# Identification of *Campylobacter jejuni* in poultry products by Real-Time PCR in Al-Muthanna province

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## Abstract

The study was undertaken due to the potential contribution of poultry products is less well known in Iraq. In this study, *Campylobacter jejuni* identified by application SYBR green based Real-Time PCR technique by amplification the specific ***hipo*** gene in (96) chicken tissue samples. The results show no significant difference in detection rates between fresh (77.8%) and frozen (83.3%) poultry tissue samples. In freshly slaughtered poultry samples, the liver detection rate (93.75 %) was higher than skin (62.5%) and meat (75%) while in frozen poultry samples, the detection rate was higher in meat (93.75 %) than skin (87.5%) and liver (68.75%). In conclusion the study showed high contamination rates of poultry with *Campylobacter jejuni* in retail markets and the direct Real-Time PCR is suitable assay for screening poultry products and identification of *Campylobacter jejuni* pathogen.

**Key words: *Campylobacter jejuni*, poultry products, Real-Time PCR, *hipo* gene.**

# تشخيص *Campylobacter jejuni* في منتجات الدواجن باستخدام تقنية الوقت الحقيقي لتفاعل سلسلة البلمرة في محافظة المثنى

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## الخلاصة

اتخذت هذه الدراسة نتيجة لقلة المعلومات حول الدور المحتمل لمنتجات الدواجن كمصدر للإصابة في العراق. في هذه الدراسة جرثومة *Campylobacter jejuni* شخصت باستخدام تقنيةِ الوقت الحقيقي لتفاعل سلسلة البلمرة المعتمد على مجس صبغة SYBR الخضراء باستخدام بادئات تفاعل جين ***hipo*** الخاص في (96) عينة دجاج نسيجية. أظهرت الدراسة بأن ليس هناك فرق معنوي في نِسَبِ الكشفِ لعينات الدواجن النسيجية بين الدجاج المذبوح حديثا 77.8 % والمجمّد 83.3 %. في العينات النسيجية للدواجنِ المذبوحةِ حديثا ، كانت نسبة الكشفِ في الكبدَ 93.75 % وكَانَ أعلى مِنْ الجلدِ 62.5 % واللحم 75 % بينما في عيناتِ الدواجنِ المجمّدةِ، نسبة الكشفَ كَانتْ أعلى في اللحمِ 93.75 % مِنْ الجلدِ 87.5 % والكبد 68.75%. نستنتج من الدراسة بان هناك نسب عالية من تلوث لحوم الدواجن في الاسواق ب *Campylobacter jejuni* وان تقنية الوقت الحقيقي لتفاعل سلسلة البلمرة هي اختبار مناسب لفحص منتجات الدواجن و لتشخيص *العامل المرضي Campylobacter jejuni* .

**الكلمات المفتاحية: منتجات الدواجن ، الوقت الحقيقي لتفاعل سلسلة البلمرة ، جين *hipo* .**

## Introduction

Campylobacteriosis is a common zoonotic disease that affect human and cause gastrointestinal disturbances (1). The main sources of *Campylobacter jejuni* infection in human are believed to be the handling and consumption of contamination meat, especially poultry meat (2), where poultry is the natural host for zoonotic *Campylobacter* and infected birds carry a very high *Campylobacter jejuni* load in their gut and consider as a commensal, most bird flocks are colonized within several days and still so until slaughter, contaminated broiler chicken meat with *Campylobacter* is an important source of food-borne gastroenteritis (3). On the other hand contact with pets and livestock, the consumption of raw milk and untreated water and traveling in high

prevalence area considered risk factors in human disease (2). This research was undertaken to detect the contamination in poultry products as a cause of human infection by direct Real-Time PCR assay by using ***hipo*** gene which is encode a species - specific hippurate hydrolase enzyme (4) that hydrolyze hippurate (N-benzoyl glycine) to benzoic acid and glycine is regularly used to differentiate *Campylobacter jejuni* from other *Campylobacters* species (5). There is no previous study seek about zoonotic importance or detection of *Campylobacter jejuni* in chicken meat in Iraq.

