



**UNIVERSITI PUTRA MALAYSIA**

***OCCURRENCE OF EXTENDED SPECTRUM BETA-LACTAMASE  
PRODUCING *Escherichia coli* IN DAIRY CATTLE,  
FARM ENVIRONMENT AND MILK***

**EMELIA AINI KAMARUZZAMAN**

**FPV 2015 14**



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FARM ENVIRONMENT AND MILK**

By

**EMELIA AINI KAMARUZZAMAN**

**Thesis Submitted to the School of Graduate Studies, Universiti  
Putra Malaysia, in Fulfillment of the Requirements for the Degree  
of Master of Veterinary Science**

**May 2015**

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## **DEDICATIONS**

This work is dedicated especially to

My beloved Parents

and

My beloved Husband and Children



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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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**EMELIA AINI KAMARUZZAMAN**

**May, 2015**

**Chair: Professor Saleha Abdul Aziz, PhD**  
**Faculty: Veterinary Medicine**

Emerging of newer groups of antibiotic resistant bacteria is widely reported as a result of the persistent use of antibiotic either for therapeutic or prophylactic purposes. Extended-Spectrum Beta Lactamase (ESBL) is an enzyme produced by gram-negative bacteria which developed resistance to beta lactam antibiotics ranging from penicillin to third and fourth generation cephalosporin; however its activity is inhibited by clavulanic acid. Due to less therapeutic options, human infections caused by ESBL-producing organisms are associated with treatment failure, increased morbidity, increase in length of hospital stay and therapeutic cost, and mortality. Three objectives in the study were first, to determine the occurrence of Extended Spectrum Beta-Lactamase producing *E. coli* (ESBL-producing *E. coli*) in dairy cattle, farm environment and milk; secondly to determine the risk factors associated with the occurrence, and thirdly to determine the antibiotic resistance patterns of the ESBL-producing *E. coli*. Samples collected comprised fecal samples (n=229), farm environment (n=77) including stall floors, feed and water trough, house flies (*Musca domestica*), feed, drinking water and source of drinking water; and milk (n=71) from 10 dairy farms located within Selangor and Negeri Sembilan. Phenotypic detection of ESBL-producing *E. coli* was carried out. The overall occurrence of ESBL-producing *E. coli* was 4.8%. There was a significant difference in the occurrence of ESBL-producing *E. coli* ( $p=0.00$ ) in all three sample groups. The highest occurrence of ESBL-producing *E. coli* was in milk (66.7%) followed by farm environment (27.8%) and cattle (5.5%). Molecular detection of four ESBL genes namely TEM, SHV, CTX-M and OXA was done using multiplex

Polymerase Chain Reaction (m-PCR) method. The CTX-M gene was predominantly detected in 12 out of 18 isolates (66.7%).

The risk factors associated with the occurrence of ESBL-producing *E. coli* in dairy cattle, farm environment and milk were investigated through questionnaires seeking information related to farm management and husbandry practices, records on animal health including antibiotic usage by the farm owners. Two factors found to be statistically significant ( $p < 0.05$ ) were “presence of other animal in the farm compound” ( $\chi^2 = 5.173$ ,  $p = 0.023$ ) and “previous history of disease outbreak” ( $\chi^2 = 3.869$ ,  $p = 0.049$ ). Beef cattle, goats, poultry species including native chickens, duck and geese as well as companion animals such as cats and dogs have been reported to be possible sources of ESBL-producing *E. coli* in the farms.

Antibiotic susceptibility test was conducted using disk diffusion method against 12 antibiotics belonging to six classes which included beta-lactams, chloramphenicol, macrolides, aminoglycoside, quinolones and sulfonamides. Sixteen (88.9%) of 18 ESBL-producing *E. coli* isolates showed resistance to all six beta-lactam antibiotics, with only one isolate (5.6%) from the drinking water was found to be resistant to all 12 antibiotics. All isolates (100%) were found to be resistant against cefotaxime, ceftriaxone and aztreonam. ESBL-producing *E. coli* showed highest susceptibility to trimethoprim-sulphamethoxazole (88.9%) followed by gentamicin and ciprofloxacin (83.3% each). Ten isolates (55.6%) were found to be multi-drug resistant, that is, resistant to three or more antibiotic classes. It is crucial to determine the resistance patterns of ESBL-producing *E. coli* isolates for the purpose of antibiotic selection for treatment options.

