UNIVERSITI PUTRA MALAYSIA

OCCURRENCE OF CAMPYLOBACTER SPP. AND THEIR ANTIBIOTIC RESISTANCE PROFILES IN CATTLE AND FARM ENVIRONMENT

WINT WINT AUNG

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By

WINT WINT AUNG

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Veterinary Science

June 2014

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DEDICATION

This thesis is especially dedicated to:

My beloved parents**,**

U AUNG MYINT
and
DAW THAN AYE

My beloved husband and daughter**,**

DR. SWE MYINT OO
KAY ZIN LEI

Who always supported and encourage me to do the best

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Veterinary Science

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Chairman: Prof. Saleha Abdul Aziz, PhD

Faculty : Veterinary Medicine

Campylobacter, principally *C. jejuni* and *C. coli*, have been recognizedas one of the important causal agents of gastrointestinal infections in humans all over the world. The major source of human infection is raw or undercooked poultry meat but beef, pork, raw milk and water have also been associated with the infection. Most of the studies in Malaysia were on poultry and poultry products. The work on occurrence of *Campylobacter* in cattle, beef and milk is very scarce. Thus, the objectives of this study were to determine the occurrence of *Campylobacter* in cattle, farm environment, milk and meat,to identify the *Campylobacter* isolates by phenotypic method and multiplex PCR assay and to study the antibiotic resistance patterns of the isolates. One hundred and eighty (180) rectal swab samples from cattle, 68 samples from cattle farm environments, 36 raw milk samples from six farms and 30 beef samples from four markets were collected. All samples were cultured on selective media and isolated *Campylobacter* species were confirmed and identified using multiplex PCR. The overall prevalence of *Campylobacter* in dairy and beef cattle was 47 (26.1%) out of 180 samples. Eleven cattle were colonized by two *Campylobacter* species. The prevalence was higher in beef cattle 18 out of 57 samples (31.6%) compared to dairy cattle 29 out of 123 samples (23.6%) but the difference was not significant (p=0.256). The prevalence was significantly higher in calves 16 out of 40 samples (40%) than adult cattle 31 out of 140 samples (22.1%) (p=0.023). The isolation of *Campylobacter* from cattle was more at incubation temperature of 42˚C (25.0%) compared to at 37˚C (21.1%), however the difference was not significant (p=0.381) and kappa test statistic showed almost perfect agreement between the two different temperatures (kappa>0.8). Six *Campylobacter* species were identified at both temperatures; the most frequent isolated species was *C. jejuni*23 (39.6%) and followed by *C. fetus*13 (22.4%), *C. upsaliensis*8 (13.8%), *C. coli*5 (8.6%),*C. hyointestinalis* subsp. *hyointestinalis* 4 (6.9%) and the least prevalent species was *C. lari*3 (5.2%). However, two isolates were unidentified *Campylobacter* species. From a total of 68 environmental samples, 19 (27.9%)*Campylobacter* isolates were isolated, namely from 10 out of 27 water samples (37.0%), four out of 16 flies samples (25.0%), one out of seven feed samples (14.3%), three out of nine

floors of the cattle houses samples (33.3%) and one out of nine water trough samples (11.1%) which are considered as the risk factors for *Campylobacter* in cattle. Flies could be an essential vector for transmission of *Campylobacter* from contaminated environment to cattle in the farms or from infected animals to the environment. The occurrence of *Campylobacter* in feed, floor, drinking water and water trough could be contaminated via flies and animal faeces.Ten (10) isolates (27.8%) of the 36 raw milk samples were *Campylobacter* positive, however none of the 30 retail beef samples were positive. The occurrence of *Campylobacter* in milk could have resulted from contamination during milking. The absence of *Campylobacter* in retail beef probably suggests they were not contaminated at processing and poor resistance of *Campylobacter*to atmospheric oxygen and other environmental pressures during storage, transportation and retailing may cause *Campylobacter* to convert to viable but non culturable (VBNC) form.The overall isolation rate of *Campylobacter* from cattle, environment samples, beef and milk when incubated under two different temperatures was higher at 42˚C (22.6%) when compared to 37˚C (18.5%); however, the difference was not significant (p=0.199) and kappa test statistic showed good agreement between the two different incubation temperatures ($0.6 \leq k < 0.8$) and six *Campylobacter* species were isolated at both temperatures.

