Comparison between stander isolation method, conventional PCR and real time PCR for detection of *Campylobacter* from frozen chicken

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Abstract

A total of 30 samples from frozen imported chickens were collected from samples which submitted to RLQP was used in comparison between 3 different methods for detection of *Campylobacter* stander isolation method, conventional PCR and real time PCR. The results 8 of 30 (26.7%) were detected by stander isolation method, 12 of 30 (40%) samples were detected by conventional PCR, 13 of 30 (43.3%) sampled were detected by real time PCR, one sample was positive for *Campylobacter coli* and *Campylobacter jejuni* at the same time by using real time PCR.

Key words: *Campylobacter, frozen chicken, Isolation, PCR, real time PCR .*

Introduction

The genus *Campylobacter* family *Campylobacteriaceae*, class Epsilonproteobacteria phylum Proteobacteria consists of sixteen species and eight subspecies, all of which are natural inhabitants of the intestinal tract of poultry and warm-blooded domestic animals where microaerohpilic conditions and the warm body temperature constitute an ideal environment for their continuous growth. The consumption of contaminated food and water by some species causes gastrointestinal illness in human, **(El-jakee *et al*., 2008).** *Campylobacter* enteritis in humans has been linked to consumption of poultry meat. Surveys show that 30–100% of poultry harbor *Campylobacter* as normal flora of the digestive tract which indicates a need to identify prevalent organism types in flocks and trace their epidemiology **(Sullivan *et al.,* 2000).** In general the occurrence of human *Campylobacter* gastroenteritis has been largely attributed to consumption of contaminated food of animal product especially poultry origin because of the high prevalence of *Campylobacter* in this animal product **(Gibreel and Taylor 2006).** Polymerase chain reaction (PCR) is molecular biology technique which has taken up an increasingly significant space in the field of laboratory diagnostics, allowing the detection of various pathogens such as *Campylobacter* species and *Salmonella* species in different kinds of food. PCR can reduce the time required to detect and identify the agent **(Santos *et al.,* 2001).** A limited number of real time PCR methods have been reported for the specific detection of *Campylobacter jejuni* further more the real time PCR method reported here is based on a specific and robust conventional gel –based PCR method validated in an international collaborative trail **(Josefsen *et al.,* 2004).**

Material and Method

Samples

A total of 30 samples were collected from different kinds of imported frozen chicken which were submitted to central laboratory for veterinary quality control on poultry production (RLQP) in a period between (2010 to 2011). We used thawed fluid (drip) for *Campylobacter* isolation

Isolation of and identification of *Campylobacter* was done according to **ISO 10272**

(1995)

PCR protocol

Positive control

Campylobacter jejuni (WHO C 10-1) (EQAS)

Campylobacter coli (WHO C 10-2) (EQAS)

Extraction of genomic DNA of Campylobacter was done by using boiling method **(De Medici *et al*., 2003).**

1-One ml of the 24 hours *Campylobacter* Preston culture was centrifuged at 14.000 xg for 10 minutes.

2-The pellet was resuspended in 300µl of DNase -RNase free distilled water and was vortexed. The cells were washed two times using DNase-RNase free distilled water.

3-The tube was centrifuged at 14.000 xg for 5 minutes then carefully the supernatant was discarded.

4-The pellet was resuspended in 200 µl of DNase-RNase-free distilled water then was vortexed.

5-The cells were boiled for 15 minutes at 100˚C and immediately chilled on ice.

6-Centrifugation was applied at 14.000 xg for 5 minutes.

7- - Two hundred µl supernatant (contain the DNA) was carefully transferred to a new sterile ependroff tube.

Table (1). Oligonucleotide primers sequences used in PCR for amplification of *ceuE* gene for *Campylobacter coli* and *mapA* gene for *Campylobacter jejuni*. **(El- Jakee*et al.,* 2008)**.

