KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY KUMASI

COLLEGE OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

COMPARATIVE DIAGNOSIS OF *ESCHERICHIA COLI*-INDUCED URINARY TRACT INFECTION USING DIPSTICK, MICROBIOLOGICAL CULTURING AND PCR METHODS IN SCHOOL-GOING ADOLESCENTS

BY

SAMUEL NKANSAH DARKO

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THIS DISSERTATION IS PRESENTED TO THE DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF AN M.Sc. DEGREE IN BIOTECHNOLOGY

BY

SAMUEL NKANSAH DARKO

NOVEMBER, 2012

DECLARATION

I hereby declare that this thesis is the outcome of my own original research and that, it has neither in part nor whole, been presented for another degree in this university or elsewhere.

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Candidate's Name (PG 5012110)	Signature	Date

Certified by

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Supervisor	Signature	Date

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Supervisor	Signature	Date

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Head of Department	Signature	Date

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ABSTRACT

Urinary tract infection (UTI) poses serious future clinical repercussions, such as hypertension, anaemia, kidney failure and death. Therefore, there is the need for the early detection and diagnosis of UTI. Currently, the use of the standard microbiological culture diagnostic method is hindered by the microbial tendency of being viable but non-culturable. On the other hand, direct Polymerase Chain Reaction (PCR) has proven to be more sensitive in detecting pathogens in various biological samples, although interference by specimen components and the possibility of contamination are some challenges. This work was thus aimed at the design of a simple and reliable PCR protocol for detecting uropathogens and comparing it with dipstick and culture, using *Escherichia coli* as the indicator uropathogen. This study, a cross-sectional one, involved the collection of urine samples from 272 adolescent students, aged between 13 and 18 years. The PCR protocol targeted a more specific *pap C* gene for fimbriae formation and the bacteriocin *usp* gene sequences for amplification. A general prevalence of 30.9% UTI was found. Using a sub-population made up of 195 subjects, for the comparative diagnosis of the three methods, the prevalence were 42.1%, 39.8% and 72.7% for dipstick, microbiological culturing and PCR respectively. PCR had a higher sensitivity of 71% and Receiver Operator Characteristic curve area of 0.7231± 0.0262, $p< 0.0001$, compared to the microbiological culture method. In addition, the ROC curve area for PCR was significantly higher than dipstick (0.7205± 0.0262, $p< 0.0001$) indicating PCR's superiority as a diagnostic tool. On the other hand, there was no statistical difference between the microbiological culturing method and dipstick as diagnostic tools (0.5026± 0.02927, $p= 0.9302$). The leukocyte esterase parameter in the dipstick method was found to be more useful to the physician in making diagnosis with a positive predictive value of 52% and a negative predictive value of 76%. The *usp* gene was also found to be more significantly distributed in urine isolates than *pap* C with *P* value of 0.0020. This suggests that most of the strains of *Escherichia coli* implicated in UTI in the sample population employ more of bacteriocin production to compete with other microbes in the urinary tract than the production of adhesion structures. The PCR is therefore, a good complementary tool for diagnosis of UTI.

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CHAPTER ONE

1.0 INTRODUCTION

Microorganisms form a bulk of the earth's biomass and their ability to adapt to newly found environments makes them beneficial or pathogenic (Singh *et al.*, 2009). Many human diseases are as a result of infections caused by bacteria pathogens, either external or internal of the human host. One of such bacterial infection is the Urinary Tract Infection (UTI), involving the presence of bacteria in the urinary tract (UT),which is naturally sterile (Zorc *et al.*, 2005).UTI mostly occurs in patients with anatomically and functionally normal UT and usually results from spontaneous ascent of bacteria from the urethra to the bladder. Occasionally, the bacteria progresses to the kidney and bloodstream. However, since asymptomatic colonization of the UT can occur, other features such as the presence of inflammatory markers or follow-up cultures are needed to correctly diagnose a person with UTI.

Bacteria colonization of the UT is predominantly caused by Gram-negative species, such as *Escherichia coli*, *Klebsiella*, *Proteus* and *Pseudomonas* and rarely, by Gram-positive organisms such as haemolytic *Streptococci* and *Staphylococcus saprophyticus* (Cheesbrough, 2006).

Global records on the disease show that among children, the infection is more common in young girls, except in the neonatal age group where boys predominate (Foxman, 2002). It is also estimated that about 20% of women develop an UTI during their lifetime; the incidence increases at puberty and remains high throughout adult life (Hooton *et al.*, 2000). Furthermore, UTIs account for approximately 23% of all hospital-acquired infections (Emmerson *et al.*, 1996). In Ghana, 7.3% of pregnant women attending antenatal care have been identified to have significant bacteriuria (presence of bacteria in urine). *E. coli* as dominant bacteria isolate, account for about 37% of all cases (Turpin *et al.*, 2007).

Unfortunately, in Ghana there is paucity of UTI prevalence data for other categories of the population, aside pregnant women.

The most commonly employed technique worldwide for detecting UTI is by using diagnostic dipstick, which measures parameters like protein, leukocyte esterase, blood and nitrate reductase levels (WHO, 2005). A more reliable but time-consuming technique of bacterial culturing is preferred, involving the use of appropriate media for selected bacterial growth (Zorc *et al.*, 2005). However, this microbiological assay has the limitation of requiring a minimum of 48 hours to make observations from the culture plates, to ascertain the presence and levels of bacteriuria. Furthermore, the inability of this technique to detect and identify viable but non-culturable microbes may exclude some pathogenic bacteria, which might later cause symptomatic bacteriuria (Colwell, 2000). The occurrence of such viable but non-culturable microbes has been reported for many Gram-negative bacteria**,** including human pathogenic *E. coli* which is the commonest pathogen, causing more than 80% of UTIs (Ronald, 2003). Therefore, there is the need for a more reliable and faster method for UTI diagnosis.

Polymerase chain reaction (PCR) is a versatile and sensitive molecular technique for amplification of minute quantities of cellular DNA (template), using a pair of primers at both ends of target region of the DNA involved. This sensitive method can amplify DNA of a single cell to a level that can be visualized on electrophoretic gels. Due to the high specificity, PCR is not only effective in detecting species of microorganisms, but it also identifies their various strains. Since the method requires minute quantities of template DNA, it has the potential to effectively detect infectious microbes, especially non-culturable bacteria or microbes in biological samples.

Gene sequencing has made it possible to identify allelic gene sequences and their effects on gene expression of various pathogenic bacteria. Studies on specific genes responsible for pathogenicity of uropathogenic *E. coli* strains have revealed the genes belong to independent clusters, organized as operons (Le Bouguenec, 1992). These virulence genes have been classified variously as adhesive, toxic or iron acquisition genes. Adhesion-mediated virulence, caused by the *pap* adhesin gene is known to be prevalent in many uropathogenic strains of *E. coli* (Marrs *et al.,* 2005). The *usp* gene, found to be homologous to a toxin gene of *Vibrio cholerae* is also a dominant marker for detecting uropathogenic strains of *E. coli* in some studies in different geographical locations (Kurazono *et al.,* 2000). These unique markers and others, present a great potential for developing PCR protocol to assess microbiological activities in urine samples.

However, the application of PCR to clinical specimens has some potential pitfalls, due to the susceptibility of PCR to inhibitors, contamination and experimental conditions (Yamamoto, 2002).

1.1 Problem Statement

The conventional means of diagnosing UTI has long been the use of urine culture and antibiotic sensitivity testing. This microbiological technique has time limitation, requiring 2 to 3 days, which delays commencement of any treatment intervention. As a common practice, in the absence of supportive laboratory evidence, due to lack of a high resolving and fast laboratory diagnosis to detect microbial infection, clinicians usually initiate empirical antibiotic treatments. But such an approach could also unnecessarily expose patients to antibiotics with little or no success in the containment of infections. Therefore, a faster, more sensitive and specific diagnostic tool for microbial detection in urine samples for assessing UTI is required.

1.2 Justification

The PCR has proven to be a powerful and rapid tool for amplifying and detecting minute quantities of DNA in various biological samples. It takes a maximum of six hours to obtain results. Small amounts (micrograms or nanograms) of samples are needed to detect microorganisms present. The diagnostic uniqueness of PCR is the ability to detect some pathogens that are undetectable by microbiological culturing methods. Specimens such as blood, sputum, cerebrospinal fluid and urine have been successfully screened for bacteria and other microbes, using the PCR technique (Yamamoto, 2002; Starcic-Erjavec *et al.*, 2008).

1.3 Main Objective

To determine the prevalence of UTI caused by *E. coli*, using different diagnostic tools within a population of school-going adolescents in Kumasi

1.4 Specific Objectives

- To use dipstick and classical microbiological culturing method to determine presence of *E. coli* in urine of subjects
- To design a PCR assay, based on dominant virulence genes (*pap* and *usp*) of uropathogenic *E. coli* strains for diagnosis of Urinary Tract Infection.
- To determine the performance characteristics of the diagnostic methods.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Urinary tract infections

Urinary tract infection (UTI) is the presence of microorganisms, predominantly bacteria, in the urinary tract, which is usually sterile (Zorc *et al.*, 2005). These uropathogens generally infect cells in the urinary tract by initial attachment and subsequent ascension from the urethra, to the bladder, kidney and through the systemic circulation, causing bacteraemia, as a result of the renal cells being compromised (Kaper *et al.*, 2004). Uropathogenic organisms are more likely to colonize anatomically and functionally normal urinary tracts; however individuals with obstructed and abnormal urinary tract structures have a higher risk of UTI (Zorc *et al.*, 2005). Infections of the lower urinary tract, is a cause of morbidity, with increasing incidence of urinary tract infections predisposing the host to more severe renal cell damage (Crain and Gerschel, 1990). The more severe renal damage frequently leads to renal scarring and hypertension with high mortality rate (Jacobson *et al.*, 1989). Some studies confirm individuals with compromised immune systems like diabetics, are more susceptible to urinary tract infections (Deresinski, 1995; Geerlings *et al.*, 2000) .

2.1.1 Epidemiology

Prevalence of urinary tract infections is dependent on factors like gender, age, disease, race and nutritional status. Females are generally more predisposed to renal infections, compared to males, except at the extremes of early childhood and geriatric stage (Akinkugbe *et al.*, 1973). This is basically due to the shorter length of the urethra in females and its closeness to the excreta passage, which is a source of pathogens that can colonize the urethra. Again, sexual intercourse, pregnancy and childbirth contribute to the increase in UTI in females (Duerden *et al.*, 1990; Geerlings *et al.*, 2000). It is estimated that about 50% of all women are likely to experience an UTI in their lifetime (Foxman *et al.*, 2000).

At the first three months after birth, boys have a higher UTI prevalence of 2.7% as compared to 0.7% in girls (Riccabona, 2003). However, just before puberty, girls are at higher risk with 3-5% UTI prevalence while it remains unchanged at 2.7% among males of the same age (Hoberman *et al.*, 1993; Hoberman and Wald, 1997). During the pubertal stage and adulthood, females maintain a higher prevalence than males (Hooton *et al.*, 2000). Other studies indicate a 10-12 fold increase in risk of UTI in uncircumcised male infants and this is thought to be likely due to the colonization of the mucosal surface of the foreskin with bacteria (Wiswell and Roscelli, 1986; Roberts and Akintemi, 1999; Wiswell, 2000).

There has been conflicting views concerning the prevalence of UTI and diseases that lead to a compromise in the immune system. Individuals with compromised immune systems, as a result of diseases like diabetes mellitus were thought to be more predisposed to UTI (Boyko *et al.*, 2005). However, recent works disprove this assertion (Geerlings *et al.*, 2000; Rizk *et al.*, 2001; Sotiropoulos *et al.*, 2005).

White children have been estimated to have a relatively higher incidence, compared to black children (Shaw *et al.*, 1998). Shaw *et al* (1998) identified the prevalence of UTI in white children to be higher in both sexes with white girls having rates as high as 16-17 %. This racial difference in UTI prevalence among girls is attributed to genetic differences in secretion of carbohydrates like mannose that inhibit the adherence of bacteria to the urinary tract. White girls have been found to lack the gene responsible for the secretion of these carbohydrates (Sheinfeld *et al.*, 1989; Jantausch *et al.*, 1994).

Individuals, especially children who are malnourished are more susceptible to UTI. Various studies in developing countries have presented data that indicate the prevalence of UTI to be 8-35 % higher in malnourished children (Banapurmath and Jayamony, 1994; Reed and Wegerhoff, 1995; Bagga *et al.*, 2003). The severity of malnourishment also has a strong

correlation with the level of bacteriuria and this is thought to be due to a weakened immune system which is overwhelmed by infections (Bagga *et al.*, 2003).

2.1.2 Aetiology

Most urinary tract infections are caused by bacteria, although viruses, fungi and some other parasites do infect the urinary tract of mostly immuno-compromised individuals (Zorc *et al.*, 2005). Gram-negative bacteria are the commonest species of bacteria associated with UTI. *Escherichia coli, Klebsiella, Proteus* and *Pseudomonas* form the predominant gram-negative bacterial, with haemolytic *Streptococci* and *Staphylococcus saprophyticus* comprising the common gram-positive species isolated in UTIs (Orenstein and Wong, 1999; Lutters and Vogt, 2000). Some aerobic gram-negative bacteria of the family Enterobacteriaceae, including *Salmonella* and *Citrobacter* have been found in UTI (Ejaz *et al.*, 2006). *Escherichia coli* is the commonest bacteria isolate detected in UTI, accounting for 70 to 90 % of all infections in both children and adults, in developed countries (Schalger, 2001; Riccabona, 2003). This has a positive correlation with UTI isolates in developing countries, although *Escherichia coli* accounts for about 39 % of infections (Longe *et al.*, 1984). Further studies have shown that the prevalence of *Escherichia coli* in UTI isolates is independent of the individual's immuno-competence status (Asharam *et al.*, 2003).

