TITLE PAGE

INCIDENCE OF ASYMPTOMATIC BACTERIURIA AMONG PREGNANT WOMEN IN SABON-GARI LGA – ZARIA, NIGERIA AND THE ANTIMICROBIAL SUSCEPTIBILITIES OF THE ISOLATES

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DEPARTMENT OF PHARMACEUTICS AND PHARMACEUTICAL MICROBIOLOGY,
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MARCH, 2013

DECLARATION

I, **Rebecca Olajumoke BOLAJI,** hereby declare that the research work and written up in this dissertation was carried out by me under the supervision of Professor J.A Onaolapo and Professor Y.K.E. Ibrahim and Professor J.O. Ehinmidu of the Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria-Nigeria.

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CERTIFICATION

This project dissertation titled INCIDENCE OF ASYMPTOMATIC BACTERIURIA AMONG PREGNANT WOMEN IN SABON-GARI LGA – ZARIA, NIGERIA AND THE ANTIMICROBIAL SUSCEPTIBILITIES OF THE ISOLATES meets the regulations governing the award of the degree of DOCTOR OF PHILOSOPHY (PHARMACEUTICAL MICROBIOLOGY) OF THE Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

TO THE IMMORTAL, INVISIBLE, THE ONLY WISE GOD BE GLORY, HONOUR AND ADORATION NOW AND FOR EVERMORE.

To the ever green memory of my beloved

Mrs Maria Mopelola Ojo

Dr (Mrs) Mary Olabanke Owonubi

Mr. Isaac Sowole Sowemimo Coker

Miss Lydia Kehinde Omoyeni Ojo

Professor Samuel Olusegun Alabi

Akpulu Ignatius Nnaemeka

May their gentle loving souls, rest in the bosom of our Lord Jesus, till we meet to part no more.

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ABSTRACT

Urinary tract infection (UTI) occurs frequently in pregnancy with a large number of infections being asymptomatic. This study was therefore conceived to determine the incidence of asymptomatic bacteriuria in pregnant women in Sabon- Gari LGA – Zaria, Nigeria using questionnaire as well as isolate and characterize any bacteria present in urine of apparently healthy pregnant women and to determine the antibiogram of such isolates.

Urine samples from two hundred and fifty (250) apparently healthy pregnant women, who were attending ante-natal clinics in Sabon-Gari Local Government Area Zaria, Nigeria, were interviewed and urine samples were collected for isolation of any possible bacteria present. Isolation was done by culturing on standard laboratory media and identification of isolates were subsequently done using standard biochemical tests and confirmed using API (bioMerieux, USA) rapid kits. The antibiogram of the isolates were determined using selected antibiotics and using disc agar diffusion technique. Plasmid isolation using gel electrophoresis was carried out on selected isolates that were multiple antibiotics resistant. Molecular characterization using multiplex Polymerase Chain Reaction (PCR) was carried out to detect nature of resistance in the resistant isolates. Primers such as SHV, OXA, TEM, aaclla, gyrA and gyrB, that codes for resistant genes for aminoglycosides, penicillins, cephalosporins and fluoroquinolones were used. Tests were also carried out to detect the production of β-lactamase and extended spectrum β-lactamase enzymes in the resistant isolates.

A total of 146 (58.4%) had levels of bacteria indicative of asymptomatic bacteriuria. Higher incidences were found among 21-30 years age group 92 (36.8%), those in

their second trimester 77 (30.8%) and among third parity (number of pregnancy) patients. Standard biochemical tests revealed the presence of 197 isolates comprising of *Staphylococcus spp* 50 (20.0%), *Klebsiella spp* 49 (19.6%), *Proteus spp* 46 (18.4%), *Escherichia spp* 33 (13.2%), *Pseudomonas spp* 18 (7.2%) and *Streptococcus* spp 1 (0.4%). Using API Staph kits a total of 20 *Staphylococcus* species made up of *Staph. aureus*, *Staph. lentus*, *Staph. xylosus*, *Staph. cohnii*, *Staph. auriculous, Staph. epidermidis, Staph. heamolyticus* and *Micrococcus spp* 4(10.3%) were identified. The API 20E kit revealed the presence of 22 Enterobacteriaceae made up of *Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii*, *Citrobacter braakii*, *Providencia rettgreri*, *Klebsiella pneumonia* while API 20NE kit revealed the presence of *Pseudomonas aeruginosa*, *Aeromonas aerohydrophila/caviae*, *Pseudomonas leutola*, *Burkholderia pseudomallei*.

The susceptibility profile of the isolates to selected antibiotics shows that all the isolates were resistant to the β-lactam antibiotics (Ampicillin (AMP) and Methicillin (MET) but relatively sensitive to Ofloxacin (87.5%), Gentamicin (71.4%), Nitrofurantoin (69.4%), and Ciprofloxacin (87.0%). A large number of the isolates were multiple antibiotics resistant. Some of the isolates were β-lactamase and Extended Spectrum β-lactamases (ESBLs) producers.

Molecular characterization of the plasmid DNA showed the presence of resistant gene determinants that code for resistance to ESBLs.

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CHAPTER ONE

INTRODUCTION

1.1 URINARY TRACT INFECTION (UTI)

Urinary tract infection (UTI) occurs frequently in pregnancy with a large number of infections being asymptomatic. The frequency of asymptomatic bacteriuria (ASB) in pregnancy accounts for the rationale of urinalysis throughout pregnancy period (Stamm and Hooton, 1993).

Considered clinically unimportant in non-pregnant women, asymptomatic bacteriuria is the most common complications of pregnancy, occurring in 4-7% of normal pregnancies. It can be associated with varieties of adverse obstetric outcome and medical conditions such as the development of acute and chronic pyelonephritis, pre-term labour, low birth weight, pre-eclampsia, chronic renal disease, and prenatal mortality (Naeye *et al.*, 1986; Stenqvist *et al.*, 1989; Mittendorf *et al.*, 1992). Many of the complications of asymptomatic bacteriuria in pregnancy can be prevented with early identification and treatment.

Urine is the product of filtration of blood excreted by the kidneys which generally consist of water and water soluble waste products which have been removed from the blood stream. The urine of normal healthy human being is composed of approximately 95% water and the rest are nitrogeneous metabolites (urea, uric acid, and creatinine), sodium chloride, and other substances in trace amounts. It is amber in colour with pH ranging from 4.5-8.0. The average adult passes about 1.419 litres of urine daily, although this volume may vary with individual's liquid intake and health conditions (e.g. pregnancy and diabetes) due to changes in the anatomical and physiological structure.

1.2 ASYMPTOMATIC BACTERIURIA

Asymptomatic is a condition in which urine of an apparently healthy individual reveals a significant number of bacterial pathogens that is greater than 10^5cfu/ml but without the patient showing symptoms of urinary tract infection, such as burning during micturition or frequent urination (Imade *et al*., 2010). This is common during pregnancy (Gilbert *et al*., 2005). The apparent reduction in the immunity of pregnant women appears to encourage the growth of both commensal and non-commensal micro-organisms (Scott *et al*., 1990). The physiological increase in plasma volume during pregnancy decrease urine concentration up to 7%. Pregnant women develop glucosuria, which encourage bacterial growth in the urine (Patterson and Andriole, 1987; Lucas and Cunningham, 1993).

Pregnancy enhances the progression from asymptomatic to symptomatic bacteriuria which could lead to pyelonephritis and adverse obstetric out comes such as premature birth, low birth weight (Connolly and Thorp, 1999) and higher foetal mortality rates (Nicolle, 1994; Delzell and Lefevre, 2000). Acute pyelonephritis, foetal growth restriction and still birth in pregnant women have been associated with asymptomatic bacteriuria (Ryan *et al*., 1990; Zhao and Wu, 2004; Hill *et al*., 2005). Asymptomatic bacteriuria has also been implicated in post partum endometritis (Lindsay, 2003). Bacteria are typically introduced into the urinary tract during intercourse or when wiping after a bowel movement (Dominic, 2006). The bacterium *E. coli* is responsible for at least 75-80% of asymptomatic bacteriuria. *Klebsiella pneumonia*, *Proteus spp., Enterococci* and group *B Streptococci* can also establish colonization. Since asymptomatic bacteriuria occurs without symptoms, it is important to know what increases infection risk. This can help avoid the consequences of untreated asymptomatic bacteriuria by early detection and treatment. Researchers have

identified a number of risk factors which include; immunosuppressive disorders, urinary tract obstructions (from stones) loss of bladder control due to neuromuscular disease, need for chronic instrumentation of the bladder (self-catheterization).

1.3 STATEMENT OF RESEARCH PROBLEM

Urine is normally sterile (i.e. contains no bacteria), but small amount of bacteria may be found in the urine of many healthy people which are usually considered to be harmless. However, certain level of bacteria can mean that the bladder, urethra or kidney is infected and colonized. Urine is the specimen most frequently submitted for medical analysis. It is used to detect and identify organisms that may be causing urinary tract infection. It also presents major problems in terms of proper specimen collection, transportation, culture techniques and inter-relations of results (Vandepitte *et al.*, 1991). Analysis of urine is commonly used in the diagnosis of diseases such as diabetes and urinary tract infection.

Bacteriuria implies the presence of significant number of bacteria in urine which indicate that there is an infection somewhere in the urinary tract (kidneys, ureter bladder or urethra). The presence of bacteria may be detected with microbiological analysis, though a number of other techniques have been employed in routine analysis. Concerning the etiology of bacteriuria, most organisms recovered by routine culture techniques continue to be coliform bacteria with *E. coli* being the most common (Uncu *et al.,* 2002; Al-Haddad, 2005; Akinloye *et al.,* 2006; Khattak *et al.,* 2006; Kutlay *et al.,* 2006; Hazhir, 2007).

