

Biosafety of *Campylobacter jejuni* from Raw Vegetables Consumed as *Ulam* with Reference to their Resistance to Antibiotics

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Abstract: Antibiotic resistance in campylobacter is an emerging global public health problem after MRSA and VRE. Fluoroquinolone and macrolide resistance have been found to be more common in this world leading foodborne pathogen. A total of fifty-six isolates of *Campylobacter jejuni* obtained from raw vegetables which are consumed as *ulam* (salad) in Malaysia, were tested with 12 antibiotics used clinically and agriculturally. The resistance was determined using the disk diffusion method. Results were determined by hierarchic numerical methods to cluster strains and antibiotics according to similarity profiles. Fifty five *C. jejuni* isolates from different isolation sites were all clustered together into ten groups. This indicates that the commodities (raw salad vegetables/*ulam*) where the isolates originated might share a similar source of cross-contamination along the production route. All antibiotics tested correlated and there were four groupings reflecting their mode of actions. Generally, *C. jejuni* isolates were found to be highly resistant to erythromycin (91.1%) and tetracycline (85.7%). Both agents are popular antibiotics used clinically to treat bacterial infections. On the other hand, the *C. jejuni* isolates showed high percentage (80.4%) of resistance towards enrofloxacin, an extensively used antimicrobial agent in agriculture practices. This study showed that *C. jejuni* isolates were highly multi-resistance to as many as 10 antibiotics. Therefore, in terms of biosafety, the presence of antibiotic resistance strains in the food chain has raised concerns that the treatment of human infections will be compromised.

Keywords: *Campylobacter jejuni*, clustering analysis, *ulam*, vegetables

INTRODUCTION

Antimicrobial resistance is a growing public health threat and has been designated by the World Health Organization as an emerging public health problem. The scenario is getting complicated with the tremendous growth in international trade of agricultural commodities in recent decades. Antibiotic resistance in pathogen is not in itself a problem. The problem arises when bacteria causing disease withstand therapy. Thus, the issue on biosafety with regard to antibiotic resistance must be addressed at a global level.

Campylobacter jejuni (*C. jejuni*) has been recognized since the late 1970s as the major cause of gastroenteritis throughout the world.

Campylobacteriosis infected approximately 1% of the United States population each year (Tauxe, 1992). Contaminated food is the well-known source of human campylobacteriosis. Undercooked chicken, unpasteurised milk and contaminated water are common sources for epidemic and sporadic food-borne cases (Butzler and Oosterom, 1991; Alterkruse *et al.*, 1999). Although it is not common, there were also outbreaks of campylobacter illness associated with consumption of contaminated fruits, vegetables, or other produce-related products (Centers for Disease Control, 2000). In most cases of human campylobacteriosis, antibiotic treatment is not required due to clinically mild and often self-limiting infection (Blaser *et al.*, 1995; Dryden *et al.*,

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1996). However, antibiotic treatment is indicated for severe and prolonged enteritis, septicemia, and for immuno-compromised patients. Normally, erythromycin and tetracycline are the first line antibiotics prescribed for campylobacter infections. Nevertheless, with the introduction of fluoroquinolones in the 1980s, these broad spectrum antibiotics have offered a new approach to antibiotic intervention (Winstrom and Norrby, 1995).

Currently, resistance to fluoroquinolones in campylobacter enteritis has caught more global attention than others. Smith *et al.* (1999) reported that patients infected with resistant *C. jejuni* had longer duration of diarrhea compared to patients with fluoroquinolone sensitive strains. As campylobacter infections can be very serious in immuno-compromised patients, the identified treatment failure raises concerns that the fluoroquinolone resistant strains may increase campylobacter associated deaths in this particular group of patients. The blame of antibiotic resistance in *Campylobacter jejuni* has been put on both clinical and as well as agricultural usage of antibiotics. It has been suggested that the use of fluoroquinolones as chemoprophylaxis in poultry has led to fluoroquinolone resistance in human isolates (Endtz *et al.*, 1991; Sanchez *et al.*, 1994). Engberg *et al.* (2001) also concluded that antibiotic resistance in human isolates can be related to exposure of animal strains to antibiotics used in farming. However, there are also reports suggesting opposite findings. According to Piddock (1999), antibiotic resistance commonly emerges when campylobacter infection is treated in humans.

