PHYLOGENETIC GROUP OF ESCHERICHIA COLI IN URINE OF CHILDREN ATTENDING A MATERNAL AND CHILD CENTRE, LAGOS

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ABSTRACT

Urinary tract infection (UTI) is one of the most common bacterial infections causing significant morbidity in children. This study determines the phylogenetic group of *Escherichia coli* isolated from the urine of children attending Maternal and Child Centre, Amuwo-Odofin, Lagos State.
Clean voided midstream urine sample were collected from 215 children aged five years and below into sterile universal bottles. The urine samples were culture on Mac Conkey and blood agar, bacterial counts greater than or equal to 1 x 10^5CFU/ml were regarded as positive for UTI. Identification of isolates and antibiotic susceptibility test was performed using standard methods. Polymerase Chain Reaction was used to classify the isolated *E. coli* into A, B1, B2 and D phylogenetic groups using presence or absence of ChuA, Yja A and TspE4C2. The prevalence of UTI was 51.2% with preponderance in male 62.7% aged 24 – 35months 75.0%. *Staphylococcus aureus* 19.1% followed by *Pseudomonas aeruginosa* 12.7% and *Escherichia coli*10.0% were common bacterial isolates. The isolates were highly resistant to Augmentin (71.8%) and Ampicillin 91.8%; the *Escherichia coli* were resistant to Augmentin 54.5% and Ampicillin 100%. The *E. coli* were classed into B1,A, and D phylogenetic groups with percentages of 54.5% 27.3% and 18.2% respectively.
The prevalence of bacteria UTI among children in this study was very high, the isolates were highly resistant to the antibiotics tested, the *E.coli* belong to phylogenetic groups A, B1 and D and all were resistant to Ampicillin.

Keywords: Urinary Tract Infection (UTI), Molecular characterization, *Escherichia coli*, Children

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INTRODUCTION

The urine is normally sterile but the presence of inflammatory cells, fungi or bacteria is indicative of a urinary tract infection (UTI). Various studies have reported different incidences and prevalence of UTI in relation to age, gender, community or hospital setting, geographical distribution (Bitsori *et al.,* 2005; Swerkersson *et al.,* 2014; Sorlózano-Puerto *et al.,* 2017).The infection accounts for 1- 3% of hospital consultations (Shaw *et al.,* 1998; Foxman *et al.,* 2002; Marcus *et al.,* 2005; Ghauor & Salehzadeh, 2017). UTI is mostly cause by fungi, Gram positive and Gram negative bacteria (Ronald, 2003). *Escherichia coli* is the most predominant Gram negative bacterial associated with 80 -90% of community and hospital acquired UTI (Shaw *et al.,* 1998;Ronald, 2003; Iranpour *et al.,* 2015).

E. coli is responsible for the majority of acute community-acquired and uncomplicated urinary tract infections (Ronald, 2003). *Escherichia coli* aside causing UTI have been reported to be the aetiology of other infection ranging from intestinal to extra-intestinal gastrointestinal such as wound, bacteremia (Akhtardanesh *et al.,* 2016; Ghauor and Salehzadeh, 2017). *E. coli* strains have been grouped into distinct phylogenetic groups based on ecological habitat, characteristics and pathogenicity (Clermont et al. 2000; Gordon *et al.* 2008; Mustak *et al.,* 2015). Four phylogenetic groups A, B1, B2, or D were initially recognized with the use of triplex PCR method which detects the presence/absence of chuA, yjaA, and TspE4.C2 genes in *E. coli* strain (Iranpour *et al.,* 2015). The introduction of a new marker arpA in addition to the previous three in a quadruplet PCR increased the

phylogenetic groups of E. coli strain from four to eight by (Clermont *et al.* 2013; Iranpour *et al.,* 2015; Mustak *et al.,* 2015).The continuous increase of bacteria resistance to multiple antibiotics of unrelated families has consistently been considered a serious health concern (Domínguez *et al.,* 2002; Sa´enz *et al.,* 2004). The increase in multi-resistant of bacteria to antibiotics has been attributed to the transference of resistance determinants such as plasmids, transposons, and gene cassettes in integrons by mobile genetic elements (Hall & Collis, 1998; Guardabassi *et al.,* 2000). The increases multi-resistant of bacteria have contributed greatly to limited therapeutic options for treatment of urinary tract infections. Although, Adenipekun *et al.* (2016) confirmed the presence of multidrug resistance *E. coli* belonging to phylogenetic groups A, B1, B2, C, and E circulating in the community acquired infections in Lagos, Nigeria, there is paucity of information on the phylogenetic groups of E. coli from urine, This study therefore determines the bacteria, antibiotic resistance and phylogenetic group of *Escherichia coli* isolated from urine of children attending Maternal and Child Centre, Amuwo-Odofin, Lagos State.

