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OCCURRENCE OF *CAMPYLOBACTER* SPECIES IN CHICKENS BY MULTIPLEX POLYMERASE CHAIN REACTION

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ABSTRACT

The genus *Campylobacter* is one of great importance to public health because it includes several species that may cause diarrhea. Poultry and poultry products are known as important sources of human campylobacteriosis. 225 samples were collected from (75) chickens including intestinal content (75), liver (75) and skin (75). The overall occurrence of *Campylobacter jejuni* and *Campylobacter coli* in chicken by PCR were (5.3% & 17.8%). Multiplex PCR targeting *23S rRNA* specific for genus *Campylobacter*, *hip O* gene specific for *C. jejuni* and *glyA* gene specific for *C. coli* was used for the confirmation of phenotypically identified *C. jejuni and C. coli* isolates. It is concluded that PCR was determined to be more specific and rapid than biochemical tests.

Key words: *Campylobacter*, chickens, multiplex PCR.

INTRODUCTION

Campylobacter food borne illness incidence is considered the major cause of diarrhea in developed and developing countries. The high incidence of *Campylobacter* diarrhea, as well as its duration and possible sequelae, makes it highly important from a socio-economic perspective (World Health Organization, 2011). *Campylobacter jejuni* accounts for the majority of food borne *Campylobacter* enteritis in human, followed by *Campylobacter coli* and to a lesser extent by *Campylobacter lari* (Skirrow and Blaser, 2000). *Campylobacter* species are widely distributed in most warm-blooded animals. They are prevalent in food animals such as poultry, cattle, pigs, sheep, ostriches, shellfish and in pets, including cats and dogs (World Health Organization, 2011). The major routes of transmission in humans are consumption of contaminated or undercooked meat (especially poultry), (Center for Food Security and Public Health, 2013). Most people who become ill with campylobacteriosis have diarrhea, abdominal pain, and fever. The diarrhea may be bloody and can be accompanied by nausea and vomiting. Some infected persons do not have any symptoms. In persons with compromised immune systems, *Campylobacter* occasionally spreads to the blood stream and causes a serious life-threatening infection (Centers for Disease Control and Prevention, 2013). Although most *Campylobacter* associated diarrhea is self-limited, complications can occur. One complication is Guillain-Barre´ Syndrome (GBS), an acute, symmetric, ascending paralysis that is estimated to occur 30 times for every 100, 000 *Campylobacter* cases Wierzba (2008), and the case fatality ratio approaches 10% (Nachamkin *et al*. 1998). The aim of the current work was to isolate and characterize of *Campylobacter* isolates from chickens by conventional methods and confirm the results by using multiplex PCR.

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MATERIALS AND METHODS

This study was carried out during the period between August 2014 and January 2016 in Reproductive Diseases Department, Animal Reproduction Research Institute El Haram, Giza.

1. Samples: 225 samples from (75) chickens including intestinal content (75), liver (75) and skin (75) were examined for *Campylobacter* from various markets in Giza Province.

2. Samples preparation

2.1. Chicken samples:

Intestinal content: about 10 gm of the intestinal content were homogenized in sterile thioglycolate broth and incubated at 42 ^{o} C For 48 hrs under microaerobic condition (5% O_2, 10% CO_2 and 85% N_2) (Gebhart *et al*. 1985).

Liver and skin samples: twenty five grams from each incised skin or liver parts were aseptically transferred to a sterile tube containing thioglycollate enrichment broth for homogenization of the sample (Sallam, 2001).

3. Isolation of campylobacters species (Smibert, 1974): a loopfull from each sample were cultured directly onto thioglycollate broth medium for 24-72 hours in sterile tubes, and then a loopfull from each tube were cultured on 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^{o}C for 48 hours and were demonstrated daily for the characteristics colonies. Then the suspected colonies were purified on blood agar media with defibrinated blood sheep containing *Campylobacter* growth supplement for 24 hours. Suspected colonies were subjected to Gram staining and motility test.

4. Identification of the isolates: The suspected colonies were identified by:

4.1. Morphological identification: Suspected growing colony on the specific agar plates were examined carefully for their morphological characters according to Koneman *et al.* (1995). A single suspected colony was stained with Gram's stain to demonstrate the characteristics morphology of the isolates. *Campylobacter* species are Gram negative.

4.2. Motility (Smibert, 1974): Direct smear from 3 days old culture of *Campylobacter* organisms were made and examined under phase contrast microscope to demonstrate the corkscrew like motion characteristic to *Campylobacter* species.

4.3. Biochemical identification: The purified colonies were identified biochemically by the following tests:

4.3.1. Catalase production test: (Laing, 1960): A small amount of pure growth were placed onto the surface of a clean, dry glass slide by sterile loop then a drop of 3% hydrogen peroxide was added into a portion of colony on the slide. Production of gas bubbles indicating the production of catalase enzyme.

