International Journal of Research
Available at https://edupediapublications.org/journals
p-ISSN: 2348-6848
e-ISSN: 2348-795X
Volume 04 Issue 06
May 2017

Isolation and Molecular Characterization of Bacterial Isolates from Diabetic Patients with Urinary Tract Infections

SAIF ALI MOHAMMED[1]
M.Sc.,
DR.K.AMMANI[2]
Research Supervisor
Department of Microbiology
[1,2] Acharya Nagarjuna University

ABSTRACT

Diabetes mellitus is one of the most challenging health problems of 21st century and is the fifth leading cause of death in developed countries. Asian patients are considered to have a higher risk of developing diabetes and potentially worse prognosis. By 2025, the number of individuals with diabetes is expected to be more than doubled. Urinary tract infection (UTI) poses serious future clinical repercussions, such as hypertension, anemia, 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-cultural. On the other hand, molecular detection by direct Polymerase Chain Reaction (PCR) technique has proven to be more sensitive in detecting pathogens in various biological samples, this work was thus

Adolescent students, aged between 13 and 18 years. Pathogens were identified by using morphological and biochemical tests. The PCR protocol targeted a more specific pap C gene for fimbriae formation and the bacitracin 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.

INTRODUCTION

One of the common complications of diabetes is its effect on genito-urinary system. Diabetic patients are at increased risk of urinary tract infections. Diabetes causes several abnormalities in the host system that increases the risk of urinary tract infection. These include immunologic

impairments such as defective migration, phagocytic alteration of chemotaxis in polymorph nuclear leukocytes[1]. Diabetics are at increased risk of developing acute pyelonephritis, renal abscess, bladder scaring and myelitis. Emphysematous pyelonephritis is almost exclusively an infection of diabetic patients and carries a grave prognosis; papillary necrosis complicates 21% of cases. Treating UTIs with broad spectrum antibiotics is widely practiced; however, because of concerns about infection with resistant organisms, treating with a narrow spectrum antibiotic may be more appropriate[2]. It is more preferred to use fluoroquinolones as initial agent for empiric therapy of UTI in an area where there is a high concern about antibiotic resistance. The reason for this is their high bacteriological and clinical cure rates, and low resistance rates among most uropathogens.

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[3].

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[4]. 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[5].

MAIN OBJECTIVES OF THE STUDY

1. Isolation of pathogens from diabetic urinary tract infection patients.

2. Identification by using morphological and biochemical tests.
3. To use dipstick and classical microbiological culturing method to determine presence of E. coli in urine of subjects
4. To design a PCR assay based on dominant virulence genes (pap and usp) of uropathogenic E. coli strains for diagnosis of Urinary Tract Infection.
5. To determine the performance characteristics of the diagnostic methods.

MATERIALS AND METHODS

1- COLLECTION OF SAMPLES

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. Urine samples were collected from near hospitals. 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 27 sub-population were analyzed with the dipstick, microbial culture and PCR methods.

2 URINE DIPSTICKS

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.

MICROBIOLOGICAL CULTURE METHOD

MacConkey broth media was employed to determine the Most Probable Number (MPN) of microbes in all 27 samples. Serial dilutions of 10^{-6}, 10^{-7} in addition to the raw sample, 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 and add three drops of Kovac's reagent were added to the test tubes. A positive test for *E. coli* presence was indicated by colour change from yellow to pink. 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)[6].

PCR METHOD

1 PREPARATION OF BACTERIAL DNA.

This test was carried out on 27 of the urine samples among the one patient sample did PCR who showed positive both URINE

DIPSTICK and microbial method. 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[7]. 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 [8].

2 AMPLIFICATION PROCEDURES

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 MgCl2, 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)

RESULTS

PREVALENCE OF UTI

A total of 27 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 years) in Table 3 below was higher than that of females (14) 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. 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.

International Journal of Research
Available at https://edupediapublications.org/journals
p-ISSN: 2348-6848
e-ISSN: 2348-795X
Volume 04 Issue 06
May 2017

Table**1**:OverallprevalenceofUTIcausedbyuropathogenic*E.coli*,usingthestandard microbiological culturemethod.

PARAMETERS	Total	Males	Females	*p* value
	N=27	N=14	N=13	
Mean age (years)	14.84± 0.08	15.04± 0.11	14.62± 0.11	**0.2130**
No. of positives	8	3	5	
Prevalence (%)	**30.88**	**27.27**	**34.88**	**0.1903**

Table**2**:Various parameters determined fora sub-populationof27subjects.