## Materials and methods

**Sample collection:** A total of (48) fresh alive chicken purchased from different local markets and a total of (32) frozen packaged chicken without liver and (16) frozen liver samples purchased from different super markets from different trademarks in Al-Muthanna province from October 2013 to February 2014. Quickly, the skin, meat (thigh and breast) and liver samples were took aseptically and placed in sterile plastic container and frozen at – 20 ˚C until DNA extraction time.

**DNA Extraction:** Bacterial genomic DNA was extracted from poultry tissue samples by using Genomic DNA Mini Kit (Tissue), (Geneaid, USA) and done according to manufacturer instructions. The extracted genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA) that check DNA concentration and estimation of DNA purity through reading the absorbance in at (260 /280 nm).

**Primers:** By using an Oligonucleotide primers were selected to amplify the unique specific DNA sequence of *Campylobacter jejuni* ***hipo*** gene, and designed by NCBI-Gene Bank data base (**Gene bank accession number AY944164.1**) and Primer 3 plus online. The primers were synthesized by (Bioneer company \ Korea). The target sequence of primers as in table (1), were subjected to a BLAST search in nucleic acid database (http://.ncbi.nlm.nih.gov/ BLAST). To confirm the specificity of the assay, the specific primers were subjected to empirical screening in which a total of (5) bacterial DNA extracted including, *Salmonella typhimurium, E. coli, Staph. aurous, vibrio cholera* and *proteus.*

**Table (1): The primers sequence of *Campylobacter jejuni hipo* gene.**

| Primer | Sequence | | Product size |
|---|---|---|---|
| *Hipo* | F | GGTGCGATGATGGCTTC | 84bp |
| | R | CTTTTCTGGAGCACTTC | |

**Real-Time PCR Assay:** Real-Time PCR was achieved for detection of *Campylobacter jejuni* in poultry tissue samples by using SYBR green chemistry and carried out as previously described (6). Real-Time PCR master mix was prepared by using Accu Power ® 2X Green star qPCR master mix kit (Bioneer. Korea), and performed in accordance with manufacturer instructions as table (2).

**Table (2): Real-Time PCR master mix components.**

| Real-Time PCR master mix | Volume |
|---|---|
| DNA template | 2.5 µl |
| Forward primer(10 pmol) | 1 µl |
| Reverse primer (10 pmol) | 1 µl |
| 2X SYBR Green master mix | 12.5 µl |
| DEPC water | 8 µl |
| Total | 25µl |

The Real-time PCR master cycler (Eppendorf \Germany) parameters was adjusted according to the primer annealing temperature and Real-Time PCR SYBR Green kit instructions as following: Pre-Denaturation 95 °C for 5 min. for1cycle, Denaturation 95 °C for 15 sec. and Annealing 57.8 °C for 30 sec. repeated 45 time. Real-Time PCR result were analyzed by using realplex Real- Time PCR software system by calculation the threshold cycle number (CT value) that represented the positive amplification of ***hipo*** gene and melting curve that represented primer specify for *Campylobacter jejuni* detection.

**Agarose gel electrophoresis:** Real-Time PCR products (84 bp) as specific for ***hipo*** gene were examined by 1.5% agarose gel electrophoresis stained with Ethidium bromide and were visualized by using UV trans illuminator (VilBer Lourmat\ France).

**Statistical analysis:** The chi square was used to assess statistical significance (7).

## Results

All poultry tissue samples were extracted and evaluated by Nano-drop were at the accepted range where the accepted concentration and purity of genomic DNA for Real-Time PCR assay is (5-100) ng\µl and (1.2-2.4) respectively. The primers tested at BLAST (http://.ncbi.nlm.nih.gov/BLAST). The specificity of the primers were also tested against *Salmonella*, *E. coli*, *Staphylococcus aurous*, *vibrio cholera* and *proteus*, all of which were found to be negative in this Real-Time PCR assay. In the present study *Campylobacter jejuni* detected by Real-Time PCR in poultry tissue samples directly from part which eaten and not all parts of carcass, including skin, meat (thigh and breast) and liver with comparing between fresh slaughtered and frozen poultry. Fig. (1); represented amplification plots of 24 positive samples, the results showed different positive reaction cycles of threshold (Ct). The target amplicon distinguished from other PCR products by melting curve analysis, where the melting curve analysis performed in this study after SYBR®green I assay that showed melting peak about 80˚C (Fig. 2). The positive percentages in all poultry tissue samples were (80.2%); the positive percentage in fresh slaughtered chicken was (77.08 %) while in frozen chicken products were (83.3 %). There is no significant difference at (P< 0.05) between the positive percentage of both fresh and frozen poultry samples (Table 3). In tissue samples of freshly slaughtered chicken, the liver results (93.75%) were higher than skin and meat which was (62.5%) and (75%) respectively, with significant difference at (p< 0.05) (Table 4).

