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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Prevalence of *Enterobacteriaceae* in Table Eggs with Particular Reference to Enterovirulent *Escherichia coli* Strains

Maha A.M. AL-Ashmawy
Department of Food Hygiene and Control, Faculty of Veterinary Medicine,
Mansoura University, Mansoura - 35516, Egypt

Abstract: A survey of the microbial quality based on enterobacteriaceae counts of the table eggs sold in Egyptian markets was conducted to evaluate their quality and the possibility of presence of Enterovirulent Escherichia coli strains. Six hundred random table egg samples were collected from different shops, supermarkets and homes. Each six pooled eggs constituted a composite sample. Higher enterobacteriaceae count/g was recorded in the content ($1.1x10^2$, 7.9x10) and in the shell ($1.2x10^2$, $2.6x10^2$) log_{10} cfu/g of Baladi hen and Duck eggs respectively. Lower enterobacteriaceae counts in brown shell and white shell hen eggs (4.9x10, 6.3x10 log_{10} cfu/g) in the content and the shell of both types. Twenty (%) of white shell and brown shell, 20, 36% of baladi hen eggs; and 36, 68% of duck eggs (content and shell) were marginally exceeded the maximum permissible count of enterobacteriaceae by European Communities Standards. *Escherichia coli, Enterobacter, Citrobacter, Klebsiella, Protus, Providencia and Shigella* had been recovered from the content and the shell of different types of table eggs. *E. coli* strains isolated from different types of table eggs were serotyped into 7 different serotypes included O_{44}, O_{111}, O_{114}, O_{125}, O_{126}, O_{127} and O_{128}. Most of these isolates (37/39) were stx2 positive. Interstingly, all stx2 positive isolates were negative for stx1 and eae genes. Enterobacteriacae count limits should be added to the microbiological criteria for fresh table eggs as regulation in Egyptian standards. Hygienic measures should applied in home produced hen and duck eggs to lower bacterial load in egg shell and subsequently in egg content.

Key words: Table eggs, enterobacteriaceae, *E. coli,* stx1 gene, stx2 gene, eae genes

INTRODUCTION

Eggs have been recognized as an important food article from the time primitive men first snatched them from the nest of wild bird. They provide a unique, well balanced source of nutrients for persons of all ages. The availability, modest cost, ease of preparation, popular taste appeal and low caloric value give eggs a deserved place in the diets especially in children diet (Layman and Rodriguez, 2009).

It is generally agreed that the microbial flora of hen eggs at the time of laying is very low. The shell acquires its first load of microorganisms at oviposition, a few are delivered from the vent and others from the nesting material, trays, soil, dust, feces, etc. The soiling microorganisms which penetrate the shell to the magma causing economic losses or public health hazards (Bruce and Drysdale, 1994).

Examining egg for the presence of members of the family enterobacteriaceae instead for *coliforms* may give a better indication of the likelihood of their presence, as well as providing more accurate information about the handling and storage of the food commodity (Roberts *et al*., 1995).

There are 30 genera in the bacterial family enterobacteriaceae (Holt *et al*., 2000). The well known enteric pathogens particularly Salmonella and *Escherichia coli* have been isolated from table eggs and their contents (Adesiyum *et al*., 2005; Hope *et al*., 2002). Other members of the family Enterobacteriacae such as *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp. and *Providencia* spp. have all been isolated from whole or cracked eggs with a potential to cause spoilage and enter the food chain through table eggs causing infection in consumers (Musgrove *et al*., 2004; Musgrove *et al*., 2008).

Escherichia coli can cause diarrheal disease in humans, referred to as diarrheagenic *E. coli.* These including enteropathogenic (EPEC), enterohaemorrahgic (EHEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), Shiga toxin-secreating (STEC), diarrhea-associated haemolytic (DHEC), entero-aggregative (EAAggEC) and cytolethal distending toxin-recreating (CDTEC) *E. coli* strains. WHO (2009) reported also that each year, infections and persistent diarrhea in children in developing countries are not rare, as observed in infants living in the Nile delta area, who experienced between 4.6 and 8.8 diarrheal episodes, with ETEC accounting for 66% of these episodes.

