UNIVERSITI PUTRA MALAYSIA

COMPARATIVE STUDY ON ISOLATION TECHNIQUES AND CHARACTERIZATION OF Helicobacter pullorum IN BROILER CHICKENS (Gallus sp.) AND THEIR FARM ENVIRONMENT IN SELANGOR, MALAYSIA

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By

SOE SOE WAI

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy.

July 2012

DEDICATION

This thesis is specially dedicated to:

My beloved parents,

U HLA AUNG

and

DAW TIN MYAING

My beloved husband and daughter,

KYAW SWAR TUN
SU YEE LIN

Who always supported and encourage me to do the best.

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfilment of the requirement for the Degree of Doctor of Philosophy.

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July 2012

Chairman: Prof. Saleha Abdul Aziz, PhD

Faculty : Veterinary Medicine

Helicobacter pullorum is becoming important as an emerging zoonotic pathogen. It has been isolated from poultry in many countries but reports are lacking in developing countries, including Malaysia. *Helicobacter pullorum* is a fastidious organism, and not readily culturable. They are generally misdiagnosed as *Campylobacter* and currently are identified by molecular method. This study was conducted to determine the appropriate isolation method, the prevalence of *H. pullorum* in broiler chickens and farm environment, antibiotic resistance and plasmid profiles of *H. pullorum* isolated and to characterize the *H. pullorum* isolates. The first part of the study showed 14 of 57 (24.6%) chickens from five farms were positive for *H. pullorum* of which 8 (14%) were co-colonized with *Campylobacter* species. In this study, three methods reported by other researchers were used (Method I, II & III) that is, based on Ceelen et al. (2006c) as method I, Zanoni et al. (2007) as method II and Miller et al. (2006) as method III. In two methods (method I & II), discrete colonies of *H. pullorum* were not obtained but were mixed with *Campylobacter* species, while (one) method III gave both discrete colonies of *H. pullorum* and mixed

colonies. In terms of better isolation, ease of preparation and recovery of pure isolates, this (Method III) third method was the method of choice. To improve the *H. pullorum* isolation percentage, a further modification was done to this Method III by adding an enrichment step and modifying the incubation temperature. Using this modified method (Method IV), 24 of 30 (80.0%) chickens from three farms were positive for *H. pullorum* while only 17 of 30 (56.7%) were positive using Method III. Method IV gave a better recovery of *H. pullorum* from chicken caecal contents. The overall prevalence in the second part of the study showed *H. pullorum* was present in 51 % broilers using method IV alone in chickens from another ten farms, eight open-house farms and two close-house farms. From a total of 18 farms, 89 out of 187 (47.6%) chickens in which *H. pullorum* were isolated. Among them, 15 (8.0%) were co-colonized with *Campylobacter* spp. From environmental samples, all water samples were negative for *H. pullorum* while flies (17.5 %) and floor swab (30 %) samples were positive for *H. pullorum* and *Campylobacter* species, which are the risk factors for *H. pullorum* in chickens. The *H. pullorum* isolates were subjected to antibiotic susceptibility test using diffusion technique and M.I.C Evaluator method (M.I.C.E). All isolates were sensitive to polymyxin B but resistant to cephalothin. The pattern of resistance of the *H. pullorum* isolates to ciprofloxacin was 65.5% and 66.7%, and erythromycin 54.5% and 38.9%, cefotaxime 50.9% and 55.5%, ampicillin 25.5% and 38.9%, tetracycline 21.8% and 16.7%, and to gentamicin 10.9% and 5.6% by disc diffusion method and M.I.C.E, respectively. The overall difference in frequency of resistance was not significant between the two methods. The isolates showed 19 different antibiograms and were resistant to were ciprofloxacin and cefotaxime. Plasmids were detected in 12 out of 53 (22.6 %) antibiotic resistant isolates. Each isolate harboured two plasmids and the size ranged

