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Recovery of Resistant Thermophilic *Campylobacters* on Farm and Market Vegetables

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A B S T R A C T

This study focused on establishing the presence of *Campylobacter* at the farm and retail level and evaluated the antibiogram profile of isolated species. *Campylobacter spp*. were isolated on mCCDA agar and confirmed by API Campy (biomereaux, France) and the susceptibility profile determined by Kirby-Bauer disk diffusion method. A total of 124 market vegetables comprising 59 lettuce and 65 cabbages and 117 farm vegetables made up of 94 lettuce and 23 cabbages were analysed. Prevalence of 41.9% (52/124) and 23.9% (28/117) were respectively documented in market and farm vegetables. *Campylobacter jejuni* was the dominant species keyed out from market vegetables (61.5%) followed by *C. coli* (23.0%), *C. lari* (11.5%) and *C. jejuni sub sp. doylei* (3.8%). Fifty percent (50%) of farm vegetable isolates were *C. jejuni*, 25% constituted *C. jejuni sub sp. doylei* and 21.4%, 3.6% were *C. coli* and *C. lari* respectively. All isolates (100%) were multidrug resistant, with highest resistance observed against cephalexin, cefotaxime, ampicillin, erythromycin and chloramphenicol (86-100%), 64-75% against tetracyclines, 32-49% to ciprofloxacin, nalidixic acid and norfloxacin and 57-67% to trimethoprim sulphamethoxazole. Against kanamycin and gentamicin resistance of 4-46% was obtained while no resistance (0%) was observed against imipenem. The results provide baseline information on *Campylobacter* in vegetables and the possible risk it poses to consumers in the face of high level resistance in the species.

Introduction

Campylobacter are zoonotic pathogens that frequently cause diarrhoea in humans often surpassing salmonellosis and shigellosis with *C. jejuni* and *C. coli* species mostly implicated (EFSA, 2013; WHO, 2015). Consumption of undercooked meat is the main source of human infections and the most significant risk factor. Nevertheless, environmental routes such as faecally contaminated water serving as conduits for the dissemination of pathogens to foods of non-animal origin have been recognized (Kumar *et al.*, 2001).

Campylobacter resilience to harsh environmental conditions have been proven by several studies contrary to earlier reports of its environmental fragility indicating non-survival on vegetables for a long period of time (Solomon and Hoover, 1999).

Campylobacter presence in the environment, particularly in water as well as soil is well documented (Chai *et al.,* 2007; Chai *et al.,* 2009). The use of *Campylobacter*-contaminated water to wash vegetables may result in banking the organism on the surface of the product. Also, leafy vegetables irrigated with untreated water or cultivated in *Campylobacter* contaminated soils are likely to carry the pathogen (Thomas *et al.,* 1999).

The significance of vegetables as important source of campylobacteriosis is supported by the numerous outbreaks linked with raw vegetables, as salad vegetables are considered to be the second highest risk factor for *Campylobacter* infection after consumption and preparation of chicken (CDC, 2000; Evans *et al.,* 2003; Abadias *et al.,* 2008;Verhoeff-Bakkenes *et al.,* 2010).In Ghana most water bodies from which most vegetable farmers depend on as irrigation sources, have been greatly polluted from human activities such as indiscriminate waste disposal. Some studies from Africa have determined the presence of other pathogenic microorganisms on raw vegetables at the farm and retail level (Amoah *et al.,* 2005) but very limited information is available on the occurrence of *Campylobacter* on this product.

This study reports on the presence of *Campylobacter spp*. on vegetables at the farm (Pre-harvest) and market stage (Post- harvest) and the resistance profile of isolated species.

Materials and Methods

Sampling

Fresh vegetables (cabbage and lettuce) were randomly purchased from the Central market as well as six other satellite markets in the Kumasi metropolis. Eleven (11) major vegetable farms were also visited early mornings (before 9:00am) where lettuce and cabbages were obtained for study. At each farm visit, fresh vegetables (either cabbage or lettuce) were randomly picked from vegetable beds depending on the availability at the time of visit. All samples were returned on ice packs to the laboratory for analysis. Sampling took place from May 2013 to February 2014.