Asymptomatic bacteriuria has been defined as a condition in which there is a large or significant number of bacteria (at least 10^5cfu/ml) that can be detected in urine

without any of the usual symptoms of urinary tract infection. Asymptomatic bacteriuria is most common in women and in people with diabetes, the elderly and those with indwelling catheters. Asymptomatic bacteriuria occurs in up to 6% of healthy individuals. It affects 18% of people with diabetes (especially women) and 20% of the elderly (often more women than men) and does not require treatment except in high risk groups like diabetics, the elderly and pregnant women (Kinningham, 1993). Asymptomatic bacteriuria usually clears up after antibiotic treatment, but can reoccur, in some people and if not treated, it can progress to full-blown urinary tract infection. In others, it can continue for years without causing obvious illness or discomfort.

Prevalence rates of asymptomatic bacteriuria vary from study to study for example, 6% (Kass, 1962, in MacLean, 2001), 5.3% (Little, 1966), 4.4% (Gruneberg, 1969), 4.6% (Lawson, 1971), 2.6% (Campbell-Brown *et al,* 1987, in MacLean, 2001), 12% (Chng *et al,* 1980), 8.3% (Robertson and Duff, 1988), 7.6% (Hagay *et al,* 1996), 2.5% (Sheiner *et al,* 2009) and 15% (Mignini *et al,* 2009). The causes of asymptomatic bacteriuria are unclear but it is more frequent in diabetics and women of low socioeconomic status and can vary by ethnic group, for example Versi *et al* (1997) found a rate of 6.3% in White women and 2.0% in Bangladeshi women. Bailey (1972) reported a rate of 2% in private clinic patients and 18.5% in urban Maori women. In Ethiopia, a prevalence of 7% (Gabre-Selassie, 1998), while Ghana reported a rate of 7.3% (Turpin *et al.,* 2007), Iran had a rate of 6.1% and 8.9% (Hazhir, 2007; Enayat, *et al.,* 2008) and Uganda has reported an incidence rate of 13.3% (Andabati and Byamugisha, 2010). In Nigeria however, studies have been conducted in different parts of South-east region where the incidence of asymptomatic bacteriuria was found to be 18.2%, 23.9% and 78.7% respectively (Olusanya, *et al.,* 1993; Amadi *et al.,*

2007; Oli *et al,* 2010), South-west region 21%, 26%, 47.5% and 19.5% respectively (Aboderin *et al,* 2004; Ogbolu *et al,* 2006; Okonko *et al.,* 2009; Taiwo *et al.,* 2009), South-south region 86.6% and 58% respectively (Akerele *et al.,* 2001; Onifade *et al.,* 2005), and North-west region 8% (Omole-Ohonsi and Nwokedi, 2009).

The most commonly identified organisms by earlier reported researchers include *E. coli,* with percentage range of 4-51.6% (Akerele *et al.,* 2001; Ogbolu *et al.,* 2006; Daniyan, *et al.,* 2010; Ajayi *et al.,*2012), *Staph. aureus* 4.8-72% (Daniyan and Abalaka, 2010; Amadi *et al,* 2007; Ajayi *et al,* 2012), *Proteus spp* 3.6 - 14% (Oyetunji *et al.,* 2005; Oli *et al.,* 2010; Ajayi *et al.,* 2012) *Klebsiella spp* 4 - 33.3% (Akerele *et al.,* 2001; Ogbolu *et al.,* 2006; Daniyan and Abalaka, 2010; Ajayi *et al.,* 2012). Other implicated organisms include some *Coliforms, Streptococcus,* and *Ps. aeruginosa*.

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 (Abraham and Chain, 1940). The age of penicillin saw the rapid emergence of resistance in *Staph. aureus* due to a plasmid-encoded penicillinase. This β-lactamase quickly spread to most clinical isolates of *Staph. aureus* as well as other species of *Staphylococci*.

Many genera of Gram-negative bacteria possess a naturally occurring, chromosomally mediated β-lactamase. 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 (Ghuysen, 1991). The first plasmid-mediated β-lactamase in Gram-negatives, TEM-1, was described in the early 1960s (Datta and Kontomichalou, 1965). The TEM-1 enzyme was originally found in a single strain of *E. coli* isolated

from a blood culture of a patient named Temoniera, in Greece (Medeiros, 1984). Being plasmid and transposon mediated, the spread of TEM-1 to other bacteria within a few years worldwide of its isolation has been rapid. TEM-1 β-lactamase has spread worldwide and is now found in many different species of members of the Family *Enterobacteriaceae, Ps. aeruginosa, Haemophilus influenzae, and Neisseria gonorrhoeae.* Another common plasmid-mediated β-lactamase found in *K. pneumoniae* and *E. coli* is SHV-1 (sulphydryl variable gene) which is chromosomally encoded in the majority of isolates of *K. pneumonia* but is usually plasmid mediated in *E. coli.*

Extended-spectrum β-lactamase (ESBLs) was first identified in the early 1980s Hamamami *et al,* 1991). Since then, they have been identified worldwide and have been found in a number of different organisms including *K. pneumoniea, E. coli, Pr. mirabilis,* and *Salmonella spp*. (Phillipon *et al.,* 1994; Thomson *et al.,* 1996; Jacoby, 1997). These strains are widespread throughout the world, but the prevalence and phenotypic characteristics of clinical isolates vary from area to area (Coque *et al.,* 2008; Falagas and Karagorgopoulous, 2009). Extended-spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum cephalosporins, such as cefotaxime, ceftriaxone, ceftazidime and aztreonam (Jacoby and Medeiros, 1991; Livermore, 1995; Medeiros, 1997; Diaz *et al.,* 2006; Ogunleye and Carlson, 2011; Tumbarello *et al,* 2011).

Several studies suggest that infections caused by ESBL-producing bacteria have an important clinical impact, and the increasing prevalence of these organisms in hospitals has been well documented (Giske *et al.,* 2008; Falagas and Karagorgopoulous, 2009; Tumbarello *et al.,* 2010). Recently they have been reported to cause urinary tract and bloodstream infections in non-hospitalized patients

(Apisarnthanarak *et al.,* 2008; Kang *et al.,* 2008; Laupland *et al.,* 2008; Rodríquez-Baño *et al.,*2008; Ben-Ami *et al.,* 2009; Friedman *et al.,* 2009; Azap *et al.,* 2010; Hawser *et al.,* 2010; Rodríquez-Baño *et al.,* 2010).

1.4 JUSTIFICATION FOR THE STUDY

Undiagnosed and untreated bacteriuria in pregnancy represents a considerable risk factor for acute pyelonephritis in approximately 5% of pregnant women with increased risk of low birth weight and prenatal fatal death (Hazhir, 2007). Detection and treatment of bacteriuria has been reported to prevent up to 80% cases of pyelonephritis (Schnarr and Smaill, 2008). The risk to acquire bacteriuria during pregnancy increases with gestational age from 0.8% in the 12^{th} week to 1.93% at the end of pregnancy. Bacteriuria in pregnancy usually reflects prior colonization e.g. at the time of onset of sexual activity rather than acquisition during pregnancy itself (Smaill and Vanquez, 2007. Although asymptomatic bacteriuria may not require treatment because the bacteria may not be causing harm, certain group of people such as pregnant women and their unborn feotuses may be at risk of complications (Guyton, 1996; Lindsay, 2003; Bloomberg *et al.*, 2005). Furthermore, there is obviously a substantial risk that the infecting pathogens are becoming resistant to empirically prescribed antimicrobial agents normally used in the community.

1.5 AIM OF THE STUDY

The main aim of this study is to determine the incidence of asymptomatic bacteriuria in pregnant women in Sabon-Gari Local Governmet Area-Zaria, Nigeria with the view of assessing their overall health status.

1.6 OBJECTIVES OF THE STUDY

a) To determine the incidence of asymptomatic bacteriuria in pregnant women in Sabon-Gari Local Government Area-Zaria, Nigeria by administering questionnaire

b) To isolate and characterise possible bacteria organisms from urine using standard bacteriological methods and rapid identification test kits.

c) To determine the antibiogram of isolated bacteria by the agar diffusion techniques.

d) To determine the presence of β-lactamase and Extended β-lactamases (ESBLs)

e) To characterise the resistant genes in some of the isolates by the plasmid DNA analysis using Polymerase Chain Reaction (PCR) and analysing by gel electrophoresis.

1.7 NULL HYPOTHESIS

H0 1) There is no incidence of asymptomatic bacteriuria among pregnant women in Sabon-Gari LGA- Zaria, Nigeria.

2) Bacteria isolated from asymptomatic pregnant women are very sensitive to at least 90% of antimicrobial agents used in the study area.

