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QUANTITATIVE RISK ASSESSMENT OF Campylobacter AND ITS BIOLOGICAL CONTROL OF FOODBORNE PATHOGENS

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By

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Campylobacter foodborne diseases are a major current public health challenge faced by the world and often *Campylobacter* infections are under reported. Furthermore, reduced efficacy of antibiotics against bacterial pathogens has complicated the status of foodborne pathogens. In Malaysia, foodborne infections show an upward trend and multidrug-resistant foodborne pathogens were reported in the country. Therefore, estimation of risk and developing new alternatives for antibiotics are greatly concerned. In view of all these, the aims of this study were to determine the prevalence and concentration of *Campylobacter* in beef food system at farm and retail level using most probable number (MPN)-multiplex Polymerase Chain Reaction (PCR) method. *Campylobacter* isolates were characterized based on the antibiotic susceptibility pattern. Additionally, the multilocus sequence typing (MLST) was used for the first time in Malaysia for molecular characterization of *Campylobacter*. A microbiological quantitative risk assessment was conducted to estimate the potential of human campylobacteriosis in Malaysia with consumption of contaminated beef. A risk assessment model was developed based on the framework proposed by the Codex Alimentarius Commission, created in an Excel spreadsheet and was simulated using @Risk, version 5.5 (Palisade, USA). Correspondingly, bacteriophages were isolated to biocontrol *Campylobacter*. All isolated bacteriophages were characterized based on the morphology, host range, physiochemical properties and type of nucleic acid. Consequently, the efficacy of the isolated phages were assessed on respective host pathogens on chicken and beef meat to determine the applicability of those as bio-control measures. The prevalence of *Campylobacter* spp. in cattle was 33%, while raw beef purchased from local wet markets and hypermarkets were contaminated at 14.2% and 7.5% respectively. The most prevalent *Campylobacter* species isolated from both cattle and beef were *Campylobacter jejuni* (58.57%), the remainder was *Campylobacter coli* (27.14%) and other *Campylobacter* species (14.28%). The microbial load of *Campylobacter* on cattle and beef ranged from 3-436 MPN/g and 3-75 MPN/g

respectively. The *Campylobacter* isolates were highly resistant to tetracycline (76.9%) and ampicillin (69.2%), whereas was susceptible to gentamicin (84.6%) and chloramphenicol (92.4%). Notably, multidrug resistance (MDR) was apparent in 53.8% of the isolates. The MLST indicated that *C. jejuni* and *C. coli* sequence types associated with human infections. The developed model simulated the contamination of beef with *Campylobacter* from the retail to table continuum. The model indicated that the probability of contamination of beef with *Campylobacter* species ranged from 0.08 to 0.14 within a 90% confidence interval. According to the model prediction, 483 cases per 100, 000 population were estimated due to consumption of contaminated beef while 0.06 cases per 100, 000 population was associated with inadequately cooked beef. The sensitivity analysis indicated that domestic food safety measures such as cross contamination, insufficient cooking temperature and washing beef can increase the risk of human campylobacteriosis. Five different virulent bacteriophages namely ØEC1, ØEC2 (*E. coli*), ØLM3 (*L. monocytogenes*), ØMRSA1 (*S. aureus*) and ØCC1 (*C. jejuni* and *C. coli*) were isolated from various food commodities. The samples harboured $8.1 \times 10^{10} \pm 4.02$ to $4.7 \times 10^{2} \pm 1.04$ PFU/ml phages concentration. Morphological characteristics observed through the transmission electron microscopy (TEM) revealed that four of the isolated bacteriophages (ØEC1, ØLM3, ØMRSA1, and ØCC1) belonged to family *Myoviridae* while ØEC2 belonged to the *Podoviridae* family. The optimal multiplication of infection was observed at 0.01 for ØEC1, ØLM3, ØMRSA1 and ØCC1 bacteriophages while 0.001 for ØLM3 phage. One-step growth kinetics of the bacteriophages showed a latent period of 15 -55 mins and 10-40 mins eclipse period. The burst size of isolated bacteriophages ranged 29-185 for phage particles/infected cell. Bacteriophages ØEC1, ØMRSA1, and ØCC1 were stable between pH 5-9. However, limited pH stability was observed for ØLM3 (pH 6-8) and ØEC2 (pH 7-8). The phage ØCC1 was able to survive up to 40 °C temperature while the remainder was stable at 4-37 °C. In the efficacy study, isolated bacteriophages significantly (2-3 $\log_{10}$ CFU/mL, $P \leq 0.05$) decreased numbers of *E. coli, L. monocytogenes*, and *S. aureus* on chicken and beef after 3 h of application. Following ØCC1 phage application to *C. jejuni* and *C. coli* contaminated chicken and beef, a significant (2-3 $\log_{10}$ CFU/mL, $P \leq 0.05$) reduction was observed after 12 h. The host bacterial decline persisted during the experimental study and none of the host pathogens recuperated into the initial contamination levels. In conclusions, this study emphasis on the high potential risk areas to be concerned in implementing food safety decisions and provides the basis for future experiments in the utilization of bacteriophages as biocontrol agents in the food industry.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENILAIAN RISIKO KUANTITATIF OF *Campylobacter* DAN BACTERIOPHAGES KAWALAN BIOLOGI MENGGUNAKAN ITS

