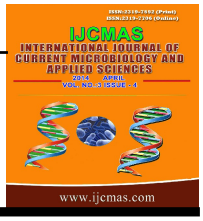
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 4 (2014) pp. 861-875**
http://www.ijcmas.com



Original Research Article

Multiplex PCR for Identification and Differentiation of *Campylobacter* Species and their Antimicrobial Susceptibility Pattern in Egyptian Patients

Samia A. Girgis*, Samar S. Rashad, Hala B. Othman, Hadia H. Bassim, Nevine N. Kassem and Fatma M. El-Sayed

Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
Corresponding author

Keywords

Campylobacter jejuni, *Campylobacter coli*, diarrhea, PCR, genotyping, *IpxA gene*, antibiotic.

A B S T R A C T

Campylobacter is an important food-borne diarrheal disease in the whole world. Post-infection complications and antibiotic resistance are increasing. The aim of this study was to detect the prevalence of *Campylobacter* species among diarrheic patients at Ain Shams University hospitals, compare between conventional methods and PCR in identifying *Campylobacter* to the species level and determine their antibiotic susceptibility pattern. 327 stool samples were subjected to conventional culture. Identification of *Campylobacter* was done phenotypically and genotypically by multiplex PCR *of lpxA* gene. Minimal inhibitory concentrations (MICs) of antibiotics were determined by E-test. *Campylobacter* is isolated from 19/327 (5.8%) diarrheic samples: 17 (89.5%) are *C. jejuni* and 2 (10.5%) are *C. coli*. The results of phenotypic and genotyping identification are identical. The antibiotic susceptibility is 94.7% to macrolides, and 42.1% ciprofloxacin. Ampicillin and nalidixic acid are 100% resistant. One *C. coli* is multidrug resistant to all tested antibiotics. In conclusion, Multiplex PCR with *lpxA* gene is rapid, sensitive and specific for identification and genotyping of *Campylobacter* and is comparable to conventional methods. *C. jejuni* is more prevalent. *Campylobacter* show best susceptibility to Macrolides. Resistant trends are emerging especially with *C. coli*. Elimination of risk factors and controlled use of antibiotics are recommended.

Introduction

Campylobacter infection is one of the most common causes of diarrhea in human all over the world. In the US, about 2.5 million people are infected each year (Mead *et al*., 1999). About 1% of the Western Europe population is infected with *Campylobacter* annually. In 2000 in UK, *Campylobacter* infection represented 27% of all food-borne diseases and about 82% of people admitted to hospital with food poisoning (Adak *et al*., 2002). Thus *Campylobacter* infection causes a large economic burden. *Campylobacter jejuni* represents 90% of species causing human infections, more than *Salmonella spp*., *Shigella spp*. or *E.coli O157H7*

(Humphrey *et al.,* 2007*).* Many factors affect the epidemiology of human *Campylobacter* infection including food, water and environment. Handling raw poultry and eating undercooked chicken are the main risk factors for *Campylobacter* infection (Forbes *et al.,* 2009*).*

The clinical picture of *Campylobacter* infection includes bloody diarrhea, abdominal pain, fever, malaise, nausea, and rarely vomiting. Complications include intestinal haemorrhage, toxic megacolon, haemolytic uraemic syndrome and mesenteric adenitis (Humphrey *et al.,* 2007*).* In the longer term infection, other clinical manifestations are meningitis, bacteremia, localized extra intestinal infections, immune-reactive complications such as reactive arthritis and neurological sequelae as Guillain-Barré syndrome (GBS). It is a serious post infection complication with acute and progressive neuromuscular paralysis (Jeon *et al.,* 2010*).*

Campylobacter identification and differentiation using the conventional culture methods is challenging because of the biochemical inertness or the fastidious growth requirements of the bacteria (Lubeck *et al*., 2003; On and Jordan, 2003). Also phenotypic differentiation between *C. coli* and *C. jejuni* is difficult as some strains of *C. jejuni* do not hydrolyze hippurate. The use of molecular methods as polymerase chain reaction (PCR) increases the sensitivity and specificity of *Campylobacter* differentiation (Koneman, 2006).