Materials and methods

Study area and population

This study was carried out at the Amuwo-Odofin Maternal and Child Centre (AOMCC), Festac-town, Ojo Local Government Area, Lagos State, Nigeria. AOMCC is an advanced Secondary Health centre with an attendance average of between 150 and 200 children daily (Medical Record Department, AOMCC, 2018). Children less than five years of age who presented at AOMCC with ill health and whose parents/guardians gave their consent to participate were recruited for this study.

Sample collection and processing

Well-labeled sterile screw cap universal bottles were given to consented parents/ guardians and were educated on how to collect midstream urine aseptically. The samples were transported to the laboratory in ice packs and processed within 4 hours of collection. The urine samples were inoculated on MacConkey and Blood agar (Oxoid) using calibrated inoculating wire loop to deliver 0.001mL. All bacteria colonies that appeared after 18 - 24 hours incubation at 37°C for were counted and bacterial counts $\leq 10^5$ cfu/mL considered as significant. The bacteria isolates were identified using colonial morphology, Gram reaction and biochemical with Microbact test kits (MICROBACT GNB 12A/B/E, 24E-Oxoid).

Antimicrobial susceptibility testing

The identified isolates were sub-cultured on nutrient agar to obtain 18 – 24 hours old culture, 3 - 5identical colonies were inoculated into sterile normal saline, the turbidity was adjusted to match 0.5 McFarland Standard and was inoculated on well dried sterile Mueller-Hinton Agar Biotec, UK) plate. Antibiotic discs were applied on the inoculated plate and incubation was done at 35°C for 18 – 24 hours in the incubator (Gulfex Medical and Scientific, England). The diameter of the zone surrounding each antimicrobial agent was measured in millimeter (mm) using a transparent ruler. The results were interpreted as susceptible, intermediate or resistant according to Clinical Laboratory Standards Institute guidelines (CLSI, 2013).

DNA extraction, molecular identification and phylogenetic group of *E. coli*

DNA extraction

Chromosomal DNA of the identified *E. coli* was extracted using the crude boiling method. One milliliter of the overnight bacterial culture in Tryptose Soy Broth was centrifuged at 8,000 rpm for 2minutes; the sediment was washed with nuclease-free water, homogenized, and heated at 95 °C for 15 minutes. The tube was placed on ice pack for 15minutes, centrifuged at 14,000rpm for 5 minutes and the supernatant which contain the DNA was transferred into another tube and stored at -20°C (Ribeiro Junior *et al.*, 2016).

Identification of *Escherichia coli*

The 16SrRNA gene specific to *E. coli* was amplified to confirm the isolates as *E. coli* using *E. coli*-specific PCR primers Tecol553 (5-TGGGAGCGAAAATCCTG-3) and TEcol754 (5-CAGTACAGGTAGACTTCTG-3) with 258 base pairs described by Maheux *et al.* (2009). The PCR was performed in 20uL volume reaction mixture of 5X PCR Master mix (Solis Biodyne) containing 1X PCR Buffer (0.4 M Tris-HCl, 0.1 M $(NH4)_2SO_4$, 0.1% w/v Tween-20), 1.5mM Magnesium Chloride, 200uM of each dNTP, 2U Taq DNA polymerase, 20pMol of each primer (Biomers, Germany), and 2ul of DNA. The PCR amplification was carried out in an Eppendorf Master cycler (Nexus series) with an initial denaturation at 95°C for 3 minutes, followed by 30 consecutive cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step of 72°C for 10 minutes was then done. The PCR products and 100bp DNA Ladder molecular weight marker were separated by electrophoresis in 1.5% agarose gel at 80V for 1 hour 30 minutes, stained with Ethidium Bromide and visualizes under UV light using Cleaver Photo documentation system.

