ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICINE
DEPARTMENT OF MICROBIOLOGY IMMUNOLOGY AND PARASITOLOGY

PHENOTYPIC DETECTION OF EXTENDED SPECTRUM BETA LACTAMASE IN UROPATHOGENS AMONG PREGNANT WOMEN ATTENDING ANTENATAL CARE IN ALERT HOSPITAL, ADDIS ABABA, ETHIOPIA.

By: Molla Getie (BSc)

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THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES, ADDIS ABABA UNIVERSITY, SCHOOL OF MEDICINE, DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY IN PARTIAL FULFILLMENTS OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL MICROBIOLOGY

MARCH, 2019
ADDIS ABABA, ETHIOPIA

ACKNOWLEDGEMENTS

It is a pleasure to thank a lot of people who assisted me in one way or another. I would like to express my thanks to the following next to God for the material support, knowledge, guidance and encouragement throughout my study.

First I am thank full to my advisors Dr. Solomon Gebre–Selassie from Department of Microbiology, Immunology and Parasitology, Addis Ababa University (AAU), for his unreserved advice and continuous comments I received throughout my research work.

I am immensely grateful to my Co-advisor Yemserach Getu (Microbiologist in All Africa Leprosy and Tuberculosis Eradication, Rehabilitation and Training (ALERT) hospital), for her guidance, continuous support of all laboratory work and encouragement from preparation of the research proposal to the write up of the thesis paper.

I would like to thank all the study participants for their willingness during sample collection. Accomplishment of this thesis would have been hardly realized without their cooperation.

I would also like to acknowledge all College of medical and health sciences staff of Samara University for their moral support and my special gratitude goes to Ato Abay Woday (Lecturer in Samara University, Department of public health) for reviewing and providing necessary comments on earlier drafts of this thesis.

I express my sincere gratitude to the staff members of laboratory, ALERT hospital especially Bacteriology unit for providing me all the necessary laboratory reagents, media and other laboratory facilities together with the practical support while I was conducting this study. Many thanks to all the staff of the department of laboratory for being the family unit that is supportive, encouraging and helpful.

I would also like to acknowledge the staff member of Anti Natal Care (ANC) unit, particularly the Gynecologists, the Nurses and Midwifes for their continuous collaboration in collecting urine samples.

Finally, my deepest gratitude go to my family who sacrificed themselves to me. My special thanks goes to my elder brother Zegeye Getie who encourage me to be better and successful.

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AAU	Addis Ababa University
ABU	Asymptomatic Bacteriuria
AHRI	Amauer Hansen Research Institute
ALERT	All Africa Leprosy and Tuberculosis Eradication, Rehabilitation and Training
ANC	Anti Natal Care
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CLED	Cysteine Lactose Electrolyte Deficient
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase Negative Staphylococcus
DDCT	Double disc combination test
DDST	Double disc synergy test
DERC	Department Research and Ethics Committee
DM	Diabetes Mellitus
DNA	Deoxyribonucles
ESBL	Extended spectrum beta lactamase
ESBL-E	Extended spectrum beta lactamases- *Enterobacteriaceae*
EUCAST	European Committee of Antimicrobial Susceptibility Testing
H_2O_2	Hydrogen peroxide
ICU	Intensive care unit
ID	Identification
KPC	Klebsiella pneumoniae carbapenemase
LIA	Lysine Iron Agar
MDR	Multi Drug Resistant

MGEs	Mobile Genetic Elements
MSU	Mid-stream urine
NIHCE	National Institute for Health and Clinical Excellence
PBP	Penicillin-Binding Protein
SB	Significant Bacteriuria
SOP	Standard Operating Procedures
SPSS	Statistical Package for Social Science
TEM	Temoneira
TSI	Triple Sugar Iron
USA	United state of America
UTI	Urinary Tract Infection
WHO	World Health Organization

ABSTRACT

Background: Nowadays magnitude of Extended Spectrum β- Lactamases (ESBL) producing bacteria which cause urinary tract infection is the most worrying issue in the world. The occurrence of ESBL producers especially in pregnant women can result in life threatening condition and morbidity for both the mother and the newborn due to unavailability of diagnostic method and very limited drug options for treatment of these pathogens in our country. Therefore, evidence regarding the magnitude of ESBL producers among pregnant women is demanding.

Objectives: To determine Extended Spectrum Beta Lactamase in uropathogens and antimicrobial susceptibility pattern of Gram negative bacilli isolates among pregnant women attending antenatal care in ALERT hospital, Addis Ababa, Ethiopia.

Methods: A cross sectional study was conducted from July to September 2018 on a total of 177 pregnant women with and without symptoms of urinary tract infection at ALERT hospital, Addis Ababa, Ethiopia. All urine samples were inoculated onto Cysteine Lactose Electrolyte Deficient medium (CLED) and MacCkonkey agar. Colonies were counted to check the presence of significant bacteriuria. Pure isolates of bacterial pathogen were characterized and identified at species level by colony morphology, gram-stain and standard biochemical procedures. All Gram negative isolates were put into Muller-Hinton agar plates for antibiotic susceptibility test by Kirby-Bauer disc diffusion technique. ESBL was detected using double disk synergy methods on Muller Hinton agar. The data were double entered into EPI INFO and analyzed using Statistical Package for Social Science (SPSS) version 26.

Results: The overall prevalence of UTI among pregnant women was 14.7% (n=26/177). *K. pneumoniae* was the predominant bacterial etiologic agent of UTI (26.9% (n=7/26)). The prevalence of ESBL among gram negative isolates was 50% (n=6/12). Among ESBL producing isolates all (100%) were resistance to Amikacin, Gentamicin and Ceftriaxone while intermediate level resistance rate of 66.7% was observed among Trimethoprim-sulphamethoxazole and Cefixime. They were susceptible for some limited drugs and these were Nitrofurantoin (83.3%) and Chloramphenicol (83.3%).

Conclusions:- Majority of ESBL producing isolates exhibited co-resistance to other commonly prescribed antibiotics. This indicates that the option of treatment for these pathogens rapidly decreased from time to time which results serous life threating conditions especially in mother and newborn unless the appropriate measure is taken.

Key words:- Extended spectrum beta lactamases, Antibiotic resistance, Uropathogen, Pregnancy, Urinary tract infection, Addis Ababa, Ethiopia

1. INTRODUCTION

1.1 Back ground

The second most common infectious disease in the community is Urinary tract infection which can affect all age group across the life span (Foxman *et al*., 2000). As many as 35% of nosocomial infections is due to Urinary Tract Infection (UTI), thus it is the common nosocomial infection and it is the second most common cause of bacteremia in hospitalized patients (Kolawole *et al*., 2009). The infection is usually due to bacteria that transfer from the digestive tract to the opening of the urethra and start to multiply to cause infection (Okonko *et al*., 2009). Women are more susceptible to UTI than men are, and the main reasons for this are pregnancy, short urethra, absence of prostatic secretion and easy contamination of the urinary tract with normal flora in the feces (Haider *et al*., 2010). It was reported that up to 15% of women would have one episode of UTI at some time during their life (Janine *et al*., 2014).

The factors that contribute for high occurrence of UTI among pregnant women include ureteral dilatation, increased bladder volume and decreased bladder tone, along with decreased ureteral tone which contributes to ureterovesical reflux and increased urinary stasis. In addition, pregnant women develop glycosuria, which encourage bacterial growth in the urine (Haider *et al*., 2010).

Asymptomatic and symptomatic clinical presentation of UTI has adverse effect on pregnant women and their fetuses; in addition, recurrence of the disease even after successful treatment, complicates the management. Unlike in non-pregnant women, in pregnant women asymptomatic bacteriuria is the most common complications, occurring in 4-7% of normal pregnancies. It can result in a variety of negative obstetric outcome and medical conditions such as preterm labor, the development of acute and chronic pyelonephritis, preeclampsia, low birth weight, chronic renal disease and prenatal mortality (McCormick *et al*., 2008).

Early identification and treatment can prevent many complications of bacteriuria in pregnancy. The most frequently prescribed antibiotics are β- Lactam agents such as Cephalosporin, Penicillin, Monobactams and Carbapenems (Pitout *et al*., 2005). Cephalosporins are the most commonly prescribed class of antimicrobials due to broad spectrum activity, favorable safety profile and proven efficacy (Laudano, 2011). Beta-lactamases are the most important enzyme that contribute to β-lactam resistance in Gram-negative pathogens. β- Lactamases are bacterial

enzymes that inactivate β lactam antibiotics by hydrolysis, that result in ineffective compounds (Pitout *et al*., 2005).

Extended-Spectrum β-Lactamases (ESBLs) are a rapidly emerging groups of β lactamases which have the ability to hydrolyze third-generation Cephalosporins and Aztreonam, yet are inhibited by Clavulanic acid (Paterson and Bonomo, 2005). ESBL-producing bacteria are associated with severe infections such as, urinary tract infections, bacteremias, intra-abdominal infection and respiratory tract infections (Dhillon and Clark, 2012).

Due to the spread of strains producing Extended-Spectrum β-Lactamases (ESBLs), resistance in Gram-negative bacteria is increasing. Many of the isolates producing these enzymes are also resistant to Quinolones, Trimethoprim and Aminoglycosides, often plasmid has co expression of other resistance mechanisms. Choice in the treatment of ESBL-producing bacterial infections are extremely rare; Carbapenems are the treatment of choice for serious infections due to such organisms (Pallett and Hand, 2010).

1.2 Statement of the problem

Nowadays the most worrying issue in the worldwide is, increase in prevalence of ESBL producers among different bacterial strains and species especially in hospitals and health care settings. Gram negative *Enterobacteriaceae* expressing ESBL are among the most multi-drug resistant pathogens in hospital and spreading worldwide. Infections caused by ESBL-producing organisms have resulted in poor outcomes, reduced rates of microbial and clinical response, greater hospital expenses and longer hospital stays (Dhillon and Clark, 2012).

Increased Extended spectrum beta lactamases- *Enterobacteriaceae* (ESBL-E) associated UTI during pregnancy have negative impact on pregnant women and neonates, including low birth weight, intrauterine growth restriction, fetal death, premature rupture of the membranes and neonatal infections (Loh and Sivalingam, 2007).

Nowadays there is study that indicates transmission of ESBL from pregnant mother to the fetus (Chan *et al*., 2013). Study done by Chan and his partners showed that cross-transmission of maternal pathogens results in neonatal colonization or infection. This is due to close relationship of mother and infant. Several studies have confirmed the role of maternal colonization in the subsequent development of neonatal sepsis (particularly for group B Streptococcus, and also for ESBL-E) (Chan *et al*., 2013).

An overall prevalence of colonization or infection with ESBL-E in pregnant and post-partum women in Africa determined by Meta-analysis of eligible published studies indicates prevalence of 17% (Bulabula *et al*., 2017). The ESBL-E rate reported from this Meta-analysis is substantially higher than the rates found in high and middle income countries, e.g, Norway (2.9%) and Argentina (5.4%) (Pitout *et al*., 2005). Increased ESBL-E carriage among African populations (both in community and hospital settings) is due to poverty, suboptimal hygiene, contamination of drinking water (faces), antibiotics, water sewage, communal toilets, easy access to antibiotics among pregnant women in Africa, and also as a result of increased use of antibiotics in livestock in Africa. Lack of trained health care workers and weak laboratory and infection control capacity contribute to healthcare associated transmission of ESBL-E to pregnant women (Bulabula *et al*., 2017).

According to the meta-analysis conducted by Bulabula and his partners, the proportion of ESBL-E among pregnant women was higher than post-partum women (22% vs 7%). The difference is due to high prevalence of UTIs with ESBL-E during pregnancy than in the post-partum period. It

also represent differences in asymptomatic bacteriuria during pregnancy, differences in antibiotic prescribing practices (Bulabula *et al*., 2017).

It is possible to prevent up to 80% cases of pyelonephritis by early Detection and treatment of bacteriuria (Schnarr and Smaill, 2008). Asymptomatic bacteriuria may not require treatment. But pregnant women and their unborn fetuses have to be treated due to the possibility of risk of complications (Bloomberg *et al*., 2005).

In Ethiopia, urine for culture and sensitivity is not requisited routinely for pregnant women suffering from UTIs. Usually antibiotics are prescribed without identifying the causative agent and evaluating its drug- resistance patterns. This inappropriate prescribing practice finally result in failer of treatment and complication of pregnancy. It has been estimated that globally symptomatic UTIs result in as many as seven million visits to outpatient clinics, one million visits to emergency departments, and 100,000 hospitalizations annually (Kripke, 2005).

Therefore, it is necessary to determine the prevalence of Uropathogens among asymptomatic and symptomatic pregnant women and drug susceptibility pattern to selected antimicrobial Agents in isolates of Gram negative bacilli.

Studies are needed to determine ESBL-E colonization and infection rates among pregnant women and their determinants in Ethiopia. Interventions have to be carried out to reduce ESBL-E colonization and carriage and it should focus on preventing both community and nosocomial ESBL-E acquisition. Some of the interventions should include clean water supplies and the provision of safe sanitation, education of mothers on personal hygiene, restricted use of antibiotics in pregnancy and strengthening of infection prevention efforts in health care facilities (appropriate disinfection of obstetric equipment and the environment).

So far many studies have been conducted concerning bacteriological profile and drug susceptibility of UTI among pregnant women in our country (Assefa *et al*., 2008; Alemu *et al*., 2012; Getachew *et al*., 2012; Tadesse *et al*., 2018). However, to the best of our knowledge there is no documented study concerning magnitude of ESBL producing Gram negative uropathogens among pregnant women especially in our study site. Laboratory detection of ESBL producing Gram-negative bacteria will help pregnant women to get effective treatment and reduce the subsequent complications of UTIs. Thus the aim of this study was To determine ESBL in uropathogens among pregnant women attending antenatal care in ALERT hospital.

1.3 Significance of the study

This study delivers present information on the prevalence of bacteria that cause UTI among pregnant women. In addition, our investigation shows the present pattern of antimicrobial resistance on Gram negative uropathogens. This will enhance physicians to administer and prescribe drugs appropriately and based on local antimicrobial susceptibility pattern. Besides this, it will help decision makers and health officials to formulate and amend policies.

