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Validation of PCR for Detection of Campylobacters Isolated from Chicken

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Abstract: Bacteriological examination of a total of 600 chicken samples either apparently healthy (228) or diseased (372) suffered from watery to bloody diarrhea, distended bile and cecum with accumulation of watery fluid in intestine and hemorrhage on the wall of intestine was investigated. 90 samples were found to be bacteriologically positive for campylobacters with an incidence of 15%. The highest recovery rates were obtained during the warmer months of the year, from May to September (Summer season) with an incidence of 23%. The lowest recovery rate was obtained in January and February (0% each), followed by December (3.1%), November (4.6%), October (6.3%), March (6.7%) and April (10%). The incidence of campylobacters among diseased chickens was 15.3% while it was 14.5% among apparently healthy chickens.The incidence of campylobacters was recorded in bile, cecal content, fecal and duodenum samples. Out of the examined 228 apparently healthy chickens, 8 isolates were identified as *C. jejuni* and 25 as *C. coli* with incidence of 3.5% and 10.9% respectively. Meanwhile *C. jejuni* (10 isolates) and *C. coli* (47 isolates) were isolated from diarrheic chickens with incidence of 2.7 and 12.6% respectively. Specificity testing of PCR among the isolates was performed using *mapA* and *ceuE* primers. All of *C. jejuni* and *C. coli* isolates examined produced bands at the expected positions (589 & 462 bp respectively) after PCR steps. The sensitivity and detection limits of PCR assay to numbers of *C. jejuni* and *C. coli* were determined. As few as 110 and 240 cells were detected by the PCR assay when DNA template extracted by phenol chloroform isoamyl alcohol or kit method from a serial dilution of a single colony of *C. coli* and *C. jejuni*, respectively.

Key words: *Campylobacter jejuni* · *Campylobacter coli* · Polymerase Chain Reaction · *map A* gene · *ceu E* gene

INTRODUCTION

The genus *Campylobacter*, family *Campylobacteriaceae* [1], class *Epsilonproteobacteria* of phylum *Proteobacteria,* currently consists of sixteen species and eight subspecies, all of which are natural inhabitants of the intestinal tracts of poultry and warm-blooded domestic animals where microaerophilic 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. Most reported *Campylobacter*-related human illnesses are caused by *C. jejuni* and, to a lesser extent, by other *Campylobacter* such as *C. coli, C. lari, C. hyointestinalis*, *C. upsaliensis* and *C. fetus* [2]. *C. jejuni* is the most commonly reported bacterial cause of food borne infection in the United States. Adding to the human and economic costs are chronic sequelae associated with *C. jejuni* infection Güillain-Barré syndrome and reactive arthritis. In addition, an increasing proportion of human infections caused by *C. jejuni* are resistant to antimicrobial therapy. Mishandling of raw poultry and consumption of undercooked poultry are the major risk factors for human campylobacteriosis. Efforts to prevent human illness are needed throughout each link in the food chain[3]. The prevalence of *Campylobacter* in fresh chicken meat and chicken by-products on retail level in Sapporo, Japan was studied by Sallam [4]. The fastidious growth requirements, complex taxonomy and unreliable biochemical tests present significant challenges in the identification of *Campylobacter* species [5]. Furthermore, *C. coli* and *C. jejuni* are closely related by phylogenetic and genetic criteria [6] making identification of *Campylobacter* at species level difficult. Defined *Campylobacter*-specific target genes may provide a valuable means for the detection and/or identification of the species of this genus by PCR methodologies [7].

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So the present study was conducted to evaluate the use of the *ceuE* or *mapA* gene as a target for the development of a simple, reliable and specific PCR-based method for the rapid identification and discrimination of *C. coli* and *C. jejuni.*

MATERIALS AND METHODS

Samples: Sixty hundred samples (263 cecum, 229 duodenum, 18 bile samples and 90 fecal swabes) were collected from 228 apparently healthy and 372 diarrheic chickens from various markets in Giza and Cairo governorates for isolation of *Campylobacter* spp. as shown in Table 1.

Isolation of *Campylobacter* species: A loop full from each sample was cultured directly onto modified *Campylobacter* blood free selective medium with antibiotics. All inoculated plates were incubated in anaerobic jar with kits which generates CO_2 (10%), O_2 (5%) and nitrogen (85%) in 37°C and 42°C for 3 days and examined daily to demonstrate the characteristics colonies. The suspected colonies were identified according to Hunt *et al.* [8] and Quinn *et al.* [9].

Extraction of genomic DNA from *Campylobacter* bacterial cell: The DNA was extracted by phenol chloroform isoamyl alcohol extraction [10], kit extraction [11] and heat treatment extraction methods [12] to compare the results.