Oleh

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Penyakit bawaan makanan merupakan cabaran besar terkini dalam kesihatan awam yang dihadapi oleh dunia. Walau bagaimanapun, seringkali jangkitan bawaan makanan tidak dilaporkan, dan agen-agen penyebab kepada 50-60% jangkitan bawaan makanan ini tidak dikenalpasti. Tambahan pula, pengurangan keberkesanan antibiotik-antibiotik terhadap patogen-patogen bakteria telah merumitkan status patogen-patogen bawaan makanan. Di Malaysia, jangkitan bawaan makanan menunjukkan kecenderungan peningkatan. Sebagai tambahan, patogen-patogen bawaan makanan yang rintang terhadap pelbagai ubat telah dilaporkan dalam negara ini. Oleh itu, penaksiran risiko dan pembangunan alternatif-alternatif antibiotik-antibiotik baru menjadi kepentingan utama. Memandangkan kesemua ini, tujuan kajian ini adalah untuk menentukan kelaziman dan penumpuan *Campylobacter* dalam sistem makanan daging di ladang dan aras runcit menggunakan kaedah nombor paling barangkali (MPN)- tindak balas berantai polimerase (PCR) multipleks. Pencilan-pencilan *Campylobacter* telah dicirikan berdasarkan pola kerentanan antibiotik. Tambahan lagi, penjenisan jujukan multilokus (MLST) telah digunakan buat pertama kali di Malaysia untuk pencirian molekular *Campylobacter*. Suatu penilaian risiko kuantitatif mikrobiologi telah dijalankan bagi menganggarkan potensi kampilobakteriosis manusia di Malaysia akibat daripada pemakanan daging yang tercemar. Penilaian risiko tersebut telah dibangunkan berdasarkan rangka kerja yang dicadangkan oleh Suruhan Jaya Codex Alimentarius, diwujudkan di dalam lembaran Excel dan disimulasi menggunakan @Risk, versi 5.5 (Palisade, USA). Berpadanan dengan itu, bakteriofaj-bakteriofaj telah dipencilkan sebagai biokawalan patogen-patogen bawaan makanan yang biasa seperti *Campylobacter, E. coli, Listeria*, dan *S. aureus*. Kesemua bakteriofaj-bakteriofaj yang telah dipencilkan dicirikan berdasarkan morfologi, julat perumah, sifat-sifat fisiokimia dan jenis asid nukleik. Oleh yang demikian, keberkesanan faj-faj yang telah dipencilkan telah ditaksirkan ke atas patogen-patogen perumah masing-masing pada daging bagi menentukan kebolehgunaan langkah-langkah biokawalan tersebut. Kelaziman *Campylobacter* spp. dalam ternakan lembu adalah agak tinggi (33%), manakala daging

