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PREVALENCE OF *CAMPYLOBACTER SPP.* AND ITS PATHOGENIC GENES IN POULTRY MEAT, HUMAN AND ENVIRONMENT IN ASWAN, UPPER EGYPT

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ABSTRACT

A total of 250 samples including 100 samples from chicken meat, 75 samples human diarrhea, 75 samples from environment were collected from Aswan, Egypt. All samples were bacteriologically and biochemically examined for isolation, identification and differentiation of campylobacter spp., multiplex PCR detection of *23S rRNA, hipO, glyA* gene for identification and differentiation of campylobacter spp. and detection of some pathogenic virulence genes include *Iam, cdtB* and *cadF* genes. Prevalence of campylobacter in chicken meat, human diarrhea and environmental samples by conventional methods were 32%, 14.7% and 13.3%, respectively. Prevalence of campylobacter in chicken meat, human diarrhea and environmental samples by mPCR were 6%, 5.33% and 4%, respectively. About 64.3%, 21.4% and 14.3 of examined samples were *Campylobacter jejuni*, *Campylobacter coli* and mixed culture, respectively. Most positive samples contain high prevalence of pathogenic virulence genes. Poultry meat and environment could be a dangerous source for pathogenic campylobacter for human. Most campylobacter isolates have a lot of pathogenic genes which increase the invasiveness and pathogenicity of Campylobacter.

Key words: Chicken meat, *Campylobacter jejuni*, *Campylobacter coli*, pathogenic genes.

INTRODUCTION

Campylobacter is the most common cause of gastroenteritis worldwide especially in children (Ruiz-Palacios, 2007, Kaakoush *et al.,* 2015), *C. jejuni* infection frequently causes an acute enteritis with diarrhea, malaise, fever and abdominal pain, vomiting and/or bloody diarrhea specific for the more sever end of the disease spectrum and may affected by the host susceptibility and/or infective dose (Gillespie *et al.,* 2006). Campylobacter enteritis signs can lasts for more than a year and may contribute to post-dysenteric irritable bowel syndrome (PD-IBS) (Spiller *et al.,* 2000) and may causes Guillain-Barre Syndrome (GBS) during the subsequent 2-month period post infection (Tam *et al.,* 2006). Guillain-Barre Syndrome (GBS) and Miller-Fisher Syndrome (MFS) are related to prior infection by *C. jejuni* in up to 40% of cases (Dingle *et al.,* 2001).

The majority of outbreaks of acute campylobacteriosis in human worldwide were associated with consumption of chicken and chicken meat products (Corry and Atabay, 2001, Friedman *et al.,* 2004) also, contact with cattle, consumption of beef and milk were responsible for more than 90% of all sporadic human cases. Other sources such as sheep, contact with wild birds, contaminated water and pet animals were contribute for human infection but in much lesser extent (Wilson *et al.,* 2008). The use of the same cutting board for chicken meat and salad without intermediate cleaning, spreading of pathogens via the kitchen environment and contaminated tools and equipments may causes cross-contamination more than the risk associated with undercooking of poultry meat (Luber, 2009). Drinking water may conform the common reservoir linking infection between humans and animals, including poultry and wild birds (Kapperud *et al.,* 2003). Poultry meat and its products are the most implicated food and the most significant risk factor for acquiring infection in human campylobacteriosis. Genotyping with multilocus sequence typing (MLST) and relatedness study of large number samples of *C. jejuni* isolated from humans and broilers was carried out, all results of source attribution analysis confirmed the strong linkage between broiler *C. jejuni* and human cases (Griekspoor *et al.,* 2015).