1.8 LIMITATIONS

i) Sampled population is limited to only pregnant women that visited Sabon-gari and Samaru Family Health Centres from February to April, 2011

ii) Only subjects with urine microbial levels > 10^5cfu/ml were included in the study.

iii) Retrospective analysis of incidence of bacteriuria in pregnant women in the health centres could not be carried out due to lack of records on patients.

iv) Follow up study was not carried out due to cultural practices that hinder contact with subjects outside the health centres.

v) Questionnaires were administered through the Nursing officials and interpreted to the un-educated Subjects. Some of the Subjects were uncooperative in giving their age and parity status

CHAPTER TWO

LITERATURE REVIEW

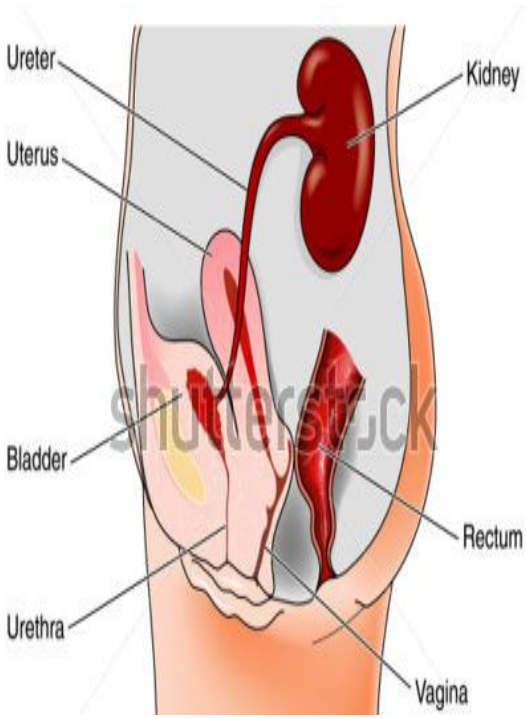
2.1 URINARY TRACT INFECTION (UTI)

The urinary tract is a passage from the urethra to the kidneys. Any form of infection along this tract is referred to as urinary tract infection. It could be upper (pyelonephritis) or lower (cystitis) tract infection. In the kidney is called pyelonephritis; in the bladder, cystitis; and in the urethra, urethritis. Urinary tract infections may result from direct or indirect infection; direct infection involves introduction of microorganisms via the use of indwelling catheters, unhygienic practices or from sexual partners. Indirect infection may be from hematogenous spread of pathogenic organisms from other infected organs or tissues (Jawetz *et al.*, 1982).

Blood, lymph and urethra are the three major routes by which bacteria can gain access to urinary tract. The heamatogenous and lymphatic routes are relatively uncommon. The primary route by which bacteria gain access to the urinary tract is the intraluminal ascent of the urethra. This ascent from the urethra to the bladder can be the result of bacteria migration, urinary uretho-vesical reflux or mechanical trauma. Poor micturition habits such as incomplete voiding or delayed urination can result in reflux or migration of bacterial to the bladder. Mechanical trauma such as insertion of the catheters or urethral milking which occurs during sexual intercourse, can also force bacteria into the bladder (Nicolle, 2005; Newman, 2010). With the exception of urethra, the urinary tract is sterile, the distal one third of the urethra, and in women, the vaginal vestibule is contaminated with enteric bacteria.



A



B

Fig 1.1: Representation of female urinary system; with and without pregnancy **(Source: www. Shutterstock.com).** The above figure A shows the effect of the foetus (X) on the urinary tract of the mother as it grows, it presses on the bladder (H) causing the urethra to open. Figure B shows the urinary tract of a non-pregnant female.

2.2 ETIOLOGY OF URINARY TRACT INFECTION

Urinary tract infection is usually caused by bacteria, particularly *E.coli,* which accounts for at least 80-90% of all urinary tract infections (McDowall, *et al.,* 1981; Barr, *et al.,* 1985; Patterson and Andriole, 1987), but also may be caused by other bacteria, viral, and fungal organisms. Klebsiella, Proteus, and Pseudomonas are commonly associated with urinary tract infection, particularly in recurrent cases (Aviva *et al.,* 2010). Additionally pregnant women develop glycosuria, which encourages bacterial growth in the urine. These multiple factors contribute to the development of UTIs during pregnancy. The organisms that cause UTIs during pregnancy are the same as those found in non-pregnant patients. Urine itself is normally sterile, but the moist environment of the periurethral area, the proximity of the urethral orifice to the rectum, and the short length of the urethra provide a conducive environment for the growth and ascension of potential uropathogenic microorganisms into the urinary system.

The presence of bacteria in the urine is called bacteriuria. In non-pregnant women, bacteriuria in the absence of symptoms is not considered an indication for treatment; however, during pregnancy, bacteriuria is associated with increased risk of pyelonephritis as well as prematurity and possibly other complications and therefore, UTI in pregnancy requires special consideration (Lumbiganon *et al.,* 2009). Cystitis often has a sudden onset. Pregnant women may present with contractions and suprapubic pain, with dysuria possibly having been mistaken for normal polyuria of pregnancy. Women with lower UTI often complain of feeling achy, crampy, or 'just not feeling well.' Pyelonephritis commonly has a gradual onset, although it can seem sudden if preceded by lower UTI, and is associated with not only urinary symptoms, but generalized symptoms such as fever, chills, nausea, malaise, and mild to

extremely severe lower to middle back discomfort. Patients with pyelonephritis may appear quite ill. It is critical to differentiate between the symptoms of cystitis and pyelonephritis, as the latter requires more aggressive treatment and carries greater risks, particularly during pregnancy. Cystitis may resolve spontaneously; however, effective treatment lessens the duration of symptoms and reduces the incidence of progression to upper urinary tract infection as an ascending infection. Pyelonephritis is associated with substantial morbidity. Complications include acute papillary necrosis with possible development of urethral obstruction, septic shock, and perinephric abscess. Chronic pyelonephritis may lead to scarring with diminished renal function (Aviva *et al.,* 2010).

2.3 INCIDENCE OF URINARY TRACT INFECTION

It is estimated that several hundred million women suffer from urinary tract infection annually, with costs to health care providers amounting to over $6 billion annually worldwide, a figure that may even be an underestimate (Aviva *et al.,* 2010). Additional costs to society of urinary tract infections are tremendous in terms of the personal suffering of millions of women annually, lost work days, and childcare costs. Over 56% of women will experience a urinary tract infection in their lifetimes, and among those experiencing uncomplicated acute urinary tract infections, as many as 20% will have a recurrence within 6 months. Overall urinary tract infection recurrence rate is between 27% and 48% (Aviva *et al.,* 2010). Even with treatment, symptoms typically last for an average of 6 days, with nearly 2.5 days of limited activity. The rates of urinary tract infection are slightly higher in the young, sexually active women because of factors affecting the urethra and the presence of uropathogenic organisms. Urinary tract infection rates increase during pregnancy and

with age, the former because of mechanical pressure of the growing uterus on the ureters and bladder preventing complete voiding, and the latter because of declining estrogen levels, declining mucin (a surface coating of the bladder epithelium that prevents bacterial adhesion), inability to void completely, incontinence, inadequate nutrition, and the occurrence of other diseases and excessive catheter use as a result of medical procedures (Aviva *et al.,* 2010).

2.4 RISK FACTORS FOR ASYMPTOMATIC BACTERIURIA

Different factors have been documented to contribute to UTI among pregnant women. These include age, parity, gestation age, level of education (Gilstrap and Ramin, 2001; Santos *et al.,* 2002; Dimetry *et al.,* 2007; Smaill and Vanquez, 2007). The physiologic changes related to pregnancy make otherwise healthy women susceptible to serious infectious complications, arising from conditions such as asymptomatic and symptomatic urinary tract infections. The combination of mechanical, hormonal and physiologic changes during pregnancy contributes to significant changes in the urinary tract, which has a profound impact on the acquisition, and natural history of bacteriuria during pregnancy. This includes dilatation of the ureter, decrease in ureteral peristalsis, and decrease in bladder tone. Additionally, the physiologic increase in plasma volume during pregnancy decreases urine concentration and increases urinary progestins and estrogens, which may lead to a decreased ability of the lower urinary tract to resist invading bacteria. However, risk only seems to be increased in the presence of uropathogenic microorganisms, either from a woman's own reservoir of bowel microbes, or passed from a sexual partner. Urinating after sexual activity decreases the rates of infection.

Urinary tract infection before pregnancy is a predictor for the diagnosis of asymptomatic bacteriuria at the first prenatal visit (Tugrul *et al.*, 2005). Evidence suggests that host genetic factors influence susceptibility to urinary tract infection. A maternal history of urinary tract infection is more often found among women who have experienced recurrent urinary tract infections than among controls. Susceptible patients may have a genetically increased number of receptors on uroepithelial cells to which bacteria may adhere. Additionally, non - secretors of specific blood group antigens, which are glycoproteins, are at increased risk of recurrent urinary tract infection. Mutations in host genes integral to the immune response (interferon receptors and others) also may affect susceptibility to urinary tract infection. The use of oral contraceptives (OCs) doubles the risk of urinary tract infection compared with no birth control, and the use of diaphragms and spermicides doubles the rate of urinary tract infection compared with OCs (Aviva *et al.,* 2010).

2.5 PREVALENCE OF ASYMPTOMATIC BACTERIURIA

It is estimated that 2 to 10% of pregnant woman suffer from any form of urinary tract infections (Lee and O'Brien, 2008). These infections complicate up to 20% of pregnancies and are responsible for the majority of antepartum admissions to the maternal–fetal medicine units (Sheffield and Cunningham, 2005).

The prevalence of asymptomatic forms of urinary tract infections has remained constant across countries, and most of the recent observational studies report similar rates, ranging from 2 to 10% - similar to that of nonpregnant women (Duarte *et al.*, 2008; Bahadi *et al.*, 2010). Acute cystitis is prevalent in 1 to 4% of pregnant women (Wagenlehner *et al*, 2009). Despite the relatively low prevalence of pyelonephritis during pregnancy (0.5 to 2%), it is estimated that 20% to 40% of pregnant women

with asymptomatic bacteriuria will develop this condition later in gestation (Jolley and Wing, 2010). A study showed that if urinary tract infection is left untreated, 30% of mothers will develop acute pyelonephritis compared with 1.8% of nonbacteriuric controls. Many studies have reported that pyelonephritis is more common during the second half of pregnancy, with an incidence peak during the last two trimesters of pregnancy (Gilstrap *et al.*, 2001; Hill *et al.*, 2005; Sharma and Thapa, 2007). Acute pyelonephritis may lead to adverse outcomes for the baby and the mother, such as premature delivery, low birth weight infants, preeclampsia, hypertension, renal failure and fetal death (Hill *et al.*, 2005).

