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Original article

ISOLATION AND ANTIMICROBIAL RESISTANCE OF *CAMPYLOBACTER* SPP. FROM CHICKEN FAECAL SAMPLES IN KHON KAEN AND NEARBY PROVINCE OF THAILAND

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Abstract Among 567 faecal samples collected from 2 broiler companies during January to April, 2003, 240 samples were positive for *Campylobacter* spp. and the overall incidence rate was 42.33%. Old age broilers (aged 40 ± 5 days) in company A were found heavily contaminated (57.50-97.50%) and 2.50-90.50% were infected in company B. Minimum inhibitory concentration (MIC) determinations were conducted on 5 drugs for human medicine namely azithromycin (AZ), doxycycline (DC), ciprofloxacin (CI), chloramphenicol (CL) and ceftazidime (TZ), by E-test strips. Among *Campylobacter* isolates tested, there were 52 *C. jejuni* isolates, 12 C. coli isolates, 16 *Campylobacter* spp. isolates, and 1 isolate of mix-contamination (Cj/Cc), respectively. AZ resistance (≥2 μg/ml as resistance) is minimal i.e. 6.94% (5/72). In addition, 90.54% (67/74) of the isolates tested were susceptible and only 9.46% of the isolates tested were found resistance (≥ 4 μg/ml) to DC. More resistant of *Campylobacter* spp. was found in the case of CI i.e. 30.67% (23/75). None of the isolates tested were resistant to neither CL nor TZ (≥ 32 μg/ml as resistance, both). Moreover, co - resistant to DC-CI and AZ-CI were encountered in 3 isolates and 1 isolate from one farm in Mahasarakam and Khon Kaen province, respectively. Farm in Mahasarakam province had highest co - resistant isolates. This study noted that CI resistance was significantly increased ($p < 0.001$) with the age of broilers.**Chiang Mai Veterinary Journal 2009;7(2):115-124.**

Keywords: *Campylobacter* spp., E-test, minimum inhibitory concentration (MIC), multiplex PCR

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Introduction

High prevalence of *Campylobacter* spp. in poultry products had been reported in developing and developed countries [1 - 5]. It is already known that *C. jejuni* is a frequent commensal in poultry and cattle, and *C. coli* is a frequent commensal in swine and poultry. Contamination of retail products with *Campylobacter* spp. during the slaughter of poultry is a well-known problem of product hygiene [6].

In Thailand, *Campylobacter* spp. was isolated from 12% of various food samples including pork, chickens, and vegetables in Bangkok [7]. Chickens were raised commercially in Thailand nowadays for exports and local consumptions. At present contamination of foodborne pathogens were frequently present and organisms were isolated from samples examined [8].

The purpose of this study was to examine the incidence and antimicrobial susceptibility testing of *Campylobacter* species. The MIC of 5 drugs uses in human medicine were investigated i.e. azithromycin (AZ), dedoxycycline (DC), ciprofloxacin (CI), chloramphenicol (CL) and ceftazidime (TZ), respectively.

Materials and methods

Faecal sample collections: Faecal collection was done on 11 farms in the area of Khon Kaen and Mahasarakam province of Thailand. Farm capacity was 5,000-10,000 broiler chickens per farm. It was raised for commercial purposes and farmers were distributed with 1 day old chicks, drugs and animal feeds were supplied by 2 companies. Chicken faeces were collected from January to April, 2003. Faecal samples were divided into 3 collection periods i.e. first, second and third collection was done when the chicken aged 40 to 45 days (big size chicken), 20 to 25 days (medium size chicken) and 10 to 15 days (small size chicken), respectively. Samples were transported in Tryptic Soy Broth (Oxoid, Hampshire, UK) which were used as transport media for *Campylobacter* and submitted to the Department of Veterinary Public Health laboratory, Faculty of Veterinary Medicine, Khon Kaen University, Thailand.

Cultivation and identification of Campylobacter species: Faecal samples were filtered through 0.45 µm pore size membrane filter (cellulose nitrate, Whatman®) of which were placed onto the center surfaces of Colombia Blood Agar plates containing 5% (v/v) defibrinated sheep blood. Antibiotic cocktails (Preston selective enrichment broth SR117E, Oxoid, Hampshire, UK) and growth promoters (*Campylobacter* growth supplement, SR 085E, Oxoid, Hampshire, UK) that favoured the growth of *Campylobacter* spp. were also incorporated in the media [3, 5, 8]. Incubation was done under the microaerophilic

(5% O_2, 10% CO_2 and 85% N_2) atmosphere generated from gas packs (Anaerocult®C, Merck, Germany) at 42°C for 48 hrs. Identification was done by examination of colony morphology, colony size, gram-stained for s-shaped or gull-wing shaped morphology, biochemical tests using oxidase, catalase and hippurate hydrolysis [3, 9, 10] for genus level. Then, multiplex PCR was employed for species identifications [11].

