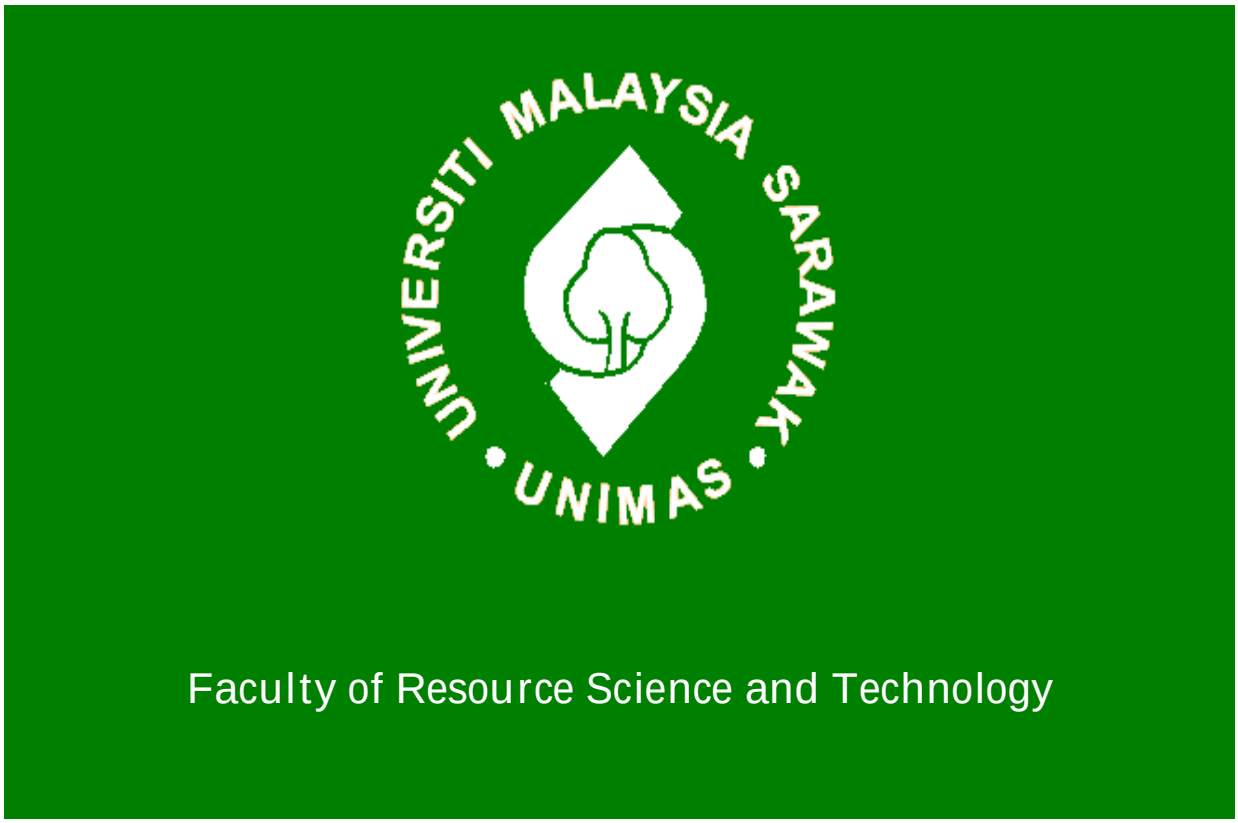

MULTIPLE ANTIBIOTIC RESISTANCE INDEX OF *Escherichia coli* ISOLATED FROM PIG FARM WASTE IN KUCHING, SARAWAK.