About 35 virulence genes were discovered in campylobacter isolates using PCR testing, include those involved in motility, chemotaxis, cell adhesion, invasion, cytotoxin production, capsule, multidrug and bile resistance, stress response/survival and the

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iron uptake system, there was no discernible difference in the virulence profiles of human and poultry isolates (Koolman *et al.,* 2015). Several authors reported that some bacterial factors are more essential for the pathogenesis of campylobacter, including the motility and adherence of bacteria to intestinal mucosa, capability to invade enterocytes and toxin production (Datta *et al.,* 2003). One of these virulence markers is the *cadF* gene, which encodes a 37 KDa protein belonging to the group of outer membrane proteins (OMPs) that functions as an adhesion protein responsible for certain steps of invasion (Konkel *et al.,* 1999). Another interesting region, designated an invasion-associated marker (*iam*), has been identified in some *C. jejuni* and *C. coli* strains (Carvalho *et al.,* 2001). CDT is composed of three subunits designated CDTs A, B and C. The B subunit targets the eukaryotic DNA and triggers a signaling pathway involving different protein kinases which results in a cell block before entering into mitosis. Until now, the individual role of the A and C subunits has not been totally elucidated. Its exact role in pathogenesis is not yet clear, but possible actions include inhibition of epithelial cell proliferation, apoptosis of immune cells and inhibition of a fibrotic response (Ceelen *et al.,* 2006). Different distribution of genetic markers between human and chicken isolates indicates that some campylobacter infections in children may have additional sources other than contaminated chicken meat (Rozynek *et al.,* 2005).

MATERIALS AND METHODS

Samples

A total of 250 samples including 100 samples from chicken meat (fresh chicken, frozen chicken and chicken meat products which include chicken luncheon, nuggets, pane, frozen liver and giblets), 75 samples human diarrhea (from hospitals and medical laboratories), 75 samples from environment (water, poultry slaughter house, meat shops, supermarkets, hospital`s kitchens and restaurants kitchens) from Aswan, Egypt were collected. Sampling box containing ice pads was used for carrying the samples maintaining low temperature from market to laboratory in the faculty of veterinary medicine, Aswan University. Samples were preserved in sterile polyethylene bags in the refrigerator. Twenty five grams of each chicken meat sample were aseptically transferred to sterile stomacher bag containing 225 ml Bolton broth with 5% lysed horse blood and antibiotic supplement. The bag content was homogenized using a Stomacher® 400 Circulator (Seward Ltd., UK) for 1 minute (FDA *et al.,* 1998). Human diarrheal samples were collected in clean sterile containers. Environmental samples were collected by swabbing and the water samples collected in clean sterile bottles then concentrated by centrifugation at 20000 rpm for 10 minutes, the pellet was re-suspended in Bolton broth (Fricker and Park, 1989).

Isolation and Identification

Aseptically transfer 25 grams of each chicken sample to a sterile 250 ml flask and incubated for 4 hours at 37°C, followed by further incubation at 42°C/48 hours under micro-aerophilic condition (5% oxygen, 10% carbon dioxide and 85% nitrogen). Also, each environmental swab was immersed into sterile flasks containing 10 ml of Bolton broth (FDA *et al.,* 1998). Loopful (10 µl) was taken from each Bolton broth enrichment culture after 48 hours and streaked on Charcoal Cefoperazone Deoxycholate Modified Agar Base (mCCDA) (Oxoid, Code: CM0739) selective solid medium with selective supplement, the culture plates were incubated micro-aerobically in a micro-aerobic atmosphere using anaerobic jar and campylobacter gas generating kits at 42°C for 48 hours (Bolton *et al.,* 1984). Diarrheal and water samples were inoculated directly on mCCDA (Maher *et al.,* 2003). Positive colonies are grayish, flat and moistened with tendency to spread and may have a metal sheen. Positive strains were confirmed with Gram's staining, oxidase, catalase, hippurate hydrolysis, Analytical Profile Index (API) Campy and other biochemical tests.

