UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISATION OF ESCHERICHIA COLI ISOLATED FROM RAW MILK AND VILLAGE CHICKEN AND BROILER LITTER

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MASTER OF SCIENCE
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By

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Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

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TABLES OF CONTENTS

		Page
ACKNOWLEDGEMENTS		**ii**
LIST OF TABLES		**vi**
LIST OF FIGURES		**viii**
LIST OF PLATES		**ix**
LIST OF ABBREVIATIONS		**xii**
ABSTRACT		**xiii**
ABSTRAK		**xv**
CHAPTER		
I	**INTRODUCTION**	1
II	**LITERATURE REVIEW**	5
	Taxonomy	5
	Habitat	7
	Cell Morphology and Cell Cycle	9
	Isolation and Identification	10
	Plasmid	11
	Basic Properties of Plasmid	11
	R Plasmid	15
	F Plasmid	17
	Isolations of Plasmid	18
	Plasmid as Cloning Vectors	20
	Antibiotics and Animal Production	21
	Use of Antibiotic in Animals	22
	Antibiotic Resistance	25
	Transfer of Antibiotic Resistance	27
	Random Amplified Polymorphic DNA (RAPD) Analysis	29
III	**ISOLATION AND IDENTIFICATION OF *ESCHERICHIA COLI***	32
	Introduction	32
	Biochemical Tests and Identification of *E. coli*	34
	Materials and Methods	35
	Samples Collection	35
	Isolation and Identification of *E. coli* from Litters of Village and Broiler Chickens and Raw Milk Samples	36

Biochemical Test..... 38
Results 39
Discussion..... 43
Conclusion..... 45

IV **PLASMID PROFILING AND ANTIBIOTIC SUSCEPTIBILITY TESTING**..... 47
Introduction..... 47
Materials and Methods..... 50
Plasmid Isolation..... 50
Agarose Gel Electrophoresis..... 51
Determination of Molecular Weight of Plasmid..... 52
Antibiotic Susceptibility Tests..... 53
Mc Farland Turbidity Standard..... 53
Inoculum Preparation..... 55
Application of Discs..... 55
Interpretation of Zon of Inhibition..... 56
Results..... 57
Plasmid Analysis of *E. coli* Isolates..... 57
Antibiotic Susceptibility among *E. coli* Isolates..... 74
Discussion..... 90
Conclusion..... 96

V **RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS**..... 98
Introduction..... 98
Materials and Methods..... 100
Genomic DNA Isolation..... 100
DNA Primers..... 101
RAPD-PCR Fingerprinting..... 101
Results..... 103
Optimisation of Concentrations of Arbitrary Primer and Mg^{2+} Ion for Ultimate Discriminatory Power..... 103
Primer Screening for Ultimate Discriminatory Power..... 103
RAPD Analysis of *E. coli* Isolates Isolated from Village Chickens..... 106
RAPD Analysis of *E. coli* Isolates Isolated from Broilers 111
RAPD Analysis of *E. coli* Isolates Isolated from Raw Milk Samples 115
Discussion..... 121
Conclusion..... 126