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Bachelor of Science with Honours
(Resource Biotechnology)
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A report submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours

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Ong Sue Ann

University Malaysia Sarawak

24 May 2011

DECLARATION

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Ong Sue Ann

24 May 2011

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LIST OF ABBREVIATIONS

Abbreviation or symbol	Term
x	Times
%	Percentage
ºC	Degree celcius
µg	Microgram
µl	Microliter
µm	Micrometer
A/E	Attaching and effacing
ADP	Asenosine diphosphate
AST	Antimicrobial Resistance Test
Bp	Base pair
dH_2O	Distill water
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDL	Extensor digitorum longus
E.R	Endoplasmic recticulum
Eae encodes intimin	*E. coli* attaching and effacing that
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E.coli*
EIEC	Enteroinvasive *E. coli*
EMB	Eosin Mthylene Blue
EMBA	Eosin Mthylene Blue agar
EPEC	Enteropathogenic *E. coli*
*esc*s /Esp	*E. coli* secretion
*esp*s	*E. coli*-secreted protein

ETEC	Enterotoxigenic *E. coli*
Gb3	Globotriaosylceramide
H_2S	Hydorgen sulphite
HUS	Hemolytic uremic syndrome
IMViC	Indole/MR/VP/citrate
kbp	Kilo base pair
kDa	Kilo Dalton
KOH	Potassium hydroxide
LBA	Luria-Bertani Agar
LT	Enterotoxins heat-labile toxins
MAR	Multiple antibiotic resistance
MDa	Molar Dalton
MHA	Muller-Hinton agar
min	Minute
ml	Mililiter
MR	Methyl Red
Mxi	Membrane expression of ipfl-surface
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
sec	Second
sep	Secretion of *E. coli* proteins
S.I.M	Simmon citrate/Indole/Motility
Spa	Surface presentation antiges
spp	Species
SrRNA	Subunit ribosomal ribonucleic acid
ST	Enterotoxins hear-stable toxins
Stx	Shiga toxin

TBE	Tris/Borate/EDTA
tir	Translocated intimin receptor
TSA	Tryptic soy agar
UV	Ultraviolet
V	Volt
VP	Voges-Proskauer

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Multiple Antibiotic Resistance Index of *Escherichia coli* Isolated From Pig Farm Waste in Kuching, Sarawak.

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ABSTRACT

E. coli has been known as one of the most common bacteria found in the intestinal tract of human and warm blooded animals. Most strains are harmless, however some strains have been identified as the serious causal agents of various illnesses. In this study, out of 47 isolates from samples from three different pig farms located at 10th miles Kuching, only 12 of the isolates were found to be *E. coli* and 2 out of 12 isolates were identified as EPEC since they were tested positive for Shiga toxin-producing *E. coli* (STEC) virulence gene *stx*1 in PCR. Molecular detection was done by PCR and the PCR product was analyzed using 1.0% agarose gel electrophoresis. EMB agar was used to selectively grow *E. coli* isolated from the pig waste and further identification was carried out through biochemical tests such as IMViC test, S.I.M test and also citrate utilization test. In addition, an antimicrobial susceptibility test had been conducted as to find out the MAR index value of *E. coli*, it was found that MAR index of *E. coli* isolated from pig farm waste is significantly high (all above 0.6) and this shows that the pig waste was a high risk sources of contamination which may posed hazard to farmers and the public.

Keywords: Pig waste, *E. coli*, antimicrobial susceptibility test, MAR index, *stx*1

ABSTRAK

E. coli telah dikenali sebagai salah satu bakteria yang paling umum ditemui pada saluran pencernaan manusia dan haiwan berdarah panas. Kebanyakan strain adalah tidak berbahaya, namun beberapa strain telah dikenalpasti sebagai agen penyebab pelbagai penyakit serius. Dalam kajian ini, 47 isolat dari sampel dari tiga ladang babi berbeza yang terletak di Kuching batu 10, hanya 12 daripada isolat dipastikan sebagai *E. coli* dan 2 dari 12 isolat dikenalpasti sebagai EPEC kerana mereka mengandungi Shiga racun-menghasilkan *E. coli* (STEC) gen virulensi *stx*1 di PCR. Pengesanan molekular dilakukan dengan PCR dan produk PCR dianalisis dengan menggunakan 1,0% agarose gel elektroforesis. EMB agar digunakan untuk pertumbuhan sesetengah jenis *E. coli* dari kotoran babi dan pengesahan terlanjut boleh dilakukan melalui ujian-ujian biokimia seperti ujian IMViC, uji S.I.M dan juga uji penggunaan sitrat. Selain itu, ujian kerentanan antimikrob telah dilakukan untuk mengetahui nilai indeks MAR *E. coli*, didapati bahawa indeks MAR *E. coli* yang diisolasi dari sisa ladang babi adalah tinggi (semua di atas 0.6) dan ini menunjukkan bahawa sisa babi adalah sumber risiko tinggi pencemaran yang boleh menyebabkan bahaya kepada petani dan awam.

