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OCCUERRENCE OF THERMOTOLERANT *CAMPYLOBACTER*S IN TABLE EGGS

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ABSTRACT

Thermotolerant *Campylobacter* spp. particularly *C. jejuni* and *C. coli* are recognized as the leading causes of bacterial foodborne diarrheal disease throughout the developed and developing world for more than century. A total of 500 eggs of Baladi and large scale eggs (250 eggs each) were collected from farmers' houses and markets in Qena Governorate. Samples were divided into pools (each pool contained 5 eggs obtaining 100 pools) and were examined for existence of thermotolerant *Campylobacter* spp. on their egg shells and in their inner content. The biochemically identified *C. jejuni* and *C. coli* isolates were confirmed using multiplex PCR. Thermotolerant *Campylobacter* spp. were isolated from 15 and 5% of egg shell and egg content samples, respectively. Isolates from egg shell samples were distributed as *C. jejuni* (9%), *C. coli* (4%), *C. lari* (1%) and *C. upsaliensis* (1%), while isolates from egg content samples were identified as *C. jejuni* (4%) and *C. lari* (1%). Large scale egg samples which are produced by poultry farms lacked thermotolerant *Campylobacter* spp. Whereas, 30 and 10% of Baladi egg samples collected from farmers' houses were contaminated with thermotolerant *Campylobacter* spp. on their egg shell and in their content, respectively. This study focuses on the risk of transmission of thermotolerant *Campylobacter* spp. through table eggs, especially that produced by farmers' houses (Baladi egg), in Qena.

Key words: Thermotolerant *Campylobacter* spp., Table eggs, *C. jejuni*, *C. coli*, mPCR.

INTRODUCTION

The significance of thermotolerant *Campylobacter* as a foodborne pathogen of major public health concern is well recognized worldwide due to the high burden of disease caused by this species (EFSA, 2014). *Campylobacter* is one of the most frequently isolated bacteria from infants with diarrhea in developing countries (Coker *et al*., 2002). *C. jejuni* and *C. coli* particularly among thermotolerant spp. have become the most common cause of human bacterial gastroenteritis which is the main disorder caused by it (Robyn *et al*., 2015). *C. jejuni* and *C. coli* have been implicated in most foodborne cases, *C. jejuni* is responsible for 90% of the outbreaks, whereas *C. coli* only accounts for 5% of the outbreaks (EFSA, 2005). The majority of human infections are self-limiting (Wei *et al*., 2016) but in some cases, it causes other serious immunoreactive complication such as Rieter's and Guillain-Barré syndrome (EFSA, 2011, Adzitey *et al*., 2012 and Bolton, 2015).

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Poultry, specifically broilers and laying hens is the main important reservoir of thermotolerant *Campylobacter* spp. (EFSA, 2010 and Josefsen *et al*., 2015). Therefore, chicken meat is the major source of food-borne Campylobacteriosis (Ganan *et al*., 2012). Despite of accumulation of circumstantial evidences that favor horizontal transmission from the environment as the most probable source of infection by *Campylobacter* through old litter, untreated drinking water, other farm animals, domestic pets, wildlife species, house flies, insects, equipment and transport vehicles, and farm workers (Sahin *et al*., 2002). There is increasing evidence suggests that vertical transmission of Campylobacter to the egg may occur through reproductive tract (Maruyama and Katsube, 1990, Jacobs-Reitsma, 1997 and Camarda *et al*., 2000).

Eggs are recognized as an exceptional nutritive complete product whose technological and flavoring characteristics countenance their use as a multifunctional element in the food industry (Espina *et al*., 2014). Although the role of the eggs as a vehicle for *Campylobacter* transmission has been studied, egg wasn't mentioned by EFSA (2015) amongst food related to Campylobacteriosis. There is only some information available about the predominance of thermotolerant *Campylobacter* spp.

on the eggshell or in the egg inner content itself. These data can be used for a hazard evaluation concerning potential human foodborne Campylobacteriosis through cross-contamination of ready-to eat food or through ingesting undercooked eggs or food produced with uncooked egg (Messelhäusser *et al*., 2011). The goal of this study was to detect thermotolerant *Campylobacter* spp. in the table egg hence evaluating it as a risk for *Campylobacter* transmission.

MATERIALS AND METHODS

Samples:
A total of 500 eggs of Baladi and large scale eggs (250 eggs each) were collected from farmers' houses and markets in Qena Governorate. Samples were transferred to the laboratory without delay to be examined for the presence of thermotolerant *Campylobacter* spp. Eggs were divided into pools, and each pool contained 5 eggs obtaining 100 pools.

