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Antibiotic resistance profile and RAPD analysis of *Campylobacter jejuni* isolated from vegetables farms and retail markets

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ABSTRACT

Objective: To investigate antibiotic resistance profile and characterize *Campylobacter jejuni* (*C. jejuni*) isolates using random amplified polymorphic DNA (RAPD) analysis.
Methods: Ninety eight *C. jejuni* isolates from farms and retail outlets were screened against 10 antibiotics commonly used clinically and agriculturally by using disk diffusion method. RAPD analysis was done to characterize 98 *C. jejuni* isolates.
Results: Fifty-one percent of the isolates had multiple antibiotic resistance index 0.2 and below. This indicated that the isolates in the vegetables were not from the high risk environment or extensive farming practices. *C. jejuni* isolates found resistant towards penicillin G (93%), vancomycin (86%), ampicillin (35%), erythromycin (28%), gentamycin (4%), amikacin (3%), enrofloxacin (1%), norfloxacin (1%) and no resistance towards ciprofloxacin. RAPD clustering analysis showed that the contamination of *C. jejuni* in vegetables was likely due to cross contamination at retail markets.
Conclusions: *C. jejuni* contamination in vegetables at retail markets was due to cross contamination. Current finding proved that *C. jejuni* in small scale vegetables production was less expose towards antibiotic abuse.

1. Introduction

Campylobacter jejuni (*C. jejuni*) is a Gram-negative, spiral-shape bacterium and requires microaerophilic growth condition [1,2]. *C. jejuni* nutrient utilisation has been fully elucidated, but its metabolic flexible processes allow survival in the environment which eventually causes infection in human [1,2]. *C. jejuni* is one of the most frequently implicated causative agent of Campylobacteriosis in human [1,2]. Major risk factors for causing Campylobacteriosis in humans are consumption of undercooked poultry, untreated or contaminated water and raw milk [2].

Campylobacter becomes more resistant toward antibiotics and some of it have formed multiple drug resistance [3,4]. Erythromycin and tetracycline are commonly administered in cases of *Campylobacter* infections, but high resistance among *Campylobacter* towards them has been reported [3,4]. Fluoroquinolones resistant *C. jejuni* was thought to be biologically stronger than susceptible strain and the usage of fluoroquinolones as prophylaxis in poultry has caused increase in resistance towards fluoroquinolones [3,4]. Chai *et al*. suggested *C. jejuni* resistance towards fluoroquinolone group of antibiotics is related to farming practices [4]. Krumperman reported the usefulness of multiple antibiotic resistance (MAR) indexing to identify bacteria isolates from high risk environment or fecal contamination [5].

The demand for ready-to-eat fresh produce has risen in recent years [6]. This might be due to the health awareness on the benefits of fresh produce intake. Several studies have reported the presence of *Campylobacter* spp. in fresh produce and the number of foodborne outbreaks associated with raw fruits and vegetables has also increased due to cross-contamination from fertilizer, soil and irrigation water [7–9]. Besides being reported presence in fresh produce that available at retail markets, *Campylobacter* spp. also detected to be present in vegetables at farms [9]. However, whether the presence of *Campylobacter* in

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vegetables at retail market originated from farms has been rarely studied.

Therefore the goal of present study is to characterize *C. jejuni* isolates by antibiotic resistant profiles and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) to determine the genetic relatedness of *C. jejuni* isolates.

2. Materials and methods

2.1. C. jejuni isolates

A total of 98 *C. jejuni* isolates from various types of samples (vegetables and soils) from 5 small scale local vegetables farms and 12 retail markets in Terengganu, Malaysia from January 2013 to April 2014. It was comprised of 9 *C. jejuni* isolates from farms and 89 *C. jejuni* isolates from retail markets. All the isolates were confirmed by PCR using species specific primers targeting 23S rRNA [10]. The primers used were 23S rRNA F (5′-TATACCGGTAAGGAGTGCTGGAG-3′) and 23S rRNA R (5′-ATCAATTAACCTTCGAGCACCG-3′). The PCR method was performed in 25 μL of reaction mixture as described in our previous study with a final concentration of 1× Green GoTaq Flexi buffer, 0.2 mmol/L concentration of the deoxynucleotide triphosphate mix, 0.2 mmol/L concentrations of each primer, 3 mmol/L $MgCl_2$ solution, 2 IU of GoTaq DNA polymerase, and 2 μL of DNA boiled lysate. All items used in the PCR were purchased from Promega (Madison, WI, USA) [8]. PCRs were performed on a Veriti 96-well Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA), with an initial denaturation step of 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. PCR products were electrophorised using 1.5% agarose gel at 70 V for 90 min. Bands were visualized with UV transilluminator (AlphaImager HP, Alpha Innotech, CA, USA) after staining with GelRed nucleic acid gel stain (Biotium, Hayward, CA, USA). A 100-bp DNA ladder (NL1405, Vivantis, Oceanside, CA, USA) was used as a DNA molecular ladder.

