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Isolation and Characterization of *Campylobacter jejuni* from Broiler Chickens in Malaysia

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Abstract: Very few studies on *Campylobacter* in chickens were done in Malaysia. This study was carried out to determine the prevalence of *Campylobacter* in broiler chickens and to characterize the isolates to species level, their antibiotic resistance patterns and plasmid profiles. Ten broiler chicken farms were studied. The chickens in nine of the farms were found colonized with *campylobacters*; the prevalence rates ranged from 46.3 to 93.3%, with a mean of 72.6%; one farm was negative. *Campylobacter jejuni* was the predominant species. Antibiotic susceptibility test revealed that all the 76 *C. jejuni* isolates tested were resistant to tetracycline; 82.9, 55.3 and 34.2% were resistant to streptomycin, kanamycin and ampicillin, respectively while resistance to gentamicin, erythromycin and chloramphenicol ranged from 26.3 to 22.4%. It was also found that 22.4% of the isolates were resistant to one antibiotic while 13.1% to all seven antibiotics. 59% of *C. jejuni* isolates harboured between one to four plasmids, demonstrating 14 plasmid profiles with size of the plasmids ranged from 4.5 to 70.3 kb. More isolates containing plasmids were observed to be resistant to tetracycline, chloramphenicol and kanamycin than those isolates without plasmids. The numbers of plasmid bands present were not consistent with the antibiotic resistance patterns.

Key Words: *Campylobacter*, broiler chickens, antibiotic resistance, plasmid analysis

Introduction

Several studies have shown that poultry, in particular chicken, is the major source of *Campylobacter* and chicken meat is predominantly associated with *Campylobacter* infection in man (Harris *et al.,* 1986; Humprey *et al.,* 1993). *Campylobacter jejuni* is the most frequently isolated species from poultry and *C. coli* is less commonly isolated. *Campylobacter jejuni* affects poultry often with high counts of 10^6 to 10^8 cfu/g of faeces (Grant *et al*., 1980; Jacobs-Reitsma, 1992). The colonization of broiler flocks with campylobacters is dependent mostly upon the introduction of the organisms to the flocks. Regardless of the source of the organisms, once *C. jejuni* appeared in a flock, most of the chickens in the flock becomes rapidly colonized (Lindblom *et al*., 1986; Genigeorgis *et al*., 1986) with eventual 100% isolation rates, either before or at slaughter (Genigeorgis *et al*., 1986).

The occurrence of resistance to several antibiotics in *Campylobacter* and that some strains possess plasmid DNA has been reported. The presence of antibiotic-resistant campylobacters in chickens may lead to their presence on chicken carcasses and their products.

The present study was conducted:

i to determine the prevalence of *Campylobacter* spp. in broiler chickens in a state of Selangor in Malaysia,
ii to determine the antibiotic resistance patterns of *C. jejuni*
iii to determine the presence of plasmid DNA of the *C. jejuni* isolates and
iv to seek correlation between antibiotic resistance and plasmid carriage among these isolates.

Materials and Methods

Sampling Procedures: Ten broiler farms situated in five different localities in Selangor were selected. Five of the farms (farms A-E) were considered as large farms with 4-6 broiler houses with between 12,000 - 25,000 birds and the other five farms (farms F-J) were small farms with 1-2 houses and raised about 5000 birds or less. A total of seventeen flocks was chosen. In each flock, cloacal samples were taken randomly from 25-30 chickens by means of sterilized cotton swabs. Thus, a total of 508 chickens were sampled.

Isolation and Identification Procedures: Each cloacal swab was streaked directly onto plates of *Campylobacter* Blood Free Selective agar (Oxoid) which was supplemented with CCDA Selective Supplement (Oxoid) containing cefoperazone (32 mg/l) and amphotericin B (10 mg/l). All the streaked plates were incubated at 42 °C for 48 h under microaerobic condition which was achieved by placing the plates in anaerobic jar together with a gas pack, Campy Pak Plus (BBL).

