Isolation, biotyping and antimicrobial susceptibility of *Campylobacter* isolates from raw milk in Erbil city, Iraq

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Abstract

This study aimed to determine the prevalence and antimicrobial sensitivity of *Campylobacter* species in raw milk sold at retail vending in Erbil city. Three hundred and fifty (350) samples were aseptically collected from retail raw milk shops between January and June 2019. For isolation of *Campylobacter* spp., samples were cultured on selective media and tested for biotype and antimicrobials susceptibility by disk diffusion assay. The overall prevalence of *Campylobacter* spp. was 12.6%. *Campylobacter jejuni* was significantly prevalent (65.9%) among other *Campylobacter* species. Antimicrobial susceptibility testing showed complete sensitivity to tetracycline, rifampicin, and neomycin. On the other hand, total resistance to ampicillin and trimethoprim was observed. Strikingly, as low as 56.8% and 72.7% of isolates are still sensitive to the drugs of choice in campylobacteriosis treatment; ciprofloxacin and erythromycin respectively. This resistance pattern of *Campylobacter* found in this study is critically alarming owing to the insusceptibility to the aforementioned antibiotics commonly used as the drugs of choice for campylobacteriosis treatment. Increase in *Campylobacter* prevalence in raw milk was associated with warm season. These levels prevalence and resistance worth further investigations and effective countermeasures owing to potential public health hazards.

Introduction

Campylobacter is a zoonotic gram negative small curved or S-shaped bacterial pathogen. It is recognized globally as the most public cause of bacterial milk-borne diseases. Since its first taxonomic validation, the genus *Campylobacter* has developed to comprise several vital human and animal pathogens. It has been the most recurrent pathogen isolated in outbreaks in both developed and developing countries throughout the past decades (Kaakoush *et al.*, 2015). *Campylobacter* species are hyperendemic in several developing countries due to abundant natural reservoirs, poor environmental sanitation, reduced food hygiene and safety, close contact with animals at domestic settings in rural and agricultural populations, among various other factors (Isabel, 2019; Kaakoush *et al.*, 2015). Various species of *Campylobacter* are common component of the intestinal microbiota of a wide range of hosts, such as farm and wild mammals, and birds. *Campylobacter* infections result mostly from oral ingestion of contaminated food or water. The frequent presence of *Campylobacter* in undercooked food and raw milk or dairy products indicates its risk of zoonotic transmission to humans (Bolton, 2015; Chlebicz & Śliżewska, 2018; Chukwu *et al.*, 2019).

Epidemiologically, *Campylobacter* has been estimated to be responsible for diarrhea affecting 400-500 million people with 37,600 deaths worldwide annually (Mughal, 2018; WHO, 2018). However, much of its epidemiological aspects in middle east countries are still unknown (Kaakoush *et al.*, 2015). Transmission of *Campylobacter* from its natural reservoirs occurs mostly via contaminated food and water, person-to-person, and contact with infected animals (Kaakoush *et al.*, 2015; Backert *et al.*, 2017). Around 80% of campylobacteriosis cases are transmitted by food. Raw milk-associated outbreaks are reported from different countries (Burakoff *et al.*, 2018; Castrodale *et al.*, 2013; Evans *et al.*, 1996; Heuvelink *et al.*, 2009; Korlath, *et al.*, 1985; Longenberger *et al.*, 2013; Mungai, *et al.*, 2015; Porter & Reid, 1980; Weltman *et al.*, 2013).

Several *Campylobacter* species have been implicated in human infections, with 95% of infections due to *C. jejuni, C. coli,* and *C. fetus* (Kaakoush *et al.*, 2015; Backert *et al.*, 2017). However, other species are also known as gastrointestinal pathogen in both developing and developed countries such as *C. lari, C. helveticus, C. upsaliensis, C. hominis, C. gracilis, C. lanienae, C. peloridis, C. concisus, C. mucosalis, C. hyointestinalis, C. sputorum, C. insulaenigrae, C. curvus, C. rectus, C. showae,* and *C. ureolyticus* (Nachamkin & Fitzgerald, 2015).