Multiplex PCR for species identifications: *Campylobacter* spp. was subjected to species identification using multiplex PCR [11] with some modifications. DNA templates were prepared by Miniprep protocol according to the manufacturer's guideline. PCR products were purchased from Takara BIO INC, Japan and used according to the manufacturer's instruction. Briefly, PCR preparation for 1 reaction included 10xPCR buffer (Mg^{2+} free), 2.5 µl; 25 mM$MgCl_2$ (20 mM), 4 µl; dNTP (2.5 mM each), 2.5 µl; CjF 0.5 µM, 1 µl; CjR 0.5 µM (for *C. jejuni*), 1 µl; CIF 0.5 µM, 1 µl; CIR 0.5 µM (for *C. lari*) , 1 µl; CcF 1.0 µM, 1 µl; CcR 1.0 µM (for *C. coli*), 1 µl; CfF 1.0 µM, 1 µl; CfR 1.0 µM (for *C. fetus*), 1 µl; CuF 2.0 µM, 1 µl; CuR 2.0 µM (for *C. upsaliensis*), 1 µl; 23SF 0.2 µM, 1 µl; 23SR 0.2 µM (the internal control), 1 µl; Taq (1.25 U) 0.4 µl, DNA template 2.5 µl and DW 1.1 µl to make up the 25 µl reaction volume. PCR conditions were 95°C, 6 min, 95°C, 30s, 59°C, 30s, 72°C, 30s, 72°C, 7 min, 4°C, infinity for 30 cycles. Gel electrophoresis of PCR amplified products was done and 100 bp DNA ladder (Takara, Japan) was used as a molecular weight marker.

MIC determinations: E-test is recommended by many investigators for assessing the antimicrobial tests of fastidious bacteria and slow growing organisms which required specific incubation atmosphere for growth such as high CO_2 atmosphere [12-15]. E-test method (AB BIODISK, Solna, Sweden) was employed in this study for identifying the MIC of this bacterium against azithromycin (AZ), ciprofloxacin (CI), chloramphenicol (CL), dodoxycycline (DC) and ceftazidime (TZ). Inoculum was standardized to 0.5% McFarland. Sterile swab was used to produce lawns of bacterial growth then E-test strip was placed in the center of the petri dishes. The plates were then incubated under the above mentioned condition. MIC was examined via hand lens or light sources. Tilt the plates for proper reading of its interception with the strip. *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used as control organism to respectively represent gram negative and gram positive bacteria. No reference strains of *C. jejuni* and *C. coli* were available in the national culture collection, Department of Medical Science, Ministry of Public Health, Thailand at the time of investigations.

Results

Among 567 faeces of chicken, 240 samples were contaminated with *Campylobacter spp.* (42.33%) from 11 farms of both companies. Table 1 shows the percentage of the isolation including numbers positive and numbers tested by age of birds. Incident rate of company A was higher than company B in every batch of sample collections. Number of chickens raised in company A was smaller than that of company B. Among the *Campylobacter* isolates identified by multiplex PCR, there were 170 *C. jejuni* (86.29%), 23 C. coli (11.68%), mix contamination (Cj/Cc) was found in 1.02% (2 isolates) and 1.02% (2 isolates) were unidentified. No other species of *Campylobacter* found in this study.

Azithromycin resistance (≥ 2 μg/ml as resistance) is minimal i.e. 6.94% (5/72). More than half of the isolates (51.38%, 37/72) were susceptible (≤ 0.025 μg/ml as susceptible) to this drug. Isolates in the intermediate category is 41.67% (30/72). The most frequently found MIC of DC was 0.032 μg/ml (17.57%, 13/74) and 90.54% (67/74) of the isolates tested were susceptible to this drug. Nine point five percent of the isolates tested were found resistance (≥ 4 μg/ml) to DC. More resistant of *Campylobacter spp*. was found in the case of CI i.e. 30.67% (23/75) and only 2 isolates were categorized in intermediate group (2.67%, 2/75). Nevertheless, majority of the isolates tested were susceptible to this drug (66.67%, 50/75). None of the isolates tested in this study were resistant to neither CL nor TZ (≥ 32 μg/ml as resistance, both). Moreover, co - resistant to DC-CI was encountered among 3 isolates from farm B1 and AZ-CI was found merely in 1 isolate from farm A3. Farm B1 had highest co-resistant isolates compared to other farms. This indicated 5.06% (4/79) of the isolates tested had co-resistant profiles to 2 drugs. There is difference in antimicrobial resistance distributions among *Campylobacter* species. More details are outlined in Table 2 and 3. Trends of resistance seem to increase with the increasing age of birds. In case of CI, resistance was significantly increased ($p < 0.001$) with the age of broilers.

