COMPARATIVE STUDY ON DIAGNOSIS OF URINARY TRACT INFECTION AMONG ADOLESCENTS IN THE KUMASI METROPOLIS USING DIPSTICK, CULTURE AND PCR METHODS

Twumasi P., Darko D.N. and Nsiah K.
Department of Biochemistry and Biotechnology,
College of Science,
Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana

ABSTRACT
Urinary tract infection (UTI) is associated with clinical conditions such as hypertension, anaemia, kidney failure and even death. Diagnosis and early detection of UTI are critical measures in the management of the disease. The classical microbiological culture method is hindered by long diagnostic time and characteristic features of certain microbes becoming viable-but-non-culturable (VBNC). Direct PCR is a more sensitive and rapid diagnostic tool for detecting pathogens in clinical samples. This work aimed at developing a simplified PCR assay for detection of uropathogenic Escherichia coli, an index microorganism for UTI and the results compared to two common diagnostic markers – i.e., dipstick and microbiological culture methods. The cross-sectional study involved collection of urine samples from 195 adolescents, aged between 13 and 18 years. DNA segments were amplified using specific primers targeting papC gene for fimbriae formation and the bacteriocin usp gene of the uropathogenic E. coli. Comparing the three diagnostic tools, the PCR produced the highest sensitivity of 71%, and the nitrite-based dipstick method recording the least sensitivity of 3%. Leukocyte esterase-based dipstick recorded the highest specificity of 76% as against 24% specificity by the PCR method. In addition, leukocyte esterase detection dipstick produced the highest values for both positive and negative predictive values of 52% and 76%, respectively. From the receiver operator characteristic (ROC) analyses, PCR showed an area of 0.7231±0.0262 (age in years X number of positives) over culture method with P value 0.0001, compared to 0.7205±0.02623 (age in years X number of positives) of culture method over the dipstick method (p<0.0001). Although the PCR diagnostic method for UTI uropathogenic indicator (E. coli) was found to be superior method as compared to culture and dipstick methods, it should be made a complementary UTI diagnostic tool due to the low specificity.

Keywords: Dipstick, Escherichia coli, PCR, papC gene, urinary tract infection (UTI), usp gene.

INTRODUCTION
Urinary tract infection (UTI) is bacterial infection of the urinary tract, which otherwise is naturally sterile (Zorc et al., 2005). 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 (Akinkugbe et al., 1973; Hoberman and Wald, 1997; Gorelick and Shaw, 1999; Foxman et al., 2000). It is also estimated that about 20%
of women develop a UTI during their lifetime, with the disease incidence increasing at puberty and remaining high throughout adult life (Hooton et al., 2000). Furthermore, UTI accounts for approximately 23% of all hospital-acquired or nosocomial infections (Emmerson et al., 1996). Bacteria colonization of the urinary tract 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). E. coli is the commonest bacterial isolate detected in UTI, accounting for 70 to 90% of all infections in both children and adults (Simerville et al., 2005; Schalger, 2001; Riccabona, 2003).

Currently, the most commonly employed technique for detecting UTI in clinical laboratories is the use of the diagnostic dipstick method. It measures parameters such as protein, leukocyte esterase, blood cells and nitrate reductase levels in the urine. The dipstick method has limitations of false positives and false negatives (Ronald, 2003; Gallagher et al., 1990; Hurlbut and Littenburg, 1991; Hooton et al., 2000). Furthermore, a meta-analysis concludes that detection of nitrite and leukocyte esterase in the urine serves as reliable indicators for UTI diagnosis (Gorelick and Shaw, 1999). However, the use of bacterial culture consisting of the use of appropriate culture media for selected bacterial growth is generally preferred (Zorc et al., 2005). Unfortunately, culture assays have the limitation of being time-consuming, requiring a minimum of 48 hours to make observations from the culture plates. Additionally, the method does not detect and identify “viable-but-non-culturable” (VBNC) microbes, and thus may exclude potential pathogenic bacteria which could later result in symptomatic bacteriuria (Colwell, 2000).