The *lpxA* gene in *Campylobacter* is a housekeeping gene that encodes an essential protein of cell function. It encodes the LpxA enzyme that catalyzes the first step of the lipid A and lipopolysaccharide synthesis. It is considered an excellent target for species-specific probes and can be used for phylogenetic analysis. Identification of the *Campylobacter* species is important for detection of source of infection, transmission routes and antimicrobial pattern of susceptibility (Klena *et al*., 2004).

Campylobacter infection is usually self-limiting and requires no antimicrobial therapy except in severe infections. However, increasing resistance of *C. jejuni* to antimicrobial agents is increasing throughout the world and it is thought to be pushed by the frequent use of antibiotics in animals farmed for meat (Wilson *et al*., 2009; Albert, 2013).

The aim of the study was to detect the prevalence of infection with *Campylobacter* species among diarrheic patients at Ain Shams University hospitals and to compare between conventional methods and PCR in identifying *Campylobacter* to the species level. Also to study the determination of antibiotic susceptibility pattern for the isolated *Campylobacter* spp., so as to recommend antibiotics being used in the empiric treatment of *Campylobacter* infection.

Materials and Methods

Stool samples were collected from patients suffering from diarrhea at Ain Shams University Hospitals, Cairo, Egypt, over the period from September 2010 to September 2011. The patients were informed about the study, a consent form was signed and questionnaire was filled by the patient or his/her family for children about ingestion of milk, type of food intake, contact with birds and clinical

condition of the patient. History was taken from the patients. A total number of 327 stool specimens were collected. Hundred and fifty (150) samples were taken from pediatric department, (50) samples from Hematology department, (70) from the Hepatology department, (30) from the Outpatient clinic, (20) from Nephrology department and (7) from the Rheumatology department. The patients were 207 males (63.3%) and 120 females (36.7%). Their ages ranged from 3½ to 35 years with a median of 20.0 (± 9.5).

Inclusion criteria for patients

All patients developed gastro-enteritis like symptoms and presented with diarrhea and/or (fever, headache, abdominal pain, myalgia, vomiting, blood in stool).

Stool sample collection:

Stool samples were collected in a sterile, disinfectant-free, screw capped wide necked containers and submitted immediately to the Central Microbiology Laboratory, and Medical Research Center Ain Shams University Hospitals.

Stool sample processing

All clinical stool samples were subjected to the following:

Physical examination of stool specimens: colour, consistency, odour, and presence of blood. Microscopic examination to exclude parasitic causes of diarrhea and perform WBCs count/HPF in stool. Immediate inoculation on Skirrow's media; which is formed of blood agar base (Oxoid, UK) with added Campylobacter Supplement-III. All media were incubated at 42ºC for 48-72hrs under micro-aerophilic conditions created by evacuation-replacement system (5% O_2, 10% CO_2, 10% H_2, and 75% N_2) (Anoxmat, Mart, UK).

Phenotypic characterization of Campylobacter species:

Identification of *Campylobacter* was performed by colony morphology, darting motility, Gram stain and oxidase test. Further biochemical tests were done for species identification; hippurate hydrolysis: for differentiation of *C. jejuni* from *C. coli* (Simga-Aldrich, Germany), indoxyl acetate test: for identification of *C.coli and C. upsaliensis* from other species (Oxoid, UK), and catalase test: for differentiation between *C. coli and C. upsaliensis* (Koneman *et al.,* 2006).

Genomic DNA extraction:

Confirmation of the species of *C. coli, C. jejuni, C. lari* and *C. upsaliensis* was done by Multiplex Polymerase Chain Reaction (PCR) to detect different lipid A gene (*lpxA*) The genomic DNA was extracted from tryptone soya broth culture of *Campylobacter* isolates using a DNA Purification, QIA amp DNA Mini Kit (Qiagen, USA) following the manufacturer's instructions (Yamazaki *et al.,* 2007).