from 2.2 to 54 kb. The presence of plasmids in *H. pullorum* isolates did not correlate with the antibiotic resistance pattern. This study also showed that *H. pullorum* isolates were multi-resistant to four or more antibiotics at 18.2% and 11.1% as observed by disc diffusion method and M.I.C. Evaluator strips, respectively. From pulse field gel electrophoresis (PFGE), all 25 field isolates from eight different farms had distinct genotypes and most strains showed a high degree of genetic diversity. All isolates from different farms showed different fragment patterns; some were closely related while some were heterogeneous in the same farms. Thus, *H. pullorum* colonization in a farm may occur with a single strain that disseminated in the same flocks; clonal relationship may derived from the same sources and heterogeneous in isolates may be distributed from a common source of clonal origin. The PFGE analysis using *Sac*II analysis was more discriminatory than using *Sma*I. To the author's knowledge, this is the first study on *H. pullorum* in chickens in Malaysia. It is concluded from this study that *H. pullorum* is prevalent in chickens in the farms. The organisms may contaminate the carcasses during processing and at retailing and may be transmitted to humans through consumption of undercooked chicken meat or cause cross contamination of ready-to-eat food. The detection of multiantibiotic resistant isolates poses a threat to humans and further limits therapeutic options. Thus, with the increase in production of chicken meat, the contamination of such meat with foodborne pathogens including with *H. pullorum* is of considerable concern with regards to public health. Hence, it is recommended that farmers and processing operators be made aware of this and other foodborne pathogens that may be found in chickens. Therefore, the farmers need to adhere to good animal husbandry practices (GAHP) and meat processing operators to good manufacturing procedures (GMP).

Abstrak tesis dikenukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi saperluan untuk ijazah Doktor Falsafah

KAJIAN KOMPARATIF TEKNIK PEMENCILAN DAN PENCIRIAN *Helicobacter pullorum* PADA AYAM PEDAGING (*Gallus* sp.) DAN DI PERSEKITARAN LADANG DI SELANGOR, MALAYSIA

Oleh

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Julai 2012

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Helicobacter pullorum semakin dikenali sebagai patogen zoonotik yang baru muncul. Organisma tersebut telah diasingkan daripada unggas di banyak negara tetapi laporan mengenainya adalah kurang di negara membangun, termasuk Malaysia. *Helicobacter pullorum* adalah sukar di kultur. Mereka mudah disalah diagnosis sebagai *Campylobacter* dan pada masa ini dikenalpasti dengan mengguna kaedah molekular. Kajian dilakukan untuk menentukan kaedah pemencilan, kadar prevalens *H. pullorum* pada ayam pedaging dan persekitaran ladang, kerintangan antibiotik dan profil plasmid isolat *H. pullorum* dan pencirian *H. pullorum.* Bahagian pertama kajian menunjukkan 14 daripada 57 (24.6 %) ayam di lima ladang didapati positif yang mana 8 (14 %) dijangkiti bersama dengan *Campylobacter*. Pada kajian ini, tiga kaedah oleh penyelidik lain telah digunakan (Kaedah I, II dan III) iaitu berdasarkan kaedah Ceelen et al. (2006c) sebagai kaedah I, Zanoni et al. (2007), sebagai kaedah II dan Miller et al. (2006) sebagai kaedah III. Koloni diskrit *H. pullorum* tidak diperolehi dalam kaedah I dan II tetapi bercampur dengan *Campylobacter* spesies. Dalam hal pemencilan yang lebih baik, kemudahan

persiapan, dan perolehan isolat murni, kaedah III adalah kaedah pilihan. Untuk meningkatkan kadar pemencilan *H. pullorum*, pengubahsuaian lanjutan dilakukan ke atas kaedah III dengan menambah langkah pengkayaan dan diubahsuai suhu pengeraman (kaedah IV). Didapati *H. pullorum* dikesan pada 24 daripada 30 (80.0%) ayam daripada tiga ladang dengan mengguna kaedah IV sedangkan 17 daripada 30 (56.7%) menggunakan kaedah III didapati positif. Kaedah pemencilan yang diubah dalam kajian ini memberikan pemencilan *H. pullorum* yang lebih baik daripada kandungan sekum ayam. Kajian ini menunjukkan bahawa prevalens keseluruhan dalam bahagian ini *H. pullorum* hadir dalam 51 % ayam dengan hanya menggunakan kaedah IV pada ayam di sepuluh ladang lain (lapan mempunyai rumah tebuka dan dua dengan rumah tertutup). Daripada sejumlah 18 ladang, 89 daripada 187 (47.6 %) ayam dapat dipencil *H. pullorum*. Diantaranya 15 (8 %) dijangkiti bersama dengan *Campylobacter* spp. Daripada analisis sampel persekitaran, semua sampel air didapati negatif untuk *H. pullorum* dan sampel lalat (17.5 %) dan usapan lantai (30 %) menunjukkan jangkitan *H. pullorum* bersama *Campylobacter* spp. yang mungkin bertindak sebagai faktor risiko bagi *H. pullorum* pada ayam. Pola kerintangan antibiotik dilakukan pada isolat *H. pullorum* dengan menggunakan kaedah “disc diffusion” dan kaedah “M.I.C Evaluator” (M.I.C.E). Kadar rintangan isolat *H. pullorum* terhadap siprofloxacin adalah 65.5% dan 66.7%, eritromisin 54.5% dan 38.9%, cefotaxim 50.9% dan 55.5%, ampisilin 25.5% dan 38.9%, tetrasiklin 21.8% dan 16.7%, gentamisin 10.9% dan 5.6% masing-masing mengguna kaedah “disc diffusion” dan M.I.C.E. Perbezaan frekuensi kerintangan didapati tak signifikan antara kedua kaedah. Semua isolat adalah seusitif terhadap polimisin B tetapi tahan terhadap cefalotin. Isola *H. pullorum* menunjukkan 19 antibiogram yang berbeza dan antibiotik yang pada umumnya mereka tahan terhadap adalah ciprofloxacin dan