Table 1. Percentage of *Campylobacter* spp. isolated from chicken faecal samples among 11 farms in Mahasarakam and Khon Kaen, Thailand

Farm no.	Age of birds		
	10-15 days	20-25 days	40-45 days
	% (no. positive/no. tested)	% (no. positive/no. tested)	% (no. positive /no. tested)
1	ND	90 (9/10)	100 (10/10)
2	ND	85 (17/20)	95 (19/20)
3	0 (0/40)	90 (9/10)	90 (9/10)
4	ND	65 (13/20)	60 (12/20)
5	ND	50 (10/20)	55 (11/20)
6	10 (4/40)	ND	100 (10/10)
7	ND	ND	100 (20/20)
8	ND	60 (24/40)	90 (9/10)
9	ND	0 (0/40)	95 (38/40)
10	ND	0 (0/40)	2.50 (1/40)
11	ND	0 (0/40)	30 (12/40)
Total	5.00 (4/80)	34.20 (82/240)	62.92 (151/240)

ND = not determine, empty houses at the time of sample collections

Table 2. Resistant of *Campylobacter* species by farms in company A and B to 5 drugs tested

Farm		No. of Resistant					
	No of isolates tested/ total no. of isolates	AZ	DC	CI	CL	TZ	Total
A1(3 farms)	21/73	0/21	0/21	0/21	0/21	0/21	0/21
A2 (2 farms)	10/48	1/10	0/10	2/10	0/10	0/10	3/10
A3 (3 farms)	20/69	1/20	0/20	9/20	0/20	0/20	10/20
B1 (1 farm)	26/38	2/26	10/26	13/26	0/26	0/26	25/26
B2 (1 farm)	1/1	1/1	0/1	0/1	0/1	0/1	1/1
B3 (1 farm)	1/11	0/1	0/1	0/1	0/1	0/1	0/1
Total	79/240	5/79	10/79	24/79	0/79	0/79	39/79

Note: AZ=azithromycin, DC=doxycycline, CI=ciprofloxacin, CL=chloramphenicol, TZ=ceftazidime; company A and B comprised of 8 farms, and 3 farms, respectively

Table 3. Distribution of MICs for the *Campylobacter* spp. isolated from chicken faeces, 2003, Mahasarakam and Khon Kaen province, Thailand

Drug	Resistance (%)	Distribution (%) of MICs (µg/ml)														
		≤ 0.125	0.125-0.24	0.25-0.49	0.5-0.99	1-1.99	2-3.99	4-7.99	8-15.99	16-31.99	32-65.99	64-127.99	128-255.99	256-511.99	512	>512
Azithromycin	6.90	77.77	8.33	2.77	4.17	2.77	1.39	2.77	0.00	1.39	0.00	0.00	0.00	1.39	0.00	0.00
(N = 72)	(5/72)	(56/72)	(6/72)	(2/72)	(3/72)	(2/72)	(1/72)	(2/72)	(0/72)	(1/72)	(0/72)	(0/72)	(0/72)	(1/72)	(0/72)	(0/72)
Doxycycline	9.46	59.46	10.81	5.41	1.35	9.46	4.05	6.76	1.35	1.35	0.00	0.00	0.00	0.00	0.00	0.00
(N = 74)	(7/74)	(44/74)	(8/74)	(4/74)	(1/74)	(7/74)	(3/74)	(5/74)	(1/74)	(1/74)	(0/74)	(0/74)	(0/74)	(0/74)	(0/74)	(0/74)
Ciprofloxacin	30.67	52.00	1.33	1.33	1.33	6.67	6.67	16.00	2.67	1.33	10.67	0.00	0.00	0.00	0.00	0.00
(N = 75)	(23/75)	(39/75)	(1/75)	(1/75)	(1/75)	(5/75)	(5/75)	(12/75)	(2/75)	(1/75)	(8/75)	(0/75)	(0/75)	(0/75)	(0/75)	(0/75)
Chloramphenicol	0.00	21.05	15.79	21.05	17.11	17.11	5.26	2.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(N = 76)	(0/76)	(16/76)	(12/76)	(16/76)	(13/76)	(13/76)	(4/76)	(2/76)	(0/76)	(0/76)	(0/76)	(0/76)	(0/76)	(0/76)	(0/76)	(0/76)
Ceftazidime	0.00	8.57	1.43	0.00	0.00	12.86	11.43	51.43	8.57	5.71	0.00	0.00	0.00	0.00	0.00	0.00
(N = 70)	(0/70)	(6/70)	(1/70)	(0/70)	(0/70)	(9/70)	(8/70)	(36/70)	(6/70)	(4/70)	(0/70)	(0/70)	(0/70)	(0/70)	(0/70)	(0/70)
Total	35/76	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Note: The white fields denote range of dilutions tested for each substance, MICs above the range are given as the concentration closest to the range, MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration; Bold vertical lines indicate microbiological cut-off values defining resistance; The highlighted fields indicate resistance; NA = not applicable; total number of isolates examined was 79, however, some plates were contaminated so data were omitted, hence, total numbers of isolates for each antimicrobial tested were not equal