Genotyping of Campylobacter species by PCR

The oligonucleotide primers of the *lpxA* gene were obtained from Promega (USA) (Table 1) (Klena *et al*., 2004). The primer pair lpxAF0301 and lpxARKK2m was used for detection of *Campylobacter* genus. Forward primers complementary to the *lpxA* nucleotide sequence of *C. coli (lpxAC. coli), C. jejuni (lpxAC. jejuni), C. lari (lpxAC. lari),* and *C. upsaliensis*

(lpxAC. upsaliensis) were used in combination with the reverse primer lpxARKK2m, for detection of *Campylobacter* species by Multiplex PCR. The reaction mixture composed of 15 µL Dream Taq Green PCR Master Mix (2X) (Green Buffer, 4 mM MgCl2, dNTP's (0.4 mM each), 2X Dream Taq polymerase) (ThermoScientific, UK), 10 pmol of each primer and 5µL of the genomic DNA template. The reaction tubes (25µL) were placed in the thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, USA) for 35 cycles, each cycle consists of: 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, for 35 cycles with a final extension time of 5 min. For multiplex PCR assays, 10 pmol of each forward primer was added to the reaction mixture with 30 pmol of the lpxARKK2m reverse primer (Klena *et al.*, 2004). The PCR products were analyzed by 3% agarose gel (Sigma, Germany) electrophoresis and bands were visualized with UV light after staining with ethidium bromide (Promega, USA) Images were captured on a Kodak Camera (Japan). The molecular size marker (Promega, USA), gave different bands ranging from 100bp-1000bp (Promega, USA). The negative control was examined to exclude any source of contamination (Figure 1 and 2).

Antimicrobial Susceptibility testing of *Campylobacter* Isolates

Minimal inhibitory concentrations (MICs) of antibiotics; erythromycin, azithromycin, ciprofloxacin, ampicillin and nalidixic acid were determined using E-test strips (AB Biodisk, Solna, Sweden) and Muller-Hinton agar (Oxoid, UK) supplemented with 7% sheep blood. The inoculum turbidity was adjusted to 0.5 McFarland. Then the agar plates were inverted and incubated at 35°C for 48h under micro-aerophilic conditions. The concentration gradient of each antimicrobial agent on the E-test strips was 0.016–256.0 mg/L, with the exception of ciprofloxacin, for which the gradient was 0.002–32.0 mg/L. Antimicrobial MIC breakpoint concentrations were recommended by CLSI 2008. The MIC resistance breakpoints were: ≥8 mg/L for erythromycin, ≥2µg/mL for azithromycin, ≥4 µg/mL for ciprofloxacin, ≥32µg/mL for ampicillin and nalidixic acid (Varela *et al*., 2008; Lehtopolku *et al*., 2010; Silva *et al*., 2011).

Quality Control

The reference strain *Campylobacter coli* ATCC 43474 and *Campylobacter jejuni* NCTC 11168 were used as positive controls. They were supported by the U.S. Naval Medical Research Unit No.3 (NAMRU-3), in Cairo, Egypt.

Statistical Analysis

Categorical variables were expressed as number (%). Chi-square test was used to study the association between each 2 variables or comparison between 2 independent groups as regards the categorized data. The probability of error at 0.05 was considered significant, while at 0.01 and 0.001 were highly significant. The sensitivity, specificity and the positive and negative predictive values were calculated for determining the diagnostic validity of the test. All the analyses were performed with commercially available software (SPSS version 20, SPSS, Inc., Chicago, IL, USA).

Results and Discussion

Direct isolation and identification from culture and biochemical reactions Nineteen

(5.8%) *Campylobacter* spp. out of 327 stool samples are isolated on Skirrow's media and are oxidase positive. 17 (89.5%) out of the 19 positive isolates are *C. jejuni* tested positive by hippurate hydrolysis while the other 2 (10.5%) samples are *C. coli* as they were positive by Indoxyl acetate and Catalase tests.

Molecular Identification and species differentiation

The PCR of the lipid A *(lpxA)* gene of the *Campylobacter* isolates confirmed the culture diagnosis of the 19 isolates (Figure1). Multiplex PCR identified 17/19 (89.5%) as *Campylobacter jejuni* detected at 331bp, whereas the other 2/19 (10.5%) isolates are confirmed to be *Campylobacter coli* detected at 391bp (Figure 2). Culture method and biochemical reactions were 100% concordant with the results of PCR.

Age and Sex

A significant association (P <0.05) is found between the positive *Campylobacter* cases and age, being highest in the youngest age group 9/19 (47.37%) between 3½-10 years. No significant difference is found as regard sex (Table 2).

Risk factors

Among the risk factors, a highly significant association between *Campylobacter* cases and consumption of unpasteurized milk 7/19 (36.84%) (P <0.001), and an almost significant association with ingestion of under cooked chicken 3/19 (15.79%) (P =0.055) are detected. No significant association is found for the contact with birds (Table 2).