asid nalidixik. Plasmid dikesan pada 12 daripada 53 (22.6%) isolat yang tahan antibiotik. Setiap isolat mengandungi dua plasmid dan julat saiz antara 2.2 hingga 54 kb. Kehadiran plasmid dalam *H. pullorum* isolat tidak berkorelasi dengan pola kerintangan antibiotik. Kajian ini menunjukkan isolat *H. pullorum* didapati multitahan (keatas empat atau tebih antibiotik), 18.2 % (kaedah "disc diffusion") dan 11.1 % (M.I.C. Evaluator strips). Dari analisis "Pulse field gel electrophoresis" (PFGE), semua 25 isolat daripada lapan ladang mempunyai strain genotip yang berlainan dan kebanyakan menunjukkan kepelbagaian genetik yang tinggi. Ladang yang berbeza menujukkan pola serpihan yang berbeza. Ini adalah pertama kalinya sebuah kajian tentang *H. pullorum* pada ayam dilakukan di Malaysia. Dapat disimpulkan dari kajian ini bahawa *H. pullorum* adalah prevalen pada ayam di ladang. Organisma tersebut dapat mencemarkan daging ayam semasa pemprosesan dan dipasar dan boleh disebarkan kepada manusia melalui makan daging ayam yang kurang dimasak atau pencemaran silang keatas makanan siap dimakan. Pengesanan isolat multitahan antibiotik dapat menimbulkan ancaman bagi manusia dan dapat menghad pilihan terapeutik. Dengan meningkatnya pengeluaran daging ayam, pencemaran daging tersebut dengan patogen bawaan makanan termasuk dengan *H. pullorum* juga dapat meningkat yang merupakan faktor yang cukup prihatin berkaitan dengan kesihatan masyarakat. Oleh kerana itu, disarankan bagi penternak dan pemproses daging agar menyedari hal ini dan mengenai patogen bawaan makanan lain yang boleh ditemui dalam ayam. Oleh kerana itu, penternak harus mengikuti amalan-amalan ladang yang baik (GAHP) dan operator pemprosesan daging mengikuti amalan pemprosesan yang baik (GMP).

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I certify that a Thesis Examination Committee has met on 3 July 2012 to conduct the final examination of SOE SOE WAI on her thesis entitle "Comparative Study on Isolation Techniques and Characterization of *Helicobacter pullorum* in Broiler Chickens (*Gallus* sp.) and their farm Environment in Selangor, Malaysia." 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 Doctor of Philosophy. Members of the Examination Committee are as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that is has not been previously, and is not concurrently submitted for any other degree at Universiti Putra Malaysia or at other institution.

SOE SOE WAI

Date : 3 July 2012

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LIST OF ABBREVIATIONS

AFLP : Amplified fragment length polymorphism

ATCC : American Type Culture Collection

BSA : Bovine Serum Albumin

ºC : Degree Celcius

CCUG : Culture Collection University of Goteborg

CHEF : Clamped Homogeneous Electric Fields

CLSI : Clinical Laboratory Standard Institute

DDT : Disc diffusion test

DNA : Dexoribonucleic acid

DVS : Department of Veterinary Services

EDTA : Ethylenediaminetetraacetic acid

HACCP : Hazard critical control point

EHS : Enterohepatic Helicobacter s

FAO : Food and Agriculture Organization

h : Hour(s)

