CHAPTER II

LITERATURE REVIEWS

1. Lactobacillus

1.1 Role of Lactobacillus in female genital and urinary tracts

Döderlein found that the microflora was homogenously colonised with gram - positive rods, which were designated the name "Döderlein's bacilli". Over the years, these bacilli have been identified as *Lactobacillus* spp. and are still believed to be the dominant components of the genital microflora; however, periodically in combination of other anaerobic and aerobic bacteria species and yeast, among other microbes (19, 20). The ecological role of lactobacilli in the female genital tract has been thoroughly studied and there is a strong global belief that lactobacilli are part of the defence against pathogenic species. Several means of action have been suggested, but the specific mechanisms and the interaction between these defence mechanisms are not fully understood. The last decade's increased global interest in women's health has led to reports concerning the microbial ecology of the female genital area.

Lactobacillus spp. is consistently reported to be the predominant species found in the vagina in the majority of healthy menstruating women (20, 21). Lactobacilli are non - pathogenic, gram positive, rod - shaped, facultative anaerobic bacteria that, as well as colonizing the genital tract, are often present in the gut and the oral cavity. The genus is large and comprises more than 100 described species. In recent years, *Lactobacillus iners, Lactobacillus crispatus, Lactobacillus gasseri* and *Lactobacillus*

jensenii have been reported to be the most common species found in the vagina of healthy females (22, 23). Vaginal samples often contain only one or a few species, and lactobacilli can be found in concentrations of up to 10^8 cells per ml vaginal fluid (24). Besides *Lactobacillus* spp., the bacterial flora in the genital tract is characterised by a mix of gram - positive cocci and gram - negative rods. Despite evidence that the *Lactobacillus* flora plays an important protective role against pathogens within the genital ecological niche, healthy women who are not colonised with lactobacilli are not uncommon (25 - 27). This is, however, not considered abnormal or as a sign of disease. Moreover, the normal flora changes during different phases of life and during the menstrual cycle (28). A difference can be seen in the flora as well as the vaginal pH in premenarcheal, premenopausal, postmenopausal and pregnant women (29). For example, the vaginal *Lactobacillus* flora is foremost prominent in women of reproductive age and the vaginal pH is often low. However, in fertile women, the numbers of protective *Lactobacillus* are reduced during menses (30), possibly as a result of a changed physiological environment. Furthermore, the changes in the microbiological flora have been associated with behavioral variables, for example, the number of sex partners and vaginal douching (30, 31).

Defence mechanisms

In addition to the influence of the genital normal flora, several systems are believed to collaborate in order to resist the overgrowth of potential pathogenic exogenous microbes, but the combined system of interaction is still unsolved. The vaginal wall is lined by a stratified squamous epithelium, which is kept moist by vaginal fluid. The fluid contains secretions from cervical and vestibular glands and a plasma transudate secreted through the vaginal wall (32). In addition to components

secreted by the normal flora, the vaginal fluid has a selective antimicrobial activity towards potential pathogenic microbes. Organic acids, peroxides and polypeptides are major elements found in significant concentrations that are high enough to be microbicide (33). Furthermore, the acidic milieu (pH < 4.5) contributes to the continuance of a stable and specific flora. Despite the fact that certain microbes grow at a broad range of pH levels, certain enzymatic systems may be corrupted when they grow in unfavourable milieus, such as low pH (34). The low pH is maintained by a continuous metabolism of glycogen by *Lactobacillus* species and/or vaginal epithelial cells (VECs) and lactic acid is the end product (25). In fact, women with high vaginal lactobacilli counts do have a lower vaginal pH (35 - 37). Furthermore, lactic acid present in the supernatant of cultured lactobacilli has been shown to cause growth inhibition of *Gardnerella vaginalis*, irrespective of the present pH (38). The impact of hydrogen peroxide producing *Lactobacillus* spp. in the vagina has also been investigated in several studies (32, 39). Al - Mushrif *et al.* showed that in women without a history of bacterial vaginosis (BV), 75% percent were colonised with lactobacilli that produced hydrogen peroxide. Only 14% of the women with BV had H_2O_2 - producing lactobacilli (38). In other study, Valore *et al.* reported antimicrobial polypeptides in the vaginal fluid among healthy women (32). Lysozyme and calprotectin were present in concentrations that were previously found to be antimicrobial. In the same study, other polypeptides were also identified but not in significant concentrations. However, a better effect of these polypeptides may be achieved by synergistic actions. For example, Singh *et al.* identified a significant antimicrobial effect when lactoferrin and secretory leukoprotease inhibitor were combined (40). Additionally, non - specific antimicrobial peptides released by VECs,

called defensins, and an immune triggering heat - shock protein, called hsp70, contribute to a healthy vaginal status (41, 42). Alongside the protective role of the genital normal flora, the innate and acquired immune systems play a considerable role in the prevention of pathogenic microbes. This protection is not fully understood and is attributed to several means of action. In short, the invading microbe expresses certain pathogen associated molecular patterns (PAMPs). Pathogenic, gram - positive bacteria present peptidoglycan on their surface and gram - negative bacteria present lipopolysaccharide. The PAMPs are recognized by different Toll - like receptors on the surface of VECs, several of which have been previously identified (43). The interaction initiates a sequence of events that finally leads to the release of pro - inflammatory cytokines and the activation of the acquired immune system.

1.2 Antimicrobial substances affected to Enterobacteriaceae from UTI.

This group includes organic acids, fatty acids, hydrogen peroxide, biosurfactants, bacteriocins and bacteriocin - like substances (BLSs) (25). Antimicrobial acids, H_2O_2 and BLSs, are through different mechanisms, the primary metabolites produced by probiotics associated with inhibition and the control of pathogens. Acids with growth inhibiting effect on different microbes, and in particular molds, are produced by specific *Lactobacillus* strains and have been identified and characterised previously.

Hydrogen peroxide is produced by many *Lactobacillus* strains and in various amounts. Kaewsrichan *et al.* showed that a number of cultured H_2O_2 - producing lactobacilli generate hydrogen peroxide at inhibitory levels to *G. vaginalis* and *E. coli*.

(44). The particular inhibition mechanism is divided into two parts : (i) toxicity to microbes or (ii) as a catalyst in the peroxidase system (34).

Acid

Lactobacillus species can produce metabolites such as lactic and acetic acid, and thus lowering the pH, a large number of lactobacilli inhibit the growth of bacterial pathogens (45). Hudault *et al.* (46) reported that the mechanism by which lactobacilli impeded the invasion of host cells by bacterial pathogens was abolished after the *Lactobacillus* culture had been neutralized to pH 7, which suggested a pH - dependent mechanism. Alakomi *et al.* (47) reported that the lactic acid produced by lactobacilli acts as a permeabilizer of the gram - negative bacterial outer membrane, allowing other antimicrobial substances produced by the host to penetrate and increase the susceptibility of pathogens to these antimicrobial molecules. However, the inhibition of the growth of bacterial pathogens is not due to pH alone, but there were the presence of other *Lactobacillus* - inhibiting substances that are extracellular and diffusible (48).

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) - producing lactobacilli predominate in the normal vagina but are seldom found in the vagina of patients with bacterial vaginosis (32). The production of H_2O_2 by *Lactobacillus* spp. may be a non - specific antimicrobial defence mechanism of the normal vaginal ecosystem (49). Optional H_2O_2 - producing *Lactobacillus* spp. are found in the vagina of most normal women but much less often in that of women with bacterial vaginosis, whereas anaerobic *Lactobacillus* spp.

which do not produce hydrogen peroxide have been found to be increased in women with bacterial vaginosis (48).

Bacteriocins

Bacteriocins are the bactericidal proteinceous molecules produced by bacteria. The bacteriocin family includes a wide variety of peptides and proteins in terms of their size, source of production, microbial targets, and mechanism of action (50). The bacteriocin comprises a large and diverse group of ribosomally synthesized antimicrobial proteins or peptides some of which undergo posttranslational modifications. Most bacteriocins from lactic acid bacteria exert their antibacterial effect by permeabilizing the target cell membrane, where the cells lose their viability (51, 52). The mechanisms of bacteriocins for possessing the bactericidal activity include enzyme activity modulation, inhibition of over growth of spores and anion carrier activity to the formation of selective or non - selective pores (53). However, the common mechanisms of the activity are membrane insertion and pore formation.

2. Enterobacteriaceae

An important bacterial family in human medicine is the Enterobacteriaceae. This family includes genera and species that cause well - defined disease with typical clinical symptoms as well as many opportunities that cause mainly nosocomial infections. Enterobacteriaceae are the most significant contributors to intestinal infection, which are among the most frequent diseases of all among the developing world population (54).