Detection of phylogenetic group of *E. coli* isolated from urine

The phylogenetic group of the identified *E. coli* was determined by multiplex PCR using the primers (*ChuA*, *YjaA*, *TspE4.C2*)as described by Clermont, *et al.* (2000). The multiplex PCR was performed in a 20µL reaction mixture containing 1X Blend Master mix buffer Buffer (2.0 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne)), 20 pMol of each primer (Biomers, Germany), 2units of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 2µL of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Eppendorf Vapo Protect Thermal Cycler (Nexus Series) for an initial denaturation of 95°C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 30 seconds at 58°C and 1 minute at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 2% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by Ethidium Bromide staining. 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker. The strains were classified into phylogenetic groups using presence or absence of ChuA, YjaA, and TSPE4.C2 genes according to Clermont *et al.* (2000), Carlos *et al*. (2010) and Iranpour *et al*. (2015).

ETHICAL CONSIDERATION

Approval for the study was obtained from Olabisi Onabanjo University Teaching Hospital Health Research Ethics Committee (OOUTH-HREC) with Approval No. OOUTH/HREC/223/2018. The consent of the parents/guardians were sought and obtained. The participants were assured of the safety and benefit of the research to the children and the right to opt out of the study.

RESULTS

A total of 215 children 5 years and below were recruited for this study, of these 110 (51.2%) have positive urine culture, majority were males 69 (62.7%) within the age group 24 - 35 months 15 (75.0) (Table 1). The Bacteria isolated from the urine includes *Pseudomonas aeruginosa* 14(12.7%), *Escherichia coli* 11 (10.0%), *Klebsiella oxytoca* 9 (8.2%), *Klebsiella pneumonia* 8 (7.3%)*and Staphylococcus aureus* 21 (19.1%) (Table 2). The bacteria were resistant to Ceftazidine 54(49.1%), Ceftriazone 68(61.8%), Gentamycin 64(58.2%), Ciprofloxacin 54(49.1%), Ofloxacin 22(20.0%), Nitrofurantoin 42 (38.2%), Augmentin 79(71.8%) and Ampicillin 101(91.8%) (Table 3). It is noteworthy that almost all isolated bacteria were resistant to Ampicillin; the *Escherichia coli* were resistant to Augmentin 6 (54.5%) and Ampicillin 11 (100%). All the isolate identified biochemically as *Escherichia coli* were confirmed molecularly as *E. coli* (Figure 1). Figure2 shows the amplification products of the three genes and the identified *Escherichia coli*. Isolates 2, 3, 4, 5, 6, 9 and 10 amplifies at 152 base pair (bp) indicating the presence of the TspE4.C2 gene, while isolates 6 and 7amplifies at 279 bp indicating the presence of the ChuA gene, isolates 6 carried both TspE4.C2 and ChuA genes. None of the isolate carried the YjaA gene which amplifies at 211 bp. Majority of the *E. coli* 6 (54.5%) belongs to phylogenetic group B_1, 3 (27.3%) belongs to phylogenetic group A and 2(18.2%) belongs to group D (Table 4).

Table 1: Age and Gender Distribution of Urine Tract Infectionamong Children attending Maternal and Child Centre, Lagos

Age (Month)	Children	Positive Urine culture		
		Male	Female	Total
	N (%)	n (%)	n (%)	n (%)
< 12	3 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)
12 – 23	95 (44.2)	42 (60.0)	28 (40.0)	70 (60.9)
24 – 35	45 (20.9)	15 (75.0)	5 (25.0)	20 (17.4)
36 – 47	29 (13.5)	3 (50.0)	3 (50.0)	6 (20.7)
48 -60	43 (20.0)	9 (64.3)	5 (35.7)	14 (32.6)
Total	215 (100)	69 (62.7)	41 (37.3)	110 (51.2)