The *Campylobacter* isolates were tested for antibiotic resistance using standard disc diffusion method and Minimum Inhibitory Concentration (M.I.C) method. The *Campylobacter* isolates were tested against 12 antibiotics and showed resistance to clindamycin and nalidixic acid (50.9%) each, cefotaxime (49.1%), sulfamethoxazole-trimethoprim (40%), ampicillin (38.2%), ciprofloxacin (23.6%), enrofloxacin and streptomycin (21.8%), tetracycline (20%), erythromycin (18.2%), chloramphenicol (16.4%) and gentamicin (12.7%) by disc diffusion method. For M.I.C method using M.I.C.Evaluator strips, the isolates were tested against four antibiotics. The isolates were found resistant to ampicillin and tetracycline (26.3%), ciprofloxacin (21%) and erythromycin (15.8%). All the isolated *Campylobacter* spp. in this study were resistant to five antibiotics namely ampicillin, clindamycin, nalidixic acid, streptomycin and cefotaxime. The resistance rates between the two methods for four antibiotics were found comparable. There is almost perfect agreement of kappa test statistic for ampicillin, erythromycin and ciprofloxacin (kappa>0.8) and also good agreement for tetracycline ($0.6 \leq k < 0.8$) between both methods. Multidrug resistance, that is resistant to three or more antibiotic classes, was high, at 52.7%. Multidrug resistant *Campylobacter* isolates poses a significant risk if they are resistant to the drugs of choice and alternative drugs for treatment.

It can be concluded from this study that *Campylobacter* species are quite prevalent at 26.1% in cattle in the farms. The presence of *Campylobacter* in cattle and milk could be a potential source of human infections and environmental contamination. Hence, it is recommended that good animal husbandry practices (GAHP) and good milking procedures must be practiced at the farms and good manufacturing procedures (GMP) at abattoirs where it may reduce the risk to humans through meat, milk and environment. The use of antibiotics in animals should also be controlled and monitored to reduce antibiotic resistance.

Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan bagi Ijazah Sarjana Sains Veterinar