This study shows the magnitude of ESBL producing gram negative in the study area. The information concerning this problem will be very important for policy makers to alert them and to implement intervention strategies.

Moreover, this study generates data that indicated the need of the routine application of ESBLs screening and confirmation in the laboratory. Furthermore, this study helps the researchers since it generates baseline information and evidence.

2. LITERATURE REVIEW

2.1 Definition of Urinary Tract Infection in Pregnancy

Urinary tract infection (UTI) is a term applied to a variety of clinical conditions ranging from asymptomatic presence of bacteria in the urine to severe infection of the kidney with resultant sepsis. It is the presence of more than 100,000 cfu/ml of urine after doing urine culture (Sawalha, 2009). Moreover, according to the National Institute for Health and Clinical Excellence (NIHCE) guidelines, urinary tract infection is defined by a combination of clinical features and the presence of bacteria and/ or fungi in urine (NIHCE, 2007).

2.2 Etiologic agents of Urinary Tract Infection

UTI Infections usually occur due to bacteria, viruses, parasite and yeasts (Zorc *et al.,* 2005). Common bacterial pathogens include gram-negative species such as *Escherichia coli, Proteus, Klebsiella, Pseudomonas, Enterobacter* and *Serratia* spp; and gram-positive organisms, including *Enterococcus spp,* group *B streptococci* and *Staphylococcus aureus*. Among these *E.coli* is the major causative organism of UTI (Zorc *et al.,* 2005).

2.3 Treatment and prevention of UTI in pregnant women

Pregnant women should receive more strong treatment than non-pregnant women due to the risk that are associated with pregnancy (Macejko and Schaeffer, 2007). American College of Obstetricians and Gynecologists recommend screening of Asymptomatic bacteriuria (ASB) in all pregnant women. In order to detect ASB, urine cultures are recommended early in pregnancy. If discovered, antibiotic treatment is given for three to seven days. It is possible to prevent the development of UTI by 80% to 90% by early detection and treatment of ASB. Two weeks after treatment is completed, a repeat culture is obtained to ensure the infection has been eradicated. Cultures are then obtained monthly until delivery to confirm that another infection has not developed (Macejko and Schaeffer, 2007).

There is no common understanding on either the duration of therapy or choice of antibiotics and as a result, practice is more guided by national patterns of practice and local resistance pattern than by evidence from clinical trials (Schnarr and Smaill, 2008). The antibiotic should be safe for the mother and fetus. Historically, ampicillin has been the drug of choice, but the emergence of resistant strains to most frequently used antibiotics such as ampicillin and amoxicillin has

been discovered. Sulfonamide or Sulfonamide containing combination, Cephalosporin, Penicillin or Nitrofurantoin, based on the results of susceptibility testing are appropriate drugs for the treatment of bacteriuria (Smaill and Vazquez., 2007). Increasing antibiotic resistance, however, complicates the option of regimens and is likely to become an increasing problem (Smaill and Vazquez., 2007).

2.4 ß-lactam antibiotics

Group of antibiotics which contain ß-lactam ring in their structure are known as ß-lactam antibiotics (Kohanski *et al*., 2007). The mechanism of action of ß-lactam antibiotics is by inhibition of cell wall synthesis, and this antibiotics exert their effect through covalent attachment to Penicillin-Binding Protein (PBP), which is a peptidoglycan trans peptidase enzyme that catalyzes the final steps in cell wall formation. Several PBPs have been identified, and they are unique to bacteria. Furthermore, the spectrum and effects of the different ß-lactams are determined by the PBPs to which these antibiotics bind (Kohanski *et al*., 2007).

2.5 ß-lactamases

ß-lactamases are group of enzymes that open and break the ß-lactam ring by adding a water molecule to the ß-lactam bond (Jose and Cesar, 2016). Emergence of resistance to β-lactam antibiotics began before the first β-lactam, Penicillin was developed. The first β-lactamase was identified in *Escherichia coli* prior to the release of penicillin for use in medical practice (Jose and Cesar, 2016).

Genes encoding for β-lactamases are generally termed bla, followed by the name of the specific enzyme (e.g. bla KPC) and they have been found in the chromosome or localized in Mobile Genetic Elements (MGEs) as part of the accessory genome. These genes can also be found forming part of integrons, a situation that facilitates their dissemination (Jose and Cesar, 2016). In terms of their expression, transcription of these genes can be constitutive or it may require an external signal to induce their production. To date more than 1,000 different β-lactamases have been described and many more are likely to continue to be reported, as part of the normal process of bacterial evolution (Jose and Cesar, 2016).

Many genera of Gram-negative bacteria possess a naturally occurring, chromosomally Mediated β-lactamase (Tham, 2012). These enzymes were thought to have evolved from penicillin binding proteins, with which they show some sequence homology. This development is likely due to the selective pressure exerted by β-lactam-producing soil organisms found in the

environment. The first plasmid-mediated ß-lactamase was detected in Gram negative in Greece and was designated as TEM after the name of the patient (Temoneira) who carried the pathogen. The most common β-lactamase in Gram-negative bacteria is TEM-1, and it can hydrolyze penicillins(Tham, 2012). In additions, the transmission of β-lactamase from one bacteria to other bacteria is fast, and soon, after changes in only one or a few amino acids, these enzymes were able to hydrolyze narrow spectrum cephalosporin and were found in *Enterobacteriaceae, Haemophilus influenzae* and *Neisseria gonorrhoeae*. Original TEMs and SHVs can not hydrolyze third-generation cephalosporin (Tham, 2012).

Resistance to beta-lactam antibiotics can be acquired or intrinsic; most gram- negative bacteria are intrinsically resistant to penicillin. Bacteria have evolved to counter the adverse effects of beta-lactam antibiotics in the following four diverse ways: production of new penicillin binding proteins that have low affinity to beta-lactams, mutations leading to loss or under-expression of porins that disallow entry of beta-lactams, expulsion of beta-lactams from periplasmic space mediated by efflux pumps and production of enzymes that hydrolyze beta-lactam rings. From all of these methods, the enzymatic inactivation by beta-lactamases is the most common strategy adopted by the bacteria. In case of Gram-positive bacteria, these enzymes are excreted outside the cell whereas in Gram negative bacteria, they are present in the periplasmic space (Rao *et al*., 2014).

2.6 Extended Spectrum Beta-Lactamases (ESBL)

2.6.1 Historical background and Definition

In 1988 Jarlier *et al* used the term ‘extended broad spectrum beta-lactamases’ to refer to those enzymes which had extended activity compared to the broad spectrum activity of classical TEM or SHV enzymes (Varaiya *et al*., 2008). In subsequent publications, the term ‘broad’ was dropped and the name Extended Spectrum Beta-Lactamase became established. In 1983 a single nucleotide mutation was found in an SHV that represented the first plasmid encoded ß-lactamase that could hydrolyze the extended spectrum cephalosporins in an isolate of *K. ozaenae*, and this type was named SHV-2. Outbreaks of primarily *Klebsiella spp* with mutated TEM and SHV enzyme derivate were reported from French hospitals at the end of the 1980s, and, to distinguish these enzymes from broad spectrum ß-lactamases (mainly TEM-1and SHV-1), the term extended spectrum ß-lactamase (ESBL) was coined by Philippon in 1989 (Varaiya *et al*., 2008).

Enzymes which can hydrolyze extended spectrum beta lactam antibiotics such as, Cefotaxime, Ceftazidime or Aztreonam were known as extended Spectrum Beta-Lactamases. However, beta-lactam inhibitors such as Clavulanic acid, Tazobactam, or Sulbactam inhibit these enzymes. In addition to this these enzyme have no hydrolytic activity against Carbapenems and Cephamycins (Bhattacharjee *et al*., 2008). Using the current definition, ESBLs have been detected among several bacteria, although members of *Enterobacteriaceae* remain their chief hosts. They have also been encountered among a few non-*Enterobacteriaceae* bacteria such as, *Stenotrophomonas spp, Pseudomonas spp, Haemophilus spp, Acinetobacter* spp and Vibrio *spp* are among others (Kingsley and Verghese, 2008).

2.7 Epidemiology of UTI

According to the study conducted in Iran by Mohammad *et al*., (2016) on prevalence and risk Factors associated with Extended Spectrum Beta Lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* Isolates in Hospitalized Patients, from 250 strains (134 *E. coli* and 116 *K. pneumoniae*) one hundred and two (40.8%) of all strains were ESBL producers, of which 54 (52.9%) were *E. coli* and 48 (47.1%) were *K. pneumoniae*. The most common antimicrobial resistance was to ampicillin; and no imipenem resistance was detected (Mohammad *et al*., 2016).

The study done by Krishnakumar *et al*., (2012) aimed to investigate Antimicrobial susceptibility pattern of Extended Spectrum Beta Lactamase (ESBL) producing uropathogens from pregnant women in clinical samples in India, and detected ESBL in *E. coli* 44.4% followed by *K. pneumoniae* 37% and *P. aeruginosa* was 18.5%.

Prevalence and Antimicrobial Susceptibility of Urinary Pathogens Among Pregnant Women in the Lagos University Teaching Hospital, Nigeria a study done by Ezeamaramu *et al*., (2015) aimed to determine the prevalence and assess antimicrobial susceptibility of Extended Spectrum β-Lactamases producing Urinary Pathogens isolated from clinical specimens of patients at Lagos University Teaching Hospital, Nigeria; showed that Cotrimoxazole presented high rate of resistance of 79.0%, 77.3%, 82.4% on *E.coli*, *Enterobacter spp.* and *Klebsiella spp*. respectively. *Pseudomonas aeruginosa* is 100% resistant to Augmentin, Cotrimoxazole, Amoxicillin. *Providencia spp*. and *Klebsiella spp*. had the highest Extended spectrum β lactamase prevalence rate of 57.1% and 35.7% respectively. Others are *E.coli* and *Enterobacter spp*. with 14.3% for both (Ezeamaramu *et al*., 2015).

In Uyo, Nigeria, Onwuezobe and Orok, (2015) detect ESBL production in *E. coli* 38%, *Klebsiella. spp* 56% and *Enterobacter cloacae* (6%) out of total of 300 urine samples processed for culture and antimicrobial sensitivity testing (Onwuezobe and Orok, 2015).

In study done by Chaula *et al*., (2017) carried out in Mwanza City, Tanzania to evaluate emergence of ESBL and UTI among HIV-Positive Pregnant Women, UTI produced by 50. *E. coli* 30 (57.7%) and *Klebsiella pneumoniae* 12 (23.1%) were the most frequent bacterial species isolated. Observed rates of resistance among *E. coli* were ampicillin 93.3%, trimethoprim sulphamethoxazole 90.0%, nitrofurantoin 16.7%, gentamicin 10.0%, ceftriaxone 13.3%, and meropenem 3.3%. For *K. pneumoniae* isolates, the resistance rates were 100%, 72.7%, 33.3%, 0.0%, and 0.0% to ampicillin, trimethoprim sulphamethoxazole, nitrofurantoin, ceftriaxone, and meropenem respectively. The proportion of extended spectrum beta-lactamase producing Gram-negative bacteria was 8.2% (4/49), all of which were *E. coli*, 13.3% (4/30) (Chaula *et al*., 2017).

Sudan study done by Mohammad, (2016) (Unpublished study) showed that: Among the Gram negative bacilli isolates (n=18), ESBL was detected in 8 (44.4%) of these isolates. the most prevalent ESBL isolates were *E. coli* accounting, 75%. Antibiotics susceptibility patterns of Gram negative bacilli isolates appeared resistance to some antibiotics as follow, Ciprofloxacin (22.2%), Cotrimoxazole (66.7%) and Amoxicillin (83.3%) (Mohammad, 2016 Unpublished study).

The study done in eastern Ethiopia by Seid and Asrat, (2005) aimed to investigate the occurrence of extended spectrum Beta-lactamase enzymes in clinical isolates of Klebsiella species from Harar region isolated a total of 57 (15%) *Klebsiella* spp. from 384 patients. Of the 57 isolates, 33 (58%) were from sputum, 18 (31.5%) from urine and 6 (10.5%) from pus. Of the 57 *Klebsiella* spp, 54 (94.7%) were identified as *K. pneumoniae* and 3 (5.3%) as *K. oxytoca*. Resistance was found against cephalosporins [cefotaxime (39.0%), cefoxitin (39.0%), ceftazidime (40.0%), ceftriaxone (40.0%), cephalothin (42.0%)], chloramphenicol (70.0%), gentamicin (61.0%) and trimethoprim–sulphamethoxazole (65.0%). ESBL was detected in 19/57 (33.3%) of the *Klebsiella* isolates (Seid and Asrat, 2005).

A study conducted in Gonder teaching hospital on Bacterial profile and drug susceptibility pattern of urinary tract infection in pregnant women showed the overall prevalence of UTI was 10.4 %. The predominant bacterial pathogens were *Escherichia coli* (47.5 %) followed by coagulase-negative staphylococci (22.5 %), *Staphylococcus aureus* (10 %), and *K. pneumoniae*

(10 %). Gram negative isolates were resulted low susceptibility to cotrimoxazole (51.9 %) and tetracycline (40.7 %) (Alemu *et al*., 2012).

Another study conducted in Gonder teaching hospital indicated that 10.2% and 15.9%, had significant bacteriuria in asymptomatic and symptomatic pregnant women respectively. Prevalence of urinary tract infection was statistically significant associated with previous history of catheterization and urinary tract infection ($p<0.05$). The result showed that the most frequently isolated Gram-negative species were *Escherichia coli* (41.5%) followed by coagulase negative *staphylococcus* (25%). Gram negative and positive bacteria accounted for (58.3%) and (41.7%) respectively. Rate of susceptibility for all isolated bacteria showed, ceftriaxone and gentamicin (87.5%) for each, amoxicillin–clavulanic acid (83.3%), ciprofloxacin (75%), and norfloxacin (70.8%). However, most resistance was to ampicillin (91.7%), amoxicillin (79.2%), tetracycline (58.3%), cotrimoxazole (50%), and chloramphenicol (33.3%) (Getachew *et al*., 2012).