2.2 Clinical Manifestations of Urinary tract infections

There are varying clinical manifestations, including asymptomatic bacteriuria, cystitis and acute pyelonephritis

2.2.1 Asymptomatic bacteriuria

This clinical manifestation of urinary tract infection is commonly associated with females; from the pubertal to adult stages. Asymptomatic bacteriuria has been found to have 4 to 6% prevalence in healthy young adults with an increase to 20% in ambulatory elderly adults

(Hooton *et al.*, 2000). It is a condition characterized by a positive urine culture test without the female individual experiencing common infection symptoms, such as fevers and especially, abdominal or flank pains, linked to urinary tract infections. Asymptomatic bacteriuria has also been found in some studies to be facilitated by diabetes mellitus in female subjects, as a result of glucosuria (Patterson and Andrriole, 1987; Lucas and Cunningham, 1993). Contraction of asymptomatic bacteriuria in diabetic females is further compounded by other risk factors, such as sexual intercourse, the duration of diabetes and its complications and insulin use. Pregnant women are mainly predisposed to asymptomatic bacteriuria, as they experience a decrease in immuno-competence, coupled with risk factors, such as sexual intercourse, which permits both commensal and non-commensal bacteria to colonize and multiply in the urogenital canals (Scott *et al.*, 1990). Although this condition is benign, some researchers suggest that it might progress to symptomatic if not diagnosed, and in pregnant women might lead to pyelonephritis, premature births and higher foetal mortalities (Nicolle, 1994; Connolly and Thorp, 1999; Delzelle and Leferre, 2000).

2.2.2 Cystitis

This is the most prevalent UTI found in women, although not absent in men (Hanno, 2007). Cystitis is localized in the bladder and can lead to the more complicated acute pyelonephritis due to the continual ascension of causative microorganisms through the ureter into the kidney, with the aid of adherence structures (Ottem *et al.*, 2007). Symptoms, such as suprapubic pain, abdominal or flank pains, urine incontinence and malodorous urine have been associated with cystitis (Zorc *et al.*, 2005). Individuals with cystitis could also experience dysuria, urgency and change in frequency of urination (Rabin *et al.*, 2000). However, fever has not been linked with this condition and is known not to cause any renal injury.

2.2.3 Acute Pyelonephritis

Pyelonephritis is a progressed result of urinary tract infection due to the ascending of bacteria that have colonized the urethra and the bladder (Bass *et al.*, 2003). This is an upper urinary tract infection, involving the ureter, collecting system and finally, the renal parenchyma (Bergeron, 1995). The ability of uropathogens to cause upper tract infections has been attributed to bacteria adhesion, virulence factors and motility, coupled with the host's anatomic, humoral and genetic factors (Kaper *et al.*, 2004). Acute pyelonephritis is commonly associated with symptoms such as flank and abdominal pains, fevers and chills. However, children may express non-specific symptoms, such as poor feeding, irritability and jaundice in newborns.

2.3 *Escherichia coli*

Escherichia coli (*E. coli*) are a Gram negative lactose fermenting bacillus found mainly in the gastrointestinal tract of humans and other mammals. This microorganism can colonize the peri-urethral region of infants a few hours after birth (Linshaw, 1996). *E. coli* is mainly a commensal, cohabiting with its animal host (Scott *et al.*, 1990). Several strains of this organism exist without causing disease, except when found in individuals with immuno-compromised conditions, such as diabetes mellitus or when the gastrointestinal tract has been breached as in peritonitis (Epoke *et al.*, 2000). The bacteria successfully persist in the mucosal layer of the mammalian colon and form the most abundant facultative anaerobes in the human microflora (Kaper *et al.*, 2004).

There are two proposed models for the mechanisms that enable *E. coli* to successfully occupy the colon as its niche. The first model suggests the more efficient utilization of gluconate in the colon than other resident microbes and thus dominating the niche (Sweeney *et al.*, 1996). A more recent model is based on the ability of several clones of the bacterium acquiring specific virulence factors, giving them the ability to adapt to various biological environments

and cause several diseases (Marrs *et al.*, 2005)**.** However, only a few of the pathogenic *E. coli* with some combinations of these virulence factors turn to be successful in causing disease in healthy individuals (Kaper *et al.*, 2004).

E. coli is a diverse species of bacteria grouped into two subsets; enteric/diarrhoea causing and the extra-intestinal disease causing type, including urinary tract infection. These subsets can further be divided into groups, based on the use of specific combinations of virulence factors, the molecular mechanisms employed in infections and also occasional peculiar symptoms associated with the diseases caused. Such groups are termed pathotypes (Marrs *et al.*, 2005).

2.3.1 Enteric/diarrhoea *E. coli*

Six different pathotypes of *E. coli,* known to cause diarrhoea have been identified. These are the enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EIEC) and diffused adherence (DAEC) *E. coli* (Kaper *et al.*, 2004; Marrs *et al.*, 2005).

2.3.1.1 Enteropathogenic *E. coli*

This pathotype of enteric *E. coli* has been tagged as the most important cause of potential fatal infant diarrhoea (Nataro and Kaper, 1998). This pathotype causes diarrhoea by attaching and effacing; having intimate adherence to the intestinal epithelial cells and inducing drastic change in the cytoskeleton of these cells. The condition is further enhanced by the accumulation of the polymerized protein, actin, beneath the attached bacterium, leading to the formation of a pedestal-like structure. A 35-kb pathogenicity island, called the locus of enterocyte effacement (LEE) is responsible for this pathogenic trait (McDaniel *et al.*, 1995). Homologues of the LEE have also been detected in other pathogens in humans, including the EHEC. The LEE also encodes the protein, intimin, which serves as a ligand for epithelial cell attachment and further stimulates T-helper cell response (Jerse *et al.*, 1990; Higgins *et al.*,

1999). However, the mechanism for causing diarrhoea is not well understood and has been attributed to active ion secretion, increased intestinal permeability, intestinal inflammation or loss of absorptive surface (Nataro and Kaper, 1998).

2.3.1.2 Enterohaemorrhagic *E. coli*

Bloody diarrhoea which requires an extremely low *E. coli* infectious dose has been associated with enterohaemorrhagic *E. coli* (Kaper *et al.*, 2004). The key virulence factor used by EHEC is the *Stx*, also known as the verocytotoxin (VT). It consists of five identical B subunits and a single A subunit. The B subunits bind the holotoxin to the glycolipid receptors on target cells, while the A subunit cleaves ribosomal RNA, inhibiting protein synthesis (Melton-Celsa and O'Brien, 1998). *Stx* is produced in the colon, where it mediates local damage, which results in bloody diarrhoea, haemorrhagic colitis and intestinal perforation. This factor travels by blood transport to the kidney where it damages renal endothelial cells and leads to inflammation (Andreoli *et al.*, 2002).

2.3.1.3 Enterotoxigenic *E. coli*

ETEC causes watery diarrhoea, through colonizing of the mucosa of the small intestine and activating synthesis of enterotoxins, leading to intestinal secretions (Nataro and Kaper, 1998). These enterotoxins are either heat-labile (LT) or heat-stable (ST), with the heat-labile related to the cholera enterotoxin of *Vibrio cholerae* (Spangler, 1992*)*.The LT enterotoxin transfers ADP-ribosyl moiety from NAD to the α subunit of the regulatory G protein for adenylate cyclase. This permanent activation leads to increased levels of cAMP, which uncontrollably activates kinases responsible for chloride channeling in epithelial cells. The net effect of this action is an increased chloride secretion, which leads to diarrhoea (Sears and Kaper, 1996).

2.3.1.4 Enteroaggregative *E. coli*

This enteric *E. coli* is differentiated from the other pathotypes by its characteristic adherence to each other and the cells of the intestinal walls. These pathogens form piles of microbial layers to the intestinal walls, in a manner known as auto-aggregation (Nataro and Kaper, 1998). Experiments have shown that this phenomenon is made possible by fimbrial structures for adhesins (Nataro *et al.*, 1992; Nataro *et al.*, 1994; Czeczulin *et al.*, 1997). Thus, the main mechanism of EAEC infection is due to their ability to associate with each other and colonize the intestinal mucosa, secreting enterotoxins and cytotoxins (Nataro *et al.*, 1998).

2.3.1.5 Enteroinvasive *E. coli*

The *Shigella spp* have been found to be biochemically and pathogenically related to this pathotype of *E. coli* (Pupo *et al.*, 2000). The afore-mentioned microorganisms share essential virulence factors. EIEC infects in the early stage by penetration through the epithelial cells of the intestines, followed by endocytic vacuolation. Once in the cytoplasm, the pathogen multiplies, followed by directional movement of the progenies, which is facilitated by concentration of cellular actin into a 'tail', extending from one pole of the bacteria to the other (Sansonetti, 2002). This enables EIEC to invade adjacent cells and cause inflammatory colitis and mainly small bowel syndrome.

2.3.1.6 Diffused adherent *E. coli*

Diffused adherent *E. coli* (DAEC) have a characteristic diffuse pattern of adherence to target cells and have been associated with several cases of diarrhoea, especially among infants (Nataro and Kaper, 1998). The mechanism involves a cytopathic effect that is characterized by the host cells developing long cellular extensions, which wrap around the bacteria. Further studies have found the presence of adhesion virulence factors as essential for this pathotype of *E. coli* to induce this characteristic sequence, in order to cause diarrhoea (Bernet-Camard *et al.*, 1996).

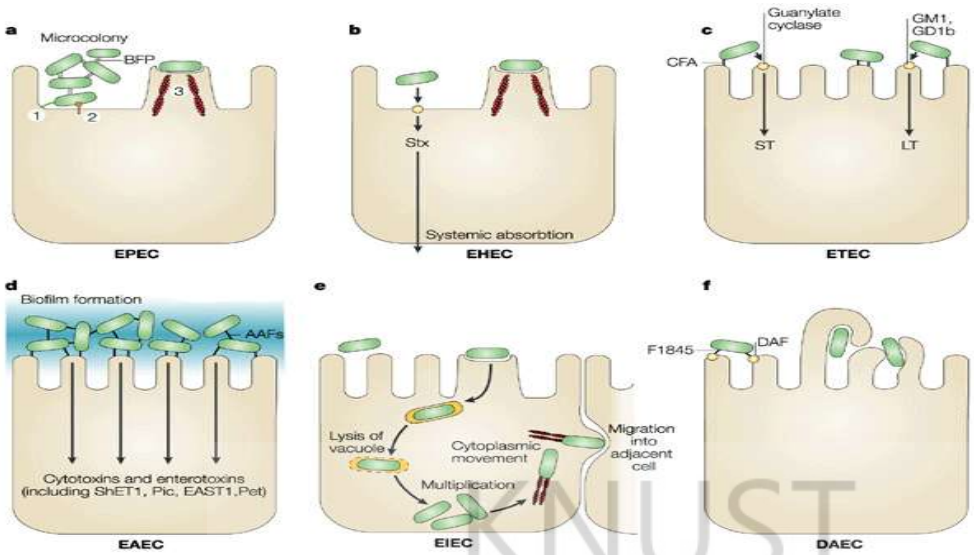

Figure 1: Pathotypes of Entero-*E. coli*; a) Enteropathogenic, b) Enterohaemorrhagic, c) Enterotoxigenic, d) Enteroaggregative, e) Enteroinvasive and f) Diffused adherence *E. coli*. Source; Nature Microbiology Review (Kaper *et al.*, 2004).

2.3.2 Extra-intestinal *E. coli*

The non-intestinal infecting strains of *E. coli* are mainly involved in causing urinary tract infections and meningitis. This class of *E. coli* has not been extensively studied and classified into pathotypes like the enteric/diarrrhoea *E. coli* (Kaper *et al.*, 2004). However, urinary tract infection-causing *E. coli* is loosely referred to as uropathogenic *E. coli* and has been studied, based on virulence factors whose protein products enable the microorganism to interact with the host tissues.

2.3.2.1 Uropathogenic *E. coli*

Non-pathogenic and pathogenic *E. coli* which migrate from the colon of the human host can colonize the urinary tract system and persist (Sweeney *et al.*, 1996). These microorganisms may possibly evolve through mutations or transformation into DNA sequences carrying virulence factors or introducing pathogenicity islands with associated virulence factors (Kaper *et al.*, 2004). These changes or additions in the genetic make-up of *E. coli* enable its adaptation and ability to persist in its new urine-abundant environment.

2.3.3 Virulence factors

Virulence is the ability of an organism to cause disease in a host and this depends on specific characteristics, due to inherent or acquired genes which enables the pathogen to infect its host (Johnson, 1991). Uropathogenic *E. coli* interacts with its host, through three major pathways facilitated by three major groups of genes, called virulence factors. These virulence factors are gene products, such as structural tissues, exotoxins or membrane-bound toxins and iron acquisition structures. No single virulence factor has been identified in isolation but various combinations of these virulence factors have been found in isolated cultures of uropathogenic *E. coli* as the cause of pathogenicity (Brooks *et al.*, 1981; Lomberg *et al.*, 1984; Johnson *et al.*, 1988). Studies have further shown that this is predominant in UTI than faecal isolates of *E. coli* (Brooks *et al.*, 1981; Hacker *et al.*, 1983). This observation therefore, suggests synergistic action of virulence factors as the mechanism of uropathogenic *E. coli* invading host defence system to cause disease (Johnson, 1991). However, variation in the expression of these virulence factors in isolated uropathogenic *E. coli* have been identified in several studies, attributed possibly to geographical differences and strain types.