Polymerase chain reaction for amplification of campylobacters *mapA* and *ceuE* gene segments [13]: In a PCR tube, 200ng of bacterial DNA, extracted from different campylobacters isolates, was mixed with 10µl of 10 X buffer, 50 picomole of both primers *mapA* and *ceuE* (Table 2), 0.5 µl (2.5 units)Taq polymerase and 2µl dNTP mix (10mM) then complete by distilled water to a final volume of 50 µl. After overlaying the mixture with mineral oil, tubes were placed in the thermocycler and the amplification was performed under the following program: initial denaturation at 94°C for 5 minutes followed by 34 cycles of denaturation at 94°C for 30 sec., annealing step at 58°C for 30 sec. and extension at 72°C for 1 minutes. A final extension step was done at 72°C for 10 minutes.The PCR products were electrophoresed in 1.5 % agarose gel using Tris-acetate EDTA buffer. The gel containing separated DNA was stained with ethidium bromide. Standard marker containing known fragments of DNA either 100 bp or 250 bp ladders was used (Stratagene, USA).

Table 1: Number and types of the examined samples

Type of examined samples	No. of apparently healthy chickens	No. of diseased chickens	Total
Cecal samples	83	180 (distended cecum and watery fluid accumulation)	263
Duodenum samples	79	150(Red-spots and hemorrhage)	229
Bile samples	8	10 (distension)	18
Fecal swabs	58	32 (semi liquid fecal content)	90
Total	228	372	600

Table 2: Shows Oligonucleotide primers [14]

Gene	Primer sequence	Amplicon
mapA (membrane associated protein A) specific for *C. jejuni*	5'CTA TTT TAT TTT TGAGTG CTT GTG3' 5'GCT TTA TTT GCC ATT TGT TTT ATT A3'	589 bp
ceuE (Siderophore binding protein) (lipoprotein component of enterocholin) specific for *C. coli*	5' AAT TGA AAA TTG CTC CAA CTA TG 3 ' 5'TGA TTT TAT TAT TTG TAG CAG CG 3'	462 bp

Sensitivity of PCR with pure culture [15]: *C. jejuni* or *C. coli* isolate was streaked onto CCDA agar plates, a colony from each plate was picked after 24h incubation and resuspended in thioglycolate broth. One ml was resuspended in 9 ml of broth and ten fold serial dilutions in thioglycolate broth were prepared. The inoculum levels were determined by surface plating of the serial dilutions onto CCDA agar. The plates were incubated microaerobicalloy at 37°C for 48 h before counting. 1 ml from each dilution were centrifuged at 16,000 x g. The pellets were used for PCR and the DNA concentrations were measured on a spectrophotometer.

Sensitivity of PCR in contaminated meat and fecal samples: From the plate which had (1-50) colonies, the original broth tube of it was taken and divided its broth into 2 test tubes first containing 10 g of chicken breast meat and second one containing 10 g feces. The samples were homogenized and incubated for one hour. The sensitivity of PCR was compared to the convention culture method with or without pre-enrichment as described by Oliveira *et al.* [15].

RESULTS AND DISCUSSION

From the results presented in Table 3 it is elucidated that the highest recovery rates were obtained during the warmer months of the year, from May to September (Summer season) with an incidence of 23%. The lowest recovery rate was obtained in January and February (0% each), followed by December (3.1%), November (4.6%),

Table 3: Incidence of *Campylobacter* microorganisms regarding to seasonal variations

Season	Month	No. of examined samples	Positive number	Percent %
Summer	May	66	12	18.20
	June	98	25	25.50
	July	70	19	27.20
	August	84	19	22.60
	September	26	4	15.40
Total		344	79	23.00
Autumn	October	32	2	6.30
	November	65	3	4.60
Total		97	5	5.20
Winter	December	32	1	3.10
	January	32	_	_
	February	40	_	_
Total		104	1	0.96
Spring	March	15	1	6.70
	April	40	4	10.00
Total		55	5	9.10
Total	Year	600	90	15.00

% was calculated according to the number of examined samples

October (6.3%), March (6.7%) and April (10%). There was seasonal variation in the level of *Campylobacter* contamination of fresh chicken, with a peak in June and the lowest positive rates in January, March and December [16]. Flocks were more frequently colonized in the warmer months and younger birds were less frequently colonized than were older slaughtered birds [17].