The prevalence of urinary tract infection in pregnancy is closely related to socioeconomic factors (Turck *et al.*, 1962). Predictors of asymptomatic forms of urinary tract infections include: welfare status, increasing maternal age, multiparity, risky sexual behavior, history of childhood urinary tract infections and history of recurrent urinary tract infections. It has been reported that indigent women have a five-fold greater incidence of bacteriuria than non-indigent populations (Turck *et al.,* 1962; Golan *et al.,* 1989). The prevalence is also markedly increased if women present certain pre-existing medical conditions, such as diabetes mellitus, sickle cell disease, immuno-deficiency states, urinary tract anatomic anomalies, spinal cord injuries and psychiatric illnesses (Ovalle *et al.,* 1989). Nevertheless, there is some controversy on the effects of these host factors as predictors of UTIs (Fatima and Ishrat, 2006). Neglecting the treatment of UTI in pregnant women may result in some health and economic problems. Due to the increase in sex hormones and the anatomic and physiologic changes during pregnancy, bladder and kidney infection is more likely and may result in hypertension, preeclampsia, low birth weight, prematurity,

septicemia, and maternal death (Abyad, 1991; Delzell and Lefevre, 2000; Saidi *et al.,* 2005; Mittal and Wing, 2005).

The predictors of asymptomatic bacteriuria include sex (mainly females especially during pregnancy), age, and urgency to void, leukocyturia, increased bacteria flora, genotype, parity and trimester.

2.6 CLASSIFICATION OF URINARY TRACT INFECTION

Actually, the term UTI represent a wide variety of conditions, including asymptomatic forms of UTIs, urethritis, cystitis, acute pyelonephritis and pyelonephritis with bacteremia or sepsis (Joseph-DiPiro *et al.,* 2011). Asymptomatic bacteriuria is defined as the presence of more than 10^8 colonies/L of urine, without the symptoms of an acute UTI (Schnarr and Smaill, 2008); there are several classification methods for these infections.

2.6.1 Lower UTIs

The anatomical site of the infection can be the bladder (cystitis) and/or the urethra (urethritis) (Joseph-DiPiro *et al.*, 2011)

2.6.2 Upper UTIs

The kidneys are the anatomical site of the infection (pyelonephritis associated with inflammation of the renal parenchyma, calices and pelvis (Wagenlehner *et al.*, 2009)

2.6.3 Uncomplicated UTIs

Infections occurring in individuals who lack structural or functional abnormalities of the urinary tract that interfere with the normal flow of urine or voiding mechanism (Joseph-DiPiro *et al.*, 2011)

2.6.4 Complicated UTIs

Infections occurring in individuals, with predisposing lesions of the urinary tract that interferes with the normal flow of urine and urinary tract defences, common causes of predisposing lesions are birth defects, kidney stones, catheters, or obstruction (Masson *et al.*, 2009. During gestation, untreated urinary tract infections can lead to several pregnancy complications, such as low birth weight infants, premature delivery, and occasionally, stillbirth. Prompt and efficacious treatment of symptomatic urinary tract infections is warranted in pregnant women. Nevertheless, there is still some controversy regarding the screening and treatment of asymptomatic forms during gestation (Lumbiganon *et al.,* 2009; Lin and Brown 2010; Schmiemann *et al.*, 2010). Given the prevalence and the potential impacts of urinary tract infections on the health of the mother and her child, the objective of this chapter is to cover the most important clinical aspects of the epidemiology, etiology, physiopathology, pregnancy outcomes, and the treatment risks and benefits associated with urinary tract infections during pregnancy.

2.7 OUTCOME OF ASYMPTOMATIC BACTERIURIA

Urinary tract infections are one of the most common medical complications of pregnancy (Mittal and Wing, 2005). It is estimated that one in three women of childbearing age will have a urinary tract infection (Duarte *et al.*, 2008). Because of the normal physiologic changes induced by gestation, pregnant women are especially susceptible to these infections. Urinary tract infections are characterized by the presence of infectious agents in the genito-urinary tract that cannot be explained by contamination. Asymptomatic bacteriuria in pregnancy may lead to less favorable pregnancy outcomes and complications like preterm delivery, low birth weight, pre-

eclamptic toxemia and anemia of pregnancy, so they must always be screened and treated. But it is not practiced in most countries, including Nigeria as screening for asymptomatic bacteriuria in pregnancy is not considered as an essential part of antenatal care like routine checkup for albumin and sugar in urine in some cases, it is generally done only in the first visit of antenatal care. The necessity and benefit of this strategy is depending on the prevalence of asymptomatic bacteriuria in the population.

2.8 TREATMENT OF ASYMPTOMATIC BACTERIURIA

Bacteria sensitivity to various antimicrobial agents varies from place to place and in the same place from time to time. Although understanding of the pathogenesis of bacteriuria has improved, selection of an appropriate antibiotic has become complex for increasing incidence of resistant pathogens. There is no clear consensus in the literature 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 emergence of resistant strains to most frequently used antibiotics such as ampicillin and amoxicillin has been reported in many studies (Smaill and Vanquez, 2007). Newer antimicrobial agents are being added to the list of drugs used in bacteriuria. Pregnant women should be treated when bacteriuria is identified (Krieger, 1986; Duff, 1993). The choice of antibiotic (Table 2.1) should address the most common infecting organisms (i.e., gram-negative gastrointestinal organisms). The antibiotic should also be safe for the mother and fetus.

Historically, ampicillin has been the drug of choice, but in recent years *E. coli* has become increasingly resistant to ampicillin (Peddie *et al.,* 1987). Ampicillin resistance

is found in 20 to 30 percent of *E. coli* cultured from urine in the out-patient setting (Sanders and Sanders, 1992) Nitrofurantoin (Macrodantin) is a good choice because of its high urinary concentration. Alternatively, cephalosporins are well tolerated and adequately treat the important organisms. Fosfomycin (Monurol) is a new antibiotic that is taken as a single dose. Sulfonamides can be taken during the first and second trimesters but, during the third trimester, the use of sulfonamides carries a risk that the infant will develop kernicterus, especially preterm infants. Other common antibiotics (e.g., fluoroquinolones and tetracyclines) should not be prescribed during pregnancy because of possible toxic effects on the fetus.

Table 2.1 Antibiotic Choices for Treatment of UTIs during Pregnancy

Antibiotic	***Pregnancy category***	***Dosage***
Cephalexin	B	250 mg two or four times daily
Erythromycin	B	250 to 500 mg four times daily
Nitrofurantoin	B	50 to 100 mg four times daily
Sulfisoxazole	C*	1 g four times daily
Amoxicillin-clavulanic acid	B	250 mg four times daily
Fosfomycin	B	One 3-g sachet
Trimethoprim-sulfamethoxazole	C†	160/180 mg twice daily

**—contraindicated in pregnant women at term*, *†—Avoid during first trimester and at term* (*Information from Duff,* 1993 and *Krieger,* 1986).

Pregnancy has been categorized by the United States of America Food and Drug Administration (FDA) into various groups represented by alphabets as guidelines for drug companies to follow in regards to labeling medications about their effect on reproduction and pregnancy.

Category B represents drugs that show no evidence of risk in humans- adequate, well controlled studies in pregnant women have not shown increased risk of feotal abnormalities despite adverse findings in animals **OR** in the absence of adequate

human studies, animal studies show no faetal risk. The chance of faetal harm is remote, but remains a possibility.

Category C represent risk can not be ruled out – adequate, well-controlled human studies are lacking and animal studies have shown a risk to the faetal or are are lacking as well. There is a chance of faetal harm if the drug is administered during pregnancy, but the potential benefits may outweigh the potential risk

A seven- to 10-day course of antibiotic treatment is usually sufficient to eradicate the infecting organism(s). Some authorities have advocated shorter courses of treatment—even single-day therapy. Conflicting evidence remains as to whether pregnant patients should be treated with shorter courses of antibiotics. Masterton (1985), demonstrated a cure rate of 88 percent with a single 3-g dose of ampicillin in ampicillin-sensitive isolates. Several other studies have found that a single dose of amoxicillin, cephalexin or nitrofurantoin was less successful in eradicating bacteriuria, with cure rates from 50 to 78 percent (Harris *et al.,* 1982; Campbell-Brown and McFadyen, 1983; Jakobi *et al.,* 1987; Patterson and Andriole, 1987).

Other antibiotics have not been extensively researched for use in UTIs, and further studies are necessary to determine whether a shorter course of other antibiotics would be as effective as the traditional treatment length (Patterson and Andriole, 1987). After patients have completed the treatment regimen, a repeat culture should be obtained to document successful eradication of bacteriuria (ACOG, 1998).

For appropriate treatment and to avoid over-treatment that would increase both costs and incidence of side effects, there is a need to stay abreast of information about the pathogens causing bacteriuria and their sensitivity and resistance pattern.

2.9 COMMONLY IMPLICATED ORGANISMS IN ASYMPTOMATIC BACTERIURIA

2.9.1 *Escherichia coli*

Escherichia coli are a facultative anaerobic Gram-negative bacillus. The urinary tract is the most common site of *E. coli* infection, and more than 80% of all uncomplicated urinary tract infections are caused by *E. coli* infection. Uropathogenic strains of *E. coli* have an adherence factor called P-fimbriae, or pili which bind to the P-blood group antigen (Warren, 1996). These P-fimbriae mediate the attachment of *E. coli* to uroeptithelial cells. *Escherichia coli* bacteremia is usually associated with urinary tract infection, especially in cases of urinary tract obstruction of any origin. The systemic reaction to endotoxins (cytokines) or lipopolysaccharides can lead to disseminated intravascular coagulation and death. *E. coli* is the leading cause of both communities acquired and nosocomial UTI, up to 50% of females eventually experience at least one episode of UTI (Kahlmeter, 2003; Ronald, 2003).