After incubation, plates were examined for *Campylobacter* as follows: wet mount preparation of each suspect colony was examined under phase contrast microscope for characteristic morphology and corkscrew-like motility. Isolates with characteristic morphology and motility were then tested for oxidase, catalase and ability to hydrolyse indoxyl acetate. Presumptive *Campylobacter* colonies were transferred onto Columbia Blood agar (Oxoid) plates and streaked to ensure purity. The plates were incubated at 37 °C for 24 h under microaerobic condition. The colonies typical of *Campylobacter* were subjected to the following tests based on the scheme of Skirrow *et al*. (1982): hippurate hydrolysis, H_2S production in FBP medium and susceptibility to nalidixic acid. The scheme differentiated *Campylobacter* into three species, namely *C. jejuni, C. coli* and *C. lari*.

Antibiotic Susceptibility Test: The antibiotic sensitivity patterns for 76 *C. jejuni* isolates were determined using the disc diffusion method. Mueller-Hinton agar (Oxoid) was supplemented with Campylobacter Growth Supplement (Oxoid). The following antibiotic discs were used: ampicillin (10 ug), chloramphenicol (30 ug), erythromycin (15 ug), gentamicin (10 ug), kanamycin (30 ug), streptomycin (10 ug) and tetracycline (30 ug).

Analysis for Plasmid DNA Profile: Seventy one *C. jejuni* isolates were examined for the presence of plasmid DNA. The method of Close and Rodriguez with slight modifications by Ansary and Radu (1992) was used for plasmid isolation. Agarose gel precipitation was used to resolve the extracted plasmids; a horizontal 0.75% agarose gel in Tris-Borate-EDTA (TBE) buffer was used. *Escherichia coli* strain V517 containing seven plasmids, of sizes 54.0, 7.3, 5.6, 5.1, 3.9, 3.0 and 2.7 kb, was used as a standard marker for molecular weight. The electrophoresis was run for 2 h, the gel was then stained with ethidium bromide, visualised on a 302 nm UV transilluminator and photographed.

Results

Prevalence of *Campylobacter* in broiler chickens: The prevalence of campylobacters in the ten broiler chicken farms ranged from 0 to 98.2%, with a mean of 72.6% (Table 1). In seven farms, the proportion of *Campylobacter*-positive birds was more than 50.0%. One farm was found negative for *Campylobacter*.

Upon biotyping the *Campylobacter* isolates, 73.2% were *C. jejuni* and the remaining 26.8% were *C. coli*. *Campylobacter lari* was not isolated.

Prevalence and patterns of antibiotic resistance in *C. jejuni* isolstes: All the 76 *C. jejuni* isolates were found to be resistant to at least one of the seven antibiotics. Resistance to a single antibiotic was seen in 18 (23.7%) of the resistant isolates whereas multiple resistance (to two or more antibiotics) was observed in 58 (76.3%) isolates. Ten (13.1%) isolates were resistant to all seven antibiotics (Table 3). All (100%) of the *C. jejuni* isolates were resistant to tetracycline and the least resistance was to erythromycin (23.7%). The percentage of *C. jejuni* to each of the seven antibiotics is also shown in Table 3.

Plasmid occurrence and profiles of *C. jejuni* isolates : Approximately 59.0% of the *C. jejuni* isolates harboured plasmids, with sizes ranging from 4.5 to 70.3 kb. Fifty-five percent (55.0%) of these isolates possessed a single plasmid and the other 45.0% had two to four plasmids. Table 4 shows the antibiotic resistance patterns and the plasmid profiles of the 71 *C. jejuni* isolates that were studied. Overall, fourteen different plasmid profiles were observed – 23.3; 61.3; 65.8; 66.0; 68.9; 70.3; 27.5, 12,6; 68.9, 5.0; 61.3, 11.5; 66.0,6.5,4.5; 68.9, 27.2, 5.0; 61.3, 27.2, 11.5; 70.3, 14.6, 5.0; and 50.0, 23.3, 14.6, 6.0 kb.

