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Molecular identification of *C. jejuni* and *C. coli* in chicken and humans, at Zagazig, Egypt, with reference to the survival of *C. jejuni* in chicken meat at refrigeration and freezing temperatures

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Abstract

Chicken meat is considered the primary source of infection with *Campylobacter* spp. in humans. A total of 125 cloacal swabs, 61 chicken skin and 122 chicken meat (thigh and breast meat, 61, each) samples obtained from retail outlets and 110 stool swabs from 10 diarrhiac and 100 apparently healthy persons were examined. The isolation rates of *Campylobacter* spp. in chicken skin, thigh meat, breast meat, cloacal swabs and human stool samples were 47.5%, 47.5%, 25.9%, 21.6% and 2.7%, respectively. *Campylobacter jejuni* was isolated from cloacal swabs, skin, and thigh meat with the isolation rates of 3.7%, 3.4% and 6.9%, respectively, while, *Campylobacter coli* were isolated from 7.4% and 6.7% of cloacal swabs and breast meat, respectively. In humans, 5.2% *C. jejuni* and 3.2% *C. coli* were identified. Quantitative PCR targeting the species specific virulence gene *cadf* showed that all *C. jejuni* and *C. coli* isolates harbored the gene. The influence of refrigeration and freezing storage on the survival of *C. jejuni* in chicken breast meat was evaluated by qPCR. The results showed a significant decline in the number of bacterial cells after storage at 4°C and -20°C for a duration ranging from 3-20 days. However, storage of chicken meat at freezing temperature is preferred to refrigeration.

Introduction

Campylobacter species are primarily zoonotic pathogens that are frequently isolated from a variety of animal species such as poultry, cattle, pigs, sheep, pets, wild birds and rodents (Modolo and Giuffrida, 2004; Meerburg *et al*., 2006). The ingestion and handling of contaminated poultry meat is supposed to be the major infection route for humans (Corry and Atabay, 2001). Since the 1970s, *Campylobacter* have been shown to be an important cause of enteritis in humans (Anonymous, 2007), and it has become a more frequently recognized cause of gastroenteritis than *Salmonella* species (EFSA-ECDC, 2009). Among the 17 validly named species in the genus *Campylobacter*, *C. jejuni* ssp. *jejuni*, *C. coli*, *C. fetus* ssp. *fetus*, *C. upsaliensis*, *C. lari*, and *C. hyointestinalis* ssp. *hyointestinalis* are the recognized cause of intestinal infections in humans (Fitzgrald and Nachamkin, 2007; Lastovica and Allos 2008). *C. jejuni* is the most frequently reported *Campylobacter* species (80-90%) followed by *C. coli* (5-10%) (Fitzgerald *et al*., 2008).

Conventional biochemical tests for discrimination between *C. jejuni* and *C. coli* rely mainly on hippurate hydrolysis which is the only phenotypic test for differentiating the two species. However, both false positive and false negative results have been reported (Waino *et al*., 2003). Therefore, PCR applications have been developed for species identification. The verification of virulence factors in *C. jejuni* and *C. coli* is a useful tool to assess the potential risk of poultry as sources for *Campylobacer* infection (Melo *et al*., 2013).

Preservation of food by refrigeration and freezing is carried out to maintain a safe product by lowering the rate of growth of pathogenic and spoilage bacteria (James *et al*., 2006). *Campylobacter* spp. have an optimal growth temperature range of 37°C to 42°C and do not grow below 30°C (Lee *et al*., 1998). However, previous studies have shown that *C. jejuni* can survive for several weeks at 4°C and freezing temperature despite the decrease in their count (Bhaduri and Cottrell, 2004).

The overall aim of the current work was to investigate the contribution of chicken as potential sources of *C. jejuni* and *C. coli* infections in humans at Zagazig, Egypt. This aim was achieved by using conventional and molecular tools to investigate the occurrence of *C. jejuni* and *C. coli* in chicken and human samples. Moreover, the effect of refrigeration and freezing on the survival of *C. jejuni* in chicken breast meat samples over several time durations mimicking the real storage situations was studied.

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Material and Methods

Sampling and sample processing

A total of 125 cloacal swabs, 61 chicken skin and 122 chicken meat (thigh and breast meat, 61, each) samples were obtained from freshly slaughtered chicken at retail outlets in Zagazig, Egypt. Moreover, 110 stool swabs from 10 diarrhiac and 100 apparently healthy persons attending the outpatient clinic of Al-Ahrar general hospital, Zagazig city, Egypt, were examined. During sampling, all human subjects were asked about chicken meat consumption and contact with poultry. The samples were collected during the period from September 2012 to April 2014.

Sterile swabs were inserted into the cloaca and voided human stool samples and then directly immersed into tubes containing sterile Preston enrichment broth base containing *Campylobacter* growth supplement (Oxoid, SR 0232) (Ellerbroek *et al.*, 2010).

Twenty five grams from each incised skin and chicken meat (thigh and breast) were aseptically transferred to a sterile blender containing 225 ml of Preston enrichment broth for homogenization of the sample (Kiss, 1984).

Bacteriological examination

The collected samples in Preston enrichment broth were incubated at 37ºC for 24 hours. After enrichment, 0.1 ml of the broth was streaked onto modified *Campylobacter* selective agar base Cefoperazone Charcoal Desoxycolate Agar (mCCDA) (Oxoid, CM 0739) containing antibiotic supplement (Oxoid, SR 0155). The plates were then incubated at 42ºC for 48 hours under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) using *Campylobacter* gas generating kits (Oxoid, BR56) (Skirrow, 1977). Suspected colonies were purified on blood agar plates (Oxoid CM0271) and subjected to biochemical identification using catalase test, oxidase test, urea hydrolysis test, hydrogen sulphide (H_2S) production, citrate utilization test and rapid hippurate hydrolysis test (Nachamkin, 1999).

Molecular identification of C. jejuni and C. coli

DNA extraction from the biochemically identified isolates was performed according to the manufacturer guidelines using Bacterial DNA Extraction Kit (Spin-column) (BioTeke Corporation, China). Two real time probe based PCR (qPCR) reactions were used separately for the confirmation of *C. jejuni* and *C. coli* biochemically identified isolates. Species-specific primers and TaqMan probe sets targeting *hipO* gene specific for *C. jejuni* and *glyA* gene specific for *C. coli* (LaGier *et al.*, 2004) were synthesized by AlphaDNA (Canada) and the sequences of hipO primers and probe are: Cj-F1 forward: 5'-TGCTAGTGAGGTTGCAAAAGAATT-3', Cj-R1 reverse: 5'-TCATTTCGCAAAAAAATCCAAA-3', Cj-FAM probe: 5'-ACGATGATTAAATTCACAAT TTTTTTCGCCAAA-3'. For glyA, sequences of the primers and probe are as follows: Cc-F1 forward: 5'-CATATTGTAAAACCAAAGCTTATCGG-3', Cc-R1reverse:5'-AGTCCAGCAATGTGTGCAATG-3', Cc-FAM probe: 5'-TAAGCTCCAACTTCATCCGC AATCTCTCTAAATTT-3'. Each qPCR assay using primers and probes specific for *C. jejuni* and *C. coli*, separately, was carried out in a 25 ml volume using QuantiTect® Probe RT-PCR kits (Qiagen) in Applied Biosystem StepOne Real Time PCR System machine. Each qPCR reaction contained 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix (containing HotStart Taq® DNA polymerase, QuantiTect Probe RT-PCR buffer [Tris-Cl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$], dNTP mix including dUTP, ROX™ passive reference dye and 8 mM $MgCl_2$), 0.1 units AmpErase [Uracil N-glycosylase] (Qiagen), 500 nM of relevant primers and 500 nM of relevant probe and 5 µl DNA template. Nuclease free water was added to a final volume of 25 µl. Non template DNA and positive controls of *C. jejuni*, *C. coli*, *E. coli*, *S.* Typhimurium, *Staph. aureus* and two biochemically identified *Campylobacter* isolates other than *C. jejuni* and *C. coli* were also run to determine the specificity of each reaction. The reaction conditions were 50ºC for two minutes to activate UNG, 95ºC for 15 min then 40 cycles at 94ºC for 15 sec and 60ºC for 60 sec followed by plate read for fluorescence acquisition. FAM fluorogenic signal was collected and the cycle threshold of the reactions was detected by MJ OpticonMonitor™ Analysis software version 3.1 (Bio-Rad).

