Incidence of Quinolones and Macrolides-resistant *Campylobacter* spp. from Diarrheal Patients, Chicken and Pig Feces in the Upper Northeast Thailand

การหาอุบัติการณ์ดื้อยาในกลุ่ม ควิโนโลนและมาโครไลด์ในเชื้อสกุลแคมไพโลแบคเตอร์จากผู้ป่วยโรคอุจจาระร่วง ขี้ไก่และขี้หมู ในภาคตะวันออกเฉียงหนือตอนบนประเทศไทย

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ABSTRACT

This study aims at determining antibiotic resistance of phenotype by Disk diffusion method based on the standard from The Clinical and Laboratory Standards Institute (CLSI 2007) and detection of genotypic by Multiplex PCR assay in *Campylobacter* species By Isolation of *Campylobacter* spp. with rectal swab from chicken (200 samples), swine (150 samples) and Stool from Central laboratory of Srinagarind hospital (150 samples) using Modified Charcoal Cefoperazone Deoxycholate gar (mCCDA) added with SR 0122E (Amphotericin B) found that *Campylobacter* spp. from swine 46 isolates and then antimicrobial susceptibility testing by CLSI (2007) use disk diffusion method, that were tested for resistance to 8 antimicrobial agents. This result *Campylobacter* species strains isolated from pig and human (total 46 isolated) have percent of resistant isolates to Amoxicillin (91.30%), Nalidixic acid (86.96%), Norfloxacin (80.43%), Gentamicin (39.13%), Erythromycin (36.96%), Cephalothin (30.43%), Ciprofloxacin (23.91%), and Azithromycin (21.74%). We devised a multiplex PCR assay based on the CDT gene detect and discriminate 6 *Campylobacter* species. The multiplex PCR assay validated with 46 strains, including 5 *C. coli*, 3 *C. fetus*, 2 *C. hyointestinalis* and 36 other than *C. jejuni*, *C. coli*, *C. fetus, C. lari*, *C. Upsaliensis*, *C. hyointestinalis*.

บทคัดย่อ

ในการศึกษานี้มีวัตถุประสงค์การหาแบบแผนความไวแลการดื้อยาปฏิชีวนะทางฟีโนทัยป์ด้วยวิธี Disk diffusion โดยอาศัยเกณฑ์มาตรฐานจาก The Clinical and Laboratory Standards Institute (CLSI 2007) และตรวจหาการดื้อยาทางจีโนทัยป์โดยวิธี Multiplex PCR assay ในเชื้อแคมไพโลแบคเตอร์ โดยคัดแยกเชื้อแคมไพโลแบคเตอร์ ด้วยวิธี Rectal swab จาก ไก่ (200 ตัวอย่าง), สุกร(150 ตัวอย่าง) และอุจจาระเหลือทิ้งจากห้องปฏิบัติการทางการแพทย์จากโรงพยาบาลศรีนครินทร์ (150 ตัวอย่าง) โดยใช้อาหารเลี้ยงเชื้อ Modified Charcoal Cefoperazone Deoxycholate gar (mCCDA) เพิ่มด้วย SR 0122E (Amphotericin B) พบว่ามี *Campylobacter* spp. จากสุกร 46 ไอโซเลท เมื่อนำเชื้อดังกล่าวมาทดสอบการดื้อยาปฏิชีวนะทางฟีโนทัยป์ด้วยวิธี Disk diffusion โดยอาศัยเกณฑ์มาตรฐานจาก The Clinical and Laboratory Standards Institute (CLSI 2007) พบว่า *Campylobacter* spp. 46 ไอโซเลท มีความไวต่อยา Amoxicillin (ร้อยละ 91.30), Nalidixic acid (ร้อยละ 86.96), Norfloxacin (ร้อยละ 80.43), Gentamicin (ร้อยละ 39.13), Erythromycin (ร้อยละ 36.96), Cephalothin (ร้อยละ 30.43), Ciprofloxacin (ร้อยละ 23.91) และ Azithromycin (ร้อยละ 21.74) และตรวจหาการดื้อยาทางจีโนทัยป์โดยวิธี Multiplex PCR assay พบว่าเป็น *Campylobacter coli* 5 ไอโซเลท, *Campylobacter fetus* 3 ไอโซเลท , *Campylobacter hyointestinalis* 2 ไอโซเลท และอีก 36 ไอโซเลทเป็นเชื้อแคมไพโลแบคเตอร์ชนิดอื่นที่ไม่ใช่ *C. jejeuni*, *C. coli*, *C. fetus, C. lari*, *C. Upsaliensis*, *C. hyointestinalis*.