Correlation between antibiotic resistance and plasmid carriage: The resistance to some antibiotics are plasmid-, or chromosome- mediated or both. In *C. jejuni*, it was reported that the resistance to chloramphenicol, kanamycin and tetracycline were plasmid-mediated. Thus, the plasmid distributions among *C. jejuni* isolates resistant to chloramphenicol, kanamycin and tetracycline were examined and compared. Table 2 showed that 59.2 to 66.7% of *C. jejuni* resistant to the three antibiotics harboured plasmids.

Table 1: The prevalence of *Campylobacter* species in broiler chickens in ten farms

Farms	No. of samples	No. positives	Percentage of positives	*C. jejuni* (%)	C. coli (%)
A	90	66	73.3	50.0	50.0
B	55	54	98.2	59.3	40.7
C	34	21	61.8	71.4	28.6
D	90	63	70.3	92.1	7.9
E	60	56	93.3	100	0
F	25	12	46.3	66.7	33.3
G	34	27	79.4	70.4	29.6
H	30	0	0	0	0
I	30	14	46.7	78.6	21.4
J	60	56	93.3	57.1	43.9
Total	508	369	72.6	73.2	26.8

Table 2: Resistance to chloramphenicol, kanamycin and tetracycline in *C. jejuni* isolates with and without plasmids

Antibiotics	Resistant With Plasmids	Isolates Without Plasmids
Chloramphenicol	10 (66.7%)	5 (33.3%)
Kanamycin	24 (60.0%)	16 (40.0%)
Tetracycline	42 (59.2%)	29 (40.8%)

Discussion

Very few studies on the occurrence of *Campylobacter* in chickens have been carried out in Malaysia compared to *Salmonella*. One study has reported the prevalence of *Campylobacter* spp. was 97.1% in broiler chickens and 51.5% in village chickens (Zeenathul, 1994) and in another, *Campylobacter* spp. was isolated in 81.9% village chickens (Saleha *et al*., 1996). In this present study, it was found that 72.6% of the broiler chickens were colonized by *Campylobacter*. Among these *Campylobacter* spp., *C. jejuni* (73.2%) was more frequently isolated than *C. coli* (26.8%). Aho and Hirn (1988) tabled the prevalence of campylobacters in several developed and developing countries which ranged from 6% in Sweden to 100% in Italy. Other studies also showed that *C. jejuni* is more commonly found in chickens (Humprey *et al*., 1993; Aho and Hirn,1988) compared to other *Campylobacter* species.

There is a concern that there may be a link among antibiotic use in feeds, the development and presence of antibiotic resistance among bacteria in food-producing animals and antibiotic-associated bacterial infection in humans (Bradbury and Munroe, 1985). Today, in the Report of the Joint Expert Advisory Committee on Antibiotic Resistance or JETACAR in Australia (1999), the committee agreed that there was evidence for the emergence of resistant bacteria in human and animal following antibiotic use, the spread of resistant bacteria from animal to human, the transfer of antibiotic-resistant genes from bacteria in animal to human pathogens and that strains of resistant bacteria which are zoonotic can cause disease in human.

All the isolates in this study showed resistance to at least one antibiotic, namely tetracycline and 13.1% were resistance to all the seven antibiotics. Overall, *C. jejuni* isolates in this study showed high resistance to a number of antibiotics, ranging from 23.7 to 100%, compared to *C. jejuni* isolates in other countries, such as Portugal where the antibiotic resistance of *C. jejuni* from chickens to six antibiotics ranged between 0 to 5.1% (Cabrita *et al*., 1992) and in Sweden, the overall resistance rates to four antibiotics were 0.5 to 4.5% (Berndtson *et al*., 1996). This high antibiotic resistance rates could be due to the widespread use of antibiotics in chickens, particularly in feed, as well as due to being use indiscriminately. Also, there are evidence to indicate that tetracycline survives longer in the environment than do other antibiotics which maybe critical in maintaining the level of tetracycline resistance at a high level (Frost, 1991). The low antimicrobial resistance among *C. jejuni* isolated from chickens was probably the result of restrictive use of antibiotics in chicken production (Berndtson *et al*., 1996). Mamber and Katz (1985) reported that the antimicrobial-resistant aerobic and facultative gram-negative enteric bacilli in chicken intestinal tracts are not necessarily selected for by antimicrobial supplementation of the feed, but rather on their common presence in the environments from which they can colonize the intestinal tracts of newly hatched chicks. These resistant enteric bacilli proliferate in the intestine and may transfer their resistance to campylobacters.