**Table (3): The rate of positive results in fresh chicken samples in comparison with frozen chicken samples.**

| Samples | No. of samples | Positive samples | Positive percentage |
|---|---|---|---|
| Fresh | 48 | 37 | 77.08 [a] |
| Frozen | 48 | 40 | 83.3 [a] |
| Total | 96 | 77 | 80.2 |

Similar letters refer to the non-significant differences among type of chicken samples at (p< 0.05).

**Table (4): *Campylobacter jejuni* positive samples of fresh chicken.**

| Tissue | No. of samples | Positive samples | Positive percentage |
|---|---|---|---|
| Skin | 16 | 10 | 62.5[a] |
| Meat | 16 | 12 | 75[a] |
| Liver | 16 | 15 | 93.75[b] |
| Total | 48 | 37 | 77.08 |

Similar litters refer to the non-significant differences among chicken parts while different litters refer to significant differences at (p<0.05).

**Table (5): *Campylobacter jejuni* positive samples of frozen chicken.**

| Tissue | No. of samples | Positive samples | Positive percentage |
|---|---|---|---|
| Skin | 16 | 14 | 87.5[ab] |
| Meat | 16 | 15 | 93.75[a] |
| Liver | 16 | 11 | 68.75[b] |
| Total | 48 | 40 | 83.3 |

Similar litters refer to the non-significant differences among chicken parts while different litters refer to significant differences at (p<0.05).

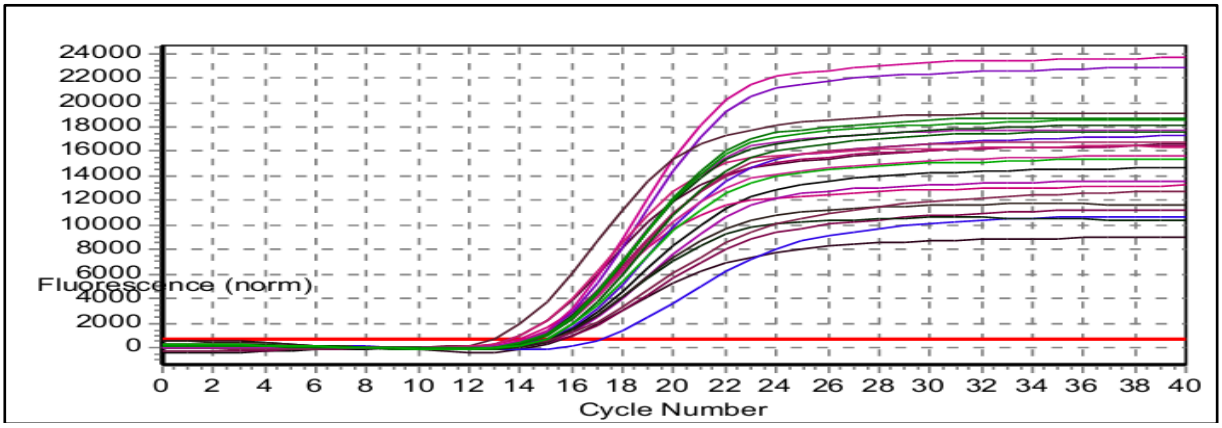


**Fig. (1): Real-Time PCR amplification plot shown the positive results of *campylobacter jejuni* in poultry tissue samples.**