In conclusion, the occurrence of ESBL-producing *E. coli* in dairy cattle, farm environment and milk is of public health significance, although it was low. The presence of antibiotic-resistant bacteria may possibly be due to imprudent use of antibiotic, or acquire from the environment. Such imprudent use of antibiotics in animal production may contribute to the persistence of zoonotic resistant organisms in food producing animals as well as in the environments and food products, hence pose serious risks to human health.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra  
Malaysia sebagai memenuhi keperluan untuk  
Master Sains Veterinar

**KEHADIRAN *EXTENDED SPECTRUM BETA-LACTAMASE*  
*Escherichia coli* PADA LEMBU TENUSU,  
PERSEKITARAN LADANG DAN SUSU**

Oleh

**EMELIA AINI KAMARUZZAMAN**

**Mei 2015**

**Pengerusi: Professor Saleha Abdul Aziz, PhD**  
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Kemunculan kumpulan baru bakteria rintang antibiotik dilaporkan hasil daripada penggunaan antibiotik yang berterusan samada untuk kegunaan terapi atau profilaksis. *Extended-Spectrum Beta Lactamase* (ESBL) adalah sejenis enzim yang dihasilkan oleh bakteria gram-negatif yang membentuk kerintangan terhadap antibiotik beta-laktam terdiri daripada *penicillin* sehingga generasi ketiga dan keempat *cephalosporin*; walau bagaimanapun aktivitiya direncat oleh asid clavulanik. Berikutan kurangnya pilihan antibiotik untuk terapi, jangkitan oleh organisma yang menghasilkan ESBL pada manusia dikaitkan dengan kegagalan rawatan, peningkatan morbiditi, peningkatan tempoh rawatan di hospital dan kos rawatan, dan kematian. Tiga objektif kajian ini adalah pertama, menentukan kehadiran *Extended Spectrum Beta-Lactamase E. coli* (ESBL *E. coli*) pada lembu tenusu, persekitaran ladang dan susu; kedua, menentukan faktor risiko yang dikaitkan dengan kehadiran ESBL *E. coli*, dan ketiga, menentukan corak kerintangan antibiotik ESBL *E. coli*. Sampel terdiri daripada sampel tinja (n=229), persekitaran ladang (n=77) yang termasuk lantai kandang, palung makanan dan air, lalat rumah (*Musca domestica*), makanan haiwan, air minuman dan punca air minuman; dan susu (n=71) yang dikumpul daripada 10 ladang tenusu yang terletak di sekitar Selangor dan Negeri Sembilan. Pengesanan fenotip ESBL *E. coli* telah dilaksanakan. Secara keseluruhan kehadiran ESBL *E. coli* ialah 4.8%. Terdapat perbezaan yang signifikan ke atas kehadiran ESBL *E. coli* ( $p=0.00$ ) dalam tiga kumpulan sampel. Kehadiran tertinggi ESBL *E. coli* adalah dalam susu (66.7%) diikuti oleh persekitaran ladang (27.8%) dan lembu (5.5%). Pengesanan molekul ke atas empat gen ESBL iaitu TEM, SHV, CTX-M dan OXA telah dilakukan dengan menggunakan kaedah PCR

multipleks (m-PCR). Gen CTX-M adalah yang paling kerap dikesan dalam 12 daripada 18 (66.7%) isolat.

Faktor risiko dikaitkan dengan kehadiran ESBL *E. coli* pada lembu tenusu, persekitaran ladang dan susu telah disiasat melalui soal selidik bagi mendapatkan maklumat berkaitan pengurusan ladang dan amalan penternakan, rekod kesihatan ternakan termasuk penggunaan antibiotik oleh pemilik ladang. Dua faktor didapati signifikan secara statistik ( $p < 0.05$ ) adalah “kehadiran haiwan lain dalam kawasan ladang” ( $\chi^2 = 5.173$ ,  $p = 0.023$ ) dan “sejarah terdahulu wabak penyakit” ( $\chi^2 = 3.869$ ,  $p = 0.049$ ). Lembu daging, kambing, spesis unggas termasuk ayam kampung, itik dan angsa dan juga haiwan kesayangan seperti kucing dan anjing telah dilaporkan boleh menjadi sumber ESBL *E. coli* dalam ladang.