KEHADIRAN *CAMPYLOBACTER* SPP. DAN PROFIL KERINTANGAN ANTIBIOTIK DI DALAM LEMBU DAN PERSEKITARAN LADANG

Oleh

WINT WINT AUNG

Jun 2014

Pengerusi: Prof. Saleha Abdul Aziz, PhD

Fakulti: Perubatan Veterinar

Campylobacter, terutama *C. jejuni* dan *C. coli* telah dikenali sebagai salah satu agen penyebab jangkitan gastrousus pada manusia di seluruh dunia. Sumber utama jangkitan pada manusia adalah daging ayam mentah atau kurang dimasak, tetapi daging lembu, daging babi, susu segar dan air telah juga dikaitkan dengan jangkitan tersebut. Kebanyakan kajian yang telah dilakukan di Malaysia adalah pada ayam dan produk ayam. Kajian kehadiran *Campylobacter* pada lembu, daging lembu dan susu adalah kurang dan amat sukar diperolehi. Objektif kajian ini adalah untuk menentukan kehadiran *Campylobacter*pada lembu, persekitaran ladang, susu dan daging, mengenalpasti isolat *Campylobacter* mengguna kaedah fenotipik dan asei m-PCR dan juga mengkaji corak kerintangan antibiotik isolat. Satu ratus lapan puluh (180) sampel calitan rektal lembu, 68 sampel persekitaran ladang lembu, 36 sampel susu segar daripada enam buah ladang dan 30 sampel daging lembu di empat pasar basah telah diambil. Kesemua sampel telah dikultur pada media selektif dan spesis *Campylobacter* yang diasingkan telah dikenalpasti dan dispesis menggunakan PCR multipleks.Prevalenkeseluruhan spesis *Campylobacter* pada lembu tenusu dan lembu pedaging adalah 26.1%. Prevalen adalah lebih tinggi pada lembu pedaging (31.6%) berbanding lembu tenusu (23.6%) tetapi perbezaannya adalah tidak signifikan (p=0.256). Prevalen adalah secara signifikan lebih tinggi pada anak lembu (40%) daripada lembu dewasa (22.1%) (p=0.023). *Campylobacter* lebih banyak diasingkan pada suhu 42˚C (25.0%) berbanding pada 37˚C (21.1%), walau bagaimanapun perbezaannya adalah tidak signifikan (p=0.381) dan ujian statistik kappa menunjukkan persetujuan hampir sempurna di antara dua suhu tersebut (kappa>0.8). Enam spesis *Campylobacter* telah dikenalpasti pada kedua-dua suhu; spesis yang paling kerap diasingkan adalah *C. jejuni* (39.6%) dan diikuti oleh *C. fetus* (22.4%), *C. upsaliensis* (13.8%), *C. coli* (8.6%), *C. hyointestinalis* subsp. *hyointestinalis*(6.9%) dan paling kurang adalah *C. lari* (5.2%). Walau bagaimanapun, dua isolat spesis *Campylobacter*tidak dapat dikenalpasti.Daripada sampel persekitaran, sejumlah 27.9%spesis *Campylobacter* telah diasingkan, iaitu daripada air (37.0%), lalat (25.0%), makanan ternakan (14.3%), lantai kandang (33.3%) dan bekas minuman (11.1%) yang telah dianggap sebagai faktor-faktor berisiko bagi

Campylobacter pada lembu. Lalat boleh menjadi vektor penting bagi pemindahan*Campylobacter*dari persekitaran tercemar kepada tenakan lembu di ladang atau dari haiwan terjangkit kepada persekitaran. Kehadiran *Campylobacter* pada makanan haiwan, lantai, air minuman dan bekas minuman boleh melalui pencemaran lalat dan tinja haiwan. Dua puluh tujuh perpuluhan lapan (27.8%) sampel susu segar didapati positif *Campylobacter*, walau bagaimanapuntiada satu pun daripada30 sampel daging lembu yang positif. Kehadiran *Campylobacter* pada susu boleh terhasil daripada pencemaran semasa pemerahan susu. Ketiadaan *Campylobacter* pada daging lembu mungkin ianya tidak dicemari semasa pemprosesan, dan juga kerintangan lemah *Campylobacter* terhadap atmosfera oksigen dan lain-lain tekanan persekitaran semasa penyimpanan, pengangkutan dan penjualan boleh menyebabkan *Campylobacter* bertukar kepada bentuk berdaya hidup tetapi tidak boleh dikultur (VBNC). Kadar keseluruhan pengasingan *Campylobacter* pada lembu, sampel persekitaran, daging lembu dan susu apabila diinkubasi di bawah dua suhu berbeza adalah lebih tinggi pada 42˚C (22.6%) berbanding 37˚C (18.5%); walaubagaimanapun, tiada perbezaan signifikan (p=0.199) dan ujian statistik kappa menunjukkan persetujuan baik di antara dua suhu inkubasi yang berbeza ($0.6 \leq k < 0.8$). Enam spesis *Campylobacter* telah diasingkan pada kedua-dua suhu.