After an incubation period of usually 3 days, human campylobacteriosis manifests by gastroenteritis and other extraintestinal manifestations, mostly as sequelae. The gastrointestinal symptoms include abdominal pain, vomiting, acute watery or bloody diarrhea especially in toddlers under 3 years as well as in elderly accompanied by fever, dehydration, and nausea. Other complications include septicemia, urinary tract infections (UTI), reactive arthritis, Guillain-Barre syndrome, and Miller-Fisher syndrome, among others (Backert *et al.*, 2017).

In Kurdistan region Iraq, raw milk and dairy products are most commonly served in food outlets especially in retail vending, restaurants, street vendors, school, hotel, canteen and also in small outlets. No information available or published data on *Campylobacter* contamination level in milk or other dairy products in Erbil governorate. Therefore, this work was conducted in order to monitor the prevalence, biotypes, and antimicrobial susceptibility of *Campylobacter* spp. in raw milk sold at retail vending in Erbil city.

Materials and Methods

Study design and sampling

A total of 350 raw milk samples (120 cattle, 115 sheep, and 115 goats) were collected under aseptic conditions during January to June 2019 in Erbil city according to previously published method (Kazemeini, *et al.*, 2011). Samples were transported in cooled bags within approximately half an hour to the Research Center Laboratory, Knowledge University.

Isolation of *Campylobacter* spp.

Samples were process according to previously published isolation method (Salihu *et al.*, 2010). In brief, *p*H of milk samples was adjusted to 7.5 and 20 ml of milk was centrifugation at 14,000 rpm for 20 min. at 4°C. The pellet was suspended in 45 ml of Brucella broth base containing Butzler supplement (HiMedia, India) and 5-7% (v/v) lysed horse blood in 100 ml sterile flask, mixed properly and incubated at microaerophilic environment (85% N_2, 5% O_2 and 10% CO_2) at 42°C for 48 h. After the enrichment, a loopful of enriched culture was streaking on *Campylobacter* agar plates containing Butzler supplement (HiMedia, India) and 5-7% (v/v) lysed horse blood. The inoculated plates were incubated at microaerophilic atmosphere at 42°C for 48 h. For purification, suspected colonies showing a typical drop-like appearance were further subcultured on the same medium and on blood agar with 5% defibrinated sheep blood and incubated for 24 h at 37°C in microaerophilic conditions (Al-Dulaimi, 2013).

Identification of *Campylobacter* species

Campylobacter species was identified by colonies morphological characteristic on the plate, modified Gram stain (by counterstaining the smear with safranin for 3 minutes and use of carbol fuchsin instead of safranin stain), motility test by wet mount smear, and biochemical tests; catalase, oxidase, urease, growth in 3.5% NaCl, TSI reaction and growth on MacConky´s agar (Marinou *et al.*, 2012).

Biotyping tests

All biotyping reactions were performed according to standard methods described in MacFaddin manual (MacFaddin, 2000), unless stated otherwise.

Growth at 25, 37 and 42°C

Campylobacter isolates were streaked on *Campylobacter* selective agar plates, and were divided into three groups. First group were incubated in 25°C, the second in 37°C and the third in 42°C for 48 hours in microaerophilic conditions (Marinou *et al.*, 2012).

Cephalothin and nalidixic acid susceptibility

A Muller Hinton agar plates were evenly inoculated and two discs were placed on the agar surface, one of cephalothin (30 µg) and the other was nalidixic acid (30 µg). After the incubation period, sensitivity to nalidixic acid and cephalothin was inferred by a zone of clearing (Medeiros & Hofmann, 2002).

DNA hydrolysis test

DNA agar plates (HiMedia, India) were inoculated and incubated at 37°C for 24-48 hours. *Campylobacter jejuni* biotype IV, *C. coli* biotype II and *C. lari* biotype II, produce DNase and lyse medial DNA, but other biotypes show negative reaction.

H_2S production

Hydrogen sulfide production was tested by two methods; Triple Sugar Iron (TSI) medium and lead acetate paper. All isolates were subjected to H_2S production by leads acetate method.