Molecular identification of virulence factors

Primers targeting the species specific virulence gene *cadf* using SYBR Green I based qPCR were synthesized by AlphaDNA (Canada). The sequences of the primers are cadF-Forward 5'-TTGAAGGTA ATTTAGATATG-3' and cadF-Reverse 5'-CTAATACCTAAAGTTGAAAC-3' (Nayak *et al.*, 2005). The amplification mixture of 25 µl contained 5 µl DNA template, 300 nM of each primer, 12.5 µL SYBR Green I ready-made master mix QuantiTect® SYBR® Green PCR kits (Qiagen) (containing HotStarTaq DNA Polymerase, Quantitect SYBR Green I PCR Buffer [Tris-Cl, KCl, $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, pH 8.7], 0.2 mM dNTP mix, SYBR Green I dye, ROX dye), 0.1 units AmpErase [Uracil N-glycosylase] (Applied Biosystems). Nuclease free water was added to a final volume of 25 µl. The reaction was performed in Applied Biosystem StepOne Real Time PCR System machine.

The reaction conditions were 50ºC for two minutes to activate UNG, 95ºC for 15 min then 40 cycles at 94ºC for 15 sec, 60ºC for 30 sec and 72ºC for 30 sec followed by plate read for fluorescence acquisition. A temperature gradient between 55ºC and 95ºC was run to obtain the dissociation curve. No template controls were also used to check the presence of contamination. SYBR Green I fluorogenic signal was collected and the cycle threshold of the reactions was detected by MJ OpticonMonitor™ Analysis software version 3.1 (Bio-Rad).

Survival of C. jejuni in chicken meat at refrigeration and freezing temperatures

Sampling and sample preparation

Skinned and deboned chicken breast samples were purchased from a local outlet in Zagazig city, Egypt, a day before conducting the experiment. Each breast meat sample was cut into pieces (each piece weighted 30 grams) to provide similar weights for bacterial inoculation. Each piece was then wrapped in aluminum foil and subjected to decontamination and cooking by autoclaving at 121°C for 15 minutes (Eideh and Al-Qadiri, 2011).

Preparation of C. jejuni inoculum

C. jejuni strain, obtained during the current study from chicken breast meat samples, was prepared from blood agar plates. A loopful from the plates was inoculated into Preston enrichment broth and incubated at 42°C for 48 hours under microaerophilic conditions. After 48 hours, bacterial count of serially diluted broth culture was enumerated using surface plating method (Thatcher and Clark, 1968). After serial dilution of the original broth culture, 100 µl from each dilution was aseptically plated onto mCCDA plates and incubated at 42°C for 48 hours under microaerophilic conditions in anaerobic jars (Eideh and Al-Qadiri, 2011). The dilution that had a microbial load of 10^7 CFU/ml (equals 7 $\log_{10}$ CFU/ml) was used for the inoculation of chicken breast meat samples.

Preparation of C. jejuni standards

One ml from the strain stock broth (7 $\log_{10}$ CFU/ml) was aseptically serially diluted using 9 ml sterile saline solution as diluent in order to obtain 6 $\log_{10}$ CFU/ml, 5 $\log_{10}$ CFU/ml, 4 $\log_{10}$ CFU/ml, 3 $\log_{10}$ CFU/ml, 2 $\log_{10}$ CFU/ml and 1 $\log_{10}$ CFU/ml. Extraction of DNA from each concentration was performed using Bacterial DNA Extraction Kit (Spin-column) (BioTeke Corporation, China) as previously described.

SYBR Green I based qPCR using *cadf* gene as a target was used to estimate the amount of *C. jejuni* in the standards (measured in triplicates). The primer sequences, amplification mixture and reaction conditions are fully described in molecular identification of virulence gene section. The cycle threshold of the reactions was detected by the MJ OpticonMonitor™ Analysis software version 3.1 (Bio-Rad). The mean Ct and standard deviations were calculated for each individual standard using data from the triplicates, and graphs of mean Ct against standard concentration were plotted to obtain a line of best fit.

Inoculation of samples with C. jejuni

Each cooked chicken breast meat sample was placed in a sterile Petri-dish and 100 µl of the strain stock broth (7 $\log_{10}$ CFU/ml) was aseptically inoculated into the surface and subsurface of chicken sample (Eideh and Al-Qadiri, 2011). The samples were kept for 30 minutes in the covered Petri-dishes to allow enough time for bacterial diffusion into the samples.

Storage of inoculated samples

The inoculated samples were divided into two groups 25 samples each, group I was stored at refrigeration temperature (4°C) and group II was stored at freezing temperature (-20°C), a control group of 25 untreated samples was also kept in each storage temperature. Examination of the samples kept at each storage temperature was carried out after one, 3, 5, 7, 10, 14 and 20 days. Each sampling was conducted in triplicate.

Recovery and enumeration of C. jejuni

Each sample was homogenized in Preston enrichment broth and then incubated at 42°C for 48 hours under microaerophilic conditions in anaerobic jars. One ml from the sample was centrifuged at 10000 rpm for 5 minutes and DNA was extracted from the bacterial pellet using Bacterial DNA Extraction Kit (Spin-column) (BioTeke Corporation, China) as previously described. The bacterial load in the sample was then determined by SYBR Green I based qPCR.

Statistical analysis

The qPCR amplification efficiency (E) determined by linear regression of the standard curve was calculated from the slope (s) using the equation:

$E = 10^{-1/s}-1$ (Klein *et al*., 1999). The acceptable efficiency of the qPCR assay should be between 90-110%. The difference between the refrigeration and freezing groups was estimated using two way ANOVA test (Factorial design) and LSD (Least significant difference) according to Snedecor and Cochran (1982). The test results were calculated by the computer program SPSS, Inc. version 22 (2012). Data were presented as mean ± SD and significance was considered at ($P < 0.05$).

Results

Prevalence of Campylobacter species in chicken and human samples

The prevalence rates of *Campylobacter* species in chicken cloacal swabs, skin and meat samples collected from Zagazig, Egypt are listed in Table 1. The occurrence of *Campylobacter* species was identified by bacteriological examination, while molecular confirmation by real time PCR was applied only to biochemically suspected *C. jejuni* and *C. coli* isolates. The results demonstrate a high prevalence rate of *Campylobacter* species in chicken skin and thigh meat samples (47.5%, each), followed by chicken breast meat (25.9%) and cloacal swabs (21.6%). *C. jejuni* was isolated from cloacal swabs, skin, and thigh meat with the isolation rates of 3.7%, 3.4% and 6.9%, respectively, while, *C. coli* were isolated from 7.4% and 6.7% of cloacal swabs and breast meat, respectively. In humans, only 2.7% of the stool samples were positive for *Campylobacter* spp., of which, *C. jejuni* and *C. coli* were identified in 5.2% and 3.2%, respectively. None of the diarrheic patients were positive for *Campylobacter* species. All humans subjected to examination during the present study have a history of chicken meat consumption.