Kata kunci: Pembuangan sisa babi, *E. coli*, uji kerentanan antimikrob, indeks MAR, *stx*1

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Diarrhea can be considered as one of the most important diseases in constituting death cases in the world (Nweze, 2009). Generally there are wide populations of enteric bacteria that can be found in a normal pig gut which are normally do not causes diseases, but some of the enteric bacteria are pathogenic such as *Escherichia coli*, *Salmonella* spp., *Yersinia* spp. and others are foodborne pathogens that cause food poisoning to human (Clark, 2007).

According to Clark (2007), pathogenic enteric bacterial *E. coli* serotypes that are involved in several reports of outbreaks due to consumption of contaminated foods and drinks are capable to produce toxic particle which is the Shiga toxin (Stx), it is named after *Shigella* dysenteria type 1 which produces similar toxin to the *E. coli* and also causes bloody diarrhea and hemolytic uremic syndrome (HUS). However, *E. coli* are important to all of us and it colonize in our colon as well as the intestinal after the birth, it helps to outcompete gut pathogens in addition to produce some crucial vitamins such as a variety of B vitamins which serve as a coenzymes and K vitamin which aid in blood-clotting (Anonymous, 2011). Basically, the diarrhea-causing bacteria can be divided into 5 categories: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and lastly enteroaggregative *E. coli* (EAEC) (Nataro & Pickering, 2004).

Since *E. coli* plays a significant role in the human body and the environment, this research study has been carried out to isolate, detect and identify the enteric bacteria found in pig wastewater and feces. Over 25% of groundwater samples were polluted with *E. coli* from years 2004 to 2006 had been reported by Environmental Protection

agency in Ireland, hence *E. coli* has been used as a most important indicator for water pollution (Teagasc, 2010). But somehow, this study will only be focusing into one type of enteric bacteria which is the *E. coli*. All the isolates were taken from the pig wastes and waste water were further characterized and revealed on their multiple antibiotic resistant (MAR) index values. MAR index is normally used to identify the risk of contamination with its probability of transferring harmful microorganism to human and hence was hazardous to the human.

1.2 Objectives

The main objectives of this research study are:

a. To detect the presence of *Escherichia coli* in pig wastes from pig farms in Kuching, Sarawak.
b. To detect the presence of Shiga toxin producing gene in *E. coli* isolates.
c. To characterize the *E. coli* based on their susceptibility to different types of antibiotics and to determine its MAR index value.

CHAPTER TWO

LITERATURE REVIEW

2.1 Swine industry

Meat from pigs is one of the most important food sources in the world which accounts for about 40% of the total meat consumption because of their high nutritional value and great deliciousness (McGlone & Pond, 2003). According to Kyriazakis & Whittemore (2006), the total world production of pig meat in year 2003 was about 96 million tonnes with a constantly increase of amount by 2.4% every year in the past 10 years, where China, USA, Brazil and the European Union-15 are the key pig production country. Pigs can be catogorized into 11 principal breeds: The Berkshire, Cucumberland, Essex, Gloucestershire Old Spots, Large Black, Large White, Lincolnshire Curly-Coated, Middle White, Old Glamorgan, Tamworth and Wessex Saddleback (Jaques, 2008). Pigs can be sold in many ways such as suckling pigs, porkers, baconers, sausage pigs or larders to gain profit for farmers, only healthy pigs can get profit whereas growing sick pigs is just a waste (Prolit, 2004).