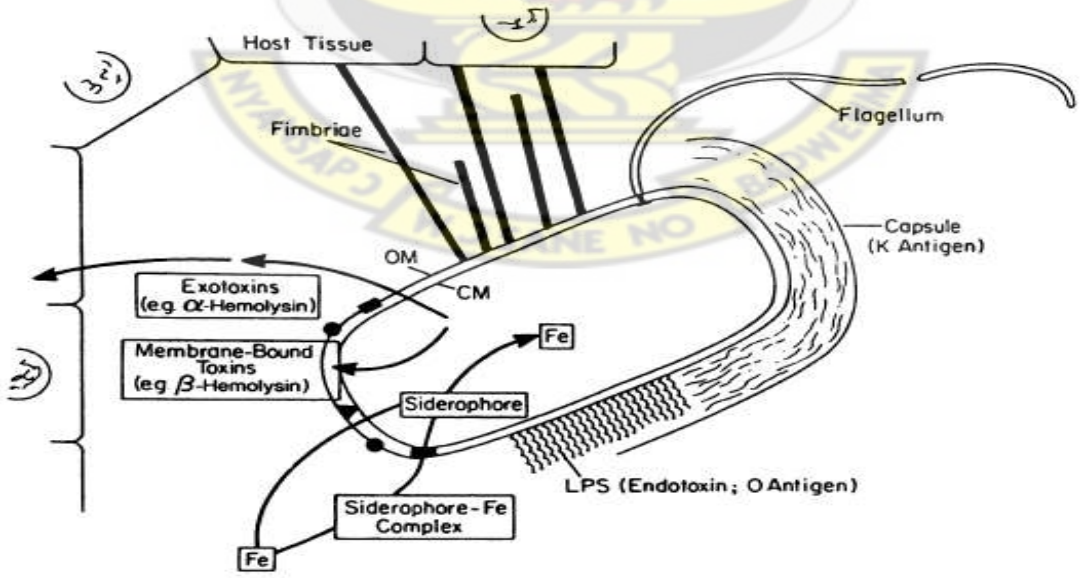

Figure 2: Interaction of uropathogenic *E. coli* with host tissue (Source; Eisenstein *et al.*, 1988).

2.3.3.1 Adherence

This is the primary interaction route the *E. coli* adopts in order to infect its host (Baddour *et al.*, 1990). Attachment to uro-epithelial cells enables the pathogen to persist and form reservoirs for subsequent infections. The mechanism is partially inhibited by the shedding of host uro-epithelial cells in the high current flow of urine (Hooton and Stamm, 1996). Fimbriae, a morphologically and functionally distinct structure from flagella, have been found to be responsible for adherence of the uropathogen to the host cells and other surfaces like pollen, latex beads and spores (de Man *et al.*, 1987). Studies have found the attachment and agglutination of erythrocytes by *E. coli* isolates to be a function of the presence of fimbrae. However, this process of agglutination of erythrocytes is influenced by the exposure of the *E. coli* to antibiotics, like ampicillin, trimethoprim and tetracycline (Hales and Amyes, 1985). Fimbrae is made up of repeating protein subunits and their ability to adhere to cells have been assessed and characterized, based on their resistance to the sugar, mannose in attaching to substrates (van Die *et al.*, 1988).

Adherence also depends on the host epithelial cells, as patients with recurrent urinary tract infections tend to promote fimbrae adherence, as compared to same cells from control patients (Bruce *et al.*, 1983). Other factors such as the antibacterial properties of UTI-free patients, stages of menstrual cycle and hormonal treatments also influence the degree of adherence by the uropathogen to host uro-epithelial cells (Reid, 2001).

2.3.3.1.1 Mannose resistant adhesins

This is a trait of most strains of uropathogenic *E. coli*, which enables them cause agglutination of erythrocytes by way of attachment and cross-linking, even in the presence of mannose and has a strong correlation with haemolysin-production (Evans *et al.*, 1980). Diversity in this trait is characterized, based on receptor specificity. Adhesins that recognize

the P blood group antigen receptors are termed P fimbriae, while others that identify antigens in the absence of P fimbriae called X adhesins (Johnson, 1991).

2.3.3.1.1.1 P fimbriae

Evidence show that this type of adhesin is specific for the Gal (α 1-4) Galβ moiety present as carbohydrate components in glycosphingolipids and glycoproteins in mammalian cells, avian eggs and on some bacteria, collectively called P blood group antigen (Marcus *et al.*, 1981). Strains exhibiting this kind of adherence either attach to surfaces naturally bearing the Gal (α 1-4) Galβ moiety or by possibly secreting substances that contain this moiety to serve as a receptor to facilitate adherence (Kroncke *et al.*, 1990). These P fimbriated strains further show slight variance as some adhere more specifically to the Gal (α 1-4) Galβ moiety than to its ceramide derivative, the globotetraosylceramide (Kroncke *et al.*, 1990). Receptors for the P fimbriae are of different densities and distribution in the cells they are found. They are mainly present on the erythrocytes of humans, pigs, pigeons, goats and dogs but not on these cells from horses, cow and guinea pigs (Parry and Rooke, 1985). Uro-epithelial cells of humans also express the Gal (α 1-4) Galβ receptor but with similar densities expressed in both males and females (Lomberg *et al.*, 1986).

P fimbriae is made up of four subunits, together with some accessory proteins; one major subunit, *Pap A*, and three minor subunits, *Pap E, Pap F* and *Pap G* (Lindberg *et al.*, 1987; Lund *et al.*, 1987). The *Pap A* forms the bulk of this protein structure and is essential for fimbriae formation but not a determinant for Gal-Gal adherence as studies show the adherence complex can form on the *E. coli* cell surface without fimbriae formation (Lindberg *et al.*, 1984). The minor subunits are minutely localized at the tip of the fimbrial structure with a more elaborate *Pap C* found on the outer membrane, forming the assembly platform for fimbrial growth (Lindberg *et al.*, 1987). These fimbriae components are arranged on a multicistronic gene cluster called *pap* (Baga *et al.*, 1984). A recent work showed the gene *pap*

C, having a 94 and 45% distribution in *E. coli* isolates from patients of non-immuno-compromised and immuno-compromised conditions and also more prevalent in isolates from pyelonephritis and prostatitis than in cystitis (Ruiz *et al.*, 2002; Rijavec *et al.*, 2008). Again, a study conducted in Brazil recorded a distribution of 21.1% of *pap C* in *E. coli* isolates from UTI patients (Santo *et al.*, 2006).

Expression of the P fimbriae is subject to both genetic and environmental factors. Fimbrial expression is favoured by a temperature of 37^{o}C but inhibited between 18-22^{o}C (Abraham *et al.*, 1986; Gander and Thomas, 1987). In addition, concentrations of trimethoprim also inhibit the expression of fimbriae. Findings of the absence of P fimbriae in isolates of pyelonephritis adult patients have led to investigators speculating it is as a result of an underlying anatomic abnormality in the host renal tissues (Dowling *et al.*, 1987).

2.3.3.1.1.2 X adhesins

Non-fimbrial adhesins that are able to cause mannose resistant hemagglutination in the absence of fimbriae are collectively called X adhesins. They are mainly of the Dr family, S fimbriae and others (Johnson, 1991).

Adhesins of the Dr family are so called due to their hybridizing with DNA probes that bind to certain parts of the Dr blood group antigen and these include the O75X and the afimbrial adhesin I(AFA-I) and afimbrial III (AFA-III) (Labigne-Roussel and Falkow, 1988; Nowicki *et al.*, 1990). Structurally, Dr adhesins appear as a fine mesh, a coil-like or filamentous coating structure on the cell membrane (Arthur *et al.*, 1989). Receptors for this type of adhesin are located on the decay accelerating factor on cells making up the Bowman's capsule, basement membrane and uro-epithelial cells, which prevent erythrocyte lysis by complement (Nowicki *et al.*, 1989). However, different strains bearing the Dr adhesins bind to different portions of the Dr antigen receptor with adhesion inhibited by the antibiotic

chloramphenicol. Genetically, the Dr adhesin is made up of five closely packed genes arranged in a cluster, which may be single or have several copies (Labigne-Roussel *et al.*, 1985). Moreover, the amino acid sequence of this gene cluster differs from one strain to the other (Labigne-Roussel and Falkow, 1988).

2.3.3.1.1.3 S fimbriae

Adhesins that cause mannose resistant haemagglutination by specifically attaching to the terminal sialyl-galactoside residues on human erythrocytes are termed S fimbriae (Parkkinen *et al.*, 1989). The gene cluster for this fimbriae comprises a structural subunit which bears similarities to the *pap A* of the P fimbriae, an adhesin and accessory proteins. Expression of S fimbriae is also regulated genetically (Riegman *et al.*, 1990).

2.3.3.1.1.4 Other X adhesins

Some less common X adherence strains have been identified, based on specific binding to other erythrocyte group antigens. The M adhesin binds to the terminal amino acid sequence of the M blood group and the G fimbriae also binds to the *N*-acetylglucosamine moiety of the erythrocyte (Vaisanen-Rhen *et al.*, 1983).

2.3.3.1.2 Mannose-sensitive adhesins

This type of adhesin is unable to effect haemagglutination in the presence of mannose and one of such has been identified in *E. coli* strains.

2.3.3.1.2.1 Type 1 fimbriae

Adherence by type 1 fimbriae is competitively inhibited by mannose and some of its conjugates like α-methylmannoside but not by other monosaccharide molecules (Johnson, 1991). This has left researchers to speculate that the receptors for type 1 fimbriae have a mannose component (Neeser *et al.*, 1986). It has also been found that nitrophenol and its derivatives inhibit adherence of type 1 fimbriae to epithelial tissues but not

haemagglutination of erythrocytes (Falkowski *et al.*, 1986). Receptors for this adhesin are distributed through the buccal, intestinal and vaginal cells of humans and other mammals (Falkowski *et al.*, 1986; Wold *et al.*, 1988). However, most investigators have identified type 1 fimbriae to be a lesser contributory factor to *E. coli* being uropathogenic (Duncan, 1988; Parkkinen *et al.*, 1989).

The gene cluster for type 1 fimbriae encodes a structural subunit, an adhesin and accessory proteins, similar to that of P fimbriae and X adhesins and regulatory proteins (Abraham *et al.*, 1987; Abraham *et al.*, 1988). In addition, isolated strains exhibit only a single copy of the gene cluster which has the expression of the structural subunit independent of the adhesin subunit. Moreover, most isolated strains identified to possess these genes are either fully fimbriated with type 1 fimbriae or non-fimbriated at all (Hultgren *et al.*, 1986; Gander and Thomas, 1987). Growth of the fimbriae is promoted by limiting oxygen concentrations and prior exposure to low concentrations of cephalosporins with chloramphenicol inhibiting its expression. Some *E. coli* cells in a population also have the tendency to genetically switch from non-fimbriated to fimbriated and vice versa (Nowicki *et al.*, 1984; Korhonen *et al.*, 1986).

2.3.3.2 Iron complexing structures/ siderophores

Microorganisms like *E. coli*, require iron for most metabolic processes, such as oxygen transportation, the electron transport system and DNA synthesis. The iron required is obtained from host cells (Neilands *et al.*, 1985; Bagg and Neilands, 1987b). A deficit of this mineral is detrimental to a pathogen as it would not be able to reproduce efficiently to persist in the host. This lack of iron may be due to the insolubility of iron and some defence mechanism employed by the host to starve the pathogen of iron (Gross *et al.*, 1984). Almost all biological forms of iron are complexed with iron proteins within cells, requiring its separation in order to be shuttled into the bacteria cell (de Lorenzo and Martinez, 1988).

Additionally, host organisms respond to bacteria infection by decreasing the absorption of iron in the intestines to reduce iron concentration available for survival of the pathogenic cells (Schaible and Kaufmann, 2004). These pathogens however, have adopted mechanisms to efficiently scavenge for iron from hosts with the aid of iron acquisition structures, called siderophores. Two main siderophores are employed by *E. coli*, namely aerobactin or hydroxamate siderophore; made up of two lysine amino acid units and a citrate molecule and the catecholate, enterobactin (Neilands *et al.*, 1985).

2.3.3.2.1 Aerobactin/Hydroxamate siderophore

Hydroxamate siderophore has been found to be the most efficient iron complexing protein structure, employed by *E. coli* to keep up a high iron concentration of 10^5 to 10^6per cell (Bagg and Neilands, 1987b; de Lorenzo and Martinez, 1988). Although a small molecule of about 616 molecular weight, its structure enables the effective chelating of iron to be shuttled into the bacterial cell, due to its high affinity constant of 10^{23} for iron (Neilands *et al.*, 1985; de Lorenzo and Martinez, 1988). After synthesis of this structure, *E. coli* secretes it to the surface of the cell, where it extracts Fe^{3+} in its environment, made possible by haemagglutination or cytolysis of host cells, caused by fimbriae attachment or cytotoxicity of secreted toxins respectively. The Fe^{3+} is taken up into the pathogen through an outer membrane protein receptor (Carbonetti and Williams, 1984). Strains of *E. coli* with the aerobactin system presents an advantage over similar ones as they are uninhibited by other proteins found in serum and dilute urine and thus are able to cleave iron from its iron-protein transferrin and make it readily available (Williams and Carbonetti, 1986; Bagg and Neilands, 1987b). Again, the aerobactin siderophore protein is recycled for iron complexing and shuttling, instead of it being hydrolyzed and resynthesized (Braun *et al.*, 1984).

Aerobactin is genetically made up of five genes; four encoding the enzymes for synthesis of aerobactin and the fifth for the outer membrane protein (Bindereif and Neilands, 1983;

Carbonetti and Williams, 1984). The four genes are termed *iuc*, found in a long sequence, designated *DBAC,* whose gene products catalyze the hydroxylation of lysine, acetylation of hydroxyl group to hydroxamic acid and condensation of two hydroxamic acids with citrate (de Lorenzo and Neilands, 1986). Synthesis is however, inhibited by very high concentrations of intracellular iron, whereby iron complexes with a ferric regulatory protein, *fur*, and binds to the promoter region of aerobactin operon, blocking transcription (Bagg and Neilands, 1987a).

2.3.3.2.2 Enterobactin siderophore

This is the primary iron complexing protein, utilized by *E. coli*. The catecholate component of enterobactin contributes to its high affinity constant of 10^{52} for iron (Williams and Carbonetti, 1986). Enterobactin also possesses a higher ability to remove iron from the iron-binding protein, transferrin, in buffered solutions (Bagg and Neilands, 1987b). However, this property is reversed in serum or urine where there are proteins which bind and reduce or possibly abolish the activity of the siderophore. In contrast to aerobactin, the enterobactin siderophore is hydrolyzed after extraction of iron to be resynthesized to repeat the formation of iron complexes (Braun *et al.*, 1984; de Lorenzo and Martinez, 1988). In addition, the enterobactin system leaves free iron in the cytosol of the bacteria, instead of delivering it to bacteria iron centres in the cytoplasm (Williams and Carbonetti, 1986).