Genetic Characterization

DNA extraction was carried out by using boiling method; DNA was prepared by the whole-cell procedure. Each DNA template was prepared by using approximately half a loopful of culture transferred to 1 ml of brain heart infusion broth (Oxoid, Code: CM1135). The optimized whole-cell DNA preparations from all campylobacter species were further diluted 1:500 in distilled water and were heated at 100°C for 10 minutes. A five µl aliquot was directly used as a template for PCR amplification (Shah *et al.,* 2009). Primer sequences were used as forward and reverse for *23S rRNA*, *hipO*, *glyA*, *cadF*, *cdtB* and *iam* genes used for identification of *Campylobacter species* and for pathogenic genes (Table). Multiplex PCR amplification of *Campylobacter species* genes (*23S rRNA, hipO, glyA*) and the second multiplex PCR for amplification of pathogenic genes (*cadF, cdtB, iam*). Each reaction consists of 2.5 µl of 10x buffer, 2.5 µl master mix, 2.5 µl Taq polymerase, 1 µl each primer, 0.5 µl DNA template and nuclease free water till 25 µl volume. Thermacycler (Eppendorf, Germany) was used with initial denaturation step at 95 for 6 minutes followed by 30 cycles (denaturation at 95°C for 0.5 minute, annealing at 59°C for 0.5 minute and extension at 72°C for 0.5 minute for species genes) (Wang *et al.,* 2002), (denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute for pathogenic genes) ending with final extension at 72°C for 7 minute. Amplified products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized on UV transilluminator (Andrzejewska *et al.,* 2011).

RESULTS

Prevalence of campylobacter in chicken meat, human diarrhea and environmental samples were 32%, 14.7% and 13.3%, respectively. Results showed that prevalence rates were 50% for fresh chicken carcasses and 12.5% for frozen chicken carcasses. Prevalence rates of human diarrheal were 17.5% in children and 11.4% in adults while these rates were 18.75% in males and 11.62% in females. Prevalence rates in environmental samples were 44.4% in slaughter houses, 40% in restaurant`s kitchens while poultry meat shops, supermarkets, hospital`s kitchens and tape water were free from campylobacter. Multiplex PCR analysis of *23S rRNA* gene to identify campylobacter species of positive isolates revealed lower prevalence rates which were 6% for chicken meat, 5.3% for human diarrhea and 4% for environmental samples. Genetic analysis of positive isolates for differentiation of campylobacter species through detection of *hipO* and *glyA* genes revealed that 64.3% were Campylobacter jejuni, 21.4% were Campylobacter coli and 14.3% were mixed culture. Prevalence of Campylobacter jejuni were 83.3%, 50% and 66.6% and of Campylobacter coli were 16.7%, 50% and 0% in chicken meat, human diarrhea and environmental isolates, respectively. Prevalence of virulence genes, *Iam*, *cdtB* and *cadF* genes in all examined samples were 61.5%, 69.2% and 84.6%, respectively. Prevalence of *cadF*, *cdtB* and *Iam* genes presence in Campylobacter jejuni isolates were 100%, 77.8% and 55.5%, respectively, while in Campylobacter coli were 33.3%, 33.3% and 66.6%, respectively.

Table 1: Prevalence of campylobacter species in chicken meat, human diarrhea and environmental samples.

Samples	Prevalence of Campylobacter			Percentage of Campylobacter species	
	No.	%		*C. jejuni*	*C. coli*
		Classic	PCR		
Chicken meat	100	32	6	83.3	16.7
Human diarrhea	75	14.7	5.3	50	50
Environmental samples	75	13.3	4	66.6	0

Table 2: Prevalence of campylobacter virulence genes in examined samples.

Gene	Prevalence		*C. jejuni*	*C. Coli*
	No.	%		
Iam	250	61.5	100	33.3
*cdt*B	250	69.2	77.8	33.3
*cad*F	250	84.6	55.5	66.6

Table 3: Primer sequences of campylobacter species genes and pathogenic genes.