Molecular characterization of C. jejuni and C. coli

The results in Table (1) show that out of 8 biochemically suspected *C. jejuni* isolates, 6 were confirmed by qPCR. For *C. coli*, 11 isolates were biochemically suspected isolates, of which 4 were confirmed by the amplification of *glyA* gene. The specificity of each reaction was characterized because primer and probe sets specific for *C. jejuni* did not amplify DNA from *C. coli* positive controls and other positive controls, also primer and probe sets specific for *C. coli* did not amplify DNA from *C. jejuni* positive controls and other positive controls.

Characterization of virulence genes

Real time PCR targeting the species specific virulence gene *cadf* was performed using 6 *C. jejuni* and 4 *C. coli* isolates obtained during the study. The results showed that all the examined isolates harbored the *cadf* gene. Moreover, the dissociation curve of the amplified products show only one peak at 82°C which confirms the amplification of only one product.

Table 1. Prevalence of *Campylobacter* species in different samples collected from Zagazig, Egypt [Number (proportion, 95% CI)]

Type of samples		Number examined	*Campylobacter* spp*	*C. jejuni**** Suspected	Confirmed	*C. coli**** Suspected	Confirmed
Chicken	Cloacal swabs	125	27 (21.6,14.7-29.8)**	1 (3.7, 0.1-19)	1 (3.7, 0.1-19)	5 (18.5, 6.3-38.1)	2 (7.4, 0.9-24.3)
	Skin	61	29 (47.5, 34.6-60.7)	3 (10.3, 2.2-27.4)	1 (3.4, 0.1-17.8)	2 (6.9, 0.8-22.8)	0 (0-11.9)
	Breast	58	15 (25.9, 15.3-39)	0 (0-21.8)	0 (0-21.8)	2 (13.3, 1.7-40.5)	1 (6.7, 0.2-31.9)
	Thigh	61	29 (47.5, 34.6-60.7)	2 (6.9, 0.8-22.8)	2 (6.9, 0.8-22.8)	1 (3.7, 0.1-17.8)	0 (0-11.9)
Humans	Stool	110	3 (2.7, 0.6-7.8)	2 (66.7, 9.4-99.2)	2 (66.7, 9.4-99.2)	1 (33.3, 0.8-90.6)	1 (33.3, 0.8-90.6)
Total		572	116 (20.3, 17.1-23.8)	8 (6.9, 3-13.1)	6 (5.2, 1.9-10.9)	11 (9.5, 4.8-16.3)	4 (3.4, 0.9-8.6)

*Isolation was identified by bacteriological examination

**Isolation rate is indicated between brackets

****C. jejuni* and *C. coli* were considered suspected by biochemical examination and confirmed by qPCR, their isolation rate was calculated from the total *Campylobacter* positives

Table 2. Count of *C. jejuni* in chicken breast meat at refrigeration and freezing storage (Mean±SD)

Storage duration (day)	Number of *C. jejuni* (CFU/ml) Refrigeration	Freezing
0	$7 \log_{10} \pm 0.01^{a}$	$7 \log_{10} \pm 0.01^{a}$
1	$7 \log_{10} \pm 0.01^{a}$	$6.35 \log_{10} \pm 0.03^{d}$
3	$6.9 \log_{10} \pm 0.06^{b}$	$6.35 \log_{10} \pm 0.02^{d}$
5	$6.79 \log_{10} \pm 0.03^{c}$	$5.8 \log_{10} \pm 0.07^{e}$
7	$6.76 \log_{10} \pm 0.07^{c}$	$5.48 \log_{10} \pm 0.01^{g}$
10	$6.37 \log_{10} \pm 0.01^{d}$	$4.9 \log_{10} \pm 0.02^{h}$
14	$6.35 \log_{10} \pm 0.01^{d}$	$4.52 \log_{10} \pm 0.14^{i}$
20	$5.9 \log_{10} \pm 0.39^{f}$	$4.51 \log_{10} \pm 0.02^{i}$

Means carrying different superscripts are significantly different at (P-value < 0 .05), while means carrying similar superscripts are insignificantly different based on LSD

Survival of C. jejuni in chicken meat at refrigeration and freezing temperatures

Quantitative PCR was used in the current study to evaluate the influence of refrigeration and freezing storage on the survival of *C. jejuni* in chicken breast meat.

The efficiency of the quantitative PCR reaction for quantification

Quantitative PCR using SYBR Green I targeting *cadf* gene in *C. jejuni* was used for quantification of *C. jejuni* in chicken meat samples. The amplification efficiency was estimated by plotting the Ct values of the assays versus the input colony forming units at a range from 7 $\log_{10}$ CFU/ml to 2 $\log_{10}$ CFU/ml (Figure 1). Figure 1 shows that a high Pearson correlation coefficient ($R^2 = 0.997$) was obtained. The reaction efficiency was calculated from the slope and it was found to be 101.7%. The inter assay precision was calculated in 7 repeats of standards and found to be less than 10% (4.8-6.07%). The sensitivity of the assay was evaluated using different amounts of *C. jejuni* DNA by serial dilution of the starting amount over 7 orders of magnitude; however, R^2 value was too low indicating low linearity. By excluding the lower concentration, the linearity was sufficient and the sensitivity of the reaction was found to be 2 $\log_{10}$

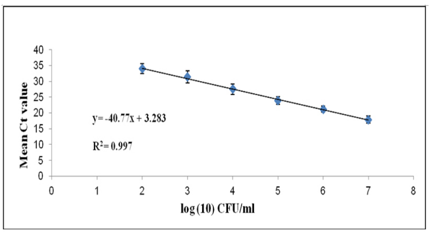

Figure 1. Average standard curve of cycle threshold (Ct) versus log (10) CFU/ml of *C. jejuni.* Data points were obtained by calculating the mean Ct across sample sets for each standard concentration. Vertical bars represent standard deviations.

CFU/ml.

Survival of C. jejuni during storage at refrigeration and freezing

All control samples kept at refrigeration and freezing were negative during the course of the experiment indicating the efficiency of autoclaving in decontamination and sterilization of the samples. The results in Table 2 show the mean colony forming units count of *C. jejuni* in the examined samples stored at refrigeration and freezing temperatures for time ranging from one day to 20 days. At refrigeration temperature, there was a significant decline of *C. jejuni* count from 7 $\log_{10}$ CFU/ml to 6.9 $\log_{10}$ CFU/ml after 3 days of storage ($p < 0.05$). The decrease in *C. jejuni* count after 5 and 7 days were insignificantly different ($P > 0.05$) from each other, while they were significantly lower than the count after 3 days of storage ($p < 0.05$). Increasing the storage duration at 4^{o}C to 14 days resulted in a significant decline to 6.35 $\log_{10}$ CFU/ml ($p < 0.05$) and 5.9 $\log_{10}$ CFU/ml after 20 days ($p < 0.05$).