Most cases of *Campylobacter* enteritis do not require antibiotic treatment. However, in many cases which require treatment, erythromycin has been the most effective and commonly used for such treatment. In this study, about 24.0% of the isolates were found resistant to erythromycin; other studies also found erythromycin resistance in *C. jejuni* and the rates were higher in *C. coli* isolates (Engberg *et al*., 2001). To prevent further development of resistance, since July 1999, the European Union

Table 3: Resistance of *C. jejuni* isolates to number and type of antibiotics

	% of isolates resistant to No. of antibiotics						
No. of antibiotics	1	2	3	4	5	6	7
	23.7	14.5	25.0	6.6	11.8	5.3	13.1
	% of isolates resistant to type of antibiotics						
Types of antibiotics	Ac	Cm	Em	Gm	Kn	Sm	Tc
	34.2	22.4	23.7	26.3	55.3	82.9	100.0

Table 4: Antibiotic resistance patterns and plasmid profiles of *C. jejuni* isolates

Isolate	Antibiotic resistance patterns	Plasmids No.	Plasmids size (kb)
A 2/13	AcGmKnSmTc	3	66.0, 6.5, 4.5
A 2/11	KnSmTc	1	61.3
A 2/5	AcCmEmGmKnSmTc	1	61.3
A 3/8	AcGmKnSmTc	3	66.0, 6.5, 4.5
A 3/9	SmTc	1	61.3
A 3/12	KnSmTc	1	61.3
B 1/14	KnSmTc	0*	
B 2/22	CmEmKnSmTc	1	66.0
B 1/5	AcCmEmGmKnSmTc	0	
B 1/7	AcCmEmGmKnSmTc	0	
B 2/7	GmKnSmTc	0	
B 2/9	AcCmEmGmKnSmTc	0	
C 1/9	AcGmGmKnSmTc	1	65.8
C 1/17	AcCmEmGmKnSmTc	0	
C 1/1	AcEmSmTc	1	70.3
C 1/6	AcGmKnSmTc	0	
C 1/11	AcKnSmTc	1	65.8
C !/14	AcSmTc	1	65.8
D 4/7	KnSmTc	3	68.9, 27.2, 5.0
D 3/7	SmTc	3	68.9, 27.2, 5.0
D 3/2	Tc	1	68.9
D 2/1	SmTc	2	68.9, 5.0
D 4/14	SmTc	1	68.9
D 3/1	Tc	1	68.9
D 1/5	Tc	1	68.9
D 2/7	SmTc	0	
D 2/3	CmKnSmTc	0	
D 2/4	SmTc	0	
D 2/5	SmTc	0	
D 2/2	SmTc	1	68.9
E 1/3	Tc	1	61.3
E 1/11	Tc	0	
E 1/13	Tc	0	
E 1/12	Tc	2	27.5,12.6
E 1/25	Tc	0	
E 1/15	Tc	0	
E 1/23	AcCmEmGmKnSmTc	4	50.0,23.3,14.6,6.0
E 1/29	Tc	0	
E 1/30	SmTc	0	
E 1/16	Tc	0	
E 1/17	Tc	0	
E 1/8	Tc	1	23.3
G 2/17	KnSmTc	0	
G 2/12	KnSmTc	0	
G 2/8	KnSmTc	0	
G 2/7	KnSmTc	0	
G 1/5	KnSmTc	0	
G 1/7	Tc	0	
G 1/1	KnSmTc	0	
G 2/15	KnSmTc	0	
G 2/11	Tc	1	61.3
G 2/1	Tc	1	61.3
G 2/5	Tc	0	
G 1/10	KnSmTc	1	61.3
I 1/1	KnSmTc	3	70.3, 14.6, 5.0
I 1/3	KnSmTc	3	70.3, 14.6, 5.0
I 1/14	KnSmTc	3	70.3, 14.6, 5.0
I 1/10	KnSmTc	3	70.3, 14.6, 5.0
J ½	SmTc	3	61.3, 27.2, 11.5
J 1/10	AcGmKnSmTc	3	61.3, 27.2, 11.5
J 1/14	AcSmTc	1	61.3
J 1/15	AcCmEmGmKnSmTc	1	61.3
J 1/12	AcCmEmGmKnSmTc	3	61.3, 27.2, 11.5
J 1/7	AcCmEmGmKnSmTc	3	61.3, 27.2, 11.5
J 4/2	AcCmGmKnSmTc	1	61.3
J 4/6	AcEmKnSmTc	3	61.3, 27.2, 11.5
J 4/1	AcCmEmSmTc	3	61.3, 27.2, 11.5
J 1/1	AcCmEmGmKnSmTc	1	61.3
J 1/5	EmGmKnSmTc	2	61.3, 11.5
J 4/3	AcEmKnTc	0	
J 4/12	AcCmEmKnSmTc	3	61.3, 27.2, 11.5