According to the study conducted in Adigrat General Hospital by Tadesse *et al*., (2018) on Prevalence, antimicrobial susceptibility profile and predictors of asymptomatic bacteriuria among pregnant women, out of 259 pregnant women included in the study, the prevalence of asymptomatic bacteriuria was 55 (21.2%). Gram negative bacteria, specifically *Escherichia coli* were the predominant isolates followed by *Klebsiella species* and *Proteus mirabilis*. Of the Gram positive identified bacteria, *Staphylococcus aureus* was main isolate. Age of the mother (18–25 years old) with [AOR = 8.5, 95% CI (2.2, 32.9)], family income (< 1000 ETB) with [AOR = 7.5, 95% CI (2.4, 23.1)] and gestational period at 1st trimester [AOR = 11.9, 95% CI (4.4, 32.4)] and 2nd trimester [AOR; 5.6, 95% CI (2.0, 15.5%)] were predictors significantly associated with asymptomatic bacteriuria. All Gram negative isolates were found 100% resistance to Ampicillin (Tadesse *et al*., 2018).

Study done in Mekelle by Tsegay, (2014) (Unpublished study) in titled with bacterial profile and drug susceptibility pattern of urinary tract infection in pregnant women attending antenatal care at Mekelle hospital, indicates 11.9% overall prevalence of UTI. Of this 11.3% and 15.4%, had significant bacteriuria in asymptomatic and symptomatic group respectively. Prevalence of UTI was significantly associated with previous history of catheterization and urinary tract infection ($p<0.05$). *Escherichia coli* was the most frequently isolated organism 6(30%) followed by coagulase negative *staphylococcus* 5(25%). Gram-negative isolates showed resistance rate of 100% to Ampicillin. However, all Gram negative bacterial isolates

revealed low level of resistance (16.7%) against nitrofurantoin and ceftriaxone (Tsegay, 2014 Unpublished study).

The study done at Tikur Anbessa Specialized Hospital by Desta *et al*., (2016) aimed to investigated the gastrointestinal colonization rate and antibiotic resistance patterns of Extended-Spectrum Beta-Lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in hospitalized patients admitted at Ethiopia's largest tertiary hospital found 52% (95%CI;46%–58%) overall gastrointestinal colonization rate of ESBL producing *Enterobacteriaceae* (ESBL-E) in hospitalized patients of which, ESBL-*E.coli* and *K.pneumoniae* accounted for 68% and 32% respectively. ESBL positivity rate was much higher in *K.pneumoniae* (76%) than *E.coli* (45%). Fecal ESBL-E carriage rate in neonates, children and adults was 74%, 59% and 46% respectively (Desta *et al*., 2016).

Another study conducted in Tikur Anbessa hospital by Legese *et al*., (2017) showed that from a total of 322 study participants who were suspected for septicemia and/or UTI, the overall prevalence of bacteria isolates from blood and urine cultures was 17.1%. From 177 blood samples 13.0% (n=23/177) and from 145 urine samples 22.1% (n=32/145) were culture positives. Coagulase negative *Staphylococci* and *Klebsiella ozaenae* were the predominate bacteria isolated in blood and urine cultures respectively. Multiple resistances were observed in 71.42% of Gram positive and 95.11% of Gram negative isolates. Prevalence of ESBL producing *Enterobacteriaceae* was 78.57% (Legese *et al*., 2017).

Previous study conducted in Tikur Anbessa by Assefa *et al*., (2008) revealed that 39/369 (10.6%) and 9/45 (20%) had significant bacteriuria in asymptomatic and symptomatic pregnant women, respectively. The overall prevalence of urinary tract infection was 48/414 (11.6%). Prevalence of urinary tract infection was significantly associated with past history of urinary tract infection and maternal educational level ($p < 0.05$). The bacterial pathogens isolated were predominantly *E. coli* 22 (44%), followed by *S. aureus* 10 (20%), coagulase-negative *staphylococci* 8 (16%), and *K. pneumoniae* 4 (8%). The rates of susceptibility of the Gram negatives to antibacterial agents tested ranged from 30-93.3% (Assefa *et al*., 2008)

2.8 Risk factor for acquisition of ESBL producers

Several studies have been undertaken to find the association between several well characterized risk factors and colonization or infection by the ESBL producers. Many studies have reported isolation of ESBL producing organisms from raw vegetables, fruits as well as food of animal

origins (Raphael *et al*., 2011). It is most likely that these animals might have acquired the ESBL producers from human contacts (EFSA, 2011). Some of the risk factors that have been positively associated with increased colonization or infection by ESBL producers include Asian/African country of birth, travel to Asian/Middle-East countries, hospital stay of more than seven days, transfer from another health care facility, stay in surgical ward, low birth weight, intra-abdominal surgery, invasive procedures, presence of central venous catheter, hemodialysis, diabetes mellitus, and antibiotic use (especially third-generation cephalosporins and quinolones) for more than a week (Demirdag and Hosoglu, 2010).

2.9 Modes of Spread of ESBL-Producing Organisms Within Hospitals

A common environmental source of ESBL-producing organisms has occasionally been discovered. Examples have included contamination of ultrasonography coupling gel, bronchoscopes, blood pressure cuffs, and glass thermometers (used in axillary measurement of temperature) (Rogues *et al*., 2000). ESBL-producing organisms have been isolated from patients' soap, sink basins, and babies' baths, but the contribution of this environmental contamination to infection was impossible to determine. Evidence suggests that transient carriage on the hands of health care workers is a more important means of transfer from patient to patient. Health care workers Hand carriage is usually eliminated by washing with chlorhexidine or alcohol based antiseptics. The use of artificial nails may also promote long term carriage and has been associated with at least one outbreak (Gupta *et al*., 2004).

2.10 Transfer of genes coding for ESBL in a microbial ecosystem

Plasmids are usually circular but rarely linear replicons of extra chromosomal DNA in bacterial cells. They play an important role in the evolution of microbes. Plasmids are mobile genetic mosaics that spread multiple traits (e.g., drug resistance and virulence) that are beneficial to the bacteria and are indeed necessary for rapid adaptation to any changes in their environment. Plasmids are found in most bacteria, and they can vary in size and are often self-transferable. ESBLs are spread by plasmids (Stokes and Gillings, 2016). Horizontal transfer of genes in a microbial ecosystem can occur through three different mechanisms: These are Transformation - direct uptake of naked DNA, Conjugation - transfer of Deoxyribonucles (DNA) from a donor cell to a recipient cell after plasmid-mediated contact (e.g., through a sex pilus) and Transduction - bacteriophage-mediated transfer of DNA between bacteria (Tham, 2012).

Resistance genes can be further incorporated into the recipient chromosome by recombination. These genes may contain single mutations or more severe sequence changes. Horizontal transfer of resistance genes is a mechanism for the dissemination of multiple drug resistance because resistance genes can be found in clusters and transferred together to the recipient. This is enabled by the existence of specific DNA structures called integrons (Ploy *et al*., 2000). Integrons are DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site specific recombination. These elements are located either on the bacterial chromosome or on broad host range plasmids (Boucher *et al*., 2015).

2.11 Clinical significance of ESBL-producing organisms

ESBL-producing organisms have many clinical and microbiological significance. These bacteria are associated with severe infections such as intra-abdominal infection, bacteremias, urinary tract infections, and respiratory tract infections [Laudano, 2011]. They inactivate cephalosporins, which are often used in treating the septic patient in a variety of clinical settings. Therefore, this often renders empiric antibiotic treatment ineffective. The delay in laboratory diagnosis and time to appropriate antibiotic therapy has been strongly associated to an increased mortality in these cases. Many ESBL genes have the propensity to jump between organisms, thus leading to outbreaks of infection if this occurs in an easily transmissible pathogen. It is also known that organisms producing ESBLs also have the ready capacity to acquire resistance to other antimicrobial classes such as the tetracyclines, quinolones, trimethoprim, cotrimoxazole, and aminoglycosides, which further limits therapeutic options. The mechanism behind this multi-drug resistance phenomenon is genetic; the gene encoding for resistance for both ESBL and other classes (e.g. quinolones) are often associated on the same mobile DNA element which is plasmid. When plasmid propagate during conjugation multidrug resistance developed in previously sensitive organisms (Macejko and Schaeffer, 2007).

2.12 Laboratory tests for detection of ESBL-producing Enterobacteriaceae

ESBL detection involves two important steps. The first is a screening test with an indicator cephalosporin which looks for resistance or diminished susceptibility, thus identifying isolates likely to be harboring ESBLs. The second step is a confirmation test which evaluates the synergy between an oxyimino cephalosporin and clavulanic acid, distinguishing isolates with ESBLs from those that are resistant for other reasons (CLSI, 2014).

ESBL Disc Screening

Disk-diffusion method for ESBL screening can be performed using cefpodoxime, ceftazidime, aztreonam, cefotaxime and ceftriaxone, according to Clinical and Laboratory Standards Institution (CLSI) guidelines. Disk diffusion methods were used for antibiotic susceptibility testing aid for screening for ESBL production by noting specific zone diameters which indicate a high level of suspicion for ESBL production. Since the affinity of ESBLs for different substrates is variable, the use of more than one of these agents for screening improves the sensitivity of detection (CLSI, 2014). However, it is adequate to use Cefotaxime, which is consistently susceptible to CTX-M; and Ceftazidime, which is a consistently good substrate for TEM and SHV variants (Rawat and Nair., 2010).

ESBL Disc Confirmation

Enterobacteriaceae suspected to be producers of ESBLs enzymes may be submitted to the following confirmation tests: Combination Disc Test (CDT) and/or Double-Disc Synergy Test (DDST). These tests permit to evaluate the inhibition of ESBL activity by Clavulanic acid (EUCAST, 2013).

Double Disc Combination Test

For each test discs containing cephalosporin alone (cefotaxime, ceftazidime, cefepime) and in combination with clavulanic acid are applied. The inhibition zone around the cephalosporin disc combined with clavulanic acid is compared with the zone around the disc with the cephalosporin alone. The test is positive if the inhibition zone diameter is $\geq$ 5 mm larger with clavulanic acid than without (EUCAST, 2013).

Double Disk Synergy Test

Isolates with zone diameters suspicious of ESBL production as pre-determined by the susceptibility test results will be subjected to the Double Disk Synergy Test to test for the presence of ESBL producing enzymes [Loh and Sivalingam, 2007]. In this, test disks of third-generation Cephalosporins and Amoxicillin/clavulanate (Augmentin) are kept 30 mm apart, center to center, on inoculated Mueller Hinton agar. A clear extension of the edge of the inhibition zone of cephalosporin towards Augmentin disk is interpreted as positive for ESBL production. Evaluations of the double disk diffusion test have revealed sensitivities of the method ranging from 79% to 97% and specificities ranging from 94% to 100% (Rawat and Nair., 2010). In isolates which are suspicious for harboring ESBLs but are negative using the

standard distance of 30 mm between disks, the test should be repeated using closer (for example, 20 mm) or more distant (for example, 40 mm) spacing (Rawat and Nair., 2010).

2.13 Treatment of ESBL producing organism

Carbapenems are considered to be the treatment of choice against serious ESBL associated infections (Pitout and Laupland, 2008). This is mainly because they are not inactivated by these enzymes in vitro, and have demonstrated adequate effectiveness for the treatment of serious Gram-negative infections at various body sites. However, specific data for their clinical use against ESBL-associated infections are rather limited, although generally supportive of their effectiveness (Zanetti *et al*., 2003).

Tigecycline, a derivative of minocycline, is the first member of the glycylcycline class of antibiotics available for clinical use (Falagas *et al*., 2009). It has the property to evade common mechanisms of resistance to tetracyclines expressed in Gram-negative and Gram-positive bacteria. Specifically, excellent activity of tigecycline has been shown against ESBL-producing *E. coli* isolates. Additionally, substantial antimicrobial activity of tigecycline has been demonstrated against ESBL-producing *K. pneumoniae* isolates, although this depends on the interpretive breakpoints of susceptibility elected. Data regarding the antimicrobial activity of tigecycline against other ESBL-producing *Enterobacteriaceae* organisms are rather limited (Falagas *et al*., 2009).

Tazobactam has been found to be more potent compared with clavulanic acid against certain CTX-M type ESBLs, while both of the above agents appear to be more potent than sulbactam in inhibiting TEM and SHV type ESBLs. The available clinical evidence regarding the utility of beta-lactam/beta-lactamase inhibitor combinations for the treatment of ESBL-associated infections is rather limited. Specifically, favorable patient outcomes have been related to piperacillin/ tazobactam treatment in some small studies, although such findings have not been consistently reproduced (Peterson, 2008).

2.14 Prevention and control of ESBL producing organism

Measures to limit the emergence of ESBL-producing Enterobacteriaceae in the hospital and the community can be divided in actions for limiting the spread of the ESBL-producing *Enterobacteriaceae* and actions to reduce the selection pressure through use of antibiotics (Harris *et al*., 2007). The most important measure to prevent spread is adherence to good hand hygiene. More extensive use of alcohol based hand rub was associated with a lower incidence

of such strains. The risk of cross transmission is higher in *K. pneumoniae* than in *E. coli*, and the reservoir of *K. pneumoniae* can in some cases be environmental. Hence ways for limiting the chance of spread through the environment are: implementation of barrier precautions, control of environmental causes and various types of equipment (e.g., bronchoscopes, gels, and cloths), putting patients with ESBL producing Enterobacteriaceae in single rooms and/or cohort isolation, and to avoid overcrowding. Knowledge of whether a patient is a carrier of an ESBL producing *Enterobacteriaceae* or not enables the health care personal to take action and hence screening programs and journal alert systems are of importance (Harris *et al*., 2007).

3. OBJECTIVE

3.1 General objective

- To determine Extended Spectrum Beta Lactamase in uropathogens and antimicrobial susceptibility pattern of Gram negative bacilli isolates among pregnant women attending antenatal care in ALERT hospital, Addis Ababa, Ethiopia.

3.2 Specific objectives

- To determine the overall prevalence of urinary tract infection among pregnant women attending antenatal care.
- To determine antimicrobial susceptibility pattern of Gram negative bacilli isolates.
- To determine Extended Spectrum β- Lactamases in uropathogens among pregnant women attending antenatal care.