Hippurate hydrolysis test: C. jejuni gives a positive result of this test, while all other species are negative.

Antibiotics susceptibility testing

Modified Kirby-Bauer disk diffusion method was employed to evaluate the susceptibility of *Campylobacter* isolates to twelve antibiotics according to CLSI guidelines (CLSI, 2011). The Enterobacteriaceae breakpoints published by CLSI were used to interpret the inhibition zones diameters around antibiotic disks. The tested antibiotics (Mast diagnostics, UK) were: amoxicillin, ampicillin, cephalothin, cefotaxime, ciprofloxacin, erythromycin, gentamycin, neomycin, rifampicin, streptomycin, and trimethoprim.

Statistical analysis

Data were analyzed via version 21 of SPSS software (SPSS Inc., Chicago). Confidence intervals were calculated by normal approximation method. Differences between groups were evaluated by Chi square test at alpha level of 0.05.

Results

Prevalence of *Campylobacter* spp.

Out of 375 raw milk samples, 12.6% were positive for the presence of *Campylobacter* spp. (Table 1). Up to 15.8% of the positive samples were derived from cow milk. Based on statistical inference, it is estimated that 9.12% to 16.08% (95% confidence interval) of raw milk sold in Erbil retail markets is contaminated by *Campylobacter* species. There is no significant difference between milk types in terms of *Campylobacter* occurrence (χ^2=0.985, *p*=0.370).

Detected *Campylobacter* spp. and biotypes

The detected species of *Campylobacter* isolated from raw milk are summarized in Table 2. *C. jejuni* was the most common species comprising 65.9% (29/44) of the total isolates. Three biotypes of *C. jejuni* were detected; biotype II (24.2%), biotype III (17.2%), and biotype IV which was the most common (58.6%). Only the biotype II of *C. coli* was found, while two biotypes of *C. lari* were detected; biotype I (66.7%) and biotype II (33.3%). *C. jejuni* is significantly more prevalent species in milk samples than other *Campylobacter* species (*p*=0.0195).

Temporal distribution of *Campylobacter* spp.

The change in prevalence rate of *Campylobacter* species was monitored throughout study period. The highest rate of isolation was observed in June (25.0%%)

Table 1. Prevalence of *Campylobacter* species among raw milk samples.

Type of milk	No. of samples	No. positive (%)	95% CI
Cow milk	120	19 (15.8)	9.27 – 22.33
Sheep milk	115	11 (9.6)	4.22 – 14.98
Goats milk	115	14 (12.2)	6.22 – 18.18
Total	350	44 (12.6)	9.12 – 16.08

Table 2. Species of *Campylobacter* isolated from raw milk samples.

Biotypes	No. of isolates	% of isolates	95% CI
C. jejuni	29	65.91	51.9 – 79.92
C. coli	7	15.91	5.1 – 26.72
C. lari	3	6.82	0.00 – 14.27
C. fetus	4	9.09	0.6 – 17.58
C. gracilis	1	2.27	0.00 – 6.67
Total	44	100	100

and May (20.3%%), while the lowest rate was found in February (3.4%). Table 3 summarizes detected proportions in temporal scale. There is a good association (R2= 0.8397) between *Campylobacter* presence in milk and warm season progress (spring – summer) (Figure 1).

Antimicrobial susceptibility of *Campylobacter* spp.

Campylobacter isolates (n=44) were evaluated against a panel of twelve commonly used antibiotics. The results of antimicrobial susceptibility testing showed a complete resistance to ampicillin and trimethoprim. On the contrary, total sensitivity was found to neomycin, rifampicin and tetracycline. The detailed antibiogram profile is summarized in Figure 2.