Ujian kerintangan antibiotik telah dijalankan menggunakan kaedah *disk diffusion* terhadap 12 antibiotik daripada enam kelas antibiotik termasuk *beta-lactams*, *chloramphenicol*, *macrolides*, *aminoglycoside*, *quinolones* dan *sulfonamides*. Antibiotik iaitu *ceftazidime* (30  $\mu\text{g}$ ), *cefotaxime* (30  $\mu\text{g}$ ), *cefepodoxime* (10  $\mu\text{g}$ ), *ceftriaxone* (10  $\mu\text{g}$ ), *ampicillin* (10  $\mu\text{g}$ ), *aztreonam* (30  $\mu\text{g}$ ), *chloramphenicol* (30  $\mu\text{g}$ ), *tetracycline* (30  $\mu\text{g}$ ), *gentamicin* (10  $\mu\text{g}$ ), *nalidixic acids* (30  $\mu\text{g}$ ), *ciprofloxacin* (5  $\mu\text{g}$ ) dan *trimethoprim-sulphamethoxazole* (25  $\mu\text{g}$ ) telah digunakan. Enambelas daripada 18 (88.9%) isolat ESBL *E. coli* menunjukkan kerintangan kepada kesemua enam antibiotik beta-laktam, dengan satu isolat (5.6%) daripada air minuman didapati rintang kepada kesemua 12 antibiotik. Kesemua isolat (100%) didapati rintang terhadap *cefotaxime*, *ceftriaxone* dan *aztreonam*. ESBL *E. coli* menunjukkan kerintangan tertinggi pada *trimethoprim-sulphamethoxazole* (88.9%) diikuti oleh *gentamicin* dan *ciprofloxacin* (83.3% setiap satu). Sepuluh isolat (55.6%) didapati rintang multi-drug, iaitu, rintang terhadap tiga atau lebih kelas antibiotik.

Kesimpulannya, kehadiran ESBL *E. coli* pada lembu tenusu, persekitaran ladang dan susu mempunyai kepentingan kesihatan awam, walaupun ianya rendah. Kehadiran bakteria rintang antibiotik berkemungkinan berikutan penggunaan antibiotik yang tidak berhemah, menyebabkan peningkatan insiden kerintangan antibiotik. Penggunaan antibiotik yang tidak berhemah ini boleh menyumbang kepada organisma zoonotik yang rintang yang hadir secara berterusan pada haiwan ternakan dan juga pada persekitaran dan produk haiwan dan oleh itu menimbulkan risiko yang serius terhadap kesihatan manusia.



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**EMELIA AINI KAMARUZZAMAN**

I certify that a Thesis Examination Committee has met on (date of viva voce) to conduct the final examination of Emelia Aini Kamaruzzaman on her thesis entitled "Occurrence Of Extended Spectrum Beta-Lactamase (ESBL) Producing *Escherichia coli*, Farm Environment and Milk in accordance with the Universities and University Colleges 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.

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## LIST OF ABBREVIATIONS

AmpC	Class C Cephalosporinase
ATCC	American Type Culture Collection
<i>bla</i>	Beta-lactamase
$\beta$ -lactam	Beta-lactam
bp	base pair
BPW	Buffered Peptone Water
$^{\circ}$ C	degree Celcius
CLSI	Clinical Laboratory Standard Institute
DDS	Double disk synergy
DDT	Disc diffusion test
DNA	Deoxyribonucleic acid
DVS	Department of Veterinary Services
<i>E. coli</i>	<i>Escherichia coli</i>
ESBL	Extended Spectrum Beta-Lactamase
FAO	<i>Food and Agriculture Organization</i>
h	Hour(s)
kg	Kilogram
L	Liter
ml	milliliter
mg	miligram
M.I.C.	Minimum inhibitory concentration
MLST	Multilocus Sequence Typing
m-PCR	Multiplex polymerase chain reaction
OIE	World Organization for Animal Health

μg	Micro gram
μl	Micro liter
PCR	Polymerase chain reaction
rpm	round per minute
TBE	Tris-borate-EDTA (TBE) buffer
TSB	Tryptone Soy Broth
V	Volt



## CHAPTER ONE

### INTRODUCTION

The use of antibiotic in animal production plays a significant role in growth promotion, disease prevention as well as for therapeutic purposes in food animal. It is part of a necessity in food animal practice as the use of this wonder drug has been proven to save animal lives, which is related to animal welfare. However, prolong and inappropriate widespread use of antibiotic compounds has posed the risk for emergence and dissemination of resistant microorganisms. Antibiotic resistant bacteria causing diseases in human and animal has long becoming public and animal health issues of major concern globally. The emergence, selection and dissemination of antibiotic-resistant microorganisms have been associated with antibiotic usage in both veterinary and human medicine (Hawkey, 2008; Marshall & Levy, 2011).