Keywords: *Campylobacter* species, quinolones and macrolides-*resistant*, pigs
คำสำคัญ: เชื้อสกุลแคมไพโลแบคเตอร์ การดื้อยาในกลุ่มควิโนโลนและมาโครไลด์ สุกร

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Introduction

Campylobacter species are gram-negative spiral, rod-shaped, or curved bacteria with a single polar flagellum, bipolar flagella, or no flagellum, depending on the species. *Campylobacter* species are non-spore-forming, are approximately 0.2 to 0.8 by 0.5 to 5 µm. The *Campylobacter* genus was established in 1963 following the renaming of *Vibrio fetus* to *Campylobacter fetus*, forming the type species of this genus. The *Campylobacter* genus belongs to the family *Campylobacteraceae*, the order *Campylobacterales*, the class *Epsilonproteobacteria*, and the phylum *Proteobacteria*. Since its first description, the genus has grown to include several important human and animal pathogens that are primarily classified through phylogenetic means. The genus *Campylobacter* consists of 26 species, 2 provisional species, and 9 subspecies (as of December 2014). (Kaakoush et al., 2015)

Campylobacter species cause acute diarrhea, 30 percent were reported in the Western of world. However, in Thailand, there were limited reported on the infections. This genus needs a little oxygen concentration than that normal atmosphere for their growth (microaerophilic). More than 90% of all human Campylobacteriosis caused by *Campylobacter jejuni* and *Campylobacter coli*. It is well acknowledged that chickens and pigs are the two major reservoirs. Currently antibiotics have been widely used in the of chickens and pigs - production. From the worldwide scale, *Campylobacter* isolated from these two sources are increasing resistance to many drugs. In Thailand, reports showed that both species were 66-87 % and 1.8-23 % resistance to quinolones and macrolides. However, pig isolates were 84-89 % and 66 % resistance to aforemationed antimicrobials. *Campylobacter* resistance to these drugs are of clinical concern in human medicine, and lead to the search for other drug of choice. Drug resistance *Campylobacter* isolated are of clinical concern, and new drugs susceptibility are required. (Alfredson & Korolik, 2007; Engberg et al., 2001)

Currently, antibiotics have been widely used in the chicken and pig production. From wide scale, *Campylobacter* isolated from these two sources are increasing in resistance to many drugs. In Thaialand, reports showed that both species were 66-86% and 9.4-66.0% resistance to quinolones and macrolides. (Ekkapobyothin et al., 2008; Chokboonmongkol et al., 2013) However, pig isolates were 84-86% and 66% resistance to the aforementionrd antimicrobials. *Campylobacter* resistance to these drugs are of clinical concern in human medicine, and lead to the search for other component drug of choices. Drug resistance *Campylobacter* isolates are of clinical concern, and new drug susceptibility testing are required. (Aarestrup, 2015; Luangtongkum, 2009) This study aims at determining the incidence of *Campylobacter* quinolone resistance (nalidixic acid, ciprofloxacin) and macrolide resistance (erythromycin, azithromycin) and also extending to including gentamicin, amoxicillin plus clavulanic acid, tetracycline and imipenem resistance profiles among chicken , pig and diarrheal human faecal samples in the upper Northeast Thailand from the year 2016 to 2017. Isolation and identification of *Campylobacter* species are conducted by plating method, biochemical tests and multiplex PCR assay. Phenotypic characteristics of drug resistance are performed by disk diffusion.

Objectives of the study

This study aimed (1.) To examine the incidence of *Campylobacter* spp. and the prevalent of each causative agents such as *C. jejuni, C. coli, C. lari, C. fetus, C. hyointestinalis, C. upsaliensis* and *C. consisus* from diarrheal

diseases and healthy animals in upper Northeast Thailand. (2.) To determine the rate of drug resistance to quinolones and macrolides in *C. jejuni, C. coli* compare to the other *Campylobacter* species. (3.) To determine drug susceptibility.