Isolation of Campylobacter spp. from Egg Samples
Preparation of the samples
Egg shells were tested by a surface rinse method (Musgrove *et al*., 2005). Egg shell rinses were obtained by putting each sample in a sterile bag containing 500 ml of Buffered Peptone water and shaking for about 5 min. Then the eggs were removed and the surface of the eggs was sterilized and aseptically cracked and egg contents were received in a sterile beaker and mixed with a sterile fork.

Isolation of Campylobacter spp. (Adzitey *et al*., 2012)
Twenty five ml of each egg rinse solution and egg content sample (25 ml each) were added to Bolton broth (supplemented with Bolton broth Selective Supplement and Laked Horse Blood, Oxoid) which was then incubated at 42 °C for 48 h in an anaerobic jar containing a gas mixture of 10% Co_2, 5% O_2 and 85% N_2 that provided by gas generating kit (Oxoid). The mCCDA agar (Campylobacter blood free selective agar, Oxoid) which was supplemented with CCDA selective supplement, (Oxoid), were then streaked with a loopful of each enrichment broth and subsequently incubated at 42 °C for 48h under microaerobic condition. From 2-3 presumptive *Campylobacter* colonies were purified on Columbia Blood Agar (containing 7% defibrinated sheep blood) without supplement. *Campylobacter* isolates were submitted to Gram stain, oxidase, catalase, inability to grow aerobically at 25 °C, hippurate hydrolysis and resistance to nalidixic acid and cephalothin.

Identification of *C. jejuni* and *C. coli* using Multiplex Polymerase Chain Reaction (mPCR):
DNA extraction
DNA extraction from isolates was operated using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with remodeling of the manufacturer's recommendations. In brief, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. Following the incubation, 200 µl of 100% ethanol was added to the lysate. The sample was thereafter washed and centrifuged according to the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer afforded with the kit.

Multiplex Polymerase Chain Reaction (mPCR)
Multiplex PCR was used to confirm *C. jejuni* according to the primer sequence 5′ACTTCTTTATTGCTTGCTGC3′ and 5′GCCACAACAAGTAAAGAAGC3′, while the primer 5′GTAAAACCAAAGCTTATCGTG3′ and 5′TCCAGCAATGTGTGCAATG3′ used to confirm *C. coli* isolates (Wang *et al*., 2002). Primers used were supplied from Metabion (Germany).

PCR amplification and analysis of the PCR Products
The PCR mixture reaction (50 µl) consisted of 25 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 9 µl of water, and 12 µl of DNA template. Amplification of DNA was accomplished with 35 cycles of the following: primary denaturation at 94°C for 10 min, annealing at 55°C for 30 s and extension at 72 °C for 30 s with a final extension time of 72°C for 7min in an Applied biosystem 2720 Thermal Cycler. Then separation of PCR products was performed by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 30 µl of the products were loaded in each gel slot. A 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

RESULTS

In the present study thermotolerant *Campylobacter* spp. could be isolated from 15% of the total egg shell samples and 5% of the total egg content samples (Table 1). *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* identified in 9, 4, 1 and 1% of the egg shell samples, respectively and 4, 0, 1 and 0% of the egg content samples, respectively (Table 2).

From data illustrated in Table 2 it is obvious that neither the egg shell nor the egg content of poultry farms egg samples harbored thermotolerant *Campylobacter* spp., while Baladi egg samples obtained from farmers' houses were found to be contaminated with thermotolerant *Campylobacter* spp. in percentage of 30 and 10% for the egg shell

and inner egg content, respectively. *Campylobacter* spp. recovered from Baladi egg samples was distributed as follow: *C. jejuni* isolated from 18 and 8% of egg shell and egg content, respectively (photo1), *C. Lari* detected in 2% of each egg shell and egg content samples, while *C. coli* and *C. upsaliensis* isolated from egg shell samples only in a percent of 8 and 2%, respectively (Table 2 and photo 1).

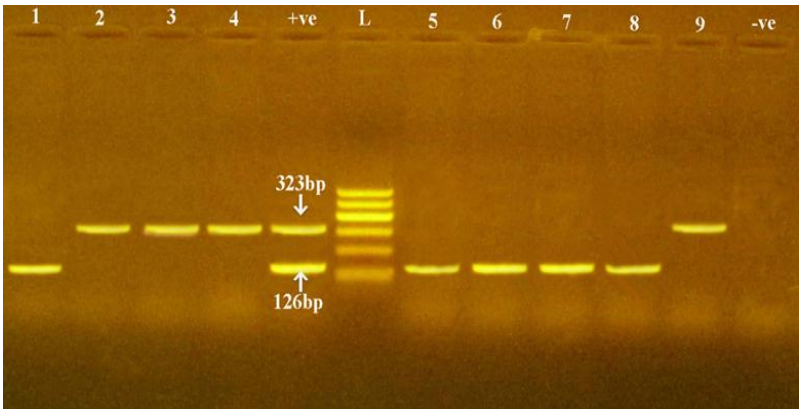

Photo-1: Multiplex-PCR of *C. jejuni* and *C. coli* strains isolated from egg sgell and egg content samples. Lane (+ve): positive control. Lane (-ve): negative control. Lane (L): 100 bp ladder as DNA marker. Lanes 1, 5, 6, 7 and 8 are positive for *C. jejuni*. Lanes 2, 3, 4 and 9 are positive for *C. coli*.