PARAMETERS	Total N=27	Males N=14	Females N=13	*p* value
Mean age (years)	14.97±0.10	15.16±0.14	14.74±0.14	**0.0351***
Leukocyte esterase (%)	6(31.79)	2(24.30)	4(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 (%)	6(31.79)	3(25.23)	4(39.77)	**0.0320***
papC(%)	16(38.97)	9(43.93)	8(32.95)	**0.1406**
usp (%)	10(52.82)	4(50.47)	6(55.68)	**0.4755**
papC or *usp*(%)	14(76.41)	7(79.44)	6(72.72)	**0.3108**
papC and *usp* (%)	**6(18.46)**	**4(20.56)**	**2(15.90)**	0.4609

[*=significant difference *p*< 0.05 at95%confidenceinterval]

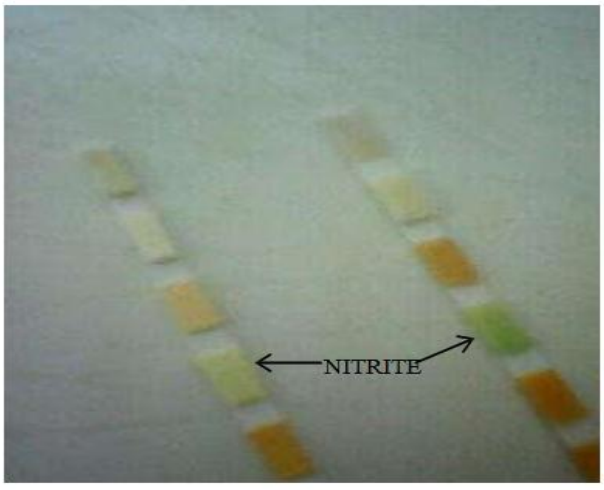

Figure**1**: Urinalysis dipstickshowing positive nitritetest.

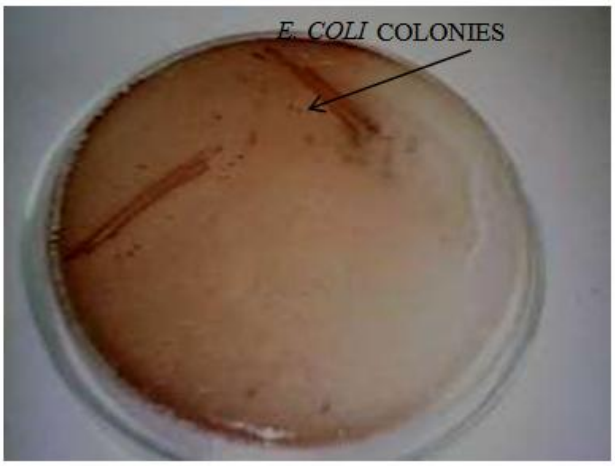

Figure**2**: Apetri dishshowing colonies of*E. coli*, growing on Salmonella Shigella agar.

Growthof*E.coli*asrose-redcolonies(Figure5)afterstreakingofurinesamplesonSSagar and incubatingfor24 hours at 37^{o}C.

Dipstick test

UTI prevalence of 31.79% was found using the dipstick method. Females had a higher prevalence (42.05%) than males (23.36%). The highest prevalence for females was recorded at age 14 (42.05%). Prevalence for the total and that of males were highest at age 15 with 23.36 % respectively.

PCR results

A general prevalence of 75.88% was recorded for UTI using the PCR method. Females contributed a higher percentage of UTIs

(42.05%) than males (23.36%). Figure 8 shows the different distribution of papC and usp genes in E. coli isolate from one of the female patients who have positive on urinary tract infection showed positive on PCR