There are a few reports on application of cluster analysis in analyzing bacterial antibiotic resistance profile (Berge *et al.*, 2003; de Fatima Silva Lopez *et al.*, 2005). Cluster analysis is a classification method that can be used to describe the complex multi-dimensional nature of antibiotic resistance in bacteria. It can efficiently evaluate large data sets so that objects having similar characteristics are grouped together and differentiated from groups (clusters) exhibiting contrasting characteristics. Although there are a large number of cluster algorithms, they do not all result in the same classification scheme and the approach taken for classification must be chosen carefully (Kaufman and Rousseeuw, 1990; Everitt, 1993).

The objective of this study was to apply clustering analysis as a method to determine the biosafety of the *C. jejuni* isolates based on their antibiotic resistance phenotypes.

MATERIALS AND METHODS

Campylobacter jejuni Isolates

A total of 56 isolates of *C. jejuni* were isolated from raw salad vegetables in a previous study (Chai *et al.*, 2007) comprised of 40 isolates from supermarkets (supermarket A located in Seri Kembangan and supermarket B located in Putrajaya) and 16 isolates from an outdoor market. All isolates were revived from glycerol stocks. Bolton enrichment broth supplemented with Bolton supplement (Merck KGaA, Darmstadt, Germany) and 5% lysed horse blood were used to revive the cultures. They were incubated at 37°C for 48 hours under microaerophilic conditions produced using the Anaerocult C system (Merck KGaA, Darmstadt, Germany).

Antimicrobial Susceptibility Testing

Antibiotic resistance patterns were determined using the disk diffusion method, according to the guidelines of The National Committee for Clinical Laboratory Standards (NCCLS, 2003). All isolates were grown in Brain heart infusion (BHI; Oxoid, Hampshire, United Kingdom) for 24 hours and were swabbed using a sterile non-toxic swab on Mueller-Hinton (MH) agar plates (Merck KGaA, Darmstadt, Germany) to form a uniform lawn of bacterial growth. Antibiotic disks were placed on the surface of the agar using a disk dispenser. Twelve antibiotics were selected for the tests. The 12 antibiotics were: akamicin (30 µg), ampicillin (10 µg), cephalothin (30 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), erythromycin (5 µg), gentamycin (10 µg), nalidixic acid (30 µg), norfloxacin (10 µg), penicillin (10 µg), tetracycline (30 µg), and tobramycin (5 µg). Antibiotic cartridges with commercially prepared antibiotic disks were purchased from Oxoid (Hampshire, United Kingdom). All plates were incubated at 37°C for 48 hours under microaerophilic conditions produced using Anaerocult C system (Merck KGaA, Darmstadt, Germany). After incubation, the size of the inhibition zones was recorded and the levels of susceptibility (sensitive and resistant) were determined according to the NCCLS guidelines.

Statistical Analysis

Associations between the resistance profiles obtained for each isolates were analyzed by hierarchic numerical methods. The numerical matrix obtained was computed using the BioNumerics version 4.5 software package