Primer	Target gene	Specificity	Primer sequence (5 - 3)
Forward	ceu*E*	*Coli*	5' AAT TGA AAA TTG CTC CAA CTA TG 3'
Reverse	ceu*E*	*Coli*	5' TGA TTT TAT TAT TTG TAG CAG CG 3'
Forward	map*A*	*Jejuni*	5' CTA TTT TAT TTT TGA GTG CTT GTG 3'
Reverse	map*A*	*Jejuni*	5' GCT TTA TTT GCC ATT TGT TTT ATT A 3'

Product size of map*A* is 589bp and of ceu*E* is 462bp

map*A* (membrane associated protein A) specific for *C. jejuni*.

ceu*E* (siderophore binding protein) (lipoprotein component of enterocholin) specific for *C. coli*

Amplification of genes (ceuE) and (mapA) from DNA of Campylobacter isolates:

***Campylobacter coli* (ceu*E*) (El-Jakee*et al.*, 2008**)

Each reaction was performed in a final volume 25µl in PCR tube **(ependorff).** Each reaction contained a mixture consisted of 3 µl of the extracted DNA template from the bacterial cultures plus 20 µl 1.1X PCR master mix, and (1) µl of each primers forward and reverse **(ceu*E*).**

The thermal cycler was adjusted as the following

Initial denaturation 94˚C for 5 min- **Followed by 34 cycles (**Denaturation 94˚C for 30s, Annealing 58˚C for 30s-, Extension 72˚C for 60 s-) **Followed by Final extraction** 72˚C for 10 min- .The PCR product may still at thermal cycler at 4˚C for storing until used.

***Campylobacter jejuni* (map*A*)**

The thermal cycler was adjusted as the following

Initial denaturation 94˚C for 5min- **Followed by 30 cycles of (**Denaturation 94˚C for 30s- ,Annealing 55˚C for 30s- Extension 72˚C for 2 min) **Followed by Final extraction** 72˚C for 10 min-. The PCR product may still at thermal cycler at 4˚C for storing until used.

Real time PCR

Table (2). Oligonucleotide primers and probes sequences used in Real time PCR for amplification of Taq*HipO* gene specific for *Campylobacter jejuni* and Taq*orfA* gene specific for *Campylobacter coli* **(Benson *et al.*, 2002).**

Primer	Target gene	Specificity	Primer sequence (5̀ - 3̀)
Forward	Taq*HipO*-1754F	*jejuni*	5'- TGG TGC TAA GGC AAT GAT AGA AGA -3'
Reverse	Taq*HipO*-1924R	*jejuni*	5'-TGA CCA CCT CTT CCA ATA ACT TCA- 3'
Probe	TaqJej- 1864s	*jejuni*	5' Fam – AAC TAT CCG AAG AAG CCA TCA TCG CAC CTT – BHQ-1-3'
Forward	Taq*OrfA*-4024F	*coli*	5'- GCA CTC ATC CAA TAC TTA CAA GA-3'
Reverse	Taq*OrfA*-4129R	*coli*	5' - CAT TAT GGT GTA TTC CGC CCA -3'

Taq*HipO* hippurate activity gene for *Campylobacter jejuni*
Taq*OrfA* open reading fram gene for *Campylobacter coli*

Amplification:

1. The number of PCR tubes required for the samples was determined and one tube for negative control and one for positive control were included.
2. 23 µl of the amplification reagent buffer were transferred to each PCR tube. The amplification reagent tube was immediately stored at 4˚C and in darkness.
3. For each sample, 2 µl DNA extract were added in to the 23 µl of the amplification reagent buffer
4. For the positive control tube, 2 µl of positive solution were added to the 23 µl of the amplification reagent buffer

3.2.4.1.4. Adjustment of real time PCR machine for detection of *Campylobacter jejuni and coli*

Once all tubes had been prepared, the real time PCR amplification was performed. The *Campylobacter* target was read in FAM. The quencher was non fluorescent.