Polymerase chain reaction (PCR) has proven to be a powerful and rapid tool for amplifying and detecting minute quantities of DNA in various biological samples (Yamamoto, 2002; Starič-Erjavec et al., 2008). Its diagnostic uniqueness in amplifying specific segments of pathogenic DNA of otherwise undetected pathogens using earlier methods, i.e., microbiological culturing and dipstick methods, is an advantage (He et al., 1994; Kurazono et al., 2000; Marrs et al., 2005). All the three aforementioned diagnostic tools have performance characteristics requiring constant assessment by laboratory technologists and clinicians (Steurer et al., 2002; Loong, 2003; Akobeng, 2006; Lelkhen and McCluskey, 2008). The current study was designed to assess the performance of a simplified PCR technique for UTI diagnosis against two other standard UTI diagnostic tools, i.e. dipstick and microbiological culture involving urine samples from adolescent population in the Kumasi Metropolis of Ghana.

MATERIALS AND METHODS

Sample collection

Early morning midstream urine specimens of 10-15 ml were aseptically collected from 195 adolescent students within the age range of 13 to 18 years from Junior and Senior High Schools in the Kumasi Metropolis. The urine samples were analyzed or stored in the freezer at -20 °C within 1–2 hours after collection to avoid overgrowth of inherent bacteria and other microbes. Subjects undergoing treatments with antibiotics in the past 72 hours were excluded from the study. Ethical approval for the study was granted by the Committee on Human Research Publications and Ethics (CHRPE) of the School of Medical Sciences of KNUST and the Komfo Anokye Teaching Hospital in Kumasi.

Dipstick analysis

Urine samples were preliminarily analyzed for UTI by the urine test strips (DIRUI A10, P.R. China) according to the manufacturer’s instructions. Dipped strips were matched with the urinalysis colour panel on the container to determine the selected parameters. The nitrite measurement was read after 60 seconds, leukocyte esterase after 2 minutes. Positive tests for either leukocyte esterase or nitrite were scored as 1, and negative tests by both parameters were scored as 0.
Urine Culture

MacConkey broth media (Oxoid Limited, England) was employed to determine the most probable number (MPN) of microbes (Cochran, 1950). Serial dilutions of $10^{-1}$, $10^{-2}$, in addition to the raw sample, were used. One milliliter of each dilution was added to 5 ml of the broth in triplicates and incubated at 44°C for 48 hours. About 1 ml of each positive test tube (i.e., a tube with microbial growth measuring more than $10^4$ cfu/mL), was transferred into 5 ml tryptophan broth (Scharlau Chemie S.A. Spain) and incubated at 44°C for 24 hours. Three drops of Kovac’s reagent (Mercks, Germany) were added to the test tubes. This is the Indole Test, in which a positive test for *E. coli* was indicated by a reddish ring formed at the surface of the broth. Simultaneously, urine samples were cultured, using *Salmonella Shigella* Agar (Oxoid Limited, England). An inoculating loop was used to deliver 1 µl of urine onto the medium and incubated at 37°C. A significant bacterial count (positive test) was taken as any count equal to or greater than 10,000 cfu/mL (Stamm et al., 1982; Stark and Maki, 1984). Positive tests were scored for samples that formed red indole ring and gave bacterial counts equal to or more than 10,000 cfu/mL as 1 and negative test 0.

Preparation of bacterial DNA

The template DNA for amplification 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, a method similar to the protocol described by Darko et al (2013). Resultant template DNA solutions were stored as template DNA stock and short-spinned for use in PCR reaction.

PCR amplification of *papC* and *usp* genes

PCR was done in a total volume of 10.5 µl, containing 1.5 µl of the template DNA, a pair of the primers each at a concentration of 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, South Korea). The primer sequences (Wageningen University and Research Center, The Netherlands) used were as follows:

- **Usp Forward** 5’- CGGCTCTTACATCGGTGCGTTG -3’,
- **usp Reverse** 5’-GACATATCCAGCCAGCGAGTTC -3’ and
- **papC Forward** 5’-ACGGCTGTACTCGAGGTTGTCG -3’,
- **papC Reverse** 5’-ATATCCTTCTGCAAGGATGCAATA -3’ (Starčič-Erjavec et al., 2008).