Immune-compromising medical conditions

A highly significant association (P <0.001) is found between Campylobacter positive cases and immune-suppression. 10/19 (52.63%) of *Campylobacter* positive cases were immune-compromised; 6 (31.6 %) had leukemia, 3 (15.8%) had renal failure and one (5.2%) was on chemotherapy (Table 2).

Clinical manifestations

The main clinical manifestations are fever 18/19 (94.74%), abdominal pain (84.21%), vomiting (47.37%), myalgia (21.05%), and bloody diarrhea (10.53%). There is a highly significant association (P<0.001) between these manifestations and Campylobacter positive cases, while there is no significant association found between headache and Campylobacter positive cases (P>0.05) (Table 2).

Stool examination

Table 2 shows that there is a highly significant association (P <0.001) between stool WBC $\geq$50/HPF and positive stool culture for *Campylobacter* 16/19 (84.21%) (Table 2). Stool WBC$\geq$50 /HPF has 84.2% sensitivity, 77.6% specificity, 18.8% PPV, 98.8% NPV, and 87% efficacy for diagnosis of Campylobacter.

Antibiotic susceptibility by E-test

All the 19 Campylobacter positive isolates show 100% resistance to ampicillin and nalidixic acid with MICs ranges from 48->256 µg/mL, and 32->256 µg/mL respectively. 11/19 (57.9%) of the tested isolates are resistant to ciprofloxacin with MIC 6->32 µg/mL. 1/19 (5.3%) *C. coli* isolate is multi-drug resistant (MDR) to all

the tested antibiotics with MIC greater than the highest level in the E-test for all the tested antibiotics (Table 3).

In the present study, the prevalence of *Campylobacter* was 19/327 (5.8%) in the tested stool samples of the diarrheal patients admitted at Ain Shams University Hospitals. Our results were comparable to those obtained by Kulkarni and his colleagues (2002), who found that the prevalence rate was 4.9 % at the Central Middlesex Hospital, London, UK. Similarly, Yang and his colleagues in (2008) found that the prevalence rate of *Campylobacter* in children in northern Taiwan was 6.8%. Also, Samuel and his associates (2006) at Ilorin (Nigeria), Bessède and colleages (2011) at Pellegrin Hospital (Bordeaux, France), Havaei and his associates (2006) in Iran, and Singh *et al.,* (2011) in India detected a prevalence rate of *Campylobacter* of 8.2%, 9.5%, 9.5% and 10.5% respectively in diarrheic fecal specimens. Higher prevalence of *Campylobacter* (19.1%) was reported by Aboderin and his colleagues (2002) at Ile-Ife (Nigeria). On the other hand, Adekunle and his associates (2009) found low prevalence rate only 3/602 (0.5%) of diarrheic samples in patients between the ages of 0 and 36 months, who presented with foul-smelling diarrhea, fever and abdominal pain, were due to *Campylobacter* infection at Osogbo (Nigeria). This different prevalence could be explained by the differences in targeted population (selected or randomly collected, different age), methods used and possible geographic factors indicating different infection patterns in different population groups as well as the sample size (Samie *et al*., 2007). In the present study, conventional culture methods and biochemical reactions were 100% concordant with the results of PCR for identification and differentiation of *Campylobacter* species. As 17/19 (89.5%) *Campylobacter* positive samples were identified as *C. jejuni* (positive Hippurate test), whereas the other 2/19 (10.5%) isolates were *C. coli* (negative Hippurate test, positive indoxyl acetate and catalase test). These results were similar to those obtained by Klena and colleages (2004), who applied multiplex PCR method isolates obtained from 108 clinical and environmental thermo-tolerant *Campylobacter* isolates from New Zealand, which showed 100% correlation with biochemical typing methods. Also, Fitzgerald and colleagues (2011) reported that the culture method showed specificity of (100%), sensitivity (94.6%), PPV (100%) and NPV (99.8%).