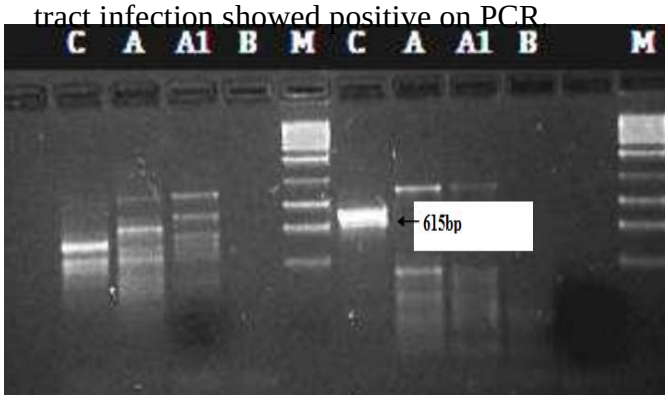

Figure**3**:Anethidiumbromide-stainedagarosegelelectrophoregramofamplified *usp* genesofuropathogenic*E. coli*DNA ofdifferent template preparation.[C= Control,A andA1=Denaturedsamplesat120^{o}C,B= Samplewithout denaturationandM=DNA Marker]

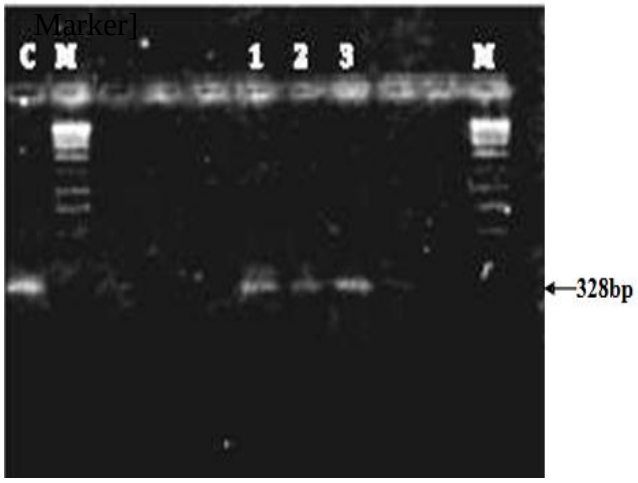

Figure 4: An ethidium bromide-stained agarose gel electrophoregram of amplified papC genes in uropathogenic E. coli from UTI samples. [C= Control, 1-3= Denatured samples at 120o C and M= DNA Marker]

DISCUSSION

Thegeneralprevalence ofUTI causedby*E.coli*was30.88%(Table2),whichislowerthan the 52.77% value realized. The prevalenceforthisstudy isalsolowerthanthe44-53%rangeobservedforyoungadults betweentheagesof14to15yearsinthesub-region.Thisdifference inprevalencecouldbebasedondifferencesinsanitary conditionsandobservedpersonal hygiene.Again,thehigherprevalenceof*E.coli*-causingUTIinadultsthantheobserved value foradolescent samplepopulation maybedueto sexual activity.Accordingtotable4,themeanageofmales (15.16±0.14)wassignificantly higherthan females(14.74±0.14)with*p*value0.0351.This suggestsmalesareenrolledatolderages comparedtofemalesatthejuniorhighschoollevel. Femaleshavingastatistically significant (*p*=0.0142)positivetests(40.90%)forleukocyteesteraseparameterofthedipstickthan males(24.3%)indicatesthehigherprevalenceofUTI infemalesthaninmales.Thisfactis supportedbyasignificantdifference(*p*=0.0320)in microbialculturewhichindicateda39.77%prevalencein females to 25.33%in males.The ages14and15ashaving highestprevalence ofUTI, usingboth thedipstick and microbial culturemethods. This observation could be explained as beingduetoanincreaseinthenumberofuropathogensduetore-infectionswhichpeaksat14and15years.Thus,these highnumbersof uropathogenic*E.coli*were enoughtogenerate leukocyteesteraseandnitritelevelsdetectablebyurinedipstickandforming coloniesgreaterthanthe10^{4}cut-offfordiagnosingUTIusingmicrobialculturemethod.PrevalenceofUTIby *E.coli*indownwardtrendfrompeakatages14and15formalesand femalesrespectively.

