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Antibiotic susceptibility and genotyping by RAPD of *Campylobacter jejuni* isolated from retailed ready-to-eat sushi

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Abstract: A study to determine the antibiotic sensitivity pattern and genotyping using RAPD-PCR was performed on 50 *C. jejuni* isolated from sushi retailed in different supermarkets. With less than half of the isolates susceptible to the antibiotics tested, resistant to two or more antibiotics were observed in most of the isolates. The banding patterns obtained from RAPD-PCR revealed that no predominant clone exists and the bacterial population is rather diverse. Hence, the resistance of the *C. jejuni* to different classes of antibiotic as well as their diverse genotypes suggests that these *C. jejuni* isolates were generated from different sources in the contaminated supermarkets where sushi were retailed. Our data showed that *C. jejuni* can be an important reservoir for resistance genes and that study with comprehensive collections of samples are urgently required to establish better measures to reduce or eliminate the risk from antibiotic resistant and pathogenic bacteria originating from minimally processed ready-to-eat food.

Keywords: *Campylobacter jejuni*, antimicrobial resistance, ready-to-eat food, RAPD, genotyping

Introduction

Campylobacter is a genus of Gram-negative, motile bacteria with a rod-like appearance (Snelling *et al*., 2005). Many species of *Campylobacter* have been implicated in human diseases, especially gasteoenteritis, with *C. jejuni* accounting for approximately 90% of the human isolate. Although most of the *Campylobacter* infections are self-limiting, sporadically a more serious illness can occur which require effective antimicrobial therapy (Jain *et al*., 2005; Andersen *et al*., 2006). Currently, macrolides and fluoroquinolones are the antimicrobials of choice for the treatment of life-threatening *Campylobacter* gastroenteritis (Lucey *et al*., 2002; Griggs *et al*., 2005). Taiwan and Spain have reported that approximately 10% of their *Campylobacter* strains were found to be resistant to macrolides (Neimann *et al*., 2003). Studies in Canada reported an erythromycin resistance rate of 12% (Dionisi *et al*., 2004; Rodriguez-Avial *et al*., 2006). The prevalence of these antibiotic resistant strains heightens the risk of treatment failure, posing a serious concern to public health (Corcoran *et al*., 2006).

Several factors may lead to the resistance of bacteria to antibiotic. Among them are the inappropriate uses of antimicrobials such as administering sub-therapeutic doses or early discontinuation of treatment (McDermott *et al*., 2002; Roe and Pillai, 2003). However, this is not the only reason to resistance (Gellin *et al*., 1989). There are many other reasons that influence antimicrobial resistance. These reasons vary among farms, depending on health status of the herd, farm management, and environmental factors (Van der Wolf *et al*., 1999; Regula *et al*., 2003; Schuppers *et al*., 2005).

The methods for genotyping *Campylobacter* species are limited because of the difficulty to obtain standard antisera and phage reagents, and the lack of standardized protocols. Recently, several genotyping methods have been described. One of them is the RAPD-PCR assay that is considered as one of the most cost effective method for the investigation of large numbers of isolates (Acik and Cetinkaya, 2005).

RAPD assay uses a single 10-mer primer (Madden *et al*., 1996; Ertas *et al*., 2004; Acik and Cetinkaya, 2005) that is able to anneal and prime to multiple

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locations randomly distributed throughout the whole genome. Therefore it can produce a spectrum of amplified products characteristic of the template DNA (Hilton *et al*., 1997). Thus, the entire genome is potentially accessible to priming and amplification, and polymorphisms can be detected. The objectives of this study are to determine the antibiotic resistant properties of *C. jejuni* isolates, and to genotype the *C. jejuni* isolates by using RAPD-PCR.

Materials and methods

Bacterial strains

This study included 50 isolates of *C. jejuni* isolated from five types of retailed ready to eat sushi from three different supermarkets in Kuala Lumpur, Malaysia from May to August 2007. The sushis selected were salmon sushi, fish roe sushi, octopus sushi, eel sushi, and omelette sushi. Only one isolate was successfully obtained from Supermarket I, 24 isolates were obtained from Supermarket II and 25 isolates from Supermarket III. All isolates were previously identified as *C. jejuni* as described previously (Tan *et al*., in press).