Discussion

Campylobacter is an important etiology in gastrointestinal bacterial outbreaks worldwide. According to the European Centers for Disease Control and Prevention (ECDC) and the Global Enteric Multicentre Study (GEMS), *Campylobacter* spp. are now considered to be the leading cause of bacterial gastroenteritis worldwide with higher occurrence rates in children under 5 years old (ECDC, 2019; Levine *et al.*, 2012). The overall prevalence of *Campylobacter* spp. found in the present study is 12.6%. These results are consistent with a previous study in Pakistan which found the highest occurrence (11.6%) of *Campylobacter* in butter and raw milk (Mahmood *et al.*, 2009). Additionally, similar prevalence rates ranging from 12% to 18% were also reported from Italy (Bianchini *et al.*, 2014), Tanzania (Kashoma *et al.*, 2016), and Yemen (Al-Zailay, 2017). However, lower rates were also reported in other studies from Iran (6.25%) (Rahimi, Sepehri, & Momtaz, 2013), Turkey (7.2%) (Elmalı *et al.*, 2019), Egypt (4.44%) (Barakat *et al.*, 2015), and India (2.9%) (Modi *et al.*, 2015). These

variations may be attributed to differences in geographical locations, sensitivity of detection method, level of hygiene, food practice, availability of natural reservoirs of *Campylobacter*, among other factors

(Kaakoush *et al.*, 2015). Regarding *Campylobacter* spp. isolated in this study (Table 2), *C. jejuni* was the most prevalent (65.9%) which is consistence with previous studies from Iran (Rahimi *et al.*, 2013),
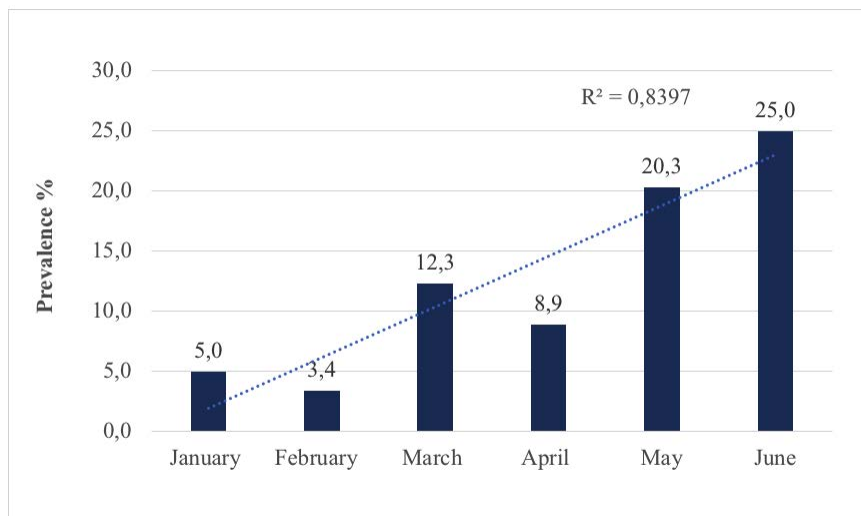

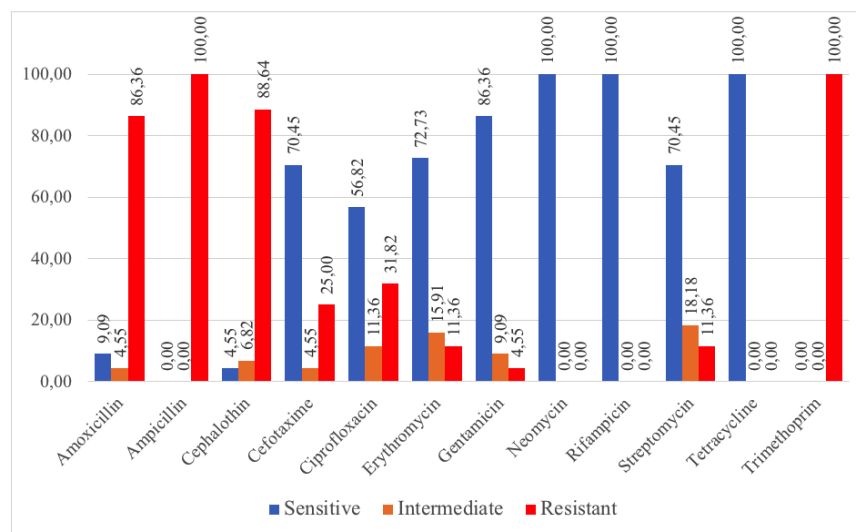**Figure 1. Association between months and prevalence of *Campylobacter* spp. in raw milk.**

Figure 2. Antibiogram profile of *Campylobacter* spp. against twelve antibiotics.