They are gram - negative, straight rods, some of which are motile and some have a capsule. Most species grow well at 37 °C, although some species grow better at 25 - 30 °C (55). Members of the family Enterobacteriaceae are primary identified by their colonial appearance and other distinguishing characteristics on some differential media such as MacConkey agar, Eosin methylene blue (EMB) agar which most of them grow well on these media. The use of biochemical profiles obtained with triple sugar iron (TSI) agar and/or lysine iron (LIA) agar are important tests. The characteristics of carbohydrate fermentation, hydrogen sulfide and gas production on TSI are listed in Table 1.

Table 1 Characterization of fermentative Enterobacteriaceae on TSI (56)

A/A or A/AG	**A/A$^+$ or A/AG$^+$**	**K/A or K/AG**	**K/A$^+$ or K/AG$^+$**
Cedecea spp.			
C. davissae			
C. neteri			
Citrobacter spp.	*C. freundii*	*C. diversus*	*C. freundii*
C. diversus			
C. amalonaticus			
			Edwardsiella tarda
Enterobacter spp.		*E. agglomerans*	
E. aerogenes			
E. agglomerans			
E. cloacae			
E. gergoviae			
E. sakazaki			
Escherichia spp.		*E. coli*	*E. coli*
E. coli		*E. hermanii*	
E. hermanii		*E. fergussonii*	
Hafnia alvei		*H. alvei*	
Klebsiella spp.		*K. ozaenae*	
K. ornitholytica		*K. rhinoscleromatis*	
K. oxytoca			
K. ozaenae			
K. rhinoscleromatis			
K. pneumoniae			
Kluyvera spp.			
K. ascorbata			
K. cryscrescens			
		Morganella morganii	

Table 1 (continued)

A/A or A/AG	**A/A$^+$ or A/AG$^+$**	**K/A or K/AG**	**K/A$^+$ or K/AG$^+$**
Proteus penneri	*P. mirabilis* *P. penneri* *P. vulgaris*		*P. mirabilis* *P. vulgaris*
		Providencia spp. *P.alcalifaciens* *P.rettgeri*	
	Salmonella subspecies III	*S.* Paratyphi A	*S.*Gallinarum *S.*Pullorum *S.*Typhi
Serratia sp.		*S. odorifera* biogroup 1 *S. odorifera* biogroup 2	
		Shigella spp.	
Yersinia spp.		*Y. pestis* *Y. pseudotuberculosis*	

* A/A, Acid slant, Acid butt; K/A, Alkaline slant, Acid butt; G, gas production; $^+$, hydrogen sulfide production

After TSI experiments, each isolate must be diagnosed with various biochemical tests for its differentiation and identification as exhibited in scheme below.

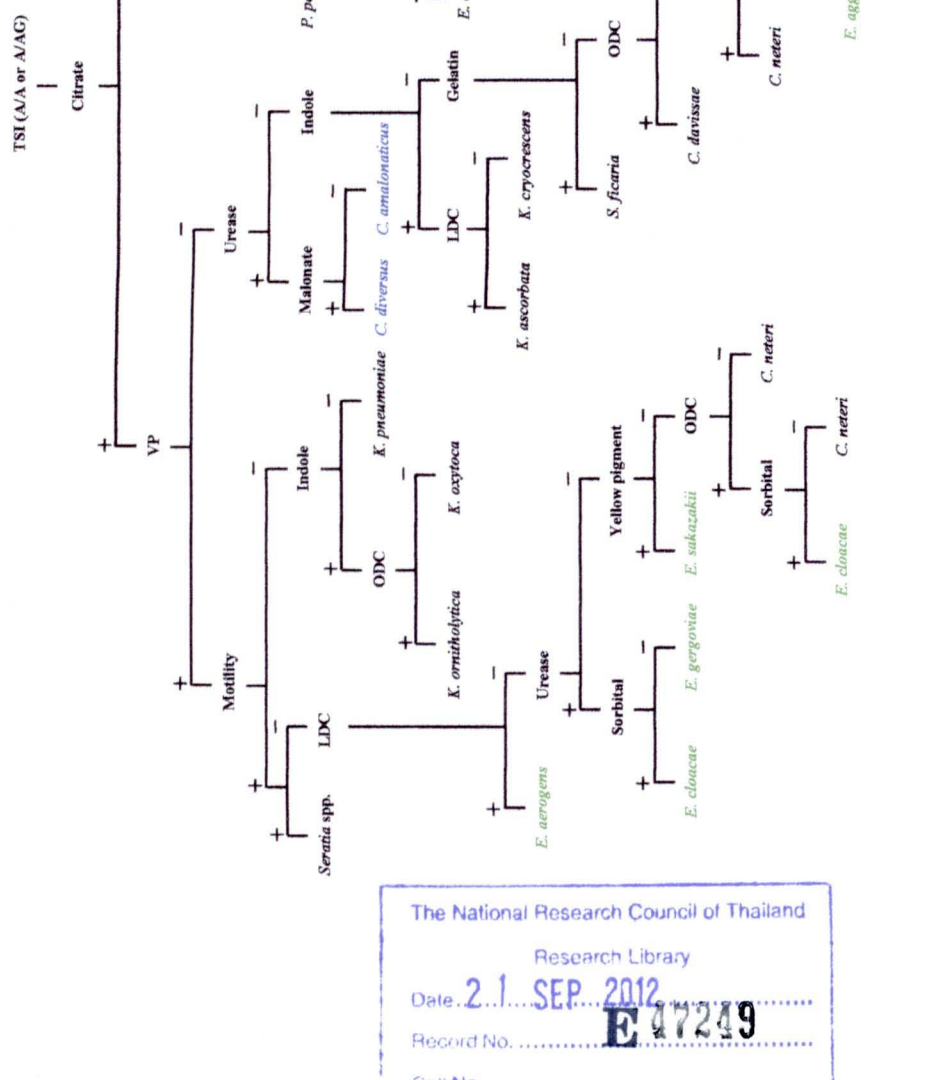

Figure 1 Identification of members of Enterobacteriaceae in the characteristics with A/A or A/AG (56)

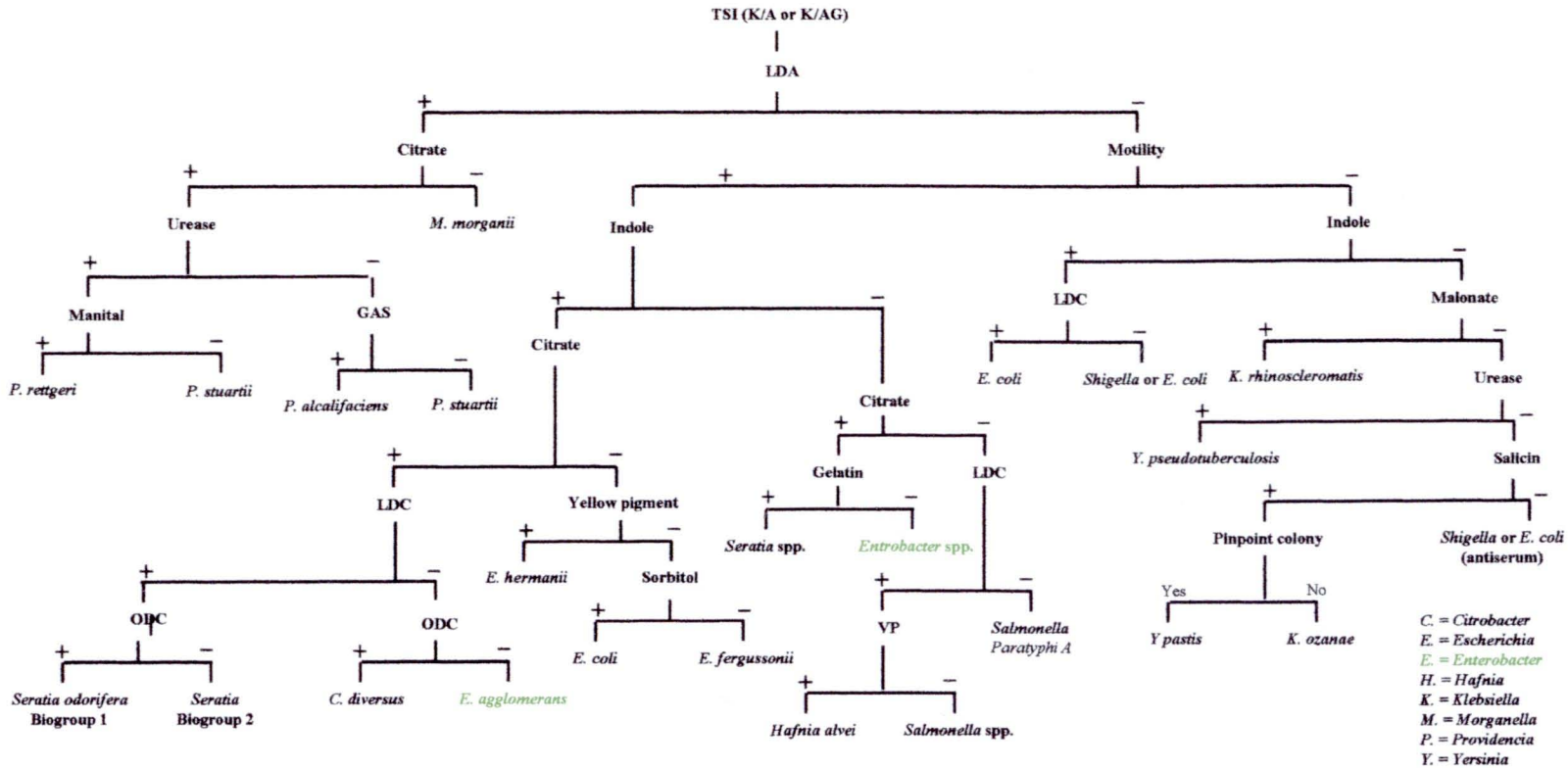

Figure 2 Identification of members of Enterobacteriaceae in the characteristics with K/A^- or K/AG^- (56)