VI GENERAL DISCUSSION AND CONCLUSION..... 127

REFERENCES..... 132

APPENDIX
A: MEDIA AND SOLUTIONS..... 146

VITA..... 155

LIST OF TABLES

Table		Page
1	Differentiation of *E. coli* from Other Species of *Escherichia*	6
2	A List of Biochemical Tests for the Identification of *E. coli* according to Edwards and Ewing (1972)	10
3	Some Phenotypic Traits Exhibited by Plasmid-carried Genes	13
4	A Comparison of Chromosomal and Plasmid-mediated Antibiotic Resistance	16
5	Prevalence of *E. coli* in Litters of Village and Broiler Chicken and Raw Milk Samples	40
6	Mean Counts of *E. coli* in Litters of Village and Broiler Chicken and Raw Milk Samples	41
7	Sizes in Megadaltons (MDa) of V517 Size Reference Plasmids used to Estimate the Molecular Weight of CCC Plasmids in *E. coli* Isolates	52
8	Plasmid Profile of *E. coli* Isolates Isolated from Litter of Village Chickens	58
9	Plasmid Profile of *E. coli* Isolates Isolated from Litter of Broilers	59
10	Plasmid Profile of *E. coli* Isolates Isolated from Raw Milk Samples	61
11	Prevalence of Resistance to Antimicrobial Agents amongst *E. coli* Isolates Isolated from Village Chicken, Broiler and Raw Milk Samples	75
12	Antibiotic Resistance and MAR Index of *E. coli* Isolates Isolated from Village Chickens	76
13	Antibiotic Resistance and MAR Index of *E. coli* Isolates Isolated from Broilers	78
14	Antibiotic Resistance and MAR Index of *E. coli* Isolates Isolated from Raw Milk Samples	80
15	Correlation Between Antibiotic Resistance Pattern and Plasmid Profile among *E. coli* Isolates Isolated from Village Chickens	82

16 Correlation Between Antibiotic Resistance Pattern and Plasmid Profile among *E. coli* Isolates Isolated from Broilers 85

17 Correlation between Antibiotic Resistance Pattern and Plasmid Profile among *E. coli* Isolates Isolated from Raw Milk Samples ... 88

18 Typical Profiles of Times and Temperatures used for RAPD Analysis using Perkin-Elmer GeneAmp PCR Instrument System..... 102

19 The Primers (10 nt) Tested for RAPD Analysis of *E. coli* 107

20 RAPD Patterns of *E. coli* Isolates Isolated from Village Chickens Obtained with Primer Genl-50-09..... 108

21 RAPD Patterns of *E. coli* Isolates Isolated from Village Chickens Obtained with Primer Genl-50-10..... 109

22 RAPD Patterns of *E. coli* Isolates Isolated from Broilers Obtained with Primer Gen1-50-09..... 112

23 RAPD patterns of *E. coli* Isolates Isolated from Broilers Obtained with Primer Gen1-50-10..... 113

24 RAPD Patterns of *E. coli* Isolates Isolated from Raw Milk Samples Obtained with Primer Genl-50-09..... 116

25 RAPD Patterns of *E. coli* Isolates Isolated from Raw Milk samples Obtained with Primer Gen1-50-10 117

LIST OF FIGURES

Figure		Page
1	Possible steps in F plasmid transfer.....	19
2	A schematic diagram of isolation and identification of *E. coli* from litters of village and broiler chicken.....	37
3	The graphical method of relating the molecular weight (MDa) of plasmid DNA molecule to its electrophoretic mobility (mm).....	54

LIST OF PLATES

Plate		Page
1	Characteristics of *E. coli* colonies on EMBA plate.....	42
2	Characteristics of *E. coli* colonies on MCA plate.....	42
3	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from village chickens.....	62
4	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from village chickens.....	62
5	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from village chickens.....	63
6	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from village chickens.....	63
7	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from village chickens.....	64
8	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from village chickens.....	64
9	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from village chickens.....	65
10	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from village chickens.....	65
11	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from broilers	66
12	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from broilers	66
13	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from broilers.....	67
14	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from broilers.....	67
15	Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from broilers	68

16 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from broilers 68

17 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from broilers..... 69

18 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from broilers 69

19 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from raw milk..... 70

20 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from raw milk..... 70

21 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E· coli* isolated from raw milk..... 71

22 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from raw milk..... 71

23 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E· coli* isolated from raw milk..... 72

24 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from raw milk..... 72

25 Agarose gel (0.7%) electrophoresis of plasmid DNA of *E. coli* isolated from raw milk..... 73

26 Optimisation of $MgCl_2$..... 105

27 Optimisation of primer..... 105

28 Reducing the amount of *Taq* polymerase..... 106

29 Genomic fingerprints of representative isolates of *E. coli* from village chickens generated by arbitrary primer PCR with primer Gen1-50-02... 107

30 Genomic fingerprints of representative isolates of *E. coli* from village chickens generated by arbitrary primer PCR with primer Gen1-50-09... 110