Antibiotic resistance test

Antibiotic resistance test was carried out using the Kirby-Bauer assay. The eight antibiotics chosen were ampicillin (AMP, 30 µg per disc), erythromycin (E, 15 µg per disc), enrofloxacin (ENR, 5 µg per disc), gentamicin (CN, 10 µg per disc), amikacin (AK, 30 µg per disc), ciprofloxacin (CIP, 5 µg per disc), nalidixic acid (NA, 30 µg per disc) and tetracycline (TE, 30 µg per disc) (Oxoid, England). The plates were incubated for 48 hours at 37°C under microaerophilic conditions by using Anerocult C system (Merck, Germany). After 48 hours, the diameter of the zone of inhibition for each antibiotic disc was measured and the sensitivity of the bacteria to each antibiotic was then determined

Genotyping by RAPD-PCR

DNA was extracted using boiled-cell method. 500 µl of the broth from the turbid tubes were centrifuged at 12,000 rpm for 3 min in order to pellet the bacterial cells. The supernatant was discarded and the pellet was then resuspended with 400 µl of sterile distilled water and boiled for 10 min followed by freezing in -20°C for 10 min. It was then centrifuged at 10,000 rpm for 5 min to pellet the cell debris. The supernatant was then kept for use in PCR. Four 10-mer primers (OPA-CAATCGCCGT, OPA 8- CCGCAGCCAA, OPA 10- GTGACGTAGG, OPA 11- GTGATCGCAG) from Research Biolabs, Singapore were used. PCR pre-mix (Intron, Korea) with 3.0 mM MgCl2 and 1.5 units of Taq polymerase were used. The final volume of 20 µl were subjected to PCR amplification with the initial denaturation of 94°C for 2 min, followed by 45 amplification cycles of denaturation at 94°C for 1 min, annealing at 32°C for 1 min, and extension at 72°C for 1 min, finishing with the final extension at 72°C for 5 min. All the PCR assays were performed with Veriti 96 well Thermal Cycler (Applied Biosystems, USA). The PCR amplicons were then ran on 2% agarose gel at 80V for 4 hours. The gel images were then analyzed using GelCompar II version 5.1 by Applied Maths, Belgium and a dendrogram of the 50 isolates of *C. jejuni* was constructed.

Results

The results of the antibiotic susceptibility testing are shown in Table 1 and 2. Even though 38% of the *C. jejuni* were susceptible to all antibiotic tested, the remaining isolates showed maximum resistant to ampicillin (62%) followed by erythromycin (54%), nalidixic acid (46%), enrofloxacin (8%), tetracycline (6%) and ciprofloxacin (4%). All 50 isolates studied were reported to be susceptible to both gentamicin and amikacin. Overall the *C. jejuni* isolates from supermarkets II and III showed a greater degree of

Table 1. Antibiotic resistance among 50 isolates of *C. jejuni* from different locations

Locations	Resistance to															
	NA		AMP		E		TE		CN		CIP		ENR		AK	
Supermarket I	-	-	1	2%	1	2%	-	-	-	-	-	-	-	-	-	-
Supermarket II	15	30%	18	36%	13	26%	-	-	-	-	-	-	-	-	-	-
Supermarket III	8	16%	12	24%	13	26%	3	6%	-	-	2	4%	4	8%	-	-
Total	23	46%	31	62%	27	54%	3	6%	-	-	2	4%	4	8%	-	-

NA = nalidixic acid, AMP = ampicillin, E = erythromycin, TE = tetracylcine, CN = gentamicin, CIP = ciprofloxacin, ENR = enrofloxacin, AK = amikacin

Table 2. Percentage of isolates with multiple antibiotics resistance

Strains	Multiple antibiotics resistance[a]	Percentage (%)
I131, I513, J422	E, AMP	6
I533	E, TE	2
C113, C413	NA, AMP	4
I522	NA, ENR	2
I532	CIP, ENR	2
I431, I512	TE, E, AMP	4
C111a, C111b, C111c, C112a, C112b, C112c, C121, C122, C132, C133, C222, C413, C532, I232, C223a, C223b, I422b, I423b	NA, E, AMP	36
I423a	NA, CIP, ENR	2
I322	NA, E, AMP, ENR	2

NA = nalidixic acid, AMP = ampicillin, E = erythromycin, TE = tetracylcine, CIP = ciprofloxacin, ENR = enrofloxacin, J = Supermarket I, C = Supermarket II, I = Supermarket III

[a] The isolates not listed in the table were susceptible to all antibiotics tested

resistance than those from supermarket I. According to Table 2, only a single isolate was resistant to four antibiotics, which were nalidixic acid, erythromycin, ampicillin, and enrofloxacin. Majority of the multi-resistant isolates displayed resistant to three different antibiotics, mostly the nalidixic acid-erythromycin-ampicillin combination (36%), with 15 of the 18 isolates originating from sushi samples in supermarket II.