4. MATERIALS AND METHODS

4.1 Study setting

The study was conducted at ALERT Hospital which is found in Kolfe-keraniyo Sub-city, Addis Ababa, Ethiopia. ALERT hospital is one of the specialized tertiary referral hospitals in the country. It is located in Addis Ababa at 7 km south west on the way to Jimma. ALERT is a medical facility in Addis Ababa, specializing in Hansen's disease, also known as 'leprosy'. It was originally the All Africa Leprosy Eradication, Rehabilitation and Training Center (hence the acronym), but the official name is now expanded to include tuberculosis: All Africa Leprosy and Tuberculosis Eradication, Rehabilitation and Training Centre. ALERT's main mission was to provide training in multiple aspect of Leprosy including prevention, treatment and rehabilitation in an African context. There is currently a 240-bed teaching hospital, which includes dermatology, ophthalmology, surgery, an orthopedic workshop and a rehabilitation program. ALERT also provides Anti Natal Care and post natal care service for women who were referred from different health institutions. It is estimated that the hospital offers ANC service for approximately 4800 pregnant women yearly. Daily in average 20 pregnant women visit this hospital.

4.2 Study design and period

A Hospital based cross-sectional study was conducted from July to September 2018.

4.3 Population

4.3.1 Source Population

- The source population comprise of pregnant women of all age group who sought medical service at ALERT Hospital, Addis Ababa.

4.3.2 Study Population

- The study population was pregnant women attending antenatal care with symptoms and without symptoms of urinary tract infection during the study period.

4.4 Inclusions and Exclusions criteria

4.4.1 Inclusion criteria

- Pregnant women attending antenatal care with or without signs and symptoms of urinary tract infection and who were willing to participate in the study.

4.4.2 Exclusion criteria

- Pregnant women who have taken antibiotics for the last 10 days.

4.5 Sample size determination

The sample size was calculated using the single population proportion formula:

$$n = \frac{Z^2 p(1-p)}{d^2}$$

Where n = sample size, Z = Z statistic for a level of confidence, P = expected prevalence or proportion and d = precision.

By considering estimated prevalence, P=12% (Getachew *et al*., 2012), 5% precision (d=0.05) and 95% level of confidence (z=1.96),

$$n = \frac{(1.96)^2}{(0.05)^2} 0.12(0.88), \qquad n = 162$$

The estimated sample size calculated to be 162. By Adding 10% contingency the final estimated sample size was 178.

4.6 Sampling technique

A Convenient sampling method was applied to recruit study participants. The study participants were selected based on the inclusion criteria and all available study participants were included until sample size achieved during the study period.

4.7 Study variables

4.7.1 Dependent variables

- Prevalence of uropathogens
- Prevalence of Extended Spectrum β- Lactamases (ESBLs) producing Gram negative bacilli
- Antibiotic susceptibility pattern of Gram negative bacilli

4.7.2 Independent variables

- Socio- demographic status: age, marital status, residence, religion, educational status, occupational status
- Pregnancy related factors: Gestational period, Gravidity
- Clinical variables: signs and symptoms of UTI, history of UTI
- Health facility related factors: inpatient, outpatient

4.8 Data collection method

A predesigned questionnaire was developed. The questionnaire consisted of socio-demographic factors, pregnancy related factors, Clinical variables and health facility related questions. Data on the etiological agents (uropathogens), ESBL production and antibiotic susceptibility pattern was obtained using standard laboratory tests.

4.8.1 Socio-demographic data

After obtaining informed consent socio-demographic data were collected from pregnant women

4.8.2 Sample Collection, Handling & Transport

Ten ml of first morning, clean-catch Mid-Stream Urine (MSU) samples were collected. Pregnant women cleaned their genital tract before sample collection in the toilet. The participants were instructed how to collect the urine sample by cleansing the gentile with soap and water using leak proof, wide mouth, sterile plastic universal containers. The urine specimens then were delivered to microbiology laboratory using an ice box and processed as

soon as possible. Sample processing and lab work was done in ALERT hospital bacteriology laboratory.

4.8.3 Bacterial Culture and Identification

Using calibrated wire inoculating loop (0.001ml), all urine samples were inoculated to Cysteine Lactose Electrolyte Deficient medium (CLED) (Oxoid, England) and MacCkonkey agar (BD, USA). Cultures were incubated in aerobic atmosphere at 37ºC for 24 hrs. Colonies were counted to check the presence of significant bacteriuria. All positive cultures from urine samples were characterized by colony characteristics, Gram stain and biochemical tests. Gram stain was done for all positive cultures with SB in order to categorize the bacteria as gram negative or gram positive by determining their reactions, cell shape and arrangement. All Gram negative bacteria were then identified at species level by the pattern of biochemical profiles using standard procedures. The Gram negative bacteria were identified by H_2S production in TSI agar, indole production, motility test, citrate utilization, urease test, LIA and Mannitol fermentation test. The Gram positive bacteria were identified using coagulase and catalase tests [Annex IV].

4.8.4 Drug Susceptibility Patterns

Antimicrobial susceptibility testing was done by disk diffusion method. All the isolated gram negative bacteria were inoculated onto Muller-Hinton agar plates (100 mm plate) (pH 7.2-7.4) for antibiotic susceptibility test by Kirby-Bauer disc diffusion technique. Disc diffusion tests was performed and interpreted according to the recommendations of the Clinical and Laboratory Standards Institute, 2018 (CLSI 2018) [Annex IV].

The following antibiotics were used: Ampicillin (10µg, BD), Amikacin (30µg, BD), Gentamicin (10µg, BD), Meropenem (10µg, Oxoid), Nitrofurantoin (300µg, BD), Trimethoprim-sulphamethoxazole (1.25/23.75µg, BD), Cefixime (5µg, BD), Ceftriaxone (30µg, BD), Cefotaxime (30µg, BD), Ceftazidime (30µg, BD), Chloramphenicol (30µg, BD) and Amoxicillin-clavulanic acid (20/10µg, BD).

4.8.5 Extended spectrum beta-lactamase detection

4.8.5.1 Screening method

Screening method was done by detection of resistance to any of the following third generation cephalosporin antibiotics: Cefotaxime, Ceftazidime and Ceftriaxone by disk diffusion method. Organisms were screened for ESBL by using CLSI (CLSI, 2018) recommendation [Annex IV].

4.8.5.2 Confirmatory method

Isolates which were suspected for ESBL production by ESBL disk screening method were undergo ESBL disk confirmation test by Double Disk Synergy method (DDST). In this, test disks of third-generation Cephalosporins (Cefotaxime, Ceftazidime and Ceftriaxone) and Amoxicillin/clavulanate (Augmentin) were kept 30 mm apart, center to center, on inoculated Mueller-Hinton agar. A clear extension of the edge of the inhibition zone of cephalosporin towards Augmentin disk was interpreted as positive for ESBL production. (Annex IV).

4.8.6 Quality control

Quality of Laboratory test was maintained by following Standard Operating Procedures (SOP) for all laboratory test. Sample containers were appropriately labeled. Expiration date, evidence of freezing, unequal fill, contamination and bubbles was checked for all media. Positive and negative controls were performed for media used based on CLSI (CLSI, 2018) recommendation. Quality control was done for each batch of the medium by using *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 as standard strains. *K. pneumoniae* ATCC 700603 (ESBL positive) and *E. coli* ATCC 25922 (ESBL negative) control strains were used for ESBL detection (CLSI, 2018). All control strains were taken from Ethiopian public health institute (EPHI). All reagents used for gram stain were filtered before use and quality control slides were also used when performing gram stain.

In order to cheek the disc quality, representative discs were tested against the reference strains of *S. aureus* ATCC 25923, *E. coli* ATCC 25922 (ESBL negative), *K. pneumoniae* ATCC 700603 (ESBL positive) and *P. aeruginosa* ATCC 27853. The zone of inhibition was compared with standard value as recommended by CLSI (CLSI, 2018).

4.9 Data quality Assurance

The questioner was translated from English to Amharic and also translated back to English. Training was given for data collectors. Pre-test was done on 5% of participants getting service other than the study hospital and some amendment was made based on the findings of the pretest. SOP were strictly followed for every tests in this study. All test results were recorded appropriately before entry to statistical analysis and prior to this data was double checked.

4.10 Statistical analysis and interpretation

The collected data was checked for completeness, errors and missing values before data entry to statistical tool. Socio-demographic, clinical and laboratory data were double entered in to EPI INFO. Then the data was exported to SPSS version 26 for analysis. Descriptive statistics was done. Association between the dependent variables and independent variables was checked by using bivariate logistic regression analysis. Then, those variables having P-value <0.2 in the bivariate analysis was entered into multivariable logistic regression analysis to control the influence of possible confounding variables and to see the strength of association. Statistically significant level was declared at p-value <0.05. Finally, the results were presented by text, tables and figures.

4.11 Operational definitions

- **Symptomatic UTI**: is a condition which is characterized by presence of significant bacteriuria in clean-voided midstream urine specimens that yield positive cultures ($\geq 10^5$cfu/ml) with symptoms such as urgency, dysuria, incontinence, frequency, supra-pubic pain, flank pain, tenderness and fever.

- **Asymptomatic UTI**: is a condition which is characterized by presence of significant bacteriuria in two consecutive clean-voided midstream urine specimens that yielding positive cultures ($\geq 10^5$cfu/ml), in a patient without classical symptoms of UTI.

- **Significant Bacteriuria**: Colony count yielding bacterial growth of $\geq 10^5$cfu/ml of urine in culture media.

- **Multidrug Resistance (MDR)**: a bacterium that is simultaneously resistant for two or more antimicrobials belonging to different chemical classes

4.12 Ethical consideration

The research project was approved and ethically cleared by Department Research and Ethics Committee (DERC) of Department of Microbiology, Immunology and Parasitology, School of Medicine, College of Health Science, Addis Ababa University. Also the study was approved and ethically cleared by ALERT/AHRI Ethics Review Committee. Supportive letters were taken from both the department of Microbiology, Immunology and Parasitology, School of Medicine, College of Health Science, Addis Ababa University and ALERT hospital. All Participants were informed about objectives and aspects of the study and signed on written informed consent statements. Confidentiality was maintained. The result of each study participant were communicated to their attending physicians.

5. RESULTS

5.1 Socio-demographic characteristics of the study subjects

A total of 177 pregnant women with and without symptoms of UTI, both admitted in ward and those who were in outpatient level were investigated during the study period. The age of the study participants were ranged from 19 to 40 years with majority 40.7% (n=72/177) in age group of 25 to 29 years. The mean age was 27.71±4.908 (Table 1). Most of the study participants were married 98.9% (n=175/177). Regarding to the residence of the study participants, 93.2% (n=165/177) were from urban and 6.8% (n=12/177) from rural. Majority of the study participants followed Orthodox Christianity 49.7% (n=88/177) and seconded with Muslim 43.5% (n=77/177) (Table 1).

Table 1. Socio-demographic characteristics of pregnant women attending ANC in ALERT Hospital, Addis Ababa, Ethiopia (July– September, 2018)

Variables		**Frequency**	**Percentage**
Age	<25	46	26.0
	25-29	72	40.7
	30-34	39	22
	>34	20	11.3
Marital status	Single	2	1.1
	Married	175	98.9
	Divorced	0	0
	Widowed	0	0
Residence	Rural	12	6.8
	Urban	165	93.2
Religion	Orthodox	88	49.7
	Muslim	77	43.5
	Others	12	6.8
Educational status	Literate	148	83.6
	Illiterate	29	16.4
Occupational status	House wife	113	63.8
	Merchant	35	19.8
	Student	4	2.3
	Government employee	25	14.1

5.2 Prevalence of urinary tract infection

Of the 177 cultured urine samples, significant bacteriuria was detected in 26 urine specimens. The overall prevalence of UTI was 14.7 % ((n=26/177), 95%CI = 9.6 – 19.8). Of the total 26 isolates, Gram-negative bacteria were prevalent (57.7% (n=15/26)) than Gram-positive bacteria (42.3% (n=11/26)).

High prevalence of UTI were observed among pregnant women of age greater than 34 (25.0% (n=5/20)). Out of 26 pregnant women who were admitted in the hospital, 30.8% (n=8/26) had significant bacteriuria while 69.2% (n=18/26) were negative for significant bacteriuria; and there was a significant association between being out patient or inpatient with UTI (AOR = 4.875, 95%CI = 1.628 –14.598, P = 0.005) **(**Table 2). In this study the prevalence of UTI among pregnant women in first trimester was18.2% (n=2/11). In our study prevalence of UTI was also different among primigravida (19.6% (n=10/51)) and multigravida women (12.7% (n=16/125)). The prevalence of bacteriuria in symptomatic and asymptomatic infection were 19.4% (n=14/72) and 11.4% (n=12/105), respectively. There was no statistical significant association between significant bacteriuria and maternal age, religion, educational status, occupational status, gestational period, gravidity, symptom of UTI and history of UTI **(**Table 2).