The pig's intestinal consist of much more beneficial bacteria than the harmful bacteria and even when pigs are infected by pathogens they will protect themselve by producing antibodies, however disease can occur depending on specific causal agents, host factors and also enviromental factors (Harris, 2000). There are several new diseases had evolved in the pig industry during the past forty years and these diseases were mainly due to earlier unknown agents and other predisposed by changes in husbandry (Jackson & Cockcroft, 2007). Jackson & Cockcroft (2007) mentioned that there was a new disease known as transmissible gastroenteritis which causes death of many pigs aged under 14 year

old, besides that, there were lots of newly emerging diseases such as dysentery, classical swine fever, procine reproductive and respiratory diseases and etc.

2.2 Enteric bacteria

Enteric bacteria are Gram-negative, rod-shaped bacteria that are related with gastrointestinal disease. They can be divided into 6 families which are: Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae, Bacteriodaceae, Campylobacteraceae and Helicobacteraceae. Enterobacteriaceae can be further categorized into 8 genera: *Escherichia, Salmonella, Shigella, Klebsiella, Proteus, Enterobacter, Serratia and Yersinia* (Diallo *et al.*, 2005). Janda & Abbott (2006) reported that enterobacteriaceae are associated with gastrointestinal tract of human and other animals and infection of gut as a result of pathologic processes. Besides that, enterobacteria also play importance role in extraintestinal disease including blood-borne infections, respiratory and urinary tract infections, infectious processes of wounds or surgical sites and disease that involve the ear, eye, nose and throat.

2.3 *Escherichia coli*

2.3.1 Characteristic of *E. coli*

E. coli is one of the member of Enterobacteriaceae which inhabitat in the bowel system of humans and also animals, it has characteristics such as (Fratamico & Smith, 2006):

i. Facultative anaerobic gram-negative
ii. Non-spore-forming
iii. Straight rod in shape, either organized in group or single
iv. Usually in size of 1.1-1.5µm x 2.0-6.0µm
v. Motile if associated with peritrichous flagella and perhaps non-motile

vi. May have capsule or microcapsules

vii. Chemo-organotrophic microorganism grow optimumly in 37ºC

viii. Oxidase negative, catalase positive, fermentative, reduce nitrate, β-galactosidase positive, about 95% are indole and methyl red positive but are Voges-Proskauer and citrate negative.

Some of the biochemical characteristic of *E . coli* are shown in Table 1 below.

Table 1: Summary of characteristics and reactions of *E . coli* (Sussman, 1997).

Mole % G+C	48–52
Optimum growth temperature	37°C
Indole production	+
Methyl red reaction	+
Voges Proskauer reaction	−
Citrate utilisation	−
90–100 per cent positive: Glucose (mixed acid + gas); lactose; D-mannitol; D-mannose; D-sorbitol; L-arabinose; maltose; D-xylose; trehalose; mucate; nitrate → nitrate reduction; β-galactosidase	
76–89 per cent positive: Lysine decarboxylase; motility; L-rhamnose; melibiose	
26–75 per cent positive: Ornithine decarboxylase; sucrose; dulcitol; salicin; raffinose; aesculin hydrolysis	
11–25 per cent positive: Arginine dihydrolase	
0–11 per cent positive: H_2S; urease; phenylalanine deaminase; gelatine liquefaction; growth in CN^-; malonate utilisation; D-adonitol; *myo*-inositol; cellobiose; α-methyl-D-glucoside; D-arabitol; lipase; DNase; oxidase; pigment	