Program concerning the MX3005P of Stratagene: Cycle (Benson *et al.*, 2002)

Initial denaturation 94˚C for 30 sec **Followed by 40 cycles of** Denaturation 94˚C for 30sec Annealing and Extension ranged from 57˚C to 64˚C for 60 sec.

Results

Table (3). Comparison between detection of *Campylobacter* by stander isolation method, conventional PCR and real time PCR.

Total samples	Stander isolation method		Conventional PCR		Real time PCR		
30	8		12		13		
%	26.7		40		43.3		
Campylobacter species.	*coli*	*jejuni*	*coli*	*jejuni*	*coli*	*jejuni*	Mixed
	5	3	7	5	7	5	1
%	62.5	37.5	58.3	41.7	53.8	38.5	7.7

Table (3) shows the comparison between different methods of detection of *Campylobacter* those 8 samples out of 30 samples (26.7 %), 5 out of 8 (62.5%) was *C. coli* and 3 out of 8 (37.5%) was *C. jejuni*. It was detected by conventional isolation method, 12 samples out of 30 (40%), 7 out of 12 samples (58.3%) was coli and 5 out of 12 samples (41.7) was *C. jejuni* samples was detected by conventional PCR and 13 out of 30 samples (43.3%) 7 out of 13 samples (53.8%) was *C. coli*, 5 out of 13 samples (38.5) was *C. jejuni* and one out of 13 samples was mixed infection samples by real time PCR.

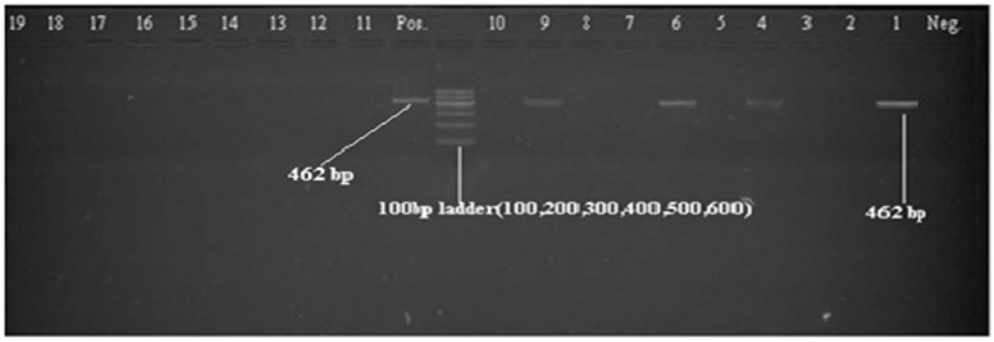

Photo (1). Agarose gel electrophoresis result of conventional PCR for detection of *C.coli* isolates (1-19)

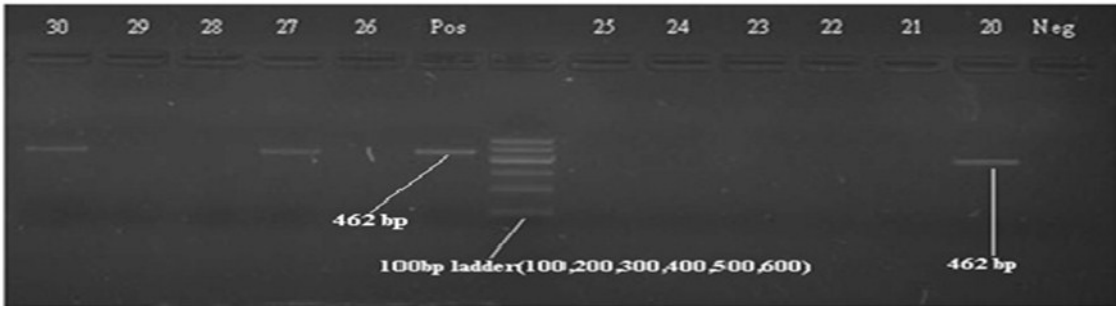

Photo (2) Agarose gel electrophoresis result of conventional PCR for detection of *C. coli* isolates (20-30)

Results of detection of field isolates of *C. coli* by conventional PCR :

PCR technique was used for detection of genes (ceu*E*) of ***C. coli*** from bacterial culturing. Results of conventional PCR revealed presence of (ceu*E*) gene in (7) isolates recovered from (30) samples (**photo 1, 2)**.