Gene	Primer	Oligonucleotide sequence (5′ → 3′)	Product size(bp)	References
23S rRNA (C. spp.)	23S rRNA (F)	5′ TATACCGGTAAGGAGTGCTGGAG ′3	650	(Wang et al., 2002)
	23S rRNA (R)	5′ ATCAATTAACCTTCGAGCACCG ′3		
hipO (C. jejuni)	hipO (F)	5′ ACTTCTTTATTGCTTGCTGC ′3	323	(Wang et al., 2002)
	hipO (R)	5′ GCCACAACAAGTAAAGAAGC ′3		
glyA (C. coli)	glyA (F)	5′ GTAAAACCAAAGCTTATCGTG ′3	126	(Wang et al., 2002)
	glyA (R)	5′ TCCAGCAATGTGTGCAATG ′3		
cadF	cadF (F)	5′ TGGAGGGTAATTTAGATATG ′3	400	(Konkel et al., 1999a)
	cadF (R)	5′ CTAATACCTAAAGTTGAAAC ′3		
ctdB	ctdB (F)	5′ GTTAAAATCCCCTGCTATCAACCA ′3	495	(Ripabelli et al., 2010)
	ctdB (R)	5′ GTTGGCACTTGGAATTTGCAAGGC′3		
iam	iam (F)	5′ GCGCAAAATATTATCACCC ′3	518	(Carvalho et al., 2001)
	Iam (R)	5′ TTCACGACTACTATGCGG ′3		