The production of cytotoxin in *E. coli* isolates have been extensively studied among isolates from humans and several animal species including poultry (Donnenberg, 2002; Zahraei Salehi *et al*., 2007). Little work has been done to investigate the production of cytotoxin by *E. coli* isolated from table eggs. The present work was planned

to enumerate Enterobacteriaceae in content and shell of Egyptian market eggs. The study comprised a qualitative analysis of microorganisms in the family Enterobacteriaceae including pathogenic bacteria as *Escherichia coli* and to detect Stx (stx1, Stx2) and eae genes among isolated *Escherichia coli* strains.

MATERIALS AND METHODS

Sample preparation: Six hundred hen table eggs (brown shell, White shell or Baladi eggs) and duck egg samples, 150 each, were randomly collected from different shops, supermarkets and homes. Samples were immediately transferred in sterile plastic bags (6 eggs in each) to the laboratory for examination. Each 6 eggs considered as one composite sample. 150 mL sterile buffered peptone water were poured into the egg samples in plastic bags and thoroughly mixed, then 25 mL of rinse buffered peptone water transferred to another sterile jar containing 225 mL of Tryptone Soya Broth (TSB) and mixed well. Egg shells were sterilized using ethyl alcohol 70% then were broken using a sterile blade and the contents transferred to a sterile beaker. Contents from 6 samples were pooled to form one sample. Transfer 25 mL of egg contents into sterile jar containing 225 mL of TSB, then homogenized for 30 sec.

Enterobacteriacae count: Ten fold dilutions were obtained aseptically in 0.1% peptone water up to 10^6 from homogenized egg contents or egg shells. Enterobacteriaceae were enumerated according to Roberts *et al*. (1995) by duplicate plating of 1 mL aliquots sample onto Violet Red Bile Glucose agar (VRGG). Plates were poured with overlay with a further 10 mL of molten, cooled VRBG agar to assist in the recovery of injured organisms.

Plates were incubated overnight at 37°C and observed for colony formation. Following incubation, dark red to purple colonies with red-purple haloes were counted. As many as 5 isolates for each positive sample were randomly selected from presumptive Enterobacteriaceae. These randomly selected isolates were streaked for purity onto Tryptone Soya agar plates and incubated at 37°C overnight. The procedure was repeated twice with an isolated colony to ensure purity. An isolate from third streak plate was saved on brain heart infusion agar slants incubated at 37°C then stored at 4°C, until identification analysis were performed.

Identification and characterization of isolated strains: Each stored isolate was streaked onto count agar plates and incubated overnight at 37°C. Suspected colonies were identified microscopically after gram staining and using biochemical tests according to Health Protection Agency (2010).

Serological identification of *E. coli*: The isolates proved biochemically to be *E. coli* were serological identified.

Table 1: Oligonucleotide primers used for amplification of the various targeted genes in *E. coli*

Gene	Oligonucleotide primer sequences	Size of amplified DNA (bp)
eae	Forward: 5'-GGCGGATTAGACTTCGGCTA-3'	150
	Reverse: 5'-CGTTTTGGCACTATTTGCCC-3'	
Stx1	Forward: 5'-TCCTGGTACAACTGCGGTTAC-3'	505
	Reverse: 5'-ACGCACTCTTCCATCTACCG-3'	
Stx2	Forward: 5'-CTGGCGTTAATGGAGTTCAGTGG-3'	318
	Reverse: 5'-CCTGTCGCCAGTTATCTGACA-3'	

Each isolate was first tested for its agglutinability to the diagnostic OK polyvalent sera, (Welcome) which are intended for use by slide agglutination technique. Once the pathogenic type has been indicated by the use of polyvalent sera, further serotyping was made with appropriate OK monovalent sera.