Table 2. Association of independent variables with culture results among pregnant women attending ANC at ALERT Hospital (July– September, 2018)

Variables	Total tested No. (%)	Significant Bacteriuria Positive No. (%)	Significant Bacteriuria Negative No. (%)	P value	COR	95% CI	P value	AOR	95% CI
Age									
<25	46(26.0)	9(19.6)	37(80.4)	0.620	0.730	(0.210 – 2.539)	0.796	0.839	(0.222 – 3.176)
25-29	72(40.7)	7(9.7)	65(90.3)	0.083	0.323	(0.090 – 1.159)	0.119	0.348	(0.092 – 1.312)
30-34	39(22.0)	5(12.8)	34(87.2)	0.245	0.441	(0.111 – 1.754)	0.248	0.417	(0.095 – 1.838)
>34	20(11.3)	5(25.0)	15(75.0)		1			1	
Religion									
Orthodox	88(49.7)	15(17.0)	73(83.0)	0.504	0.616	(0.149 – 2.550)	0.524	0.608	(0.131 – 2.808)
Muslim	77(43.5)	8(10.4)	69(89.6)	0.167	0.348	(0.078 – 1.550)	0.241	0.385	(0.078 – 1.896)
Others	12(6.8)	3(25.0)	9(75.0)		1			1	
Patient type									
Out patient	151(85.3)	18(11.9)	133(88.1)		1			1	
Inpatient	26(14.7)	8(30.8)	18(69.2)	0.016	3.284	(1.248 – 8.641)	0.005	4.875	(1.628 –14.598)
Educational status									
Literate	148(83.6)	21(14.2)	127(85.8)		1				
Illiterate	29(16.4)	5(17.2)	24(82.8)	0.672	1.260	(0.433 – 3.667)			
Occupation									
House wife	113(63.8)	15(13.3)	98(86.7)	0.391	0.612	(0.200 – 1.877)			
Merchant	35(19.8)	5(14.3)	30(85.7)	0.560	0.667	(0.171 – 2.604)			
Student	4(2.3)	1(25.0)	3(75.0)	0.819	1.333	(0.113 –15.704)			
Government employ	25(14.1)	5(20.0)	20(80.0)		1				
Gestational period									
1st trimester	11(6.2)	2(18.2)	9(81.8)		1				
2nd trimester	28(15.8)	5(17.9)	23(82.1)	0.981	0.978	(0.160 – 5.989)			
3rd trimester	138(78.0)	19(13.8)	119(86.2)	0.687	0.718	(0.144 – 3.583)			
Gravidity									
Primigravida	51(28.8)	10(19.6)	41(80.4)		1				
Multigravida	125(70.6)	16(12.7)	110(87.3)	0.243	0.596	(0.250 – 1.420)			
Symptom of UTI									
Yes	72(40.7)	14(19.4)	58(80.6)	0.143	1.871	(0.809 – 4.324)	0.119	2.100	(0.827 – 5.328)
No	105(59.3)	12(11.4)	93(88.6)		1			1	
History of UTI									
Yes	61(34.5)	10(16.4)	51(83.6)	0.643	1.225	(0.519 – 2.893)			
No	116(66.5)	16(13.8)	100(86.2)		1				

COR---------Crude odds ratio, AOR---Adjusted odds ratio, CI----------Confidence Interval

5.2.1 Distribution of uropathogens

The percentage of each uropathogen isolated from mid-stream urine samples are presented as shown in Table 3. The predominantly isolated bacteria were *K .pneumoniae* 26.9% (n=7/26) followed by *S. aureus* 23.1% (n=6/26), *E. coli* 15.4 % (n=4/26) and *S. epidermidis* 15.4 % (n=4/26). Other UTI isolates were *C. diversus* 7.7% (n=2/26), *K. oxytoca* 3.8% (n=1/26), *E. cloacae* 3.8% (n=1/26) and *S. saprophyticus* 3.8% (n=1/26).

Table 3. Percentage and types of bacterial isolates from culture positive pregnant women at ALERT Hospital from (July - September 2018)

Bacterial isolates	Frequency (n)	Percentage (%)
K .pneumoniae	7	26.9
E. coli	4	15.4
C. diversus	2	7.7
K. oxytoca	1	3.8
E. cloacae	1	3.8
S. aureus	6	23.1
S. epidermidis	4	15.4
S. saprophyticus	1	3.8

The predominant bacteria *K .pneumoniae* (26.9% (n=7/26)) constitute the highest number (n=4) among bacteria isolated from symptomatic pregnant women with significant bacteriuria where as *E. cloacae, S. epidermidis and K. oxytoca* constitute the lowest (n=1). *S. aureus,* the major isolates from Gram positive bacteria (23.1% (n=6/26)) constitute the highest number (n=4) among bacteria isolated from asymptomatic pregnant women with significant bacteriuria where as *E. coli* and *S. saprophyticus* constitute the lowest (n=1). Distribution of bacterial isolates in relation to symptom is shown in figure 1.

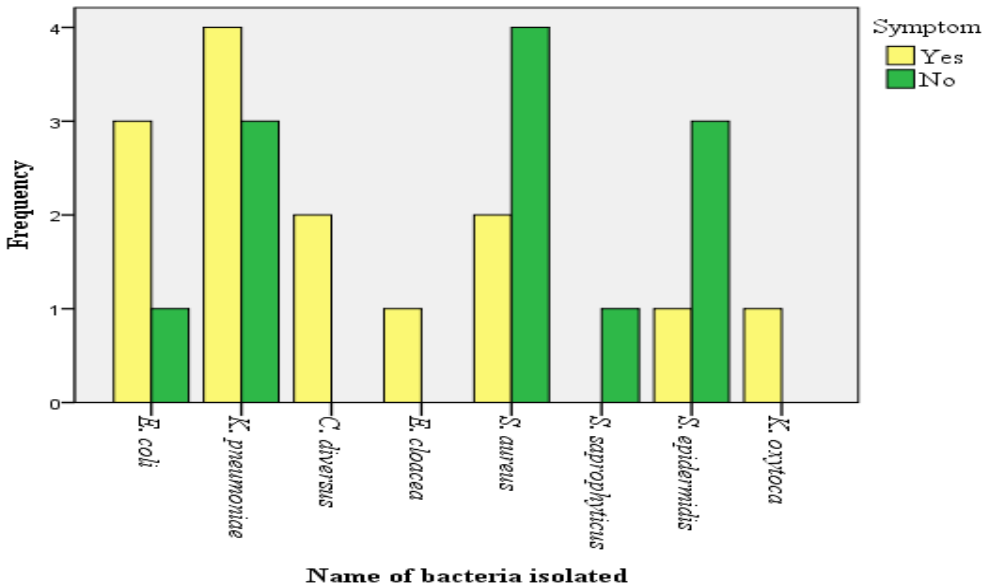

Figure 1 Frequency of bacteria isolated from urine in relation to symptom at ALERT Hospital from July to September 2018

K .pneumoniae (26.9% (n=7/26)) accounts the highest number (n=5) among bacteria isolated from pregnant women in outpatient department with significant bacteriuria. On the other hand, *S. aureus, S. epidermidis* and *K .pneumoniae* (n=2) were the most common isolates among bacteria isolated from pregnant women in inpatient department with significant bacteriuria (figure 2).

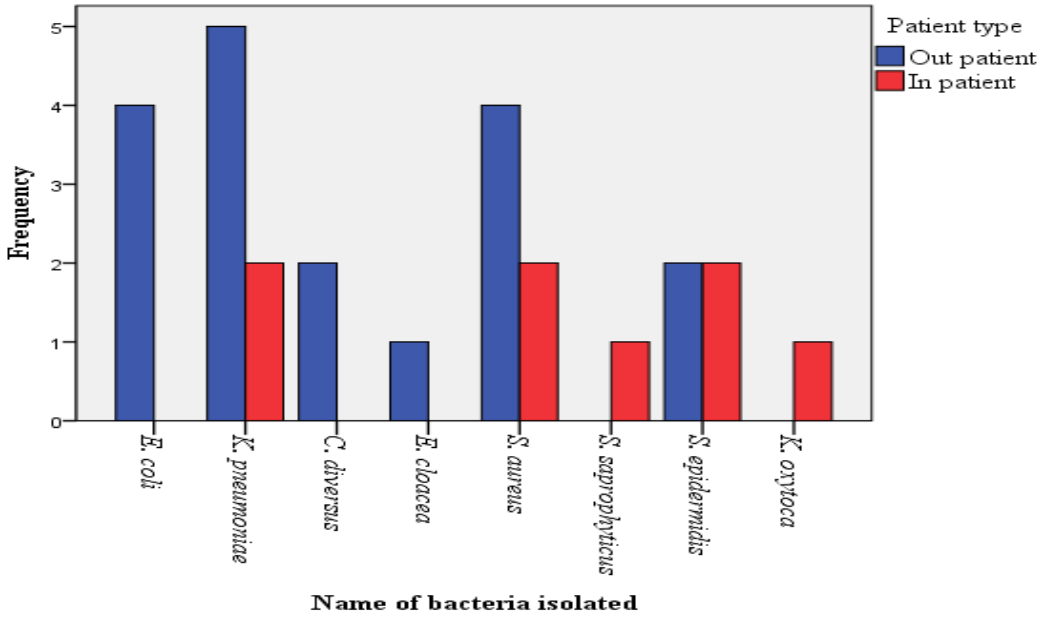

Figure 2 Frequency of bacteria isolated from urine accordingly patient type at ALERT Hospital from July to September 2018

5.3 Antimicrobial susceptibility pattern of Gram-negative bacteria

The result of antimicrobial susceptibility pattern of bacterial isolates is shown on Table 4. In general, all Gram-negative isolates (n=15) showed resistance rate of 100% to Amikacin and Gentamicin. On the other hand, all Gram-negative isolates showed sensitivity rate of 100% to Meropenem. Resistance against Ampicillin, Trimethoprim/sulphamethoxazole, Ceftriaxone and Cefixime were 11(73.3%), 9(60.0%), 6(40.0%) and 4(26.7%) respectively. However, Gram negative bacterial isolates showed low level resistance against Nitrofurantoin (6.7%), Chloramphenicol (6.7%) and Cefotaxime (6.7%).

K. pneumonia, which constitutes 46.7% of gram negative bacteria, showed 100% resistance to Ampicillin, Amikacin and Gentamicin but 100% susceptible to Meropenem, Nitrofurantoin, Chloramphenicol and Cefotaxime. *E. coli* which accounted for 26.7% of gram negative bacteria was 100% resistance to Amikacin and Gentamicin while 75% resistance to

Trimethoprim/sulphamethoxazole. However, it was sensitive to Meropenem, Nitrofurantoin and Chloramphenicol (100%), Ampicillin and Cefixime (25%). *K. oxytoca* which accounted 6.7% of gram negative bacteria was 100% resistant to Nitrofurantoin, Amikacin, Gentamicin, Ceftriaxone, Cefotaxime and Cefixime but it was also 100% sensitive to Meropenem and Chloramphenicol. *C. diversus* which constitutes 13.3% of gram negative bacteria showed 100% resistance to Ampicillin, Amikacin and Gentamicin while 50% resistance to Cefixime and Trimethoprim/sulphamethoxazole. However it was sensitive to Meropenem, Nitrofurantoin, Chloramphenicol, Ceftriaxone and Cefotaxime (100%). *E. cloacae* which accounted for 6.7% of gram negative bacteria was 100% resistant to Ampicillin, Amikacin, Gentamicin, Chloramphenicol and Trimethoprim/sulphamethoxazole also it showed 100% sensitivity to Meropenem, Nitrofurantoin and Cefixime.

Table 4. Antimicrobial susceptible pattern of gram negative bacteria isolated from urine culture in pregnant women at ALERT Hospital, Addis Ababa, Ethiopia (July-September 2018).

		Antimicrobial agents tested									
Bacterial Isolates	**Patern**	**AMP**	**MER**	**AMK**	**GEM**	**NIT**	**STX**	**CHL**	**CTR**	**CTX**	**CXM**
		No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)
E. coli	**S**	1(25.0)	4(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	4(100)	2(50.0)	3(75.0)	1(25.0)
(n=4)	**I**	2(50.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(25.0)	0(0.0)	0(0.0)	1(25.0)	2(50.0)
	R	1(25.0)	0(0.0)	4(100)	4(100)	0(0.0)	3(75.0)	0(0.0)	2(50.0)	0(0.0)	1(25.0)
	S	0(0.0)	7(100)	(0.0)	(0.0)	7(100)	1(14.3)	7(100)	4(57.1)	7(100)	3(42.9)
K. pneumonia	**I**	0(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	2(28.6)	(0.0)	0(0.0)	0(0.0)	3(42.9)
(n=7)	**R**	7(100)	(0.0)	7(100)	7(100)	(0.0)	4(57.1)	(0.0)	3(42.9)	0(0.0)	1(14.3)
	S	0(0.0)	1(100)	0(0.0)	0(0.0)	0(0)	0(0.0)	1(100)	0(0.0)	0(0.0)	0(0.0)
K. oxytoca	**I**	1(100)	0(0.0)	0(0.0)	0(0.0)	0(0)	1(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
(n=1)	**R**	0(0.0)	0(0.0)	1(100)	1(100)	1(100)	0(0.0)	0(0.0)	1(100)	1(100)	1(100)
	S	0(0).0	2(100)	0(0.0)	0(0.0)	2(100)	1(50.0)	2(100)	2(100)	2(100)	0(0.0)
C. diversus	**I**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(50.0)
(n=2)	**R**	2(100)	0(0.0)	2(100)	2(100)	0(0.0)	1(50.0)	0(0.0)	0(0.0)	0(0.0)	1(50.0)
	S	0(0.0)	1(100)	0(0.0)	0(0.0)	1(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(100)
E. cloacae	**I**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(100)	1(100)	0(0.0)
(n=1)	**R**	1(100)	0(0.0)	1(100)	1(100)	0(0.0)	1(100)	1(100)	0(0.0)	0(0.0)	0(0.0)
	S	1(6.7)	15(100)	0(0.0)	0(0.0)	14(93.3)	2(13.3)	14(93.3)	8(53.3)	12(80.0)	5(33.3)
Total	**I**	3(20.0)	0(0.0)	0(0.0)	0(0.0)	0(0)	4(26.7)	0(0.0)	1(6.7)	2(13.3)	6(40.0)
(n=15)	**R**	11(73.3)	0(0.0)	15(100)	15(100)	1(6.7)	9(60.0)	1(6.7)	6(40.0)	1(6.7)	4(26.7)

AMK**=** amikacin, GEN = gentamicin, CTR = ceftriaxone, CTX = cefotaxime, CXM = cefixime, MER = meropenem, CHL = chloramphenicol, NIT = Nitrofurantoin, AMP = Ampicillin, STX = trimethoprim/sulphamethoxazole., R = Resistant **S** = Sensitive I = Intermediate

5.3.1 Multiple drug resistance patterns of gram negative isolates

Among the total gram negative isolates (n=15), multi drug resistance (MDR) (resistance to two or more drugs) were observed in 80% (n=12/15) of the isolates. Among all isolates of *K. pneumoniae* which is predominant from Gram negative bacteria (n=7), MDR were observed in 71.4%(n=5/7) of the isolates (Table 5).