Table 2: Distribution of Bacteria Isolated from Urine

Bacterial Species	Frequency	
	N	(%)
Escherichia coli	11	10.0
Escherichia coli inactive	2	1.8
Salmonella subtype 3B	4	3.6
Citrobacter sedlakii	5	4.5
Citrobacter amalonaticus	1	0.9
Citrobacter diversus	2	1.8
Klebsiella ornithinolytica	1	0.9
Morganella morganii biogroup 1	2	1.8
Citrobacter freundii	1	0.9
Pseudomonas flourescens 25	1	0.9
Burkholderia pseudomallei	7	6.4
Aeromonas hydrphylia	2	1.8
Enterobacter hormaechei	2	1.8
Providencia rettgeri	2	1.8
Proteus vulgaris	2	1.8
Hafnia alvei	2	1.8
Serratia liquefaciens complex	2	1.8
Seratia odorifeciens biogroup 2	2	1.8
Enterobacter aglomorans complex	1	0.9
Klebsiella oxytoca	9	8.2
Klebsiella pneumonia	8	7.3
Klebsiella orazai	6	5.5
Pseudomonas aeruginosa	14	12.7
Staphylococcus aureus	21	19.1

Table 3: Antibiotics resistance of Bacteria Isolated from the Urine of Children with UTI

Antibiotics(µg) Isolates	N	CRO n (%)	CAZ n (%)	GEN n (%)	CIP n (%)	OFL n (%)	AUG n (%)	NIT n (%)	AMP n (%)
E.coli	11	5(45.5)	4(36.4)	3(27.3)	0(0.0)	0(0.0)	6(54.5)	1(9.1)	11(100.0)
E.coli inactive	2	0(0.0)	2(100.0)	0(0.0)	0(0.0)	1(50.0)	2(100.0)	1(0.0)	2(100.0)
Salmonella sub 3B	4	3(75.0)	4(100.0)	0(0.0)	0(0.0)	1(25.0)	2(50.0)	0(0.0)	4(100.0)
C.sedlakii	5	4(80.0)	4(80.0)	4(80.0)	1(20.0)	2(40.0)	5(100.0)	0(0.0)	4(80.0)
C.amalonaticus	1	1(100.0)	1(100.0)	1(100.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(100.0)
C.diversus	2	2(100.0)	1(50.0)	1(50.0)	0(0.0)	0(0.0)	2(100.0)	2(100.0)	2(100.0)
K.ornitholytica	1	1(100.0)	0(0.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)
M. morganiibiogp 1	2	2(100.0)	1(50.0)	1(50.0)	1(50.0)	1(50.0)	2(100.0)	2(100.0)	2(100.0)
C.freundii	1	1(100.0)	0(0.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)
Ps.flourescene 25	1	1(100.0)	1(100.0)	1(100.0)	0(0.0)	0(0.0)	1(100.0)	0(0.0)	1(100.0)
B. pseudomallei	7	7(100.0)	1(14.3)	1(14.3)	3(42.9)	3(42.9)	6(85.7)	1(14.3)	1(100.0)
A.hydrphylia	2	2(100.0)	1(50.0)	2(50.0)	0(0.0)	0(0.0)	2(100.0)	0(0.0)	2(100.0)
E. hormaechei	2	2(100.0)	2(100.0)	2(100.0)	0(0.0)	0(0.0)	2(100.0)	2(100.0)	2(100.0)
P.rettgeri	2	2(100.0)	1(50.0)	2(100.0)	1(50.0)	1(50.0)	1(50.0)	1(50.0)	2(100.0)
P.vulgaris	2	2(100.0)	2(100.0)	2(100.0)	1(50.0)	0(0.0)	1(50.0)	0(0.0)	2(100.0)
H.alvei	2	2(100.0)	1(50.0)	1(50.0)	0(0.0)	1(50.0)	1(50.0)	0(0.0)	2(100.0)
S.liquefaciens complex	2	2(100.0)	1(50.0)	2(100.0)	0(0.0)	0(0.0)	2(100.0)	0(0.0)	2(100.0)
S.odorifeciens biogp 2	2	2(100.0)	2(100.0)	0(0.0)	0(0.0)	0(0.0)	1(50.0)	1(50.0)	2(100.0)
E.aglomorans complex	1	1(100.0)	1(100.0)	1(100.0)	0(0.0)	0(0.0)	1(100.0)	1(100.0)	1(100.0)
K.oxytoca	9	7(77.8)	2(22.2)	5(55.6)	2(22.2)	0(0.0)	9(100.0)	1(11.1)	9(100.0)
K.pneumonia	8	3(33.3)	3 (33.3)	7(87.5)	0(0.0)	0(0.0)	7(87.5)	2(25.0)	8(100.0)
K.orazai	6	3(50.0)	2(33.3)	2(33.3)	2	2(33.3)	3(50.0)	3(50.0)	6(100.0)
P.aeruginosae	14	8(57.1)	8(57.1)	11(78.6)	5(35.7)	8(57.1)	12(85.7)	9(64.3)	14(100.0)
Staphylococcus aureus	21	5(23.8)	6(28.6)	16(76.2)	0(0.0)	0(0.0)	9(42.9)	13(61.9)	13(61.9)
TOTAL	110	68(61.8)	54(49.1)	64(58.2)	54(49.1)	22(20.0)	79(71.8)	42(38.2)	101(91.8)