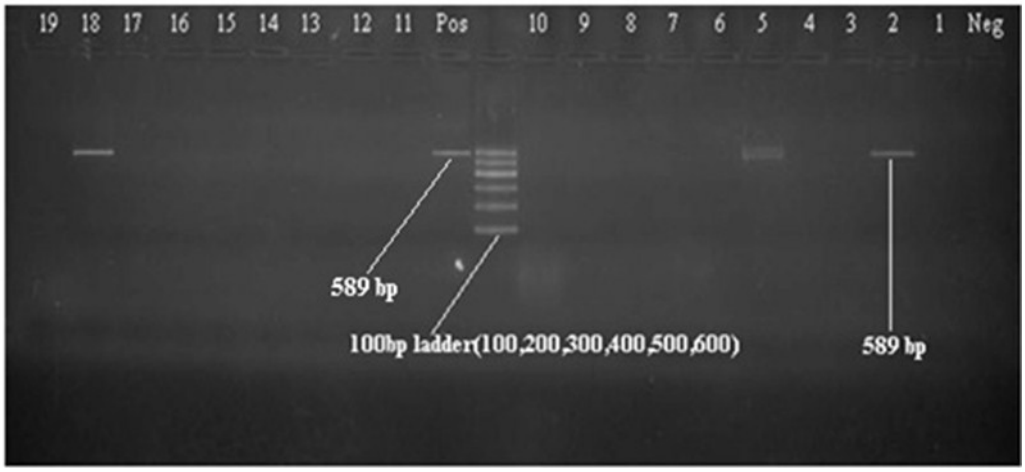

Photo (3) Agarose gel electrophoresis result of conventional PCR for detection of *C. jejuni* isolates (1-19)

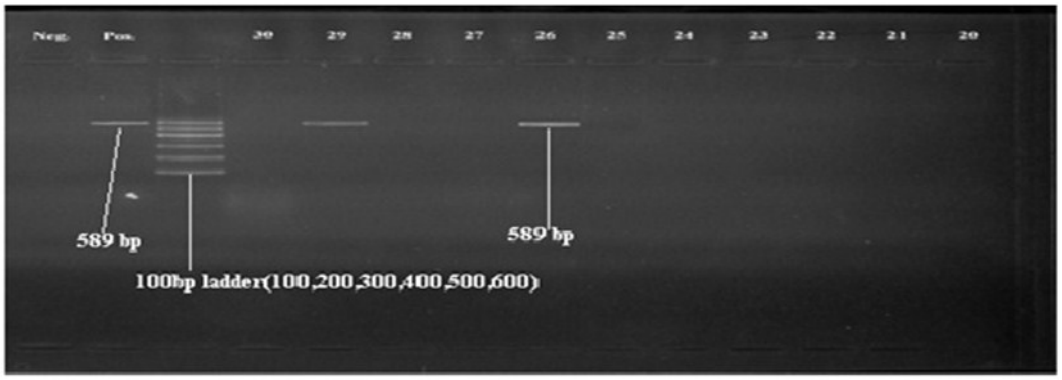

Photo (4) Agarose gel electrophoresis result of conventional PCR for detection of *C. jejuni* isolates (20-30).

Results of detection of field isolates of *C.jejuni* by conventional PCR :

PCR technique was used for detection of gene (map *A*) of ***C. jejuni*** from bacterial culturing .