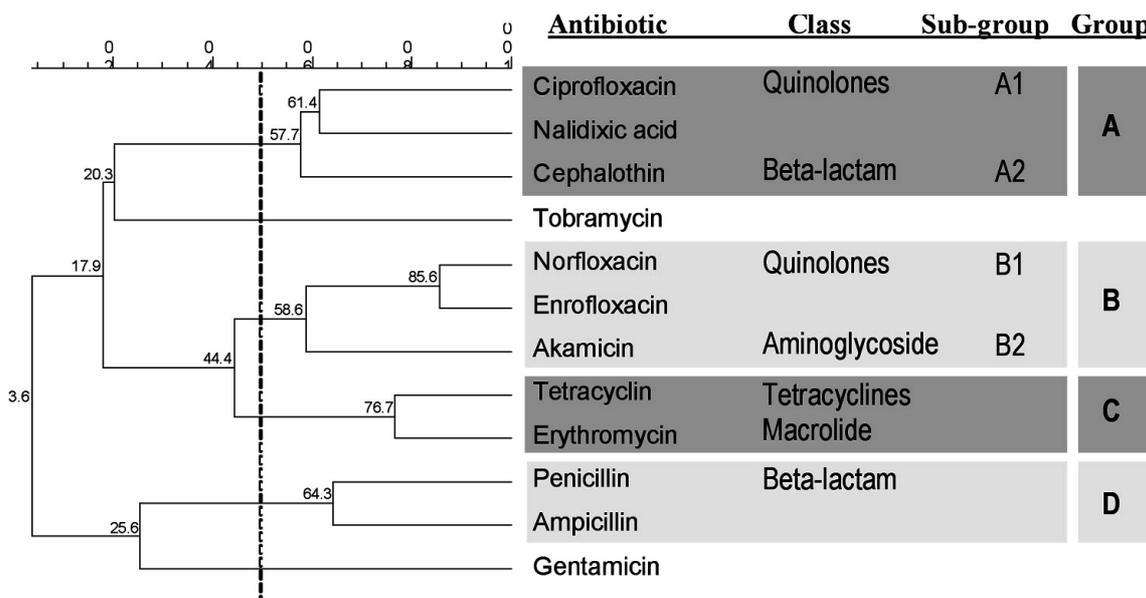


Figure 1: Dendrogram based on the clustering analysis of the resistance patterns of twelve antimicrobial agents tested against 56 isolates of *C. jejuni*

(Applied Maths, Kortrijk, Belgium) and analyzing for the Pearson correlation coefficient. Agglomerative clustering was performed with the unweighted average linkage (UPGMA).

RESULTS

A dendrogram (Figure 1) showing the clustering of 12 antimicrobial agents tested was obtained by the computed results of the resistance patterns of the 56 isolates of *C. jejuni* toward the 12 antibiotics used. The clustering of antimicrobial agents in the dendrogram demonstrated the correlation of the antibiotics from different classes and different modes of action. Using the cut off value at 50% similarity, 10 out of 12 antibiotics tested in this study correlated. Tobramycin and gentamicin correlated with antibiotics group A and antibiotics group D, respectively, at similarity level lower than 30%. Both tobramycin and gentamicin were grouped in single-member clusters and therefore failed to show any correlation with other antimicrobial agents tested in this study. Norfloxacin and enrofloxacin (group B1), ciprofloxacin and nalidixic acid (group A1) were correlated with each other at 85.6% and 61.4%, respectively. Both clusters were found to be members of the quinolones class. Another cluster with antibiotics from the same class was penicillin and ampicillin in group D (64.3%), the beta-lactam antibiotics.

The only cluster that did not belong to a single class of antibiotic was group C, which was occupied by tetracycline (tetracyclines) and erythromycin (macrolide), at similarity level as high as 76.6%.

Cluster analysis of 56 *C. jejuni* isolates using the software package of BioNumerics version 4.5 provided results in a dendrogram (Figure 2). Fifty-five out of 56 isolates could be observed as groupings into ten discriminatory clusters with a cut off value at 50% similarity. The remaining one isolate was grouped in a single-member cluster at 8.3% similarity with other clusters. Isolates from various locations and sources seemed to cluster together into nine clusters with only one exception, cluster C8, occupied only by isolates from supermarkets. Both dendrograms showing the clustering of the 12 antibiotics (Figure 1) and the 56 isolates of *C. jejuni* (Figure 2) were combined and summarized in Table 1. It was obvious from Table 1 that cluster C9 was resistant to the least group of antibiotics, being resistant toward only antibiotics in group D.

The *C. jejuni* isolates from raw vegetables which were consumed as *ulam* were highly multi-resistance to as many as 10 antibiotics (Figure 3). All isolates were found to be multi-resistance with minimum resistance to 2 antibiotics. About ninety one percent ((91.1%) of isolates were resistant to 3 or more antibiotics. Majority of the *C. jejuni* isolates (53.6%) were resistant to 6 to 8 antibiotics tested. A total of 14.3% of *C. jejuni* isolates

Table 1: Characterization of *Campylobacter jejuni* clusters defined in the hierarchic analysis performed with antibiotic resistant profile

Cluster	Similarity (%)	Locations		Antibiotics							
		SM	OM	A1	A2	B1	B2	C	D	TOB	CN
C1	62.2	6	1	-	-	+	+	+	+	-	-
C2	77.2	2	1	-	-	+	+	+	-	+	-
C3	79.6	10	3	-	+	+	+	+	-	-	-
C4	64.2	3	1	-	+	+	+	+	-	+	-
C5	100.0	1	1	-	+	+	+	+	+	-	+
C6	69.0	5	4	+	+	+	+	+	-	-	-
C7	72.7	2	2	+	+	+	+	+	-	+	-
C8	54.3	5	0	-	-	-	+	+	+	-	-
C9	77.5	2	2	-	-	-	-	-	+	-	-
C10	51.2	3	1	-	±	-	-	+	+	-	-