Table 5. Multi drug resistance pattern of Gram negative bacterial isolates (n=15) from pregnant women at ALERT Hospital, Addis Ababa, Ethiopia (July– September, 2018)

Bacterial isolates	**Total N (%)**	**Antimicrobial resistance pattern**								
		R_0 N (%)	**R_1 N (%)**	**R_2 N (%)**	**R_3 N (%)**	**R_4 N (%)**	**R_5 N (%)**	**R_6 N (%)**	**R_7 N (%)**	**MDR N (%)**
K pneumoniae	7(46.7)	1(14.3)	1(14.3)	3(42.9)	2(28.6)	0(0)	0(0)	0(0)	0(0)	5(71.4)
E. coli	4(26.7)	0(0)	1(25)	3(75)	0(0)	0(0)	0(0)	0(0)	0(0)	3(75)
C. diversus	2(13.3)	0(0)	0(0)	1(50)	1(50)	0(0)	0(0)	0(0)	0(0)	2(100)
K. oxytoca	1(6.7)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)
E. cloacae	1(6.7)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)
Total	15(100)	1(6.7)	2(13.3)	9(60)	3(20)	0(0)	0(0)	0(0)	0(0)	12(80)

R_0 - No antibiotic resistance, R_1- Resistance to one drug, R_2-Resistance to two drugs, R_3-Resistance to three drugs, R_4- Resistance to four drugs, R_5- Resistance to five drugs, R_6-Resistance to six drugs, R_7- Resistance to seven drugs, MDR- Resistance to two and more drugs

5.4 Extended spectrum Beta-lactamase producing gram negative bacilli

Fifteen (15) members of enterobacteriaceae have been isolated from 177 pregnant women. These isolates were *K. pneumoniae* 26.9% (n=7/26), *E. coli* 15.4 % (n=4/26), *C. diversus* 7.7% (n=2/26), *E. cloacae* 3.8% (n=1/26) and *K. oxytoca* 3.8% (n=1/26). However, *E. cloacae* (3.8% (n=1/26)) and *C. diversus* (7.7% (n=2/26)) were excluded from further screening for ESBL though they were suspected because as this methods were not validated for these groups.

Therefore, all the 12 Enterobacteriaceae isolates suspected for ESBL by screening method and tested for ESBLs using the phenotypic confirmatory double disk synergy method according to EUCAST. The overall prevalence of ESBL producing gram negative bacilli was 50% (n=6/12). The most prevalent ESBL isolates were *K. pneumoniae* and *E.coli* accounting for 50% (n=3/6) and 33.3% (n=2/6), respectively. The least prevalent ESBL isolates was *K.*

oxytoca which accounts 16.7% (n=1/6). Type of ESBL producer Gram negative bacilli isolates is shown in Table 6.

Table 6. Distribution of ESBL producers in gram negative isolates in ALERT Hospital (July-September 2018)

Gram negative bacilli	**ESBL producers no. (%)**
E. coli	2(33.3)
K. pneumoniae	3(50)
K. oxytoca	1(16.7)

5.4.1 Antimicrobial susceptibility pattern of ESBL producing gram negative bacilli

The result of antimicrobial susceptibility pattern of ESBL producing Gram negative isolates is shown on Table 7. In general, among ESBL producing isolates all (100%) are resistant to Amikacin, Gentamicin and Ceftriaxone while intermediate level resistance rate of 66.7% was observed among Sulphamethoxazole-Trimethoprim and Cefixime. They were susceptible for some limited drugs and these were Nitrofurantoin (83.3%) and Chloramphenicol (83.3%). All ESBL producing isolates were susceptible to Meropenem.

K. pneumoniae which accounted, for 50% of ESBL producers was 100% resistant to Amikacin, Gentamicin and Ceftriaxone; 33.3% resistance to Chloramphenicol, Sulphamethoxazole Trimethoprim and Ampicillin. However, 100% susceptibility level was observed for Meropenem and Cefotaxime. *E. coli,* the second common ESBL producers (33.3%), was also 100% resistance to Amikacin, Gentamicin and Ceftriaxone while 50% resistance for Trimethoprim/sulphamethoxazole and Cefixime. However, it was sensitive to Meropenem, Nitrofurantoin and Chloramphenicol (100%). The least ESBL producers which was *K. oxytoca* (16.7%) showed 100% resistant rate for Nitrofurantoin, Amikacin, Gentamicin, Ceftriaxone and Cefixime but it was also 100% sensitive to Meropenem, Cefotaxime and Chloramphenicol.

Table 7. Antimicrobial susceptible pattern of ESBL producing gram negative bacilli at ALERT Hospital, Addis Ababa, Ethiopia (July-September 2018).

		Antimicrobial agents tested									
Bacterial Isolates	**Patern**	**AMP**	**MER**	**AMK**	**GEM**	**NIT**	**STX**	**CHL**	**CTR**	**CTX**	**CXM**
		No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)
E. coli	**S**	0(0.0)	2(100)	0(0.0)	0(0.0)	2(100)	0(0.0)	2(100)	0(0.0)	1(50.0)	0(0.0)
(n=2)	**I**	2(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(50.0)	0(0.0)	0(0.0)	1(50.0)	1(50.0)
	R	0(0.0)	0(0.0)	2(100)	2(100)	0(0.0)	1(50.0)	0(0.0)	2(100)	0(0.0)	1(50.0)
	S	2(66.7)	3(100)	0(0.0)	0(0.0)	2(66.7)	0(0.0)	2(66.7)	0(0.0)	3(100)	0(0.0)
K. pneumonia	**I**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(33.3)	2(66.7)	0(0.0)	0(0.0)	0(0.0)	3(100)
(n=3)	**R**	1(33.3)	0(0.0)	3(100)	3(100)	0(0.0)	1(33.3)	1(33.3)	3(100)	0(0.0)	0(0.0)
	S	0(0.0)	1(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(100)	0(0.0)	1(100)	0(0.0)
K. oxytoca	**I**	1(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
(n=1)	**R**	0(0.0)	0(0.0)	1(100)	1(100)	1(100)	0(0.0)	0(0.0)	1(100)	0(0.0)	1(100)
	S	2(33.3)	6(100)	0(0.0)	0(0.0)	4(66.7)	0(0.0)	5(83.3)	0(0.0)	5(83.3)	0(0.0)
Total	**I**	3(50.0)	0(0.0)	0(0.0)	0(0.0)	1(16.7)	4(66.7)	0(0.0)	0(0.0)	1(16.7)	4(66.7)
(n=6)	**R**	1(16.7)	0(0.0)	6(100)	6(100)	1(16.7)	2(33.3)	1(16.7)	6(100)	0(0.0)	2(33.3)

AMK**=** amikacin, GEN = gentamicin, CTR = ceftriaxone, CTX = cefotaxime, CXM = cefixime, MER = meropenem, CHL = chloramphenicol, NIT = Nitrofurantoin, AMP = Ampicillin, STX = trimethoprim/sulphamethoxazole., R = Resistant **S** = Sensitive I = Intermediate

6. DISCUSSION

Increased ESBL-E associated with UTI during pregnancy have negative impact on pregnant women and birth outcomes, such as low birth weight, intrauterine growth restriction, fetal death, premature rupture of the membranes and neonatal infections (Loh and Sivalingam, 2007). Studies are needed to determine ESBL-E colonization and infection rates among pregnant women and their determinants in Ethiopia. Thus this study intended to determine the magnitude of Gram negative bacilli producing ESBLs and causing urinary tract infections among pregnant women.

The overall prevalence of UTI in this study was 14.7%. This was a similar finding to the study done in Khartoum North Hospital in Sudan (14.0 %) [Hamdan *et al.,* 2011]. Our result was also similar to the local findings reported previously in Mekelle (11.9%) [Tsegay, 2014 Unpublished study] and Gondar (12%) [Getachew *et al*., 2012]. However, the finding in this study was lower than the study done in Sudan (22%) [Mohammad, 2016 Unpublished study]. The explanation for lower finding of our study than the study done in Sudan by Mohammad, (2016) would be, the participants in the Sudan study were only hospitalized pregnant women which increases the prevalence of UTI due to the occurrence of nosocomial infection in admitted patients.

In the present study, high prevalence of UTI among pregnant women of age greater than 34 (25.0%) were observed which was higher than different local studies (Alemu *et al.*, 2012; Getachew *et al*., 2012) which reported 13.6% and 10.0%, respectively. The discrepancies might be due to difference in sample size. There was no statistical significant association between significant bacteriuria and maternal age, religion, educational status, occupation, gestational age, gravidity and history of UTI. This was in agreement with several studies in Ethiopia [Alemu *et al.,* 2012; Getachew *et al.,* 2012; Tazebew *et al.,* 2012] and Sudan [Hamdan *et al.,* 2011].

In this study, 30.8% of admitted pregnant women had significant bacteriuria while 69.2% of them were negative for significant bacteriuria; and there was a significant association between being out patient or inpatient with UTI (COR = 3.284, 95%CI = 1.248 – 8.641, P = 0.016). Multivariable analysis showed that hospitalization was an independent risk factor for UTI infections. In this study inpatients are almost 5 times more likely to develop UTI infections

than outpatients (AOR = 4.875, 95%CI = 1.628 –14.598, P = 0.005). This was supported by study done in Tikur Anbessa University Hospital (Hailu *et al*., 2017).

In this study the prevalence of bacteriuria in symptomatic infection was 19.4% which was higher than previous studies reported from Ethiopia [Alemu *et al.*, 2012; Tazebew *et al.*, 2012] and study done in Sudan (7.3%) [Mohammad, 2016 Unpublished study]. The possible explanation for this is, most of the study participants in the present study (93.8%) were in the 2^{nd} and 3^{rd} trimester of pregnancy. The risk to acquire bacteriuria during pregnancy increases with gestational age from 0.8% in the 12th week to 1.93% at the end of pregnancy (Bloomberg *et al*., 2005). On the other hand the prevalence of (11.4%) bacteriuria in asymptomatic infection was in line with previous local report from Ethiopia [Assefa *et al.*, 2008] and elsewhere in the world such as in Ghana [Obirikorang *et al*., 2012], but lower than other finding in Sudan (14.7%) [Mohammad, 2016 Unpublished study]. The difference from latter study may be due to difference in study populations. In our study there was no statistical significant association between symptom of UTI with significant bacteriuria (AOR =2.100, 95%CI = 0.827 – 5.328, p=0.119). From 72 pregnant women with symptoms that suggest urinary tract infection, only 14(19.4%) were found to have culture confirmed significant bacteriuria. Symptomatic patients whose urine culture didn't show significant growth might be due to other less frequent UTI causing microorganisms, such as parasites, viruses and fungi [Bonadio *et al.*, 2001].

The predominant etiological agents in our study were Gram negative organisms (57.7%) while Gram positive accounted, for relatively small proportion (42.3%). A previous study done in Mekelle showed similar finding [Tsegay, 2014 Unpublished study]. Reports from Sudan also showed the same findings [Mohammad, 2016 Unpublished study].. This could be due to the presence of unique structure in Gram negative bacteria (pilus adhesions) which help for attachment to the uroepithelial cells and prevent bacteria from urinary lavage, allowing for multiplication and tissue invasion resulting in invasive infection and pyelonephritis in pregnancy.

In our investigation *K. pneumoniae* was the first most common bacterial etiologic agent of UTI which accounted for 26.9%. This finding was in contrast with other study in which *E. coli* was the predominant bacteria [Yitayal *et al*., 2013]. Bacterial etiologies of UTI can show geographic variation and may even vary over time within a population [Mulugeta and Bayeh, 2014]. The second dominant isolate was *S.aures* (23.1 %). This was not supported by report

from Addis Ababa, Tikur Anbessa hospital, (Hailu *et al*., 2017) in which *K. pneumoniae* was the second dominant isolate. Both *E. coli* and *S. epidermidis* were the 3rd common bacteria isolates (15.4%).

The present study revealed that gram negative isolates had shown a higher prevalence rate of resistance to the commonly prescribed antibiotics. Antimicrobial resistance levels for the Gram negative isolates range from 0-100% which was also described by study done in Tikur Anbessa hospital (Hailu *et al*., 2017).

All Gram negative isolates showed resistance to Amikacin and Gentamicin (100%). Different finding was reported from a study done in Gonder University hospital (Alemu *et al*., 2012) in which resistance rate of 7.4% was observed. The level of resistance reported from study in Tikur Anbessa hospital (70.9 %) was higher than report from Gonder University hospital but still it is lower than our investigation (Hailu *et al*., 2017). The possible explanation for lower findings from previous studies in our country as compared to the present study is, Amikacin and Gentamicin are injectable drugs which was not easily available. So, the selective pressure which was created by misuse and over use of this drugs was reduced in earlier time in our country. However, our result showed that the custom of drug administration changed from time to time which could be one of the reasons for production of resistance to these drugs in this study.

Ampicillin, with 73.3% resistance rate, was the second drug that showed high level of resistance following Amikacin and Gentamicin in this study. Higher finding were reported from Gonder with 100% resistance rate (Alemu *et al*., 2012). However, slightly lower result (70%) was reported in a study at Tikur Anbessa hospital (Assefa *et al*., 2008). The discrepancy might be due to difference in drug prescribing practice and etiologic agent across different geographic areas.

Among the drugs, cefotaxime, Nitrofurantoin and Chloramphenicol with resistance rate of 6.7% had a better susceptibility for most of Gram negative bacteria than other drugs tested. Our finding was supported by previous studies done in Ethiopia (Getachew *et al*., 2012, Tazebew *et al.,* 2012), in Tanzania (Rakaa *et al.,* 2004) and Iran (Farajnia *et al.,* 2009).

Meropenems had 100% efficacy against all gram negative bacteria isolated in our study. This was supported by (Agamy *et al*., 2014). However, this result was higher than what had been found in other study which was 26.08% [Sanjay *et al*., 2012]. Since these drugs were not sold

in our country and also they were expensive, this probably had restricted their procurement and indiscriminate use, therefore making the organisms susceptible to it.