Isolat *Campylobacter* telah diuji kerintangan antibiotik dengan menggunakan kaedah disc diffusion dan kaedah *Minimum Inhibitory Concentration (M.I.C)*. Isolat *Campylobacter* telah diuji terhadap 12 antibiotik dan menunjukkan kerintangan terhadap setiap satuclindamycin dan nalidixic acid (50.9%), cefotaxime (49.1%), sulfamethoxazole-trimethoprim (40%), ampicillin (38.2%), ciprofloxacin (23.6%), enrofloxacin dan streptomycin (21.8%), tetracycline (20%), erythromycin (18.2%), chloramphenicol (16.4%) dan gentamicin (12.7%) melalui kaedah disc diffusion. Bagi kaedah *M.I.C*menggunakan strip *M.I.C. Evaluator*, isolat telah diuji terhadap empat antibiotik. Isolat telah didapati rintang terhadap ampicillin dan tetracycline (26.3%), ciprofloxacin (21%) dan erythromycin (15.8%). Kesemua isolat *Campylobacter* spp. di dalam kajian ini adalah rintang terhadap lima antibiotik iaitu ampicillin, clindamycin, nalidixic acid, streptomycin dan cefotaxime. Kadar kerintangan di antara dua kaedah bagi empat antibiotik didapati setanding. Terdapat persetujuan hampir sempurna bagi ujian statistik kappa bagi ampicillin, erythromycin dan ciprofloxacin ($kappa > 0.8$) dan juga persetujuan baik bagi tetracycline ($0.6 \leq k < 0.8$) di antara kedua-dua kaedah. Kerintangan multidrug, iaitu rintang kepada tiga atau lebih kelas antibiotik, adalah tinggi, 52.7%. Isolat multidrug rintang *Campylobacter* boleh menyebabkan risiko signifikan sekiranya ia rintang kepada drug pilihan dan drug alternatif untuk rawatan.

Daripada kajian ini, dapat disimpulkan bahawa spesis *Campylobacter*adalah agak prevalen 26.1% pada ternakan lembu di ladang. Kehadiran *Campylobacter* pada lembu dan susu boleh menjadi sumber yang berpotensi menyebabkan jangkitan pada manusia dan pencemaran persekitaran. Oleh yang demikian, Amalan Penternakan Haiwan Baik (GAHP) dan prosedur pemerahan susu yang baik perlu dilaksanakan di ladang serta Prosedur Pengeluaran Baik (GMP) di rumah sembelih yang dapat mengurangkan risiko kepada manusia melalui daging, susu dan persekitaran. Penggunaan antibiotik pada haiwan perlu dikawal dan dipantau bagi mengurangkan kerintangan antibiotik.

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I certify that a Thesis Examination Committee has met on 13th June 2014 to conduct the final examination of WINT WINT AUNG on her thesis titled "Occurrence of *Campylobacter* in Cattle and Its Farm Environment and their Antibiotic Resistance Profiles" in accordance with Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Veterinary Science. Members of the Examination Committee are as follows:

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This thesis was submitted to the Senate of University Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Veterinary Science. The members of supervisory committee were as follows:

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
bp	Base pairs
CBA	Columbia Blood Agar
CCUG	Culture Collection of the University of Goteborg
ceuE	Siderophore enterochelin
CLSI	Clinical Laboratory Standard Institute
cstA	Cystatin-A
˚C	Degree Celcius
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
flaA	Flagellin A gene
glyA	Serine hydroxyl methyl transferase gene
g	Gram (s)
h	Hour (s)
hip	hippuricase gene
lpxA	UDP-N-acetyl glucosamine acetyltransferase
ml	Milliliter
mg	Milligram (s)
min	Minute (s)
mm	Millimeter
MDR	Multidrug resistance
mCCDA	Modified Charcoal Cefoperazone Deoxycholate Agar
MIC	Minimum inhibitory concentration
MICE	Minimum Inhibitory Concentration Evaluator
mPCR	Multiplex Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
s	Second (s)
Spp.	Species
TBE	Tris-borate EDTA
UV	Ultraviolet

V	Volt
WHO	World Health Organization
µL	Micro Liter
µg	Micro Gram
µM	Micro Molar

CHAPTER 1

INTRODUCTION

Campylobacter species are important in veterinary and public health due to their zoonotic nature, colonizing a large variety of reservoir hosts and being environmental persistence(Hannon et al., 2009).In humans, these *Campylobacter* species are well-known causes of food-borne gastroenteritis (Allos, 2001), thus of major public health importance worldwide particularly in industrialized countries(Adhikari et al., 2004).In many European countries, the prevalence of campylobacteriosis continues to increase and today it exceeds the number of salmonellosis cases (Silva et al., 2011). Most of the human foodborne diseases are caused by*Campylobacter jejuni* and *Campylobacter coli* (Uaboi-Egbenni et al., 2012). *Campylobacter* species can be found in the reproductive organs, gastrointestinal tracts, and oral cavities of animals and humans (Dadi &Asrat, 2008). *Campylobacter* species colonize various species of wild and farm animals, principally poultry and birds, as part of their gut microbiota (Neimann et al., 2003; Van Vliet & Ketley, 2001) without causing infection. *Campylobacter* infection is also one of the causes of reproductive disorders in cattle such as poor calving in southern Africa (Schmidt et al., 2010).