Table 1: Incidence of thermotolerant *Campylobacter* spp. in the examined egg pools.

Egg Samples	No. of Samples	Egg Shell		Egg Content	
		No.	%	No.	%
Poultry Farms	50	0	0	0	0
Farmers' houses (Baladi)	50	15	30	5	10
Total	100	15	15	5	5

Table 2: Distribution of thermotolerant *Campylobacter* spp. in egg shells and content

Source	No. of samples	Egg shell No. (%)					Egg content No. (%)				
		C. jejuni	*C. coli*	*C. lari*	*C. upsaliensis*	Total No./50 (%)	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*	Total No./50 (%)
Poultry Farm	50	0	0	0	0	0	0	0	0	0	0
Farmers' houses (Baladi)	50	9 (18)	4 (8)	1(2)	1 (2)	15(30)	4(8)	0	1(2)	0	5(10)
Total	100	9 (9)	4(4)	1(1)	1(1)	15(15)	4 (4)	0	1 (1)	0	5 (5)

DISCUSSION

Thermotolerant *Campylobacter* spp. could not be isolated from commercial eggs produced by large poultry farms giving an image of the hygienic measures applied in the farm and its role in the freedom of eggs from *Campylobacter* contamination as eggs are frequently collected and separated from hens plus there is no potential transport of *Campylobacter* from wild birds or other animals to the flock and subsequently their eggs. This result is identical with Moyle *et al*. (2016) who didn't isolate *Campylobacter* spp. from egg shell wash or internal contents of egg samples collected from 2 farms. While, Adesiyun *et al*. (2005) found 2 out of 46 samples were positive for *Campylobacter* spp., both isolates belonged to the contents of eggs.

On the other hand, in this study all *Campylobacter* isolates obtained from egg samples that collected from farmers' houses (Baladi egg). In farmers' houses birds are subjected to be in contact with wild birds that is considered natural reservoirs of *Campylobacter* spp. and are frequently mentioned as

possible vectors for transmission to poultry (Waldenström *et al.*, 2002). Also eggs are laid in unhygienic environment includes damp places contaminated with feces, old liter, and untreated drinking water.

It is very obvious from results recorded in this study that the egg shell was more subjected to contamination with thermotolerant *Campylobacter* spp. than the egg content as 15 of eggshell versus 5% of egg content collected from both poultry farms and farmers' houses were contaminated. The existence of the environmental conditions in which eggs are laid as mentioned before facing the fact that *Campylobacter* must compete forcefully with different microflora that present at high numbers in feces to penetrate through the eggshell, considering its week competitive nature, in addition to the adverse impact of high pH and the presence of bactericidal compounds, like lysozyme and conalbumin, in the albumen on it if penetration happened (Sahin *et al.*, 2003) resulted in that diversity in the incidence of the egg shell contamination and egg content contamination.

Regarding the total prevalence of *Campylobacter* spp., it was noticed that Jonaidi-Jafari *et al*. (2016) recorded lower incidence of egg shell samples contaminated by *Campylobacter* spp. (7%) which distributed as *C. jejuni* (5%) and *C. coli* (2%). In the same time, they registered weakly higher prevalence of *Campylobacter* spp. (6%) and *C. jejuni* (5%) in the egg content. Unlike this study *C. coli* (1%) could be isolated by them from the internal content of the egg. Also lower incidence recorded by Messelhäusser *et al*.(2011) who detected *Campylobacter* spp. in 4.1% of the egg shell samples. Eight and 3 isolates were confirmed as *C. coli* and *C.jejuni*, respectively.

Detection of *Campylobacter* spp. in the internal egg content of farmers' houses could be explained as follow: in farmers' houses the eggs remain in contact with hens, fecal materials and damp environment until they are collected. During this period there is a chance for the microbe entrance through the egg shell's pores, which have an average diameter of 11 μm to 12 μm (Fonseca *et al*., 2014) due to its motility and its size which is smaller than the diameter of the pores (Vandamme *et al*., 1992), thus rendering the consumption of the contaminated egg a potential risk to human health.