Escherichia coli typically produce positive tests for indole, lysine decarboxylase, and mannitol fermentation and produces gas from lactose. It is a Gram – negative rod. And isolates from urine can be quickly identified as E–coli by its heamolysis on blood agar, typical colonial morphology with a iridescent sheen on differential media such EM13 agar. It has motile, flat and non-viscous colonies. Strains of *E. coli* grow well on non-selective media, forming smooth, colourless colonies 2-3min in diameter in 18h., on nutrient agar, and large, red colonies on MacConkey agar. They grow over a wide range of temperature (15-45˚C). Many characteristic biochemical reactions such as indole production and the formation of acid and gas from lactose and other carbohydrates take place at 44˚C as well as 37˚C. *Escherichia coli* is a member of the

normal intestinal flora and does not cause disease. It becomes pathogenic only when it reaches tissues outside the intestinal tract, particularly the urinary and biliary tract, lungs, peritoneum and menninges, causing inflammation at these sites. It is the most common course of acute, uncomplicated UTI outside the hospitals and the signs and symptoms include:- Urinary frequency, dysuria, hematuria and sometimes pyuria. Clinical specimens (urine, blood, pus, spinal fluid, sputum, or other materials, as indicated by the localisation of the disease process) may be stained by Gram's method for microscopical examination and are cultured on suitable media so that the organism may be isolated for identification and sensitivity testing. In the case of suspected urinary tract infection, culture is semi quantitative and in acute *E. coli* infection the organism is generally present in virtually pure culture and a count of 10^5cfu per ml of urine or higher (Jawetz *et al.*, 1982).

2.9.2 *Staphylococci*

The S*taphylococci* are Gram-positive spherical cells usually arranged in grape like irregular clusters, non-motile, non-spore forming and catalase positive bacteria, but some, especially when examined in pathological specimens occur as single cells or pairs of cells. They grow readily on many types of media and are active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. Some are members of the normal flora of the skin and mucous membranes of humans, others cause suppurations, abscess formation, a variety of pyogenic infectious, and even fatal septicaemia.

The ability to clot plasma is the most widely used and generally accepted criterion for the identification of *S. aureus* (coagulase positive). *Staphylococcus aureus* is

ubiquitous, and may be a part of human flora found in the maxillae, the inguinal and perinea areas, and the anterior nares. Community-associated methcillin-resistant *S. aureus* (CA-MRSA) is less often found in the anterior nares than are methicillin-sensitive *S. aureus* (MSSA) and hospital-acquired methicillin resistant *S. aureus* (HA-MRSA); rather, it colonizes the skin, particularly in the perinea area and the rectum. It also colonizes the pharynx, gut and vagina. The organism may cause disease through tissue invasion and toxin production. The toxins liberated by the organism may have effects at sites distant from the focus of infection or colonization. *Staphylococcus aureus* bacteriuria can lead to subsequent invasive infection. The efficacy of anti staphylococcal therapy in preventing late-onset staphylococcal infection in patients with persistent staphylococcal bacteriuria should be tested in controlled trials (Muder *et al.*, 2006). The pathogenic staphylococci often heamolyse blood, coagulate plasma, and produce a variety of extracellular enzymes and toxins.

Young cocci are Gram-positive; on aging many cells become Gram-negative. Under the influence of certain chemicals (e.g penicillin), Staphylococci are lysed or changed into L-forms, but they are not affected by bile salts or optochin. Staphylococci are salt tolerant and can be selectively isolated from materials such as food and feaces by use of media containing 7-10% sodium chloride. *Staphylococcus aureus* appear as golden yellow colour on mannitol salt agar. The presence of *Staphyhococci* can often be demonstrated by examination of a Gram stained smear. The organism can be cultured readily under aerobic or microaerophilic condition on nutrient, blood or milk agar. Plates are inspected for characteristic golden, cream-coloured or white colonies. Staphylococci grow most rapidly at 37˚C but form pigment best at room temperature (20-25˚C). Colonies on solid media are round, smooth, raised and glistering (Jawetz *et al.*, 1982). The S*taphylococci* produce catalase, which differentiates them from the

S*treptococci*. *Staphylococci* slowly ferment many carbohydrates, producing lactic acid but not gas. *Staphylococcus aureus* is coagulase positive.

2.9.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacillus, non-sporing, non-capsulate, and occurs as single bacteria, in pairs and occasionally in short chains. *Pseudomonas aeruginosa* is an obligate aerobe that grows readily on many types of culture media, sometimes producing a sweet or grape-like odour. Some strains hemolyze blood. *Pseudomonas aeruginosa* form smooth round colonies with a fluorescent greenish colour. It often produces the non fluorescent bluish pigment pyocyanin, which diffuses into the agar. *Pseudomonas aeruginosa* in a culture can produce multiple colony types giving the impression of a culture of mixed species of bacteria. Cultures from patients with cystic fibrosis often yield *Ps. aeruginosa* organisms that form very mucoid colonies. On selective media such as cetrimide agar, it appears as bluish-green pigment. *Pseudomonas aeruginosa* grows well at 37-42˚C, its growth at 42˚C helps differentiate it from other *Pseudomonas* species. It is oxidase positive, does not ferment carbohydrate, but many strains oxidize glucose. Identification is usually based on colonial morphology. It is a frequent cause of nosocomial infections, urinary tract infection and bacteraemia. *Pseudomonas* infections are complicated and can be an opportunistic pathogen. It rarely causes disease in healthy persons. The pathogenesis of *pseudomonas* infections is multi factorial and complex.

Pseudomonas spp. is both invasive and toxigenic. The three stages according to Pollack (2000) are:

- Bacterial attachment and colonization

- Local infection and
- Bloodstream dissemination and systemic disease.

Production of extra cellular proteases adds to the organism's virulence by assisting in bacterial adherence and invasion. *Pseudomonas* urinary tract infections are usually hospital- acquired and are associated with catheterization, instrumentation, and surgery. These infections can involve the urinary tract through an ascending infection or through bacteraemia spread. In addition, these infections are a frequent source of bacteraemia. No specific characteristics distinguish this type of infection from other forms of urinary tract infection. *Pseudomonas aeruginosa* is the third most common pathogen associated with hospital-acquired catheter associated UTIs (Jarvis and Martone, 1992).

2.9.4 *Proteus*

Proteus species are part of the Enterobacteriaceae Family of Gram-negative bacilli. *Proteus* isolates are implicated in serious infections in human, along with *E. coli, Klebsiella, Enterobacter*, and *Serratia spp*. *Proteus mirabilis* causes 90% of *Proteus* infections, and can be considered as a community acquired infection. Patients with recurrent infections, those with structural abnormalities of the urinary tract, those who have had urethral instrumentation, and those whose infections were acquired in the hospital have an increased frequency of infection caused by *Proteus* and other organisms (e.g *Klebsiella, Enterobacter, Pseudomonas, Enterococci, Staphylococci)* (Mobley and Warren 1987, Mobley and Chippendale, 1990). *Proteus mirabilis* is one of the most common causes of urinary tract infections among individuals with long term indwelling catheters, complicated urinary tract infections, and bacteraemia

among the elderly (Mobley and Warren, 1987; Mobley and Chippendale, 1990). Infection depends on the interaction between the infecting organism and the host defence mechanisms. Certain virulence factors have been identified in the bacteria. The first step in the infection process is adherence of the microbe to host tissue. Fimbria facilitate adherence and thus enhance the capacity of the organism to attach. Attachment of *Proteus spp.* to uroepithelial cells initiates several events in the mucosal endothelial cells. Production of urease has also been shown to increase the risk of pyelonephritis in experimental animals. Urease production, together with the presence of bacterial motility and fimbriae may favour the production of upper urinary tract infections. Enterobacteriaceae (of which *Proteus* is a member) are organisms most commonly responsible for Gram-negative bacteremia. When these organisms invade the bloodstream, an endotoxin (a component of Gram-negative bacteria cell walls) apparently triggers a cascade of host inflammatory responses and leads to major detrimental effects. The ability of *Proteus* to produce urease and to alkalinize the urine by hydrolyzing urea to ammonia makes it effective in producing an environment in which it can survive. This leads to precipitation of organic stone formation (Antoni *et al.*, 1997).

2.9.5 *Klebsiella*

The genus *Klebsiella* is a member of the Family Enterobacteriacea. *Klebsiella* are non-motile, rod-shaped, Gram-negative bacteria with a prominent polysaccharide capsule. Members of the *Klebsiella* genus typically express 2 types of antigens on their cell surface. The first is a lipopolysaccharide (O-antigen); the other is a capsular polysaccharide (K-antigen). Capsules and lipopolysaccharides are essential to the virulence of *Klebsiella* (Highsmith and Jarvis, 1985). Both of these antigens

contribute to pathogenicity. The bacteria also produce multiple adhesins. These may be fimbrial or non-fimbrial, each with distinct receptor specificity. These help the micro-organism to adhere to host cells, which is critical to the infectious process. *Klebsiella* are also ubiquitous in nature, gastro intestinal tract, and the urinary tract. They are opportunistic pathogens; the principal pathogenic reservoirs of infection are the gastrointestinal tract of patients and the hands of hospital personnel. *Klebsiella* can rapidly spread leading to nosocomial outbreaks. *Klebsiella* accounts for 6 to 17% of all nosocomial urinary tract infections and shows an even higher incidence in specific groups of patients at risk, e.g., patients with neuropathic bladders or with diabetes mellitus (Lye *et al*., 1992; Benneth *et al.*, 1995). *Klebsiella* can cause a variety of clinical syndromes. Common *Klebsiellae* infections in humans include;

- Community-acquired pneumonia
- Urinary tract infection
- Nosocomial infection
- Rhinoscleroma and ozena
- Colonization.