*Only antibiotics that were discriminatory are presented. A1, A2, B1, B2, C and D correspond to Figure 1. TOB, tobramycin; CN, gentamicin. +, when at least 60% of the isolates in the cluster behaved as resistant; -, when at least 60% of the isolates in the cluster behaved as susceptible; ±, when an equal numbers of isolates in the cluster behaved as resistant or susceptible. SM, supermarkets; OM, Outdoor market.

demonstrated multi-resistance toward more than 9 antibiotics tested in this study. Overall, the highest percentage of resistance was observed toward erythromycin (91.1%) and tetracycline (85.7%). About 80.4% of the *C. jejuni* isolates were resistant to enrofloxacin (Figure 3). Only 16 out of 56 *C. jejuni* isolates (28.6%) were resistant to ciprofloxacin (Figure 3). Most of the *C. jejuni* isolates were susceptible to tobramycin and gentamicin.

DISCUSSION

The emergence of bacterial resistance to antimicrobial agents is always related to exposure of bacteria to antibiotics available in the environment. However, the origin and spread of antibiotic resistance in a particular population is complex. It is well known that the spread of antibiotic resistance genes are due to mutations, transposition of the bacterial genome and genetic exchange between bacteria (O'Brien, 2002). Multi-drug resistance as observed in this study is a growing public concern. It is viewed as a consequence of resistance traits being linked and expressed as a unit (Clockaert and Schwarz, 2001). To understand this dynamics of resistance, these linked characteristics of resistance in *C. jejuni* were captured and described using cluster analysis.

The twelve antimicrobial agents investigated in this study were clustered into four groups by cluster analysis. Majority of the groups comprised

members of antimicrobial agents from different classes. The clustering of erythromycin and tetracycline in group C at similarity level as high as 76.6% suggested that the clustering may go according to the antimicrobials mechanism of action. Both erythromycin and tetracycline share the same mechanism of action (inhibition of protein synthesis). Elsewhere, Lopes *et al.* (2005) and Da Costa *et al.* (2006), who worked on antibiotic resistance in enterococci, reported on the grouping of erythromycin and tetracycline in their work and concluded that the clustering of antibiotics followed a functional pattern according to their mode of action. The clustering of cephalothin with ciprofloxacin and nalidixic acid (group A); akamicin with norfloxacin and enrofloxacin (group B) were observed in our study and this could neither be explained by clustering according to antimicrobial classes nor the mechanism of actions. The most probable explanation could be due to the occurrence of cross-resistance.

C. jejuni isolates investigated in this study were isolated in our previous work from raw salad vegetables (locally known as *ulam*) (Chai *et al.*, 2007). According to published reports, the major reservoir of *C. jejuni* is poultry (Harris *et al.*, 1986; Humphrey *et al.*, 1993). The prevalence of *Campylobacter* spp. in fresh produce is generally believed to be due to cross-contamination from other sources. To take into account for the possibility that the isolates may have originated from poultry, agriculturally used antimicrobial