No *Campylobacter* was isolated from the shell surface of two Baladi egg samples which had the organism in their internal content. This may be contributed to 2 probabilities; the first one is the death of *Campylobacter* on the shell due to its sensitivity to the dry conditions and atmospheric oxygen (Park, 2002). Shane *et al*. (1986) affirmed that when they reported that the viability of *C. jejuni* on the fecally contaminated shell egg was retained for only 16 h due to desiccation of the fecal suspension. While, the second one is contamination of the egg content with *Campylobacter* was caused by systemic infection of the hen's reproductive tract. *C. jejuni* has been isolated from the ovaries and oviducts of healthy laying chicken hens by Jacobs-Reitsma (1997); Camarda *et al*. (2000) and from matured yellow follicles and lower oviduct of experimentally infected laying quails by Maruyama and Katsube (1990) unveiling the role of infected reproductive organs in the contamination of eggs.

There is a fake conviction that eggs produced by farmers' houses (Baladi) are further nutritious and microbiologically safe however the findings of this investigation proved the microbiological hazard associated with it. Faraway from laboratory conditions, in the ordinary kitchen throughout cooking, it is approximately impossible to crack eggs aseptically. Therefore, polluted eggshells constantly create a threat of cross-contamination of the egg content with pathogens and of subsequent initiation of food-borne infections when producing ready-to eat food with uncooked or undercooked egg content. The other prospect is cross-contamination from the eggshell to other ready-to-eat meals or products which do not include the egg content itself. During the cooking or production process, the threat of cross-contamination can be excluded only through application of special and very strict hygienic measures. Accordingly, contamination of the eggshell has to be of much more importance for a human food-borne infection than the presumed very low contamination rate and short viability of thermotolerant *Campylobacter* spp. that reported by many authors like Doyle (1984), Neill *et al*. (1985), Clark and Bueschkens (1986), Shane *et al*. (1986) and Maruyama *et al*. (1995), in particular with the fast movement of the egg in the market, thus rendering egg a hazard for Campylobacteriosis in humans.

Finally, safe handling and processing techniques should not only be implemented into food safety and quality control systems of the food business operators, but also be communicated to the consumers. Egg washing with sanitizers is a common procedure to minimize shell contamination, however; this process is noticeably variable as it isn't always obligatory to wash eggs. Regulations prohibiting the sale of dirty and/or cracked eggs should be developed and activated.

CONCLUSION

Table eggs sold in Qena represent a risk for transmission of thermotolerant *Campylobacter* spp. in which *C. jejuni* followed by *C. coli* are superior species. All contaminated samples belonged to Baladi egg samples accentuating that eggs obtained from large poultry farms are safer due to the good hygienic conditions applied in these farms. Primary

disinfection of the egg surface with disinfectant, separation of dirty eggs from clean one and application of safe handling procedures can reduce the hazard of human Campylobacteriosis.

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مدى تواجد ميكروبات الكامبيلوباكتر المتحملة للحرارة فى بيض المائدة

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ان ميكروب الكامبيلوباكتر وخاصة الكامبيلو باكتر جيجوناي وكامبيلوباكتر كولاي من احد اهم الميكروبات المسببة للاسهال. الهدف من هذا البحث هو دراسة دور بيض المائدة المنتج من المزارع الكبرى و بيوت المزارعين (البيض البلدي) في قنا في نقل هذا الميكروب. لذلك تم تجميع عدد ٢٥٠ بيضة منتجة بواسطة المزارع الكبري وعدد ٢٥٠ بيضة تم تجميعها من بيوت المزارعين بواقع عدد كلي ٥٠٠ بيضة و تم تقسيم هذا العدد الي ١٠٠ مجموعة تمثل ١٠٠ عينه وكل عينة تحتوي على عدد ٥ بيضات وتم فحصها لمعرفة مدي تواجد ميكروبات الكامبيلوباكتر على سطح القشرة وكذلك داخل محتويات البيضة. وقد تم عزل ميكروب الكامبيلوباكتر من على سطح القشرة بنسبة ١٥% ومحتويات البيض بنسبة ٥% من العدد الكلي للعينات وتم تصنيف العزلات المتحصل عليها من سطح القشرة كالأتي: كامبيلوباكتر جيجوناي (٩%)، كامبيلوباكتر كولاي (٤%)، كامبيلوباكتر لاري (١%) وكامبيلوباكتر ابسالينسيس (١%). بينما العزلات المعزولة من محتويات البيضة الداخلية كانت كامبيلوباكتر جيجوناي (٤%) وكامبيلوباكتر لاري (١%). من ابرز النتائج هو خلو البيض المنتج بواسطة المزارع الكبرى من ميكروب الكامبيلوباكتر. أما بالنسبة للبيض البلدي فان ٣٠ و ١٠% من عينات قشرة البيض وعينات محتوى البيض الداخلى كانت ملوثة بميكروب الكامبيلوباكتر على التوالي. كما أن عزلات الكامبيلوباكتر جيجوناي والكامبيلوباكتر كولاي تم التاكد منها باستخدام تفاعل البلمرة المتسلسل المتعدد.