Culture, identification and confirmation

A 20-g sample of lettuce or cabbage was cut into pieces and placed into a sterile ziplock bag containing 180 ml sterile 0.1% peptone water and pulsified for 30s using a Microgen Pulsifier (Bioproducts, UK). One ml (1ml) aliquots were transferred into 5ml of blood-free Campylobacter enrichment broth (Oxoid CM0963) supplemented with CCDA supplement (Oxoid SRO155E) in a bijou bottle and incubated at 37^{o}C overnight. A loopful of the overnight culture was plated directly onto CCDA agar plates and incubated microaerophilically (CampyGen Oxoid CN0025A) at 42^{o}C for 48h. Typical colonies were cultured on Nutrient agar from which Gram stain, catalase and oxidase test were performed. Small, curved, catalase and oxidase positive Gram negative rods were presumptively identified as *Campylobacter spp*. These isolates were further confirmed and characterized by API CAMPY system (bioMerieux, France).

Antibiotic susceptibility test

Antibiotic susceptibility tests were carried out by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Liofilchem-Italy) supplemented with 5% sheep blood following CLSI guidelines. Tested antibiotics and their corresponding concentrations were: Ampicillin (10 µg/disc), chloramphenicol (30µg/disc), ciprofloxacin (5µg/disc), kanamycin (30µg/disc), erythromycin (15µg/disc), gentamicin (10µg/disc), nalidixic acid (30 µg/disc), tetracycline (30µg/disc),

cephalexin (30µg/disc), trimethoprim sulfamethoxazole (25µg/disc), norfloxacin (10µg/disc), cefotaxime (30µg/disc) and imipenem (10µg/disc). Mueller-Hinton agar plates were inoculated with 0.5Mcfarland suspension and incubated under microaerophilic condition at 48^{o}C for 24hours. The zones of inhibition were recorded and results interpreted according to EUCAST and CLSI breakpoints for *Campylobacter*. Quality control was attained by *E. coli* (ATCC25922) and *S. aureus* (ATCC25923) strains.

Statistical analysis

Percentages were used for the descriptive analysis. Associations were determined using the Chi-square test at a significance level of < 0·05. Stata 14.0 software was used for statistical analysis.

Results and Discussion

Of the 124 market vegetable samples (59 lettuce and 65 cabbage) and the 117 farm vegetables (94 lettuce and 23 cabbage), 52 (27 cabbage, 25 lettuce) and 28 (7 cabbage, 21 lettuce) isolates were confirmed as *Campylobacter* with a prevalence rate of 41.9% (52/124) in market vegetables and 23.9% (28/117) in farm vegetables. There were statistically significant (p=0.003) differences in the isolation frequency of *Campylobacter* from market and farm vegetable samples (Table 1).

In the market vegetable samples, *Campylobacter jejuni* was most prevalent (61.5%) followed by *C. coli* (23.0%), *C. lari* (11.5%) and *C. jejuni sub sp. doylei* (3.8%). Similarly, 50% of farm vegetable isolates were *C. jejuni*, 25% *C. jejuni sub sp. doylei*, 21.4% *C. coli* and 3.5% *C. lari* (Table 2). Market vegetable strains showed resistance of 98%, 96%, 90%, 75% and 67% respectively to Ampicillin, erythromycin, chloramphenicol, tetracycline and trimethoprim sulfamethoxazole. Against the cephalosporins; resistance was 100% each to cephalexin and cefotaxime. Resistance to the quinolones was 49% to ciprofloxacin, 44% to nalidixic acid, and 35% to norfloxacin. Against the aminoglycosides, resistance was 46% to gentamicin and 25% to kanamycin; as 0% resistance was observed against imipenem (Table 3).