mentah yang dibeli dari pasar basah tempatan dan pasar raya besar telah dicemari sebanyak 14.2% dan 7.5% masing-masing. Spesies *Campylobacter* yang paling lazim dipencilkan dari kedua-dua lembu dan daging adalah *Campylobacter jejuni* (58.57%), manakala yang selebihnya adalah *Campylobacter coli* (27.14%) dan spesies *Campylobacter* yang lain (14.28%). Beban mikrob *Campylobacter* pada lembu dan daging berada dalam lingkungan 3-436 MPN/g dan 3-75 MPN/g masing-masing. Pencilan-pencilan *Campylobacter* mempunyai kerintangan tinggi terhadap tetrasiklin (76.9%) dan ampisilin (69.2%), manakala rentan terhadap gentamisin (84.6%) dan kloramfenikol (92.4%). Lebih ketara, kerintangan terhadap pelbagai ubat (MDR) adalah jelas dalam 53.8% daripada pencilan-pencilan tersebut. MLST menunjukkan bahawa jenis-jenis jujukan *C. jejuni* dan *C. coli* adalah berkaitan dengan jangkitan manusia. Model yang telah dibangunkan mensimulasi pencemaran daging oleh *Campylobacter* dari jualan ke hidangan yang selanjar. Model tersebut menunjukkan bahawa kebarangkalian pencemaran daging dengan spesies *Campylobacter* adalah 0.08 dan 0.14 masing-masing. Berdasarkan ramalan model tersebut, pelbagai 483 per 100, 000 kes-kes populasi dianggarkan akibat pemakanan daging yang kurang masak. Analisis kepekaan menunjukkan penumpuan *Campylobacter* pada daging dan langkah-langkah keselamatan makanan rumah seperti pencemaran silang, suhu memasak yang tidak mencukupi dan masa meningkatkan risiko kampilobakteriosis manusia. Lima bakteriofaj-bakteriofaj virulen yang berbeza iaitu ØEC1, ØEC2 (*E. coli*), ØLM3 (*L. monocytogenes*), ØMRSA1 (*S. aureus*) dan ØCC1 (*C. jejuni* dan *C. coli*) telah dipencilkan daripada pelbagai jenis barangan makanan. Sampel-sampel tersebut mempamerkan $8.1 \times 10^{10} \pm 4.02$ to $4.7 \times 10^{2} \pm 1.04$ penumpuan faj-faj. Ciri-ciri morfologi yang dicerap melalui mikroskopi elektron transmisi (TEM) memperlihatkan bahawa empat daripada bakteriofaj-bakteriofaj yang telah dipencilkan itu (ØEC1, ØLM3, ØMRSA1, dan ØCC1) tergolong dalam famili *Myoviridae* manakala ØEC2 tergolong dalam famili *Podoviridae*. Penggandaan jangkitan yang optima telah dicerap pada 0.01 bagi faj-faj ØEC1, ØLM3, ØMRSA1 dan ØCC1 manakala 0.001 bagi faj ØLM3. Kinetik satu-langkah faj-faj tersebut menunjukkan tempoh pendam selama 15 - 55 min dan 10-40 min tempoh gerhana. Saiz letusan bakteriofaj-bakteriofaj yang telah dipencilkan adalah dalam lingkungan 29-185 bagi partikel faj/sel dijangkiti. Baktriofaj-bakteriofaj ØEC1, ØMRSA1, dan ØCC1 adalah stabil antara pH 5-9. Walau bagaimanapun, kestabilan pH yang terhad dicerap bagi ØLM3 (pH 6-8) dan ØEC2 (pH 7-8). Faj ØCC1 mampu bertahan sehingga suhu 40 °C manakala yang selebihnya stabil pada 4-37 °C. Dalam kajian keberkesanan, bakteriofaj-bakteriofaj yang dipencilkan telah mengurangkan dengan nyata (2-3 $\log_{10}$ CFU/mL, $P \leq 0.05$) bilangan *E. coli*, *L. monocytogenes*, dan *S. aureus* pada ayam dan daging selepas 3 jam penerapan. Berikutan penerapan faj ØCC1 pada ayam dan daging yang telah dicemari *C. jejuni* dan *C. coli*, pengurangan yang ketara (2-3 $\log_{10}$ CFU/mL, $P \leq 0.05$) telah dicerap selepas 12 jam. Setelah semua perkara diteliti, penolakan bakteria perumah didapati berterusan sepanjang kajian eksperimen dan tiada patogen-patogen perumah pulih kepada tahap pencemaran yang asal. Sebagai kesimpulan, kajian ini menekankan ruang-ruang berpotensi tinggi yang perlu diberi kepentingan dalam melaksanakan keputusan-keputusan keselamatan makanan dan menyediakan asas bagi eksperimen-eksperimen akan datang dalam penggunaan bakteriofaj-bakteriofaj sebagai agen biokawalan dalam industri pemakanan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AGST	Antigen Gene sequence Typing
ATCC	American Type Culture Collection
Ø	bacteriophage
BHI	Brain heart infusion
CCLS	National Committee for Clinical Laboratory Standards
CDC	Centres for Disease Control and Prevention
CFU	Colony Forming Units
cm	centimeter
cm^2	square centimeter
°C	Degree Celsius
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	deoxynucleotide triphosphates
DVS	Department of Veterinary Services, Malaysia
ETDA	ethylenediaminetetra-acetic acid
FAO	Food and Agricultural Organization
FDA	Food Drug Administration
g	centrifugal force in gravity
g	gram
h	hour
ICMSF	International Commission on Microbiological Specifications for Foods