Freezing of chicken meat samples for one and 3 days resulted in a significant reduction to 6.35 $\log_{10}$ CFU/ml ($p < 0.05$) compared to the initial *C. jejuni* count. After 7 and 10 days of storage, *C. jejuni* count decreased significantly to 5.8 $\log_{10}$ CFU/ml and 5.48 $\log_{10}$ CFU/ml ($p < 0.05$), respectively. There was no significant difference of the bacterial count survived after 14 and 20 days at freezing temperature ($P > 0.05$).

Discussion

Campylobacteriosis continues to significantly contribute to the frequently increased number of gastrointestinal illnesses worldwide (EFSA-ECDC, 2009). The primary objective of the current study was to investigate the presence of *C. jejuni* and *C. coli* in chicken and humans at Zagazig city, Egypt.

Occurrence of Campylobacter species in chicken

Poultry and poultry products are considered a common and main source of *Campylobacter* infection to humans (Humphrey *et al*., 2007). A world survey estimated the contamination of chickens with *Campylobacter* spp. to be about 58% (Suzuki and Yamamoto, 2009). Broiler carcasses could be cross-contaminated with *Campylobacter* spp. by fecal contents or ingesta (Mead *et al.*, 1995), so the consumption of undercooked poultry products and direct contact with live poultry or their feces are the possible risk pathways for human infections (Anderson *et al*., 2012).

Table (1) shows that *Campylobacter* spp. were isolated from 21.6% of the examined cloacal swabs. A Dutch study reported a prevalence ranged from 20% to 31% in poultry cecal samples (Van Asselt *et al*., 2008), which are nearly similar to the prevalence rate obtained in the present study. Higher isolation rates than that reported in the current study were previously obtained by Anderson *et al.* (2012) in New Zealand and Henry *et al.* (2011) who reported prevalence rates of 57% and 54%, respectively. The possible reason for their higher isolation rates could be the collection of fresh fecal samples from the ground rather than the sampling of cloacal swabs in the current study. Studies have suggested that the contamination of the ground near poultry houses with *Campylobacter* spp. was reported to be 68%, the possible contamination sources were wild birds, rodents and free living pets near or in farms (Studer *et al*., 1999).

Out of the 27 *Campylobacter* spp. isolated from cloacal swabs, 7.4% and 3.7% were identified as *C. coli* and *C. jejuni*, respectively (Table 1). The comparable isolation rates of *C. coli* versus *C. jejuni* were (55.5% versus 31.4%), (6.6% versus 55.5%) and (57.5% versus 0) as respectively recorded by Henry *et al.* (2011) in Reunion island, Anderson *et al.* (2012) and Marinou *et al.* (2012) in Greece. The higher isolation rate of *C. coli* in the aforementioned studies could be related to geographic regions where the studies were conducted (Marinou *et al*., 2012).

It is clear from the results in Table (1) that *C. coli* predominates *C. jejuni*, this is in contrast with other studies that reported the primarily colonization of poultry with *C. jejuni* (Ellerbroek *et al*., 2010; Anderson *et al*., 2012). A study conducted in France attributed the higher colonization of poultry with *C. coli* to the administration of β-lactam antibiotics to reared poultry and due to the type of ration (Marinou *et al*., 2012).

Poultry are exposed to *Campylobacter* spp. firstly at farm level due to insufficient biosecurity measure, secondary at market outlets due to contamination of carcasses during evisceration and scalding, thirdly during storage (Ellis-Iversen *et al.*, 2009). Countries using pluck-shop based markets have higher contamination rates of *Campylobacter* spp. from poultry than countries using modern processing plants (Parkar *et al.*, 2013). Manual slaughtering and evisceration lead to fecal contamination of carcasses, which in turn may be responsible for increased numbers of *Campylobacter* spp. in poultry meat (Parkar *et al.*, 2013). The risk of chicken meat contaminated with *Campylobacter* spp. is not only due to the consumption, but also due to the transfer of the bacteria present in chicken parts to hands, kitchen utensils and to other food either directly or via cutting boards (Guyard-Nicodème *et al.*, 2013).

Table (1) shows that *Campylobacter* spp. were isolated from 25.9% and 47.5% of the examined breast and thigh meat samples, respectively. Similarly, Luu *et al.* (2006) and Guyard-Nicodème *et al.* (2013) reported the isolation of *Campylobacter* spp. from 31% of breast meat and 47.9% of chicken legs, respectively. *C. coli* and *C. jejuni* were isolated from 6.9% thigh and 6.7% breast meat samples, respectively (Table 1). A similar isolation rate (6.7%) of *C. coli* was reported by van Nierop *et al.* (2005) from fresh chicken meat samples collected from butcheries. Moreover, a nearly similar isolation rate of *C. coli* from chicken meat (10.8%) was obtained by Rahimi and Tajbakhsh (2008) in Iran.

Poultry are colonized by high levels of *Campylobacter* spp. on their feathers, skin and intestine; consequently, defeathering and evisceration result in the contamination of carcasses (Jacobs-Reitsma, 2000). Chicken skin provides suitable microenvironment for the survival of *Campylobacter* spp. due to accumulation of water which increases the surface area available for bacterial contamination (Chantarapanont *et al.*, 2003). The higher isolation rate (47.5%) of *Campylobacter* spp. from skin samples (Table 1) highlights the risk of carcass contamination during slaughter, which in turn poses a risk to humans consuming poultry meat. Garin *et al.* (2012) and Kovalenko *et al.* (2013) reported slightly higher isolation rates of 65% and 60%, respectively, from chicken skin samples. Moreover, they recovered *C. jejuni* from 48.3% of the examined samples, which is higher than 3.4% obtained during the current study (Table 1). The relatively high isolation rate of *Campylobacter* spp. from chicken carcasses during the current study could be attributed to the fact that in Egypt, most of chicken are sold in pluck-shop markets that devoid hygienic measures leading to increased chances for contamination of slaughtered chicken carcasses with *Campylobacter* species.

Occurrence of Campylobacter species in humans

Campylobacter infection in humans, along with *Salmonella* infection, is the most common cause of bacterial diarrhea worldwide (Samuel *et al.*, 2004). It has been estimated that as few as 500 cells of *C. jejuni* could cause human illness; therefore, contamination of food with *Campylobacter* spp. represents a potential health hazard (Yang *et al.*, 2003). The estimated incidence of campylobacteriosis in European Union is 45-50 cases per 100,000 inhabitants, while in the United States; it is 13 cases per 100,000 (Scallan *et al.*, 2011). However, in developing countries, there is no estimated incidence due to absence of national surveillance programs (Coker *et al.*, 2002).

Table (1) shows that the isolation rate of *Campylobacter* spp. from human stool samples (2.7%) was nearly similar to 2.3% and 2.9% prevalence rates obtained by Varoli *et al.* (1989) and Kang *et al.* (2006), respectively. However, slightly higher prevalence of 6% in Nigeria (Aboderin *et al.*, 2002) and 6.4% in Alexandria, Egypt (Pazzaglia *et al.*, 1995) were also reported. In Cairo, Egypt, Zaghloul *et al.* (2012) reported that *Campylobacter* spp. were identified in 6.6% of human stool samples. Moreover, a higher isolation rate of 16.7% was reported in Giza, Egypt, this higher percentage could be attributed to the sampling of stool samples from human in contact with food animals (Hassanain, 2011). The low prevalence rate of *Campylobacter* species in human samples during the current study could be attributed to the low number of samples collected from diarrheic patients (only 10).