Farm vegetable isolates showed resistance of 100%, 96%, 93%, 64% and 57% respectively to erythromycin, chloramphenicol, Ampicillin, tetracycline and trimethoprim sulfamethoxazole. Against the cephalosporins; resistance was 100% to cephalexin and 86% to cefotaxime. Resistance to the quinolones was 46% each against nalidixic acid and ciprofloxacin and 32% to norfloxacin. Against the aminoglycosides; resistance was 11% to kanamycin and 4% to gentamicin; as 0% resistance was to imipenem.

About sixty five percent (65.4%) of *Campylobacter jejuni*, 23.1% of *C. coli* and 11.5%of *C. lari* strains from market vegetables were multidrug resistant. Among farm vegetable isolates 75.0% of *C. jejuni*, 21.4% of *C. coli* and 3.6% of *C. lari* strains were multidrug resistant. The difference in multidrug resistance between farm and market vegetable strains was not statistically significant (p= 1.000) (Table 4).

Globally, there is limited data on *Campylobacter* contamination in vegetables (Kumar *et al.,* 2001; Chai *et al.,* 2007). Our study found 41.8% *Campylobacter* contamination of market vegetables and 23.9% of farm vegetables. Market vegetable contamination with *Campylobacter* in our study is consistent with the work of Hussain *et al.,* (2007) who reported 40.9% in

vegetables from retailers in Pakistan and 52.6% in Malaysia by Khalid *et al.,* (2014). Much lower levels; 3.1%, 3.57%, and 7.5% have also been reported in Canada, India and Brazil, respectively (Park and Sanders, 1992; Kumar *et al.,* 2001; Carvalho *et al.,* 2013). Contamination levels of 18.8% and 35.2% have also been reported by Chai *et al.,* (2009) and Khalid *et al.,* (2014) in farm vegetables.

*Campylobacter*on vegetables at pre-harvest stage (farms) may come from faecally polluted irrigation water, use of poultry manure for soil enrichment and use of raw sewage sludge (Jones *et al.,* 1990; Kumar *et al.,* 2001; Chai *et al.,* 2009; Verhoeff-Bakkenes *et al.,* 2011). Moreover, most of the irrigation sources in this study region have been greatly polluted through indiscriminate waste disposal; it is therefore fair to partly attribute the source of *Campylobacter* contamination to these faecally polluted water sources.

Similarly, reasons for the contamination of vegetables at post-harvest stage (market) may be due to the display of vegetables on bare floors, or on old sacks laid on the floor, sprinkling of vegetables with poor quality water to keep them fresh, improper hygiene of the market women, packing and sorting and transportation in rickety public buses or taxis to the markets. These practices which are common among people involved in vegetable trade in the study region have also been confirmed by several authors as sources of contamination (Beuchat, 1996; Beuchat, 2002; Amoah *et al.,* 2007; Chai *et al.,* 2007).

Table.1 Prevalence of *Campylobacter* from farm and market vegetables

Vegetable Source	No. Samples	No. Campylobacter Identified N (%)	P-value (Chi-square, df)
Market	124	52 (41.9)	0.003
Farm	117	28 (23.9)	(8.799,1)
Total	241	80 (33.2)	

Table.2 Species specific prevalence of *Campylobacter* from vegetable farms and markets in Kumasi

Vegetable source	No. isolates (N)	*C. jejuni* (%)	*C. doylei* (%)	*C. coli* (%)	*C. lari* (%)
Market	52	32(61.5)	2(3.8)	12(23.0)	6(11.5)
Farm	28	14(50.0)	7(25.0)	6(21.4)	1(3.5)
Total	80	46	9	18	7

C. doylei= C. jejuni sub. sp. doylei

Table.3 Resistance and susceptibility patterns of *Campylobacter* from vegetable farms and market