The dendrogram shown in Figure 1 was constructed using GelCompar II version 5.1 by Applied Maths, Belgium with Pearson correlation and UPGMA clustering to determine the genetic relatedness of the 50 isolates. All 50 isolates had 100% typability in RAPD assay by all four primers used, generating a specific profile of DNA fragment which was unique among the isolates investigated. According to Figure 1, the dendrogram branched into four major clusters at a similarity level of 60%. Based on the clustering of the *C. jejuni* isolates, no predominant clone exist in the bacterial population obtained from sushi in the three different supermarkets as the isolates were clustering mainly according to the supermarket from where the sushi samples were obtained.

Discussion

Though none of the *C. jejuni* isolates were resistant to gentamicin and amikacin, more than half of the *C. jejuni* isolates examined in this study displayed resistance towards the other antibiotics tested. In contrast, an earlier report by Chai *et al*. (2008) showed that all the 56 *C. jejuni* isolated from raw salad retailed in several supermarkets displayed resistant to all 12 antibiotics tested. Until a few years ago, fluoroquinolones were the main antibiotics used for the treatment of *Campylobacter* infections. However, recent studies often reported resistance of *Campylobacter* spp. strains to quinolones (Han *et al*., 2007). Nalidixic acid, ciprofloxacin and enrofloxacin are the three quinolones tested in this study. Among the three quinolones tested, only nalidixic acid showed a significant effect to the *C. jejuni* isolates with 24% of them being resistant. This finding is lower than that of Han *et al*. (2007) who found a 92.2% resistance to nalidixic acid among isolates from retailed raw chickens in Korea, and Saenz *et al*. (2000) who found a 98.7% and 76.9% resistance to nalidixic acid from the isolates collected from broilers and foods, respectively, in Spain. On the other hand, this finding is higher than the report of Ishihara *et al*. (2004) who reported only 10.2% resistance against nalidixic acid among the isolates of food producing animals in farms in Japan.

Only 4% of the isolates were resistant to enrofloxacin in this study. This finding is consistent with the report of Ishihara *et al*. (2004) who found a 9.9% of resistance among isolates collected from food producing animals. However, Pezzotti *et al*. (2003) found a rather high resistance pattern of *C. jejuni* isolates towards enrofloxacin, which were 42.2% from broilers isolates, 36.2% from pig isolates, 25% from beef cattle isolates and 38.2% from human

isolates. None of the isolates in this study were found to be resistant to ciprofloxacin. This was unexpected as various reports often reported high resistant patterns of *C. jejuni* towards this antibiotic. Kassa *et al*. (2007) reported a 100% resistance of the isolates collected from food producing animal in Ethiopia, Han *et al*. (2007) who reported a 92.2% resistance in Korea, and Rodrigo *et al*. (2007) reported a 86.6% of resistance in Trinidad.

An epidemiological study from Australia and New Zealand reported the increasing trend of nalidixic acid resistance from 5.7% to 41%, and ciprofloxacin from 1.4% to 29% since 1998 (Moore *et al*., 2006). Higher numbers of antibiotic resistant Campylobacters were found in developing countries where the use of antibiotics for humans and animals is relatively unrestricted (Fields and Swerdlow, 1999). Several other epidemiological studies have indicated that there is a connection between the use of fluoroquinolones in veterinary medicine, especially for poultry, and the increasing percentage of quinolone-resistant *Campylobacter* species (Smith *et al*., 1999; Luber *et al*., 2003; Jain *et al*., 2005). It has been reported that *Campylobacter* can develop to be resistant to fluoroquinolones in vivo even after one or two administration of the drugs (Adler *et al*., 1999).