Detection of E. coli virulence genes by PCR: *E. coli* isolates were screened for the presence of virulence genes that included eae, stx1 and stx2 genes. The sequences of the three oligonucleotide primer sets for the three genes and the expected size are listed in Table 1. A single colony was used to grow on MacConkey agar plates and incubated at 37°C/24 h. Subsequently, 2-4 colonies from each plate were randomly selected using a sterile toothpick. Colonies were suspended in 50 µL distilled water and incubated at 95°C for 5 min. Following centrifugation at 13 000 rpm/1 min, direct colony PCR of the supernatant was performed with the Dream Tag Green PCR Master Mix (Fermentas). Cycling conditions were 95°C for 5 min, 35 cycles at 94°C for 20 sec, 60°C for 30 sec and 72°C for 50 sec with a final extension at 72°C for 7 min. PCR products were visualized using ethidium bromide stained 1.2% agarose gel electrophoresis.

Statistical analysis: The SPSS software (IBM, Armonk, NY, USA) was used for statistical data analysis. The ANOVA test was used for comparing sample means after log transformation of data to increase sample homogeneity.

RESULTS AND DISCUSSION

The Enterobacteriaceae family includes coliforms, fecal coliforms, *Echerchia coli* (Holt *et al*., 2000) and lactose negative facultative anaerobic rods (Mossel, 1978). The results of enterobacteriaceae count/g table eggs reported in Table 2 reveal that 44, 44, 44, 84 and 52%, 52, 80 and 96% of content and shell of brown shell, white shell, Baladi hen egg and duck egg samples contaminated with enterobacteriaceae organisms, respectively, with mean counts in egg content 4.9x10, 4.9x10, $1.1x10^2$ and 7.9x10 (log_{10} cfu/g) in brown shell, white shell, Baladi hen egg and duck egg samples, respectively. Counts in egg shell were 6.3x10, 6.3x10, 1.2×10^2 and 2.6×10^2 (log_{10} cfu/g), respectively.

Table 2: *Enterobacteriaceae* count on Violet Red Bile Glucose Agar medium (VRBG) from different types of table eggs (25 composite sample each)

		Positive samples		Counts (log_{10} cfu/ g)		
Type of eggs	Type of samples	No.	%	Min.	Max.	Mean±SE
White shell hen egg	Content	11	44	1.0x10	$7.0x10^2$	$4.9x10±1.7^a$
	Shell	13	52	1.0x10	$1.0x10^3$	$6.3x10±1.6^c$
Brown shell hen egg	Content	11	44	1.0x10	$7.0x10^2$	$4.9x10±1.7^a$
	Shell	13	52	1.0x10	$1.0x10^3$	$6.3x10±1.6^c$
Baladi hen egg	Content	11	44	2.0x10	$2.0x10^3$	$1.1x10^2±1.6^a$
	Shell	20	80	1.0x10	$2.5x10^3$	$1.2x10^2±1.5^b$
Duck egg	Content	21	84	1.0x10	$2.5x10^3$	$7.9x10±1.3^a$
	Shell	24	96	1.0x10	$1.1x10^4$	$2.6x10^2±1.4^a$

[a]No significant difference at p = 0.05

[a,b,c]Means with significant difference between table egg samples at p=0.05 in egg shell counts

Table 3: Frequency distribution of examined table hen eggs based on Enterobacteriaceae count (cfu/g)

	White shell egg		Brown shell egg		Baladi egg		Duck egg	
Intervals	Content No (%)	Shell No (%)	Content No (%)	Shell No (%)	Content No (%)	Shell No (%)	Content No (%)	Shell No (%)
0-<10^1	14 (56)	12 (48)	14 (56)	12 (48)	14 (56)	5 (20)	4 (16)	1 (4)
10^1<10^2	6 (24)	8 (32)	6 (24)	8 (32)	6 (24)	11 (44)	12 (48)	7 (28)
10^2<10^3	5 (20)	3 (12)	5 (20)	3 (12)	3 (12)	6 (24)	8 (32)	13 (52)
10^3<10^4	0 (0)	2 (8)	0 (0)	2 (8)	2 (8)	3 (12)	1 (4)	4 (16)
Total	25 (100)	25 (100)	25 (100)	25 (100)	25 (100)	25 (100)	25 (100)	25 (100)

<10 cfu is considered negative

The presence of enterobacteriaceae in table eggs were reported by several investigators (Adesiyum *et al*., 2005; Adesiyun *et al*., 2006; Jones *et al*., 2004; Musgrove *et al*., 2004; Musgrove *et al*., 2008).