Klebsiella urinary tract infections are clinically indistinguishable from UTIs caused by other common organisms. Clinical features include frequency, urgency, dysuria, hesitancy, low back pain and supra-pubic discomfort. Systemic symptoms such as fever and chills are usually indicative of concomitant pyelonephritis or postratitis.

2.9.6 *Candida*

Candida species are unusual causes of urinary tract infection (UTI) in healthy individuals, but common in the hospital setting or among patients with predisposing

diseases and structural abnormalities of the kidney. Candidiasis or thrush is a fungal infection (mycosis) of any of the Candida species (all yeasts), of which *Candida albicans* is the most common. *Candida albicans* is the most significant pathogenic species. The urinary tract may be invaded in either an antergrade fashion from the blood stream or retrograde via the urethra and bladder. *Candida* species employ a repertoire of virulence factor, including phenotypic switching, dimorphism, galvanomorphism and thigmotropism, and hydrolytic enzymes, to colonize and then invade the urinary tract. In addition to asymptomatic candiduria, recognized clinical forms of candidal urinary tract infections include bladder infection, renal parenchymal infection, and infections associated with fungus ball formation (Fisher *et al*., 1982).

2.10 DEVELOPMENT OF BACTERIAL DRUG RESISTANCE (BDR)

2.10.1 Incidence of Antimicrobial Drug Resistance

Antimicrobial drug resistance (ADR) has been a major problem in the chemotherapy of microbial infections, with the bacteria being extremely adept at becoming resistant to many agents (Neu, 1985) Antimicrobial resistance is recognized when the microorganism multiply at an antibiotic concentration which can be achieved in the tissues (Simon *et al.,* 1993). The microbial cells now survive and are able to thrive in hitherto microbiostatic or microbiocidal concentration of the chemotherapeutic agent, Resistance to antimicrobials has been described as a natural phenomenon (WHO, 2001).

The introduction of every antimicrobial agent into clinical practice has been followed by the detection in the laboratory of strains of microorganisms that are resistant, i.e. able to multiply in the presence of the drug concentrations in humans receiving therapeutic doses (WHO, 2001). This has been true, historically. Paul Ehrlich, from the work on

specific staining of cells and the use of these vitals stains in the treatment of protozoal infections had recognized the specific relationships between parasites and drugs and the development of what was termed drug-fastness in parasites (Jawetz *et al.,* 1974)

The changed prognosis of tuberculosis as a result of the discovery in 1944 of streptomycin; a specific antibacterial therapy which often produced a cure within months and could be given if need be on an out-patient basis suffered a blow within a year. This is because patients ceased to respond to treatment and relapsed as a result of their infecting organism developing resistance to streptomycin (Collard, 1976).

The introduction of sulfonamides in 1936 for the treatment of gonorrhea produced remarkable results, with practically all strains of gonococci being susceptible to the chemotherapeutic agent. Unfortunately, most strains were resistant to sulfonamides six years later (Jawetz *et al.,* 1974). Also, before 1962, meningococci were uniformly susceptible to sulfonamides. The subsequent emergence of sulfonamide-resistant meningococci and its spread rendered sulfonamides useless in the prevention and treatment of meningococcal infections (Jawetz *et al.*, 1974; Franklin, 1987).

Today, antimicrobial resistance is considered a serious, ever-increasing problem (Masterton, 2000) and one the greatest current challenge to the effective treatment of infections with every indication that it will become an even greater public health challenge in future (Karlousky and Sahm, 2002).

Incidences of occurrence of resistance to either a single chemotherapeutic agent or many agents at the same time (multiple-drug resistance) have been reported in many pathogenic organisms (Ribner, 1987; Oduyebo *et al.*, 1997; Gosbell *et al.*, 2001; Hryniewicz *et al.,* 2001; Pellegrino *et al.,* 2002)

Antimicrobial resistance encountered in clinical practice settings could be inherent or acquired (Franklin, 1987) or could be non-genetic or genetic (Jawetz *et al.,* 1974). This means resistance may either be a characteristic associated with the entire species or it may emerge in strains of the microorganism normally susceptible to the antimicrobial agent.

2.10.2 Non-Genetic Resistance

Some microorganisms are known to be naturally or innately resistant to certain groups of chemotherapeutic agents. Gram-negative bacteria as a group are known to be resistant to a number of antimicrobial agents that are very effective against the Gram positive organisms. The inherent resistance of G-ve bacteria is associated with impermeability of the complex outer layers of cell envelop to some drugs thus preventing the attainment of an inhibitory concentration within the cell (Lowbury and Ayliffe, 1974; Franklin, 1987). This type of- natural resistance led Ehrilic Shiga to wrongly conclude in 1902 that atoxyl (Sodium arsenilate) was ineffective in the treatment of mouse trypanosomiasis. They had used a strain of trypanosome that was naturally resistant to arsenicals (Collard, 1976)

Since active replication of bacteria is often required in most antibacterial drug actions, non-multiplying (metabolically inactive) microorganisms may be phenotypically resistant to drugs but their offspring will be susceptible (Jawetz *et al.,* 1974) For example, Mycobacteria which often survive in tissues for many years after infection are restrained by the host's defenses and do not multiply. These persisting organisms are resistant and cannot be eradicated by drugs. However, if they start multiplying as a result of corticosteroid treatment in the patient, they are fully susceptible to the same drugs.

Penicillin susceptible organisms may change to L-forms during penicillin administration. Since they lack most cell wall which is the specific target structure, they are resistant to cell wall inhibitor drugs like penicillins, cephalosporins and remain for several generations in this form as "persisters". When cell-wall production is resumed, they become fully penicillin-susceptible. These innate resistances are consistent and predictable because they reflect the natural composition of the organisms (Nester *et al.,* 1998).

The emergence of many drug-resistant microorganisms has been attributed to genetic changes followed up by the selection pressure exerted by wide spread use of antimicrobial agents (Jawetz *et al.,* 1974; Franklin, 1992; Simon *et al.,* 1993). The rapid multiplication rates and large sizes of bacterial populations confer on such populations a remarkable ability to respond to changes in their growth environment.

The introduction of a new drug initially marks a 'Victory' on the causative organisms that are not inherently resistant to the drug, such that high success rate is reported in therapy (Franklin, 1992). However, reports of treatment failures are soon encountered after months or years of continuous use of the new drug, as a result of the emergence and spread of resistant strains of the implicated organisms (Gold and Moellering Jr., 1996). This acquired ability of the microorganisms to thrive in the presence of the new drug is a reflection of the differences in the genetic composition of the resistant strains which gradually replace the sensitive strains as a result of the antibiotic selective pressure (Franklin, 1992; Simon *et al*, 1993)

The genetic information in a cell can be altered by many factors including; a change in the nucleotide sequence of DNA (mutation), transfer of sets of genes from other cells and of certain genes (transposable elements) from one location to another in the genome of the same cell (Nester *et al.,* 1998). These factors can broadly be classified as either chromosomal or extrachromosomal (Jawetz *et al.,* 1974; Simon *et al.,* 1993).

The combination of intracellular and intercellular movement of genetic materials (DNA) accounts for the rapid spread of resistance to antibiotics and heavy metals in bacterial populations.

2.11 TYPES OF BACTERIAL DRUG RESISTANCE (BDR)

The different mechanisms of BDR can be classified based on the source of genetic change(s) responsible for the 'acquired' resistance (Simon *et al.*, 1993)

2.11.1 Chromosomal Mediated

Replica plating has demonstrated that resistant cells can appear in low frequency in a bacterial genome (Franklin, 1992; Nester *et al.,* 1998). The properties of a cell depend largely on its proteins, which include both enzymes and proteins that form part of the cell structures such as the proteins in the cytoplasmic membrane and the ribosome (Nester *et al.,* 1998). The substitution of even one amino acid for another (change in genetic information) in any given protein may cause the protein to be non-functional, thereby changing the properties of the cell, Mutational genetic alterations may be expressed through various physiological (phenotypic) mechanisms which may be strain dependent.

2.11.2 Extra chromosomal (Plasmid) Mediated

A major driving force behind the increase in resistant strains is the ease with which bacteria can acquire resistance genes (Salyers and Amabile-Cuevas, 1997) present on transferable elements. The well known extra chromosomal microbial DNA may contain resistance genes that can be transferred to another bacteria are plasmids. A plasmid is a circular body of double stranded DNA which is separate from the chromosome and carries genes that encode various traits such as virulence and antimicrobial resistance (Akkina and Johnson, 1999).

There are two types of plasmids based on their ability to transfer from one bacterium to another. The self-transmissible (conjugative) plasmids can transfer to other bacteria via sex pili while the non conjugative (tranducible) plasmids are not capable of such transfer.

Various gene transfer elements have been described (Salyers and Amabile-Cuevas, 1997; Powell, 2000) and the characteristics of the different elements involved in the spread of resistance genes.

2.12 ACQUISITION OF ANTIBIOTIC RESISTANCE BY THE TRANSFER OF GENETIC INFORMATION

Chromosomal and plasmid-DNA containing resistance genes can be transferred from bacteria to bacteria of the same or different species and genus. The transfer of these antimicrobial resistance phenotypes by one or more of the DNA mobile elements may be through conjugation, transduction or transformation (Salyer and Amabile- Cuevas, 1997; Olsen, 1999; White and McDermott, 2001) and their various modifications.