2.2. Antibiotic resistance test

Antibiotics resistance patterns were determined using disk diffusion method according to Clinical and Laboratory Standards Institute [11]. All isolates were grown in Bolton Broth with supplement (Oxoid, Hampshire, England) without lyse horse blood for 48 h at 42 °C. Sterile cotton swabs were used to spread uniformly *C. jejuni* from broth into Mueller–Hinton agar plates (Merck, Germany). Ten antibiotic discs were selected to test on its susceptibility to *C. jejuni*.

All antibiotics discs were placed on the agar surface by using disc dispenser. The selected antibiotics were penicillin G (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), norfloxacin (10 μg), amikacin (30 μg), vancomycin (5 μg), ampicillin (10 μg). Inoculated plates were incubated at 42 °C for 48 h under microaerophilic condition generated by Anaerocult C system (Merck, Germany).

2.3. MAR index

MAR index of the isolates was determined as a/b, where ‘a’ represents the number of multiple antibiotics to which the particular isolates are resistant, and ‘b’ represents the number of multiple antibiotics to which the particular isolates are exposed [5].

2.4. Cluster analysis using RAPD-PCR

DNA was extracted using boiled cell method as described by Khalid *et al*. with minor modification [8]. A total of 1 mL Bolton broth from the turbid tubes was centrifuged at 12 000 r/min for 10 min in order to pellet the bacterial cells. The supernatant was discarded and the pellet was then resuspended with 300 μL of sterile distilled water and boiled for 10 min followed by freezing at −20 °C for 10 min. It was then centrifuged at 12 000 r/min for 10 min to pellet the cell debris [8]. The supernatant was then kept for use in RAPD-PCR.

A 10-mer oligonucleotide primers of OPA 11 (5′-CAATCGCCGT-3′) from Integrated DNA Technologies, Singapore were used to characterize the isolates. PCR amplification was done with following programme: initial denaturation of 95 °C (5 min); 45 cycles of denaturation at 95 °C (1 min), annealing at 36 °C (1 min), and extension at 72 °C (2 min); final extension at 72 °C (5 min). All the PCR assays were performed with Veriti 96-well Thermal Cycler (Applied Biosystems, USA). PCR products were visualized by electrophoresis in a 1.5% agarose gel at 70 V for 90 min. Bands were visualized with UV transilluminator (AlphaImager HP, Alpha Innotech, CA, USA) after staining with GelRed™ Nucleic Acid Gel Stain (Biotium, USA). A 100 bp-DNA ladder (NL1405; Vivantis, USA) was used as a DNA-molecular ladder. Cluster analysis was done using GelCompar II version 5.1 (Applied Maths, Belgium).

3. Results

From Table 1, all 98 isolates of *C. jejuni* were tested against 10 types of antibiotics that frequently used in clinical and

Table 1

Number and percentages of antimicrobial-resistant *C. jejuni* isolated from various samples ($N = 98$). *n* (%).

Antimicrobial agent	Disk content (μg)	Resistance	Susceptible
Amikacin	30	3 (3)	95 (97)
Ampicillin	10	34 (35)	64 (65)
Ciprofloxacin	5	0 (0)	98 (100)
Enrofloxacin	5	1 (1)	97 (99)
Erythromycin	15	27 (28)	71 (72)
Gentamicin	10	4 (4)	94 (96)
Norfloxacin	10	1 (1)	97 (99)
Penicillin G	10	91 (93)	7 (7)
Tetracycline	30	5 (5)	93 (95)
Vancomycin	5	84 (86)	14 (14)

Table 2

Antibiotic resistance profile and multiple antibiotic resistance index of *C. jejuni* from vegetable and soil samples.

Antibiotic resistant profiles	No. Isolates (%)	MAR index
PVaAmpE	16 (17)	0.4
PVaNorAmp	1 (1)	0.4
PVaEnr	1 (1)	0.3
PETeAk	2 (2)	0.3
PVaAmp	17 (18)	0.3
PVaE	9 (10)	0.3
PVa	40 (43)	0.2
PTe	3 (3)	0.2
PCn	2 (2)	0.2
Cn	2 (2)	0.1
Ak	1 (1)	0.1

Ak: 30 μg amikacin; Amp: 10 μg ampicillin; Enr: 5 μg enrofloxacin; E: 15 μg erythromycin; Cn: 10 μg gentamicin; Nor: 10 μg norfloxacin; P: 10 μg penicillin G; Te: 30 μg tetracycline; Va: 5 μg vancomycin.