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). At the end of the reaction, 5 µl of the reaction mixture was analyzed by electrophoresis on 2% agarose gel, and the PCR products visualized by staining with ethidium bromide. PCR product sizes were established against size markers ran in lanes alongside that of amplified DNA products in the gel. Positive test was indicated by the presence of an amplified DNA product observed as a band on the gel and scored as 1 and negative tests scored as 0 corresponding with absent of DNA band at the locus.

Statistical analysis

The data from the various diagnostic tools were analyzed with the Fisher’s exact or Chi-square test and 2x2 contingency Table. Comparison of diagnostic methods was done through the receiver operator characteristics (ROC) analysis. The statistical software used was GraphPad Prism version 5 from GraphPad Software Inc. USA. The statistical significance between treatments was tested at probability of 5%.

RESULTS

The mean age of the sample population was 14.97±0.10 years, with males being significantly older (15.16±0.14) as compared to the females (14.74±0.14) (p=0.0351) (Table 1). The study showed a UTI prevalence of 31.79% (62/195) with the dipstick method, 31.80% (62/195) with
microbiological culture method and 75.88% (149/195) with the PCR method on the basis of DNA bands scored. The UTI prevalence produced by PCR method is about twice the values given by dipstick or culture method (Figure 1 and 2).

Leukocyte esterase parameter in the dipstick diagnosis showed more females having UTI (40.90%) as compared to 24.30% of the males ($p=0.0142$). According to the microbiological culture method, a significant proportion of the females (39.77%) contracted the disease as compared to 25.23% of the males ($p=0.0320$). On the contrary, the PCR method which amplified the two genes or either of the two ($papC$, $usp$, $papC$ or $usp$ and $papC+usp$) showed no significant differences in UTI development between the male and female adolescent population. However, the male adolescents recorded slightly

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

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

[Fig. 1: 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°C, B= Sample without denaturation and M= DNA Marker]

Fig. 2: Comparison of prevalence determined by dipstick, culture and PCR methods. [*=significant difference; $p= 0.0001$ at 95% confidence interval].

Table 1: Various parameters determined for sample population
higher percentages for \(papC\) (43.93%), \(papC\) or \(usp\) (79.44%) and \(papC+usp\) (20.54%) compared to the female adolescents (Table 1).

Table 2 shows calculated performance characteristics of the three UTI diagnostic tools employed in the study. Comparatively, the PCR method was the most sensitive (71%) but least specific (24%). In contrast, the nitrite parameter of the dipstick method had the highest specificity value (99%) and the lowest sensitivity (3%). Compared to both nitrite and PCR, leukocyte esterase component of dipstick method recorded the highest values for both positive (52%) and negative predictive (76%) values. Nitrite component of dipstick method had the least positive predictive value (1%) and negative predictive value (68%). A positive predictive value of 30% and negative predictive value of 64% were calculated for the PCR method.

### Table 2: Performance characteristics of dipstick and PCR using culture as standard

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte esterase</td>
<td>52%</td>
<td>76%</td>
<td>52%</td>
<td>76%</td>
</tr>
<tr>
<td>Nitrite</td>
<td>3%</td>
<td>99%</td>
<td>1%</td>
<td>68%</td>
</tr>
<tr>
<td>PCR</td>
<td>71%</td>
<td>24%</td>
<td>30%</td>
<td>64%</td>
</tr>
</tbody>
</table>

*PPV= Positive predictive value, NPV= Negative predictive value*

The area under the receiver operation characteristic (ROC) curve of the PCR against that of the control culture (Figure 3A) shows a value of 0.7231 ± 0.0262 (age in years X number of positives) with statistical significance \((p< 0.0001)\). However, an area of 0.5026 ± 0.0293 (age in years X number of positives) with no statistical difference \((p= 0.9302)\) is observed between the microbiological culture as control and dipstick method (Figure 3B). Comparing PCR as control and dipstick as the test (Figure 3C), an area of 0.7205 ± 0.0262 (age in years X number of positives) was obtained with the dipstick, an area much lower than that of PCR \((p< 0.0001)\).
DISCUSSION
Different prevalence values of urinary tract infection (UTI) were obtained for the adolescent populations of the Kumasi Metropolis using the three diagnostic tools – microbiological culture (31.80%), dipstick (31.78%) and PCR (75.88%). Interestingly, the culture and dipstick methods gave similar UTI prevalence values, each about half the UTI prevalence determined by the PCR method. The exceptional sensitivity of the PCR diagnostic method (Yamamoto, 2002; Darko et al., 2013) has given the marker a global favorite among clinical laboratories especially in infectious disease detections (Marrs et al., 2005; Starčič-Erjavec et al., 2008). PCR works by amplification of targeted nucleotide sequences of DNA of the organism and the segment which would otherwise remain invisible detected in electrophoretic gel. Moreover, the absence of a “cut off” threshold for PCR method similar to the $10^4$ CFU/mL for culture method and colour levels established for dipstick by manufacturers might have contributed to the high prevalence observed for PCR method since it can detect the smallest cell numbers.