Methodology

Left over stool sample from diarrheal patients

One-hundread and fifty specimens were from who place in Srinagarind hospital during 2017. All patients have sign and symptoms of acute diarrhea with duration of diarrhea not more than one week, but also not healthy people who have annual physical examination.

Specimen collection from chicken and swine feces

Apply buffered Glycerol peptone for maintaining rectal swab of chickens and swine from upper Northeast Thailand and keep in an ice box or refrigerator until bacterial isolation.

Ethical approval

Human Ethical committee approved number is HE 591288 and Animal Ethical committee are accepted that this study is not bother their daily life of those animals.

Isolation and Identification by biochemical testing

Rectal swab from chickens and swine including human feces were collected from left over patients at Central laboratory from Srinagarind hospital, Khon Kaen, Thailand. Then the samples were isolated on selective agar medium, called modified Charcoal Cefoperazone Deoxycholate gar (mCCDA) supplement with SR 0122E which included Amphotericin B, placed the sterile membrane filter at center mCCDA and allotted sample approximately 200 to 300 µl onto sterile membrane filter (0.45 micron), leaved at room temperature for 30 min, then removed the filter paper and then incubated at 42° C for 48 hours under microarophilic conditions (5% O_2, 10% CO_2, and 85% N_2 ; CampyGen™). The typical colonies on fresh medium appeared grey, flat, irregular and spreading, occasionally mucoid, on less fresh medium will be 1 to 2 mm in diameter, appeared as grey round, convex and glistening with little or no spreading. Sometimes colonies can be grey to pinkish or yellowish grey and were nonhemolytic. Perform gram stain of suspected colonies using basic fuchsin or carbol fuchsin counterstain after removed from the microaerobic incubation. *Campylobacter* species appeared as slender helical or cured gram - negative bacilli sometimes in gull wing, archery bows or comma, darting motility in wet preparation and presumptive identification with positive of oxidase and catalase test. Further identification with susceptibility to 30 µg disk cephalotin and nalidixic acid including ability for hippurate hydrolysis, Indoxyl Acetate hydrolysis, H_2S production in TSI agar and nitrate to nitrite will be tested, finally the culture is stored in 20% glycerol at –20°C until needed for further experiments.

Identification genus and species of *campylobacter* by polymerase chain reaction

Chromosomal DNA extraction

DNA extraction use boiling method. Prepare bacterial suspension from 48 hours culture on sheep blood agar in 1 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), the suspensions were boiled for 10 min, incubated on ice for 5 min, and centrifuged at 12,800 × g for 5 min. The supernatants were collected and stored at –20°C until needed

for further experiments. (Kamei et al., 2016). Primer sets and condition using for identification genus and species of *Campylobacter* are shown in Table 1.

Table 1 Predicted sizes of amplified product of multiplex PCR by agarose gel electrophoresis.

Target DNA (*cdt* gene)	Primer	Sequence	PCR conditions	Product (bp)
C. jejuni	Cj- spBU5	ATCTTTTAACCTTGCTTTTGC	94°C 1 min, 30 cycle 94°C 30 s, 56°C 90 s, 72°C 90 s and Final extension 72°C 5 min	714
	Cj- spBR6	GCAAGCATTAAAATCGCAGC		
C. fetus	Cf-spBU6	GGCTTTGCAAAACCAGAAG		553
	Cf-spBR3	CAAGAGTTCCTCTTAAACTC		
C. coli	Cc- spBU10	CTGTATCAAGACCTAGCTC		433
	Cc- spBR9	TATAAAGCTGCAGTGTTGG		
C. upsaliensis	Cu- spBU5	GCCTTAGCTTTCTTTGGG		242
	Cu- spBR5	CATCGGCTTGGACGCGAC		
C. hyointestinalis	Chll- spBU8	CCTAGTAGCGCTACTTAG		215
	Chll- spBR8	CAAATACCCTACCTGTAGC		
C. lari	Cl- spBU4	GTATCCATGCTTTATCAAGA		141
	Cl- spBR4	GTAGGCCTATAAGAGAACC		