The*papC*gene recordedformalesandfemaleswere 55.29%and45.31%respectively andan

overallprevalence of51.01% (25).Thismeansthatabouthalfthenumbersof*E.coli* isolatesemploy fimbriaeformationforadherencetouro-epithelialcellstocauseUTI. Moreover,itcouldbeasaresultofmostofUTIdetectedbeing firsttimeinfections,requiring pathogenadherencetouro-epithelialcells,inordertocolonizetheurinary bladder.Hence, pathogens adaptthroughthe productionofadhesionstructureslike*papC*. The *papC* prevalenceof51.01%isgreaterthanthe21.1%determinedinaBrazilianstudyandthe45%forimmuno-compromisedpopulationsinSlovenia (26).Thesedifferencesmighthavearisen fromboththeSample populationsandthe compositenumberofmalesandfemalesinthesepopulations. Itisalsopossible,strain differencesduetogeographiclocationscanbeacontributoryfactortothedeviationfromthe94%fornon-immuno-compromised in literature (27).However, thedifference indistributionwithrespecttogendercouldbejustifiedbythefactthatisolatesfromcystitis, a conditionthatpredominates infemales, have beenfoundtohavelowprevalence of*papC*, hencethesmallerpercentageof32.95%determinedinfemales (28). Moreover,somestrains of*E.coli*isolates detectedin maleswith UTImaybeinvolved infirst timeinfectionswhichrequireadhesionstructures madepossibleby the*papC*gene, contributing toahigherprevalenceofthegene,comparedtofemales.bothmales andfemales recorded*usp*geneasthe predominantvirulencegene,with63.53% and76.56% respectively,althoughthere wasnosignificantdifferencebetweengenders.However,there wasstatisticaldifferencebetween*usp*and*papC*gene(*p*=0.0020)(29).Theoverallprevalenceof52.82%forthe*usp*genewasclosetothe68.5%determinedforwomenandthe79-93%rangeinmixedpopulationsinJapanesestudies(30).

This indicates theimportanceof the*usp*gene for *E.coli*survival and persistencein the microbialcommunityinvolvedinurinarytractinfection.Indeed,thehigherpercentageof*usp* gene,comparedto*papC*gene observedinisolatesfromfemalescouldbe due tothefactthat infection by differenturopathogenscanbegreatly enhanced by theshorterurinary tractof females.Thisincreasesthediversity ofmicrobialcommunity withintheurinary tract,leading totheacquisitionofthe*usp*geneby *E.coli*toproducebacteriocintoeliminatecompeting uropathogens.Itcanalsobearguedthatsomeofthe UTI recordedmightnothavebeenfirst episodeinfection,butre-infectionsmadepossibleby reservoiruropathogensintheurinary tract.Thus,these*E.coli forming*partofthereservoirmicrobialcommunity musthave adaptedbyresorting totheuseofthe*usp*gene,toeliminateothercompeting microbes,bythe production ofbacitracin.Thetwoparametersofdipstick,i.e.leukocyteesteraseandnitrite,andPCR were analyzedfor theirsensitivity,specificity andreliability indetectingUTIamongschoolchildreninAshanti region,using themicrobialculturemethodasthegoldstandard. Theleukocyte esterasesensitivityof 52% fellout of the67-94%range for sensitivitycomponents of dipstick urinalysis,employedby theWHO(WHO,2005).Thismighthavebeenduetosomeofthe UTIcasesdetectedbeingintheirearlystagesinthe urethra.Asaresult,theseinfections couldnottriggerenoughinfluxof leukocytes,toproduce highconcentrationsof leukocyte esteraseforapositivetestby colourgenerationonthedipstick.Moreover,thelowsensitivity (52%) couldbeattributedtodipstickdefectinthereactiveingredientsfordetecting leukocyte esterase,leading toincreaseinfalsenegativesanddecreasing itssensitivity.valueof52%andnegativepredictiv

evalue of76%for leukocyte esteraseisan indication that comparatively, the parameter better serves as an index for correctly eliminatingapatient as not havingUTIwhen the test resultis negative (30).