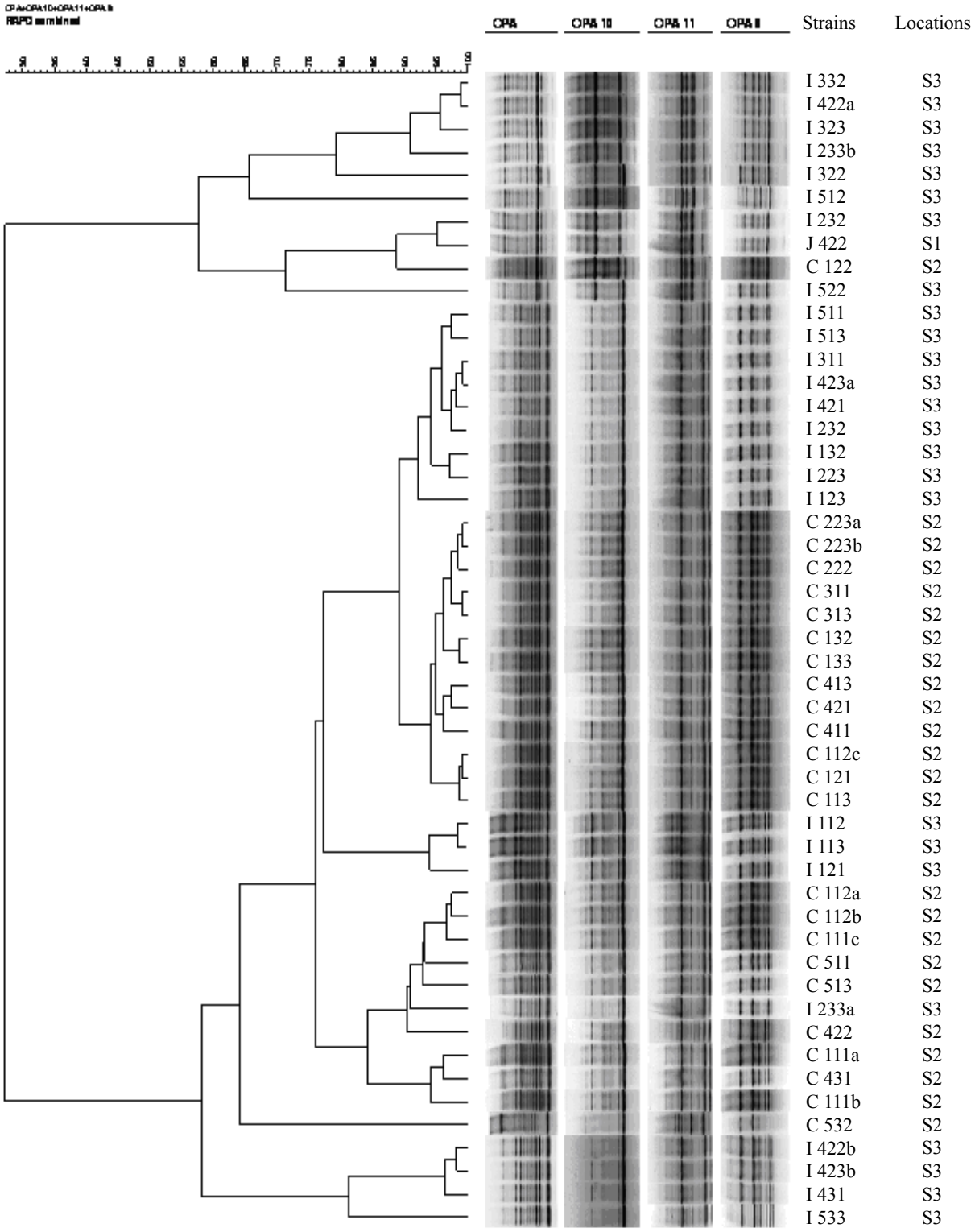
Another antibiotic tested in this study is erythromycin from the macrolide group. It was found that 52% of the *C. jejuni* isolates collected in this study were resistant to erythromycin. This is higher than the results obtained by Saleha (2002) who found a 23.7% resistance among isolates collected from broilers chicken in Malaysia. Other studies with similar results to Saleha (2002) were Rodrigo *et al*. (2007) and Olah *et al*. (2004) who reported a resistance of 26.8% and 20%, respectively. Saenz *et al*. (2000) reported a lower resistance pattern of *C. jejuni* isolates from food of 12.2%. Other studies found very low resistance among isolates towards erythromycin. Pezzoti *et al*. (2003) found a resistance among isolates that ranged from 1.4% to 8.3% from various samples whereas Luber *et al*. (2003) reported a range from 0% to 3.8%. Reports from Ishihara *et al*. (2004) and Han *et al*. (2007) found 0% resistance among the isolates collected. The erythromycin resistance is of great concern because it is considered as the optimal drug used in the treatment of human gastroenteritis (Allos, 2001; Olah *et al*., 2004; Rodrigo *et al*., 2007). The connection of the usage of this drug in the poultry industry in feed preparation to the detection of erythromycin-resistant strains of *C. jejuni* poses therapeutic problems in broiler-borne gastroenteritis in humans (Cabrita *et al*., 1992).