2.3.2 *E. coli* strains pathogenesis/clinical features

2.3.2.1 Enterotoxigenic *E. coli* (ETEC)

Normally, symptoms of the infection show up in 12-36 hours period of time after consumption of contaminated object. The symptoms might be from mild afebrile diarrhea to severe cholera-like syndrome of watery stools without blood or mucus accompany by stomachache and vomiting, the illness was continue for 2-3 days which may lead to serious dehydration (Adams & Moss, 2008). ETEC adhere and colonize the intestinal mucosa, then produce and release either enterotoxins heat-labile toxins (LT) or hear-stable toxins (ST), or else both LT and ST to generate the illness (Fratamico & Smith, 2006). LT is a protein resembling cholera toxin which is 82% amino acid homology to it, LT is 86 kDa in size and consist of five B subunit that bind GM1 ganglioside receptors found in the intestinal epithelium and also an enzymatically active A subunit, ADP-ribosylating activity activate cellular adenyl cyclas causes chloride ions efflux and watery diarrhea. Whereas ST is a greatly folded peptide and is 18-amino acid long which will resulting disruption of chloride channels in cell and secretory diarrhea (Anonymous, Diarrhoeal Diseases , 2009). The simplified pathway where it contacts with cells is shown in figure 2.

2.3.2.2 Enteroinvasive *E. coli* (EIEC)

The pathogenesis of EIEC is almost the same as the pathogenesis of *Shigella* but different from other strains, EIEC is able to attack the cells via the unique virulence component encoded by multiple genes in both the chromosome and also plasmid (Manning, 2010). Manning reveals that primary virulence genes which are *mxi* and *spa* encode a machinery that exudes multiple proteins which aid in the invasive process, other than that he also states that EIEC synthesize one or more cytotoxins that damage the cells. EIEC destroy and multipliy in the colon epithelial cells causes ulceration and inflammation to occur, besides

that, the symptoms include fever, severe stomach pain, malaise and watery diarrhea and often lead to diarrhea with blood, mucus and fecal leukocytes (Adams & Moss, 2008). Adam and Moss (2008) affirmed that the infective dose of EIEC are higher than *Shigella* due to the organism's which are more sensitive to gastric acidity, the rate of invasive is correlated to the number of outer membrane protein which encode for a large plasmid (about 140 MDa). The simplifed pathway where it contacts with cells are shown in figure 2.

2.3.2.3 Enteropathogenic *E. coli* (EPEC)

EPEC develops as microcolonies on outside of the intestinal epithelial cells and histopathological alterations of the infected cells are called attaching and effacing (A/E) lesions (Roxas *et al*., 2007). According to Goosney and friends (1999) this A/E lesion formation involve 3 stages which are: First stage, adherence of epithelial cells through the formation of type IV frimbriae named bundle forming pili and all the important genes that are involved in formation of A/E lesions are situated at a 35-kbp pathogenity island called the locus of enterocyte effacement as shown in figure 1 below, the genes including of *esp*s (*E. coli*-secreted protein), *esc*s (*E. coli* secretion), *sep* (secretion of *E. coli* proteins), *eae* (*E. coli* attaching and effacing that encodes intimin), and *tir* (translocated intimin receptor) genes. Second stage involves discharge of bacterial proteins such as EspA, EspB, and EspD into host cell and the last stage is an intimate attachment to the host cell, a 94-kDa outer membrane protein, intimin was encoded by *eae* gene (Goosney *et al*., 1999).