Similar to the aerobactin system, enterobactin requires genes for siderophore synthesis and outer membrane transport protein but involves a higher number of these genes. The system requires at least sixteen genes; seven for synthesis of the siderophore structure, eight for the outer membrane transport structure and the last for a protein responsible for cleaving iron from the siderophore in the cytoplasm (Crowley *et al.*, 1991).

2.3.3.3 Toxin genes

In order for microorganisms to obtain essential nutrients from host cells, certain cytolytic proteins are synthesized to effect the rupturing of host cells. These protein molecules are either secreted or membrane-bound. Examples of such molecules are haemolysin, cytotoxic necrotizing factor 1 and *usp* protein.

2.3.3.3.1 Haemolysin

Haemolytic *E. coli* synthesizes cytolytic proteins that rupture erythrocytes in mammals and some aquatic species. These toxins are mainly proteins of about 110 kDa, with some phospholipid components and are either secreted α-haemolysin or membrane-bound β-haemolysin (Beutin *et al.*, 1988; Wagner *et al.*, 1988).

Activity of this toxin requires calcium ions (Ca^{2+}) as a cofactor at about ≥10 mM, although activity in the absence of this ion has been reported (Bhakdi *et al.*, 1986). Haemolysin acts by inserting into lipid-containing cation-selective channels in host erythrocytes, making it more permeable to molecules, such as Ca^{2+}, K^{+} and sucrose. This leads to lysis or the erythrocytes being bleached of haemoglobin (Jorgensen *et al.*, 1986; Bhakdi *et al.*, 1988). In addition to erythrocyte lysis, haemolysins are also known to be cytotoxic to host defence cells. Exposure to haemolysin causes polymorphonuclear lymphocytes to lose their morphology, due to degranulation and loss of leukotrienes, and a resultant impaired natural defence mechanisms, such as loss of response to chemical signals and phagocytosis (Gadeberg and Larsen, 1988; Bhakdi *et al.*, 1989). Haemolysin also stimulates the generation of reactive oxygen species from renal tubular cells and histamine release from mast cells and basophils, causing the compromise of renal tissues (Keane *et al.*, 1987; Gross-Weege *et al.*, 1988).

A four-gene operon, termed *hly,* has been identified to be involved in the production of haemolysin (Johnson, 1991). The *hly A* and *hly C* are genes for synthesizing the toxin protein

structure and activation of the intracellular protein, respectively (Felmlee *et al.*, 1985). Secretion of activated toxins from the cell membrane is made possible by the activity of *hly B* and the process further aided by the protein product of *hly D* (Wagner *et al.*, 1988).

The haemolytic action of *E. coli* is regulated physico-chemically by temperature and iron concentration. Haemolytic activity is inhibited by high temperatures with a corresponding decrease in haemolysin production, when iron concentration increases (Bohach and Snyder, 1985; Mackman *et al.*, 1986).

2.3.3.3.2 Cytotoxic necrotizing factor-1(CNF-1)

This toxin was identified as a cell product of *E. coli* strains, mainly isolated from diarrhoea experiencing infants (Caprioli *et al.*, 1983). Studies have shown CNF-1 can induce cell death and cause changes in cell morphology as it caused necrosis of rabbit skin and induced multi-nucleation of tissue cultured cells (Blanco *et al.*, 1992; Capo *et al.*, 1998). Most pathogenic isolates of *E. coli* produce CNF-1 of varying percentages, based on source of bacteria strains; 40% of strains isolated in urinary tract infections and 5-30% of strains involved in diarrhoea (Blanco *et al.*, 1992; Landraud *et al.*, 2000). CNF-1 acts on host epithelial cells and leukocytes, like monocytes or macrophages, via the activation of guanosine 5′-triphosphates (GTP) binding proteins, Rho A, Rac and Cdc (Chant and Stowers, 1995; Fiorentini *et al.*, 1997; Capo *et al.*, 1998; Fiorentini C. *et al.*, 1998). The action of these GTP proteins, with specific effects on actin, is dependent on the regulation of the actin-myosin cytoskeleton. Rho A is specifically modified by CNF-1 by deamidation of glutamine 63 (Gln 63) of the protein into glutamic acid (Flatau *et al.*, 1997). Moreover, the GTP proteins serve as substrates for the deaminase activity of the CNF-1 and this has subsequently been linked to the accumulation of stress fibres in epithelial cells (Fiorentini *et al.*, 1997; Lerm *et al.*, 1999). This action leads to modification of microvilli structure and also inhibits trans-epithelial migration of polymorphonuclear leukocytes. Work on monocyte-macrophages reveal CNF-1

increases filamentous actin content, which results in a decrease in phagocytotic activity (Capo *et al.*, 1998).

2.3.3.3.3 *usp* Protein

The gene encoding this protein toxin was first isolated from uropathogenic *E. coli*, responsible for causing inflammation of the prostate gland in a patient (Kurazono *et al.*, 2000). Similar to the CNF-1, studies suggest *usp* is common amongst uropathogenic strains than faecal strains (Yamamoto *et al.*, 2001). The *usp* gene is located on the chromosome and not on plasmids of pathogenic *E. coli* (Nakano *et al.*, 2001). The gene was found to be located on a 4.2kb pathogenicity island, followed downstream by putative sequences without any functions in a mouse model (Nakano *et al.*, 2001; Yamamoto *et al.*, 2001). However, analysis of the sequence upstream revealed a recurrent sequence, identical to that responsible for the production of haemolysin-coregulated protein (Hcp protein) in *Vibrio cholerae*, which has an unknown function (Williams *et al.*, 1996).

Homology studies have drawn a relation between the *usp* protein and the S-type pyocin of some *Pseudomonas aeruginosa* strains. These pyocins are ribosomally-synthesized antimicrobial molecules which microbes direct against closely related competing strains in their environment (Riley, 1998). The S pyocin is also chromosomally encoded and synthesized as a protein complex, comprising a 'killing protein' with a nuclease activity and an immunity protein against the nuclease action on the producer cell (Kurazono *et al.*, 2000; Nakano *et al.*, 2001). It is therefore, suggested that the uropathogenic *E. coli* increases virulence by synthesizing and secreting its bacteriocin, *usp* protein, to eliminate competing microbes but persists to continually infect the host, with the help of an immunity protein against the bacteriocin. A screening of *E. coli* isolates from UTI patients identified *usp* gene prevalence of 44% (Starcic-Erjavec *et al.*, 2008). Other studies in different geographical regions, like Japan in Asia, have recorded the distribution of *usp* in uropathogenic *E. coli* to

be as high as 79-93%, giving an indication of geographical locations contributing to differences in strains (Yamamoto *et al.*, 2001; Kanamaru *et al.*, 2003). A similar work done in the United States on only women, recorded a 68.5% distribution of *usp* gene in the isolates (Bauer *et al.*, 2002)

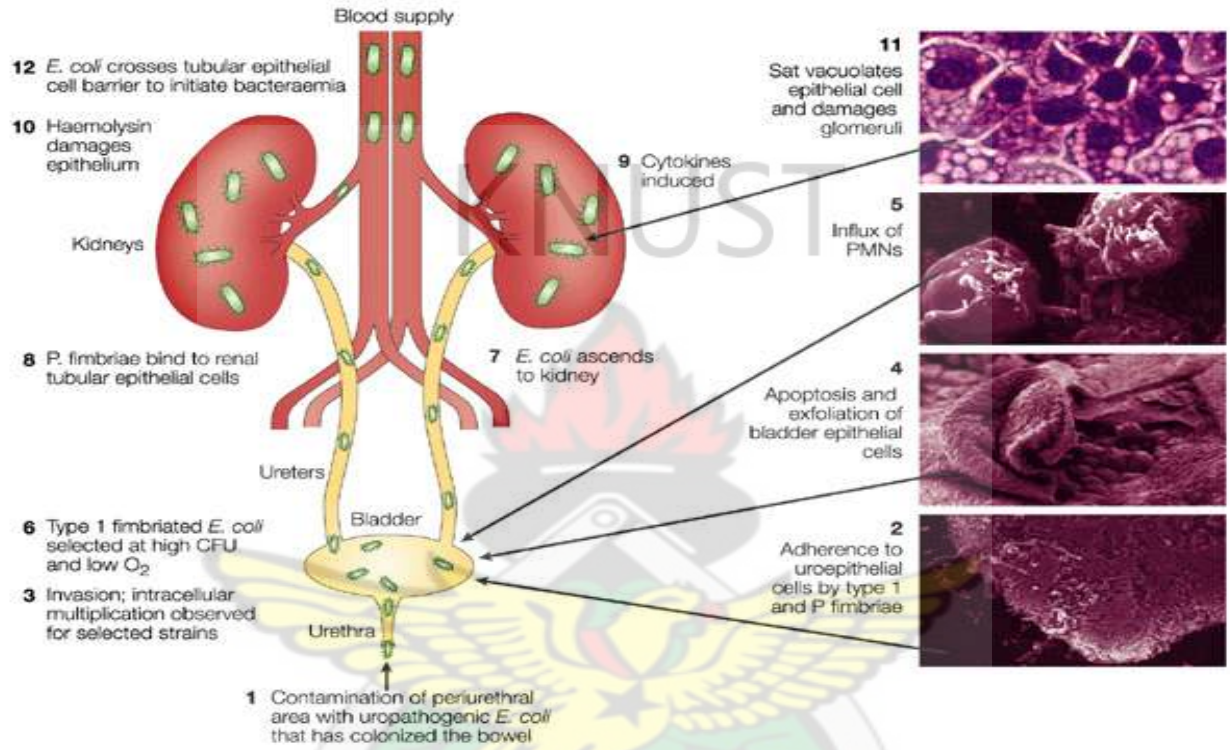

Figure 3: Mechanism of UTI caused by uropathogenic *E. coli* (Kapler *et al.*, 2004).

2.4 Detection of Urinary Tract Infection

Two major diagnostic methods are commonly used for the detection of uropathogens; microbiological culturing which is the currently accepted gold standard and dipstick urinalysis. The PCR method is also a known powerful tool for diagnosis.

2.4.1 Dipstick Urinalysis

The urine dipstick is a fast diagnostic technique for the detection of urinary tract infections. It is relatively less time-consuming and cheaper, as compared to known methods for microbial

detection in urine samples (Hurlbut and Littenburg, 1991). However, studies suggest that the sample population and the setting are factors which dictate the results obtained using dipstick (Deville *et al.*, 2004). Analysis by dipstick is preceded by urine sample collection which could be a clean catch midstream technique. External genitalia cleansing before passing of urine sample has been found to be of little benefit as there is only a 3% difference in contamination rates between samples taken with and without prior cleansing (Lifshitz and Kramer, 2000). The dipstick is impregnated with various colour-generating chemicals, used to measure parameters indicative of conditions due to UTI.

2.4.1.1 Parameters determined by Dipstick

Some parameters determined in dipstick urinalysis give good indication of infections in the urinary tract. These are haematuria, proteinuria, nitrites and leukocyte esterase.

2.4.1.1.1 Haematuria

The presence of more than two red blood cells in the urine of a patient under a high powered light microscope field, according to the American Urological Association, could be an indication of urinary tract infections (Mariani *et al.*, 1989). The dipstick measures this parameter by detecting the peroxidase activity of haemoglobin or myoglobin, with the aid of an indicator which is oxidized to change colour (Ahmed and Lee, 1997). However, there are other known causes of haematuria such as tumours, calculi and exercise-induced haematuria, which may give a false positive result for UTI (Siegel *et al.*, 1979; Brendler, 1998).

2.4.1.1.2 Proteinuria

Although the glomerulus normally secretes some urinary proteins, such as albumin and globulin, excretion above the threshold of 10-20 mg/dL is indicative of renal disease, causing the glomerulus to be porous and unable to reabsorb these proteins or the proteins overwhelming the cells' ability to reabsorb (Sheets and Lyman, 1986). The dipstick is able to

yield positive results for protein concentrations as low as 5-10 mg/dL with reliable sensitivities and specificities greater than 99% (Woolhandler *et al.*, 1989). Besides, postural proteinuria, which is a benign condition from prolonged standing could give a false positive for UTI.

2.4.1.1.3 Nitrites

Conversion of nitrates in urine to nitrite by bacteria, serves as evidence of UTI, and this can be detected by dipstick. Most Gram-negative and some Gram-positive bacteria convert nitrate to nitrite which when detected by dipstick is indicative of more than 100,000 per mL of bacteria present (Simerville *et al.*, 2005). The test is very specific for nitrite but with a low sensitivity (Pels *et al.*, 1989). Exposure of the dipstick to air has been found to alter its sensitivity, with greater numbers giving false positives, upon subsequent exposures (Gallagher *et al.*, 1990). However, non-nitrate reducing bacteria, example *Staphylococcus saprophyticus* and Enterococcus, may give false negative results.

2.4.1.1.4 Leukocyte Esterase

The localization of leukocytes is a strong indication of infection and this phenomenon is employed in dipstick urinalysis to detect the presence of bacteria infection. Leukocyte esterase, produced by neutrophils at the site of inflammation is qualitatively detected to indicate infection by bacteria. However, localization of leukocytes could also be due to urethritis, tuberculosis, viral infections, steroids and exercise (Simerville *et al.*, 2005).