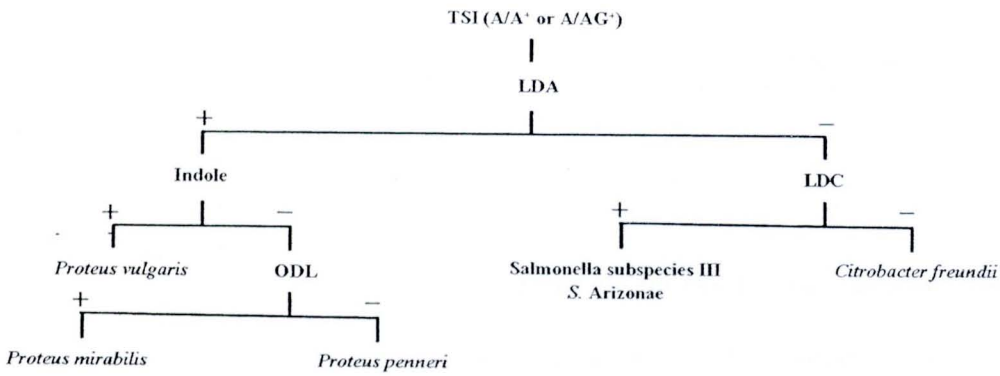

Figure 3 Identification of members of Enterobacteriaceae in the characteristics with A/A^+ or A/AG^+ (56)

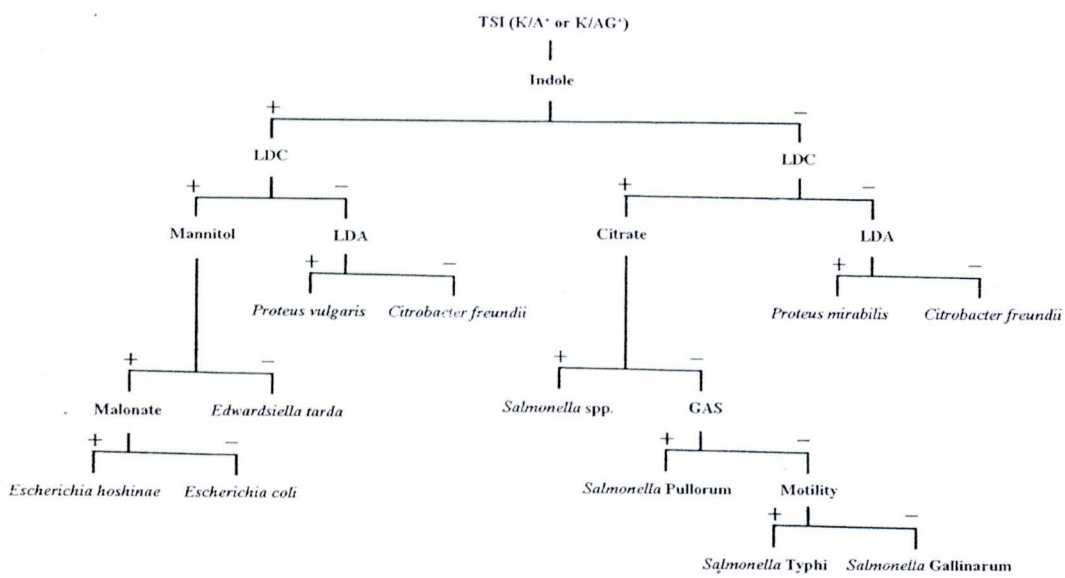

Figure 4 Identification of members of Enterobacteriaceae in the characteristics with K/A^+ or K/AG^+ (5)

2.1 Pathogenic members in the family Enterobacteriaceae

Most common pathogenic genera of the family Enterobacteriaceae include as belows.

2.1.1 *Escherichia*

There are 11 species, of which 4 species are known to cause human disease. Only the most successful combinations of virulence factors have persisted to become specific "pathotypes" of *E. coli* that are capable of causing disease in healthy individuals. Three general clinical syndromes can result from infection with one of these pathotypes: enteric/diarrhoeal disease, UTIs and sepsis/meningitis (57). While *E. coli* is responsible for the vast majority of *Escherichia* - related pathogenesis, other members of the genus have also been implicated in human disease (58, 59). Theodor Escherich first described *E. coli* in 1885, as *Bacterium coli* commune, which he isolated from the feces of newborns. It was later renamed *E. coli*, and for many years the bacterium was simply considered to be a commensal organism of the large intestine. It was not until 1935 that a strain of *E. coli* was shown to be the cause of an outbreak of diarrhea among infants. *E. coli* is facultatively anaerobic gram - negative rods that live in the intestinal tracts of animals in health and disease. It can grow in media with glucose as the sole organic constituent. Wild - type *E. coli* has no growth factor requirements, and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O_2. Under anaerobic conditions it will grow by means of fermentation, producing characteristic "mixed acids and gas" as end products. However, it can also grow by means of anaerobic respiration, since it is able to utilize NO_3, NO_2 or fumarate as final electron acceptors for respiratory electron transport

processes. In part, this adapts *E. coli* to its intestinal (anaerobic) and its extraintestinal (aerobic or anaerobic) habitats (60).

Adhesion/colonization

Pathogenic *E. coli* strains possess specific adherence factors that allow them to colonize sites that *E. coli* does not normally inhabit, such as the small intestine and the urethra (Table 2). Most frequently these adhesins form distinct morphological structures called fimbriae (also called pili) or fibrillae, which can belong to one of several different classes. Fimbriae are rod - like structures of 5 - 10 nm diameter that are distinct from flagella. Fibrillae are 2 - 4 nm in diameter, and are either long and wiry or curly and flexible (61). The Afa adhesins that are produced by many diarrhoeagenic and uropathogenic *E. coli* are described as afimbrial adhesins, but in fact seem to have a fine fibrillar structure that is difficult to visualize (62). Adhesins of pathogenic *E. coli* can also include outer - membrane proteins, such as intimin of UPEC and EHEC, or other non - fimbrial proteins. Some surface structures trigger signal transduction pathways or cytoskeletal rearrangements that can lead to disease. For example, the members of the Dr family of adhesins that are expressed by DAEC and UPEC bind to the Decay - accelerating factor (DAF, also known as CD55), which results in activation of phosphatidylinositol 3 - kinase (PI - 3 - kinase) and cell - surface expression of the major histocompatibility complex class I - related molecule (63). The IcsA protein of EIEC nucleates actin filaments at one pole of the bacterium, which allows it to move within the cytoplasm and into adjacent epithelial cells on a "tail" of polymerized actin (64). Even surface structures that are present on commensal *E. coli* strains can induce signaling cascades if the organism encounters the appropriate receptor. The LPS of *E. coli* and other gram - negative bacteria binds

to Toll - like receptor 4 (TLR4), triggering a potent cytokine cascade that can lead to septic shock and death (65). Flagellin, the main component of flagella, can bind to TLR5, thereby activating interleukin (IL) - 8 expression and an inflammatory response (66).