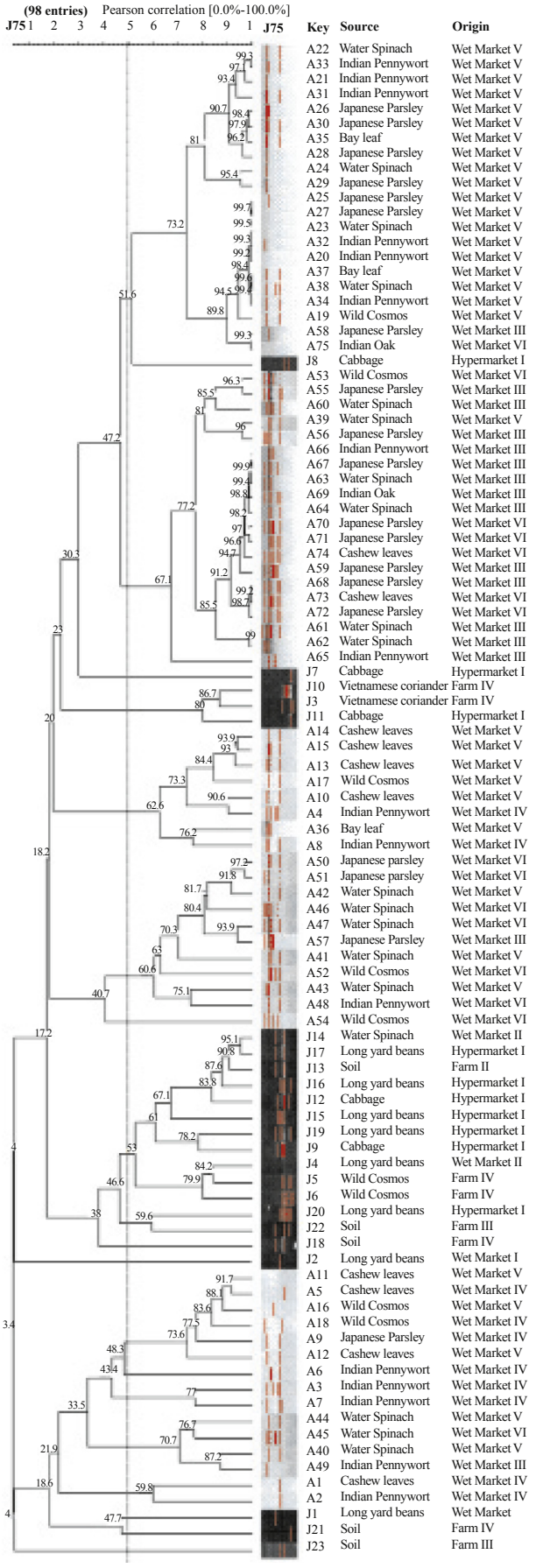

Figure 1. Dendrogram based on the hierarchic numerical analysis of the resistance profiles obtained for 98 *C. jejuni* isolates, employing the Pearson correlation coefficient and UPGMA for clustering.

Table 3

Characterization of *C. jejuni* clusters defined in the hierarchic analysis performed with location of sampling.

Cluster	Similarity (> 50%)	Location	
		Farm	Retail market
C1	51.6	0	22
C2	67.1	0	20
C3	80.0	2	1
C4	62.6	0	8
C5	60.6	0	10
C6	53.0	3	8
C7	59.6	1	1
C8	73.6	0	6
C9	77.0	0	2
C10	70.7	0	4
C11	59.8	0	2

agricultural practices. *C. jejuni* isolates showed the highest resistance towards penicillin G (93%), followed by vancomycin (86%). All *C. jejuni* isolates were susceptible towards ciprofloxacin (100%) and showed highly susceptible to enrofloxacin, norfloxacin, amikacin, gentamicin and tetracycline with each recorded susceptibility level of 99%, 99%, 97%, 96% and 95%. Other antibiotics used in this study showed moderate percentage of *C. jejuni* resistance with ampicillin (35%) and erythromycin (28%).

MAR index is shown in Table 2. Percentages of *C. jejuni* isolates recorded MAR index 0.1, 0.2, 0.3 and 0.4 were 3%, 48%, 31% and 18%, respectively.

The genetic diversity among *C. jejuni* strains isolated from various sources was investigated by RAPD-PCR using OPA 11 primer. All isolates generated bands with OPA 11 primer. The genetic diversity was observed among all strains. The dendrograms derived from the RAPD-PCR profiles generated with primers OPA 11 is shown in Figure 1. The dendrogram was constructed using GelCompar II version 5.1 by Applied Maths, Belgium with Pearson correlation and unweighted pair-group method with arithmetic means clustering to determine the genetic relatedness of the 98 isolates. According to Figure 1, the dendrogram branched into 11 major clusters at a similarity level of 50%. Ninety-one out of 98 isolates was grouped into this 11 major clusters and the remaining 7 isolates were not grouped due to similarity below 50%. Three out of 11 clusters demonstrated relatedness between isolates from farms and the retail markets is shown in Table 3.