Specificityvalueof99%fornitritewhichfellwithin therangeof90-100%(W.H.O,

2005)isastrongindicatoroftheparametercorrectl y testingnegativefornon-UTI samples. However,theextremelyhighvaluemay beduetofalsepositivescausedbyexposureofthe dipsticktoair.Lowsensitivity(3%)fornitritemay havebeenprimarilycausedbylownitrate dietsleadingtocorrespondinglowlevelsofnitrite by nitratereductase-negative microorganismswhichcouldnotshowpositivetes tsforUTI positivesamples.Inaddition,a positivepredictivevalueof1%isanindicationofni tritehavingavery lowlikelihoodofa patienthaving thediseasegiventhatthetestispositive.Ontheothe rhand,the negative predictivevalueof68%isofrelevancetoaphysicia nasitshowsnitritehas ahigher probabilityforan individual not havingUTIwhenthe test is negative.PCRbeingagoodtechniquefordetectin g the lowpresence ofuropathogensinurinesamples.However,the29 %sampleswith*E.coli* undetected byPCR could probablyhave been due to sampling of DNA template from solution (31).Moreover,alowerspecificity of24%couldbelinkedtofalsepositives arisingfromalesssensitiveculturestandard,unabl etodetectsomeuropathogensorthePCR detectingthelowpresenceof*E.coli*withoutsatisfy ingany setquantitativethreshold comparabletothe10^4 CFU/mLcut-offusedintheculturestandardfordiagnosis.This resultantrise infalsepositiveshadaneffectonthepositive predictivevalueof PCR asadiagnostictoolinUTI.

AUTIprevalenceof30.88%obtainedinthisstudy raisesconcern,asitcouldhaveserious immediateorfuturerepercussions.Feversandpain sassociatedwithsymptomsof UTI can disruptthesoundframeofmindneededbyschool-goingadolescentstostudy andthishasthe potentialtoloweracademic performanceof students.Infectedadolescentscouldalsogrow anaemicinadvancedstagesoftheinfection,duetok idney damage.Thisisasaresultof deficiency inproductionofthehormoneerythropoietininthek idneyswhichcontrolsred bloodcellformation.Currently,microbiologicalc ultureremainsthestandard methodforUTI diagnosesand prevalencedetermination.However,establishedd ataindicatethatculture resultscould be contaminatedby pathogensoffaecaloriginandthisisamajor shortcoming ofthemicrobiologicalculturemethod (32).PCRmethodfordiagnosingUTI,onthe otherhandcanbestrain-specific,dependingonthesetofprimersusedfordia gnosis,as shownbythisstudy.Moreover,performancecomp arisonbetweenPCRandmicrobiological cultureshoweditssuperiority asadiagnosticmethod (33).Thus,PCRprovidesamore reliable resultforUTIscreeningwith respect to pathogen strain specificity.

REFERENCES

1. Abraham,J.M.,FreitagC.S.,Gander R.M.,ClementsJ.R.,ThomasV.L.andEi sensteinB.I. (1986) FimbrialphasevariationandDNArearra ngementsinuropathogenic isolatesof *Escherichia coli*. *Mol. Biol. Med.*3, 495-508.

2. Abraham, S.N., GoguenJ.D. andBeachey,E.H.(1988) Hyperadhesivemutantof type 1-fimbriated*Escherichia coli*associated withformation of*FimH*organelles (fimbriosomes). *Infect. Immun.* 56, 1023-1029.

3. Abraham,S.N.,Goguen,J.D.,Sun,D.,Klemm,P. andBeachey,E.H.(1987)Identification of two ancillary subunits of *Escherichia coli* type 1 fimbriae by using antibodies against synthetic oligopeptidesof*fim*geneproducts.*J. Bacteriol.*169, 5530-5536.

4. Ahmed,Z.andLee,J.(1997)Asymptomaticurinary abnormalities.Hematuriaand proteinuria. *Med ClinNorth Am.* 81, 641-652.

5. Aiyegoro,O.A., Igbinosa,O.O.,Ogunmwonyi,I.N.,Odjadjare,E.E.,Igbinosa,O.E.and Okoh,A.I.(2007)Incidenceofurinary tractinfections(UTI)amongchildrenandadolescents inIle-Ife, Nigeria.*Afri. JMicrobiol. Res.* 1, 13-19.

6. Baga,M.,Normark,S.,Hardy,J.,O'Hanley,P.,Lark,D., Olsson,O.,Schoolnik,G. and Falkow, S.(1984)Nucleotide sequence of the*papA*geneencodingthe*pap*pilussubunitof human uropathogenic*Escherichia coli. J. Bacteriol.*157, 330-333.

7. Bagg,A.andNeilands,J.B.(1987a)Ferri cuptake regulationprotein actsasa repressor, employing iron(II)asacofactortobindtheoperatorofaniron transportoperonin *Escherichia coli*. *Biochemistry.* 26, 5471-5477.

8. Bagg,A. andNeilands,J.B. (1987b) Molecularmechanismofregulation of siderophore- mediated iron assimilation.*Microbiol. Rev.* 51, 509-518.