DISCUSSION

In this study, the prevalence of campylobacter in chicken meat samples was 32% which is similar to that found in Gauteng, South Africa, 32.3% (Van Nierop *et al.,* 2005) and lower than rates in Spain in which campylobacter isolated with percentage of 49.5% from chicken meat samples (Dominguez *et al.,* 2002). Poultry can be contaminated from a variety of sources on farms and the contaminants are spread during processing, scalding, defeathering, evisceration and giblet operations are major points of spread, further spread can occur during handling in markets and kitchens. Insufficient thermal processing or cooking allows survival, Improper handling of cooked chicken frequently results in cross contamination from previously handled raw carcasses and parts (Bryan and Doyle, 1995). Prevalence of campylobacter in fresh chicken carcasses and in frozen carcasses was 50% and 12.5%, respectively. These rates are lower than results of fresh chickens sold in Ontario and Ohio which are 62% and 54%, respectively, (Park *et al.,* 1981) and 64% in Sapporo, Hokkaido, Japan (Sallam, 2007), lower rates, 39.2% was recorded (Sproston *et al.,* 2014), also, it is noticed that prevalence of campylobacter in fresh chickens was higher than that of frozen ones, reduction in positive samples in frozen chickens may be due to killing of campylobacter spp. or sub-lethal injury with or without reduction in viable counts under investigated storage temperatures which may indicates the ability of campylobacter to survive in chicken meat stored under refrigerated and frozen conditions (Eideh and Al-Qadiri, 2011), also, may be due to oxidative stress contribute to the freeze-thaw induced killing of campylobacter (Stead and Park, 2000). Prevalence of campylobacter in human diarrheal samples was 14.7% which are higher than results of Bangkok and its suburb in Thailand, 3% (Samosornsuk *et al.,* 2015), and results of northern region of India, 2.6% (Vaishnavi *et al.,* 2015) and lower than results obtained in Indonesia which was 79.5% (Pagaya *et al.,* 2015) and that obtained in Pakistan which was 30% (Guhar *et al.,* 2015), variations of rates in various parts of the world may be due to the varying standards and styles of living conditions, water supply and feeding habits. Rate of campylobacter in children was 17.5% which was lower than results in Egypt which was 35% (Barakat *et al.,* 2015), higher than results of India which was 10% (Salim *et al.,* 2014) and similar to results of northern Thailand which was 18% (Padungtod and Kaneene, 2005). Rate in adults was 11.4% which was higher than in northern Thailand which was 5% (Padungtod and Kaneene, 2005), prevalence in children was higher than that in adults may be due to lower immunity in children and lower standard of personal hygiene in contrast to adults, in older ages, most infections by campylobacter are mild or asymptomatic, probably because of immunity that may follow frequent exposure to contaminated food or water (Havelaar *et al.,* 2009). Campylobacter infections were more common in males, 18.7%, than in females, 11.6%, and this may be due to that males are more exposed to infection than females, also, males eating food outside home more than females (Sadkowska-Todys and Hucharczyk, 2012). Prevalence of campylobacter in environmental samples was 13.3% which are lower than results of Israel which was 38.7% from environmental swabs and 100% from washing water (Rogol *et al.,* 1985) and higher than results of Turin, northern Italy which was 0% for swabs (Bellio *et al.,* 2014) and in Sao Paulo, Brazil which was 4.9% in abattoirs (Cortez *et al.,* 2006). Prevalence rates in environmental samples were 44.4% in slaughter houses, 40% in restaurant`s kitchens while poultry meat shops, supermarkets, hospital`s kitchens and tape water were free from campylobacter. These results indicate higher rates of contamination in slaughter houses and restaurants that may attributed to low level of hygiene and cleanliness, high incidence of cross contamination from other birds and from use of equipments and utensils, low personal hygiene of workers (Tang *et al.,* 2011). Transfer of campylobacter from naturally contaminated raw chicken products to cooked chicken products via cutting board occurred and that both *C. jejuni* and *C. coli* are able to transfer (Guyard-Nicodeme *et al.,* 2013). There is no campylobacter found in tape water which is similar to results obtained in Finland (Hänninen *et al.,* 2003), this may be due to well chlorination of water (Moore *et al.,* 1996). Results showed high proportion of *C. jejuni* relative to *C. coli* in all tested samples. Prevalence of campylobacter species in chicken meat was 83.3% *C. jejuni* and 16.7% *C. coli*, these findings were differ than results obtained in Yangzhou, China which was 45.5% *C. jejuni* and 30.9% *C. coli* (Huang *et al.,* 2016) and closely similar to results of Sao Paulo, Brazil which was 91.6% *C. jejuni* and 8.3% *C. coli* (Carvalho *et al.,* 2013). Prevalence of campylobacter species in human samples was 50% *C. jejuni* and 50% *C. coli* which are closely similar to results of Thailand which was 58% *C. jejuni* and 40% *C. coli* (Samosornuk *et al.,* 2015) and differs than results of Kayseri, Turkey which was 84% *C. jejuni* and 13% *C. coli* and 5% other species (Kayman *et al.,* 2013) and fifers than results of north Lebanon which was 10% *C. jejuni*, 10% *C. coli*, 20% mixed culture and 60% other species (Dabboussi *et al.,* 2012). Prevalence of campylobacter species in environmental samples was 66.7% *C. jejuni* and 33.3% mixed culture, this finding was differs than results of Brazil which was 93.3% *C. jejuni* and 6.7% *C. coli* from abattoir samples (Cortez *et al.,* 2006), while in Italy was found 0% for any of *Campylobacter spp*. in environmental samples (Bellio *et al.,* 2014). Genetic characterization of pathogenic genes results showed that in chicken meat samples, prevalence of *Iam* gene was 60% in *C. jejuni* and 100% *C. coli*, *cdtB* gene was 80% *C. jejuni* and 0% *C. coli* and *cadF* gene found in 100% *C. jejuni* and