Campylobacter spp. were then identified as *C. jejuni* and *C. coli* in 66.7% and 33.3%, of the examined human stool samples, respectively (Table 1). These results were similar to those reported in Cairo, Egypt, by Wasfy *et al.* (2000) who isolated both *C. jejuni* and *C. coli* from 63% and 37% of human stool samples, respectively. Also, Sorokin *et al.* (2007) isolated *C. jejuni* and *C. coli* in similar proportions as 69.3% and 30.7%, respectively, in Romania.

Molecular identification of C. jejuni and C. coli

The identification and discrimination of *C. jejuni* and *C. coli* is considered problematic because it only depends on a single phenotypic test based on the hydrolysis of hippurate (Steinhauserova *et al.*, 2001). Therefore, molecular identification methods have been described as an alternative to the inaccurate, time consuming, biochemical phenotypic methods (LaGier *et al.*, 2004). A number of conventional PCR assays targeting a variety of genes such as *hipO*, *glyA*, *cadf*, *ceuE* and *mapA* have been documented (On and Jordan, 2003). However, the recent development of real-time PCR removed the need to manipulate PCR products after amplification to reduce cross-contamination (LaGier *et al.*, 2004). The single copy gene *hipO* gene (benzoglycine amidohydrolase) is responsible for the hippurate activity which discriminates *C. jejuni* from other *Campylobacter* spp. (Englen *et al*., 2003). For *C. coli* identification, the genome of this species has *glyA* gene which has a unique specific nucleotide regions (Englen *et al.*, 2003). The aforementioned two genes are known to be highly conserved among *C. jejuni* and *C. coli*, respectively, enabling accurate discrimination between the two species.

Probe based qPCR reactions targeting *hipO* gene specific for *C. jejuni* and *glyA* gene specific for *C. coli* were used during the present study. The results in Table (1) show that out of 8 biochemically suspected *C. jejuni* isolates, 6 were confirmed by qPCR, while 4 *C. coli* isolates were confirmed by the amplification of *glyA* gene. These results strengthen the hypothesis that although hippurate hydrolysis test is widely used to differentiate *C. jejuni* from other species, *C. jejuni* hippurate negative strains and false positive strains have been isolated (Nayak *et al*., 2005). Furthermore, Englen *et al.* (2003) and LaGier *et al.* (2004) reported that about 10% of *C. jejuni* isolates fail to hydrolyze hippurate under laboratory conditions, resulting in misclassification of these isolates as *C. coli.* In addition, the hippurate hydrolysis assay is dependent upon the inoculums size of the bacterium, which means that the assay is unable to detect low level of hippuricase product (Linton *et al*., 1997). Therefore, the detection of the gene by PCR instead of the phenotypic detection of the hippuricase product is considered a reliable alternative method for the discrimination of *C. jejuni* isolates (Slater and Owen, 1997).

Detection of virulence factors

cadf gene is a putative virulence gene associated with adhesion of the pathogen to intestinal epithelial cells (Rozynek *et al*., 2005). This gene is 100% conserved among *C. jejuni* and *C. coli* isolates of diverse sources; therefore, it was used to detect virulent isolates of both species (Datta *et al*., 2003). In the present study, the confirmed *C. jejuni* isolates (n = 6) and *C. coli* isolates (n = 4) by probe based qPCR were examined for the presence of *cadf* gene using SYBR Green I based qPCR. The results showed that all the examined isolates were positive for *cadf* gene.

Wieczorek *et al.* (2012) reported that all *C. jejuni* and *C. coli* isolates identified from chicken meat samples were positive for *cadf* gene which is consistent with the results obtained during the present study. Moreover, Datta *et al.* (2003) identified *cadf* gene in 100% of *C. jejuni* isolates recovered from human stool, poultry meat, poultry feces and bovine feces. Nayak *et al.* (2005) reported also the identification of *cadf* gene from *C. jejuni* and *C. coli* isolates obtained from human and poultry sources.

Survival of C. jejuni in chicken meat at refrigeration and freezing temperatures

The efficiency of the quantitative PCR reaction for quantification

SYBR Green I qPCR targeting *cadf* gene was used during the current study to evaluate the influence of storage temperature on the survival of *C. jejuni* in chicken meat. Figure 1 shows that a high Pearson correlation coefficient (R^2 = 0.997) was obtained indicating a linear standard curve. This implies that the efficiency of amplification was consistent at varying template concentrations. The efficiency was calculated from the slope and it was found to be 101.7% which shows sufficient doubling of the product amount with each cycle. The inter assay precision was calculated in 7 repeats of standards and found to be less than 10% (4.8-6.07%) which is within the acceptable range showing minimal variation. This indicates the reproducibility of the assay over six orders of magnitude and high precision of the applied assay.

Survival of C. jejuni during storage at refrigeration and freezing

Poultry meat is believed to be predominantly associated with campylobacteriosis (Humphrey *et al*., 2007). This product is stored in outlets and at consumers by refrigeration and freezing in order to control microbial proliferation and spoilage (Dooley and Roberts, 2000). The ability of *Campylobacter* spp. to survive in food during storage represents a risk for human health due to the ability of the organism to produce infection with low infectious dose (Lori *et al.*, 2007). Little is known about how *Campylobacter* spp. persist in chicken meat, therefore, there is a need for quantitative data on survival of *Campylobacter* spp. at storage by refrigeration and freezing.

Previous studies on the survival of *Campylobacter* spp. at storage temperatures showed that freezing has an impact on the prevalence of the organism in chicken meat (Sampers *et al*., 2008). Although refrigeration was shown to effectively reduce the survived counts of *C. jejuni* in chicken meat, this method may not be considered the absolute preservation method since some samples showed bacterial survivors (Eideh and Al-Qadiri, 2011). Sampers *et al.* (2008) perceived that although microbial growth is absent during refrigeration and freezing of chicken meat, *C. jejuni* have been shown to survive for variable durations.

For practical purposes, storage of chicken meat at refrigeration temperature more than a week is outside their reported shelf life (Cox *et al*., 1998). However, in order to compare the results of the present experiment to previously reported ones, 20 days of storage was chosen. After 7 days of storage, a significant decline of 0.24 $\log_{10}$ CFU/ml ($P < 0.05$) was obtained at refrigeration temperature (Table 2). Likewise, previous studies of *C. jejuni* survival during refrigeration in chicken meat have demonstrated small declines after one week of storage (Bhaduri and Cottrell, 2004). For instance, in an experiment conducted by Bhaduri and Cottrell (2004), a decline in *C. jejuni* counts ranged from 0.34 to 0.81 log10 CFU/g on ground chicken meat samples kept for 7 days at 4^{o}C was observed. Eideh and Al-Qadiri (2011) and Blankenship and Craven (1982) reported a reduction of one log10 CFU/g and less than one log10 CFU/g after 7 days storage at refrigeration temperature, respectively.

The results obtained during the current study revealed that after 20 days of refrigeration storage there was a significant reduction of 1.1 $\log_{10}$ CFU/ml in the count of *C. jejuni* ($P < 0.05$). In accord with the obtained results, Kärenlampi and Hänninen (2004) reported that *C. jejuni* counts on sterile ground chicken meat declined by one $\log_{10}$ CFU/ml at 4^{o}C after storage for 17 days. Freezing exerts a lethal effect on *Campylobacter* spp., serving as a preventive measure by reducing the risk of exposing consumers to high numbers of *Campylobacter* spp. in chicken (Sampers *et al*., 2010). The effect of freezing on the survived numbers of *C. jejuni* could be explained by cell death caused by ice nucleation and dehydration during freezing (Mazur, 1970). Though, a proportion of *C. jejuni* was found frequently in frozen chicken at the retail level (Archer, 2003).