31 Genomic fingerprints of representative isolates of *E. coli* from village chickens generated by arbitrary primer PCR with primer Gen1-50-10... 110

32 Genomic fingerprints of representative isolates of *E. coli* from broilers generated by arbitrary primer PCR with primer Gen1-50-02..... 114

33 Genomic fingerprints of representative isolates of *E. coli* from broilers generated by arbitrary primer PCR with primer Gen1-50-09..... 114

34 Genomic fingerprints of representative isolates of *E. coli* from broilers generated by arbitrary primer PCR with pmer Gen1-50-10..... 115

35 Genomic fingerprints of representative isolates of *E. coli* from raw milk generated by arbitrary primer PCR with primer Gen1-50-02..... 118

36 Genomic fingerprints of representative isolates of *E. coli* from raw milk generated by arbitrary primer PCR with primer Gen1-50-09..... 119

37 Genomic fingerprints of representative isolates of *E. coli* from raw milk generated by arbitrary primer PCR with primer Gen1-50-09..... 119

38 Genomic fingerprints of representative isolates of *E. coli* from raw milk generated by arbitrary primer PCR with primer Gen1-50-10..... 120

39 Genomic fingerprints of representative isolates of *E. coli* from raw milk generated by arbitrary primer PCR with primer Gen1-50-10..... 120

LIST OF ABBREVIATIONS

A	Adenine or adenosine
AP-PCR	Arbitrary primed-polymerase chain reaction
ATP	Adenosine triphosphate
bp	Base pair
C	Cytosin
CCC	Covently closed circular
CDNA	Complementary DNA
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetra-acetic acid
EDTA	Ethylenediamine tetra-acetic acid
EMBA	Eosin methylene blue agar
G	Guanin
kb	kilobase
LB	Luria-Bertani
MAR	Multiple antibiotic resistance
MDa	Megadalton
Mr	Relative molecular weight
mRNA	Messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nt	nucleotide
PCR	Polymerase chain reaction
rpm	Revolution per minute
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
ssDNA	Single stranded DNA
Taq	*Thermus aquaticus*
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA
Tris	Tris (hydroxymethyl) methylamine
UV	Ultra violet
V	Volt

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science.

MOLECULAR CHARACTERISATION OF *ESCHERICHIA COLI* ISOLATED FROM RAW MILK AND VILLAGE CHICKEN AND BROILER LITTER

By

SAMUEL LIHAN

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Chairman: Son Radu, Ph.D.

Faculty: Food Science and Biotechnology

Thirty five litter samples of village chickens, 35 broilers and 32 samples of raw milk were examined for the presence of *E. coli*. All samples were positive for *E. coli*. Three hundred and five isolates of *E. coli* were isolated from litter samples of village chickens (105 isolates), broilers (105 isolates) and raw milk samples (95 isolates). All the isolates were examined for antibiotic resistance, plasmid profiles and polymorphism using random amplification of polymorphic DNA (RAPD) analysis. Isolates isolated from litter of village chickens and broilers had a multiple antibiotic resistance (MAR) index of 0.31 to 0.75 and 0.44 to 0.69, respectively. Isolates isolated from raw milk had a MAR index of 0.31 to 0.88. High MAR index suggests that all the isolates originated from high risk sources. The *E. coli* isolates isolated from village chickens, broilers and raw milk samples were

grouped into 34, 30 and 28 distinct antibiotypes, respectively. Eighty (76.2%) and 99 (94.3%) isolates were found to harbour plasmids ranging in size from 1.2 to 64 MDa and 1.2 to 80 MDa among isolates isolated from village chickens and broilers, respectively. Isolates isolated from raw milk harboured plasmids ranging in size from 1.4 to 68 MDa. Based on their plasmid profiles, the *E. coli* isolates isolated from village chickens, broilers and raw milk were grouped into 28, 57 and 5 plasmid patterns, respectively. Three 10-mer oligonucleotides primers (Gen1-50-02, Gen1-50-09 and Gen1-50-10) were used to amplify genomic DNA. The profiles observed after electrophoretic separation for the three primers when combined together were able to distinguish the *E. coli* isolates from village chickens, broilers and raw milk into 92, 96 and 50 RAPD patterns, respectively. The large number of subgroups within these isolates indicates that there is a high degree of diversity within *E. coli* isolates, isolated from village chickens, broilers and raw milk samples. Isolates, isolated from village chickens, broilers and raw milk are genotypically diverse as shown by RAPD pattern, suggesting that different strains have been brought into the geographic region and strains already present have continued to evolve. These results suggest that RAPD-PCR assay is more discriminating than plasmid profiling and antibiotyping, and could be a valuable tool for epidemiological studies.