0% *C. coli*. In human samples, prevalence of *Iam, cdtB* and *cadF* genes were 100 % *C. jejuni* and 50% *C. coli* in all samples. In environmental samples, prevalence of *cdtB* gene was 50% in *C. jejuni* and 100% in mixed culture, *cadF* gene was found in 100% of samples while Iam gene was not found in *C. jejuni* isolates and found in 100% in mixed culture. *Iam* gene is responsible for invasiveness of *Campylobacter species* and marker potentially associated with the severity of campylobacter-induced enteritis (Carvalho *et al.,* 2001) and this gene was prominent in *C. coli* compared to *C. jejuni* (Andrzejewska *et al.,* 2015). *cadF* gene is gene of adhesion of campylobacter to fibronectin, an outer membrane protein (Monteville *et al.,* 2003), *cadF* gene was found nearly in all campylobacter isolates (Rozynek *et al.,* 2005). The cdtB gene is the toxin-forming gene of campylobacter jejuni, seems to be important for cell cycle control ond induction of host cell apoptosis and recognized as a major pathogenicity-associated factor (Dasti *et al.,* 2010), the possible actions of this toxin are firstly inhibition of epithelial cell proliferation and apoptosis allowing bacterial invasion, secondly cell cycle arrest of immune cells ensuing local immune suppression and finally inhibition of fibrotic response (Ceelen *et al.,* 2006).CDT activity requires the function of three genes: *cdtA, cdtB* and *cdtC* (Lara-Tejero and Galan, 2001).

CONCLUSION

Poultry meat and poultry meat products were an important source of campylobacter infections, also, campylobacter contamination can occur through environmental sources and from human itself. Campylobacter food poisoning caused mainly by Campylobacter jejuni and to lesser extent by Campylobacter coli and other species and most of the isolates was Campylobacter jejuni. Most campylobacter isolates have pathogenic genes responsible for adhesion, invasion and cytolethal toxins production which increase the virulence and pathogenicity of the microorganism.

AUTHORS' CONTRIBUTIONS

Author performs collection, preparation, processing, and analysis of samples, isolation of bacteria, data acquisition, writing, preparation and revision of manuscript.

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مدى انتشار ميكروب الكامبيلوباكتر و جيناته الممرضة فى لحوم الدواجن و الأنسان و البيئة فى أسوان – مصر

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تم تجميع عدد ٢٥٠ عينة عبارة عن ١٠٠ عينة من لحوم الدواجن و ٧٥ عينة من اسهالات الأنسان و ٧٥ عينة من البيئة المحيطة. تم عمل الفحص الباكتيريولوجى والبيوكيميائى لجميع العينات وذلك لعمل عزل وتعرف على الأنواع المختلفة من ميكروب الكامبيلوباكتر. كما تم عمل أختبار تفاعل البلمرة المتسلسل المتعدد للكشف على بعض جينات التعرف مثل *23S rRNA, hipO, glyA* genes وكذلك الجينات الممرضة مثل *Iam, cdtB* and *cadF* genes . أظهرت النتائج أن النسبة المئوية لمدى انتشار ميكروب الكامبيلوباكتر فى لحوم الدواجن ومنتجاتها وفى عينات الأسهال فى الأنسان وفى عينات البيئة المحيطة بالأغذية هى ٣٢% و ١٤.٧% و١٣,٣% على التوالى وذلك باستخدام الطرق التقليدية للعزل بينما تغيرت هذه النسبة بشكل كبير باستخدام أختبار تفاعل البلمرة المتسلسل المتعدد فكانت ٦% و ٥,٣٣% و ٤% على التوالى. كما أظهرت النتائج أن ٦٤,٣٣% من العينات كانت ايجابية لميكروب الكامبيلوباكتر جيجونای وأن ٢١,٤% من العينات كانت ايجابية لميكروب الكامبيلوباكتر كولای وأن ١٤,٣% من العينات كانت ايجابية لأنواع مختلفة من ميكروب الكامبيلوباكتر كذلك أظهرت النتائج أن معظم العينات كانت ايجابية لوجود الجينات الممرضة بها. نستخلص من هذه الدراسة أن لحوم الدواجن ومنتجاتها والبيئة المحيطة بالأغذية تشكل مصدرا خطيرا لميكروب الكامبيلوباكتر الممرض للأنسان وأن معظم هذه الميكروبات تحتوى على جينات الضراوة الخطيرة.