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

1Awadallah, M. A. I., 1Ahmed, H. A., 2El-Gedawy, A. A., and 1Saad, A. M.

1Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Egypt
2Department of Bacteriology, TB unit, Animal health Research Institute, Dokki, Giza

Article history
Received: 16 February 2014
Received in revised form: 16 March 2014
Accepted: 17 March 2014

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; Meerburt 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 (Fitzgerald 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 Campylobacter 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.

*Corresponding author.
Email: heba_ahmed@zu.edu.eg
**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 diarrheic 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 37°C 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 42°C for 48 hours under microaerophilic conditions (5% O₂, 10% CO₂ and 85% N₂) 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 (H₂S) production, citrate utilization test and rapid hippurate hydrolysis test (Nachamkin, 1999).

**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 and probe are as follows: Cj-F1 forward: 5’-TGCTAGTGAGTTGCAAAAAAGATT-3’, Cj-R1 reverse: 5’-TCATTTTCGAAAAAATTCAAAA-3’, Cj-FAM probe: 5’-AGCATGATTACATTCAATTTTTTCGCCAAA-3’. For glyA, sequences of the primers and probe are as follows: Cc-F1 forward: 5’-CATATTGAAAACCAAGCTTATCGG-3’, Cc-R1 reverse: 5’-AGTCCAGCAATGTGTGCAATG-3’, Cc-FAM probe: 5’-TAAGCTCACCATTTCATCCGC AATCTCTCATAATT-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, (NH₄)₂SO₄, 8 mM MgCl₂], dNTP mix including dUTP, ROX™ passive reference dye and 8 mM MgCl₂), 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*, *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 50°C for two minutes to activate UNG, 95°C for 15 min then 40 cycles at 94°C for 15 sec and 60°C 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 OpticomMonitor™ Analysis software version 3.1 (Bio-Rad).

**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’-TGCTAGTGAGTTGCAAAAAAGATT-3’, Cj-R1 reverse: 5’-TCATTTTCGAAAAAATTCAAAA-3’, Cj-FAM probe: 5’-AGCATGATTACATTCAATTTTTTCGCCAAA-3’. For glyA, sequences of the primers and probe are as follows: Cc-F1 forward: 5’-CATATTGAAAACCAAGCTTATCGG-3’, Cc-R1 reverse: 5’-AGTCCAGCAATGTGTGCAATG-3’, Cc-FAM probe: 5’-TAAGCTCACCATTTCATCCGC AATCTCTCATAATT-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 Biosysstem 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, (NH₄)₂SO₄, 8 mM MgCl₂], dNTP mix including dUTP, ROX™ passive reference dye and 8 mM MgCl₂), 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*, *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 50°C for two minutes to activate UNG, 95°C for 15 min then 40 cycles at 94°C for 15 sec and 60°C 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 OpticomMonitor™ Analysis software version 3.1 (Bio-Rad).
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 10⁷ CFU/ml (equals 7 log₁₀ 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 log₁₀ CFU/ml) was aseptically serially diluted using 9 ml sterile saline solution as diluent in order to obtain 6 log₁₀ CFU/ml, 5 log₁₀ CFU/ml, 4 log₁₀ CFU/ml, 3 log₁₀ CFU/ml, 2 log₁₀ CFU/ml and 1 log₁₀ 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 log₁₀ 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^{15.3}-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.

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}
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°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.

**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.**
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 butchers. 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 inoculum 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 (Rozynec 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 (R² = 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 (Sampsers 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°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°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°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 log_{10} CFU/g and 1.57 log_{10} 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 log_{10} CFU/g during the experiment, and they reported a reduction of 1 log_{10} CFU/g after 20 days storage at -18°C. It was also stated that 0.9 to 3.2 log_{10} reductions were observed in *C. jejuni* counts after 14 days of storage at -20°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°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


Jacobs-Reitsma, W. 2000. Campylobacter in the food


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