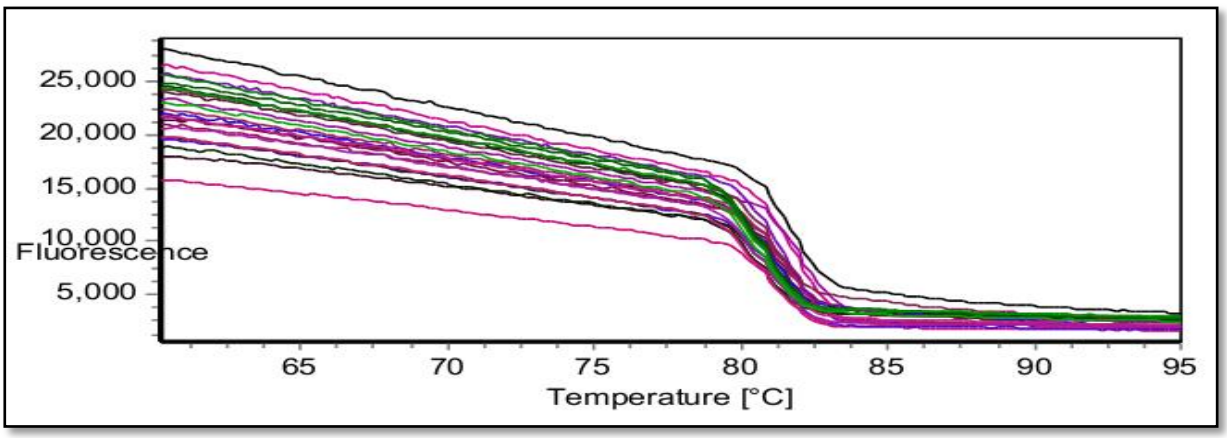


**Fig. (2): Real-Time PCR melting curve shown the positive results of *Campylobacter jejuni* in poultry tissue samples.**

The detection rate of *Campylobacter jejuni* in frozen chicken samples was (83.3%) higher than the detection rate of freshly slaughtered poultry samples, the meat (breast and thigh) (93.75%) was higher than skin and liver which were (87.5%) and (68.75%) respectively with significant difference at ($p<0.05$) between liver and meat samples but skin has no significant difference with meat and liver (Table 5). The agarose gel electrophoresis showed that *Campylobacter jejuni* amplicons matching a size of approximately 100 base pairs (Fig. 3).

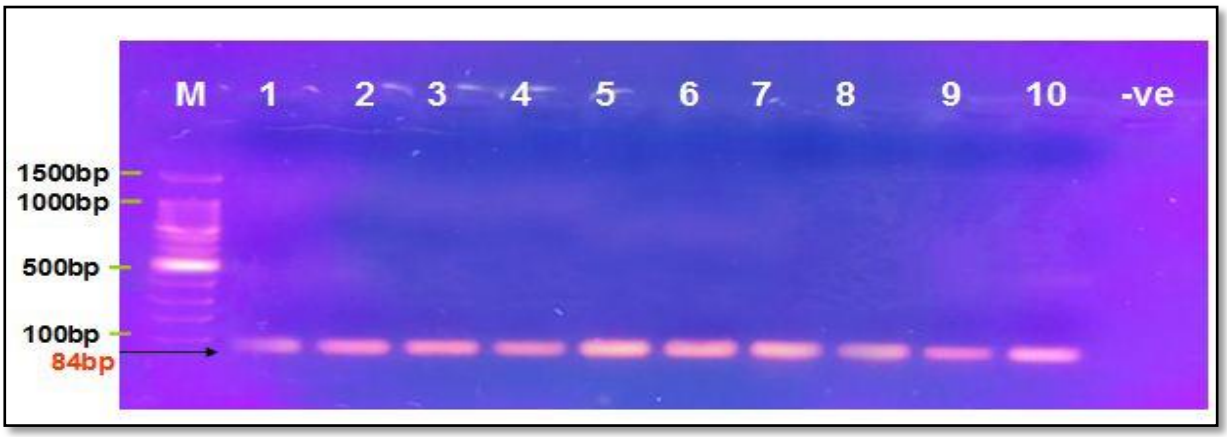


**Fig. (3): Agarose gel electrophoresis for *Campylobacter jejuni* PCR product of poultry samples.**

## Discussion

The present study describe a molecular method for assessment the retail chicken meat in Al-Muthanna local markets as a source of *Campylobacter jejuni* by direct Real-Time PCR assay because this technique confers high sensitive detection and has the ability to quantify nucleic acids at very little copies of target sequence and the reaction occurred in closed tubes that not require post manipulations where this reduce the chance of cross contamination and relatively quick (8). Real-Time PCR technique achieved by using SYBR ®green I chemistry to amplify a part of *Campylobacter jejuni* ***hipo*** gene. SYBR®green I based Real-Time PCR is highly specific, sensitive, much cheaper than probe and need a little optimization as an effective method for laboratory application (9, 10). The ***hipo*** gene is a unique species-specific sequence in *Campylobacter jejuni*, and this specificity has been reported previously (4). The extracted DNA of poultry tissue samples evaluated by Nano drop because the loss of DNA tamplet during