Table 1: Causes of false positive and false negative results of urine dipstick test

Dipstick test	*False Positive*	*False Negative*
Blood	Dehydration, exercise, haemoglobinuria, menstrual blood, myoglobinuria	Captopril (Capoten), elevated specific gravity, pH < 5.1, proteinuria, vitamin C
Protein	Alkaline or concentrated urine, phenazopyridine, quaternary ammonia compounds	Acidic or dilute urine, primary protein is not albumin
Nitrite	Contamination, exposure of dipstick to air, phenazopyridine	Elevated specific gravity, elevated urobilinogen levels, nitrate reductase-negative bacteria, pH < 6.0, vitamin C
Leukocyte esterase	Contamination	Elevated specific gravity, glycosuria, ketonuria, proteinuria, some oxidizing drugs (cephalexin , nitrofurantoin, tetracycline, gentamicin), vitamin C

(Source: Simerville *et al.*, 2005)

2.4.1.1.5 Diagnostic indicators of UTI in dipstick urinalysis

Urinalysis using dipstick has necessitated a number of studies which have utilized nitrite and leukocyte esterase parameters as good indicators of UTI. A meta-analysis in the 1990s concluded that the use of the presence of nitrite and leukocyte esterase in urine were superior to the use of microscopy for detecting the presence of leukocytes, as an indication of infection (Gorelick and Shaw, 1999). A more recent meta-analysis however, suggested a high power field microscopy for pyuria to be the best for assessing UTI (W. H.O, 2005).

Studies in Africa reveal a good correlation in positive and negative predictive values for nitrite and leukocyte esterase combination for UTI diagnosis. A rural African study gave a positive predictive value of 73.8% and a negative predictive value of 95.7% while a city study gave a positive predictive value of 90.5% and a negative predictive value of 98.4%, indicating differences in dipstick performance in UTI prevalence between both settings (Wiggelinkhuizen *et al.*, 1988; Reed and Wegerhoff, 1995).

2.4.1.2 Usefulness of diagnostic tests

Diagnostic tests are of use in their ability to detect a person with a particular disease or exclude another without the disease. The diagnostic usefulness of a test is expressed in terms of sensitivity, specificity, positive and negative predictive values (Steurer *et al.*, 2002).

2.4.1.2.1 Sensitivity

The sensitivity of a clinical test refers to its ability to correctly identify patients with a disease (Altman and Bland, 1994). Sensitivity is calculated as the number of patients with disease who tested positive (true positives) divided by patients with disease who tested positive (true positives) plus patients with disease who tested negative i.e. false negatives (Lalkhen and McCluskey, 2008). Hypothetically, a test with 80% sensitivity detects 80% of patients with the disease (true positives) but 20% with the disease are undetected (false negatives). Hence, inference can only be made on how good a test is for identifying people with disease based on sensitivity when considering only diseased patients (Akobeng, 2006).

2.4.1.2.2 Specificity

Specificity of a test is the proportion of people without the disease who will have a negative result (Akobeng, 2006). This value is calculated from the number of patients without the disease who tested negative (true negatives) divided by the number of patients without the disease who tested negative (true negatives) and the number of patients without the disease who tested positive i.e. false positives (Lalkhen and McCluskey, 2008). A test with 80% specificity correctly detects 80% of the patients without the disease as negative (true negatives) but 20% of patients without the disease are incorrectly detected as positive (false positives). The specificity value is also only important when considering only those without the disease (Loong, 2003).

2.4.1.2.3 Limitations

A test with high sensitivity but low specificity, due to increase in false positives, which is usually the case for improved methods, results in many patients without the disease being detected as having it (Loong, 2003). In the absence of the ideal but unrealistic situation of 100% for both sensitivity and specificity, the patient is subjected to a confirmatory test with a lower sensitivity and higher specificity to correct the anomaly in the initial test (Lalkhen and McCluskey, 2008).

2.4.1.2.4 Predictive values

The aim of a diagnostic test is to make diagnosis from its result; hence there is the need to determine the probability that a test result will give a correct diagnosis (Akobeng, 2006). Probabilities can therefore be determined for both positive and negative tests.

2.4.1.2.5 Positive predictive value (PPV)

The PPV indicates the likelihood of a patient having the disease, given that the test is positive. This value is obtained by dividing the number of true positives by the total number of positives i.e. true positives plus false positives (Altman and Bland, 1994).

2.4.1.2.6 Negative predictive value (NPV)

The NPV of a test indicates how likely it is that a patient does not have the disease, given that the result is negative. NPV is expressed as the number of true negatives divided by the number of true negatives and false negatives (Altman and Bland, 1994).

2.4.1.2.7 Receiver Operator Characteristic (ROC) Curves

The ROC is used to investigate to what extent test results differ from people who do or do not have a disease under study. It was a plot designed in the 1950's for radar signal detection but is now used in the medical field (Metz, 1986). An ROC plot is generated by calculation of

sensitivity and specificity of every observed data value and plotting sensitivity against 1-specificity. A test is usually considered useless when the plot gives a straight line from the bottom left corner to the top right corner (Altman and Bland, 1994). Moreover, the acceptance of a test is given by the area under the curve, which is equivalent to the probability that a person chosen at random with the disease would have a higher value than an individual without the disease (Henley and McNeil, 1982). An ROC curve is also useful when comparing two or more measurements or tests. A test curve which lies wholly to the left or above another will be a better diagnostic tool with an area closer to 1.0 confirming a better method than that closer to 0.5 (Hilden, 1991). This analysis tool is simple and gives graphical representation of the results which is easily understood. However, the number of subjects is hidden in the display of results and the higher the number of subjects, the more jagged the resultant curve (Zweig and Campbell, 1993).

Table 2: Sensitivity and specificity of dipstick components in urinalysis

Test	Sensitivity % (range)	Specificity % (range)
Leukocyte esterase	83 (67-94)	78 (64-92)
Nitrite	53 (15-82)	98 (90-100)
Leukocyte esterase or nitrite positive	93 (90-100)	72 (58-91)

(Source: WHO, 2005)

2.4.2 Microbiological Culture

Growth of pathogenic microbes in specific media has remained the gold standard for detecting infections (Zorc *et al.*, 2005). The predominant Gram-negative bacteria associated with UTI grow on specific solid media at specific temperatures, to give rise to colonies originating from a single cell or changes in the colour and/or produce gases in liquid media, due to metabolic activities, indicating presence and probable number of the microorganism(s)

(Orenstein and Wong, 1999; Lutters and Vogt, 2000). However, culturing is limited, since it cannot differentiate between the same pathogen from different origins. Hence, microbiological culture for UTI diagnosis may be contaminated by faecal bacteria that colonize the perineal and distal urethra. Moreover, problems of contamination of culture from true infection are common and require repeated culturing for good diagnosis (Zorc *et al.*, 2005). In adults, a threshold of $\geq 10^5$CFU/mL of voided urine specimen is accepted as a positive test for UTI while the cut-off is 10^4 for children (Kass, 1956). However, in some cases the growth of any number of bacteria from urine specimen is considered significant. It is also sometimes difficult to diagnose a patient with UTI based on microbiological culture without any clinical evidence due to the possibility of asymptomatic bacteriuria (Kunin, 1970). Some pathogens are fastidious and others, viable but non-culturable (Colwell, 2006). This condition of viable but being non-culturable, therefore prevents the investigator from obtaining the true presence and number of bacteria present which would affect diagnosis of the infection.

2.4.3 Polymerase Chain Reaction

The advancement of molecular biology has led to the development of highly sensitive techniques, including Polymerase Chain Reaction (PCR), which is widely applied in the field of diagnosis. PCR mimics the *in vivo* process of DNA replication. The technique, thus enables amplification of DNA sequences from any organism. Separation of the PCR products by electrophoresis allows determination of polymorphism and cloning of amplified genes. A thermal cycler is employed to run PCR reaction mixture which includes deoxynucleotide triphosphates (dNTPs), buffer containing $MgCl_2$, *Taq* polymerase, DNA template and DNA primers, through cycles of DNA denaturation, primer annealing and primer elongation (Clement and Heyman, 1997).

PCR makes diagnosis of microorganisms, like viruses and bacteria much easier, as it reduces the time for specimen analysis, ranging from a few minutes to hours, as compared to other methods, notably the culturing method, especially for those microbes with designed primers (Yamamoto, 2002). Increasing trend in genome sequencing and analysis has facilitated the increased usage of PCR in molecular diagnostics. In addition, substantial work has been done to ascertain host-pathogen information at molecular level (Marrs *et al.*, 2005). Comparatively, PCR requires much smaller quantities of specimen for analysis. In addition, the specificity of primers used for pathogen detection reduces false results due to contamination (He *et al.*, 1994).

The use of PCR, however, is not without shortcomings. The first of them is the unavailability of the thermal cycler, hindering the routine usage of the technique for pathogen detection. Several factors, such as pH, metabolites and even debris in specimens like blood, urine and cerebrospinal fluid are known to interfere with PCR reaction conditions (Yamamoto, 2002). PCR products could be subject to contamination from the environment and carryover of DNA from previously amplified DNA, if stringent measures like the preparation of specimen in a flow cabinet with ultraviolet light and the use of new pipette tips per specimen are not adhered to (Kwok and Higuchi, 1989; Kitchin *et al.*, 1990). Moreover, sensitivity of the PCR is also dependent on the DNA extraction and the PCR protocol used (Dennett *et al.*, 1991). When a procedure requires it, most specimens are boiled and extracted with organic solvents or with commercially available kits, which employ silica or glass columns for DNA extraction (Bouquillon *et al.*, 2000). The nested PCR, which involves the amplification of conventional PCR products using a second set of primers, is also known to be about 1,000 times more sensitive than the standard PCR, though not always the case (Apfalter *et al.*, 2001). In addition to the PCR protocols, the target genes used for designing primers and the primer length contribute to the sensitivity and specificity of PCR detection of pathogens

(Schmidt, 1997). Varying results for different primers design from the same sequence, have been realized in pathogen detection with a high number of target sequence copies found to increase the sensitivity of the method (Yamamoto, 2002). Moreover, most studies require the pre-culturing of microbes to increase the cell numbers before PCR runs and this prolongs the time for obtaining results (Kurazono *et al.*, 2000; Marrs *et al.*, 2005).

2.4.4 Prevalence of Urinary Tract Infection in West Africa and Ghana.

A number of studies have been done in the West African sub-region, which suggest a wide range of prevalence of UTI in some selected populations. Most of these studies were done in febrile and malnourished infants and children below the age of 12 years, with the study sites being primarily hospitals and other health facilities (Mussa-Aisien *et al.*, 2003). Some studies have also been done on male and female adults, including pregnant women (Kolawole *et al.*, 2009; Imade *et al.*, 2010).

A Nigerian study on febrile infants of 1 to 60 months, reported a UTI prevalence of 9%, with females having a significantly higher prevalence, compared to males (Mussa-Aisien *et al.*, 2003). A study has reported a prevalence rate of 43% in children below the age of 10 years (Babaoye *et al.*, 1991). Moreover, in all these investigations on UTI in children, *E. coli* was found to be the predominant causative pathogen. Again, in Nigeria, an adolescent population gave a 28% incidence of UTI, with *E. coli* the causative agent of 52.77% (Aiyegoro *et al.*, 2007). However, data on the adolescent group has been few, to draw any conclusion on the population.

Similarly, UTIs in young adults in the sub-region show the predominance of *E. coli* in culture isolates from urine samples, with the age range of 21 to 30 years having the highest infections of 44 to 53%. It has also been established that adult females have UTI prevalence twice as much as their male counterparts as shown in a study in the sub-region, with *E. coli* frequency

of 30.56% (Kolawole *et al.*, 2009). Pregnant women attending antenatal clinic have presented prevalence ranging from 7.3% in a study in Kumasi, Ghana (Turpin *et al.,* 2007), to 86.6% in Benin City, Nigeria (Akerele and Okonofua, 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1Materials. Refer to Appendix 1A

3.1.1 Study site

The study population was drawn from three basic schools in the Oforikrom Sub-Metro in Kumasi. Two hundred and seventy two students (272) were recruited from Junior High School (JHS) 1 to 3 for the study. The sample size estimate was based on a prevalence of 10% in a Nigerian study (Oladeinde *et al.*, 2011), using a confidence level of 95% and marginal error of 0.05.

3.1.2 Recruitment

First meeting was to get the participants informed about the objectives of the study and its significance. A questionnaire was administered and participants provided with the sample bottles to be used for the collection of the urine. The urine collecting methodology was carefully explained to the students. Participants were asked to seek parental consents before passing early morning mid-stream urine the next day. Urine samples were collected in the morning at schools in Kentinkrono, Ayeduase and Bomso.

3.2 Collection of samples

Early morning mid-stream urine samples of about 10-15 ml were collected; using UV-sterilized plastic bottles with air-tight screw cap tops. Each urine sample bottle was labelled with a reference code, age, sex, and time of collection. The samples were placed in a cold box for transportation to the laboratory, where it was stored until analyses were carried out. All samples were analyzed with the microbial culture method and a 195 sub-population were analyzed with the dipstick, microbial culture and PCR methods.

3.2.1 ***Inclusion criteria***: Pupils and students of 13 to 19 years, without any symptoms indicative of UTI, who consented to be part of the study, were recruited.

3.2.2 ***Exclusion criteria***: Participants diagnosed with UTI and those on antibiotic therapy were excluded from the study.

3.3 Urine dipstick

Urine samples were preliminarily analyzed for UTI by use of DIRUI A10 urine test strips. This was done within the first two hours of sample collection. Each urine strip was dipped in urine and analyzed according to the manufacturer's instructions. A positive test for leukocyte esterase and/or nitrite was scored 1 for UTI using dipstick and 0 for absence of UTI.

3.4 Microbiological Culture Method

MacConkey broth media (Oxoid Limited) was employed to determine the Most Probable Number (MPN) of microbes in all 272 samples (Cochran, 1950). Serial dilutions of 10^{-1}, 10^{-2} in addition to the raw sample (10^{o}), were used, with 1 ml of each solution added to 5 ml of the broth in triplicates and incubated at 44^{o}C for 48 hours. A positive test for *E. coli* presence was indicated by colour change from pink to yellow and colony forming units greater than 10^{4}/mL. About 1 ml of positive test tubes contents were transferred into 5 ml tryptophan broth and incubated at 44^{o}C for 24 hours. Three drops of Kovac's reagent were added to the test tubes. A positive test for *E. coli* indicated a reddish ring formed at the surface of the broth. Positive *E. coli* test tubes were then plated, using SS agar and incubated at 37^{o}C. *E. coli* presence was confirmed by identification of rose-red colonies (Stamm *et al.*, 1982; Stark and Maki, 1984). UTI diagnosed with microbial culture method was scored as 1 and 0 for UTI absence.