ISO	International Organization for Standardization
kb	kilobasepair
kb	kilo basepair
kg	kilogram
LB	Luria-Bertani
M	molarity
MAR	Multiple Antibiotic Resistance
mCCDA	modified Ceferoperazone-Charcoal Deoxycolate agar
MDR	Multidrug Resistance
min	minute
mL	milliliter
MLST	Multi Locus Sequence Typing
MoH	Ministry of Health
MOI	Multiplicity of Infection
MPN	most-probable-number
MRSA	methicillin-resistant *S. aureus*
NaCl	sodium chloride
NaHCO3	sodium bicarbonate
nm	nanometer
NZCYM	New Zealand Casamino Yeast Medium
OD	optical density
%	Percentage(s)
PBS	phosphate buffer saline

PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque Forming Unit
pH	*Puissance hydrogene*
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolution per minute
SM	Salt magnesium
spp.	species
ss	single stranded
ST	Sequence type
SVR	Short Variable Region
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
Tris-HCl	Tris-hydrochloric acid
TSA	Tryptic Soy agar
UV	ultraviolet
V	volts
v/v	volume per volume
v/w	weight per volume
VBNC	Viable but Non-Culturable

WHO	World Health Organization
WTO	World Trade Organization
XLD	Xylose Lysine Deoxycholate
µg	microgram
µl	microliter
µm	micrometer

CHAPTER 1

INTRODUCTION

1.1. Background

Foodborne diseases have become a key public health and economic issue in the world. Diarrhoeal diseases occur due to foodborne infections account for the majority of illnesses and deaths in the world, also identified as one of the leading causes of deaths in young children (Havelaar *et al*., 2015). The burden of foodborne diseases has reported being higher in the low-income countries comparative to high-income countries, leading to 1400 disability-adjusted-life-years (DALYs) per 100,000 population in low-income South-East Asian subregions and 35 DALYs per 100,000 population in high-income North American countries (WHO, 2015). According to the WHO estimates an average of 600 million foodborne infections leading to 420,000 deaths occur worldwide (Hoffman & Scallan, 2017).

Campylobacter has been identified as the most commonly reported cause of foodborne gastroenteritis (Havelaar *et al*., 2015) resultant in 96 million (95% UI 52–177 million) foodborne diseases (Kirk *et al*., 2015). Since 1990s incidence rate of human campylobacteriosis has gradually increased and continues to grow despite stringent control measures in place (WHO, 2012). Increased incidence of campylobacter cases has been identified accounting for 112 cases per 100,000 in Australia, 30-50 cases per 100,000 in Europe, 1512 cases per 100,000 in Japan and 14-50 cases per 100,000 in North America ranged (Connerton & Connerton, 2017). The majority of the human *Campylobacter* cases are sporadic, thus difficult to diagnose or trace back to the source of infection (WHO, 2012). However, *Campylobacter* outbreaks are also not infrequent (Crim *et al*., 2015; Tam *et al*., 2012). *Campylobacter* transmits through ingestion of contaminated food and water (Kaakoush *et al*., 2015; Jacobs-Reitsma *et al*., 2008). Consumption and handling of contaminated poultry remain as the principal source of human campylobacteriosis (Havelaar *et al*., 2005). While meat, vegetables, processed foods, raw milk, raw dairy products and contaminated water also have been recognised as risk factors for human *Campylobacter* infection (Kaakoush *et al*., 2015; Nachamkin *et al*., 2008). The majority of the human *Campylobacter* outbreaks have been reported due to consumption of raw milk (Mungai *et al*., 2015; Heuvelink *et al*., 2009; Peterson *et al*., 2003; CDC, 2002) and contaminated water (DeFraites *et al*., 2014; CDC, 2013; Pitkänen, 2013). While to a lesser degree through consumption of undercooked poultry (Moffatt *et al*., 2016; Scott *et al*., 2015; Edwards *et al*., 2014).

In Malaysia, *Campylobacter* spp. have isolated from various food commodities including chicken meat, duck meat, vegetables and sushi (Nor Faiza *et al.*, 2013; Tang *et al.*, 2009; Chai *et al*., 2007). More than 80% of chicken samples were contaminated with *Campylobacter* spp. and *C. jejuni* was the most frequently isolated species (Tang

et al., 2009). Saleha (2004) and Huat *et al.* (2010) reported a high prevalence of *Campylobacter* in broiler birds.