kg : Kilogram

L : Litre

LB : Lysis buffer

ml : mililitre

MDR : Multidrug resistant

M.I.C. : Minimum inhibitory concentration

MLST : Multilocus Sequence Typing

MPN-PCR: Most probable Numeration- Polymerase chain reaction

m-PCR : Multiplex polymerase chain reaction

NCCLS : National Committee for Clinical Laboratory Standards

PCR : Polymerase chain reaction

PFGE : Pulsed field gel electrophoresis

PFG : marker for pulsed field gel electrophoresis

PMSF : Phenylmethylsulfonyl fluoride

RAPD : Random amplified polymorphic DNA

RE : Restriction enzyme

RFLP : Restriction Fragment Length Polymorphism

rRNA : Ribosomal ribonucleic acid

RTqPCR: Real Time quantitive Polymerase chain reaction

SBA : Sheep blood Agar

TBE : Tris-borate-EDTA (TBE) buffer

TE : Tris-EDTA (TE) buffer

UPGMA : Unweighted pair group method arithmetic averages

uv : ultra violet

V : Volt

W/V : weight by volume

CHAPTER 1

INTRODUCTION

Food borne pathogens are of great concern with regard to consumer protection and food safety. In poultry and poultry products, the food borne pathogen such as *Salmonella* and *Campylobacter* spp., are of greatest global concern (Ducatelle et al., 2006). The past few years, *Helicobacter* and *Arcobacter* have been recognised as important threat to food safety as they have been identified as potential zoonotic food borne pathogens in raw poultry meat (Atabay et al., 1998 and Corry and Atabay, 2001).

Helicobacter pullorum was first isolated as a new species of *Helicobacter* from poultry and human faeces by Stanley et al. (1994) on the basis of 16S rRNA phylogenetic analysis. It is believed to be a zoonotic foodborne pathogen because it has been reported in various human cases of gastroenteritis (Stanley et al., 1994; Burnen et al., 1996; Atabay et al., 1998 and Young et al., 2000b; Steinbrueckner et al., 1997; Gibson et al., 1999; Melito et al., 2000; Kuijper et al., 2003 and Ceelen et al., 2005a) and its DNA has been detected in liver from patients with primary sclerosing cholangitis, cirrhosis and hepatocellular carcinoma (Fox et al., 1998; Ponzetto et al., 2000; Pellicano et al., 2004; Bohr et al., 2002; Bohr et al., 2004; Roacha et al., 2005; Castera et al., 2006; Casswall et al., 2010; Karagin et al., 2010).

Helicobacter pullorum is reported to colonize chicken flocks and is commonly isolated from the caecal contents (Ceelen et al., 2006c; Miller et al., 2006; Manfreda et al., 2006; Svobodova and Boribova, 2003 and Zanoni et al., 2007). Nuebuer and

Hess (2006); Ceelen et al. (2007) and Mohamed et al. (2010) emphasized that *H. pullorum* colonized the caeca of broilers and are excreted in their faeces. Upon processing, the organisms may contaminate the carcasses. *Helicobacter pullorum* is transmitted horizontally among chicken flocks. It has also been isolated from chicken carcasses (Atabay et al., 1998; Ceelen et al., 2006c; Mohamed et al., 2010; Gonzalez et al., 2008). Faeces from chickens containing *H. pullorum* may also contaminate eggs (Karima and Wallaa, 2010) and also possibly contaminate water (Azevedo et al., 2008), soil, litters as well as the environment (Graham et al., 2008). Thus, there is the possibility of transmission of *H. pullorum* from poultry and the environment to humans.

The isolation of *H. pullorum* from human faeces with gastroenteritis suggests it causes a foodborne disease; however, this has not been established because the period between consumption and development of symptoms makes it difficult to identify the source of infection (Tee et al., 2001). Of the known *Helicobacter* species, only *H. pullorum* has been reported to be transmitted from poultry to humans via consumption of undercooked chickens (Wesley, 2001; Karagin et al., 2010). However, it is still unclear if the organism had a causal role in infections of humans (Gibson et al., 1999). The mode of transmission may occur in many ways. Faecal-oral, oral-oral, gastro-oral, contaminated meat, food and water have been implicated as sources for enterohepatic *Helicobacter* species (EHS) infection in humans (Hegarty et al., 1999; Azevedo et al., 2008). The lower bowels are the natural reservoir of EHS, from where most commonly there but are isolates cultivated from faeces or mucosal biopsies from the colon or cecum; however, isolation from liver and gallbladder is also common. Thus, faecal-oral and gastro-oral

routes of transmission are most likely between poultry and human populations (Watson et al., 2004; Nayak and Rose, 2007; Azevedo et al., 2008).