3.5 PCR method

3.5.1 Preparation of bacterial DNA.

This test was carried out on 195 of the urine samples. DNA to be amplified was obtained from 400 µl aliquots of urine samples initially dispensed into sterile 1500 µl Eppendorf tubes and centrifuged at 11,000 rpm for 30 seconds. Aliquots of 200 µl were pipetted from the supernatant into sterile 1500 µl Eppendorf tubes and incubated at 120°C for 15 minutes in a water bath, similar to the protocol described by Le Bouguenec *et al* (1992). Resultant template DNA solution was stored as template DNA stock and short-spinned for use in PCR reaction.

3.5.2 Amplification procedure

PCR was done in a total volume of 10.5 µl, containing 1.5 µl of the template DNA, each of the primers at 10 µM, the four deoxynucleoside triphosphates (each at 250 µM), 10 mM Tris hydrochloride (pH 9), 1.5 mM $MgCl_2$, 30 mM KCl and 1U of DNA polymerase (Bioneer, . PCR amplifications consisted of an initial denaturation at 94°C for 2 minutes and 30 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 1 min, and extension at 72°C for 2 minutes and finally held at 4°C for 7 minutes in a Thermal Cycler (Eppendorf mastercycler, Germany). Five microliters of the reaction mixture was then analyzed by electrophoresis on 2% agarose gels and 3 µL of ethidium bromide. The resulting electrophoretic band was compared to a DNA size ladder corresponding to the products of the amplification, while viewing with the aid of a UV-trans-illuminator (TFM-26, USA) and image captured with a digital camera (Samsung, 12.5 megapixels, Republic of Korea)

3.5.3 Scoring of PCR products

DNA bands corresponding to PCR products for *papC* and/or *usp* primers were scored 1 for UTI presence and 0 for absence.

3.6 Statistical Analysis

The data were entered and analyzed with the Fischer's exact or Chi-square test and 2 x 2 contingency table. Comparison of diagnostic methods was done through the receiver operator characteristics analysis. The statistical software used was GraphPad Prism version 5 from GraphPad Software Inc USA. The statistical significance was set at $P \leq 0.05$.

3.7 Ethical clearance

Ethical approval for the study was obtained from the Committee of Human Research, Publications and Ethics of the Komfo Anokye Teaching Hospital and the School of Medical Sciences, KNUST. Permission was also sought from the Metro Education Office and headteachers of the schools.

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence of UTI

A total of 272 urine samples were analyzed, using microbiological culture method to determine the total UTI prevalence (30.88%) by uropathogenic *E. coli*. Mean age of males (15.04± 0.11 years) in Table 3 below was higher than that of females (14.62± 0.11) with no significant difference (p= 0.2130). Conversely, females had a higher prevalence of 34.88% and 27.27% for males with no significant difference (p= 0.1903

The sub-populations used for comparing the three diagnostic tests had a mean age of 14.97±0.10 with males being significantly older (15.16±0.14 years) than females (14.74±0.14) with p value 0.0351 (Table 4). Leukocyte esterase parameter in dipstick analysis detected more females having UTI (40.90%) than males (24.30%) with p value 0.0320. In addition, the culture method detected more females significantly having UTI (39.77%) compared to males (25.23%) with a p value of 0.0320. The PCR method parameters (*papC*, *usp*, *papC* or *usp* and *papC+usp*) showed no significant differences between males and females. Males recorded higher percentages for *papC* (43.93%), *papC* or *usp* (79.44%) and *papC+usp* (20.54%) than females.

Table 3: Overall prevalence of UTI caused by uropathogenic *E. coli,* using the standard microbiological culture method.

PARAMETER	Total	Males	Females	*p* value
	N=272	N=143	N=129	
Mean age (years)	14.84± 0.08	15.04± 0.11	14.62± 0.11	0.2130
No. of positives	84	39	45	
Prevalence (%)	30.88	27.27	34.88	0.1903

Table 4: Various parameters determined for a sub-population of 195 subjects.

PARAMETERS	Total N=195	Males N=107	Females N=88	*p* value
Mean age (years)	14.97±0.10	15.16±0.14	14.74±0.14	0.0351*
Leukocyte esterase (%)	62(31.79)	26(24.30)	36(40.90)	0.0142*
Nitrite (%)	2(1.03)	0(0.00)	2(2.27)	0.2024
Protein (%)	1(0.51)	1(0.93)	0(0.00)	1.0000
Blood (%)	8(4.10)	4(3.74)	4(4.55)	1.0000
Culture (%)	62(31.79)	27(25.23)	35(39.77)	0.0320*
papC(%)	76(38.97)	47(43.93)	29(32.95)	0.1406
usp (%)	103(52.82)	54(50.47)	49(55.68)	0.4755
papC or *usp*(%)	149(76.41)	85(79.44)	64(72.72)	0.3108
papC and *usp* (%)	36(18.46)	22(20.56)	14(15.90)	0.4609

[*=significant difference *p*< 0.05 at 95% confidence interval]

Table 5: Prevalence of uropathogenic *E. coli* infection stratified by age in both males and females based on positive tests for leukocyte esterase and/or nitrite parameters of dipstick method.

Age	Total	Male	Female
13	8/33(24.24)	2/18(11.11)	6/15(40.00)
14	17/44(38.64)	4/22(18.18)	13/22(59.09)
15	22/52(42.31)	13/27(48.15)	9/25(36.00)
16	5/37(13.51)	2/24(8.33)	3/13(23.08)
17	8/20(40.00)	3/12(25.00)	5/8(62.5)
18	2/9(22.22)	1/7(14.29)	1/2(50.00)
Prevalence	62/195(31.79)	25/107(23.36)	37/88(42.05)

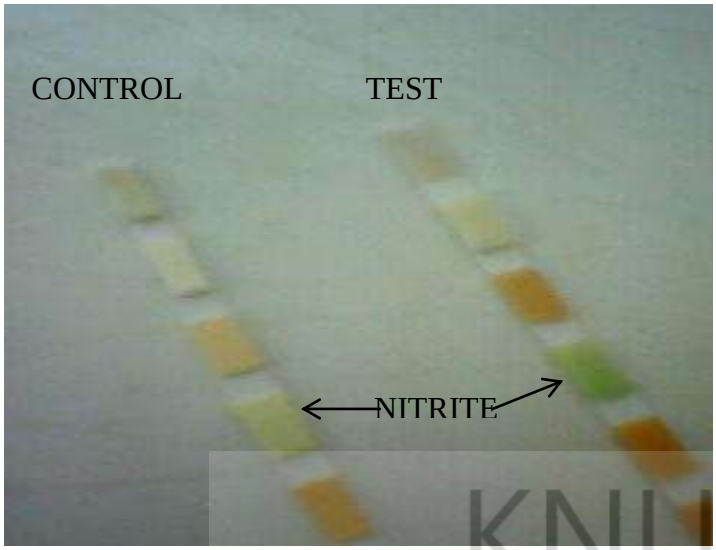

Figure 4: Urinalysis dipstick showing positive nitrite test.

Figure shows the nitrite panel on the test strip having reacted with urine to generate a green colour indicating a positive test for UTI.

Figure 5: A petri dish showing colonies of *E. coli*, growing on Salmonella Shigella agar.

Growth of *E. coli* as rose-red colonies (Figure 5) after streaking of urine samples on SS agar and incubating for 24 hours at 37^{o}C.

4.2 Dipstick test

UTI prevalence of 31.79% was found using the dipstick method (Table 5). Females had a higher prevalence (42.05%) than males (23.36%). The highest prevalence for females was recorded at age 17 (62.50%) and the least at age 16 (23.08%). Prevalence for the total and that of males were highest at age 15 with 42.31% and 48.15% respectively which declined to 22.22% and 14.29% respectively at age 18.

4.3 Culturing of Microbes

Sixty two urine samples (31.79%) tested positive for UTI using the culture method. A lower percentage of males (25.23%) were UTI positive compared to females (39.77%) in Table 6 below. The highest prevalence was recorded at age 17 for both the total and females with 45.00% and 75.00% respectively. Males recorded the highest percentage at age 15 with 40.74%.

Table 6: Prevalence of uropathogenic *E. coli* infection stratified by age in both males and females using culture method.

Age	Total	Male	Female
13	6/33(18.18)	4/18(22.22)	2/15(13.33)
14	14/44(31.82)	5/22(22.73)	9/22(40.91)
15	22/52(42.31)	11/27(40.74)	11/25(44.00)
16	10/37(27.03)	6/24(25.00)	4/13(30.77)
17	9/20(45.00)	3/12(25.00)	6/8(75.00)
18	1/9(11.11)	1/7(14.29)	0/2(0.00)
Prevalence	62/195(31.79)	27/107(25.23)	35/88(39.77)

Table 7: Prevalence of uropathogenic *E. coli* stratified by age in both males and females based on detection of *papC* and/or *usp* genes using PCR method.

Age	Total	Male	Female
13	29/33(87.88)	14/18(77.78)	15/15(100.00)
14	33/44(75.00)	18/22(81.82)	15/22(68.18)
15	40/52(76.92)	21/27(77.78)	19/25(76.00)
16	27/37(72.97)	18/24(75.00)	9/13(69.23)
17	14/20(70.00)	9/12(75.00)	5/8(62.50)
18	6/9(66.66)	5/7(71.43)	1/2(50.00)
Prevalence	149/195(75.88)	85/107(79.44)	64/88(72.73)

4.4 PCR results

A general prevalence of 75.88% (149/195) was recorded for UTI using the PCR method in Table 7 below. Males contributed a higher percentage of UTIs (79.44%) than females (72.73%). The total age-stratified prevalence decreased from age 13 (87.88%) to age 18 (66.66%). Prevalence for UTIs increased from 77.78% (13years) to 81.82% (14years) for males and gradually declined to 71.44% (18years) .Females had the highest prevalence (100.00%) at 13years with the least observed at 18years (50.00%).

Figure 8 shows the different distribution of *papC* and *usp* genes in *E. coli* isolates, according to gender. Males had a higher prevalence of the *papC* gene, compared to females. Both sexes had higher distribution for the *usp* gene, although there was no significant difference ($p > 0.05$) between sexes. There was significant difference between *usp* and *papC* genes ($p = 0.002$) and between *usp* gene and *usp* + *papC* genes ($p < 0.0001$). Both sexes also had the least prevalence for both *pap C* and *usp* gene identified in an *E. coli* isolate with no significant difference ($p > 0.05$).

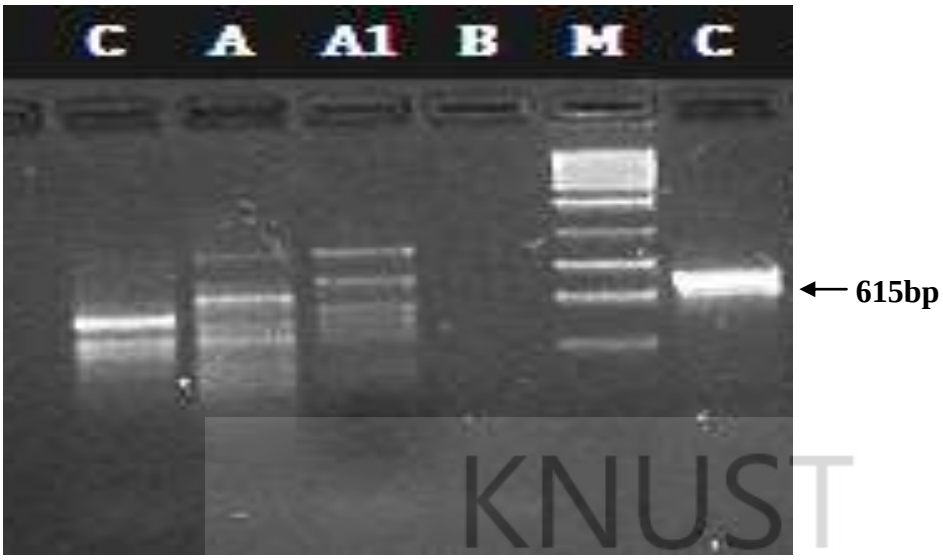

Figure 6: An ethidium bromide-stained agarose gel electrophoregram of amplified *usp* genes of uropathogenic *E. coli* DNA of different template preparation. [C= Control, A and A1= Denatured samples at 120^{o} C, B= Sample without denaturation and M= DNA Marker]

Figure 7: An ethidium bromide-stained agarose gel electrophoregram of amplified *papC* genes in uropathogenic *E. coli* from UTI samples. [C= Control, 1-3= Denatured samples at 120^{o} C and M= DNA Marker]

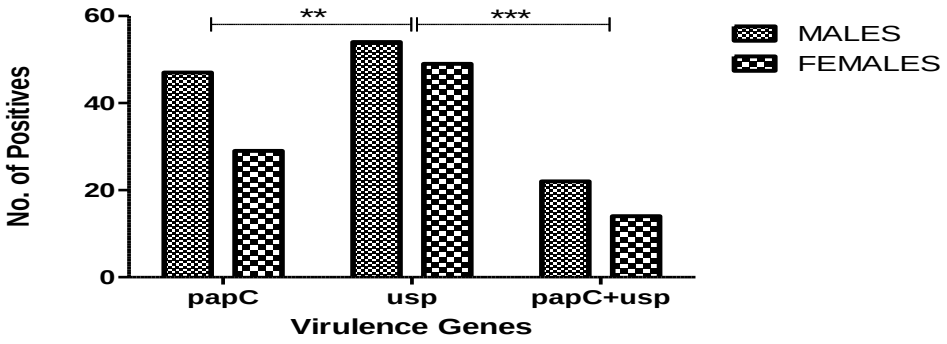

Figure 8: Distribution of virulence genes in *E. coli* isolates based on sex. [=significant difference $p< 0.002$, ***= significant difference $p< 0.0001$ at 95% confidence interval]**