Table 2 *E. coli* virulence factors : colonization and fitness factors (67)

Factors	**Pathotypes**	**Remarks**
Adhesion		
Intimin	EPEC, EHEC	induces TH1 response
Dr adhesins	DAEC, UPEC	binds to decay - accelerating factor (DAF), activates PI - 3 - kinase, induces MHC class I chain - related gene A (MICA)
(Pap) fimbriae	UPEC	induces cytokine expression
CFAs	ETEC	> 20 different factors designated colonization factor antigen (CFA), coli surface antigen (CS) or putative colonization factor (PCF)
Type - 1 fimbriae	All	UPEC adhesin; binds to uroplakin
F1C fimbriae	UPEC	
S fimbriae	UPEC, MNEC	
Bundle - forming pilus (BFP)	EPEC	type IV pilus
Aggregative adherence fimbriae	EAEC	> 4 subtypes
Paa	EPEC, EHEC	
ToxB	EHEC	
Efa - 1/LifA	EHEC	

Table 2 (continued)

Factors	Pathotypes	Remarks
Long polar fimbriae (LPF)	EHEC, EPEC	
Saa	EHEC	
OmpA	MNEC, EHEC	
Curli	Various	binds to fibronectin
Promotes invasion		
IbeA, B, C	MNEC	
AslA	MNEC	
Promotes colonization		
Dispersin	EAEC	aids mucous penetration
Iron acquisition		
Aerobactin	EIEC	siderophore
Yersiniabactin	Various	siderophore
IreA	UPEC	siderophore receptor
IroN	UPEC	siderophore receptor
Chu (Shu)	EIEC, UPEC, MNEC	haem transport
Antiphagocytic		
K antigen capsules	MNEC	> 80 K types
Induces cytokine expression		
Lipopolysaccharide	All	through TLR4; >180 O types
Flagellin	All	induces cytokine expression through TLR5; > 50 flagella (H) serotypes
Motility		
IcsA (VirG)	EIEC	

Toxins

More numerous than surface structures that trigger signal transduction pathways are secreted toxins and other effector proteins that affect an astonishing variety of fundamental eukaryotic processes (Table 3). Concentrations of important intracellular messengers, such as cyclic AMP, cyclic GMP and Ca^{2+}, can be increased, which leads to ion secretion by the actions of the heat - labile enterotoxin (LT), heat - stable enterotoxin a (STa) and heat - stable enterotoxin b (STb), respectively - all of which are produced by different strains of ETEC (68). The Shiga toxin (Stx) of EHEC cleaves ribosomal RNA, thereby disrupting protein synthesis and killing the intoxicated epithelial or endothelial cells (69). The cytolethal distending toxin (CDT) has DNase I activity that ultimately blocks cell division in the G2/M phase of the cell cycle (70). Another toxin that blocks cell division in the same phase, called Cif (cycle - inhibiting factor), does not possess DNase I activity, but might act by inhibition of Cdk1 kinase activity (71). The cytotoxic necrotizing factors (CNF 1 and CNF 2) deaminate a crucial glutamine residue of RhoA, Cdc42 and Rac, thereby locking these important signaling molecules in the "on" position and leading to marked cytoskeletal alterations, multinucleation with cellular enlargement, and necrosis (72). The Map protein of EPEC and EHEC has at least two independent activities - stimulating Cdc42 - dependent filopodia formation and targeting mitochondria to disrupt membrane potential in these organelles (73).

Table 3 Example of *E. coli* virulence factors: toxins and effectors (67)

Factors	Pathotypes	Targets	Activity/effects
AB subunit			
Heat - labile enterotoxin (LT)	ETEC	Gs	ADP ribosylates and activates adenylate cyclase resulting in ion secretion
Shiga toxin (Stx)	EHEC	rRNA	Depurinates rRNA, inhibits protein synthesis; induces apoptosis
Autotransporter			
Haemoglobin - binding protease (Tsh)	ExPEC, APEC	Haem	Degrades haemoglobin to release haem/iron
Pet	EAEC	Spectrin	Serine protease; ion secretion; cytotoxicity
Type III effector			
Map	EPEC, EHEC	Mitochondria	Disrupts mitochondrial membrane potential
Tir	EPEC, EHEC	Nck	Nucleation of cytoskeletal proteins, loss of microvilli, GAP - like activity
IpaA	EIEC	Vanculin	Actin depolymerization
IpaB	EIEC	Caspase 1	Apoptosis, IL - 1 release; membrane insertion
IpaC	EIEC	Actin	Actin polymerization, activation of Cdc42 and Rac
IpaH	EIEC	Nucleus	Modulates inflammation

Table 3 (continued)

Factors	Pathotypes	Targets	Activity/effects
RTX toxins			
HlyA	UPEC	Erythrocytes, Leukocytes	Cell lysis
Ehx	EHEC	Erythrocytes, Leukocytes	Cell lysis
Heat - stable enterotoxins			
Heat - stable enterotoxin a (STa)	ETEC	Guanylate cyclase	Activates guanylate cyclase resulting in ion secretion
Heat - stable enterotoxins			
EAST	Various	Guanylate cyclase	Activates guanylate cyclase resulting in ion secretion

*These factors have been characterized in *Shigella* species, but their presence in EIEC has not yet been established. EAST, enteroaggregative *E. coli* ST; GAP, GTPase - activating protein; IL, interleukin; PtdIns (4 - 5) P_2, phosphatidylinositol - 4, 5 - bisphosphate.

Pathotypes and pathogenesis

Enteropathogenic *E. coli* (EPEC)

EPEC induce a profuse watery, sometimes bloody, diarrhea. They are a leading cause of infantile diarrhea in developing countries. Pathogenesis of EPEC involves a plasmid - encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells and a non fimbrial adhesion designated intimin, which is an outer membrane protein that mediates the final stages

of adherence. EPEC adhere to small bowel enterocytes, but destroy the normal microvillar architecture, inducing the characteristic attaching and effacing lesion. EPEC strains are said to be "moderately - invasive", meaning they are not as invasive as *Shigella*. The diarrhea and other symptoms of EPEC infections probably are caused by bacterial invasion of host cells and interference with normal cellular signal transduction, rather than by production of toxins (Figure 5). Through volunteer feeding studies the infectious dose of EPEC in healthy adults has been estimated to be 10^6 organisms.

Enterohaemorrhagic *E. coli* (EHEC)

EHEC are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal HUS. EHEC are characterized by the production of verotoxin or Stx. Although Stx1 and Stx2 are most often implicated in human illness, several variants of Stx2 exist. There are many serotypes of Stx - producing *E. coli*, but only those that have been clinically associated with HC are designated as EHEC. Of these, O157 : H7 is the prototypic EHEC and most often implicated in illness worldwide. The infectious dose for O157 : H7 is estimated to be 10 - 100 cells; but no information is available for other EHEC serotypes. EHEC infections are mostly food or water borne and have implicated undercooked ground beef, raw milk, cold sandwiches, water, unpasteurized apple juice and vegetables. EHEC are considered to be "moderately invasive". The bacteria do not invade mucosal cells as readily as *Shigella*, but EHEC strains produce a toxin that is virtually identical to the Shiga toxin. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability

of EHEC strains to cause HUS. The toxin is phage encoded and its production is enhanced by iron deficiency.

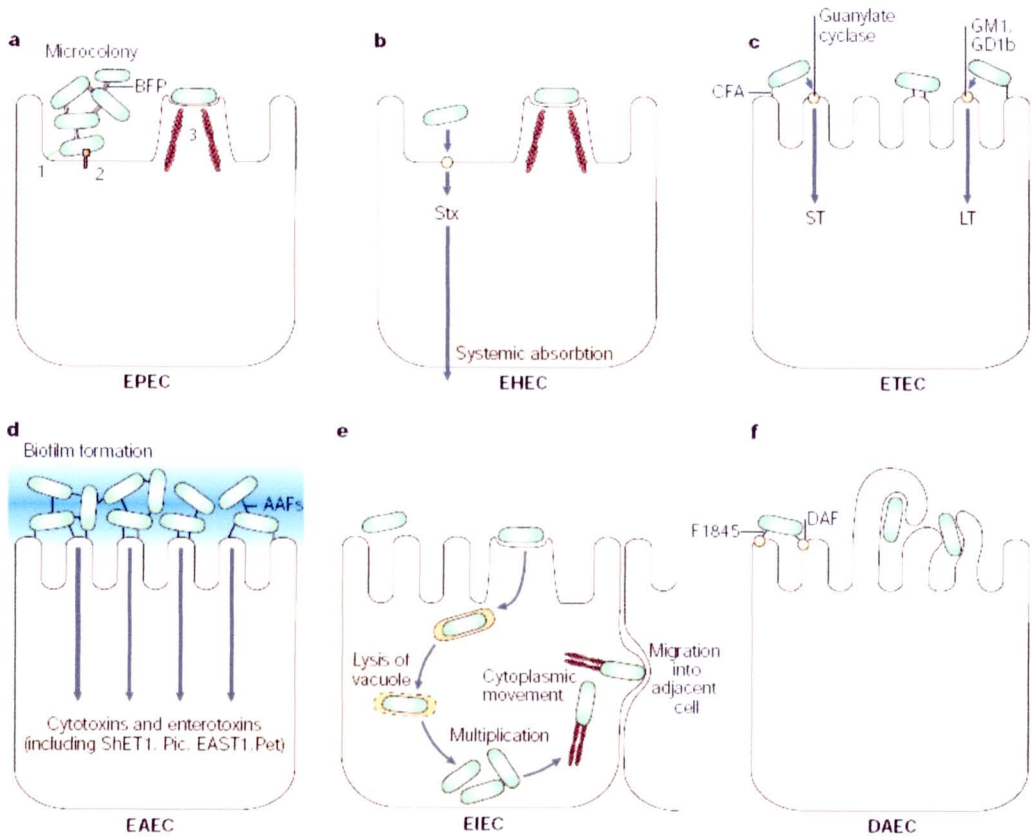

Figure 5 Pathogenic schema of diarrhoeagenic *E. coli* (67); 1, Initial adhesion; 2, Protein translocation by type III secretion; 3, Pedestal formation; BFP, bundle - forming pilus; CFA, colonization factor antigen; DAF, decay - accelerating factor; EAST1, enteroaggregative *E. coli* ST1; ShET1, *Shigell*a enterotoxin 1.