Results of conventional PCR revealed presence of (map*A*) gene in (5) isolates recovered from (30) samples (**photo 3, 4)**

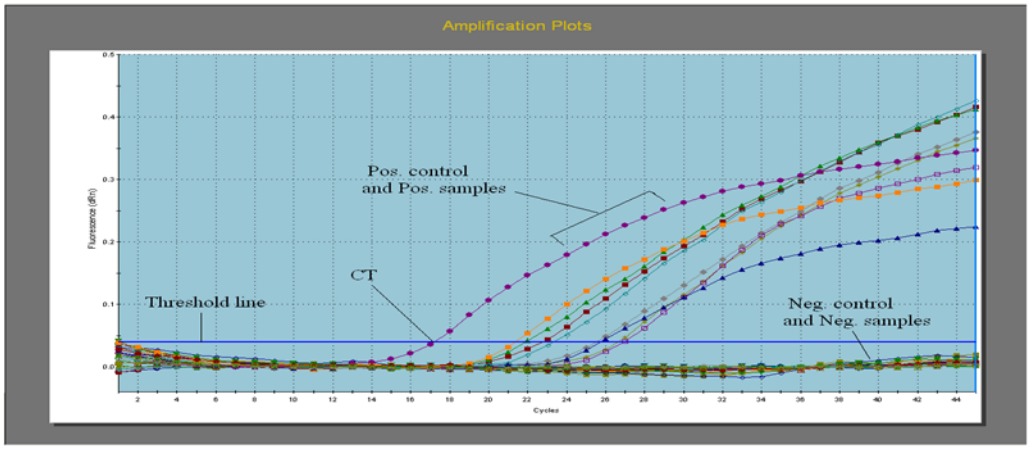

Figure (1). Amplification curves of real time PCR for detection of (Tag orf*A*) gene of *C.coli.*

Results of detection of *C. jejuni* field isolates (6) by Real time PCR:

Positive samples were detected by amplification curves of FAM dye, while negative samples show no amplification.

Table (4). Results for real time PCR for *Campylobacter jejuni* and *Campylobacter coli*:

Sample no.	Results of *Campylobacter jejuni*		Results of *Campylobacter coli*	
	Result	CT value	Result	CT value
Neg. control	Neg.	No CT	Neg.	No CT
1	Neg.	No CT	Pos.	25.53
2	Pos.	25.57	Neg.	No CT
3	Neg.	No CT	Neg.	No CT
4	Neg.	No CT	Pos.	23.31
5	Pos.	21.49	Pos.	26.68
6	Neg.	No CT	Pos.	25.75
7	Neg.	No CT	Neg.	No CT
8	Neg.	No CT	Neg.	No CT
9	Neg.	No CT	Pos.	26.94
10	Neg.	No CT	Neg.	No CT
11	Neg.	No CT	Neg.	No CT
12	Neg.	No CT	Neg.	No CT
13	Neg.	No CT	Neg.	No CT
14	Neg.	No CT	Neg.	No CT
15	Neg.	No CT	Neg.	No CT
16	Neg.	No CT	Neg.	No CT
17	Neg.	No CT	Neg.	No CT
18	Pos.	21.92	Neg.	No CT
19	Neg.	No CT	Neg.	No CT
20	Neg.	No CT	Pos.	21.43
21	Neg.	No CT	Neg.	No CT
22	Pos.	25.58	Neg.	No CT
23	Neg.	No CT	Neg.	No CT
24	Neg.	No CT	Neg.	No CT
25	Neg.	No CT	Neg.	No CT
26	Pos.	23.96	Neg.	No CT
27	Neg.	No CT	Pos.	21.89
28	Neg.	No CT	Neg.	No CT
29	Pos.	22.71	Neg.	No CT
30	Neg.	No CT	Pos.	22.74
Pos. control	Pos.	17.25	Pos.	17.21