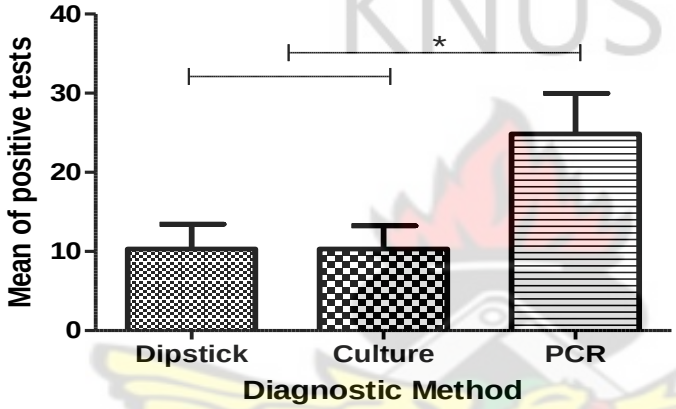

Figure 9: Mean number of positive tests for the three diagnostic methods. [Bar=SEM, N=195, *=significant difference $p< 0.05$ at 95% confidence interval]

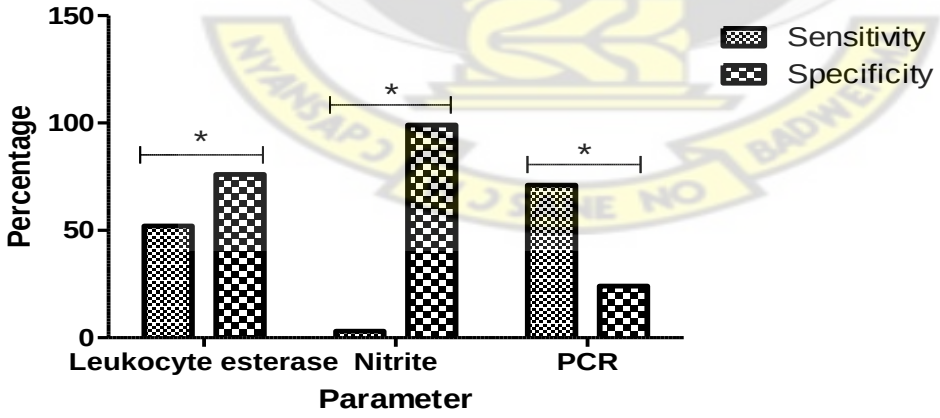

Figure 10: Sensitivities and specificities of parameters, compared with standard culture used for detecting UTI by *Escherichia coli*. [*=significant difference p< 0.05 at 95% confidence interval]

4.5 Comparison of diagnostic methods

According to figure 9, PCR had a significantly higher mean positive tests, which was more than twice t of both dipstick and culture methods. There was no difference between the means for dipstick and culture.

PCR had the highest sensitivity of 71% observed in figure 10 with significant difference ($p< 0.05$), compared to that of leukocyte esterase and nitrite. Difference between sensitivity for leukocyte esterase and nitrite was also statistically significant ($p< 0.05$). Nitrite had the highest specificity of 99%, compared to both leukocyte esterase and PCR with $p< 0.05$. Difference between sensitivity for leukocyte esterase and PCR was also statistically significant.

Comparatively, the PCR method was the most sensitive (71%) but least specific (24%), as observed in table 8. In contrast, the nitrite parameter had the highest specificity value (99%) and the lowest sensitivity (3%). Compared to both nitrite and PCR, leukocyte esterase had the highest values for both positive (52%) and negative predictive values (76%).

The ROC area under the curve of the PCR against that of the control culture (Fig 11A) shows a value of 0.7231, with statistical significance ($p< 0.0001$). Figure 11B shows an area of 0.5026 with no statistical significance ($p= 0.9302$) between the control culture and dipstick. Figure 11C also shows an area of 0.7205 with statistical significance ($p< 0.0001$) between the control PCR and dipstick.

Table 8: Performance characteristics of dipstick and PCR using culture as standard.

Parameter	Sensitivity	Specificity	PPV	NPV
Leukocyte esterase	52%	76%	52%	76%
Nitrite	3%	99%	1%	68%
PCR	71%	24%	30%	64%

PPV= Positive predictive value, NPV= Negative predictive value

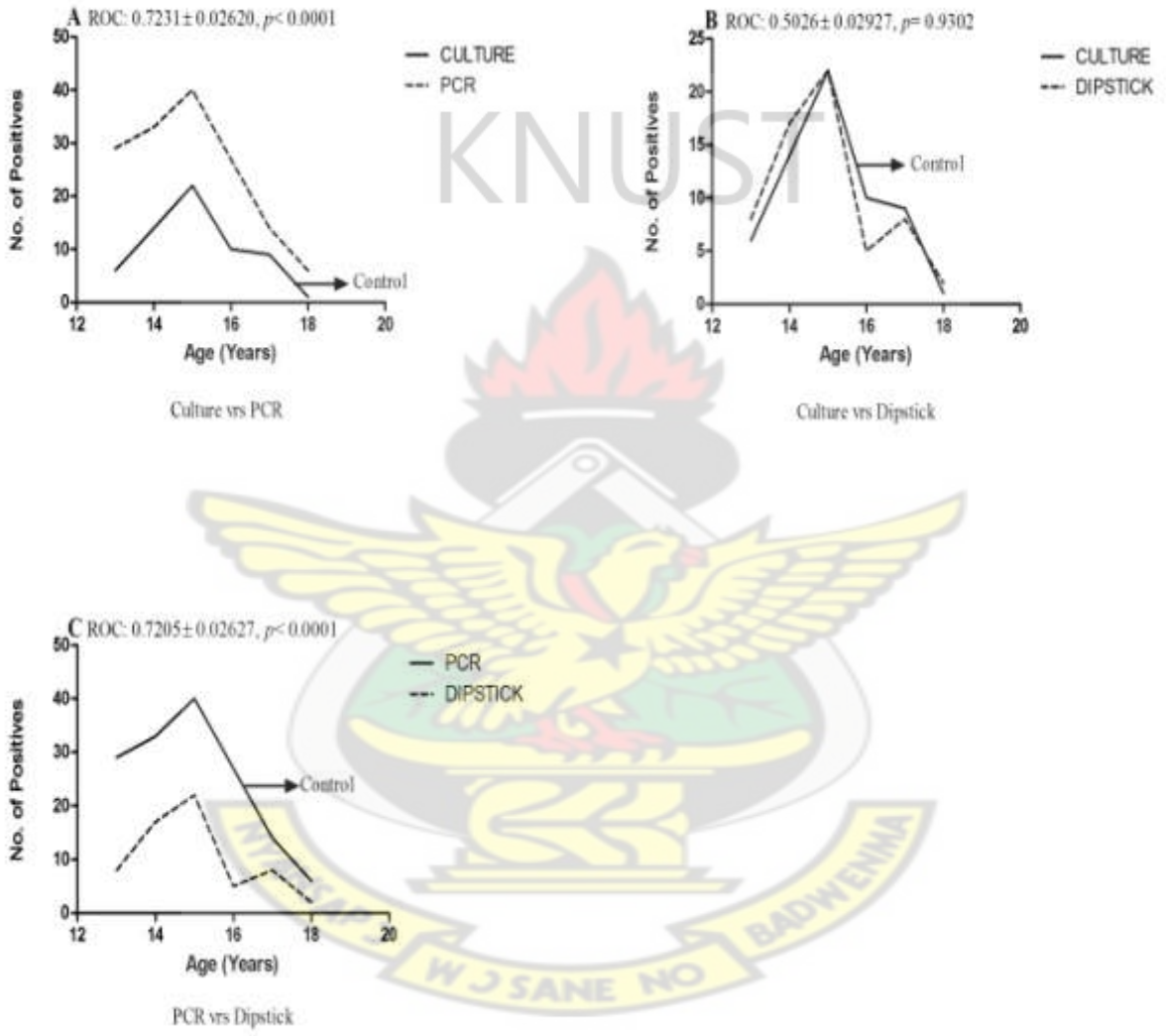

Figure 11: Receiver operator characteristics of the diagnostic methods with respect to ages of participants and number of positive UTI detections. A) Culture versus PCR. B) Culture versus Dipstick and C) PCR versus Dipstick.

CHAPTER FIVE

5.0 DISCUSSION

The general prevalence of UTI caused by *E. coli* was 30.88% (Table 3), which is lower than the 52.77% value realized in a similar work done in Nigeria (Aiyegoro *et al.*, 2007). The prevalence for this study is also lower than the 44-53% range observed for young adults between the ages of 21 to 30 years in the sub-region (Kolawole *et al.*, 2009). This difference in prevalence could be based on differences in sanitary conditions and observed personal hygiene. Again, the higher prevalence of *E. coli*-causing UTI in adults than the observed value for adolescent sample population may be due to sexual activity (Geerlings *et al.*, 2000).

According to table 4, the mean age of males (15.16±0.14) was significantly higher than females (14.74±0.14) with *p* value 0.0351. This suggests males are enrolled at older ages compared to females at the junior high school level. Females having a statistically significant (p= 0.0142) positive tests (40.90%) for leukocyte esterase parameter of the dipstick than males (24.3%) indicates the higher prevalence of UTI in females than in males. This fact is supported by a significant difference (p= 0.0320) in microbial culture which indicated a 39.77% prevalence in females to 25.33% in males.

Results from both table 5 and 6 indicate ages 15 and 17 as having highest prevalence of UTI, using both the dipstick and microbial culture methods. This observation could be explained as being due to an increase in the number of uropathogens due to re-infections which peaks at 15 and 17years. Thus, these high numbers of uropathogenic *E. coli* were enough to generate leukocyte esterase and nitrite levels detectable by urine dipstick and forming colonies greater than the 10^4cut-off for diagnosing UTI using microbial culture method. Prevalence of UTI by *E. coli* in table 7 showed a downward trend from peak at ages 14 and 13 for males and females respectively. It could be argued that the trend was due to the PCR method's ability to pick low bacteria cell counts ($<10^4$) as positive for UTI. This might have resulted in both the

dipstick and microbial culture methods having UTI prevalence of 31.79% (Tables 5 and 6) while PCR method had 75.88% (Table 7).

The *papC* gene recorded for males and females were 55.29% and 45.31% respectively and an overall prevalence of 51.01% (Figure 8). This means that about half the numbers of *E. coli* isolates employ fimbriae formation for adherence to uro-epithelial cells to cause UTI. Moreover, it could be as a result of most of UTI detected being first time infections, requiring pathogen adherence to uro-epithelial cells, in order to colonize the urinary bladder. Hence, pathogens adapt through the production of adhesion structures like *papC*. The *papC* prevalence of 51.01% is greater than the 21.1% determined in a Brazilian study (Santo *et al.*, in 2006) and the 45% for immuno-compromised populations in Slovenia (Rijavec *et al.*, 2008). These differences might have arisen from both the sample populations and the composite number of males and females in these populations. It is also possible, strain differences due to geographic locations can be a contributory factor to the deviation from the 94% for non-immuno-compromised in literature (Marrs *et al.*, 2005). However, the difference in distribution with respect to gender could be justified by the fact that isolates from cystitis, a condition that predominates in females, have been found to have low prevalence of *papC*, hence the smaller percentage of 32.95% (Fig 8) determined in females (Ruiz *et al.*, 2002). Moreover, some strains of *E. coli* isolates detected in males with UTI may be involved in first time infections which require adhesion structures made possible by the *papC* gene, contributing to a higher prevalence of the gene, compared to females. In figure 8, both males and females recorded *usp* gene as the predominant virulence gene, with 63.53% and 76.56% respectively, although there was no significant difference between genders. However, there was statistical difference between *usp* and *papC* gene (p= 0.0020). The overall prevalence of 52.82% (Table 4) for the *usp* gene was close to the 68.5% determined for women by Bauer *et al.*, (2002) and the 79-93% range in mixed populations in Japanese studies (Kurazono *et al.*,

2000). This indicates the importance of the *usp* gene for *E. coli* survival and persistence in the microbial community involved in urinary tract infection. Indeed, the higher percentage of *usp* gene, compared to *papC* gene observed in isolates from females could be due to the fact that infection by different uropathogens can be greatly enhanced by the shorter urinary tract of females. This increases the diversity of microbial community within the urinary tract, leading to the acquisition of the *usp* gene by *E. coli* to produce bacteriocin to eliminate competing uropathogens. It can also be argued that some of the UTI recorded might not have been first episode infection, but re-infections made possible by reservoir uropathogens in the urinary tract. Thus, these *E. coli* forming part of the reservoir microbial community must have adapted by resorting to the use of the *usp* gene, to eliminate other competing microbes, by the production of bacteriocin.

The two parameters of dipstick, i.e. leukocyte esterase and nitrite, and PCR were analyzed for their sensitivity, specificity and reliability in detecting UTI among school children in Ashanti region, using the microbial culture method as the gold standard. In table 8, the leukocyte esterase sensitivity of 52% fell out of the 67-94% range for sensitivity components of dipstick urinalysis, employed by the WHO (WHO, 2005). This might have been due to some of the UTI cases detected being in their early stages in the urethra. As a result, these infections could not trigger enough influx of leukocytes, to produce high concentrations of leukocyte esterase for a positive test by colour generation on the dipstick. Moreover, the low sensitivity (52%) could be attributed to dipstick defect in the reactive ingredients for detecting leukocyte esterase, leading to increase in false negatives and decreasing its sensitivity. Concentrated early morning samples may have further reduced the sensitivity of leukocyte esterase, by an increase in number of false negatives (Simerville *et al.,* 2005). It is worth noting that urine contamination may have insignificant effect since there is very minimal difference in results between clean catch and mid-stream urine (Lifshitz and Kramer, 2000). Positive predictive

value of 52% and negative predictive value of 76% for leukocyte esterase (Table 8) is an indication that comparatively, the parameter better serves as an index for correctly eliminating a patient as not having UTI when the test result is negative.