Drug susceptibility test

Determination of antimicrobial susceptibility by disk diffusion method

Disk diffusion method was performed by standardized method, recommended by Clinical of Laboratory Standard Institute (CLSI 2007). Briefly, Preparation of 0.5 Mc Farland turbidity standard equal to $0.5x10^8$ CFU/ml was performed by using 48 hour culture from blood agar plate. Sterile cotton swab was dipped into this suspension, remove excess fluid by pressed gently on the inner wall of the culture tube. The swab was streaked entire on to Muller-Hinton agar supplemented with 5% sheep blood, incubated at 42°C for 48 hours in a microaerobic environment. The standardized interpretive criteria for *Campylobacter* susceptibility to the antimicrobial was shown in Table 2.

Table 2 Interpretation criteria of antimicrobial susceptibility by disk diffusion method

Antimicrobial agent	Disc content (µg)	Zone diameter (mm)		
		R	I	S
Amoxicillin	10	≤13	14-16	≥17
Azithromycin	30	≤17	18-20	≥21
Cephalothin	30	≤14	15-17	≥18
Ciprofloxacin	5	≤15	16-20	≥21
Erythromycin	10	≤12	13-14	≥15
Gentamicin	10	≤13	14-22	≥23
Norfloxacin	10	≤12	13-16	≥17
Nalidixic acid	30	≤13	14-18	≥19

Statistical analysis

The relationship of phenotypes and genotypes have number of drug resistance less than five using Fisher exact test and if number of those up to or greater than five using Chi square test.

Results

Bacterial isolation from human and animals

Isolation of samples by the filtration method reveals that *Campylobacter* spp. positive only one patient who place in Srinagarind hospital, Khon Kaen, Thailand during 2018. Beside to rectal swab of swine from upper Northeast Thailand have forty-five *Campylobacter* spp. but not found any *Campylobacter* spp. in chicken. The percentage of positive in human, pig and chicken are 0.67, 30.67 and 0, respectively. It may due to heavy use antibiotic in production process of chickens more than swine during investigation. These lead to *Campylobacter* isolated from those animals are likely negative as shown in Table 3.

Table 3 *Campylobacter* species isolated by the filtration method.

Sample	Number sample(n)	Total number positive	% Positive
Stool from human	150	1	0.67
Rectal swab from swine	150	45	30.67
Rectal swab from chickens	200	0	0.00

Antimicrobial susceptibility by disk diffusion method

Disk diffusion method was performed by standardized method, recommended by Clinical of Laboratory Standard Institute (CLSI 2007) for exploring resistant strains to 8 antimicrobial agents. The 46 of *Campylobacter* spp. from swine isolates reveal resistant to Amoxicillin (91.30%), Nalidixic acid (86.96%), Norfloxacin (80.43%), Gentamicin (39.13%), Erythromycin (36.96%), Cephalothin (30.43%), Ciprofloxacin (23.91%), and Azithromycin (21.74%), respectively. (Table 4) The drug resistance patterns of the *Campylobacter* spp. from swine isolates are shown in Table 5.

Table 4 Antimicrobial susceptibility patterns of *Campylobacter* isolates identified by the disk diffusion method.

Antimicrobial agent	No. of Campylobacter isolates			% of resistant isolates
	S	I	R	
Amoxicillin	3	1	42	91.30
Nalidixic acid	4	2	40	86.96
Norfloxacin	6	3	37	80.43
Gentamicin	18	8	18	39.13
Erythromycin	29	0	17	36.96
Cephalothin	19	13	14	30.43
Ciprofloxacin	19	16	11	23.91
Azithromycin	34	2	10	21.74

Table 5 Multi - drugs resistant patterns of *Campylobacter* isolates identified by the disk diffusion method.

Multi - drugs Resistant Patterns	% of resistant isolates
AML, NAL, NOR	86.23
AML, NAL, GET	72.46
NAL, NOR, GET	68.84
NAL, NOR, ERY	68.12
NOR, GEN,CET	50.00
CET, AZM, ERY,CIP	28.26

*** AML=Amoxicillin, NAL = Nalidixic acid, NOR=Norfloxacin, CET =Cephalothin, GEN = Gentamycin, AZM = Azithromycin, ERY = Erythromycin and CIP = Ciprofloxacin

Isolation and identification of *Campylobacter* spp. by polymerase chain reaction

Isolation and identification of *Campylobacter* spp. by Multiplex PCR

The 46 of Campylobacter spp. from swine isolates perform by Multiplex PCR using 6 primer of *Campylobacter* spp. that predicted sizes of amplified product by the difference in size by capillary gel electrophoresis. This result found that 3 *C. fetus*, 5 *C. coli*, 2 *C. hyointestinalis* and 36 other than above six species of *Campylobacter* spp. (Table 6)

Table 6 PCR results by multiplex PCR analysis of *Campylobacter* spp.