Enterotoxigenic *E. coli* (ETEC)

ETEC is an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. The diseases vary from minor

discomfort to a severe cholera - like syndrome. ETEC are acquired by ingestion of contaminated food and water, and adults in endemic areas evidently develop immunity. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid - encoded. ETEC may produce a heat - labile enterotoxin (LT) that is similar in molecular size, sequence, antigenicity, and function to the cholera toxin (Ctx). It is an 86 kDa protein composed of an enzymatically active (A) subunit surrounded by 5 identical binding (B) subunits. It binds to the same identical ganglioside receptors that are recognized by the cholera toxin (i.e., GM1), and its enzymatic activity is identical to that of the cholera toxin. ETEC may also produce ST that is of low molecular size and resistant to boiling for 30 min. The ST enterotoxins are peptides of molecular weight about 4,000 daltons. Their small size explains why they are not inactivated by heat. ST causes an increase in cyclic GMP in host cell cytoplasm leading to the same effects as an increase in cAMP. ST1a is known to act by binding to a guanylate cyclase that is located on the apical membranes of host cells, thereby activating the enzyme. This leads to secretion of fluid and electrolytes resulting in diarrhea. The infective dose of ETEC for adults has been estimated to be at least 10^8 cells; but the young, the elderly and the infirm may be susceptible to lower numbers. ETEC adhesins are fimbriae which are species - specific. These fimbrial adhesins adhere to specific receptors on enterocytes of the proximal small intestine. Symptoms ETEC infections include diarrhea without fever. The bacteria colonize the GI tract by means of a fimbrial adhesin, e.g. CFA I and CFA II, and are noninvasive, but produce either the LT or ST toxin.

Enteroaggregative *E. coli* (EAEC)

The distinguishing feature of EAEC strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non - bloody diarrhea without invading or causing inflammation. Recently, a distinctive heat - labile plasmid - encoded toxin has been isolated from these strains, called the EAST (EnteroAggregative ST) toxin. They also produce a hemolysin related to the hemolysin produced by *E. coli* strains involved in urinary tract infections.

Enteroinvasive *E. coli* (EIEC)

EIEC closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. EIEC penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes dysentery - like diarrhea with fever. EIEC apparently lack fimbrial adhesins but do possess a specific adhesin that, as in *Shigella*, is thought to be an outer membrane protein. Also, like *Shigella*, EIEC are invasive organisms. They do not produce LT or ST toxin. There are no known animal reservoirs of EIEC. Hence the primary source for EIEC appears to be infected humans. Although the infective dose of *Shigella* is low (in the range of 10 to few hundred cells), volunteer feeding studies showed that at least 10^6 EIEC organisms are required to cause illness in healthy adults. Unlike typical *E. coli*, EIEC are non - motile, do not decarboxylate lysine and do not ferment lactose. Pathogenicity of EIEC is primarily due to its ability to invade and destroy colonic tissue.

Diffusely adherent *E. coli* (DAEC)

DAEC are defined by the presence of a characteristic, diffuse pattern of adherence to HEp - 2 cell monolayers. DAEC have been implicated as a cause of diarrhoea in several studies, particularly in children > 12 months of age (74). Approximately 75% of DAEC strains produce a fimbrial adhesin called F1845 or a related adhesin (75), F1845 belongs to the Dr family of adhesins, which use DAF, a 4cell - surface glycosylphosphatidylinositol - anchored protein, which normally protects cells from damage by the complement system, as the receptor (76, 77). DAEC strains induce a cytopathic effect that is characterized by the development of long cellular extensions, which wrap around the adherent bacteria (Figure 5). This characteristic effect requires binding and clustering of the DAF receptor by Dr fimbriae (77). Binding of Dr adhesins is accompanied by the activation of signal transduction cascades, including activation of PI - 3 kinase (78). Peiffer *et al.* have reported that infection of an intestinal cell line by strains of DAEC impairs the activities and reduces the abundance of brush - border - associated sucraseisomaltase and dipeptidylpeptidase IV (79). This effect is independent of the DAF - associated pathway described above, and therefore provides a feasible mechanism for DAEC - induced enteric disease and also indicates the presence of virulence factors in DAEC other than Dr adhesins. Tieng *et al.* (63) have proposed that DAEC might induce expression of MHC class I chain - related gene A by intestinal epithelial cells, indicating that DAEC infection could be proinflammatory; this effect could potentially be important in the induction of inflammatory bowel diseases.

Uropathogenic *E. coli* (UPEC)

The urinary tract is among the most common sites of bacterial infection and *E. coli* is by far the most common infecting agent at this site. The subset of *E. coli* that causes uncomplicated cystitis and acute pyelonephritis is distinct from the commensal *E. coli* strains that comprise most of the *E. coli* populating the lower colon of humans (Figure 6). *E. coli* from a small number of O serogroups (six O groups cause 75% of UTIs) have phenotypes that are epidemiologically associated with cystitis and acute pyelonephritis in the normal urinary tract, which include expression of P fimbriae, hemolysin, aerobactin, serum resistance and encapsulation. Clonal groups and epidemic strains that are associated with UTIs have been identified (80). Although many UTI isolates seem to be clonal, there is no single phenotypic profile that causes UTIs. Specific adhesins, including P (Pap), type 1 and other fimbriae (such as F1C, S, M and Dr), seem to aid in colonization (81). It is likely that infection begins with the colonization of the bowel with an uropathogenic strain in addition to the commensal flora. This strain, by virtue of factors that are encoded in pathogenicity islands, is capable of infecting an immunocompetent host, as it colonizes the periurethral area and ascends the urethra to the bladder (Figure 6). Between 4 and 24 hrs after infection, the new environment in the bladder selects for the expression of type 1 fimbriae (82), which have an important role early in the development of a UTI (83). Type 1 fimbriated *E. coli* attach to mannose moieties of the uroplakin receptors that coat transitional epithelial cells (84). Attachment triggers apoptosis and exfoliation; for at least one strain, invasion of the bladder epithelium is accompanied with formation of pod - like bulges on the bladder surface that contain bacteria encased in a polysaccharide - rich matrix surrounded by a shell of uroplakin (85).

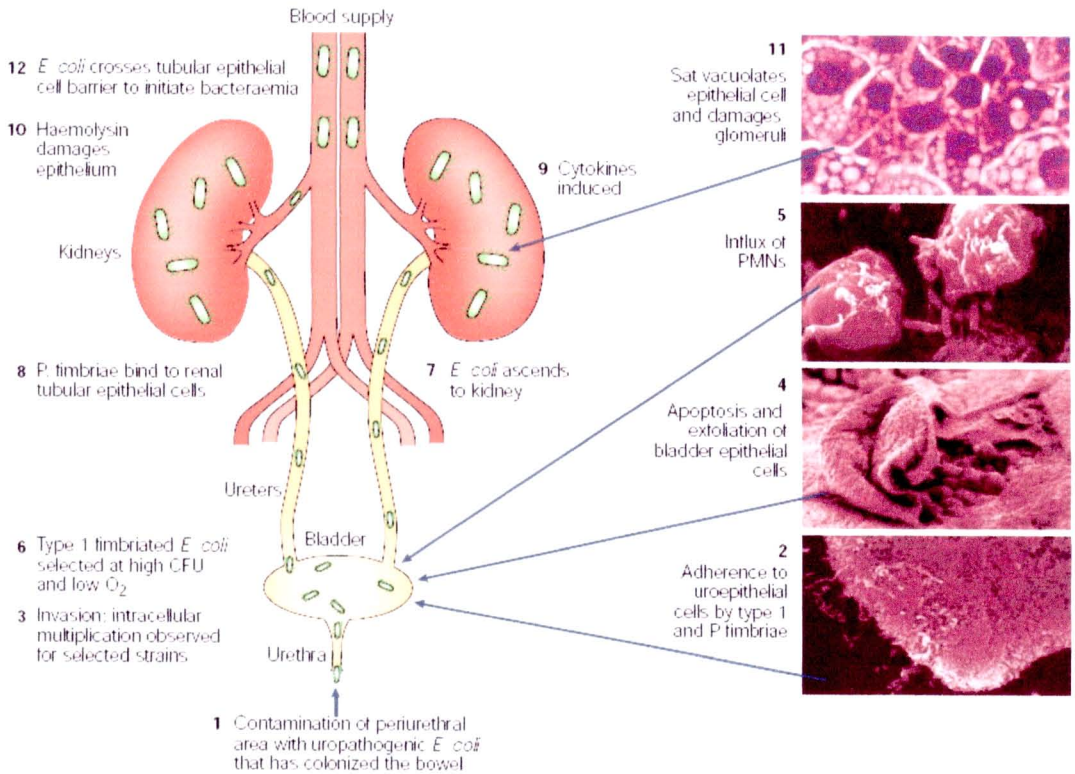

Figure 6 Pathogenesis of UTIs caused by uropathogenic *E. coli* (67)