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
		Farm (N=28)	
Nalidixic acid	54	NA	46
Tetracycline	22	14	64
Erythromycin	0	NA	100
Ciprofloxacin	25	29	46
Chloramphenicol	4	0	96
Ampicillin	4	3	93
Cefotaxime	14	0	86
Kanamycin	56	36	8
Gentamicin	93	3	4
Norfloxacin	57	11	32
SXT	43	0	57
Cephalexin	0	NA	100
Imipenem	68	32	0
		Market (N=52)	
Nalidixic acid	56	NA	44
Tetracycline	19	6	75
Erythromycin	4	NA	96
Ciprofloxacin	34	17	49
Chloramphenicol	0	10	90
Ampicillin	2	0	98
Cefotaxime	0	0	100
Kanamycin	58	17	25
Gentamicin	46	8	46
Norfloxacin	59	6	35
SXT	33	0	67
Cephalexin	0	NA	100
Imipenem	75	25	0

Table.4 Multidrug resistance in *Campylobacter* species from vegetables

Vegetable isolates	Total	Multi-drug resistance	
		Market	Farm
C. coli	18	12(23.1)	6(21.4)
C. lari	7	6(11.5)	1(3.6)
C. jejuni	55	34(65.4)	21(75.0)
Total	80	52	28

NB*: C. jejuni* sub sp. *doylei* was counted as part of *C. jejuni* isolates

Campylobacter jejuni was the dominant species identified in this study which is consistent with works from other countries. In our market vegetables, 61.5% were *C. jejuni* and 23.0% *C. coli* and 50% and 21.4% were *C. jejuni* and *C. coli* in the farm vegetables. In Canada, Park and Sanders (1992) reported 88% of *C. jejuni* and 4% *C. coli* in vegetables purchased from outdoor markets and supermarkets while in Malaysia, Chai *et al.*, (2007) reported 40.7% *C. jejuni* and 35.2% *C. coli* from supermarkets. The high isolation rate of *C. jejuni* as opposed to *C. coli* supports the theory that *C. jejuni* is more resilient to environmental stresses (Gonzalez and Hänninen, 2012; Bronowski *et al.*, 2014); as Chai *et al.*, (2009) also failed to isolate *C. coli* from soil and manure in Malaysia.

Antibiotic resistance profiles of the *Campylobacter* species were generally high with isolates showing 100% multidrug resistance. Highest resistance was observed against cephalexin, cefotaxime, ampicillin, erythromycin and chloramphenicol with resistance ranging from 86-100% in both market and farm vegetables. Resistance rates of below fifty percent (32-49%) were recorded for the quinolones (ciprofloxacin, nalidixic acid and norfloxacin), 64-75% for tetracyclines and 57-67% for trimethoprim sulphamethoxazole. However, 4-46% was obtained against the aminoglycosides while no resistance (0%) was observed against imipenem.

In Malaysia, isolates from vegetables has been reported to be 100% multidrug resistant (Chai *et al.*, 2008).

The uncontrolled use of antimicrobial agents in agriculture and in hospitals cannot be totally excluded as a lead to the high rates of resistance recorded in this study. In Ghana, reports of increasing resistance to several pathogens have been reported in both human and animals (Donkor *et al.*, 2008; Newman *et al.*, 2011). The prevalence of *Campylobacter spp*. in vegetables is generally believed to be due to cross-contamination from sources such as humans, birds, livestock, and environmental water sources. In most developing countries, despite the ban of fluoroquinolones application in agriculture, the tetracyclines and fluoroquinolones are extensively applied in farming practices (Turkson, 2008; Newman *et al.*, 2011). Besides, agricultural extension officers and veterinarians are not consulted before drug application often resulting in abuse.

In conclusion, *campylobacter* species have been established in vegetables from Kumasi at the pre- harvest (farms) and post- harvest stage (markets). Multidrug resistant strains of *Campylobacter* isolated poses direct risk to consumers since vegetables (cabbages and lettuce) are normally served raw at most food joints in Ghana. It is essential that vegetables are well decontaminated to ensure that they are free of pathogens such as *Campylobacters* prior to consumption. Much attention should be given to the increasing resistance of pathogens including *Campylobacter* to commonly used antibiotics in Ghana through comprehensive research and education to control the resistance menace.

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