In spite various food safety measures containing good hygiene practice (GHP), good manufacturing practice (GMP), and implementing hazard analysis critical control points (HACCP); still the incidence of foodborne infections tend to increase due to incorrect practices and/or poor application of these safety measures (Forsythe, 2002). Conducting a food safety risk assessment can estimate the risk associated with a food commodity and identify the most suitable approaches that could be implemented to decrease the impact of a foodborne disease (FAO/WHO, 2005). Through conducting a risk assessment can understand hazards and associated risk thereby can identify the level of activities that should be carried out at national and industry level to enhance food safety and quality (FAO/WHO, 2006). Usage of risk assessment is becoming an internationally accepted method to control biological hazards in food and ensure food safety (FAO/WHO, 2005). The World Trade Organization (WTO), the World Health Organization (WHO), the Codex Alimentarius, and the Food and Agricultural Organization of the United Nations (FAO) promote usage of risk assessment because it provides an integrated approach for controlling food safety problems (CAC, 1999).

Reduce effectiveness of antibiotics for pathogenic bacteria including *Campylobacter* have become a significant global public health risk (Laxminarayan *et al*., 2013). Excess usage of antibiotic in food production industry has led to the development of resistance in bacteria (Kemper, 2008). Further, various preservation techniques used in the food production system may have accelerated the emergence and spreading of resistance in foodborne pathogens (Walsh & Fanning, 2008). Despite effective disease control programmes, a widespread resistance reported in both Gram-negative and positive bacteria (Carlet *et al*., 2012). Annually around 25,000 deaths (ECDC/EMEA, 2009) and 90,000 illnesses (APUA, 2010) reported due to antibiotic resistant bacteria in Europe and the USA respectively. Therefore, to combat foodborne diseases and to control contamination of food with resistant bacteria, effective stratagems are necessary (Gálvez *et al*., 2010; García *et al*., 2010). From the consumer perspectives, there is a high demand for healthy food free of harmful synthetic chemicals (Sillankorva *et al*., 2012). Bacteriophages (phages) are bacterial viruses which have high specificity towards the host hence can infect and lyse the pathogenic bacteria without destroying the natural microflora (Sillankorva *et al*., 2012; Gálvez *et al*., 2010; García *et al*., 2010). These characters make bacteriophages as a good alternative and bio-control agent that can be used to prevent contamination of food with foodborne pathogens (Hagens & Loessner, 2010).

1.2. Problem of Statement

Although the prevalence of *Campylobacter* has been assessed in various food commodities in Malaysia; no studies have determined the prevalence of *Campylobacter* spp. in beef or the associated risk factors. Further, beef is a staple food in Malaysia (Ariff *et al*., 2015) that has 6.74 kg annual per capita consumption in the year 2013 (DVS,

2015). The National surveillance system of Malaysia reports an upward trend for foodborne infections (MoH, 2016). However, *Campylobacter* infections are not included in the surveillance network which hinders assessing the true burden of campylobacteriosis in the country. Lack of fundamental quantitative microbiological risk assessment on *Campylobacter* with considering various steps in the food productions system in the country presents a major challenge to decision makers in industry and public health officers (Forsythe, 2002). Further, novel effective strategies that do not contain any harmful synthetic chemicals are required to control contamination of food with resistant bacteria (Gálvez *et al*., 2010; García *et al*., 2010).

Therefore, the current study focuses on determining the prevalence of *Campylobacter* spp. in cattle and beef meat, assess the microbiological risk associated with *Campylobacter* in beef by developing a quantitative risk assessment model from retail to consumption and biocontrol of *Campylobacter* using bacteriophages. The hypothesis of this study is that determining the prevalence of *Campylobacter* spp. in the beef food system and conducting a quantitative microbiological risk assessment will enable to assess the risk of developing campylobacteriosis. Secondly, isolation and characterising a bacteriophage effective against *Campylobacter* will provide a potential biocontrol agent to prevent contamination of food with foodborne pathogens. Based on the above hypotheses, the objectives of this study are;

1.3. Objectives

1) To quantify and determine the prevalence of *Campylobacter* spp. in the beef food system and to characterise the *Campylobacter* isolates based on antibiotic resistance and molecular strain type.
2) To conduct a quantitative microbiological risk assessment for *Campylobacter* in beef.
3) To biocontrol *Campylobacter* using bacteriophages.

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