The frequency distribution of examined table egg samples (Table 3) indicate that 20% of white shell and brown shell hen eggs (content and shell) marginally exceed the desire maximum count of enterobacteriaceae (10^2/g) by European Communities (1993), while 20,36% (content and shell) Baladi hen eggs; 36, 68% (content and shell) duck egg samples exceeded these microbiological standards.

In this study, commercially produced table eggs (white shell and brown shell hen eggs) less contaminated with enterobacteriaceae organisms and showed low counts than home produced table eggs (Baladi hen eggs and duck eggs). Hannah *et al*. (2011) proved that housing hens in cages with manure removal belts results in lower bacterial load for both washed and unwashed eggs. High levels of external shell contamination can adversely affect the shelf life and food safety of eggs. De Reu *et al*. (2006), Messens *et al*. (2007) and Smith *et al*. (2000), reported that increasing numbers of microorganisms on the egg shell consequently increase the risk of microbial egg shell penetration and egg content contamination.

Random selection of colonies from VRBG plates allowed us to determine prevalent species in both shell and content. Many genera of enterobacteriaceae including *Escherichia coli, Enterobacter, Citrobacter, Klebsiella, Protus, Providencia and Shigella* have been recovered from content and shell of different types of table eggs. (Table 4). The most frequently isolated bacteria were *Enterobacter, Citrobacter, E. coli* and *Klebsilla*. These organisms have 37°C as their optimal growth temperature and are commonly isolated from the intestinal tracts of vertebrate animals (Holt *et al*., 2000). These findings similar with those reported by Adesiyum *et al*. (2005); Adesiyum *et al*. (2006).

Esherichia coli population can be used as measures of quality and sanitary processing condition (Kornacki and Johnson, 2001; Ricke *et al*., 2001). Also, it is an ideal indicator organisms of fecal contamination, in human and animal feces, 90-100% of *coliform* organisms isolated are *E.coli* (Hurst *et al*., 2002).

The conventionally identified *E.coli* strains isolated from different types of table eggs were serotyped into 7 different serotypes included O_{44}, O_{111}, O_{114}, O_{125}, O_{126}, O_{127} and O_{128}.

E. coli serotyping is an important technique for making the proper diagnosis and epidemiological investigations during food borne outbreaks. Thus serotyping alone cannot be relied on for categorizing a strain of *E. coli* and the identification of specific virulance characteristics/genes must also be performed (Barlow *et al*., 1999). Ansaruzzaman *et al*. (2007) reported that classification of ETEC strains, largely based on O-antigen type, Colonization Factor (CF) expression pattern and toxin profile.

Most of *E. coli* isolates in this study carried stx2 type gene. (37/39) were stx2-positive (Fig. 1), there was no isolate carrying stx1 gene sequence. Interestingly, all the stx2 positive isolates were negative for Stx1 and eae genes (Table 5). Available data from previous work

Table 4: Incidence of Enterobacteriaceae isolated from different types of eggs on Violet Red Bile Glucose Agar medium (VRBG)

	White shell egg		Brown shell egg		Baladi egg		Duck egg	
Isolated species	Content No (%)	Shell No (%)	Content No (%)	Shell No (%)	Content No (%)	Shell No (%)	Content No (%)	Shell No (%)
E.coli	0 (0)	1 (4)	1 (4)	8 (32)	8 (32)	8 (32)	1 (4)	4 (16)
Enterobacter	10 (40)	12 (48)	5 (20)	2 (8)	2 (8)	6 (24)	8 (32)	11 (44)
Citrobacter	0 (0)	2 (8)	5 (20)	4 (16)	4 (16)	4 (16)	8 (32)	5 (20)
Klebsiella	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	2 (8)	3 (12)
Proteus	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)
Providenciae	0 (0)	0 (0)	0 (0)	1 (4)	1 (4)	1 (4)	0 (0)	1 (4)
Shigella	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)