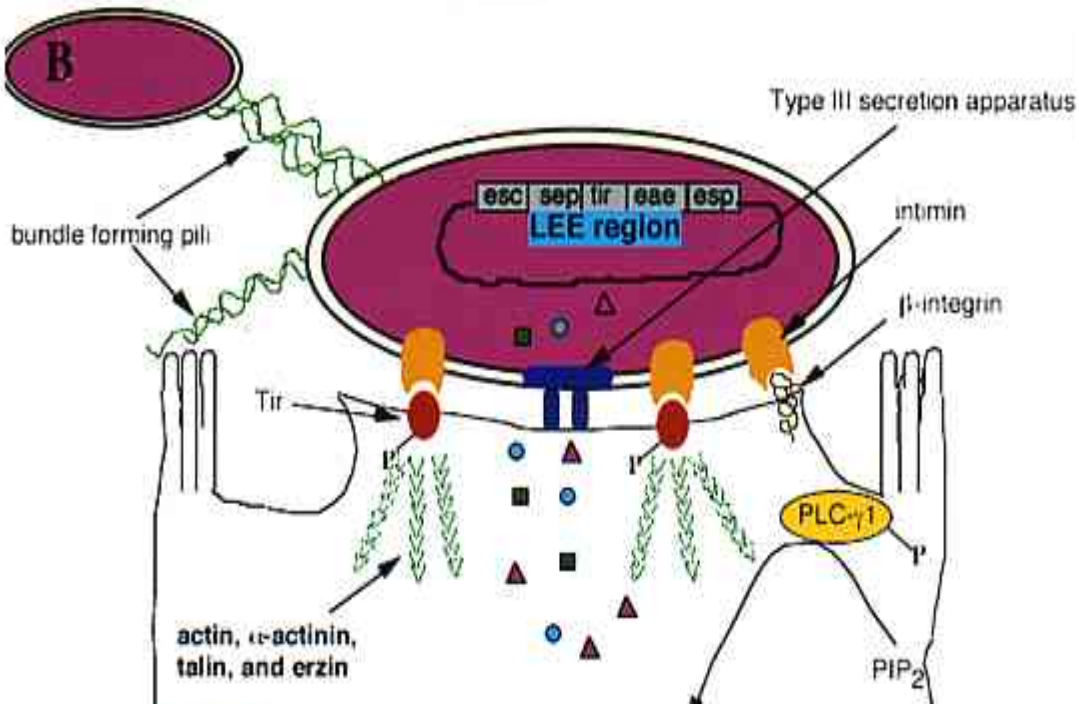

Figure 1: 35-kbp pathogenity island called the locus of enterocyte effacement (LEE) region. (Goosney *et al*., 1999)

2.3.2.4 Enterohemorrhagic *E.coli* (EHEC)

EHEC has ability to produce Shiga toxin and this can be divided into 2 types which are *stx*1 or *stx*2 only or produced both simultaneously, *stx* toxins induce submucosal immune cells to release cytokine which will causes inflammatory and also increase expression of stx receptor globotriaosylceramide (Gb3) (Garcia *et al.*, 2010). Gracia and friends pointed out that stx targets on endothelium organs where the Gb3 receptor is expressed, assembling of fibrin and thrombosis due to the damage of stx-mediated endothelial triggers coagulation and inhibition of fibrinolysis. The intimin, an outer membrane protein plays an important role in the pathogenesis of EHEC too. The symptoms were to be shown out within 1-9 days, this including of bloody diarrhea, obvious bloody tool specimen, without fever, peripheral leukocyte count above 10 000/microliter and abdominal tenderness, 65% of patient with *E.coli* O157:H7 infection show 3 or more symptoms and others maybe caused by *Shigella, Campylobacter* or *Salmonella.* As low as 1-2 percent of fatal rate and

the chances to death is higher among elderly and patients who associated with hemolytic-uremic syndrome (HUS). Mild infection will be recovered around one week time (Calderwood, n.d). The simplified pathway of various pathogenic *E . coli* where it is contacts with cells are shown in Figure 2 as below.