Also similarly, Eyigor *et al.* (1999) (USA), Havaei *et al*. (2006) (Iran), Al Amri *et al*. (2007) (Saudi Arabia and Bahrain), Yang *et al*., (2008) (China), Fernandes *et al*., (2010) (Portugal) and Chen *et al.,* (2011) (China) found that the only recovered species from *Campylobacter* isolates by culture and multiplex PCR were *C. jejuni* and *C. Coli* with higher prevalence of *C. jejuni*. Hamphrey *et al*., (2006) also indicated that these two species can cause severe disease. However, additional *Campylobacter* species were detected as *C. lari* (3.2%) and *C. upsaliensis* (1.6%) by Prasad *et al.,* (2001) (India) and *C. lari* (2%) by Jain *et al.,* (2005) (India). This may be attributed to geographic factors indicating different infection patterns in different populations with different habits of meal and contact with different animals. On the other hand, Magistrado and associates (2001) (Philippines) and Schweitzer and colleagues (2011) (Hungary), although identified only *C. jejuni* and *C. coli*, however the prevalence of *C. coli* (62.5% and 20.3%) was higher

than that of *C. jejuni* (37.5% and 12.9%) respectively by PCR. The higher rates of isolation of *C. coli*, may be due to high consumption of pork meat as *C. coli* colonize more the caeca of pigs (Allos, 2009).

The current study revealed significant association between the age group and Campylobacter infection being highest in the youngest age group between 3½-10 years of age, 9/19 (47.37%). Similar results from previous studies in Egypt were reported. Pazzaglia and his co-workers (1991) reported that *Campylobacter* was the most common bacterial enteropathogen isolated from diarrheic Egyptian children aged from 0-60 months. It was isolated from 25.9% of cases and 15.2% of controls. They found that asymptomatic shedding in controls was positively associated with a recent diarrheal episode. In Cairo, George *et al* (2003) isolated *C. jejuni* 2 out of 56 children (3.6%) presenting with diarrhea. The 2 patients were 18 months and 6 years-old. Similarly, Nour (2004) showed that the highest isolation rate of *C. jejuni* was among the 7-12 months age group in 25.9% of acute diarrheal patients in Bab El-Shaareya hospital, Cairo. Similarly, El-Saifi and his associates (2005) isolated seven (2.06%) *C. Jejuni* out of 340 children with acute diarrhea from the outpatient clinic of Abu-El-Riche children's referral Hospital, Egypt between March 2003 and September 2004. In Alexandria, Fahmy (2005), isolated *C. jejuni* from the stool of 35 out of 230 (15.2%) unselected children with diarrhea. It was apparent that the maximal incidence of *Campylobacter* infection occurs below the age of 3 years and reaching its maximum at the age of 2 years (28.6%). In another study, in Alexandria, *C. jejuni* was isolated from 71 out of 470 cases (15.1%) of infantile diarrhea. It was not isolated from any of the control group (Mazloum *et al.,* 2006).

The present work revealed no significant association between the sex and *Campylobacter* infection (P>0.5). Similarly, Inns and associates (2010) (UK) reported that there was no significant difference in age or gender between cases and controls. However, Adekunle and associates (2009) (Nigeria) reported that the *Campylobacter* infection rate was significantly higher among males (0.82%; 3/368) than females (0%; 0/368). On the contrary, Fitzgerald and colleagues (2011) found that there was a significant association between females and *Campylobacter* infection. Also, Gillespie and co-workers (2006) found that being an infant and being a female gender has an increased risk of acquiring *Campylobacter* infection.

Regarding risk factors, *Campylobacter* infection had a highly significant association with consumption of unpasteurized milk 7/19 (36.84%) (P <0.001), and an almost significant association with ingestion of undercooked chicken, 3/14 (15.79%) (P ≈0.05). This was in agreement with Rao *et al*. (2001), Tenkate *et al*. (2001), Friedman *et al*. (2004); Danis *et al*. (2009); and Doorduyn *et al.* (2010); who reported a highly significant association between *Campylobacter* infection and with these risk factors (P<0.001).

The current work revealed statistically significant association between immune suppression and infection with *Campylobacter* (P<0.001), as 10 *Campylobacter* positive cases were immune-compromised with the main underlying conditions were leukemia 6/19 (31.6 %), renal failure 3/19 (15.8%) and

1/19 (5.2%) was on chemotherapy. Also, Pacanowski and associates (2008) found that 130/167 of *Campylobacter* infected patients (78%) were immune-compromised, the main underlying conditions were liver disease (39%) and cancer (38%). While Samie and colleagues (2007) found that *Campylobacter* spp. were important pathogens associated with diarrhea among HIV positive individuals (22.8%) with a rate for *C. jejuni* and *C. coli* (18.2% and 11.4%), respectively among HIV positive patients compared to 11.4% and 6.2% in HIV negative individuals.