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PENCIRIAN MOLEKULAR *ESCHERICHIA COLI* DARI SUSU MENTAH, NAJIS AYAM KAMPUNG DAN AYAM DAGING

Oleh

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Tiga puluh lima sampel najis ayam kampung, 35 sampel najis ayam daging dan 32 sampel susu mentah telah dikaji untuk kehadiran *E. coli*. Kesemua sampel didapati positif untuk kehadiran *E. coli*. Tiga ratus lima pencilan *E. coli* telah dipencilkan dari najis ayam kampung (105 pencilan), ayam daging (105 pencilan) dan susu mentah (95 pencilan). Semua pencilan telah dikaji untuk kerintangan kepada antibiotik, profil plasmid dan polimorfik menggunakan analisis random amplification of polymorphic DNA (RAPD). Pencilan yang dipencil dari ayam kampung dan ayam daging mempunyai indeks kerintangan pelbagai terhadap antibiotik (MAR) 0.31 hingga 0.75 dan 0.44 hinga 0.69 masing-masing. Pencilan dari susu mentah mempunyai indeks MAR 0.31 hingga 0.88. Indeks MAR yang tinggi mencadangkan bahawa semua pencilan berasal dari sumber berisiko tinggi.

Pencilan *E. coli* yang dipencilkan dari ayam kampung, ayam daging dan susu mentah dapat dikumpulkan kepada 34, 30 dan 28 antibiotip masing-masing. Lapan puluh (76.2%) dan 99 (94.3%) pencilan didapati membawa plasmid bersaiz dari 1.2 hingga 64 MDa dan 1.2 hingga 80 MDa bagi pencilan yang dipencilkan dari ayam kampung dan ayam daging masing-masing. Tujuh puluh satu (74.7%) pencilan dari susu mentah membawa plasmid bersaiz dari 1.4 hingga 68 MDa. Berdasarkan profil plasmid, pencilan *E. coli* dari ayam kampung, ayam daging dan susu mentah dapat dikumpulkan kepada 28, 57 dan 5 corak plasmid masing-masing. Tiga primer oligonukleotid 10-mer (Gen1-50-02, Gen1-50-09 dan Gen1-50-10) digunakan untuk amplifikasi DNA genomik. Selepas pengasingan elektroforetik, pencilan ayam kampung, ayam daging dan susu mentah dapat dibezakan kepada 92, 96 dan 50 corak RAPD, masing-masing bila ketiga-tiga primer digabungkan. Sub-kumpulan yang besar dalam pencilan-pencilan ini menunjukkan bahawa terdapat darjah kepelbagaian yang tinggi dalam pencilan *E. coli* yang dipencilkan dari ayam kampung, ayam daging dan susu mentah. Perbezaan genotip bagi *E. coli* dari ayam kampung, ayam daging dan susu mentah seperti yang ditunjukkan oleh corak RAPD mencadangkan strain berlainan telah dibawa masuk ke kawasan geografi berkenaan dan strain yang sedia ada terus mengalami evolusi. Keputusan ini menunjukkan bahawa RAPD-PCR lebih diskriminasi dari profil plasmid dan antibiotip dan boleh digunakan sebagai alat yang amat berguna dalam kajian epidemiologi.