Iron acquisition and type 1 fimbriae are continually expressed and the infection is confined to the bladder (83). In pyelonephritis strains, the invertible element that controls type 1 fimbriae expression turns to the "off" position and type 1 fimbriae are less well expressed (82). It could be argued that this releases the *E. coli* strain from bladder epithelial cell receptors and allows the organism to ascend through the ureters to the kidneys, where the organism can attach by P fimbriae to digalactoside receptors that are expressed on ability to grow in urine are also crucial for survival. At this stage, haemolysin could damage the renal epithelium (86) and, together with other bacterial products including LPS, an acute inflammatory response recruits PMNs to the site. Haemolysin has also been shown to induce Ca^{2+}

oscillations in renal epithelial cells, resulting in increased production of IL - 6 and IL - 8 (87). Secretion of Sat, a vacuolating cytotoxin, damages glomeruli and is cytopathic for the surrounding epithelium (88). In some cases, the barrier that is provided by the one - cell - thick proximal tubules can be breached and bacteria can penetrate the endothelial cell to enter the bloodstream, leading to bacteraemia.

Meningitis/sepsis - associated *E. coli* (MNEC)

This *E. coli* pathotype is the most common cause of gram - negative neonatal meningitis, with a case fatality rate of 15 - 40% and severe neurological defects in many of the survivors (58, 59). As with *E. coli* pathotypes that have a well defined genetic basis for virulence, strains that cause meningitis are represented by only a limited number of O serogroups, and 80% of the strains are of the K1 capsule type.

2.1.2 *Klebsiella*

Common sites of colonization in healthy humans are the gastrointestinal tract, eyes, respiratory tract, and genitourinary tract (89). Five species include *Klebsiella oxytoca, Klebsiella granulomatis*, *Klebsiella variicola, Klebsiella singaporensis* and *Klebsiella pneumoniae*. While four species, previously named *K. pneumoniae, Klebsiella ozaenae, Klebsiella rhinoscleromatis* and *Klebsiella aerogenes* are now classed as subspecies of *K. pneumoniae*. *K. pneumoniae* has emerged as an important cause of hospital acquired infections, especially among patients in the neonatal intensive - care unit and mortality rates can be as high as 70% (90). Over the last two decades, the incidence of infections caused by multidrug - resistant *Klebsiella* strains has increased.

Virulence Factors

Extracellular capsules are essential to virulence; the capsular material forms thick bundles of fibrillous structures that cover the bacterial surface in massive layers (89). This protects the bacterium from phagocytosis by polymorphonuclear granulocytes and prevents killing by bactericidal serum factors via the complement mediated cascade. In addition to the capsule, there are about five somatic or O antigens, fimbrial and nonfimbrial adhesins, which serve as virulence factors. The fimbriae or pili are nonflagellar, filamentous projections on the bacterial surface that mediate attachment of the organism to respiratory, gastrointestinal, and urinary tract mucosal cells. Additional virulence determinants for *Klebsiella* include the ability of the organism to scavenge iron from the surrounding medium using secreted siderophores, that is, enterochelin and aerobactin. These are high - affinity, low molecular weight iron chelators that competitively take up iron bound to host proteins (91).

2.1.3 *Salmonella*

Serotypes of *Salmonella* and *Arizona* are now considered to belong to two species - *Salmonella* Bongori (formerly subspecies V) and *Salmonella* Enterica. Most serotype is motile. All except *Salmonella* Typhi produce gas from glucose. Most produce hydrogen sulphide except *S.* Typhi and *Salmonella* Paratyphi A (which is a weak producer). The members are huge cause of diarrhea and bacteremia. Virulence factors are fimbria and adherence proteins. All virulent strains have pathogenicity islands, including one for a Type III secretion system.

2.1.4 *Shigella*

There are four species, *S. dysenteriae* (serogroup A), *Shigella flexneri* (serogroup B), *Shigella boydii* (serogroup C), *Shigella sonnei* (serogroup D). They are highly infective, particularly *S. dysenteriae*. This genus survives in stomach and bind to colon epithelium integrins. They internalize in phagosomes, lyse phagosome membrane and multiply in cytoplasm. They release endotoxin (lipopolysaccharide), causing inflame necrosis, cramping and bloody diarrhea. *S. dysenteriae* is the only group to produce shiga toxin as a virulence factor. This AB subunit toxin is associated with HUS, an acute kidney failure mostly in children (92).

2.1.5 *Enterobacter*

There are eleven species, but only eight have been isolated from clinical material. They grow readily ordinary on agar, ferment glucose with production of acid and gas, and are motile by peritrichous flagella. Some strains with a K antigen possess a capsule. Several strains of these bacteria are pathogenic and cause opportunistic infections in immunocompromised (usually hospitalized) hosts. The urinary and respiratory tracts are the most common sites of infection. Two clinically - important species from this genus are *E. aerogenes* and *E. cloacae*. *E. aerogenes* is a nosocomial and pathogenic bacterium that causes opportunistic infections in skin and other tissues. *E. cloacae* is sometimes associated with urinary tract and respiratory tract infections (93).

2.1.6 *Proteus*

There are four species of *Proteus*, of which three cause disease. All strains are urease position and motile. They may swarm on blood agar, producing concentric zones or an even film. *Proteus* includes pathogens responsible for many human

urinary tract infections. *P. mirabilis* causes wound and urinary tract infections. *P. vulgaris* and *P. penneri* are easily isolated from individuals in long - term care facilities and hospitals and from patients with underlying diseases or compromised immune systems. The first step in the infectious process is adherence of the microbe to host tissue. Fimbriae facilitate adherence and thus enhance the capacity of the organism to produce disease. *E. coli*, *P. mirabilis*, and other gram - negative bacteria contain fimbriae (pili), which are tiny projections on the surface of the bacterium. Specific chemicals located on the tips of pili enable organisms to attach to selected host tissue sites (e.g., urinary tract endothelium). The presence of these fimbriae has been demonstrated to be important for the attachment of *P. mirabilis* to host tissue (94).

2.1.7 *Yersinia*

The genus *Yersinia* contains eleven species, three of which (*Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis*) are known pathogens of human and animal (95). *Y. pestis* is the causative agent of the plague. Rodents are the natural reservoirs of *Yersinia*; less frequently other mammals serve as the host. Infection may occur either through blood (in the case of *Y. pestis*) or in an alimentary fashion, occasionally via consumption of food products (especially vegetables, milk - derived products and meat) contaminated with infected urine or faeces. The disease caused by *Y. enterocolitica* is called Yersiniosis. Infection with *Y. enterocolitica* can cause a variety of symptoms depending on the age of the person infected. Common symptoms in children are fever, abdominal pain, and diarrhea, which is often bloody. *Y. pseudotuberculosis* sometimes but rarely causes disease (96).

2.1.8 *Citrobacter*

There are eleven species of which nine have been recovered from clinical material. They may be found in the faeces of humans and animals as part of the normal flora and grow readily on ordinary media. Colonies are generally smooth and moist although mucoid or rough strains occur. Some strains of *Citrobacter* resemble *Salmonella* species biochemically and agglutinate with Salmonella polyvalent antisera, which may lead to misidentification. This genus is rarely the source of illnesses, except for infections of the urinary tract and infant meningitis and sepsis (97, 98).

2.1.9 *Providencia*

The genus *Providencia* was originally established for organism similar to *Proteus* that was urease negative. There are five species within the genus, of which three cause disease. All are motile but do not swarm. They are resistant to polymyxin B and colistin. Some strains (*P. stuartii*, for example) are opportunistic pathogens in humans and can cause urinary tract infections, particularly in patients with long - term indwelling urinary catheters or extensive severe burns. Other strains (for example *P. burhodogranariea* and *P. sneebia*) are found in the haemolymph of *Drosophila melanogaster* fruit flies (99).