*Escherichia coli* (*E. coli*), *Campylobacter* and *Salmonella* have emerged as antibiotic resistant bacteria that colonized farm animals and can be transmitted to human in food, handling of animals or through the environment (<http://www.ciwf.org.uk/media/3758863/Antibiotics-in-Animal-Farming-Public-Health-and-Animal-Welfare.pdf>). The World Organization for Animal Health (OIE) has emphasized the importance of surveillance on antimicrobial resistance and to support one of the objectives in Fifth Strategic Plan: 2011-2015 which is to ensure the scientific excellence of information and advice available to the veterinary field ([www.oie.int](http://www.oie.int)).

Extended Spectrum Beta-Lactamase (ESBL) is an enzyme capable of conferring bacterial resistance to  $\beta$ -lactam drugs such as penicillins, 1<sup>st</sup> to 3<sup>rd</sup> generation of cephalosporins such as cefotaxime, ceftriaxone and aztreonam (except cephamycin or carbapenems); however its activity is inhibited by clavulanic acid (Paterson & Bonomo, 2005). Bush & Fisher (2011) in their review defined ESBL as an enzyme that hydrolyzes oxyimino-cephalosporins and monobactams in addition to penicillins and early cephalosporins, and its activity is inhibited by clavulanic acid or tazobactam.

The gene of ESBL is located on a plasmid (a mobile genetic element) (Carattoli, 2009), which made it easily transmitted horizontally within and between bacterial species (Carattoli, 2011). Also, it has been reported that these resistance genes are disseminated throughout the food chain or via direct contact in human and animals (Oppegaard, 2001). A study by Leverstein-van Hall et al., (2011) indicated the ESBL gene similarity between a patient, food animal and its based products. Human infected with ESBL-producing *E. coli* is often associated with limited choice of antibiotic treatment and delayed therapy which leads to prolonged hospital stay and eventually death.

In comparison to other commodities such as poultry and pig, dairy production is rather a small industry in Malaysia. In year 2011, the total local milk production was 70 million litres with 13% self-sufficient level (www.dvs.gov.my). Currently there is a small number of commercial scale dairy farming consisting of more than 50 lactating cows per farm. The majority of the farmers are practicing at medium scale (30-49) and small scale (less than 29) farm. Dairy farming industry in Peninsular Malaysia is regarded as family farming, and nearly half of the farmers inherited the farm from their fore fathers.

The last decade saw drug resistance in *Enterobacteriaceae* has risen dramatically around the globe. ESBL, known as plasmid mediated enzymes were reported to confer resistance not only to beta-lactams antibiotics including penicillins, first to fourth generation cephalosporins and monobactams, but also to other classes of antibiotics namely fluoroquinolones and aminoglycosides (Gundogan & Avci, 2013), resulting in limited therapeutic options.

Despite various reported studies of ESBL-producing *E. coli* occurrence in food animals, there were limited studies on risk factors associated with the ESBL-producing *E. coli* occurrence. The use of cephalosporins was reported as important risk factor for the spread of the resistance genes, in addition to generic antimicrobial use (Liebana et al., 2013). Farm hygiene management was also reported to be another factor associated with the occurrence of ESBL-producing *E. coli* on dairy farms in United Kingdom (Snow et al., 2012).

ESBL genes are located on plasmids, therefore it can be transferred between and within bacterial species easily (Overdeest et al., 2011). These genes were homologous in bacteria from humans, food and food animals, and the risk factors for their occurrence in food animals were complicated.

Several studies on ESBL-producing *E. coli* both in human and livestock have been reported extensively by developed regions in the world mainly from the European and American regions; and Asian region including Japan, China and Hong Kong. However, data on the prevalence of ESBL *E. coli* in food animals particularly in the South East Asia is scarce. In Malaysia, there were few studies and mainly in humans which reported the occurrence in different patient categories including adult patients with respiratory problems and paediatrics and in urban surface water. To date, there is limited information available on the ESBL occurrence in food producing animal in the country, particularly in dairy cattle. It is believed that this is the first study on ESBL-producing *E. coli* in dairy cattle to be reported.

It is hypothesized that the occurrence of ESBL-producing *E. coli* in dairy cattle, farm environment and milk is low due to less use of antibiotics in dairy production.

Therefore, the objectives of this study were:

1. To determine the occurrence of ESBL-producing *E. coli* in dairy cattle;
2. To study the risk factors associated with the ESBL-producing *E. coli* occurrence; and
3. To determine the antibiotic resistance patterns of the isolates.





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