CHAPTER I

INTRODUCTION

Escherichia coli was first described by Theobald Escherich in 1885 (Sojka, 1965). He examined the faeces of new-born breast-feeding babies and found that they contained bacteria; he called this microorganism as *Bacterium coli commune* which is now accepted as *E. coli*. The bacteria consisting the species *Escherichia coli* are commonly found in the intestinal flora of man and animals, and were until late 1950s recognised as non-pathogenic normal cohabitants. Because the bacterium's main reservoir is the intestinal tract of warm blooded animals, the presence of strains in other environments like food and water is used as an indicator of contamination from the previous indicated reservoirs. Thus, the detection of *E. coli* can be use to assess the sanitary quality of food, as well as in water and is considered primarily as an index organism for the possible presence of pathogenic bacteria. Under suitable condition, *E. coli* can grow in environments like water and food (Doyle and Padhye, 1989).

E. coli is among the most frequently isolated bacteria in human and veterinary diagnostic microbiology, but the causative role of the bacterium in a number of diseases is probably not yet recognised or fully understood. However, certain strains might induce disease, and *E. coli* should therefore be regarded as a potential pathogenic organism (Sussman, 1985). The pathogenic strains can cause

disease syndromes as distinct as different diarrheal diseases, urinary tract infections, wound infections, meningitis, septicemia, artherosclerosis, hemolytic uremic syndrome, and immunological diseases like reactive and rheumatoid arthritis (Doyle and Padhye, 1989; Sussman, 1985). In rare instances, it may produce in animals meningitis, septicemia, endocarditis and arthritis (Soltys, 1979).

The problem of drug resistance in *E. coli* has been of considerable importance to microbiologists and is now posing therapeutic problems for clinician and public health problem to concern authorities. The resistance to certain antibiotics which are the drug of choice for human infection has caused particular alarm (Anderson, 1968; Towner, 1982; Son *et al.*, 1997a). Resistance to antibiotics is known to develop as a result of widespread use of antimicrobial agents, thereby increasing the opportunity for resistant species to grow at the expense of sensitive microorganisms (Richmond, 1972). Transfer of resistance factor (R-factor) has been observed *in vivo* in subjects under chemoteraphy and also between *E. coli* and other members of enteric bacteria (Linton *et al.*, 1981). It has also been shown that antibiotic-resistant strains of *E. coli* of animal origin can be transmitted to man (Levy *et al.*, 1976; Hirsch and Wiger, 1978). Consequently, such an event may occur in the intestinal tract of consumers through ingestion of live drug-resistant organisms present in food. The genes that make bacteria resistant to antibiotics are usually encoded not on their chromosomes but on smaller self–replicating companion loops of DNA called plasmids (Saunders, 1984).

More research is needed to determine the potential characteristics of *E. coli* that can be used to identify its point of origin from various sources. Bario is a small rural village in Sarawak (Malaysia) accessible only by air transport, located in the interior on the island of Borneo. Of recent years, it has increasingly obtained its supply of poultry meat from poultry farms in Miri (a coastal town) and this has prompted this investigation since broiler chicken infected by resistant strains of *E. coli* potentially may serve as reservoirs for these bacteria and aid in their dissemination. This may influence the genetic make-up of resident bacteria. This would have public health significance since village chickens are in contact with contaminated waste discharges. In addition, bacteria from polluted environments have been reported to have higher incidence of plasmids and greater resistance to antimicrobial agents than those obtained from cleaner sites (Baya *et al.*, 1986).

The ability to identify *E. coli* quickly and reliably can be important in both livestock industry and medicine for establishing the causes of undesirable contaminations and precise determination of pathogenic strains. Limited information concerning genetic differences among the strains of *E. coli* in Malaysia is available, and consequently limited data relating genetic differences to virulence have been published (Son *et al.*, 1998a). An understanding of genetic variability in *E. coli* is important for studies of the taxonomy, epidemiology and pathogenicity of this species. The objectives of this study are:

1. Isolation and identification of *E. coli* from litters of village and broiler chicken, and raw milk samples.

2. To determine the antimicrobial resistance patterns and plasmid profiles among the *E. coli* isolates.
3. To detect the DNA sequence diversity of *E. coli* isolates by random amplified polymorphic DNA (RAPD) analysis.