2.2 Urinary tract infection caused from Enterobacteriaceae

UTI is one of the most common bacterial infections encountered in clinical practice. Uropathogenic *E. coli* (UPEC) is the causative agent in 70% - 95% of community acquired UTI and 50% of all cases of nosocomial infection (100). It is estimated that about 35% of healthy women suffer symptoms of UTI at some time in

their life. Symptoms suggestive of UTI are the reason for 0.5 - 1% of consultations, and each year about 5% of women present to their general practitioner with painful urination (dysuria) and frequency (101, 102). UTI is the presence of bacteria in the urine (bacteriuria). For epidemiological purposes, "significant" bacteriuria is defined as at least 10^5 bacteria/ml in freshly - voided urine, though symptomatic infection can occur with 10^3 bacteria/ml. Symptomatic infection is associated with inflammation and a urine WBC count higher than 8 cells/ml (pyuria, leucocyturia).

2.2.1 Complicated infections

Complicated UTI is associated with anatomical, functional, or metabolic abnormalities of the urinary tract that disable the natural innate host defences and lead to tissue injury.

2.2.2 Cystitis

Cystitis is inflammation of the bladder. Infection is the most common cause, but there are other non - bacterial causes.

Asymptomatic bacteriuria is found in 1 - 2% of girls aged 4 - 12 years and 3 - 5% of women. Symptomatic infections are rare in girls under 13 years of age, but the incidence increases during adolescence; in a study of acute UTI in young women, it was 0.5 - 0.7 per person - year. Recurrent UTIs are a problem in 20 - 25% of women; these are usually exogenous re - infections. Bacteriuria is rare in boys and young men, unless an anatomical or functional abnormality of the urinary tract is present. The incidence of UTI increases markedly in the elderly. Bacteriuria is found in 21% of women and 12% of men over 65 years of age, and in 53% and 37% of those living in institutions. UTI is the commonest cause of gram - negative septicaemia.

Bacteria most commonly enter via the urethra (ascending infection), but can enter via the bloodstream. Ascending infections account for most cases of uncomplicated cystitis and pyelonephritis, and usually involve organisms of the normal bowel flora - principally *E. coli* ($\geq$ 75% of cases). *Staphylococcus saprophyticus* is sometimes found in young women (5 - 10%), and *P. mirabilis* and *K. pneumoniae* are rare causes. The greater susceptibility of women to UTI is partly because of the short urethra and its proximity to the bowel. The most important are those that result in urine stasis. About 50% of infections are caused by *E. coli*; other causes are *Staphylococcus aureus*, coagulase - negative staphylococci, *Pseudomonas* sp. and other gram - negative organisms. Bacteria exhibit variable antibiotic resistance.

2.3 Their β - lactamase production

Bacterial resistance to β - lactam antibiotics and β - lactamase inhibitors is an ever - increasing problem that threatens the clinical utility of drugs that form the cornerstone of the antibiotic arsenal. The elaboration of structurally and mechanistically novel β - lactamase enzymes, especially among gram - negative pathogen, is the most important means by which resistance occurs.

The classification of β - lactamases was first proposed by Jack and Richmond in 1970, on the basis of phenotypic characters such as substrate profile, susceptibility to inhibitors, isoelectric focusing, molecular size, and immunological identity. This scheme divides the β - lactamases from gram - negative bacilli into five major classes (103).

Class 1: Enzymes are primarily cephalosporinases.

Class 2: Penicillinases.

Class 3: Enzymes are active against a broad spectrum of penicillins and cephalosporins, while resistant to inhibition by *p* - chloromercuric benzoate (*p* - CMB) and sensitive to cloxacillin.

Class 4: Enzymes have substrate profile similar to Class 3 but are resistant to inhibition by cloxacillin and sensitive to *p* - CMB.

Class 5: Enzymes are penicillinases and have a spectrum, which is broader than that of Class 2.

Two major groups of enzymes arose from this classification scheme:

1. Chromosomally mediated β - lactamases.

2. Plasmid mediated β - lactamases.

Plasmid - mediated enzymes in gram - negative rods are constitutively expressed while chromosomal enzymes are inducible. Most plasmid - mediated enzymes are susceptible to inhibitors while most of the chromosomal enzymes are not (104). The phenotypic classification faces the problem that point mutation can greatly alter substrate specificity and inhibitor susceptibility of the enzyme. Therefore, the β - lactamases are now classified by amino acid and nucleotide sequence. Such classification is stable and cannot be distorted by mutations. This scheme separates β - lactamases into four major classes, A, B, C, and D. Classes A, C, and D comprise evolutionarily distinct groups of serine enzymes and class B contains Zn^{2+} types (105).

2.3.1 Action of β - lactamase

β - lactamases are enzymes produced by bacteria, which can destroy β - lactam ring of antibiotics (Figure 7). The β - lactam agent becomes so changed in its

chemical structure that it is no longer recognized by the enzymes responsible for making the peptidoglycan layer of the bacterial cell wall (106). Enzymes of class A, C, and D have their active site at serine residue. These serine - based enzymes first associated non - covalently with the antibiotic to yield the non - covalent Michaelis complex. The β - lactam ring is then attacked by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester. The hydrolysis of the ester finally liberates active enzyme and the hydrolyzed inactive drug. This mechanism is followed by β - lactamases of molecular classes A, C, and D but class B enzymes utilize a Zn^{2+} ion to attack the β - lactam ring (105).

Chromosomal β - lactamases

Chromosomal β - lactamases are present in gram - positive bacteria, gram - negative bacteria, *Mycobacterium*, and *Nocardia* (107). Chromosomal β - lactamases are either inducible or constitutive (e.g., constitutively produced class A enzymes of *Klebsiella* and *Bacteroides fragilis* and inducible class B carbapenemase by *Stenotrophomonas maltophilia*) (105). Chromosomal β - lactamases are almost ubiquitous in Enterobacteria, except for *Salmonella*, but vary greatly in amount, mode of production, and consequently in their contribution to resistance (104).

Plasmid - mediated β - lactamases

During the past 20 - 50 years plasmid mediated β - lactamases have become common in *Staphylococcus, Enterobacter, Haemophilus influenzae*, and *Neisseria gonorrhoeae*. The plasmid - mediated β - lactamases confer resistance to broad - spectrum β - lactam antibiotics, including third - and fourth - generation cephalosporins, aztreonam, and extended - spectrum penicillins. Other resistances, such as aminoglycoside and trimethoprim/sulfamethoxazole resistance, are often

co - transferred on the same plasmid (109). Over 75 different plasmid - mediated β - lactamases have been recorded in gram - negative bacilli. The most common of these enzymes in Enterobacteriaceae is TEM - 1, others include TEM - 2, SHV - 1 and OXA - 1 (110), and BIL - 1, FOX - 1, LAT - 1, MIR - 1, OHIO - 1, TLE - 1, ROB - 1, LXA - 1, TLE - 2 etc are found in occasional isolates (111).

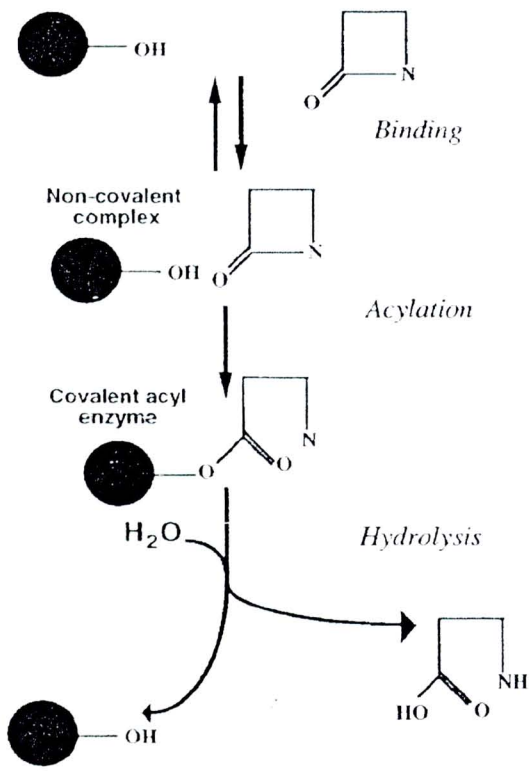

Figure 7 Action of β - lactamase (108)

2.3.2 Determinations of β - lactamase function in resistance

The ability or inability of a β - lactamase to confer resistance depends on its location, kinetics.

Location

The β - lactamases of gram - positive species are largely extracellular, although, depending on the growth conditions, some enzyme may adhere to the cytoplasmic membrane. By contrast, the β - lactamases of gram - negative species are largely periplasmic, although some extracellular release may occur, mediated by leakage rather than secretion.