In the present study, all cases had diarrhea as one of the inclusion criteria. The other accompanied main clinical manifestations were fever 18/19 (94.74%), abdominal pain (84.21%), vomiting (47.37%), myalgia (21.05%), bloody diarrhea and headache (10.53%), all of them had a highly significant association with *Campylobacter* infection except headache which had no significant association. Gillespie and co-workers (2006), showed that the most prevalent symptoms among the 11□831 cases of *C. jejuni* infection in the UK were diarrhea followed by abdominal pain, fever, vomiting and bloody diarrhea as 76.5%, 68.9%, 62.5%, 28.2% and 22.5% respectively. Whereas, Pacanowski and associates (2008) found that the main clinical manifestations were fever (42%) and diarrhea (33%). While, Jain and colleagues (2005) reported that diarrhea (13.5%) and abdominal pain (18.64%) had significant association with *Campylobacter* infection.

In the present study, Stool WBC≥50 /HPF is a value to predict *Campylobacter* infection showing a highly significant association (P <0.001) with 84.2% sensitivity, 77.6% specificity, 18.8% PPV, 98.8% NPV, and 87% efficacy. This finding was in accordance to those of *Mshana and associates (2009)* who found that there was strong association between WBC in stool and the presence of *Campylobacter* infection and WBC can be a good predictor of *campylobacter* infection. On the other hand, *Jagannathan and Penn (2005)* stated that although the likelihood of infection with *Campylobacter* or other entero-invasive pathogens may be higher in the presence of fecal leukocytes, however the absence of fecal leukocytes does not rule out the diagnosis.

In the current study, all the 19 *Campylobacter* isolates showed 100% resistance rate to ampicillin and nalidixic acid with MICs ranged from 48->256 µg/ml, and 32->256 µg/ml respectively. 11 (57.9%) of tested isolates were resistant to ciprofloxacin with MIC range 6->32 µg/ml. However, only one (5.3%) *C. coli* was multi-drug resistant (MDR) to all of the tested antibiotics with MICs greater than the highest level. In agreement with our work, Lévesque and colleagues (2008) (Québec, Canada) found that 16/289 (5.5%) *C. jejuni* isolates were resistant to erythromycin but only 21 (7.2%) isolates were resistant to ciprofloxacin. On the other hand, Lehtopolku and associates (2012) (Finland) found that out of 238 *Campylobacter* strains 19 (8%) were resistant to erythromycin (MIC ≥ 16 µg/ml), 18 (7.6%) were resistant to ciprofloxacin (MIC ≥ 4 µg/ml) and 17 (7 %) to azithromycin (MIC ≥ 64µg/ml). All erythromycin-resistant strains were multidrug resistant. Erythromycin resistance was significantly more common

Table.1 PCR primers of *lpxA* gene of Campylobacter species used in this study

Primers	Sequence (5´-3´)	Size (bp)
Campylobacter genus 0301 (F)	CTT AAA GCN ATG ATA GTR GAY AAR	521
*lpxA C. Coli (*F)	AGA CAA ATA AGA GAG AAT CAG	391
lpxA C. jejuni (F)	ACA ACT TGG TGA CGA TGT TGT A	331
lpxA C. Lari (F)	TRC CAA ATG TTA AAA TAG GCG A	233
lpxA C. Upsaliensis (F)	AAG TCG TAT ATT TTC YTA CGC TTG TGT G	206
lpxARKK2m (R)	CAA TCA TGD GCD ATA TGA SAA TAH GCC AT	

Table.2 Association of *Campylobacter* cases with patient history and clinical picture