* 0 – no plasmid

Contd.:

has banned the use of macrolides for growth promotion (Engberg *et al*., 2001).

The finding of plasmids in only 59% of the resistant isolates suggests that antibiotic resistance in some of these isolates may have been chromosome-mediated. Lee *et al*. (1994) found a high percentage (91%) of *C. jejuni* isolates harboured plasmids with the size ranged from 16 to 208 kb. The resistance to tetracycline and chloramphenicol in *C. jejuni* is reported as plasmid mediated, resistance to kanamycin is both plasmid and transposon mediated while resistance to streptomycin, erythromycin, ampicillin and nalidixic acid is chromosome encoded (Taylor and Courvalin, 1988). The correlation between tetracycline resistance and the presence of plasmids in *C. jejuni* has been quite extensively studied and Tenover *et al*. (1985) reported that all tetracycline-resistant strains harboured plasmids. However, in this study it was found that five isolates which were resistant to tetracycline did not harbour plasmids. This finding agreed with that of Sagara *et al*. (1987), Bradbury and Munroe (1985) and Lee *et al*. (1994) who found tetracycline-resistant *C. jejuni* without plasmids. The hybridization study by Lee *et al*. (1994) found that *C. jejuni* from chickens carried a tetracycline resistance determinant on plasmid (87%) or chromosome (11%) and that a 61 kb plasmid was usually associated with tetracycline resistance.

The study also revealed that there is no consistent relationship between antibiotic resistance pattern and the number of plasmid bands present – an example, two isolates of *C. jejuni* found

resistant to seven antibiotics had three plasmids while another had only one plasmid; also, there were seven isolates that were resistant to three antibiotics but contained no plasmid. Probably, the antibiotic resistance in those isolates which did not possess plasmid was associated with chromosome and / or transposons instead of being plasmid mediated.

There may some degree of plasmid relatedness among the *C. jejuni* isolates from the various farms studied because of the presence of similar size plasmids. This was shown by the *C. jejuni* isolates in four of the farms which had plasmids of the same size, 61.3 kb plasmid.

Overall, the study found that the broiler chickens were commonly colonized by *C. jejuni* and that the organisms were found resistance to at least one antibiotic, namely tetracycline and a small percentage to seven antibiotics. Also, the presence of plasmid DNA in some of the antibiotic resistant *C. jejuni* isolates demonstrated that such resistance was mediated by plasmids.

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