***Campylobacter* spp.**	**No. of test (No. of PCR positive)**
C. jejuni	46(0)
C. fetus	46(3)
C. coli	46(5)
C. upsaliensis	46(0)
C. hyointestinalis	46(2)
C. lari	46(0)
Other than above 6 species	46(36)

Discussions

In Northern India, *Campylobacter jejuni* Isolated from Poultry Meat and Related Samples at Retail Shops found that percent of resistance co-trimoxazole (84.1%), cephalothin (81.1%) and tetracycline (59.4%) (Khan et al., 2018). In Tanzania, *Campylobacter* Isolated from Pigs, Dairy, and Beef Cattle found that percent of resistance ampicillin (70.3%), erythromycin (41.4 %), ciprofloxacin (14.4 %), nalidixic acid (39.6%) and azithromycin (13.5%) (Kashoma et al., 2015). In US, *Campylobacter* isolated from feedlot cattlet hat, report that *Campylobacter coli* found that nalidixic acid (82.6%) and ciprofloxacin (77.4%), *Campylobacter jejuni* found that nalidixic acid (34.3%) and ciprofloxacin (35.6%) (Tang et al., 2017). In Italy, *Campylobacter coli* and *Campylobacter jejuni* from broiler chicken in farms and at time of slaughter, report that *Campylobacter coli* found that percent of resistance fluoroquinolones (70%), tetracycline (70%) and erythromycin (30%) and *Campylobacter jejuni* found that percent of resistance fluoroquinolone (39%) and tetracycline (10%) (Pergola et al., 2017). In Iran, *Campylobacter* species isolated from meat samples was *Campylobacter jejuni* (88.3%), the remaining isolates were *Campylobacter coli* (11.7%). All 60 Campylobacter strains identified as *C. jejuni and C. coli* were also positive by using polymerase chain reaction (PCR). (Rahimi et al., 2010)

In Iran, *Campylobacter* spp. were observed in this study in sheep (10%), goat (8%), cattle (5.3%), and camel (4%). The highest prevalence of Campylobacter spp. was found in the summer (10%) and the lowest was in winter (4%). Among the isolates from livestock, both *C. jejuni* and *C. coli* from fecal samples had the highest frequency of tetracycline (75.1%) and ciprofloxacin (57.1%) resistance (Rahimi et al., 2010)

In Kayseri, Of *Campylobacter* positive cases 71% (127/179) were children and 58% (104/179) were male. The prevalence rate was estimated as 7.5% (127/1683) for children and 3.2% (52/1604) for adults. Of the isolates, 146 (82%) were identified as *C. jejuni*, 24 (13%) were *C. coli*, 6 (3%) were *C. lari* and 3 (2%) were *C. upsaliensis* with phenotypic tests. By using mPCR, 152 (85%) and 27 (15%) of 179 isolates were identified as *C. jejuni* and *C. coli*, respectively and the rates of resistance of the isolates were 92.6% for trimethoprim- sulfamethoxazole, 79.5% for nalidixic acid, 75.6% for levofloxacin, 73.9% for ciprofloxacin, 40.3% for ampicillin, 35% for cefotaxime, 33.4% for piperacillin-tazobactam, 24% for tetracycline, 14.6% for clindamycin, 11.2% for amikacin and 6.3% for erythromycin. (Kayman et al., 2013)

Conclusions

This study Isolation of *campylobacter* spp. found that stool of human and e rectal swab of pig and *Campylobacter* isolated found that high drug resistance because used in the production process of pigs. In Upper Northeast Thailand found that high drug resistance in quinolones and macrolides. Then detect genus and species by Multiplex PCR found that *C. fetus*, *C. coli*, *C. hyointestinalis*.

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