Patient condition	**Item**	***Campylobacter* positive N=19 (%)**	***Campylobacter* negative N=308 (%)**	**Chi Squared X^2**	**P**	**S.**
Age (years)	3½-10	9 (47.37%)	74 (24.03%)	6.607	0.0368	S
	10-20	6 (31.58%)	88 (28.57%)			
	20-35	4 (21.05%)	146 (47.40%)			
Gender	Male	11 (57.89%)	196 (63.64%)	0.254	0.6143	NS
	Female	8 (47.37%)	112 (36.36%)			
Risk factors:	Unpasteurized milk	7 (36.84%)	39 (12.6%)	8.656	0.003	HS
	Undercooked chicken	3 (15.79%)	16 (5.2%)	3.67	0.055	≈S
	Contact with birds	4 (21.05%)	48 (15.6%)	0.4	0.527	NS
	Immunocompromised	10 (52.63%)	24 (7.8%)	38.62	<0.001	HS
Clinical picture	Fever	18 (94.74%)	31 (10.06%)	100.7	<0.001	HS
	Abdominal pain	16 (84.21%)	25 (8.12%)	94.5	<0.001	HS
	Vomiting	9 (47.37%)	22 (7.14%)	94.5	<0.001	HS
	Myalgia	4 (21.05%)	12 (3.90%)	11.32	<0.001	HS
	Blood in stool	2 (10.53%)	5 (1.62%)	6.771	0.009	HS
	Headache	2 (10.53%)	12 (3.90%)	1.92	0.166	NS
Stool Analysis	WBC: ≥ 50/HPF	16 (84.21%)	69 (22.4%)	35.54	<0.001	HS

P: probability; S.: Significant; HS: highly significant; NS: non-significant; ≈S: almost significant;

Table.3 MICs of the used antimicrobials for the 19 *Campylobacter* isolates

Antimicrobial agent	MIC Resistance Break Point (µg/mL)	Detected Resistant MIC (µg/mL)	Resistant isolates No. (%)
Erthromycin	8	>256	1 (5.3%)
Azithromycin	2	>256	1 (5.3%)
Ciprofloxacin	4	6->32	11 (57.9%)
Nalidixic acid	32	32- >256	19 (100%)
Ampicillin	32	48- >256	19 (100%)

MIC: Minimal inhibitory concentration

Figure.1 PCR detection of *Campylobacter lpx*A gene.
Lane M: PCR marker; Lane 1: Positive control; Lane 2: Negative control; Lane 3-8: *Campylobacter*

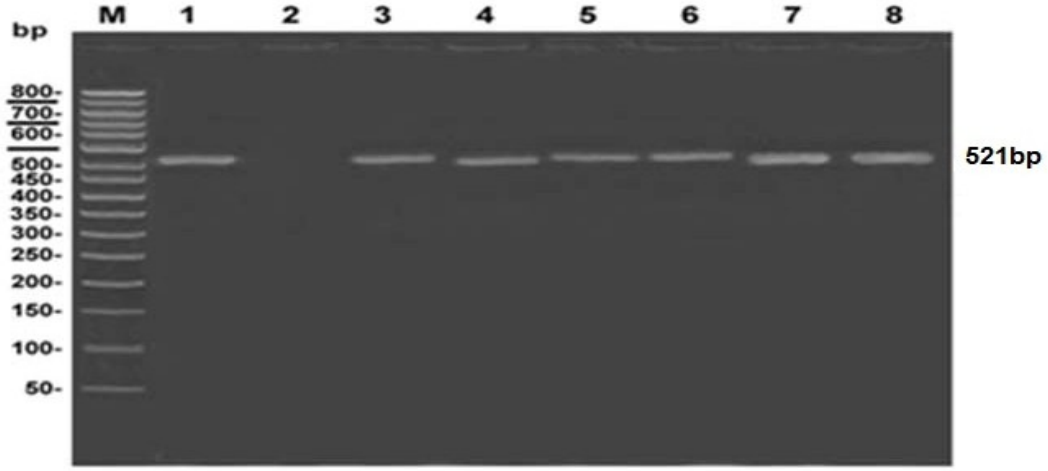
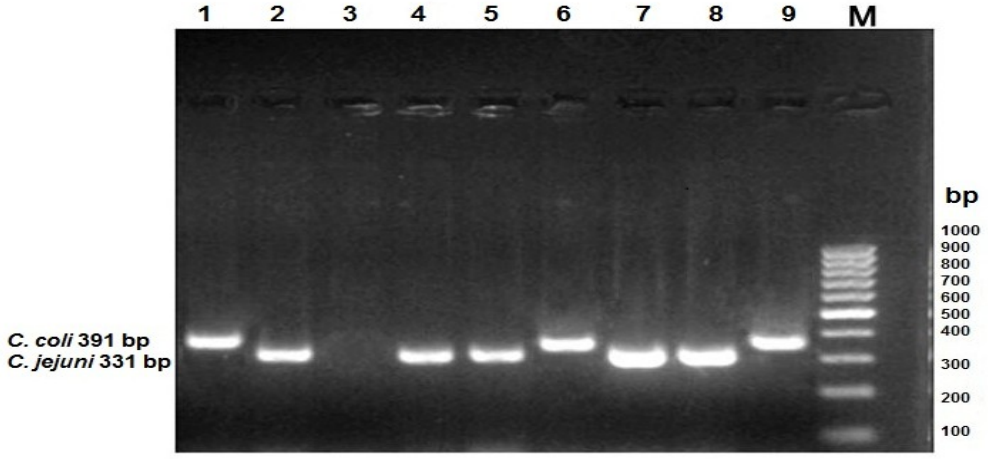