4.3.2. Nitrate reduction test: (Bryner and Frank, 1955): The isolated organisms were inoculated into nitrate broth. After 48hours 5 drops of solution A (sulfanilic acid+ acetic acid) and solution B (α-naphtylamin+ acetic acid) were added to the tube. A positive test is indicated by the development of red colour in 1-2 miutes.

4.3.3. Oxidase test: (El-Gohary, 1998): Oxidase activity was examined on filter paper with 1% aqueous solution of tetramethyl-p-phenyl-diamine-dihydrochloride as a reagent. With a wooden loop a separate well grown colony will be picked up from a fresh culture medium (24 hours) and applied to the reaction on the filter paper.A positive reaction indicated by a violet colouration within 20-60 seconds at the contact point.

4.3.4. Urease test (El-Gohary, 1998): The isolated organisms were inoculated over the entire slope surface of the urease test tube and incubated at 37^{o}C microaerophilic. Examination occurred after 4 hours and after overnight incubation. Urease positive cultures change the colour of the indicator to purple pink.

4.3.5. Hydrogen sulphide production, H_2S: (Bryner and Frank, 1955) By using lead acetate paper: The test conducted by suspending dried filter paper strips saturated with 10% lead acetate solution in tubes of thioglycollate medium and incubated at 37^{o}C for 1-2 days. Blackening of the paper is considered positive.

4.3.6. Temperature tolerance test: By using loopfull of diluted culture, streak a line across each of 3 plates of agar per isolate then inoculated and incubated one plate at 25^{o}C, one at 35-37^{o}C and one at 42^{o}C under microaerophilic atmosphere for 3 days. Positive reaction was indicated by the appearance of more growth than the initial inoculum after 72 hours of incubation.

4.3.7. Glycine tolerance test: **(Chang and Ogg, 1971)**: The isolated organism was inoculated into semisolid thiglycollate tubes containing 1% glycine and incubated at 37^{o}C for 5 days. Positive reaction was indicated by the appearance of specific colonies after 5 days of incubation.

4.3.8. Sodium chloride (Nacl) tolerance test: (Taul and Kleckner, 1968): Sodium chloride tolerance was determined by inoculation of the organism into semisolid thiglycollate medium containing 3.5% Nacl then incubated at 37^{o}C for 5 days. Positive reaction was indicated by the appearance of specific colonies after 5 days of incubation.

4.3.9. Hippurate hydrolysis test: (Carter, 1984): It is the only biochemical test to distinguish between *C.jejuni* and *C.coli*. In Wasserman test tube put 0.4 ml of thawed sodium hippurate then added large loopfull of isolated organism and emulsified. The culture was incubated for 2 hours at 37^{o}C. Then 0.2 ml of ninhydrin solution was added and the development of a deep purple color within 10-20 minutes indicated positive reaction.

5. Molecular identification:

5.1. DNA extraction: extraction of C*ampylobacter* DNA from culture using (Thermo Scientific Gene Jet Genomic DNA Purification Kit#K0721, #K0722).

5.2. The primer sequences used for detection of *Campylobacter*: The primer sequence of *Campylobacter* targeting *23S rRNA* gene were 5` TATACCGGTAAGGAGTGCTGGAG3` (forward) and 5`ATCAATTAACCTTCGAGCACCG 3` (reverse). While Species-specific primer targeting *hip O* gene specific for *C.jejuni* (Wang *et al*., 2002) were 5` ACTTCTTTATTGCTTGCTGC3` (forward) and 5`GCCACAACAAGTAAAGAAGC3`(reverse).

Species-specific primer targeting *glyA* gene specific for *C.coli* (Wang *et al*., 2002) were 5` GTAAAACCAAAGCTTATCGTG3` (forward) and 5`TCCAGCAATGTGTGCAATG 3` (reverse).

5.3. DNA amplification of Campylobacter: Cycling conditions of the primers during PCR according to (Wang *et al*., 2002) with modifications. PCR amplification was performed using thermal cycler (Biometra) with the following programme: one cycle of 6 min at 94^{o}C. 35 cycles each consisting of 30s at 95^{o}C (denaturation), 30s at 59^{o}C (annealing), 30 s at 72 ^{o}C (extension) and a final extension step at 72^{o}C for 7 min.

5.4. Detection of PCR products using Agarose gel electrophoresis (El-Adawy *et al*, 2012): The amplified PCR products were electrophosed in 1.5% agarose gel (Biometra).
The gel was photographed by a gel documentation system (Alpha Innotech).