Discussions

Incidence of *Campylobacter* species contaminations among the 2 companies were 42.33% (240/567). This number was 21% lower than that reported in central Thailand by Suwatanawiroj et al. [5] in 2002. In this study, older broilers (40 ± 5 days) were most contaminated compared to medium size broilers (20 ± 5 days). Moreover, the least contaminated or not contaminated group was younger broilers (10 ± 5 days). During sample collections, observation was made and found that company A's broilers became ill of respiratory diseases and diarrheoa or bloody faeces more than company B. Also, healthy birds were protected from *Campylobacter* species infections. It was therefore the needs for the improvement of farm management to reduce the infection of this bacterium in broilers. Also, times for faecal sample collections were between winter approaching summer month in Thailand. It is well acknowledged a higher contamination rate during summer month of the year in developed countries. It is therefore recommended that an experiment should cover other seasons of the year as well. Critical control points for exposure and contamination of chicken meat supply should be identified so that methods could be developed to protect human exposure to *Campylobacter* spp. [16].

Previous study indicated that resistance to ciprofloxacin was 82% of the human isolates by disc diffusion methods [17]. In addition, high levels of resistance to drugs such as ciprofloxacin were observed in the isolates from poultry. Likewise, these authors noted that 96% in both human and poultry isolates [18] were resistant to ciprofloxacin. The present finding noted a decrease in resistant rate of *Campylobacter* spp. (30.67%) regardless of its origin (human versus animal isolates). Azithromycin has been suggested as a replacement for quinolones for empiric treatment of travelers' diarrhea in Thailand [19]. The prevalence of azithromycin resistance remains relatively low in Thailand of which is 6.94% in the present study compared to 6% in 1999 (0.94% increase) while cirpofloxacin resistance was as high as 77% in other studies. However, this study indicated the 30.67% resistance of *Campylobacter* spp. to ciprofloxacin. This difference may be attributable to the different origin of isolates (clinical versus chicken faecal isolates). The present study showed a marginal co-resistance to more than one drug because merely 4 isolates were resistant to 2 drugs, especially DC-CI (3 isolates) and AZ-CI (1 isolate). In addition to quinolone resistance, co-resistance with other antibiotics such as macrolides has been noted in Spain and Thailand [20]. Surprisingly, 3 isolates with 2 drugs resistant profiles were evident in

the same farm of Mahasarakam province whereas 1 isolate from farm in Khon Kaen province had 2 drugs resistant profiles. Moreover, resistance to antimicrobials was more frequently found in old age broilers than theirs younger age counterparts. However, significant different was found only in case of CI ($p<0.001$).

In conclusion, the isolation of *Campylobacter* spp. from chicken faecal samples revealed the 42.33% (240/567) contamination. MIC determinations of 5 drugs tested indicated that *Campylobacter* spp. were most resistant to CI (30.67%) and only 4 isolates (5.06%, 4/79) were found harboring 2 drugs resistant profiles (DC-CI, 3 isolates and AZ-CI, 1 isolate). Resistance seems to increase with the increasing age of birds. This study indicates that 5 tested drugs were still potentially useful for treating campylobacteriosis cases of whom derived *Campylobacter* spp. from poultry origin in Thailand.

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