Among the Gram negative bacteria the predominant isolates *K. pneumoniae* showed high level of resistance to Amikacin (100%), Gentamicin (100%), Ampicillin (60%) and Sulphamethoxazole-Trimethoprim (60%). This finding was in agreement with the study done in Gondar, Ethiopia [Amare *et al*., 2012] and in Nigeria [Akingbade *et al*., 2013]. Better susceptibility can be achieved using, Meropenem (100%), Nitrofurantoin (93.3%), Chloramphenicol (93.3%) and Cefotaxime (80%), compared to other tested drugs. Based on our finding, we suggests that Meropenem is the primary drug of choice for UTI in pregnancy followed by Cefotaxime, Nitrofurantoin and Chloramphenicol.

In our study overall MDR were observed among 12(80%) of gram negative isolates. This finding was lower than what has been reported from previous study at Tikur Anbessa which was 89.1%, (Hailu *et al*., 2017). The higher record reported from Tikur Anbessa was due to high prevalence of ESBL producing isolates (78.57%). This is explained by the fact that many of the isolates producing these enzymes are also resistant to Quinolones, Trimethoprim and Aminoglycosides; often plasmid has co expression of other resistance mechanisms (Pallett and Hand, 2010).

In this study ESBL producers were found in 50% of gram negative bacilli which was similar with study done at Tikur Anbessa Specialized Hospital where the overall gastrointestinal colonization rate of ESBL producing Enterobacteriaceae in hospitalized patients was 52% (Desta *et al*., 2016). Our finding was higher than previous study done in Harar (33%) (Seid and Asrat, 2005). In addition, our finding was also higher than studies from Nigeria (20%) (Onwuezobe and Orok, 2015), northwestern Nigeria (34.3%) (Giwa *et al*., 2018) and elsewhere such as Iran (40.8%) (Mohammad *et al.,* 2016). On the other hand higher result were reported from Tikur Anbessa hospital in Addis Ababa (78.57%) (Legese *et al*., 2017), Southern Terai of Nepal (63.31%) (Yadav and Prakash, 2017). The finding of higher result from study done in Tikur Anbessa hospital by Legese *et al*., (2017) could be due to incorporation of large proportion of hospitalized study participants (80%) as compared to our study (14.7%).This is true because hospitalization is the risk factor for colonization with ESBL producing bacteria.

This study revealed that *K. pneumoniae* was the most prevalent ESBL producing Isolates (50%). This was in line with study done in Uyo, Nigeria (50%) (Onwuezobe and Orok, 2015). Lower results were obtained by study done in North western Nigeria (40%) (Giwa *et al*., 2018) and local study at Tikur Anbessa Specialized Hospital (32%) (Desta *et al*., 2016). *E. coli* was the second most prevalent ESBL producing Isolates which accounts 33.3%. Greater result was reported from North western Nigeria (50%) (Giwa *et al*., 2018) and previous study at Tikur Anbessa Hospital (68%) (Desta *et al*., 2016). *K. oxytoca* accounted, for 16.7% of ESBL producers which disagrees with study from Uyo in Nigeria where 6% of ESBL producer were *K. oxytoca* (Onwuezobe and Orok, 2015). The variation on ESBL positivity might be due to the number of isolates studied, variation in institution to institution, geographic location and also country to country. The prevalence of ESBL production is high in the referral centers, where the patients are referred from the peripheral centers and where the antibiotic use is profuse. The higher prevalence of ESBL producers in our country compared to western countries can be explained by the fact that western countries have strict infection control policies and practices, efficient and effective antibiotic audit systems, shorter average hospital stays, better nursing barriers and other important health care measures which substantially decrease the chances of acquisition and spread of ESBLs strains. The uncontrolled use of 3rd generation cephalosporin antibiotics at our study hospital could be a leading contributory factor to the high ESBLs prevalence observed in this study.

Among ESBL producing isolates (100%) all were resistant to Amikacin, Gentamicin and Ceftriaxone while intermediate level resistance rate of 66.7% was observed among Sulphamethoxazole-Trimethoprim and Cefixime. Therefore using these drugs for the infection of ESBL producing isolates can result in failer of treatment. Thus the problem of ESBLs have great clinical importance but some clinicians not give good concern yet. The choice of antimicrobial agents effective against ESBLs producing species is currently limited and cause series therapeutic problems in the future (Gabriel *et al*., 2013). In our study most of them were susceptible for some limited drugs and these were Nitrofurantoin (83.3%) and Chloramphenicol (83.3%) as compared to other tested drugs. Ampicillin (33.3%) was also an option which had lowered resistance level of 16.7% however it may not be satisfactory as needed since it had wide range of intermediate level (50.0%) with a tendency of becoming resistant. Meropenem was the 1st choice of drug for ESBL producing bacteria since all isolates responded to this drug which had susceptibility level of 100%. However meropenem is not easily accessible, available and very costy.

The high prevalence of resistance among urinary isolates from ALERT hospital to most commonly used antimicrobial agents suggests the need to perform drug sensitivity test before administering the drug to avoid selective pressure created by inappropriate use of drugs.

STRENGTH AND LIMITATION OF THE STUDY

Strength of the study

- This study shows the magnitude of Extended Spectrum β- Lactamases (ESBLs) producing Gram negative uropathogens among pregnant women
- To the best of our knowledge there was no documented study conducted similar to this in the study site
- It suggests treatment options for ESBL producing Gram negative bacteria especially in the study site

Limitations of the study

- Unavailability of certain drugs used for antimicrobial susceptibility testing

7. CONCLUSION

This study shows that overall prevalence of bacteriuria in pregnant women was 14.7%. In our investigation *K. pneumoniae* was the predominant bacterial etiologic agent of UTI. ESBLs production showed high distribution in Gram negative isolates especially in *K. pneumoniae*. In this study ESBL producers were found in 50% of gram negative isolates. This study revealed that *K. pneumoniae* was the most prevalent ESBL producing Isolates followed by *E. coli*. *K. oxytoca* was the least ESBL producers.

The finding from this study indicates that the highest level of resistance of Gram negatives was observed for Amikacin and Gentamicin with 100% rate of resistance for each. Most of the Gram negatives showed high resistance level for Sulphamethoxazole-Trimethoprim and Ampicillin. Lower resistance level was seen for Cefotaxime, Nitrofurantoin and Chloramphenicol. There was no resistance observed for meropenem (100% sensitive). MDR were observed among most of gram negative isolates. Therefore our study suggests Cefotaxime, Nitrofurantoin, Chloramphenicol and Meropenem as the treatments of choice for UIT in pregnancy with Meropenem the first choice at ALERT hospital. Among ESBL producing isolates (100%) all were resistant to Amikacin, Gentamicin and Ceftriaxone. The present study showed that some of ESBL isolates exhibited co-resistance to other antibiotics including Amikacin, Gentamicin, Sulphamethoxazole-Trimethoprim and Ampicillin. In our study most of them were susceptible for some limited drugs and these were Nitrofurantoin and Chloramphenicol as compared to other tested drugs. All ESBL producing isolates were susceptible for Meropenem. Therefore our study suggests Meropenem as the treatment of choice for ESBL producing isolates causing UTI in pregnant women in our study area.

8. RECOMMENDATIONS

- Genotypic characterization of ESBL producing isolates which causes UTI in pregnant women should be carried out especially in our study area.
- Training about ESBL detection methodologies especially double disk synergy method should be given for all laboratory personnel to help them to be familiar with this easy but valuable methods which aids them to do when it is requested.
- Further and extended investigation on ESBLS producing organisms in pregnant women that includes hospitals, different geographical zones and other population in larger sample size are needed to confirm these findings and assess possible risk factors.

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ANNEXES

Annexes I: Patient information sheet form (English version)

Title of the project:- Phenotypic detection of Extended Spectrum Beta Lactamase in uropathogens among pregnant women attending antenatal care in ALERT hospital, Addis Ababa, Ethiopia.

Purpose:-

We have planned to conduct a study with the objective of determining the magnitude of Extended Spectrum β- Lactamases (ESBLs) producing Gram negative uropathogens among pregnant women attending antenatal care. It is Important to know the type of organisms producing Extended Spectrum β- Lactamases (ESBLs) and their pattern of antimicrobial susceptibility in UTI of pregnant women, and the result of this study is believed to be helpful in appropriate management of urinary tract infection in pregnant women.

Procedures:-

We are asking you and others to participate voluntarily in this study, which would require your response to an interview and to give urine sample for laboratory examination. You will be given instruction how to collect the urine samples in clean/sterile container by health workers.

Risks associated:-

There is minimal risk by participating in the study.

Benefits:-

If there is any positive finding in laboratory examination the result will be reported to your physician for appropriate treatment and management.

Compensation:

There is no Compensation for participating in this study.

Confidentiality:-

Any information that is collected about you will be kept private and in a secured place.

Sharing the result:-

There will be a report which is written about the result of this study either through publication or any other means. The result will not bear any information relevant to your personality in anyway. Your permission is also needed to use the test results for writing a report.

I would also like to inform you that this study is approved by Department Ethical Clearance Committee, School of Medicine, Addis Ababa University.

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የጥናቱ ተሳታፊዎች የመረጃ ቅጽ

የጥናቱ ዓላማ፡ ብዙ መድሀኒት የተላመዱ ባክቴሪያ የተባሉትን ረቂቅ ህዋሳት ሽንት ቱቦ አካላት ላይ የሚያመጡትን ችግርና ስርጭታቸውን ለማጥናትና ለህዋሳቱ ተመራጭ የሆኑት መደሃኒቶች ለመምረጥ ነው፡፡

ፈቃደኝነት፡- እርስዎንና ሌሎችንም በጥናቱ በሙሉ ፍቃደኝነት እንዲሳተፉ እየጠየቅን በጥናቱ በመሳተፍ ፍቃደኛ ከሆኑ ለሚቀርብሌዎትን መጠይቅ ምላሽ ከሰጡ በኋላ የሽንት ናሙና እንዲሰጡ ይጠየቃሉ::

ልያደርስ የሚችለው አደጋ፡- የሚወሰደው ናሙና ሽንት ብቻና እራሰዎ ያለ ምንም ተጨማሪ መሳርያ የሚሰጡ ስለሆነ የሚያመጣዉ ችግር የለም፡፡

የሚያገኙት ጥቅም፡- በሽታ አምጪ ህዋሳት በላቦራቶሪ መኖራቸው ከተረጋገጠ በኋላ ተገቢውን መደሃኒት እንዲወስዱ ውጤቱ ወደ ሀኪምዎ ተልኮ መደኒቱን በሀኪምዎ ትዕዛዝ ይሰጥዎታል፡፡

ማካካሻ፡- በጥናቱ በመሳተፍዎ የሚያገኙት ማካካሻ የለም፡፡

ሚስጥራዊነት፡- የእርስዎ የግል መረጃ በሙሉ ሚስጥራዊነቱ የተጠበቀ ይሆናል፡፡

ውጤቱን ስለመጠቀም፡- ከዚህ ጥናት በኋላ የበሽታውን ስርጭት በተመለከተ ሪፖርት ይፃፋል፡፡ሆኖም የእርስዎን ማንነት የሚገልፅ መረጃ የማይካተት ሲሆን ችግሩን ለማሳወቅ ብቻ የሚውል ነው፡፡

አድራሻ

ማንኛውም ጥያቄ ወይም ጥርጣሬ ካለዎት ይህንን አድርሻ ይጠቀሙ፡

የዋናው ተመራማሪ አድራሻ

ሞላ ጌቴ

ህክምና ፋኩሊቲ አዲስ አበባ ዩኒቨርስቲ ማይክሮባይልጂ፣ኢሚዮኖልጂና ፓራሳይቶልጂ ት/ት ክፍል፡፡

የመ.ሳ.ቁ. 3042 አዲስ አበባ

ስልክ፡-0923534505

ኢሜይል፡- mollagetie2006@gmail.com

ግንቦት 2010

Annexes II: Consent form

Agreement form for data collection from pregnant women (English version)

I, the undersigned, confirm that, I give consent to participate in the study with a clear understanding of the objectives and conditions of the study.

I---------- hereby give my consent for giving the requested information because the proposal has been explained to me in the language I understand.

I ---------- hereby also agree to give urine sample.

Name of the participant---------- Signature ----

Date----------

Name of the witness---------- Signature ----

Date----------

Name of the researcher---------- Signature ----

Date----------

May 2018

የፈቃደኝነት መጠየቂያ ቅፅ

እኔ/ ተማሪ/ አቶ/ ወ.ሮ/ ወ.ት_____ የተባልኩ ለምርምሩ የሚያስፈልጉ መጠይቆችን መረጃና የሽንት ናሙና ለመስጠት በሚገባኝ ቋንቋ የተብራራልኝ በመሆኑ በጥናቱ ለመሳተፍ በሙሉ ፍቃዴ የተስማማሁ መሆኔን በፊርማዬ አረጋግጣለሁ፡፡

የተሳታፊዋ ስም_____ የተሳታፊዋ ፊርማ _____

ቀን_____

የእማኝ ስም _____ የእማኝ ፊርማ _____

ቀን_____

የተመራማሪው ስም_____ የተመራማሪው ፊርማ_____

ቀን_____

ግንቦት 2010

Annex III: Questionnaire

This questioner will be filled by the health care worker at ANC while the sample is collected from ANC attending pregnant women. Health care worker fills this questioner by asking pregnant women who are selected as study participants. This information is only used for the study and the information will be kept confidentially. Mark or write on the space provided. For any question please use the address below

Principal investigator: Molla Getie

Mobile. No 0923534505 E-mail: mollagetie2006@gmail.com Date: ----------

I. Personal/ socio-demographic data		
1	ID No	
2	Hospital card number	
3	Age	
4	Marital status	A. Single □ C. Divorced □ B. Married □ D. Widowed □
5	Residence	A. Urban □ B. Rural □
6	Religion	A. Orthodox □ C. Others □ B. Muslim □
7	Patient type	A. In patient □ B. Out patient □
8	Educational status	A. Illiterate □ B. literate □
9	Occupational status	A. House wife □ C. Student □ B. Merchant □ D. Government employee □
10	Gestational period	A. Frist □ B. Second □ C. Third □
11	Gravidity	A. Primigravidae □B. Meltigravidae □
12	History of UTI	A. Yes □ B. No □

II. Clinical data

	Clinical symptoms	Yes	No
1	Dysuria	□	□
2	Fever	□ To ( )	□
3	Urgency	□	□
4	Frequency	□	□
5	Supra pubic pain	□	□