Kinetics

The activity of most β - lactamases against most substrates can be described by the Michaelis - Menten equation:

$$v = V_{max}S/(K_m + S)$$

where v is the hydrolysis rate, S is the β - lactam concentration, and V_{max} and K_m are the kinetic constants. A low K_m reflects high affinity and may be as critical to resistance as a high V_{max} (112). Occasionally, simple Michaelis - Menten kinetics are not obeyed, either because burst kinetics arise, with acylation much faster than deacylation, or because there is a branched reaction pathway, with either the acyl - enzyme or the Michaelis complex able to isomerize between two forms with differing stabilities. Behavior of this type is common with class D β - lactamases (113) but occasionally occurs with other types, for example, for class A and C enzymes with meropenem (114, 115). Its significance in the bacterial cell is uncertain but seems likely to reduce hydrolytic efficiency.

2.3.3 ESBL - β - lactamases and their classifications

A commonly used working definition is that the ESBLs are β - lactamases capable of conferring bacterial resistance to the penicillins, first - , second - , and third - generation cephalosporins, and aztreonam by hydrolysis of these antibiotics, and

which are inhibited by β - lactamase inhibitors such as clavulanic acid. The term ESBL will be taken to mean those β - lactamases of Bush - Jacoby - Medeiros group as shown in Table 4. The ESBLs have hydrolysis rates for ceftazidime, cefotaxime, or aztreonam (aminothiazoleoxime β - lactam antibiotics) at least 10% that for benzylpenicillin. They are inhibited by clavulanic acid (116). This property differentiates the ESBLs from the AmpC - type β - lactamases (group 1) produced by organisms such as *E. cloacae* which have third - generation cephalosporins as their substrates but which are not inhibited by clavulanic acid. Selection of stably derepressed mutants which hyperproduced the AmpC - β - lactamases has been associated with clinical failure when third - generation cephalosporins are used to treat serious infections with *Enterobacter* spp. (117). In general, the fourth - generation cephalosporin, cefepime, is clinically useful against organisms producing AmpC - β - lactamases (118), but may be less useful in treating ESBL producing organisms (119).

Functional classification

Since 1970, several criteria have been used in the functional classification of the β - lactamases, including the spectrum of the antimicrobial substrate profile, enzyme inhibition profile, enzyme net charge (pI), hydrolysis rate (V_{max}), binding affinity (K_m), isoelectric focusing, protein molecular weight, and amino acid composition. The classification of β - lactamases was first proposed by Richmond and Sykes in 1973 on the basis of phenotypic characters such as substrate profile, susceptibility to inhibitors, isoelectric focusing, molecular size and immunological identity, and divides the β - lactamases into five major groups (I - V) (103). In 1995,

Bush - Jacoby - Medeiros presented the latest classification scheme based on four groups and subgroups as follows (120) (Table 4).

Group 1 consists of cephalosporinases not inhibited by clavulanic acid, belonging to molecular class C.

Group 2 are penicillinases, cephalosporinases, or both, inhibited by clavulanic acid, corresponding to molecular classes A and D reflecting the original TEM and SHV genes.

Table 4 Functional and molecular characteristics of the major groups of β - lactamases (114, 115)

Functional group (Bush - Jacoby - Medeiros)	Major group	Molecular Class (Amber)	Attributes of β - lactamases in functional group
1		C	Confer resistance to all classes of β - lactams, except carbapenems. Not inhibited by clavulanic acid.
2		A, D	Most enzymes responsive to inhibition by clavulanic acid.
	2a	A	Staphylococcal and enterococcal penicillinases included. Confer resistance to penicillins.
	2b	A	Broad - spectrum β - lactamases, including TEM - 1 and SHV - 1.
	2be	A	ESBL conferring resistance to oxymino - cephalosporins and monobactams.
	2br	A	Inhibitor - resistant TEM (IRT) β - lactamases.
	2c	A	Carbenicillin - hydrolyzing enzymes.
	2d	D	Cloxacillin - (oxacillin) - hydrolyzing enzymes, modestly inhibited by clavulanic acid.
	2e	A	Cephalosporinases inhibited by clavulanic acid.
	2f	A	Carbapenem - hydrolyzing enzymes with active site serine, inhibited by clavulanic acid.

Table 4 (continued)

Functional group (Bush - Jacoby - Medeiros)	Major group	Molecular Class (Amber)	Attributes of β - lactamases in functional group
3	3a, 3b, 3c	B	Metallo - β - lactamases conferring resistance to carbapenems and all β - lactam classes except monobactams. Not inhibited by clavulanic acid.
4			Miscellaneous unsequenced enzymes that do not fit into other groups

1. The 2a subgroup contains only penicillinases.

2. 2b enzymes are broad - spectrum β - lactamases, meaning that they are capable of inactivating penicillins and cephalosporins at the same rate. Furthermore, new sub - subgroups were segregated from subgroup 2b.

2.1 Subgroup 2be, with the letter "e" standing for extended - spectrum of activity, represents the ESBLs, which are capable of inactivating third generation cephalosporins (ceftazidime, cefotaxime, and cefpodoxime) as well as monobactams (aztreonam).

2.2 The 2br enzymes, with the letter "r" denoting reduced binding to clavulanic acid and sulbactam, are also called inhibitor - resistant TEM derivative enzymes; nevertheless, they are still susceptible to tazobactam.

3. Later, subgroup 2c was segregated from group 2b because these enzymes inactivate carbenicillin more than benzylpenicillin, with some effect on cloxacillin.

4. Subgroup 2d enzymes inactivate cloxacillin more than benzylpenicillin, with some activity against carbenicillin; these enzymes are poorly inhibited by clavulanic acid, and some of them are ESBLs.

5. Subgroup 2e enzymes are cephalosporinases that can also hydrolyze monobactams, and they are inhibited by clavulanic acid.

6. Subgroup 2f was added because these are serine - based carbapenemases, in contrast to the zinc - based carbapenemases included in group 3.

Group 3 is composed of the zinc - based or metallo - β - lactamases, corresponding to the molecular class B, which are the only enzymes acting by the metal ion zinc as discussed above. They are able to hydrolyze penicillins,

cephalosporins, and carbapenems. Thus, carbapenems are inhibited by both group 2f (serine - based mechanism) and group 3 (zinc - based mechanism).

Group 4 includes penicillinases that are not inhibited by clavulanic acid, and they do not yet have a corresponding molecular class.

Molecular classification

The molecular classification of β - lactamases is based on the nucleotide and amino acid sequences in these enzymes. To date, four classes are recognized (A - D), correlating with the functional classification. Classes A, C, and D act via a serine - based mechanism, whereas class B or metallo - β - lactamases need zinc for their action. The majority of ESBLs belong to Ambler's molecular class A (121), characterized by an active - site serine, a molecular mass of approximately 29 kDa, and the preferential hydrolysis of penicillins (122).

2.3.4 AmpC - β - lactamases

AmpC - β - lactamases have gained importance since the late 1970s as one of the mediators of antimicrobial resistance in gram negative bacilli. These enzymes are cephalosporinases capable of hydrolyzing all β - lactams to some extent (123). AmpC - β - lactamases are of two types plasmid - mediated and chromosomal or inducible *ampC*. Chromosomal *ampC* enzymes are seen in organisms such as *C. freundii*, *E. cloacae*, *M. morganii*, *H. alvei* and *Serratia marcescens* and are typically inducible by β - lactam antibiotics such as cefoxitin and imipenem but poorly induced by the third - or fourth - generation cephalosporins (124). In the late 1980s, these inducible chromosomal genes were also detected on plasmids (most without induction capabilities) and were transferred to organisms, which typically do not express chromosomal β - lactamases such as *Klebsiella* spp., *E. coli* or *Salmonella*

spp. (123). The *ampC* strains made little if any β - lactamase, suggesting that *ampC* was the structural gene for the enzyme (125). Most of the *amp* nomenclature has changed over the years, but the designation *ampC* has persisted. The sequence of the *ampC* gene from *E. coli* was reported in 1981 (126). It differed from the sequence of penicillinase - type β - lactamases such as TEM - 1 but, like them, had serine at its active site (127). In the Ambler structural classification of β - lactamases (121), AmpC enzymes belong to class C, while in the functional classification scheme of Bush *et al.* (111), they were assigned to group 1.

2.3.5 Metallo - β - lactamase

Class A, C and D enzymes of Ambler structural classification use an active site serine residue as a nucleophile, whereas class B lactamases (generically termed metallo - β - lactamases, MβLs) employ one or two Zn (II) ions to cleave the β - lactam ring (128, 129). MβLs stand as one of the main mechanisms of bacterial resistance toward carbapenems. MβLs have particular importance in the clinical setting in that they can hydrolyze a broader spectrum of β - lactam substrates (including carbapenems) than the serine - type enzymes and are resistant to most clinically employed inhibitors (129). The design of an efficient pan - MβL inhibitor has been mostly limited by a striking diversity in the active site structures, catalytic profiles and metal ion requirements for activity among different enzymes. Based on this heterogeneity, MβLs have been classified into three subclasses: B1, B2 and B3 (130).