Conjugation: Conjugation is the direct transfer of DNA from one bacterial cell to another bacterial cell. The transferred DNA is a plasmid, a circle of DNA that is distinct from the main bacterial chromosome. The F plasmid is similar to a virus or a transposon in its ability to move independently of the main chromosome. The transfer of the plasmid take advantage of the complementary nature of double stranded DNA. One strand of the plasmid is transferred and the other remains in the original cell. Both strands have the complementary stranded added so that each cell ends up with a complete plasmid.

Transduction: Transduction is the process by which DNA is transferred from one bacterium to another by a virus. It also refers to the process whereby foreign DNA is

introduced into another cell via a viral vector. Transduction does not require physical contact between the cell donating the DNA and the cell receiving the DNA (which occurs in conjugation), and it is DNAase resistant (transformation is susceptible to DNAase). Transduction is a common tool used by molecular biologists to stably introduce a foreign gene into a host cell's genome. When bacteriophages (viruses that infect bacteria) infect a bacterial cell, their normal mode of reproduction is to harness the replicational, transcriptional, and translation machinery of the host bacterial cell to make numerous virions, or complete viral particles, including the viral DNA or RNA and the protein coat.

Transduction is especially important because it explains one mechanism by which antibiotic drugs become ineffective due to the transfer of antibiotic-resistance genes between bacteria.

Transformation: Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. If the foreign DNA has an origin of replication recognized by the host cell DNA polymerases, the bacteria will replicate the foreign DNA along with their own DNA. When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules, and those bacteria can be selected for that incorporation. Bacteria which are able to uptake DNA are called "competent" and are made so by treatment with calcium chloride in the early log phase of growth. The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA. The exact mechanism of this uptake is unknown. It is known, however, that the calcium chloride treatment be followed by

heat. When *E. coli* are subjected to 42^{o}C heat, a set of genes are expressed which aid the bacteria in surviving at such temperatures. These sets of genes are called the heat shock genes. The heat shock step is necessary for the uptake of DNA. At temperatures above 42^{o}C, the bacteria's ability to uptake DNA becomes reduced, and at extreme temperatures the bacteria will die.

2.13 BIOCHEMICAL MECHANISMS OF ACQUIRED BACTERIAL ANTIMICROBIAL RESISTANCE

Alterations in genetic composition of the bacteria as a result of either mutational changes or acquisition of any of the resistance gene transfer elements are expressed through various biochemical mechanisms that protect the bacteria from the killing effects of antimicrobial agents. These basic mechanisms of acquired antimicrobial resistance can be divided into five categories,

- Alteration of target receptor
- Decreasing the concentration of the drug at the target site.
- Enzymatic inactivation or destruction of the antimicrobial agent.
- Synthesis of resistant or alternate metabolic pathways.
- Failure to metabolise the drug to its active state.

Resistance in bacteria arises through a process from low level to high level, except a plasmid is acquired which already contains genes for full blown resistance (Levy, 1998). Bacteria resistance to a particular antibiotic or related series of drugs usually depends on a single mechanism (Franklin, 1992) occasionally, multiple mechanisms of resistance can occur in a single isolate, leading to higher levels of resistance (Hawkey, 2003)

2.13.1 Alteration of Target Receptor

Many of the antibiotics inactivate specific enzyme in the microorganism or the ribosome as in the case of a large number of protein synthesis inhibitors. The antibiotic would need to bind to the target receptor molecule in the bacteria. By altering the target receptor molecule, the antimicrobial is unable to bind and therefore does not have any effect.

Altered penicillin binding proteins (PBPs) are the cause of resistance in some strains of *Streptococcus pneumoniae* to Penicillin G and it is also responsible for the resistance in certain strains of *Staphylococcus aureus* to β-lactamase stable penicillins. This resistance is believed to occur as a result of the acquisition of a chromosomal gene *(mec* A) encoding the new target enzyme penicillin-binding protein 2a (PBP 2a or PBP 2') that has much lower affinity for the antibiotic, Penicillin-binding protein 2a mediates clinically relevant resistance to all p- actam antibiotics and more specifically, methicillin resistance in *Staphylococcus aureus* (Powell, 2000).

Apart from the mutation leading to a target protein being unable to bind the antibiotic, in some situations the target receptor retains its function even after formation of the complex (Powell, 2000), Examples of such mechanisms abound. The ribosome of Staphylococci can become insensitive to erythromycin following specific enzymatic modifications of rRNA (Davies, 1992). This sequence-specific methylation of the 23S molecule is one of the best known mechanisms for developing macrolide resistance and it is catalysed by a methyltransferase enzyme *Erm(* erythromycin resistance methylase) that does not impair protein biosynthesis (Walsh, 2000; Dessen *et al.,* 2001). The *Erm* mechanism is said to be the main route in drug-resistant clinical isolates of *S. aureus* and is present in organisms which originally produce erythromycin, *Streptomyces erythreus* as a self-immunity mechanism (Walsh, 2000; Dessen *et al.*, 2001).

Another example of the reprogramming or camouflaging of the target in the now resistant bacteria is used by vancomycin-resistant enterococci (VRE) to escape from vancomycin (Yoneyama and Katsumata, 2006). The enzymes in three of the five clinical phenotypes of vancomycin resistance, VanA, VanB, and VanD are involved in the reprogramming of the peptidoglycan termini from D-Ala-D-Ala to D-Ala-D-lactate, which decreases the binding affinity of vancomycin to its target (D-Ala-D-Ala) by 1,000-fold, leading to high vancomycin resistance (Bugg *et al.,* 1991). On the other hand, two of the enzymes, VanC and VanE replace the terminal D-Ala with D-Ser, resulting in low-level vancomycin resistance (Park *et al.,* 1997; Yoneyama and Katsumata, 2006).

Resistance to flouroquinolones is frequently associated with mutations in the "quinolone resistance-determining region" (QRDR) of the A subunit of DMA gyrase, the target molecule (Jenkins, 1996; Dessen *et al.*, 2001).

Macrolide-lincomycin resistance in clinical isolates of staphylococci and streptococci is believed to have resulted from a biochemical modification (methylation) in the 5OS ribosomal subunit RNA thus reducing binding. Rifampicin resistance results from inactivation of a single target (Spratt, 1994) and require a single amino acid change in the b-subunit of the DNA-directed RNA polymerase (Powell, 2000). Unlike resistance genes which are shared between bacterial species, rifampicin resistance can result from a single mutation and is a good example of how mutations that confer antibiotic resistance are conserved (Powell, 2000).

2.13.2 Decreased Concentration of the Drug At The Target Site.

Decreasing the accessibility of the antimicrobial agent to the target site can be achieved by either altering entry of the antimicrobial into the cell (decreased uptake or influx) or

increasing the removal of the antimicrobial from the cell (increased efflux) (Yoneyarna and Katsurnata, 2006) . A third possibility is that the antimicrobial can be altered in its passage across the microbial membrane such that is cannot bind its target (Powell, 2000).

Tetracycline resistance is believed to be as a result of decrease in the levels of drug accumulation caused by decreased uptake and increased efflux of the drug molecule (Yoneyama and Katsumata, 2006). This resistance is usually plasmid-mediated.

Plasmids containing tetracycline resistance marker are known to move among members of the *Enterobacteriaceae* and have been observed between *Staphylococcus aureus, Staphylococcus epidermidis, S. pyogenes, S. pneumoniae and Strept. faecalis* (McManus, 1997).

Aminoglycoside resistance has been attributed to the ability of resistant organisms to alter the compound in their periplasmic space by bacterial enzymes that acetylate, phosphorylate or adenylate aminoglycosides (Kaye *et al.,* 2000). The enzymatic alteration of the compound leads to binding to the bacteria ribosomes and poor uptake into the cell. Research has shown that the genes coding for aminoglycoside altering enzymes are often found on transposons that have been identified in members of the *Enterobacteriaceae* and *Ps. aeruginosa, Strept. pneumoniae* and Gram-positive species such as Staph. *aureus, Strept. faecalis,* and Strept. *pyogenes* (Neu and Gootz$_r$ 1996; Kaye *et al.,* 2000).

In bacteria that acquire the ability to actively pump antibiotic out across the cytoplasmic membrane, the drug is actively pumped out faster than it can diffuse in, so the concentration of the antibiotic remains too low to be effective (Walsh, 2000). This mechanism referred to as active efflux, which may be drug-specific or multidrug efflux pump is believed to play an increasingly important role in the resistance to many different

antibiotics, including macrolides, quinolones, β-lactams and chloramphenicol (Levy, 1992; Saier *et al.*, 1998; Kaye *et al.,* 2000).

Pseudomonas aeruginosa, an opportunistic pathogen gives a vivid example of this mechanism of antibiotic resistance. Mutants deficient in an outer membrane porin channel, OprD2, decrease the uptake of carbapenem, a β-lactam antibiotic, and increase resistance to this antibiotic due to low accessibility of the drug to its target, penicillin-binding protein (Nikaido, 1994). A mutation resulting in over expression of a multidrug efflux pump, MexAB-OprM, results in a strain of this organism resistant to a wide variety of structurally unrelated antibiotics (Poole *et al.,* 1993; Morshed *et al.,* 1995). Since a loss-of-function mutation in the MexAB-OprM efflux pump causes hypersensitivity toward various antibiotics, the intrinsic resistance of this organism is believed to be due mainly to an active efflux of antibiotics (Yoneyama *et al.,* 1997; Yoneyama and Katsumata, 2006),

2.13.3 Enzyme Inactivation of the Drug

Enzyme inactivation of the antimicrobial agent remains the predominant biochemical method of resistance to a wide variety of antibiotic structural types (Anon, 2001). The strategy involves the destruction of the chemical warhead in the antibiotic. This resistance mechanism usually involves the hyper production of an enzyme that inactivates the antibiotic by enzymatic cleavage or chemical modification such that they no longer interact with the target site or are no longer taken up by the organism (Powell, 2000). The most common example of this type of resistance is that mediated by β-lactamases which are widely distributed in both Gram-negative and Gram-positive species (Hawkey, 2000). The four-membered, strained β-lactam ring is the chemically

activated functional moiety in the β-lactams that acylate and irreversibly modifies the cell-wall cross-linking PBPs (Walsh, 2000; Yoneyama and Katsumata$_r$ 2006). Hydrolytic deactivation opens the β-lactam ring producing a product that is non-functional as a PBP pseudo substrate and useless as an antibiotic. This enzymatic weapon is secreted into the penplasmic space by the β-lactamase producing strains to inactivate the β-lactam antibiotics before reaching the site of action, the PBPs in the cytoplasmic membrane (Walsh, 2000). It has been estimated that a single β-lactamase molecule can hydrolyse 10^3 penicillin molecules per-second (Walsh, 2000).