RESULTS

The overall occurrence of *Campylobacter* was 24.9% (56 out of 225) which differentiated into *C.jejuni* 5.3 % (12 out of 225) and *C. coli* 17.8% (40 out of 225) by multiplex PCR. However the percentages of *C.jejuni* and *C.coli* in the samples of intestinal content were 4% (3 out of 75) & 17.3% (13 out of 75), respectively. The percentages of *C.jejuni* and *C.coli* in the liver were 4% (3 out of 75) & 22.7% **(**17 out of 75), respectively. On the other hand the the percentages of *C.jejuni* and *C.coli* in the skin were 8% (6 out of 75) & 13.3% (10 out of 75), respectively as shown in (Table 1).

Table 1: Detection of *Campylobacter* by multiplex PCR in chicken

Type of examined samples	Number of examined samples	Positive *Campylobacter* species		*Campylobacter jejuni*		*Campylobacter coli*		Other *Campylobacter* species	
		No.	%	No.	%	No.	%	No.	%
Intestinal content	75	20	26.7	3	4	13	17.3	4	5.3
Liver	75	20	26.7	3	4	17	22.7	0	0
Skin	75	16	21.3	6	8	10	13.3	0	0
Total	225	56	24.9	12	5.3	40	17.8	4	1.8

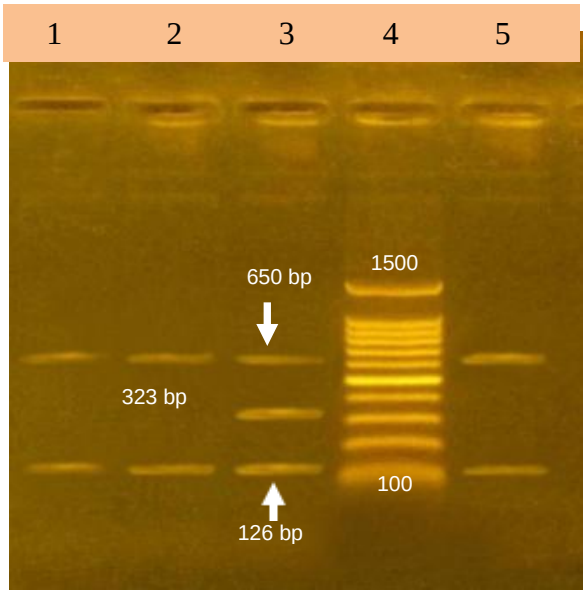

Figure (1): Multiplex PCR for detection of *Campylobacter* spp. (650 bp) using *23S rRNA* gene, *Campylobacter jejuni* (323 bp) using *hip O* gene and *Campylobacter coli* (126bp) using *gly A* gene. Lane 1, 2 and 5: positive for *Campylobacter coli*., Lane 3: Mixed infection for both *C.jejuni* and *C.coli*., and Lane 4: a 100bp molecular size marker.

DISCUSSION

Data recorded in table (1) revealed that the overall prevalence of *Campylobacter* in chickens was 24.9%. *C. jejuni* and *C. coli* were isolated from 5.3% and 17.8%, respectively from chicken samples. Higher percentages (80%, 56%, 76%, 68%, 44.4% and 48.7%) of *Campylobacter* spp in chickens were obtained by Bardon *et al*. (2009); Ellerbroek *et al*. (2010); Weber *et al*. (2014); Sandberg *et al*. (2015); Osbjer *et al*. (2016) Schallegger *et al*. (2016) and respectively. *C.jejuni* was detected in 12 (5.3%) of the examined chicken samples. Higher percentages of *C.jejuni* (22%, 17%, 36% and 73.3%) in chickens were obtained by Ansari-Lari *et al*. (2011), Henry *et al*. (2011), Khalifa *et al*. (2013) and Schallegger *et al*. (2016), respectively. Lower percentage of *C. coli* (2.7%) was obtained by Bardon *et al*. (2009). However higher percentages (53.3%, 30% and 32%) of *C. coli* were obtained by Schallegger *et al*. (2016), Henry *et al*. (2011) and Ansari-Lari *et al*. (2011). Generally, the variation in *Campylobacter* species isolation rate between different studies could be attributed to different possible reasons, such as, type of examined samples, location, climate factors, hygienic measures and isolation as well as identification techniques (Jorgensen *et al*. 2011 and Chatur *et al*., 2014).