DNA extraction act as inhibition factor to Real-Time PCR, other PCR inhibitors compounds such as urea, hemoglobin, organic and phenolic inhibitors may affect the PCR (8), but the use of buffer lysis and proteinase K (11), and the centrifugation in DNA extraction method will remove these inhibitors. Real-Time PCR described previously as a direct assay that detected thermophilic *Campylobacter* spp. in chicken feces in less than 4 hours (12), and developed as quantitative detection of *Campylobacter jejuni* in food after enrichment culture (13). The results of present study (80.2%) for all samples are lower than the results of other study surveyed retail chicken meat products in Italy where the isolation percentage of *Campylobacter jejuni* was (87%) by application of SYBR green Real-Time PCR (6). Investigation of retail poultry meat in other countries have found the occurrence of *Campylobacter jejuni* in retail meat lower or higher than the results seen in this study but all referred to *Campylobacter jejuni* as the predominant species in poultry. The difference in results may be return to the difference in sampling, season, regions and the procedure of detection. The detection rate of *Campylobacter jejuni* in freshly slaughtered chicken samples was (77.08%) which higher than study suggested a (66.7%) of fresh chicken were positive with *Campylobacter jejuni* by using ***hipo*** gene primers (14) while lower than study results where *Campylobacter jejuni* has been reported as the most prevalent *Campylobacter* species in raw chicken meat in Iran by using PCR assay with (92.9%) (15). In Portugal, the positive percentage of *Campylobacter jejuni* isolation in different sources of meat samples was (68%) and the rate of *Campylobacter jejuni* isolation in chicken meat alone was (60.2%), reflecting that chicken is a major source of Campylobacteriosis (16).The frozen poultry products in present study contaminated with (83.3%). *Campylobacter jejuni* can survives at low temperature which indicated that frozen storage of foods cannot be considered a safety assuring procedure (17). The results of frozen poultry products were higher than (30.6%) those investigated of frozen chicken meat samples in China (18), and higher than (65.2%) in study reported that *Campylobacter jejuni* was the most frequently isolated species from chilled poultry and poultry products (19). The results of fresh skin sample in agreement with (20) who detect *Campylobacter jejuni* in (66%) of freshly slaughtered neck skin samples from slaughterhouses in Switzerland by direct multiplex Real-Time PCR, (10%) contaminated with *Campylobacter coli* and (30%) of samples had both species. Malaysian study showed that (51.06%) of poultry samples from different carcass parts contaminated with *Campylobacter jejuni* where (66.67%) of breast, (75%) of thigh and (66.67%) of liver were contaminated (21). In contrast to these findings, *Campylobacter jejuni* isolated from liver and intestine sample from broiler flocks in Turkey with (14.25%) by culturing and PCR assay where (8.5%) of liver samples and (20%) of intestinal samples were contaminated with referring that *Campylobacter jejuni* is more common in poultry than *Campylobacter coli* (22). Frozen liver samples result in our study disagreed with (23) who found (22.2%) of frozen liver contaminated with *Campylobacter jejuni* at retail markets in Bulgaria. The result of meat in this study disagreed with results of breast fillets where *Campylobacter jejuni* isolated with (31.2%) of samples (24). Another study similar to this study results, it suggested the *Campylobacter jejuni* average prevalence was (66%) in retail broiler meat in Alabama\USA, in breast was (66%) and in thigh was (70%) (24). In conclusion, The high positive percentages of *Campylobacter jejuni* in poultry meat suggesting that poultry meat are a major source of human Campylobacteriosis. The high contamination rates of meat of freshly slaughtered poultry and frozen poultry products give explanation that both at the same risk as a source of *Campylobacter jejuni*. The study showed the ability on direct application of Real-Time PCR application in screening of poultry products which is rapid, sensitive, specific and time-saving technique.

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