Immediately after chicken meat freezing, a rapid decrease in *C. jejuni* count was observed (Table 2); this is consistent with those originally represented by Hänninen (1981), Stern *et al.* (1985) and Georgsson *et al.* (2006). After three days freezing, *C. jejuni* reduction rate was 0.65 $\log_{10}$ CFU/ml (Table 2), this was lower than a reported decline of 1.3 $\log_{10}$ CFU/g in chicken wings stored at -20^{o}C (Zhao *et al*., 2003). Such difference could be attributed to the inoculation of the organism into the subsurface of chicken meat during the current experiment, providing microaerophilic conditions that to some extent protect the organism from the effect of freezing (Bhaduri and Cottrell, 2004).

A significant decline of 2.48 $\log_{10}$ CFU/ml in *C. jejuni* count after 14 and 20 days of freezing storage was obtained (Table 2). Similarly, after 14 days freezing storage of chicken meat contaminated with *C. jejuni*, a decline of 2 log10 CFU/g and 1.57 log10 CFU/g were obtained by Stern and Kotula (1982) and Bhaduri and Cottrell (2004), respectively. Eideh and Al-Qadiri (2011) used an inoculum of 2.7 log10 CFU/g during the experiment, and they reported a reduction of 1 log10 CFU/g after 20 days storage at -18^{o}C. It was also stated that 0.9 to 3.2 log10 reductions were observed in *C. jejuni* counts after 14 days of storage at -20^{o}C on chicken skin, below skin and on muscle parts that were naturally contaminated with several strains of *C. jejuni* (Sampers *et al.*, 2010). The inconsistency of the current results with previously reported ones could be a reason of different initial size of inoculums. This was supported by Pearson *et al.* (1996) and Sampers *et al.* (2008) who reported that the higher the initial bacterial count, the higher is the number of survivals after exposure to a chilling temperature stress. Moreover, genetic differences between strains of *C. jejuni* have been described, so it is expected that the resistance of *C. jejuni* to temperature stress could be strain related (Martinez-Rodriguez and Mackey, 2005; Oyarzabal *et al*., 2010).

A long term holding of meat at freezing temperature for 84 days reduced the initial numbers of *Campylobacter* spp. below the detection limit of 10 CFU/g; however, *Campylobacter* spp. were still detected by culture (Sampers *et al*., 2010). Moreover, Georgsson *et al.* (2006) reported that after 220 days of chicken meat freezing at -20^{o}C, positive samples were detected. Although the existence of *Campylobacter* species in chicken meat is considered a risk for consumers, various risk assessments approved that high risk of infection was mostly attributed to the highest load of the organism in chicken meat (Nauta *et al*., 2008). These risk assessments concluded that the most effective intervention measures aim at reducing *Campylobacter* spp. concentrations, rather than reducing the prevalence (Sampers *et al*., 2010). Therefore, freezing of chicken meat preparations could be considered a preventive measure that reduces the risk of exposure to high *Campylobacter* concentrations (Sampers *et al*., 2010). Nevertheless, poultry handling during slaughter and evisceration has a significant impact on the risk of poultry meat contamination rather than storage temperature.

In conclusion, storage of poultry meat at freezing temperature is preferred to refrigeration due to the significant decline of *C. jejuni* count during freezing for a duration ranging from 3-20 days.

References

Aboderin, A. O., Smith, S. I., Oyelese, A. O., Onipede, A. O., Zailani, S. B. and Coker, A. O. 2002. Role of *Campylobacter jejuni/coli* in diarrhoea in Ile-Ife, Nigeria. East African Medical Journal 79 (8): 423-426.

Anderson, J., Horn, B. J. and Gilpin, B. J. 2012. The prevalence and genetic diversity of Campylobacter spp. in domestic backyard poultry in Centerbury, New Zealand. Zoonosis and Public Health 59 (1): 52-60.

Anonymous. 2007. Emerging infections program. Food Net News 1 (1).

Archer, D. 2003. Freezing: an under-utilized food safety technology. International Journal of Food Microbiology 90: 127–38.

Bhaduri, S. and Cottrell, B. 2004. Survival of cold-stressed *Campylobacter jejuni* on ground chicken and chicken skin during frozen storage. Applied and Environmental Microbiology 70 (12): 7103–7109.

Blankenship, L. C. and Craven, S. E. 1982. *Campylobacter jejuni* survival in chicken meat as a function of temperature. Applied and Environmental Microbiology 44: 88–92.

Chantarapanont, W., Berrang, M. and Frank, J. F. 2003. Direct microscopic observation and viability determination of *Campylobacter jejuni* on chicken skin. Journal of Food Protection 6: 2222–2230.

Coker, A. O., Isokpehi, R. D., Thomas, B. N., Amisu, K. O. and Obi, C. L. 2002. Human campylobacteriosis in developing countries. Emerging Infectious Diseases 8 (3): 237–244.

Corry, J. E. L. and Atabay, H. I. 2001. Poultry as a source of *Campylobacter* and related organisms. Journal of Applied Microbiology 90: 96S–114S.

Cox, N. A., Russell, S. M. and Bailey, J. S. 1998. The microbiology of stored poultry. In Davies, A. and Board, R. (Eds). The microbiology of meat and poultry. p. 266–267. New York: Blackie Academic & Professional.

Datta, S., Niwa, H. and Itoh, K. 2003. Prevalence of 11 pathogenic genes of *Campylobacter jejuni* by PCR in strains isolated from humans, poultry meat and broiler and bovine faeces. Journal of Medical Microbiology 52 (4): 345–348.

Dooley, J. S. G. and Roberts, T. A. 2000. Control of vegetative micro-organisms in foods. British Medical Bulletin 56 (1): 142–157.

EFSA-ECDC 2009. The community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. EFSA Journal 223, 223-240.

Eideh, A. M. F. and Al-Qadiri, H. M. 2011. Effect of refrigerated and frozen storage on the survival of *Campylobacter jejuni* in cooked chicken meat breast. Journal of Food Science 76 (1): 17-21.

Ellerbroek, L. I., Lienan, J. A. and Klein, G. 2010. *Campylobacter* spp. in broiler flocks at farm level and the potential for cross-contamination during slaughter. Zoonoses and Public Health 57 (7-8): 81- 88.

Ellis-Iversen, J., Jorgensen, F., Bull, S., Powell, L., Cook, A. J. and Humphrey, T. J. 2009. Risk factors for *Campylobacter* colonization during rearing of broiler flocks in Great Britain. Preventive Veterinary Medicine 89 (3-4): 178-184.

Englen, M. D., Ladely, S. R. and Fedorka-Cray, P. J. 2003. Isolation of *Campylobacter* and identification by PCR. Methods in Molecular Biology 216: 109–121.

Fitzgerald, C., and Nachamkin, I. 2007. Campylobacter and Arcobacter. In Murray, P. R. (Ed.). Manual of Clinical Microbiology, p. 933-946. Washington D.C.: ASM Press.

Fitzgerald, C., Whichard, J. and Nachamkin, I. 2008. Campylobacter. In Nachamkin, I., Szymanski, C., Blaser, M. (Eds.). Diagnosis and antimicrobial susceptibility of Campylobacter species, p. 227-243. Washington, DC: ASM Press.