The poultry industry is one of the largest and fastest growing agro-based industries in the world. This can be attributed to an increasing demand for poultry meat and egg products. In the last decade, the consumption of poultry meat has tremendously increased worldwide. This is because poultry products are a major source of protein which are easily available and acceptable to most or a majority of the society in any country worldwide. The Ninth Malaysia Plan (9MP) (2006-2010) projected the poultry industry to grow at an average rate of 5.7% per annum to meet the demand for broiler meat. The industry has grown at 5.95% annually with production increasing from 724,300 to 1.202 million metric tonnes from year 2000 to 2009, with total ex-farm value of RM5.468 billion or 53% of ex-farm value of the livestock industry. The total export of chicken products increased from RM54.44 million in 2007 to RM350.68 million in 2009. In addition, 510 million metric tonnes of eggs were produced in 2009 with a total ex-farm value of RM2.226 billion or 22% of ex-farm value of the livestock industry (Idris and Rahim, 2010). Thus, poultry industry in Malaysia has played its part in contributing to the realization of agriculture as one of the engines of growth and creation of a high income economy.

Chickens and their products are commonly consumed in modern Malaysian diets. Malaysia has one of the highest per capital consumption rates in the world for chicken at 34.7 kg (Idris and Rahim, 2010). As the production of poultry meat increases, contamination of such meat with foodborne pathogens is a considerable factor of concern with regards to public health. The high prevalence of

Campylobacter in poultry flocks in Malaysia is regarded as one of the main sources of infection for humans (Saleha et al., 2003), however, in comparison, data on *Arcobacter* and *Helicobacter* are very limited. Saleha et al. (2007) reported the contamination rate of *Arcobacter* spp. in chicken meat was 38.2% with warm chicken meat at 41.0% and chilled meat at 35.5% which was significantly higher than previous report (22%) in Malaysia. Amare et al. (2010) reported that the prevalence of *Arcobacter* spp. in chicken meat was 39.02% but did not find the bacteria in chickens in the farms. From very limited studies, it was reported that *H. pullorum* are increasingly recognized in poultry and poultry products and these organisms showed multiple antibiotic resistance (Mohamed et al., 2010; Karima and Wallaa, 2010).

The common characteristic features of members belonging to three related genera, namely *Campylobacter, Arcobacter* and *Helicobacter,* are their fastidious growth requirements and the fact that they are biochemically relatively inert. As a consequence, isolation and identification is often a time–consuming and cumbersome process. Moreover, limited data are available on *Arcobacter* and *Helicobacter* in poultry flocks and poultry products. There is no ideal method for the isolation of *Helicobacter* species (On et al., 2005) and the accurate identification of these bacteria is known to be a difficult task (On et al., 1996). *Helicobacter pullorum* is a urease negative organism with a monopolar, unsheathed flagella, sensitive to polymyxin B and nalidixic acid and often shared colonised sites with *Campylobacter* in poultry. Therefore, *H. pullorum* may be easily misidentified as *Campylobacter* species, in particular *C. coli* and *C. lari,* with which several key phenotypic traits are shared (Atabay et al., 1998 and Kuijper et al., 2003). Appropriate measures are

therefore required to reduce or minimize infection of broiler chickens during production, processing and distribution. In the broiler production stage, there is a need for a better understanding of the epidemiology of *H. pullorum* infection in chickens and its ecology in farm practices. Transmission mechanisms of *C. jejuni* have been well established and it may be similar for *Helicobacter* and *Arcobacter* spp. because of their phylogenetic proximity. A number of methods have been described for the detection of *H. pullorum* in chickens. Standardized isolation and identification procedures are needed to determine important clinical and epidemiological information of *H. pullorum* in chickens. The occurrence of *H. pullorum* has been reported in countries such as, Belgium, Italy, Australia, UK, USA, Switzerland, Czech Republic, Egypt and France (Ceelen et al., 2006c; Zanoni et al., 2007; Miller et al., 2006; Atabay et al., 1998; Stanley et al., 1994; Burnen et al., 1994; Svobodova and Boribova, 2003; Mohamed et al., 2010 and Pilon et al., 2005), although the information is limited. There is a lack of data on the prevalence of *H. pullorum* in poultry in Malaysia.

Understanding the modes of transmission of *Helicobacter* infection is essential for developing measures to control the spread of infection in the farms. It was hoped that a study on *H. pullorum* may provide the information to control the organism at the farm level to retailing. Such data will have public health benefits in relation to reducing potential human exposure associated with the handling and consumption of contaminated meat.

Therefore, the objective of this study was:

(1) to compare and develop on the isolation method for *H. pullorum* in chickens

(2) to determine the occurrence of *H. pullorum* in chickens in the farms and their environment

(3) to study the antibiotic resistance pattern and plasmid profile of *H. pullorum* isolates and

(4) to characterize *H. pullorum* isolates using PFGE genotyping.

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