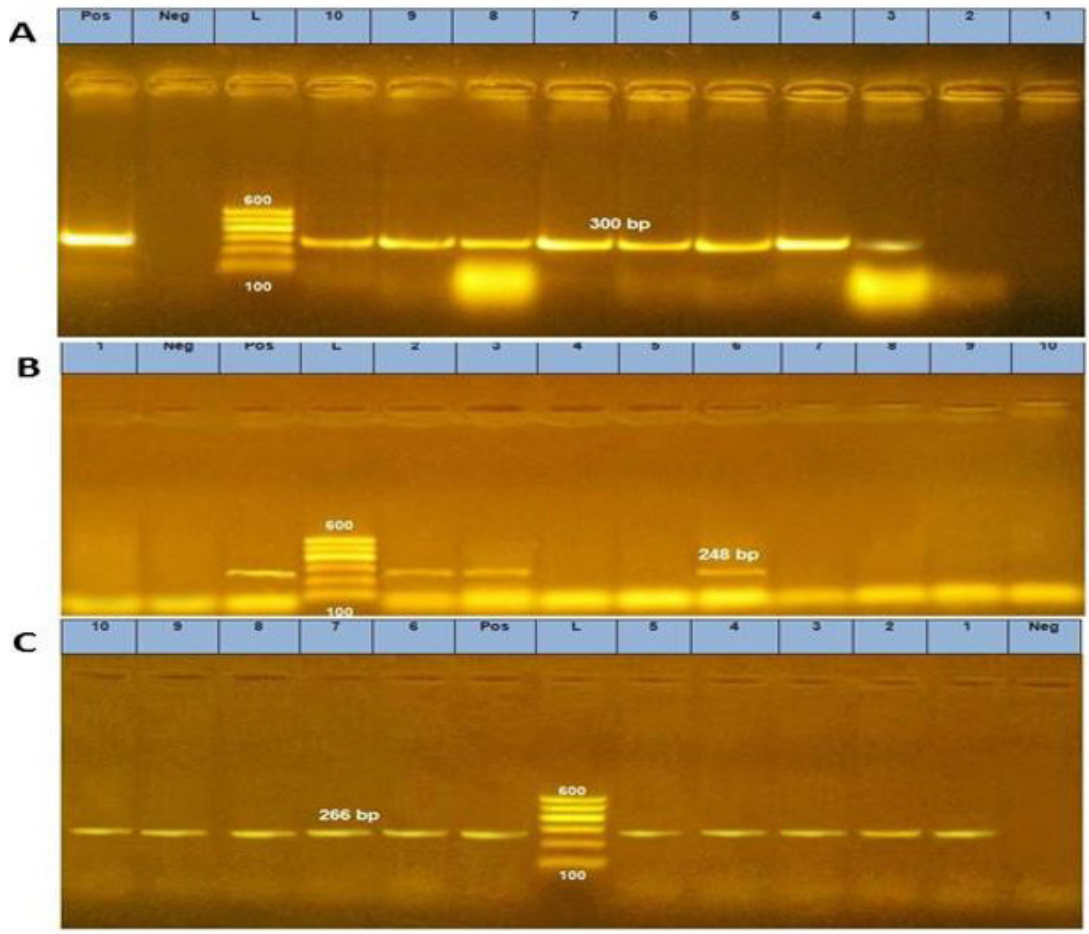

Figure 1. Electrophoretic pattern of the PCR products of (A): *IutA* gene (300 bp); (B): *eaeA* gene (248 bp; (C): *Iss* gene (266 bp); Lane L: Ladder 100 plus bp (100-600); Lanes (1-10): represents the tested strains, positive control (Lane. pos) and negative control (Lane. neg).

heme attached to haemoglobin, hemoglobin-haptoglobin, myoglobin, and hemopexin from its hosts (Wandersman and Stojiljkovic, 2000).

The attaching and effacing *eae,* gene encoding intimin responsible for the production of outer-membrane proteins, such as intimin which allows *E. coli* to adhere and colonize in the small intestine and the urethra (Oswald *et al*., 2000). The *eae* gene was detected in 30% of examined *E. coli* (Figure, 1B). Lower detection limit from beef products 9.1% was reported in USA (Hill *et al*., 2010). Meanwhile, this gene was not detected in ground beef and lamb meat cuts in Australia (Barlow *et al*., 2006). The increased serum survival gene *iss* gene was detected in 100% (Figure, 1C) of examined *E. coli* isolates. Similarly, the same gene was detected in 100% of extraintestinal pathogenic *E. coli* in Canada (Jørgensen *et al*., 2019) and in chicken from Brazil (Oliveira *et al*., 2019). However, it was detected in 20% of the extraintestinal pathogenic *E. coli* isolates from Brazil (Cyoia *et al*., 2015). and 60% of *E. coli* isolated from meat and meat products from Egypt (Hanan *et al*., 2020). Taken together, the presence of MDR *E. coli* in the examined buffalo meat and offal with high rates suggests the increased public health risk posed by these important bacteria, especially in cases of zoonotic transmission.

CONCLUSION

The obtained data suggest that buffalo meat and offal could be a potential source of MDR pathogenic *E. coli*. Therefore, cautions are necessary to decrease the incidence of multi-drug resistant *E. coli* in animals and people. To achieve this, good hygienic practices are necessary from the farm to the table, especially in the slaughterhouse to prevent contamination of buffalo products and abattoir environment with intestinal content.

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