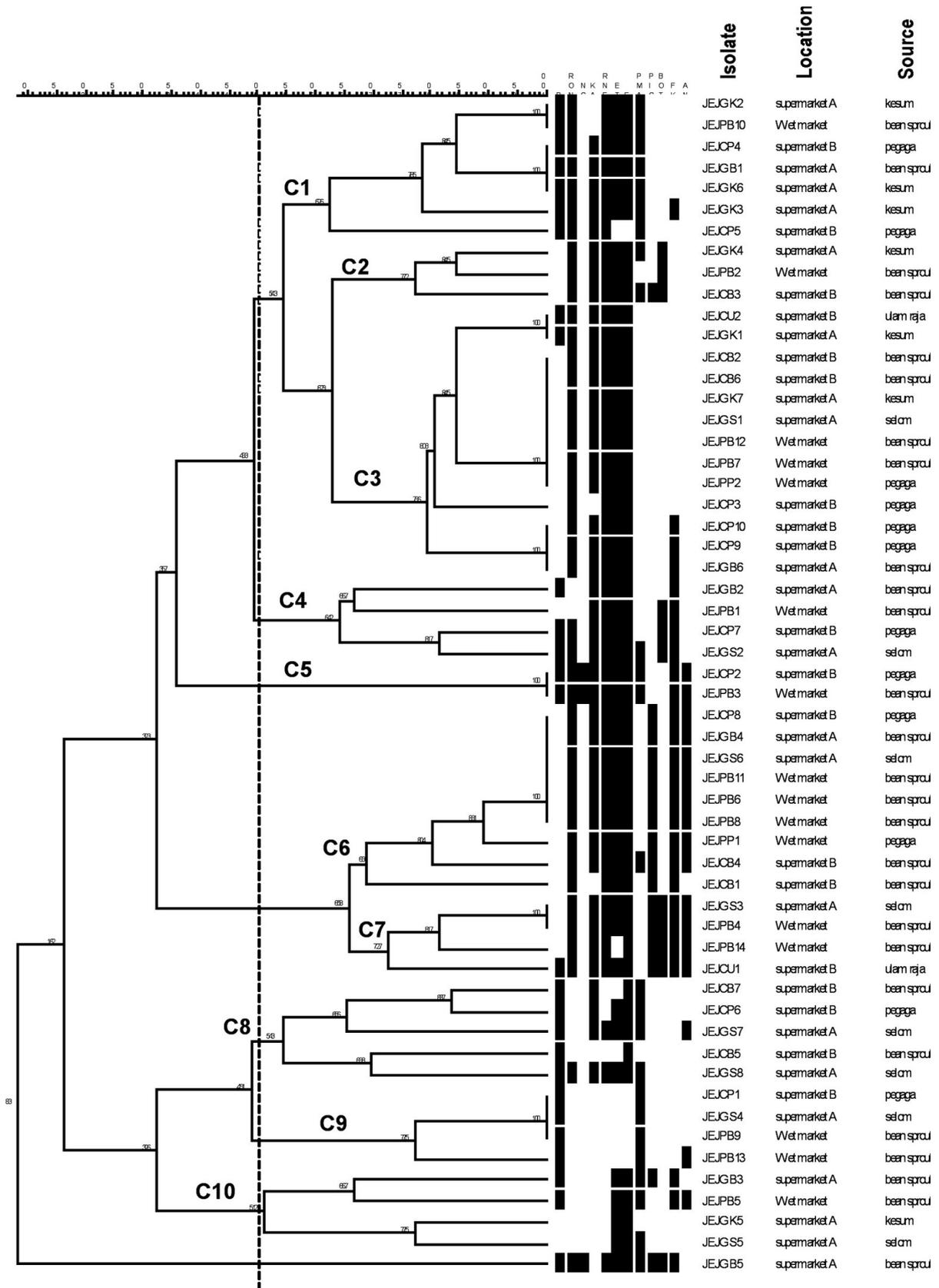


Figure 2: Dendrogram based on the hierarchic numerical analysis of the resistance profiles obtained for 56 *C. jejuni* isolates, employing the Pearson correlation coefficient and UPGMA for clustering