Garin, B., Gouali, M., Wouafo, M., Perchec, A., Thu, P. M., Ravaonindrina, N., Urbès, F., Gay, M., Diawara, A., Leclercq, A., Rocourt, J. and Pouillot, R. 2012. Prevalence, quantification and antimicrobial resistance of *Campylobacter* spp. on chicken neck-skins at points of slaughter in 5 major cities located on 4 continents. International Journal of Food Microbiology 157: 102–107.

Georgsson, F., Thornorkelsson, A. E., Geirsdóttir, M., Reiersen, J., Stern, N. J. 2006. The influence of freezing and duration of storage on Campylobacter and indicator bacteria in broiler carcasses. Food Microbiology 23 (7): 677–683.

Guyard-Nicodème, M., Tresse, O., Houard, E., Jugiau, F., Courtillon, C., El Manaa, K., Laisney a, M. and Chemaly, M. 2013. Characterization of *Campylobacter* spp. transferred from naturally contaminated chicken legs to cooked chicken slices via a cutting board. International Journal of Food Microbiology 164 (1): 7–14.

Hänninen, M. L. 1981. Survival of *Campylobacter jejuni/coli* in ground refrigerated and in ground frozen beef liver and in frozen broiler carcasses. Acta Veterinaria Scandinavica 22: 566–577.

Hassanain, N. A. 2011. Antimicrobial resistant *Campylobacter jejuni* isolated from humans and animals in Egypt. Global Veterinaria 6 (2): 195-200.

Henry, I., Reichardt, J., Denis, M. and Cardinale, E. 2011. Prevalence and risk factors for *Campylobacter* spp. in chicken broiler flocks in Reunion Island (Indian Ocean). Preventive Veterinary Medicine 100 (1): 64-70.

Humphrey, T., O'Brien, S. and MadSen, M. 2007. *Campylobacter* spp. as zoonotic pathogens: a food production perspective. International Journal of Food Microbiology 117: 237–257.

Jacobs-Reitsma, W. 2000. Campylobacter in the food supply. In Nachamkin, I. and Blaser, M. J. (Eds). Campylobacter, p. 467–4812. Washington, D.C: ASM Press.

James, C., Vincent, C., Lima, T. I. and James, S. J. 2006. The primary chilling of poultry carcasses: a review. International Journal of Refrigeration 29: 847–62.

Kang, Y. S., Cho, Y. S., Yoon, S. K., Yu, M. A., Kim, C. M., Lee, J. O. and Pyun, Y. R. 2006. Prevalence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from raw chicken meat and human stools in Korea. Journal of Food Protection 69 (12): 2915-2923.

Kärenlampi, R. and Hänninen, M. L. 2004. Survival of *Campylobacter jejuni* on various fresh produce. International Journal of Food Microbiology 97: 187–95.

Kiss, I. 1984. Testing methods in food microbiology. AkademiaKiado, Budapest, Hungary.

Klein, D., Janda, P., Steinborn, R., Muller, M., Salmons, B. and Gunzburg, W.H. 1999. Pro-viral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: influence of mismatches on quantification. Electrophoresis 20: 291-299.

Kovalenko, K., Roasto, M., Liepin, E., Mäesaar, M. and Hörman, A. 2013. High occurrence of *Campylobacter* spp. in Latvian broiler chicken production. Food Control 29: 188-191.

LaGier, M. J., Joseph, L. A., Passaretti, T. V., Musser, K. A. and Cirino, N. M. 2004. A real-time multiplexed PCR assay for rapid detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli.* Molecular and Cellular Probes 18 (4): 275-282.

Lastovica, A. J. and Allos, B. M. 2008. Clinical significance of *Campylobacter* and related species other than *Campylobacter jejuni* and *Campylobacter coli.* In Nachamkin, I., Szymanski, C., Blaser, M. (Eds.). Campylobacter, p. 123-149. Washington, DC: ASM Press.

Lee, A., Smith, S. C. and Coloe, P. J. 1998. Survival and growth of *Campylobacter jejuni* after artificial inoculation onto chicken skin as a function of temperature and packaging conditions. Journal of Food Protection 61 (12): 1609-14.

Linton, D., Lawson, A. J., Owen, R. J. and Stanley, J. 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. Journal of Clinical Microbiology 35: 2568–2572.

Lori, S., Buckow, R., Knorr, D., Heinz, V. and Lehmacher, A. 2007. Predictive model for inactivation of *Campylobacter* spp. by heat and high hydrostatic pressure. Journal of Food Protection 70 (9): 2023-2029.

Luu, Q. H., Tran, T. H., Phung, D. C. and Nguyen, T. B. 2006. Study on the prevalence of *Campylobacter* spp. from chicken meat in Hanoi, Vietnam. Annals of the New York Academy of Science 1081: 273–275.

Marinou, I., Bersimis, S., Ioannidis, A., Nicolaou, C., Mitroussia-Ziouva, A., Legakis, N. J. and Chatzipanagiotou, S. 2012. Identification and antimicrobial resistance of *Campylobacter* species isolated from animal sources. Frontiers in Microbiology 3 (58): 1-6.

Martinez-Rodriguez, A. and Mackey, B. 2005. Physiological changes in *Campylobacter jejuni* on entry into stationary phase. International Journal of Food Microbiology 101: 1–8.

Mazur, P. 1970. Cryobiology: The freezing of biological systems. Science 168 (934): 939-949.

Mead, G. C., Hudson, W. R. and Hinton, M. H. 1995. Effects of changes in processing to improve hygiene control on contamination of poultry carcasses with *Campylobacter.* Epidemiology and Infection 115: 495–500.

Meerburg, B. G., Jacobs-Reitsma, W. F., Wagenaar, J. A. and Kijlstra, A. (2006): Presence of *Salmonella* and *Campylobacter* spp. in wild small mammals on organic farms. Applied and Environmental Microbiology 72 (1): 960–962.

Melo, R. T., Nalevaiko, P. C., Mendonça, E. P., Borges, L. W., Fonseca, B. B., Beletti, M. E. and Rossi, D. A. 2013. *Campylobacter jejuni* strains isolated from chicken meat harbor several virulence factors and represent a potential risk to humans. Food Control 33: 227-231.

Modolo, J. R. and Giuffrida, R. 2004. *Campylobacter upsaliensis* isolated from young dogs with and without diarrhea. Revista da Sociedade Brasileira de Medicina Tropical 37 (1): 72-73.

Nachamkin, I. 1999. Campylobacter and Arcobacter. In Muray, P. R., Baron, E. J. et al.(Eds). Manual of clinical microbiology, p. 716-726. Washington, D. C: ASM press.

Nayak, R., Stewart, T. M. and Nawaz, M. S. 2005. PCR identification of *Campylobacter coli* and *Campylobacter jejuni* by partial sequencing of virulence genes. Molecular and Cellular Probes19 (3): 187-193.

Nauta, M., Hill, A., Rosenquist, H., Brynestad, S., Fetsch, A., van der Logt, P., Fazil, A., Christensen, B., Katsma, E., Borck, B. and Havelaar, A. 2008. A comparison of risk assessments on *Campylobacter* in broiler meat. International Journal of Food Microbiology 129 (2): 107–123.

On, S. L. W. and Jordan, P. J. 2003. Evaluation of 11 PCR assays for species-level identification of *Campylobacter jejuni* and *Campylobacter coli.* Journal of Clinical Microbiology 41: 330–6.