It is clear from Table 4 that out of the examined 228 apparently healthy chickens, 8 isolates were identified as *C. jejuni* and 25 isolates were identified as *C. coli* with incidence of 3.5% and 10.9% respectively. Meanwhile *C. jejuni* (10 isolates) and *C. coli* (47 isolates) were isolated from diarrheic chickens with an incidence of 2.7 and 12.6% respectively. Wittwer *et al.* [18] identified 24 (17%) *C. coli* from poultry farm. The majority of *Campylobacter* isolates from chickens (52%), were *C. coli.* [19]. In Denmark, the prevalence of *Campylobacter* infected broiler focks has recently been reported to be 37.7% in the year 2000 with the majority of species (86%) being identified as *C. jejuni* [20]. Contamination control at the farm level would be one of reducing consumer exposure; however, the epidemiology of *C. jejuni* in broiler flocks is still unclear [21].

Polymerase chain reaction (PCR) can provide a highly sensitive, specific and rapid method for detecting bacteria in pure culture and natural reservoirs. To date, a number of PCR assays for the detection of *Campylobacter* spp. have been developed. These assays include conventional PCR [22, 23] nested and semi-nested PCR [24], multiplex PCR [25], reverse transcriptase PCR [26], PCR enzyme-linked immunosorbent assay[27] and real-time PCR [28], Recently Wolffs *et al.* [29] described a method for *Campylobacter* spp. quantification by a flotation and real-time multiplex PCR. The detection and differentiation between the closely related species of *C. jejuni* and *C. coli* are of particular concern as they are the major human enteropathogenic campylobacters. In this study we evaluated the suitability of a simple and specific PCR method for differentiating between these two closely related species, using *mapA* and *ceuE* primers. All of *C. coli* and *C. jejuni* isolates examined produced bands at the expected positions (462 & 589 bp respectively) after PCR steps as shown in Photos (1 & 2).

Table (5) and Photos (3 & 4) show the effect of DNA extraction methods on PCR results. It is clear that phenol chloroform isoamyl alcohol extraction method and extraction by kit yielded positive results [30], concluded that the sensitivity of PCR method was increased when CTAB extraction of DNA was used, probably due to inactivation of the inhibitor substances present in the chicken rinse. On the other hand, the boiling method yielded negative PCR results for all samples containing *Campylobacter*. These results are in line with other previous study [31].

The sensitivity and detection limits of PCR assay to all numbers of *C. jejuni* and *C. coli* were determined. As few as 110 and 240 cells were detected by the assay when DNA template extracted by phenol chloroform isoamyl alcohol or kit methods from a serial dilution of a single colony of *C.coli* and C. *jejuni* respectively as shown in Table 6. The detection sensitivities obtained were slightly less than the published results for both primer sets. The detection limit of primers C442-C490 was 12.5 CFU per PCR reaction [32]. On the other hand, Ng *et al.* [33]

Table 4: Incidence of *Campylobacter* species among the examined samples

		Apparently healthy chickens (228)										Diseased chickens (372)									
		Cecum contents		Dudenum samples		Bile samples		Fecal swabs		Total		Cecum samples		Dudenum samples		Bile sampless		Fecal swabs		Total	
Campylobacter species	No.of isolates	No	%	No	%	No	%	No	%	No	%*	No	%	No	%	No	%	No	%	No	%*
C. jejuni	18	5	27.8	2	11.1	_	_	1	5.6	8	3.5	7	38.9	_	_	1	5.6	2	11.1	10	2.7
C. coli	72	9	12.5	8	11.1	1	1.4	7	9.7	25	10.9	32	44.4	4	5.6	3	4.2	8	11.1	47	126
Total	90	14	15.6	10	11.1	1	1.1	8	8.9	33	14.5	39	43.3	4	4.4	4	4.4	10	11.1	57	153

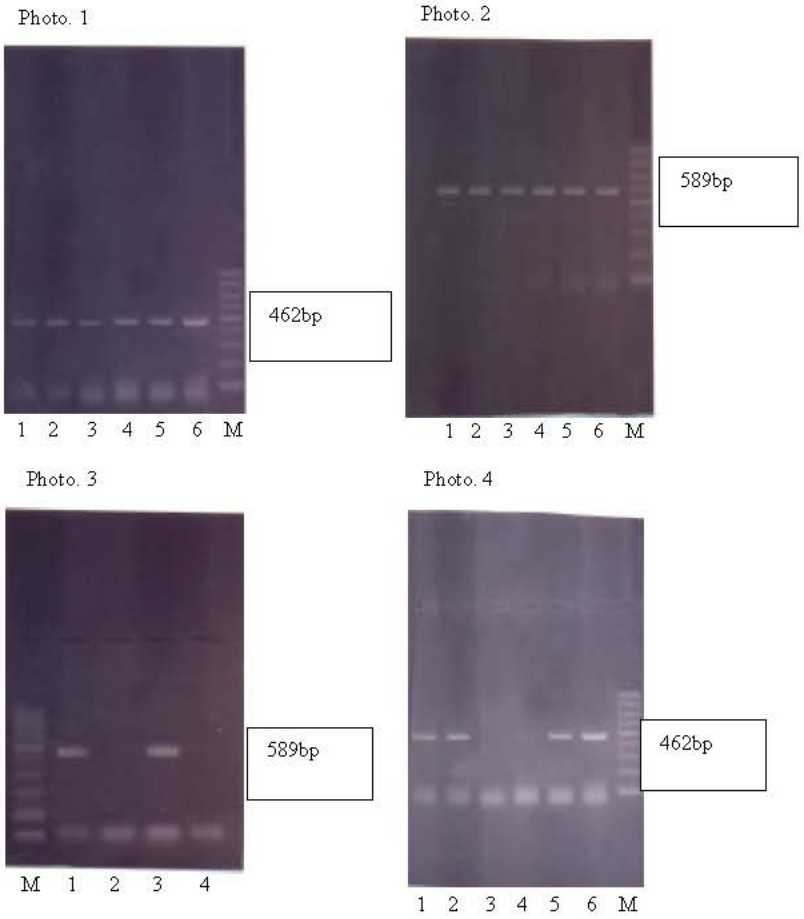
% was calculated according to the number of isolates. * % was calculated according to the number of examined samples