In human campylobacteriosis, poultry meat has long been regarded as the major source and cattle may also play an important reservoir host species (Stanley & Jones, 2003). Contamination of human food can arise at any step from the slaughter house, to processing plant to the consumer (Neimann et al., 2003). Detection of *C. jejuni* and *C. coli* on the carcasses is mainly due to contamination from the gastrointestinal contents of slaughtered healthy animals (Nonga et al., 2010). Besides thermophilic *Campylobacter* spp. which included *C. jejuni*, *C. coli*, *C. lari*, *C. hyointestinalis* and *C. lanienae* in cattle may have implication on public health (Sanad et al., 2011; Humphrey et al., 2007; Acik & Cetinkaya, 2005; Logan et al., 2000). Many studies have observed identical strain types between *Campylobacter* species isolated from cattle faeces or from contaminated bovine origin food products and those from infected human (Hakkinen et al., 2009; Gilpin et al., 2008b).

Apart from beef, the existence of foodborne pathogens in milk is also a potential hazard to public health, principally among milk manufacturers, farm workers and their families and those keen on consuming unpasteurized milk (Ryser, 1998). Besides chicken meat, cattle and beef have been implicated in human campylobacteriosis outbreaks and sporadic cases, were generally associated with drinking of unpasteurized milk and consumption of beef (Nielsen, 2002). The contact with cattle faeces via environmental contamination is also regarded as a threat to humans (Garrett et al., 2007). Furthermore, cattle have been involved in the environmental transmission of *Campylobacter* to water(Clark et al., 2003). *Campylobacter* from the faeces of warm blooded animals, birds and infected humans can get into the water and food(Scotter et al., 1993) and that water is not only common as vehicle of *Campylobacter* spread to humans but also to cattle (Besser et al., 2005).

There is increasing scientific confirmation, especially in developed countries concerning the widespread antibiotic usage in food animals that leads to the development of resistant pathogenic microorganisms that can get to humans through the food chain (Marshall & Levy, 2011; Philips et al., 2004).Treatment with antibiotics for uncomplicated*Campylobacter* infection is not common. On the other hand, *Campylobacter* have been increasingly reported to be resistant to antibiotics used for treatment (principally macrolides and fluoroquinolones) (Aarestrup & Engberg, 2001). Antibiotic therapy is mostly considered in severe cases. The frequency of resistance to macrolides among *Campylobacter* spp. is considerable since the 1990s, and it has since been identified as an emerging public health problem (Engberg et al., 2001). Numerous studies have revealed that human diseases with fluoroquinolone-resistant (FQr) *Campylobacter* have increased worldwide, corresponding with the use of fluoroquinolones in animal agriculture (Serichantalergs et al., 2007; Gupta et al., 2004; Engberg et al., 2001).

The occurrence of *Campylobacter* species in cattle has been studied in countries such as United States, Turkey, New Zealand, Nigeria, Southern Chile, Canada, UK, Tanzania, USA (Sanad et al., 2011; Grove-White et al., 2010; Nonga et al., 2010; Salihu et al., 2009; Fernández & Hitschfeld, 2009; Hannon et al., 2009; Gilpin et al., 2008b; Bae et al., 2005; Açik & Cetinkaya, 2005) but there is very few information on the occurrence of *Campylobacter* in cattle in Malaysia. There is a need to know the extent of *Campylobacter* infection in cattle and the presence of *Campylobacter* in farm environment, milk and meat.

Thus, the objectives of this study were:

1. to determine the occurrence of *Campylobacter* in dairy and beef cattle, their farm environment, milk and meat.
2. to identify the *Campylobacter* isolates by phenotypic method and multiplex PCR assay.
3. to determine theantibiotic resistance patterns among *Campylobacter* isolates.

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