Figure.2 Multiplex PCR detection of *Campylobacter lpxA* gene on agarose gel electrophoresis



Lane 1: Positive control (*C. coli*) (391bp); lane 2: Positive control (*C. jejuni*) (331bp); lane 3: Negative control; Lane 4, 5, 7, 8: *C. Jejuni* (331bp); Lane 6, 9: *C. coli* (391 bp); lane M: molecular marker.

among *C. coli* than among *C. jejuni* strains. In a study done on 1110 stool samples collected from food-producing animals at the time of slaughter in Hungary, Schweitzer and colleagues (2011) found that resistance to ciprofloxacin and nalidixic acid was (73.3%) and (77.2%) respectively. Higher erythromycin resistance rates were found among *C. coli* isolates (9.7 %) than among *C. jejuni* isolates (3.1%). Praakle and associates (2007) (Estonia) detected similar results of resistance to ciprofloxacin (66%), but lower resistance to nalidixic acid (66%) and ampicillin (34%), and higher resistance to erythromycin (14%) among 70 *Campylobacter* isolates. Agricultural Science (2012) detected *Campylobacter* lower resistance of 2.46% to erythromycin, 88.18% to nalidixic acid, while higher resistance 91.13% to ciprofloxacin among 184 *C. jejuni* and 19 *C.coli* isolates. While, Sonnevend and co-workers (2006) revealed that 41 (100%) of tested isolates of *C. jejuni* strains isolated from patients in Tawam Hospital, Al Ain, United Arab Emirat, were all sensitive to erythromycin, while higher resistance rates (85.4□%) to ciprofloxacin were detected. Also Senok and Botta (2009) reported higher levels of ciprofloxacin resistance in the Arabian Gulf. The highest levels were reported in Bahrain (69-85%) and United Arab Emirates (UAE) (84%) and similarly to our work, 53% resistance was documented in a study in Kuwait. Also, Chu and associates (2004) reported high levels of ciprofloxacin resistance in Thailand (96%), Spain (75%), Hong Kong (85.9%), and India (77.1%). In contrast to our work, Marinou and associates (2012) (Greece) identified 16 *Campylobacter* isolates among 1080 fecal samples, 14 were *C. coli* and only two were *C. jejuni*. 13/14. 93% of *C.coli* were resistant to erythromycin, all strains were resistant to ampicillin (100%) and two isolates were resistant to nalidixic acid (14%), whereas, all the strains were susceptible to ciprofloxacin. The discovery of the differences in susceptibility patterns in different countries is important for the proper treatment of the patients.

Multiplex PCR and conventional culture methods were comparable for identification and species differentiation of *Campylobater*. Using the *lpxA* gene PCR is rapid, sensitive and specific for identification and genotypic differentiation of *Campylobacter* isolates. The most common species identified were mainly *C. jejuni* and to a lesser extent *C. coli*. Risk factors for *Campylobacter* infection are young age, immune-compromised patients and consumption of contaminated under cooked food and unpasteurized milk especially for children under 10 year of age. Stool WBC≥50 /HPF may predict diagnosis of *Campylobacter* diarrhea. Macrolides remain the drugs of choice for empiric treatment of *Campylobacter*. But Macrolides and quinolones resistance trends and the development of multi-drug resistant *C. coli* strains must be tracked in human clinical isolates in relation to use of these agents in food animals.

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