Table 5: Validation of different DNA extraction methods for accuracy of PCR results

		Different DNA extraction methods		
Isolate number	Campylobacter Species	Heat treatment	Phenol chloroform isoamyl alcohol	Kit
1(Coccoid form)	*C. jejuni*	-	-	-
2	*C. jejuni*	-	+	+
3	*C. jejuni*	-	+	+
4	*C. coli*	+	+	+
5	*C. coli*	+	+	+
6	*C. coli*	-	-	+

Table 6: PCR sensitivity results of *C.coli* and *C. jejuni*

	C.coli			*C.jejuni*		
Serial dilution	O.D.	PCR	CFU/ml	O.D.	PCR	CUF/ml
1	0.215	+	60000	0.270	+	4600
2	0.200	+	6000	0.182	+	240
3	0.192	+	620	0.142	-	-
4	0.153	+	110	0.061	-	-
5	0.093	-	-	0.055	-	-
6	0.073	-	-	0.029	-	-
7	0.058	-	-	0.027	-	-
8	0.031	-	-	0.020	-	-



Photograph 1: Shows agarose gel electrophoresis showing amplification of 462 bp fragment of *C. coli* (lanes 1-6).

Photograph 2: Shows agarose gel electrophoresis showing amplification of 589 bp fragment of *C. jejuni* (lanes 1-6).

Photograph 3: Shows PCR products among DNA extracted from *C. jejuni* by different methods using *mapA* oligonucleotide primers. Lanes1&3(phenol chloroform isoamyl alcohol &Kit extraction method respectively) yielded positive PCR. Lanes 2 &4 (heat treatment extraction method) yielded negative PCR.

Photograph 4: Shows PCR products among DNA extracted from *C.coli* by different methods using *ceuE* oligonucleotide primers. Lanes 1, 2 (Kit extraction), 5&6 (phenol chloroform isoamyl alcohol method) yielded positive PCR. Lanes 3&4 (heat treatment extraction method) yielded negative PCR.

attained a detection limit of 3 CFU for the primer sets CR3 and CL2 compared to 100 CFU in Magistrado *et al.* [34]. PCR assays are sensitive since *C. jejuni* can be detected at DNA concentrations as low as 0.01-0.05 pg/PCR corresponding to 1-5 CFU/ml in the presence of DNA isolated from environmental samples at the dilution 1:100 [20].The lower sensitivities obtained in this study may be partly due to the difference in the batches of the reagents used, or to the different strains tested and the different PCR machines used.

Attempt was made during this study to identify campylobacters directly in fecal &meat samples to determine the minimum numbers of bacteria required to produce a positive result. However, there is evidence that PCR-based assays can be successfully applied to the direct detection of *Campylobacter* spp. and other pathogenic bacteria in clinical stool samples. For the experimentally infected meat and fecal samples, a positive result was obtained with PCR after one hour pre-enrichment. Meanwhile Abu-Halaweh *et al.* [2] recorded that, the earliest point at which *C. jejuni*, *C. coli* and C. *lari* was detected from growth in broth cultures was 8h. Not only does an enrichment procedure dilute any inhibitors present, but dead bacteria are diluted as well, thus reducing the probability of detecting them by the subsequent PCR assay [24]. The method described in this study is specific for detection of *C. jejuni* and *C. coli* and can be used for fecal samples having high levels of microbiological contamination or humic matter, as well as for meat samples containing high levels of background flora. The simplicity and speed of the PCR-based assays make them highly applicable in the analysis of foods for the detection and identification of *Campylobacter* species and well suited for use for routine analysis and incorporation into an automated mass screening system.

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