Another group of antibiotic studied was the aminoglycosides which include gentamicin and amikacin. Results show that all isolates of *C. jejuni* were susceptible to these antibiotics. This finding was expected due to the fact that various studies also reported similar results. Most of the studies reported that *C. jejuni* was susceptible to gentamicin (Luber *et al*., 2003; Ishihara *et al*., 2004; Andersen *et al*., 2006; Han *et al*., 2007; Little *et al*., 2008). In contrast, some studies reported a low resistance pattern of *C. jejuni* isolates towards gentamicin. A study carried out in Spain by Saenz *et al*. (2000) reported resistance in the range of 0.4% to 11.9%, whereas Kassa *et al*. (2007) reported a 0.7% resistance. The low level of resistance may be due to the fact that gentamicin is rarely used in the poultry industry either prophylactically or therapeutically, and its intramuscular route of administration may be impracticable for the large scale application in production farm (Rodrigo *et al*., 2007). Therefore, different studies have reported that gentamicin is an effective drug for the treatment of *Campylobacter* enteritis in human (Velazquez *et al*., 1995; Aarestrup *et al*., 1997; Li *et al*., 1998). Furthermore, in Japan, gentamicin has been approved for the treatment of cattle and pigs (Ishihara *et al*., 2004). Limited reports were found on the study of resistance of amikacin among *C. jejuni* isolates. According to Saenz *et al*. (2000), isolates collected from animal, food, and human showed a resistance pattern that ranged from 0% to 2.4%, which is consistent with this study.

Other antibiotics tested in this study were ampicillin and tetracycline. In this study, 50% of the isolates were resistant to ampicillin. This is consistent with the results of Han *et al*. (2007) who found that 43.1% of the isolates tested to be resistant, and Saenz *et al*. (2000) who found the resistance of isolates collected from broiler, food and human to be 47.4%, 40%, and 38%, respectively. In contrast, Luber *et al*. (2003) revealed a resistance in the range of 8.8% to 38.7% among isolates of chicken, turkey and human. Kassa *et al*. (2007) found 17% of food animal isolates to be resistant to ampicillin.

The resistance of isolates towards tetracycline in this study was not significant, with only 6% of resistant isolates reported. The resistance to tetracycline observed is much lower than that reported by Pezzotti *et al*. (2003) who found a resistance of 8.3%, 25% and 30.9% among isolates obtained from beef cattle, broiler and human, respectively. Other studies which

Figure 1. Dendrogram constructed based on RAPD with 4 primers (OPA, OPA 8, OPA 10 and OPA11), S1: Supermarket I, S2: Supermarket II, S3: Supermarket III



reported a high resistance pattern towards tetracycline are Saenz *et al*. (2000) who reported 31.8% resistance among broiler isolates and 65% among food isolates, and Han *et al*. (2007) who reported 99.1% resistance among raw retailed chicken isolates. In contrast, Kassa *et al*. (2007) reported a lower resistance of 1.5% among food animal isolates.

Genotyping using RAPD method was also performed on the 50 isolates of *C. jejuni* collected. There are quite a number of other genotyping methods available such as the PFGE and RFLP. However, they are more labor intensive (Jana *et al*., 2003). Therefore, RAPD was chosen in this study, because it is relatively simpler and rapid (Hernandez *et al*., 1995; Jana *et al*., 2003). Computational analysis of band patterns using GelCompar II program from Applied Maths, Belgium was carried out rather than visual analysis by naked eyes, in order to maintain the consistency of technique and comparison of data (Wassenaar and Newell, 2000).

The 50 isolates of *C. jejuni* collected from retailed sushi were divided into four main clusters (Figure 1). This suggests that the isolates studied are not as diverse as reported; however, more isolates should be investigated in the future. Misawa *et al*. (2000) and Ertas *et al*. (2004) also found low heterogeneity of *Campylobacter* spp. in their studies of isolates from zoo animals in Japan and commercial broiler flocks in Turkey. In this study, clustering based on location was observed for the isolates of *C. jejuni*. Cluster A comprised of isolates from Supermarket III, while cluster B contained a mixture of isolates from all three supermarkets. Cluster C contained a mixture of isolates from Supermarket II and III. All the isolates in sub-clusters C1 and C3 were from Supermarket III whereas all the isolates in sub-cluster C2 were from Supermarket II. Sub-cluster C4 contained isolate from Supermarket III whereas all isolates in cluster D were from supermarket III. However, it is difficult to conclude whether the isolates collected from different locations shared a same source of contamination sources due to the limited numbers of isolates in this study. More isolates are needed to provide a more detailed analysis on the contamination trend.

The high percentage of resistance to erythromycin is of great concern as this antibiotic is thought to be the ideal drug of choice to treat *Campylobacter* enteritis in humans. The RAPD genotyping used in this study also provides a discriminatory and rapid means of comparing *C. jejuni* isolates, and such data are valuable in the epidemiological surveillance and in the investigation of the distribution of strains in different environments.

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