CT: Threshold cycle
Pos.: Positive
Neg.: Negative

Discussion

Campylobacter is one of food poisoning microorganism which causes severe cases of gastroenteritis. Poultry is the main vehicle for transmission of *Campylobacter* to human. The organism is commensal microorganism in intestinal tract of bird with or without any clinical signs on the bird. At slaughtering of the bird the organism contaminates the water of scalding and washing containers. It was well known that the poultry carcasses had become contaminated with *Campylobacter* bacteria from their intestinal contents during the slaughter process (**Berndeston 1992**). Comparing between methods of detection of *Campylobacter* (conventional isolation method, PCR and real time PCR), the results showed that 8 samples (26.7%) out of 30 samples by isolation and 12 samples (40%) out of 30 samples were detected by using conventional isolation meth-

od and PCR. These results agreed with **(Kulkarni *et al.,* (2002)** where PCR was more sensitive than the conventional isolation method as they found 17 out of 343 samples were positive by isolation method and 20 out of 343 samples were positive by PCR and they reported that PCR result was available on the same day as the assay were performed. **(Giesendorf *et al.,* (1992)** reached to different results as they examined 45 chicken samples from local poultry shops and supermarkets for presence of *Campylobacter* species in 2g of skin samples by using the PCR- culture assay and conventional isolation method. In this survey 36 (80%) of 45 samples were positive with both PCR and conventional isolation method and 9 (20%) samples were negative with both methods. When comparing the culturing method and the real time PCR in our study we found that 8 (26.7%) positive samples *Campylobacter* were detected by culturing method and 13 (43.3%) positive samples by using real time PCR these results agreed with **(Sails *et al.,* (2003)** who examined 66 samples for presence of *Campylobacter* and found 57 samples were positive by using culture method and 63 positive samples by using real time PCR. The total time taken for detection from enrichment broth was approximately 3 h for the real-time PCR assay, as the results being available immediately at the end of PCR cycling, compared to 48 h for subculture on selective agar.

This assay significantly reduces the total time taken for the detection of *Campylobacter* in foods, where **(Lund *et al.,* (2004)** found that when comparing the real time PCR and conventional isolation method for chicken samples 41 positive samples out of 111 samples by real time PCR and 43 positive samples out of 111 samples by using cultivation method were obtained. Finally we can conclude that the real time PCR give good results in short time because it can detect the one DNA copy of *Campylobacter* in the sample. **(Suzuki and Yamamoto (2008)** stated that *Campylobacter* species are common bacterial pathogens associated with human gastroenteritis in both developed and developing countries. Contaminated raw or undercooked poultry meats and/or by-products are particularly important to cause food-borne campylobacteriosis in humans. There are many reports describing *Campylobacter* contamination in retail poultry meats and/or by-products in the world.

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المقارنه بين طرق العزل القياسيه و استخدام انزيم البلمره المتسلسل و استخدام انزيم البلمره المتسلسل فى الوقت الحقيقي في التعرف على وجود الميكروب الكامبيلوباكتر فى الدواجن المجمده

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الملخص العربي

ثلاثون عينه من الدجاج المجمد المستورد الوارده لفحص في المعمل المرجعي للرقابه البيطريه على الانتاج الداجني تم استخدامها للمقارنه بين ثلاث طرق مختلفه للكشف عن وجود الكامبيلوباكتر (طرق العزل القياسيه و انزيم البلمره المتسلسل و انزيم البلمره المتسلسل فى الوقت الحقيقي). وكانت نتائج هذه المقارنه هى كالاتى 8 من 30 (26.7%) عن طرق استخدام طرق العزل القياسيه و 12 من 30 (40%) عن طريق استخدام انزيم البلمره المتسلسل و13من 30 (43.3%) باستخدام انزيم البلمره المتسلسل فى الوقت الحقيقي وعينه واحده كان بها الكامبيلوباكتر الكولاى وجيجونای تم التعرف عليها عن طريق انزيم البلمره المتسلسل فى الوقت الحقيقي.