Oyarzabal, O. A., Oscar, T. P., Speegle, L.and Nyati, H. 2010. Survival of *Campylobacter jejuni* and *Campylobacter coli* on retail broiler meat stored at -20, 4, or 12^{o}C and development of weibull models for survival. Journal of Food Protection 73 (8): 1438–1446.

Parkar, S. F. D., Sachdev, D., deSouza, N., Kamble, A., Suresh, G. Munot, H., Hanagal, D., Shouche, Y. and Kapadnis, B. 2013. Prevalence, seasonality and antibiotic susceptibility of thermophilic *Campylobacter* spp. in ceca and carcasses of poultry birds in the "livebird market". African Journal of Microbiological Research 7 (21): 2442-2453.

Pazzaglia, G., Bourgeois, A. L., Mourad, A. S., Gaafar, T., Diab, A. S., Hebert, A., Churilla, A. and Murphy, J. R. 1995. *Campylobacter* diarrhea in Alexandria, Egypt. Journal of Egyptian Public Health Association 70 (3-4): 229-241.

Pearson, A., Greenwood, M., Felthman, R., Healing, T., Donaldson, J., Jones, D. and Colwell, R. 1996. Microbial ecology of *Campylobacter jejuni* in a United Kingdom chicken supply chain: intermittent common sources, vertical transmission, and amplification by flock propagation. Applied and Environmental Microbiology 62: 4614–20.

Rahimi, E. and Tajbakhsh, E. 2008. Prevalence of *Campylobacter* species in poultry meat in the Esfahan city, Iran. Bulgarian Journal of Veterinary Medicine 11 (4): 257−262.

Rozynek, E., Dzierzanowska-Fangrat, K., Jozwiak, P., Popowski, J., Korsak, D. and Dzierzanowska, D. 2005. Prevalence of potential virulence markers in Polish *Campylobacter jejuni* and *Campylobacter coli* isolates obtained from hospitalized children and from chicken carcasses. Journal of Medical Microbiology 54: 615-619.

Sampers, I., Habib, I., Berkvens, D., Dumoulin, A., Zutter, L. D. and Uyttendaele, M. 2008. Processing practices contributing to *Campylobacter* contamination in Belgian chicken meat preparations. International Journal of Food Microbiology 128 (2): 297-303.

Sampers, I., Habib, I., Zutter , L. D., Dumoulin, A. and Uyttendaele, M. 2010. Survival of *Campylobacter* spp. in poultry meat preparations subjected to freezing, refrigeration, minor salt concentration, and heat treatment. International Journal of Food Microbiology 137: 147–153.

Samuel, M. C., Vugia, D. J., Shallow, S., Marcus, R., Segler, S. and McGivern, T. 2004. Epidemiology of sporadic *Campylobacter* infection in the United States and declining trend in incidence, Food Net 1996–1999. Clinical Infectious Diseases 38 (Suppl 3):S165–74.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L. and Griffin, P. M. 2011. Foodborne illness acquired in the United States–major pathogens. Emerging Infectious Diseases 17: 7–15.

Skirrow, M. B. 1977. Campylobacter enteritis: a "new" disease. British Medical Journal 2: 9.

Slater, E. R. and Owen, R. J. 1997. Restriction fragment length polymorphism analysis shows that the hippuricase gene of *Campylobacter jejuni* is highly conserved. Letters in Applied Microbiology 25: 274–278.

Snedecor, G. W. and Cochran, W. G. 1982. Statistical Methods. 6th ed., Iowa State University Press, Ames, USA.

Sorokin, M., Usein, C. R., Irimia, M. and Damian, M. 2007. A laboratory-based survey of Campylobacter infections in Prahova County. Roumanian archives of microbiology and immunology 66 (3-4): 85-89.

Steinhauserova, I., Ceskova, J., Fojtikova, K. and Obrovska, I. 2001. Identification of thermophilic *Campylobacter* spp. by phenotypic and molecular methods. Journal of Applied Microbiology 90: 470–5.

Stern, N. J. and Kotula, A. W. 1982. Survival of *Campylobacter jejuni* inoculated into ground beef. Applied and Environmental Microbiology 44 (5): 1150–1153.

Stern, N. J., Rothenberg, P. J. and Stone, J. M. 1985. Enumeration and reduction of *Campylobacter jejuni* in poultry and red meats. Journal of Food Protection 48, 606–610.

Studer, E., Luthy, J. and Hubner, P. 1999. Study of the presence of *Campylobacter jejuni* and *C. coli* in sand samples from four Swiss chicken farms. Research in Microbiology 150 (3): 213–219.

Suzuki, H. and Yamamoto, S. 2009. *Campylobacter* contamination in retail poultry meats and by-products in the world: a literature survey. The Journal of Veterinary Medical Science 71 (3): 255–261.

Thatcher, P. S. and Clark, D. S. 1968. Microorganisms in foods: their significance and methods of enumeration. Volume 1. Toronto, University of Toronto Press.

Van Asselt, E. D., Jacobs-Reitsma,W. F., Van Brakel, R., Van Der Voet, H. and Van Der Fels-Klerx, H. J. 2008. *Campylobacter* prevalence in the broiler supply chain in the Netherlands. Poultry Science 87: 2166-2172.

van Nierop, W., Duse´, A. G., Marais, E., Aithma, N., Thothobolo, N., Kassel, M., Stewart, R., Potgieter, A., Fernandes, B., Galpin, J. S. and Bloomfield, S. F. 2005. Contamination of chicken carcasses in Gauteng, South Africa, by *Salmonella*, *Listeria monocytogenes* and *Campylobacter.* International Journal of Food Microbiology 99: 1–6.

Varoli, O., Gatti, M. and Piscolla, F. 1989. A one-year study on thermophilic *Campylobacter* spp. isolated from fecal specimens. Microbiologica 12 (3): 263-5.

Waino, M., Bang, D. D., Lund, M., Nordentoft, S., Andersen, J. S., Pedersen, K. and Madsen, M. 2003. Identification of *Campylobacteria* isolated from Danish broilers by phenotypic tests and species-specific PCR assays. Journal of Applied Microbiology 95: 649-655.

Wasfy, M. O., Oyofo, B. A., David, J. C., Ismail, T. F., El-Gendy, A. M., Mohran, Z. S., Sultan, Y., Peruski, L. F. 2000. Isolation and antibiotic susceptibility of *Salmonella*, *Shigella* and *Campylobacter* from acute enteric infections in Egypt. Journal of Health, Population and Nutrition 18 (1): 33-38.

Wieczorek, K., Szewczyk, R. and Osek, J. 2012. Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter jejuni* and *C. coli* isolated from retail raw meat in Poland. Veterinarnia Medicina 57 (6): 293–299.

Yang, C., Jiang, Y. Huang, K., Zhu, C., Yin, Y. 2003. Application of real-time PCR for quantitative detection of *Campylobacter jejuni* in poultry, milk and environmental water. FEMS Immunology and Medical Microbiology 38 (3): 265-271.

Zaghloul, M. Z., Farouk, N. and Galal, Z. A. 2012.

Detection of *Cambylobacter* spp. in stool samples by new methods in comparison to culture. Life Science Journal 9 (4): 2566-2571.

Zhao, T., Ezeike, G. O. I., Doyle, M. P., Hung, Y. and Howell, R. S. 2003. Reduction of *Campylobacter jejuni* on poultry by low-temperature treatment. Journal of Food Protection 66 (4): 535-709.