Specificity value of 99% (Fig 10) for nitrite which fell within the range of 90-100% (W.H.O, 2005) is a strong indicator of the parameter correctly testing negative for non-UTI samples. However, the extremely high value may be due to false positives caused by exposure of the dipstick to air. Low sensitivity (3%) for nitrite may have been primarily caused by low nitrate diets leading to corresponding low levels of nitrite by nitrate reductase-negative microorganisms which could not show positive tests for UTI positive samples. In addition, a positive predictive value of 1% is an indication of nitrite having a very low likelihood of a patient having the disease given that the test is positive. On the other hand, the negative predictive value of 68% is of relevance to a physician as it shows nitrite has a higher probability for an individual not having UTI when the test is negative.

With reference to the ROC analyses, the dipstick test, compared to the standard culture method, gave an area of 0.5026±0.02927 which is close to the 0.5 value indicative of a poor test method (Fig 11B). Moreover the *p* value of 0.9302 shows there is little difference between the performance of dipstick and culture test. When the dipstick was compared to the PCR method (Fig 11C), the ROC curve for dipstick was below that of PCR, indicating the superiority of PCR to dipstick with statistical significance ($p< 0.0001$). The PCR sensitivity of 71% (Fig 10) which falls within the range of both sensitivity ranges for leukocyte esterase (67-94%) and nitrite (15-82%) is indicative of PCR being a good technique for detecting the low presence of uropathogens in urine samples. However, the 29% samples with *E. coli* undetected by PCR could probably have been due to sampling of DNA template from solution. Moreover, a lower specificity of 24% (Fig 10) could be linked to false positives arising from a less sensitive culture standard, unable to detect some uropathogens or the PCR

detecting the low presence of *E. coli* without satisfying any set quantitative threshold comparable to the 10^4 CFU/mL cut-off used in the culture standard for diagnosis. This resultant rise in false positives had an effect on the positive predictive value (Table 8) of PCR (30%) as a diagnostic tool in UTI. The area under the curve (0.7231) for the ROC analyses (Fig 11A) for the culture versus PCR indicates PCR's higher ability to positively detect UTI since this value is closer to the 1.0000 for that of a perfect test method.

A UTI prevalence of 30.88% obtained in this study raises concern, as it could have serious immediate or future repercussions. Fevers and pains associated with symptoms of UTI can disrupt the sound frame of mind needed by school-going adolescents to study and this has the potential to lower academic performance of students. Infected adolescents could also grow anaemic in advanced stages of the infection, due to kidney damage. This is as a result of deficiency in production of the hormone erythropoietin in the kidneys which controls red blood cell formation. Undiagnosed and untreated asymptomatic UTI could also progress into kidney failure and hypertension in adulthood, which has the tendency to cause death and hence shortening of life expectancy (Zorc *et al.,* 2005). In effect, the nation's future skilled labour will be affected by morbidity and mortality, leading to reduction in productivity. Moreover, it could increase the burden on the country's health facilities. There is therefore the need to diagnose UTI with superior methods like PCR, to ensure that sub-clinical UTIs are promptly detected for appropriate intervention.

Currently, microbiological culture remains the standard method for UTI diagnoses and prevalence determination. However, established data indicate that culture results could be contaminated by pathogens of faecal origin (Simerville *et al.,* 2005), and this is a major shortcoming of the microbiological culture method. PCR method for diagnosing UTI, on the other hand can be strain-specific, depending on the set of primers used for diagnosis, as shown by this study. Moreover, performance comparison between PCR and microbiological

culture (Fig 11A) showed its superiority as a diagnostic method. Thus, PCR provides a more reliable result for UTI screening with respect to pathogen strain specificity.

5.1 CONCLUSIONS

The PCR method had the highest mean number (25) of positive detections of UTI caused by uropathogenic *E. coli,* compared to the standard culture (10) and the dipstick (10) parameters (i.e. leukocyte esterase and nitrite). Moreover, PCR had the highest sensitivity for diagnosing UTI when compared to the standard culture, and this is an indication of the PCR method being a superior diagnostic tool for detecting UTI. ROC curve area values of 0.7231± 0.0262, $p< 0.0001$ and 0.7205± 0.0262, $p< 0.0001$ when PCR was compared to the microbiological culture method and dipstick method respectively, is an indication of PCR being a superior diagnostic method. Furthermore, an ROC curve area value close to 0.5 with no significant difference (0.5026± 0.02927, $p= 0.9302$) observed upon comparing the microbiological culture method to the dipstick method shows there is no difference between the two in their ability to detect UTI. The low specificity recorded for PCR is an indication that the method requires another to augment the shortfall in its diagnostic power. Hence, the PCR method can be best used as a complementary tool rather than a single diagnostic tool for detecting UTI. The leukocyte esterase parameter in dipstick urinalysis had the highest values for both positive predictive value and negative predictive value, which makes it a single parameter of great significance to the physician in diagnosing patients with UTI.

High UTI prevalence was realized at 15 and 17years using the standard microbial culture method, which gives an indication of increasing number of uropathogenic *E. coli* in the urinary tract through the adolescent stage. This trend could be due to re-infections. In addition, age 15 was observed to have the highest prevalence of UTI by all three methods, an indication of a peak in infection at this age.

A higher distribution of the *papC* gene in uropathogenic *E. coli* isolates in the male adolescent population may be due to the use of adhesion factors to help attach to uro-epithelial cells in first time infections. It is possible most of the UTI detected in the adolescent females may have been re-infections by reservoir uropathogens from previous infections, which may had evolved to produce the bacteriocin toxin by the *usp* gene. This resulted in a significantly higher distribution of the *usp* gene compared to *papC* gene. Furthermore, males also having a high distribution of *usp* gene in *E. coli* isolates from their urine samples suggests UTI is mainly caused by different microorganisms in the urinary tract.

5.2 RECOMMENDATIONS

The study should be carried out in a population of higher age profile to compare results obtained by the PCR method.

More work should be done on other virulence genes with other populations to ascertain the distribution of these genes to help identify which treatment would best relieve patients of UTI.

A quantitative criterion for PCR work on UTI, comparable to the $\geq 10^4$ or 10^5 colony forming units per mL should be determined.

The general populace should be educated on UTI, its repercussions and how it could be prevented.

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APPENDICES

Appendix I

Table A1. Materials/equipment

EQUIPMENT/REAGENT	MANUFACTURER/MODEL	COUNTRY
Incubator	Marius Instrumenten	Netherlands
Laminar flow cabinet	Gelaire BSB6	Germany
Ice-maker	Scotsman AF 10 AE 0600	Italy
Water bath	Salm & Kipp	Germany
Centrifuge	Eppendorf 5424	South Africa
Thermal cycler	Eppendorf Mastercycler	Germany
High performance Ultraviolet Transilluminator	TFM-26	U.S.A.
Autoclave	Yamato Sterilizer SM510	Japan
Kovac's Indole reagent	Mercks	Germany
PCR Premix	Bioneer	South Korea
MacConkey broth	Oxoid Limited, Basingstoke New Hampshire	England
Tryptophan	Scharlau Chemie S.A.	Spain
Salmonella Shigella Agar (SS agar)	Oxoid Limited, Basingstoke New Hampshire	England
Digital camera (12.5 megapixels)	Samsung	Republic of Korea

Appendix II

Questionnaire

COMPARATIVE DIAGNOSIS OF URINARY TRACT INFECTION BY *ESCHERICHIA COLI* USING DIPSTICK, CULTURE AND PCR IN SCHOOL-GOING ADOLESCENTS.

QUESTIONNAIRE FOR VOLUNTEERS

PERSONAL DATA

NAME: ……. SEX: …….. CODE: ……

AGE: …… Years.

RESIDENTIAL LOCATION: ……...

PARTICIPANT'S HISTORY

1. Have you been asked to have urine test before?

Yes ☐ No ☐

2. When was it?

……

3. What treatment was given to you?

……

4. How many times do you urinate in a day?

……

5. Have there been cases where you have noticed changes in your urination in the following ways

(i) Increased frequency Yes ☐ No ☐

(ii) Decreased frequency Yes ☐ No ☐

(iii) Pain Yes ☐ No ☐

(iv)Urgency Yes ☐ No ☐

(v) Hesitancy Yes ☐ No ☐

(vi) Blood (not due to menses in girls) Yes ☐ No ☐

(vii) Urine retention Yes ☐ No ☐

(viii) Discharge Yes ☐ No ☐

6. Have you had any of the above together with abdominal pain and fever? Yes ☐ No ☐

Table B1. Column statistics for ages of sub-population used in the analysis of dipstick, culture and PCR

Number of values	195
Minimum	13
25% Percentile	14
Median	15
75% Percentile	16
Maximum	18
Mean	14.97
Std. Deviation	1.388
Std. Error	0.09941
Lower 95% CI of mean	14.77
Upper 95% CI of mean	15.17
Sum	2919

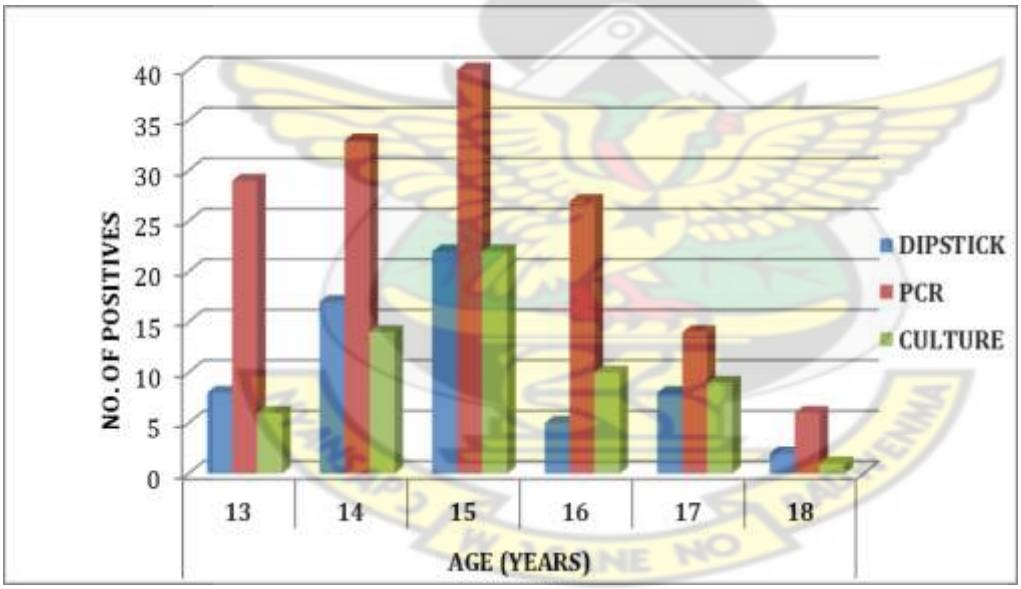

Figure A1. Age distribution of positive UTI detections using dipstick, culture and PCR.

Table C1. One-Way Analysis of Variance for the number of positive detections from dipstick, culture and PCR methods. *= *significant*

ANOVA Table	SS	df	MS	*p* value
Treatment (between columns)	841	2	420.5	0.0258
Residual (within columns)	1338	15	89.17	
Total	2179	17		

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Dipstick vs PCR	-14.5	3.761	Yes	*
Dipstick vs Culture	0	0	No	ns
Culture vs PCR	-14.5	3.761	Yes	*

Table D Distribution of amplified virulence genes

1

Virulence gene	Total No. of Positives	Males	Females
pap-C only	76	47	29
usp only	103	54	49
pap-C and *usp*	36	22	14
pap-C or *usp*	149	85	64

2*usp* gene and *usp+papC*genes

Fisher's exact test			
P value	< 0.0001		
P value summary	***		
One- or two-sided	Two-sided		
Statistically significant? (alpha<0.05)	Yes		
Data analyzed	*usp*	*usp+papC*	Total
Row 1	103	36	139
Row 2	46	113	159
Total	149	149	298

3 *papC* and *usp* genes

Fisher's exact test

P value	0.002
P value summary	**
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	Yes

Data analyzed	*papC*	*usp*	Total
Row 1	76	103	179
Row 2	73	46	119
Total	149	149	298

E. Area under the Receiver Operator Characteristic curves

1. Culture (Control) and PCR (Test)

Area	0.7231
Std. Error	0.0262
95% confidence interval	0.6717 to 0.7744
P value	< 0.0001
Data	
Controls (CULTURE)	195
Patients (PCR)	195
Missing Controls	0
Missing Patients	0

2. PCR (Control) and Dipstick (Test)

Area	0.7205
Std. Error	0.02627
95% confidence interval	0.6690 to 0.7720
P value	< 0.0001
Data	
Controls (PCR)	195
Patients (DIPSTICK)	195
Missing Controls	0
Missing Patients	0

3.Culture (Control) and Dipstick (Test)

Area	0.5026
Std. Error	0.02927
95% confidence interval	0.4452 to 0.5600
P value	0.9302
Data	
Controls (CULTURE)	195
Patients (DIPSTICK)	195
Missing Controls	0
Missing Patients	0

F. 2 x 2 contingency tables for sensitivity and specificity

1.

Leukocyte esterase			
Fisher's exact test			
P value	0.0007		
P value summary	***		
One- or two-sided	Two-sided		
Statistically significant? (alpha<0.05)	Yes		
Data analyzed	Sensitivity	Specificity	Total
Row 1	52	76	128
Row 2	48	24	72
Total	100	100	200

2.

Nitrite			
Fisher's exact test			
P value	< 0.0001		
P value summary	***		
One- or two-sided	Two-sided		
Statistically significant? (alpha<0.05)	Yes		
Data analyzed	Sensitivity	Specificity	Total
Row 1	3	99	102
Row 2	97	1	98
Total	100	100	200

3.

PCR			
Fisher's exact test			
P value	< 0.0001		
P value summary	***		
One- or two-sided	Two-sided		
Statistically significant? (alpha<0.05)	Yes		
Data analyzed	sensitivity	specificity	Total
PCR	71	24	95
Row 2	29	76	105
Total	100	100	200

Table G1. Chi-square analysis for difference in sensitivity and specificity between leukocyte esterase, nitrite and PCR. [*=*significant*]

Chi-square, df	107.1, 2
P value	< 0.0001
P value summary	***
One- or two-sided	NA
Statistically significant? (alpha<0.05)	Yes
Data analyzed	
Number of rows	3
Number of columns	2