Table 5: Serotypes and virulence genes in of identified *Escherichia coli* strains from examined egg samples

Pathotypes and serotypes	No. of strains		Type of contaminated sample				Virulence genes		
	Content	Shell	WS HE	BS HE	Baladi HE	DE	eae	Stx1	Stx2
STEC									
$O_{44}:K_{74}$	3	3	1	2	4	5	-	-	+
$O_{111}:K_{58}$	-	1	-	-	-	1	-	-	+
$O_{114}:K_{90}$	1	3	1	-	2	1	-	-	+
$O_{125}:K_{70}$	2	3	-	2	2	1	-	-	+
$O_{125}:K_{70}$	-	2	-	1	1	-	-	-	+
$O_{126}:K_{71}$	-	3	-	2	-	1	-	-	+
$O_{127}:K_{63}$	2	6	2	-	2	4	-	-	+
$O_{127}:K_{63}$	3	4	-	2	3	2	-	-	+
$O_{128}:K_{67}$	-	1	-	-	-	1		-	+
Untyped	0	2	-	0	0	2	-	-	-
Total	11	28					0	0	37

HE: Hen egg, DE: Duck egg

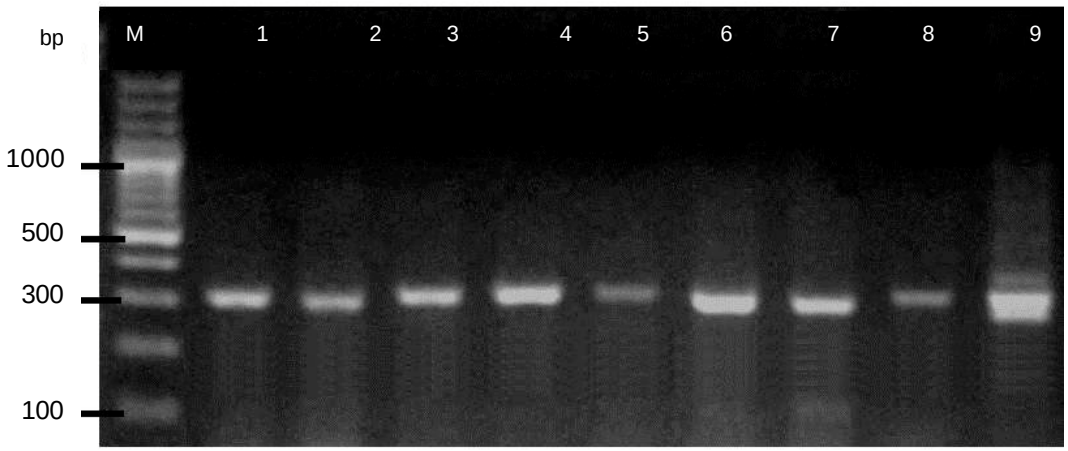

Fig 1: Agarose gel electrophoresis of stx2 gene amplicons. Lanes from 1 to 9 were amplicons from enterovirulent *E. coli* isolates

confirm that table eggs represent a source of STEC strains, characterized by the frequent presence of virulence genes associated with disease in human. Zahraei Salehi *et al.* (2007) reported that high percentage 75% (9/12) of the Avian pathogenic *Escherichia coli* isolates carried only Stx2 gene sequence. Also, Parriera and Gyles (2002) reported that all the stx-positive isolates were negative for the eae gene in their study.

The heat stable toxin (Stx) causes disruption of chloride channels in the cell and secretion of fluid and electrolytes into the intestinal lumen causing diarrhoea. (Gaastra and Svennerholm, 1996)

Conclusions: We strongly recommend that the maximum limit Enterobacteriacae count should be added to the microbiological criteria for fresh table eggs as regulation in Egyptian standards. Hygienic measures should applied in home produced hen and duck eggs to lower bacterial load in egg shell and subsequently in egg content. Also, table eggs should be refrigerated during its storage in

supermarkets and small shops. Further investigation should be applied on pathogenic isolates from table eggs and the cytotoxicity should be detected on Vero cell to determine the possible shiga toxin production.

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