CHAPTER II

LITERATURE REVIEW

Taxonomy

Escherichia coli is classified in the family *Enterobacteriaceae* and its taxonomic features includes Gram-negative, asporogenic, straight rods that may be peritrichously flagellated or non motile (Orskov, 1984). It coagulated milk, producing acid and gas. The ability to ferment certain carbohydrates, producing acid and gas was soon adapted as a basis for the differentiation of closely related enteric bacteria such as *Shigella* and *Salmonella*. The genus *Escherichia* contains six species, *E. coli*, the type species, *E. decarboxylata*, *E. fergusonii* (Farmer *et al.*, 1985), *E. hermanii* (Brenner *et al.*, 1982a), *E. vulneris* (Brenner *et al.*, 1982b) and *E. blattae* (Burgess *et al.*, 1973). The reclassification of *E. decarboxylata* in a new genus *Leclercia* has been proposed (Tamura *et al.*, 1986), but the status of this genus is uncertain (Jones, 1988). The DNAs from all above mentioned species of *Escherichia* are related to those of *E. coli* (average, 84%) (Ewing, 1986). *E. coli* is closely related to *Shigella*, and on some criteria (e.g., DNA homology) the two form a single species (Brenner, 1984). *E. coli* may be differentiated from other species by the biochemical tests as listed in Table 1.

Generally, the genus of *Escherichia* is composed of motile or non motile bacteria that conforms to the definitions of the family *Entrobacteriaceae* and the tribe *Escherichia.* Both acid and gas are formed from a wide variety of fermentable carbohydrates, but aerogenic biotypes occur; salicin is fermented by many isolates but inositol is not utilised and adonitol is utilised by members of only one species. Lactose often is fermented rapidly and the important diagnostic feature of *E. coli* is the production of indole from tryptophane. However, this and other features may be readily lost by mutation, while unusual traits may be acquired, e.g., delayed fermentation of lactose and failure to produce indole (Varnam and Evans, 1991).

Table 1: Differentiation of *E. coli* from Other Species of *Escherichia.*

Test	*E. coli*	*E. adecarboxylata*	*E. hermanii*	*E. vulneris*
Arginine dihydrolase	-	-	-	+/-
Lysine decarboxylase	+/-	-	-	+/-
Ornithine decarboxylase	+/-	-	+	-
Indole	+	+	+	-
Aesculin hydrolysis	-	+	-	-
Malonate	-	+	-	+/-
Acid from				
Adonitol	-	+	-	-
Amygdalin	-	+	+	+/-
Cellobiose	-	+	+	+
Sorbitol	+	-	-	-

Note:
+ 90% or more strains positive
- no reaction (90% or more strains negative)
+/- different reactions; some strains positive, some strains negative

Lysine and ornithine are decarboxylated by the majority of cultures, acid is formed from sodium mucate, and sodium acetate frequently is utilised as sole source of carbon.

E. coli, the type species in this family was fully reviewed by Glass (1982) as a favourite organism for all types of microbiological studies. Prominent in any list of the advantages of research on bacteria must be the rapid growth and limited nutritional requirements of those microorganisms. The genome size (including chromosomal and extrachromosomal DNA) in *E. coli* strain varies from 2.3 X 10^6 to 3.0 X 10^9 daltons. The G+C content ranges from 49 to 52% (Selander *et al.*, 1987).

Habitat

Most strains of *E. coli* are harmless commensal members of the intestinal flora of mammals and, to an undetermined extent, in birds some strains tend to adhere to the intestinal mucous while others are only temporary transients in the lumen of the colon. *E. coli* is the major facultative anaerobe of the large intestine, occurring in normal densities of about 10^6 cells per g of colon contents. It is a minor component of the total intestine flora, which consists largely of obligate anaerobes and the aggregate reaches 10^{11} cells per g of colon contents (Selander *et al.*, 1987).

E. coli is also one of the first of the intestinal bacteria to colonise the newborn, generally being derived from the faeces of the mother in both humans and animals (Selander *et al.*, 1987) and may be present in the cecum and lower intestine