Table 3. Temporal distribution of *Campylobacter* spp. during study period.

Month	No. of examined milk (no. of positive)			Total examined	Total positive (%)
	Cow	Sheep	Goat		
January	22 (2)	22 (0)	18 (1)	60	3 (5.0)
February	20 (1)	19 (1)	19 (0)	58	2 (3.4)
March	19 (2)	19 (2)	19 (3)	57	7 (12.3)
April	18 (3)	18 (1)	20 (1)	56	5 (8.9)
May	20 (5)	20 (3)	19 (4)	59	12 (20.3)
June	21 (6)	19 (4)	20 (5)	60	15 (25.0)
Total	120 (19)	115 (11)	115 (14)	350	44 (12.6)

Tanzania (Kaakoush *et al.*, 2015), Yemen (Al-Zailay, 2017), and Turkey (Elmalı *et al.*, 2019). A very recent study in Iraq (Kanaan, 2018) has found that two distinct biotypes (I & II) of two *Campylobacter* species; *C. jejuni* and *C. coli* to be the most prevalent biotypes. Additionally, biotype II of *C. coli* was the most common (25%) biotype of the species. However, biotypes I of each species were detected in the present work. The observation that *C. jejuni* and *C. coli* biotype I dominate in humans, while biotype II was more prevalent in animals suggests that the contaminating source of milk is most probably the domesticated animals including the animals from which samples were drawn.

In terms of time-based prevalence of *Campylobacter*, the highest occurrence was documented in late spring and early summer, while the lowest rate was found in winter (Table 3). These observations are in good line with a Nigerian study that reported that the number of campylobacteriosis peaks on summer seasons and then decline in winter season (Salihu *et al.*, 2010). Several studies had connected warm periods to high prevalence of *Campylobacter* (Zeleňáková *et al.*, 2012), Germany (Schielke *et al.*, 2014), Egypt (Omara *et al.*, 2015), and Lebanon (Ibrahim *et al.*, 2019). The underlying reason behind this seasonality is still unclear, but may indicate a possible association between temperature and *Campylobacter* survival and transmission of infection (Jorgensen *et al.*, 2011; Patrick *et al.*, 2004).

The antibiogram profile showed complete resistance (100%) to ampicillin and trimethoprim, while total sensitivity was recorded to neomycin, rifampicin, and tetracycline (Figure 2). There are wide variations in antibiotic resistance pattern of *Campylobacter* isolates in published literature from different countries around the globe. For instance, ciprofloxacin resistance level found in the present work is in good agreement with previously reported (30.77% and 34.4%) in Iran (Maktabi *et al.*, 2019; Rahimi *et al.*, 2013). However, the high sensitivity to gentamicin and streptomycin found in this study is lower than reported from India (Modi *et al.*, 2015). The resistance levels to ampicillin, erythromycin, and gentamicin are also in good agreement with a recent Tanzanian study of beef carcasses and raw milk samples (Kashoma *et al.*, 2016). These variations in resistance phenotypes are influenced by strains thriving in different ecological niches, geographical locations, antibiotic uses, and horizontal gene transfer of resistance determinants (Luangtongkum *et al.*, 2009; Shen *et al.*, 2018).

Conclusions

Campylobacter species prevalence in raw milk is moderately high in Erbil city, which may pose a serious threat to consumers. Warm season (summer) was found to be associated with increase in *Campylobacter* prevalence in raw milk samples. Fortunately, the isolates still have accepted sensitivity level to the drug of choice in treatment of campylobacteriosis (Macrolides, tetracyclines, and aminoglycosides). The high occurrence of *Campylobacter* spp. in raw milk could be reduced by improvement of sanitary condition applied during milking, handling, storage, and also by increase awareness of farmers and retailers. A four-season study is highly recommended to investigate the distribution of campylobacters in raw milk accompanied by antibiotic susceptibility testing to aid in control of burden and morbidity of campylobacteriosis.

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