4. Discussion

C. jejuni multidrug resistance especially towards quinolones and erythromycin had created concerns around the world [3,12]. Investigation of *C. jejuni* in vegetables was limited and most studies investigated antibiotic resistance of *C. jejuni* isolated from poultry and meat product [13–16]. Chai *et al*. had studied the biosafety of *C. jejuni* in 'ulam' and reported the same pattern which showed resistance of *Campylobacter* isolates towards erythromycin was higher compared to other antibiotics group [4]. In the present study, it has seen *C. jejuni* isolates from farms and retail outlets were susceptible towards fluoroquinolone, aminoglycosides and tetracycline groups. Our findings were contradicted with other studies that showed high resistance of *C. jejuni* towards fluoroquinolone due to the usage in clinical and animal farming [17,18]. Veterinary usage

of third generation quinolones since 1980s to combat respiratory infection due to *Escherichia coli* has induced resistance among *C. jejuni* isolates [19]. Rodrigo *et al.* reported 86.6% *Campylobacter* spp. resistance towards ciprofloxacin [19]. Usage of untreated chicken manure as fertilizer has also been thought to be one of the contributing factor high resistance on quinolone among *C. jejuni* found in vegetables [4]. Low resistance towards these types of antibiotics in the present study might be due to small scale production of the vegetables.

Clinical and agriculture excessive use of antibiotics has been thought to cause increase resistance among *C. jejuni* isolates [4,17,18]. Fluoroquinolones-resistant *Campylobacter* strains have been demonstrated to be biologically stronger than fluoroquinolone-susceptible strains [20,21]. A study discovered inoculation of mixed population consists of fluoroquinolone-susceptible and fluoroquinolone-resistant *Campylobacter* isolates resulted in highly isolation rate of fluoroquinolone-resistant strain [21]. Antibiotic-free poultry farming discovered no *Campylobacter* in the chicken, but free-range farming showed no significant difference with regards to multiple antibiotics resistance among *Campylobacter* isolates compared to conventional farming [21,22].

Besides low fluoroquinolone resistance observed in this study, *C. jejuni* isolates also demonstrated similar low resistance towards aminoglycosides group. Penicillin G had the highest level of resistance (93%) among *C. jejuni* isolates. *C. jejuni* is inherently resistant to many β-lactam drugs making the use of drugs from β-lactamase group suboptimal, especially in serious infections [23].

It is shown that erythromycin-resistance among *C. jejuni* isolates were increasing, though earlier reports showed the minimal changes after years of testing for antimicrobial resistance in many parts of the world [24–27]. In Thailand, *Campylobacter* isolates from chicken, pig, dairy and human were found to be resistant to erythromycin and tetracycline at 38.3% and 66.2%, respectively [28].

However, this study suggested that there are differences in farming practices around Terengganu. Most of the vegetables farms are small scale to meet the local demands. This explains the low MAR index among *C. jejuni* isolates found in vegetables. Low MAR index would indicate that the isolates from vegetables were from low risks of animal waste contamination [5].

Dendogram from RAPD analysis which comprised of 98 *C. jejuni* isolates from farms and retail outlets were divided into 11 clusters (Table 3). Clusters 3, 6 and 7 comprised of isolates from both farm and retail outlet with various types of salad vegetable. Present study showed that *C. jejuni* isolates from retails and farm was less correlated to each other (Figure 1 and Table 3). Since majority of the cluster was unrelated, there was a little possibility that the strain from farm's sample had associated *C. jejuni* isolates at retail outlets. Only 3/11 (27%) of the clusters possibly have correlation. Most of *C. jejuni* isolates had demonstrated location specific or source specific either in retails or farm. This proved that *C. jejuni* isolated from raw salad vegetables (ulam) from the retail markets may have been exposed to cross-contamination due to poor handling practices among workers, quality of irrigation water and wash water [7,29,30].

This study showed that small scale vegetables production resulted in lower resistance profile among *C. jejuni* isolates, though there is an increase pattern of fluoroquinolone and macrolide resistance globally in *Campylobacter* [12] and growing threat in Southeast Asia region [4]. Cross-contamination is inevitable the major route of microorganism contamination in fresh produce. Ways to decontaminate or prevent growth of microorganisms in fresh produce at retail markets would be useful to reduce the risk of human infection from consumption of raw or minimally cooked fresh produce.

Conflict of interest statement

We declare that we have no conflict of interest.

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