CRO30µg- Ceftazidime,CAZ20µg-Ceftriazone,GEN10µg -Gentamycin,CIP10µg -Ciprofloxacin, OFL 10µg -Oflozaxin,AUG30µg -Augmentin,NIT 300µg -Nitrofurantoin,AMP30µg - Ampicilin.

Table 4: Phylogenetic group of *E. coli* isolated from urine

GENE	*E. coli* isolates number										
	1	2	3	4	5	6	7	8	9	10	11
ChuA	-	-	-	-	-	+	+	-	-	-	-
YjaA	-	-	-	-	-	-	-	-	-	-	-
TspE4	-	+	+	+	+	+	-	-	+	+	-
Group	A	B_1	B_1	B_1	B_1	D_2	D_1	A	B_1	B_1	A

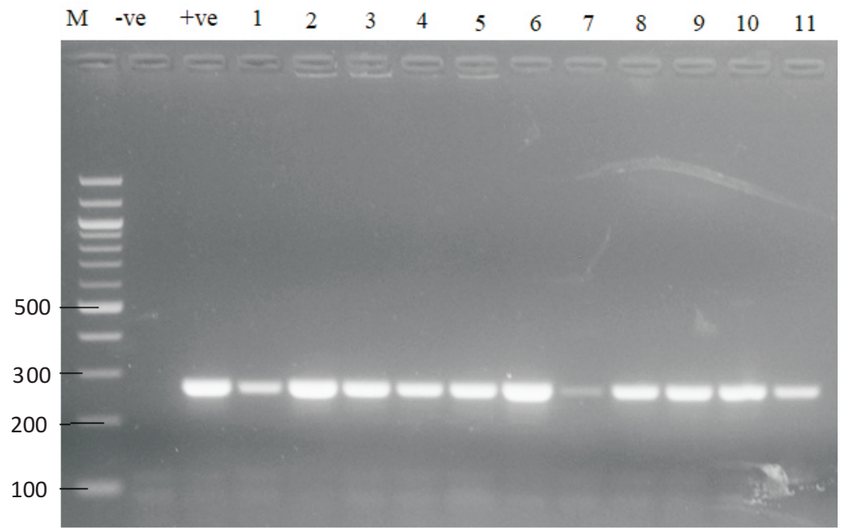

Figure 1: Gel electrophoresis showing amplification products for confirmation of *Escherichia coli* using the TEcol primer. M =Molecular weight marker, -ve = negative control, +ve = positive control(*E.coli*), lanes 1 – 11= *E.coli* isolates (258 base pairs) from urine