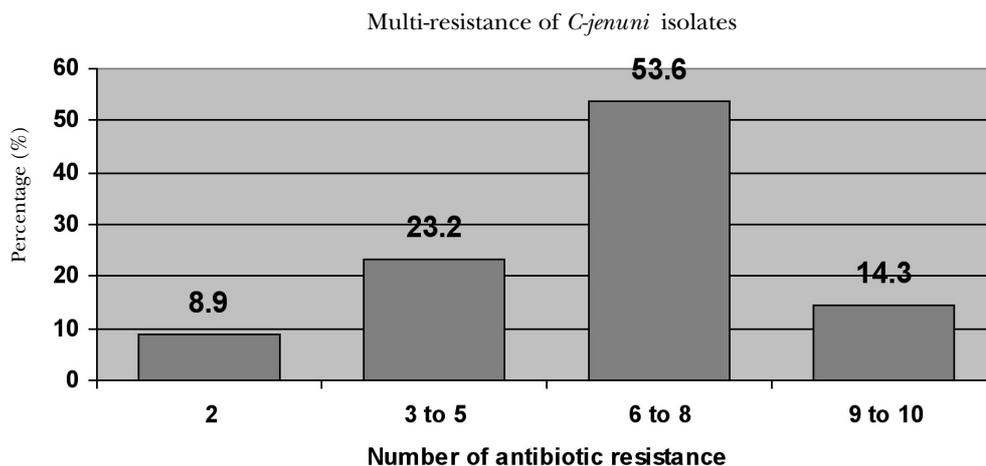


Figure 3: Resistance of *C. jejuni* isolates to number of antibiotics

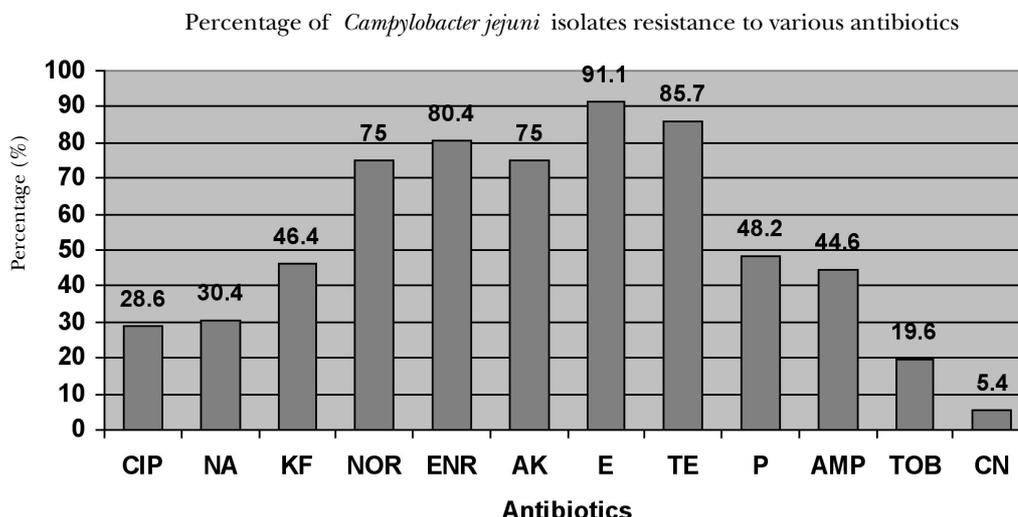


Figure 4: Resistance of *C. jejuni* isolates to number of antibiotics. CIP, ciprofloxacin; NA, nalidixic acid; KF, NOR, norfloxacin; ENR, enrofloxacin; AK, E, erythromycin; TE, tetracycline; P, penicillin; AMP, ampicillin; TOB, tobramycin; CN, gentamicin

agents especially in poultry farming were included in this study. Since 1980s, third generation quinolones such as ciprofloxacin and enrofloxacin have been used worldwide in agricultural practices (Endtz *et al.*, 1991; Jacobs-Reistma *et al.*, 1994; Hollinger *et al.*, 2000., Roissiter *et al.*, 2000). Enrofloxacin, which is commercially known as Baytril, is an extensively used antimicrobial agent in poultry farms to reduce vaccination problems and also mortality associated with *Escherichia coli* (Jacobs-Reistma *et al.*, 1994). It is limited for use in only veterinary and agricultural practices. However,

the increase in fluoroquinolones-resistance campylobacter has caused a ban on the use of fluoro-quinolones especially enrofloxacin and ciprofloxacin in the United States and Europe. In most developing countries including Malaysia, despite the banning of fluoro-quinolones application in agricultural practices in most countries, fluoroquinolones (norfloxacin and enrofloxacin) and tetracyclines are still widely used in farming practices. The control of usage of these antimicrobial agents in agricultural practices is very difficult since these antimicrobial agents are readily

available and consultation with the veterinarians before use is not compulsory. Therefore, it is believed that the abuse and misuse of antimicrobial agents in agricultural farming in Malaysia may be one of the factors that contribute to the extremely high resistance of *C. jejuni* toward enrofloxacin (80.4%) and tetracycline (85.7%).