መጠይቅ

ይህ መጠይቅ መረጃ በሚሰበስቡ የጤና ባለሙያዎች የቅድመ- ወሊድ ክትትል በሚሰጥበት ክፍል የሚሞላ ይሆናል። በመጀመሪያ ጥናቱን በተመለከተ በቂ ማብራሪያ በመረጃ ሰብሳቢዎች ይሰጥዎታል። በቂ ማብራሪያ ካገኙ በኃላ በጥናቱ ለመሳተፍ ፈቃደኛነትዎ ይጠየቃል። ፈቃደኛ ከሆኑ መጠይቁን ይጠየቃሉ። መጠይቁ ካለቀ በኃላ የሽንት ናሙና እንዲሰጡ ይጠየቃሉ። የሽንት ናሙና ከመስጠትዎ በፊት በመረጃ ሰብሳቢዎች በቂ ማብራሪያይሰጥዎታል።ማብራሪያዉን በደንብ ካዳመጡ በኃላ ናሙና ይሰጣሉ። ይህ መረጃም ለጥናት ብቻ የሚዉል ይሆናል፤እንዲሁም የመረጃው ሚስጥራዊነት የተጠበቀ ይሆናል። ማንኛዉም አይነት ጥያቄ ካለዎት ከታች የተጻፈውን አድራሻ ይጠቀሙ።

ዋናው ተመራማሪ: ሞላ ጌቴ

ስልክ: 0923534505

ኢሜይል: mollagetie2006@gmail.com

ስለትብብርዎ እናመሰግናለን።

1. ማሕበራዊ ሁኔታ		
1	መለያ ቁጥር	
2	የሆስፒታሉ ካርድ ቁጥር	
3	እድሜ	
4	የጋብቻ ሁኔታ	ሀ. ያላገባች □ ሐ. ያገባች □ ለ. አግብታ የፈታች □ መ. ባል የሞተባት □
5	መኖሪያ ቦታ	ሀ. ገጠር □ ለ. ከተማ □
6	ሀይማኖት	ሀ. ኦርቶዶክስ □ ሐ. ሌላ □ ለ. ሙስሊም □
7	የታማሚ አይነት	ሀ. ተመላላሽ □ ለ.ተኝቶ የሚታከም □
8	የትምርት ሁኔታ	ሀ. የተማረች □ ለ. ያልተማረች □
9	የስራ ሁኔታ	ሀ. የቤት እመቤት □ ሐ. ተማሪ □

		ለ. ነጋዴ □ መ. የመንግስት ሰራተኛ □
10	የርግዝና ጊዜ	ሀ. የመጀመሪያ 3 ወር □ ለ. ሁለተኛ 3 ወር □ ሐ. ሶስተኛ 3 ወር □
11	ለስንት ጊዜ ጸንሰው ያውቃሉ?	ሀ. ለመጀመሪያ ጊዜ □ ለ. ለሁለተኛና ከዚያ በላይ □
12	ከዚህ በፊት በሽንት ቱቦ በሽታ ታመው ያውቃሉ?	ሀ. አዎ □ ለ. አይደለም □

2. የበሽታው ምልክቶች			
	የበሽታው ምልክቶች	እወ	እይደለም
1	በሚሸኑበት ጊዜ የህመም ስሜት ይሰማዎታል?	□	□
2	ትኩሳት አለብዎ ?	□ To ( )	□
3	ሽንት የመቆጣጠር ችግር አለብዎ ?	□	□
4	ቶሎ ቶሎ ሽንት ቤት ይመላለሳሉ?	□	□
5	ከሰዉነትዎ ጎን አካባቢ የህመም ስሜት ይሰማዎታል?	□	□

Annexes IV: Laboratory procedures

Urine sample collection

The first urine passed by the patient at the beginning of the day should be sent for examination. This specimen is the most concentrated and therefore the most suitable for culture, microscopy, and biochemical analysis.

Mid-stream urine (MSU) for microbiological examination is collected as follows:

1. Give the patient a sterile, dry, wide-necked, leak proof container and request a 10–20 ml specimen. Wash the hands. Cleanse the area around the urethral opening with clean water, dry the area with a sterile gauze pad, and collect the urine with the labia held apart.
    - ***Important:*** Explain to the patient the need to collect the urine with as little contamination as possible, i.e. a clean-catch specimen.
2. Label the container with the date, Identification number of the patient and time of collection.
3. As soon as possible, deliver the specimen with a request form to the laboratory. When immediate delivery to the laboratory is not possible, refrigerate the urine at 4–6 °C. When a delay in delivery of more than 2 hours is anticipated, add boric acid preservative to the urine.

Gram staining technique

Method

1. After making a smear, leave the slide in a safe place for the smear to air-dry then fixed by heat, alcohol, or occasionally by other chemicals.
2. Cover the fixed smear with crystal violet stain for 30–60 seconds.
3. Rapidly wash off the stain with clean water. *Note*: When the tap water is not clean, use filtered water or clean boiled rainwater.
4. Tip off all the water, and cover the smear with Lugol’s iodine for 30–60 seconds.
5. Wash off the iodine with clean water.

6. Decolorize rapidly (few seconds) with acetone–alcohol. Wash immediately with clean water.

   Caution: Acetone–alcohol is highly flammable; therefore use it well away from an open flame.

7. Cover the smear with neutral red stain for 2 minutes.

8. Wash off the stain with clean water.

9. Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry.

10. Examine the smear microscopically, first with the 40x objective to check the staining and to see the distribution of material, and then with the oil immersion objective to report the bacteria and cells.

Biochemical tests

Catalase test procedure

Principle

Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more than 24 hours old.

Required

Hydrogen peroxide, 3% H_2O_2 (10 volume solution)

Method

1. Pour 2–3 ml of the hydrogen peroxide solution into a test tube.
2. Using a sterile wooden stick or a glass rod (not a nichrome wire loop), remove several colonies of the test organism and immerse in the hydrogen peroxide solution.

   - ***Important****:* Care must be taken when testing an organism cultured on a medium containing blood because catalase is present in red cells. If any of the blood agar is removed with the organism, a false positive reaction may occur.

3. Look for immediate bubbling.

Results

Active bubbling . . . . . Positive catalase test

No bubbles . . . . . Negative catalase test

Coagulase test procedure

Principle

Coagulase causes plasma to clot by converting fibrinogen to fibrin.

Required

EDTA anti- coagulated human plasma (preferably pooled and previously HIV and hepatitis tested) or rabbit plasma. The plasma should be allowed to warm to room temperature before being used.

Slide test method (detects bound coagulase)

1. Place a drop of distilled water on each end of a slide or on two separate slides.
2. Emulsify a colony of the test organism (previously checked by Gram staining) in each of the drops to make two thick suspensions and mix gently, look for clumping.

   Note: Colonies from a Mannitol salt agar culture are not suitable for coagulase testing. The organism must first be cultured on nutrient agar or blood agar.

3. Add a loop full (not more) of plasma to one of the organisms within 10 seconds. No plasma is added to the second suspension. This is used to differentiate any granular appearance of the organism from true coagulase clumping.

Results

Clumping within 10 seconds . . . . . *S. aureus*

No clumping within 10 seconds . . . . . no bound coagulase

Indole test

Testing for indole production is important in the identification of enterobacteriaceae. Most strain of *E. coli, P. vulgaris, P. rettgeri, M. morganii,* and *Providencia* species break down the amino acid tryptophan with the release of indole.

Principle

The test organism is cultured in a medium which contains tryptophan. Indole production is detected by Kovac's or Ehrlich's reagent which contains 4 (p)-dimethylaminobenzaldehyde. This reacts with the indole to produce a red colored compound. The indole test also can be performed by culturing the organism in tryptone water or peptone water containing tryptophan, and detecting indole production by adding Kovac's or Ehrlich's reagent to an 18-24 h culture. Kovac‟s reagent is recommended in preference to Ehrlich's reagent for the detection of indole from enterobacteriaceae.

Method

1) Transfer about 1ml of the test organism (tryptone water) into test tube.
2) Add 3-5 drops of Indole reagent (modified kovac‟s reagent).

Results

Positive test: Red color

Negative test: No red color

Urease test (Christensen's (modified) urea broth): Urea agars will be inoculated heavily over the entire surfaces of the slants in bijou bottles. The cap will be loosened and then incubated at 37^{o}C for 3-12 hours. A urease-positive culture produces an alkaline reaction in the medium, evidenced by pinkish red color of the Medium. Urease-negative organisms do not change the color of the medium, which is pale yellow-pink.

Triple Sugar Iron (TSI) Agar Slant: Using a sterile inoculating needle, stab the butt of the TSI slant twice then streak back and forth along the surface of the agar with the organism. Incubate at 37◦C for 18 to 24 h. If acid slant–acid butt (yellow–yellow): glucose and sucrose and/or lactose fermented. If alkaline slant–acid butt (red–yellow): glucose fermented only. If alkaline slant–alkaline butt (red–red): glucose not fermented. The presence of black precipitate (butt) indicates hydrogen sulfide production, and presence of splits or cracks with air bubbles indicates gas production.

Citrate utilization test using Simmon's citrate agar: Simmon‘s citrate slopes will be prepared in bijou bottles as recommended by the manufacturer (stored at 2-8^{o}C). And the slopes will be then stabbed and incubated at 37^{o}C aerobically for 48 hours. Blue color indicates a positive

reaction and if Simmon‘s citrate agar slopes remained as green in color indicate negative reaction.

Motility Test (using motility agars): Motility agar will be prepared and inoculated with a straight inoculating needle making a single stab about 1-2cm down into the medium. The motility will be examined after 35-37oC for 24 hour. Motility will be indicated by the presence of diffuse growth (appearing as coloring of the medium) away from the line of inoculation.

Lysine decarboxylase: Decarboxylation of lysine can be detected by culturing bacteria in a medium containing the desired amino acid, glucose, and a pH indicator bromcresol purple. The acids produced by the bacteria from the fermentation of glucose will initially lower the pH of the medium and cause the pH indicator to change from purple to yellow. The acid pH activates the enzyme that causes decarboxylation of lysine to amines and the subsequent neutralization of the medium. This results in another color change from yellow back to purple. Bacteria that decarboxylate lysine turn the medium purple. In addition bacteria that produce H2S appear as black colonies.

Modified Kirby-Bauer susceptibility testing technique

Method

1) Using a sterile wire loop, touch 3–5 well-isolated colonies of similar appearance to the test organism and emulsify in 3–4 ml of sterile physiological saline or nutrient broth.

2) Match the turbidity of the suspension to the turbidity standard (mix the standard immediately before use).

3) Using a sterile swab, inoculate a plate of Mueller Hinton agar. Remove excess fluid by pressing and rotating the swab against the side of the tube above the level of the suspension. Streak the swab evenly over the surface of the medium in three directions, rotating the plate approximately 60^{o} to ensure even distribution.

4) With the Petri dish lid in place, allow 3–5 minutes (no longer than 15 minutes) for the surface of the agar to dry.

5) Using sterile forceps, needle mounted in a holder, or a multidisc dispenser, place the appropriate antimicrobial discs, evenly distributed on the inoculated Mueller Hinton agar.

   Note: The discs should be about 15 mm from the edge of the plate and no closer than about 25 from disc to disc. No more than 6 discs should be applied (90 mm dish). Each disc should be lightly pressed down to ensure its contact with the agar. It should not be moved once in place.

6) Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35^0C for 16–18 h (temperatures over 35^0C invalidate results for oxacillin).

7) After overnight incubation, examine the control and test plates to ensure the growth is confluent or near confluent. Using a ruler on the underside of the plate, measure the diameter of each zone of inhibition in mm. The endpoint of inhibition is where growth starts.

Table 8 ESBL Disc screening recommendation by CLSI (CLSI, 2018).

CLSI Recommended		
Antibiotic Disc		Conduct ESBL confirmation testing if
Cefotaxime	CTX 30 μg	Inhibition zone ≤ 27 mm
Ceftriaxone	CTR 30 μg	Inhibition zone ≤ 25 mm
Ceftazidime	CAZ 30 μg	Inhibition zone ≤ 22 mm
Aztreonam	ATM 30 μg	Inhibition zone ≤ 27 mm
Cefpodoxime	PX 10 μg	Inhibition zone ≤ 22 mm

ESBL Disk confirmation test

Double Disk Synergy Test (DDST)

Method

1) Test organisms (suspected of ESBL production) were cultured overnight on nutrient agar.

2) Using a fresh, pure culture prepare a suspension of the test organism equal to 0.5 McFarland Standard.

3) Using a sterile cotton swab, spread the adjusted suspension over the entire area of a Mueller Hinton agar plate.

4) Amoxicillin (20 µg)/ clavulanic acid (10 µg) combination disc was placed at the center of each inoculated Mueller Hinton agar plate.

5) Cefotaxime (30 µg), ceftazidime (30 µg), aztreonam (30µg) cefpodoxime (10 µg) and Ceftriaxone, (30 µg) single discs were then placed 20 mm (center to center) from the amoxicillin/clavulanic acid disc.

6) Incubate at 35±2°C for 18-24 hours.

Positive test: Enhancement of the zones of inhibition of any of the cephalosporin beta-lactam antibiotic discs (i.e. cefotaxime, aztreonam, cefpodoxime or ceftazidime) towards the amoxicillin/clavulanic acid disc caused by the synergy with clavulanate was taken as an evidence of ESBL production.

Dummy table. AST for gram negative bacteria

Antibiotic disc	Susceptible	Intermediate	Resistance
Ampicillin			
Amikacin			
Gentamicin			
Meropenem			
Nitrofurantoin			
Cefixime			
Ceftriaxone			
Cefotaxime			
Chloramphenicol			
Trimethoprim sulphamethoxazole			

DECLARATION

I, the undersigned, declare that this M.Sc. Research thesis is my original work, has not been presented for a degree in other university and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. candidate Molla Getie

Signature _____

Date and place of submission _____

Addis Ababa, Ethiopia

Advisor _____

Signature _____

Date and place of submission _____

Addis Ababa, Ethiopia

Advisor _____

Signature _____

Date and place of submission _____

Addis Ababa, Ethiopia