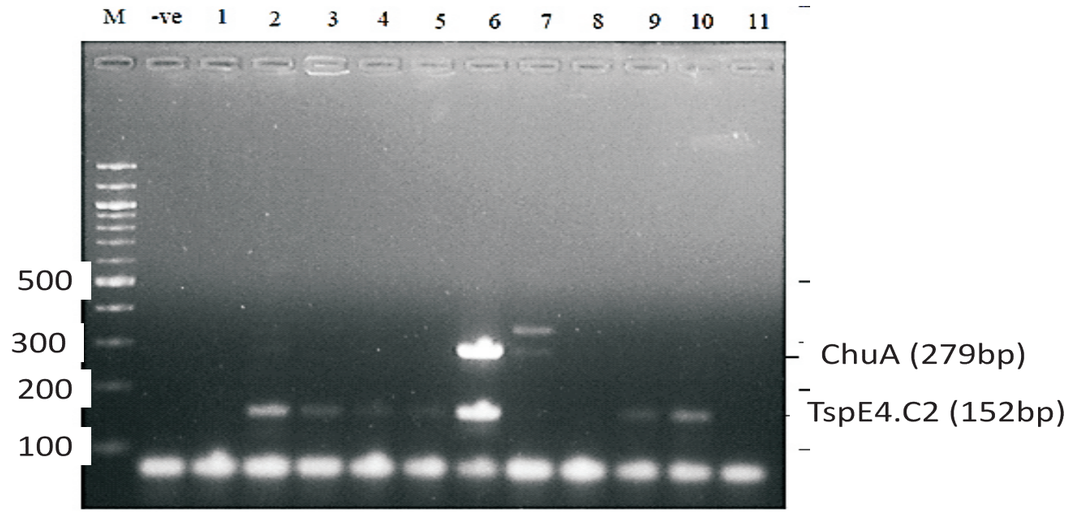

Figure 2: Gel electrophoresis amplification of *Escherichia coli* ChuA (279bp) and TspE4.C2 (152bp) in **triplex PCR.**
M = marker, -ve negative control, lanes 1-11 =*Escherichia coli*

Discussion

The prevalence of positive urine culture in children under the age of 5 years in this study population is 51.2%, this is far greater than the 11.0%, 13.7%,15.0% in febrile children less than 5 years,1 - 60 months and <2 years of age reported by Ibeneme *et al*. (2014); Rabasa & Gofama (2009) and Nair & Rai (2018) respectively. The differences in reported prevalence of UTI in different studies have been attributed to various factors such as sex, age, nutritional status, study population and size (Asinobi *et al*. 2003; Rabasa & Gofama, 2009). The recruitment of children into this study irrespective of the presenting complaints at the Center could probably be responsible for the observed high prevalence. *Staphylococcus aureus* 21 (19.1%), *Pseudomonas aeruginosa* 14 (12.7%) and *Escherichia coli* 11 (10.0%) were the most common bacterial pathogens isolated to be the cause of UTI in children in this study. These bacteria have been associated with both complicated and uncomplicated UTI (Ronald, 2003). Urinary pathogens have been known to include strains that are resistant to commonly used antibiotics (Orrett, 2001). High resistance to Ampicillin observed in the present study is a source of concern. All the *Escherichia coli* are resistant to Ampicillin, a common antibiotic that can easily be obtain over the counter and often used without prescription, these have resulted in selective pressure with resultant resistance of bacteria to the drug (Seiji *et al*., 2005). *Escherichia coli* apart from being classified into strains based on their virulence factors are also classified into A, B1, B2 or D phylogenetic groups using the presence or absence of chuA, yjaA genes and TspE4C2 anonymous DNA fragment (Akhtardanesh *et al*., 2016). Similarly, they are classified into intestinal pathogen, extra-intestinal pathogen and commensal groups based on genetic and clinical manifestations (Akhtardanesh *et al*., 2016).The observation that the *E. coli* in this study belongs to phylogenetic group A, B1and D is in agreement with Asadi *et al*. (2010); no B2 were identified. Contrary to the report of Clermont *et al*. (2000) and Ghauor & Salehzadeh (2017) that most extra-intestinal pathogenic strains were in group B2 and D, majority of the *E. coli* in this study are in phylogenetic group B_1. Most of the commensal strains belong to group A and B_1whereas groups D are the pathogenic extra-intestinal strains (Escobar Paramo *et al*. 2004; Girardeau *et al*. 2005). Although this study did not relate the phylogenetic group of the *E. coli* to antibiotic resistance, Ghauor & Salehzadeh (2017) reported that uropathogenic *E. coli* strains from phylogenetic group D were significantly resistant to the majority of antibiotics compared to other phylogenetic groups. The resistance of all the *E. coli* isolated in this study to Ampicillin (100%) is a pointer to their relatedness to the phylogenetic groups A, B1 and D

Conclusion

The prevalence of bacteria UTI among children in this study was 53.5%, *Staphylococcus aureus* was the most common bacteria in urine 19.1%,followed by *Pseudomonas aeruginosa* 12.7% and *Escherichia coli* 10.0%. The isolates were highly resistant to majority of the antibiotics tested; all the *E. coli* isolated were resistant to Ampicillin 100% and belong to phylogenetic A, B1 and D

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