The study which involved adolescent males (with an average age of 15.16±0.14) slightly older than the female adolescents (with average age of 14.74±0.14) ($P=0.0351$) showed more females (40.9%) significantly testing positive for leukocyte esterase of the dipstick as compared to the males (24.3%) ($p=0.0142$). The disparity in the UTI prevalence among the sexes was corroborated by the microbiological culture results where the prevalence was 39.77% in females as compared to 25.3% in males. Females having a higher UTI prevalence in both the dipstick and microbiological culture methods could be 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 (Gorelick and Shaw, 1999; Foxman et al., 2000; Zorc et al., 2005). In contrast, the prevalence observed by the use of PCR targeting at least one of the virulence genes showed a higher frequency in isolates from males (79.4%) than that from females (72.7%) though with no significant difference ($P=0.3108$). The high sensitivity of PCR makes it possible to detect the presence of microbes in much lower density than the $10^4$ cells/ml threshold set for other UTI diagnostic methods.

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 the school children in Ashanti region. In Table 2, 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 be due to patients being in the early stages of UTI where there is partial microbial colonization of the urethra. As a result, these infections could not trigger enough influx of leukocytes leading to high concentrations of leukocyte esterase to indicate a positive test by colour generation on the dipstick. Concentrated early morning samples may have further reduced the sensitivity of leukocyte esterase, by an increase in the number of false negatives (Simerville et al., 2005). It is worth noting that urine contamination in this study had insignificant effect on the results obtained from clean catch and mid-stream urine samples (Lifshitz and Kramer, 2000). Positive predictive value of 52% and negative predictive value of 76% for leukocyte esterase (Table 2) 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% (Table 2) for nitrite which fell within the range of 90-100% (WHO, 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 diagnosing a non-
infected person with UTI. 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 (Faraggi and Reiser, 2002), the dipstick test, compared to the standard culture method, gave an area of $0.5026 \pm 0.0293$ (age in years X number of positives) which is close to the 0.5 value indicative of a poor test method (Figure 3B). Moreover, the $P$ value of 0.9302 shows there is no significant difference between the performance of dipstick and culture test. When the dipstick was compared to the control PCR method (Figure 3C), the ROC curve for dipstick was below that of PCR indicating the superiority of PCR over dipstick with statistical significance ($P < 0.0001$). The PCR sensitivity of 71% (Table 2) which falls within sensitivity ranges for leukocyte esterase (67-94%) and nitrite (15-82%) is indicative of PCR being a good technique for detecting low presence of uropathogens in urine samples. However, the 29% samples with E. coli undetected by PCR could probably have been due to pretreatment of urine samples. Moreover, a lower specificity of 24% (Table 2) 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. However, the resultant rise in false positives could affect the positive predictive value of PCR as a diagnostic tool in UTI (Table 2). The area under the curve of $0.7231 \pm 0.0262$ (age in years X number of positives) for the ROC analyses (Figure 3A) 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.

In conclusion, this study has firmly established PCR to be most sensitive diagnostic marker for UTI compared to the other two commonly used methods - microbiological culture and dipstick methods. However, the low specificity of the PCR method makes it imperative to complement it with other methods such as dipstick. The leukocyte esterase parameter in dipstick urinalysis had the highest positive and negative predictive value, and therefore making it a single most important parameter significant to physicians in the diagnosis of UTI in patients.

REFERENCES


