UNIVERSITI PUTRA MALAYSIA

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

WINT WINT AUNG

FPV 2014 26
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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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WINT WINT AUNG

June 2014

Chairman: Prof. Saleha Abdul Aziz, PhD

Faculty: Veterinary Medicine

Campylobacter, principally C. jejuni and C. coli, have been recognized as 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. jejuni23 (39.6%) and followed by C. fetus13 (22.4%), C. upsaliensis8 (13.8%), C. coli5 (8.6%), C. hyointestinalis subsp. hyointestinalis 4 (6.9%) and the least prevalent species was C. lari3 (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≤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≤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 dikaftakan 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 menggunakan kaedah fenotipik dan aseim-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 spesies Campylobacter yang diasingkan telah dikenalpasti dan dispesis menggunakan PCR multipleks. Prevalenkeseluruhan spesies 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 spesies Campylobacter telah diikali pasti pada kedua-dua suhu; spesies yang paling kerap diasingkan adalah C. jejuni (39.6%) dan diikali 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 diikali pasti. Daripada sampel persekitaran, sejumlah 27.9% spesies 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 bagaimanapun ia tidak dicermi 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). Kedua puluh tujuh perpuluhan (27.8%) sampel daging lembu yang positif. Kehadiran Campylobacter pada susu boleh terhasil daripada pencemaran semasa pemerahan susu. Ketiadaan Campylobacter pada daging lembu mungkin ianya tidak dicermi 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). Kedua puluh tujuh perpuluhan (27.8%) sampel 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≤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 menggunaan 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≤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.

ACKNOWLEDGEMENTS

My warmest appreciation goes to my supervisor Prof. Dr. Saleha Abdul Aziz, the chairman of Supervisory Committee for her continuous encouragement, care and excellent scientific guidance during the course of this study. I deeply appreciate her patience, understanding and invaluable advice. I would like to express my special thanks to my co-supervisors, Assoc. Prof. Dr. Zunita Zakaria and Dr. Murugaiyah Marimuthu for their compassion, supervision, encouragement, valuable comments and suggestions.

I would like to take this opportunity to acknowledge my appreciation to SEARCA (South East Asian Regional Centre for Graduate Study and Research in Agriculture), for the financial assistance.

I would like to express my appreciation and special gratitude to Brigadier General U Ohn Myint (Minister, Ministry of Livestock Fisheries and Rural Development), U Khin Mg Aye, Dr. Aung Myat Oo, U Tin Ngwe (Deputy Ministers, Ministry of Livestock Fisheries and Rural Development), Colonel Dr. Myint Than (Director General, Livestock Breeding and Veterinary Department, Ministry of Livestock Fisheries and Rural Development), Prof. Dr. Myint Thein (formerly Director General, LBVD), Dr. Khin Zaw (Deputy Director General, LBVD), Dr. Win Myint (Director, Animal Health and Development Section), Dr. Khin Mg Aye (Deputy Director, Animal Health and Development Section), Dr. Khin Mg Oo and Dr. Yin Yin San (Assistant Directors, Artificial Insemination and Research and Development Section) for allowing me to pursue this postgraduate programme. My thanks to all my colleagues in department for their kind takeoverover my duties during my postgraduate study.

Grateful acknowledgement and sincere appreciation are extended to Puan Fauziah Nordin, staff of Veterinary Public Health Laboratory and all of my lab mates, Rasheed, Emelia, Teguh, Dauda Goni, Abdelrahman, Yousif, and Jalo for sharing their knowledge and experience with me, for their generous help and kindness which enabled me to finish my project smoothly. In addition, I am indeed thankful to Krish and all the staff of Veterinary Bacteriology laboratory for their kindness, guidance, teaching and generous helps to finish the project of my research. My sincere thanks to all colleagues, staff of the faculty who contributed one way or another towards completion of my study.

Last but not least, I express the most gratitude to my beloved parents, my sisters, my brother and my parent-in-laws for their love, understanding, encouragement, support and affection. Special thanks to my friend Khin Thida Khair who give me endless patience and care during the time stay together in Malaysia and also my sister-in-laws Lei Lei Swe and Toe Toe Lwin who take care of my daughter when I was studying. My deepest gratitude goes to my dearest and nearest: my husband for his endless love and encouragement, and also to my daughter for being understanding when Mum was working and not around as much as you were previously used to. Without their support, surely I would not be able to give attention on my studies.
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:

**Jalila Abu, PhD**  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Chairman)

**Abdul Aziz Saharee, PhD**  
Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Internal Examiner)

**Abdul Rahim Mutalib, PhD**  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Internal Examiner)

**Najiah Musa, PhD**  
Associate Professor  
Faculty of Agrotechnology and Food Science  
Universiti Malaysia Terengganu  
(External Examiner)

---

**NORITAH OMAR, PhD**  
Associate Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia  

**Date:**
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:

**Saleha Bt Abdul Aziz, PhD**  
Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Chairman)

**Zunita Zakaria, PhD**  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

**Murugaiyah Marimuthu, PhD**  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

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Signature: ___________________________  Signature: ___________________________
Name of Chairman of Supervisory Committee: Saleha Bt Abdul Aziz, PhD
Name of Member of Supervisory Committee: Zunita Zakaria, PhD

Signature: ___________________________
Name of Member of Supervisory Committee: Murugaivah Marimuthu, PhD
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<td>5.1</td>
<td>From Farm to Table: Risk of <em>Campylobacter</em> Infection</td>
<td>59</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

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