The antimicrobial agents to which resistance was demonstrated most frequently were erythromycin (91.1%) and tetracycline (85.7%). The finding was considered very high when compared to other available published data. Rodrigo *et al.* (2007) recorded 26.8% of *Campylobacter* spp. resistant to erythromycin while Cabrita *et al.* (1992) found only 5.1% resistant to it. In Thailand, the neighboring country of Malaysia, 38.3% and 66.2% of *Campylobacter* spp. isolates from chicken, pig, dairy and human were found to be resistant to erythromycin and tetracycline, respectively (Padungtod *et al.*, 2006). However, the level of erythromycin and tetracycline resistance was not the highest as compared to other published reports on other bacterial species from Malaysia. Comparatively, high rate of erythromycin resistance had been observed in other species such as *Aeromonas* spp. (79%), *Enterococcus* spp. (100%), *Burkholderia pseudomallei* (100%), *Klebsiella pneumonia* (100%), *Salmonella* spp. (67%), *Vibrio vulnificus* (71%), *Escherichia coli* O157:H7 (30%), and others. (Radu *et al.*, 1998; 2000; 2001; 2001; 2003; Haryani *et al.*, 2007; Tunung *et al.*, 2007). A previous study by Radu *et al.* (2002) also showed a high rate of tetracycline resistance (77.76%) in *Vibrio Cholera* O1 outbreak strains in Malaysia. According to Saleha (2002), all the *C. jejuni* strains isolated from broiler farms were found to be resistant to tetracycline. Basically, the reported rates of both erythromycin and tetracycline resistance were higher compared to reports from other countries especially from developed countries. The controlled usage of these antimicrobial agents in agricultural practices as well as in hospitals might be a reasonable explanation to the comparatively lower rates of erythromycin and tetracycline in those countries. The application of tetracycline in agricultural practices had been banned in Denmark, the United States and other developed countries due to the increasing resistance toward this agent. The resistance of erythromycin is always of deep concern globally due to its application in treatment of human campylobacter gastroenteritis.

All the isolates in this study showed resistance to at least two antibiotics. This indicated that 100% of *C. jejuni* isolates in our study were multiresistant.

As many as 14.3% of isolates demonstrated resistance to at least 9 out of 12 antimicrobial agents tested in this study. Our finding was in concordant with a report from Saleha (2002) who also found a high level of multi-resistance (76.3%) in *C. jejuni* isolated from poultry farms in Malaysia. The finding in this study is not surprising as multi-resistance in pathogens is not a new issue in Malaysia. It has been reported quite frequently by other researchers in this region (Radu *et al.*, 2000; 2001; 2002; 2003; Saleha, 2002; Tunung *et al.*, 2007). The pattern of antibiotics usage in a certain geographic area is an important selective force in bacterial antibiotic resistant. The emergence of multi-resistance as observed in this study warrant attention as it poses risk to public health due to the problem in treating patients with bacterial infection at hospitals and the increasing economic burden on the country as more expensive drugs have to be used to combat the infection.

Cluster analysis can be used to assess epidemiological relationships between variables and outcomes. The clustering analysis of antibiotic resistance data using BioNumeric version 4.5 software package categorized *C. jejuni* isolates from different isolation locations and sources into ten clusters without any significant patterns. Fifty-five out of 56 isolates were clustered into ten clusters. The findings indicated that *C. jejuni* isolates from supermarkets and a local outdoor market were highly correlated to each other as shown in the dendrogram (Figure 2). None of the isolates demonstrated location specific or source specific based on resistance phenotypes. This might indicate that *C. jejuni* strains isolated from raw salad vegetables (ulam) from the supermarkets and the local outdoor market had been exposed to a similar source of cross-contamination along the production chain of such fresh produce. Table 1 was another summarized presentation form of the data to provide a better interpretation to show the similarity and difference between clusters from the dendrograms in Figures 1 and 2. As observed in Table 1, more than 80% of the 10 clusters demonstrated resistance to antibiotics in group B1 and group C. Both antibiotic groups showed the same mode of action: inhibition of protein synthesis. However, no conclusion could be made from the clustering in this study. Extensive studies on *C. jejuni* from various sources like clinical, environmental and poultry samples have to be included in the clustering analysis to come out with a conclusion on the correlation between the sources and antibiotics and the spreading of the resistance strains in this region.

In conclusion, this study showed an extremely high level of multi-resistant strains of *C. jejuni* to as many as ten antibiotics and was relatively higher compared to published data available. In terms of biosafety, the high resistance level to fluoroquinolones and macrolide (erythromycin) were of concern as they are the drugs of choice for treatment of campylobacteriosis. Whilst fluoroquinolone and macrolide resistance in campylobacter emerge globally, this study showed that fluoroquinolone and macrolide resistant campylobacter is a growing threat in this region.

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