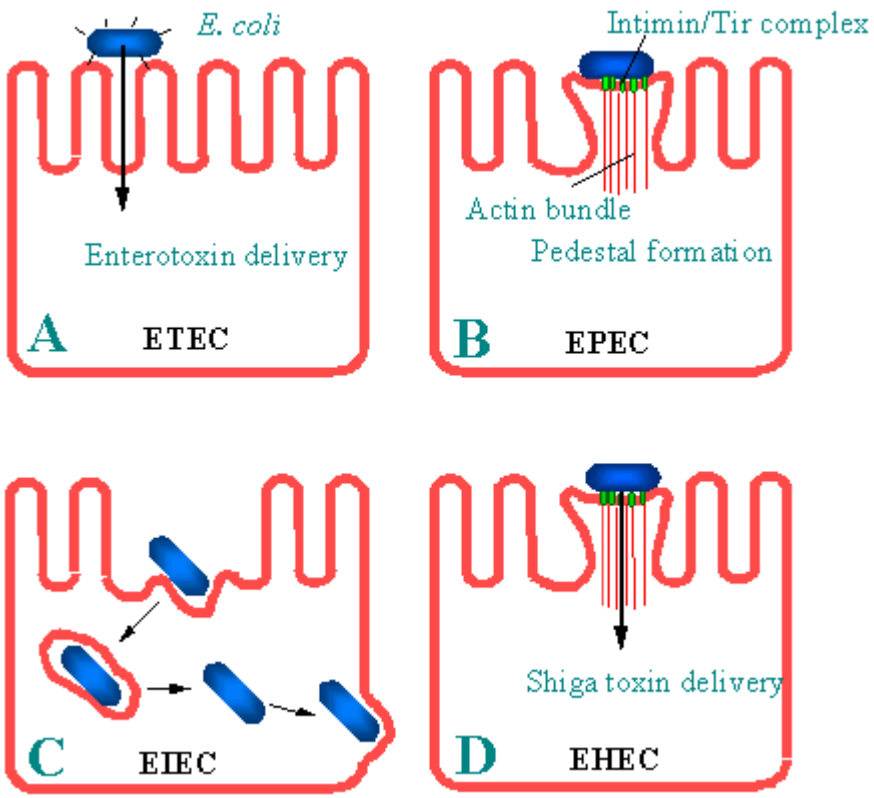

Figure 2: Pathways of various pathogenic *E. coli* contact with cells. A: ETEC binds using frimbriae and toxin released into cell without disruption of cytoskeleton. B: EPEC devastates the brush boarder microvilli and then attached tightly via pedestral which made by actin and actin binding proteins. C: EIEC enter cell then exit from immune system by digesting the phagolyosome, it grows and multiply in cell and infect surrounding cells via destroying of membranes. D: EHEC, function similarly to EPEC and Shiga toxin secretes taken up by cells in coated pits then taken to Golgi, toxins move from Golgi to E.R to destroy ribosomes by removing a adenine residue from 28SrRNA causing death of the cell. (Anonymous, The Invasion of Eukaryotic cells by Bacteria- The Role of Cytoskeleton, n.d)

2.3.2.5 Enteroaggregative *E. coli* (EAEC)

Pathogenesis of EAEC was initiated by the adherence of the bacterium to the intestinal mucosa the examination of may involve both the small and large intestines, then secrete one or more enterotoxins and causes diarrhea to be happened, adherence of EAEC can be

detected by presence of a thick, aggregating biofilm (Nishi *at el.,* 2003). For example, EAEC mucosal effects can be review through the study of strain 042 which is shown to causes diarrhea in the adult. It was found that the strain 042 was strongly adhere to the jejuna, ilea and colonic mucosa, besides that, cytotoxic effects are shown too which noticeable by exfoliation of mucosal epithelial cells (Nataro *at el.,* 1996). Symptoms of infection are such as watery, mucoid and secretory diarrhea, low or absence of fever, occasionally vomiting, 33 percent infected persons consist of bloody stools, febrile conditions presence and those who are infected with prototype EAEC strains show low volume mucoid diarrhea with no blood or fecal white blood cells (Villaseca *at el.*, 2005).

2.4 Antimicrobial susceptibility tests (ASTs)

There are 4 fundamental methods for ASTs which are: disc diffusion, agar dilution, broth dilution and gradient diffusion where disc diffusion method is the most commonly used method (Nagoba & Nagoba, 2009; Evangelista & Truant, 2002). There is a research carried out for disk diffusion testing for pathogenic enteric bacteria, it was found that pattern of antimicrobial susceptibility of *E. coli* and *Salmonella* spp. were alike although the resistance of *E. coli* towards each antibiotic was higher than the *Salmonella* spp. Both of them are resistant to penicillins, tetracycline, several cephems, carbapenem, aminoglycosides (Cho *et al*, 2008). According to Maraki *et al.* (2003) *Yersinia* spp. was found to be most resistance to amplicillin, following by chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole but resistance to gentamicin, norfloxacin, ciprofloxacin does not detected.

2.5 Polymerase Chain Reaction

The theory behind this technique is that amplification of specific DNA region in microbe of interest using a thermostable DNA polymerase to a measurable level. The amplicons