Campylobacter species were isolated from 20 (26.7%) out of the examined intestinal samples collected from chickens. Nearly similar percentage (26.3%) of *Campylobacter* species was isolated from the intestine by Bai *et al*. (2014). However lower percentages (19.7% and 4.8%) were obtained by Oyarzabel *et al.* (1995) and Hofshagen and Kruse (2005), respectively. Higher isolation rates (45.9%, 28% and 83.3%) were obtained by Atanssova and Ring (1998), Bartkowiak-Higgo *et al*. (2006) and Kramer *et al*. (2000), respectively. *C.jejuni* and *C.coli* were isolated from 4% and 17.3%, respectively from intestinal samples of chickens. The incidence of *C.jejuni* in the intestine was 3(4%) (Table 6). Higher percentage (36.3%**)** was obtained by Kang *et al*. (2006). On the other hand the percentage of *C.coli* was 13(17.3%) (Table 6). Lower percentage (1.8%) of *C.coli* was obtained by Zweifel *et al*. (2008). While higher percentages (26.4% & 18.5%) of *C.coli* were obtained by Kang *et al*. (2006) and Sallam (2007).

The overall occurrence of *Campylobacter* in liver samples of chickens was (26.7%). %). Higher percentage (53.3%) of *Campylobacter* species in liver was obtained by Stoyanchev (2004). While lower percentages (15.5%, 24% & 21%) of *Campylobacter* species in liver were obtained by Boukraa *et al*. (1991); Bartkowiak-Higgo *et al*. (2006) and Vashin *et al*. (2009) respectively. The isolation rate of *C. coli* isolated from the liver was higher than *C. jejuni*. It has been noted that liver hygiene highly concerns food safety mainly in two directions. First, there is huge risk of many people to be infected after consuming insufficient cooked liver with *Campylobacter*. Besides, the contaminated poultry liver is a potential source for transferring *Campylobacter* in further stages of poultry processing (Vashin *et al*. 2009). This result is important in food hygiene circle since it could lead to high risk of infection among consumers who might eat insufficiently-cooked chicken liver. In addition, the liver, if packed inside the carcasses, becomes a good vehicle for *Campylobacter* spread inside the body cavity the (Stoyanchev 2004).

The isolation rate of *Campylobacter* from skin samples was 16 (21.3%) of which, 8% were identified as *C. jejuni* and 12.3% were *C. coli* (Table 1). Higher percentages (47.5%, 46.6%, and 30.8%) of *Campylobacter* in skin of chickens were obtained by Garin *et al*. (2012); Saad (2014) and Abd El- Tawab *et al*. (2015), respectively. The higher incidence of *Campylobacter jejuni* in skin samples and *Campylobacter coli* in liver samples of chickens may be due to rupture of intestine during processing activities. Chicken skin provides suitable microenvironment for the survival of Campylobacters due to accumulation of water which increases the surface area available for bacterial contamination (Chantarapanont *et al.,* 2003).

CONCLUSION

It is concluded that the relatively high proportion of the examined chicken is contaminated by *Campylobacter* spp. and that consumption of undercooked or cooked contaminated poultry products consider a possible risk for consumers. Contamination of poultry by *Campylobacter* is a significant risk factor of human campylobacteriosis.

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يعد ميكروب الكامبيلوباكترمن البكتيريا الحلزونية اللاهوائية والتي تسبب المرض للحيوان و الإنسان. يعتبركامبيلوباكتر الأمعاء الوسطي وكامبيلوباكتر القولونية من أهم الأنواع التي تصيب الحيوان والطيور والإنسان. تلعب الطيور ومنتجاتها دور رئيسي في نقل العدوي للإنسان. يصاب الإنسان بإسهال والآم في البطن وحمي وغثيان وتقيؤ وأحيانا إسهال دموي. تم إجراء هذه الدراسة علي ٧٥دجاجة ، ومن كل طائر تم أخذ ثلاث عينات كالآتي : (عينة من الأمعاء- عينة من الكبد-عينة من الجلد) ليصبح عدد العينات التي تم فحصها ٢٢٥ عينة . وقد تم تجميع العينات بطريقة عشوائية ومن أماكن مختلفة. هذا وقد تم فحص العينات باستخدام الطرق التقليدية من زرع وعزل الميكروب بكتيريولوجيا ثم إجراء التفاعلات الكيميائية الخاصة بالميكروب.وبفحص الميكروب وجد أن نسبة تواجد ميكروب كامبيلوباكتر الأمعاء الوسطي و كامبيلوباكتر القولون في الدجاج (٥,٣% ، ١٧,٨). وقد تم عزل ميكروب كامبيلوباكتر الأمعاء الوسطي وكامبيلوباكتر القولون من الأمعاء بنسبة ٤% ، ١٧,٣% علي التوالي. كما تم عزل ميكروب كامبيلوباكتر الأمعاء الوسطي وكامبيلوباكتر القولون من كبد الدجاج بنسبة ٤% ، ٢٢,٧ علي التوالي. وأيضا تم عزل ميكروب كامبيلوباكتر الأمعاء الوسطي وكامبيلوباكتر القولون من جلد الدجاج بنسبة ٨% ، ١٣,٣% علي التوالي.