Staphylococci are the only common Gram-positive pathogens in which β-lactamases have caused major resistance problems (Livermore, 1995); the penicillinases increasing in occurrences from oniy about 5% of *S. aureus* isolates when benzylpenicillin was introduced, to 80 to 90% of isolates, both of *S. aureus* and the coagulase negative Staphylococci through plasmid transfer and strain selection (Livermore, 1995), However, plasmids encoding staphylococcal penicillinase have spread to *Enterococcus faecalis* isolates (Murray, 2000), Other antibiotic classes, such as the aminogiycosides which do not possess such hydrolytically labile group (like the β-lactam ring) can still be neutralized by deactivating the periphery of the aminoglycosides with three types of chemical substituent (Walsh, 2000) thus interrupting the binding of the RNA targets In the ribosome.

Resistance to the aminoglycosides through enzymatic deactivation involves three different classes of enzyme, each with many variants (Smith and Baker, 2002), This deactivation of aminoglycoside antibiotics by chemical modification is catalysed by bacterial enzymes that transfer an acetyl group (derived from acetyl-Co-A), an adenylyl group or a phosphate group (both derived from ATP), The aminoglycoside-modifying enzymes are therefore broadly classified as N-acetyl transferases (AACs), 0-adenyl-transferases (ANTs) or 0-

phosphotransferases (APHs), and together comprise over 50 different enzymes which are further divided into subtypes according to the position on the drug at which the modification occurs (Smith and Baker, 2002). Many Gram-positive pathogens possess an unusual bi-functional Aminoglycoside-modifying enzyme (Davies, 1994; Dessen *et al.,* 2001). The bi-functional AAC (6')-APH (2") is a two domain, 57kDa protein which has the capability of acetylating a variety of aminoglycoside antibiotics (Shaw and Wright, 2000). The best known O-adenyl transferase is ANT (4^{r})-la from *S.aureus* which confers resistance to a variety of aminoglycosides (Dessen *et al.,* 2001)

2.13.4 Synthesis of Resistant Metabolic Pathways

When a drug inhibits an enzyme critical for cell growth, cells that produce greater amounts of the enzyme may be able to produce sufficient amounts of the metabolic product to survive the pressure of the drug concentrations that are usually attained clinically (Powell, 2000), for example, mutants of these organisms that overproduce folic acid reductase (dihydrofolate reductase (DHFR) enzyme that is the target of trimethoprim are resistant to the drug. There can be an acquisition of the *dfr* gene that encodes the production of a resistant form of DHFR (Smith, 2004). Based on sequence homology there are 15 different DHFR enzyme types. The acquisition of *dfr* genes encoding alternative DHFR of type I, II or V is believed to be the most common mechanism of trimethoprin resistance among the Enterobacteriaceae (Smith, 2004).

The R-plasmid-mediated resistance to trimethoprim often occurs in combination with resistance to sufphonamides. Gene for one or two variants of the sulphonamide target enzyme, dihydropteroate synthetase that are sulphonamide-resistant occurs with that coding for trimethoprim resistant-DHFR (Franklin, 1992). Another example of this type

of resistance is found in the thymidine requiring Streptococci that are not inhibited by trimethoprim and sulphonamides due to the production of adequate concentrations of thymidine nucleotides by an alternative pathway (Powell, 2000).

2.13.5 Failure to Metabolize the Drug

When the activity of a drug depends on its conversion by the bacteria to the active form, mutants that do not metabolize the drug will be resistant (Pratt and Taylor, 1990). *Bacteroides fragilis* that do not metabolize nitro-imidazole, metronidazole to the active metabolite are resistant to the drug (Powell, 2000). Another example is seen with the nitrofurans.

2.14 PROPERTIES OF ANTIBIOTICS USED IN UTI

2.14.1 Penicillins

The penicillins comprise a group of natural and semi synthctic antibiotics containing the chemical nucleus 6- aminopenicillanic acid, which consist of a β-lactam ring fused to a thiazolidine ring. The naturally occurring compounds are produced by a number of *Penicilliun* spp. The penicillins differ from one another in substitution at the 6 position, where changes in the side chain may modify their pharmacokinetic and antibacterial properties (Joseph and Robert, 1991).

2.14.2 Cephalosporins

Cephalosporins are derivatives of the fermentation products of species of the fungus *Cephalosporium.* They contain a 7 aminocephalosporanic acid nucleus, which consist of

a β-lactam ring fused to a dihydrothiazine ring. Various substitutions at the 3 and 7 positions alter their antibacterial activity and pharmacokinetic properties. Addition of a methoxy groups at position 7 of the β -lactam ring results in a new group of compound called cephamycins, which are highly resistant to a variety of β -lactamases. Similar to the penicillins, cephalosporins act by binding to PBPs of susceptible organism, thereby interfering with synthesis of peptidoglycan of the bacterial cell wall. In addition, these β -lactam agent may produce bactericidal effects by triggering autolytic enzymes in the cell envelope (Waxman and Stromiger, 1983).

2.14.3 Monobactam (Aztreonam)

Aztreonam is the only monobactam antibiotics currently in clinical use. The monobactams are β-laclams with various side chains affixed to a monocyclic nucleus. Aztreonam binds primarily to PBP3 of Gram -negative aerobes, including *Ps. aeruginosa,* thereby disrupting bacterial cell wall synthesis. It is not hydrolyzed by most commonly occurring plasmid- and chromosomally mediated β -lactamases, and it does not induce the production of these enzymes (Bush *et al.,* 1982).

The antibacterial activity of aztreonam is limited to aerobic Gram-negative bacilli, inhibiting most enterobacteriaceae, Neisseria spp and *Haemophilus* spp with MIC90s of ≤ 0.5ug/ml (Jacobus *et al.,* 1982; Sykes *et al.,* 1982; Barry *et al.,* 1985). It has significant activity against *Ps. aeruginosa, Enterobacter* spp and *Serratia marcescens,* with most strain being inhibited at ≤ 16ug/ml. However, many *Acinobacter* spp., *Pseudomonas cepacia,* and *Xanthomonas maltophilia* are resistant. It shows *in vitro* synergism when combined with aminoglycosidcs against 30 to 60% of aztreonam-susccptiblc organism, including *Ps. aeruginosa* and aminoglycoside

resistant Gram-negative bacilli. Bacterial tolerance and inoculums effect are generally not seen with this agent. Aztreonam is not active against Gram positive bacteria and anaerobes.

2.14.4 Carbapenems

Imipenem (N-formimidoyl-thienamycin) is the first carbapcncm antimicrobial agent developed for clinical use. It is a semi-synthetic derivative of thienamycin which is produced by *Streptomyces* spp. Thienamycins differ from other β-lactams in having a Trans- configuration of the hydroxyethyl side chain at position 6 and lacking a sulfur or oxygen atom in the bicylic nucleus, the unique stereochemistry of the hydroxyethyl side chain provides stability against β-Iactamases (Barza, 1985). Imipenem binds to PBPl and PBP2 of Gram-negative and Gram-positive bacteria, causing cell elongation and lysis. It is stable toward most plasmid or chromosomally mediated β-lactamases except those produced by X. maltophilia and some strain *of B. fragilis* (Kropp *et al.,* 1985). Imipenem has the widest spectrum of antibacterial activity of the currently available antibiotics. It has excellent in vitro activity against aerobic gram-positive species. More than 90% of enterobactcriaeae are susceptible to imipenem, including those resistant to other β-lactams and aminoglycosides. Most *Acinetobacter* spp, *Enterobacter* spp, *Citrobacter* spp, and *Serratia* spp are inhibited by <3µg/ml. The activity against *Ps. aeruginosa* is similar to that of ceftazidime, with most strain inhibited at 1 to 6 ug/ml (Prince and Neu, 1981) while imipenem inhibits *Ps. cepacia* and *Pseudononas stutzeri,* it is inactive against *X. maltophilia.* Emergence of resistant Pseudomonas spp has been observed during therapy with imipenem (Winston *et al.,* 1984), The drug may show *in vitro* antagonism when combined with third-generation ccphalosporins or extended spectrum penicillins as a

result of its ability to induce β-lactamase production (Neu, 1985). Imipenem is the most potent β-lactam against anaerobes, with activity comparable to those of clindamycin and metronidazole. The MIC_{90} for *B. fragiiis, Bacteroides melaninogenicus,* other Bacteroides spp, fusobactcrium spp and anaerobic Gram positive cocci are ≤ l μg/ml (Kesado *et al.,* 1982) most *Clostridium* spp except some strains of *Clostridium difficile* are susceptible. Imipenem is also active against *Acinomyces* spp,t *Nocardia asteriodes,* and *Mycobacterium* spp (Cynamon and Palmer, 1982). Other members of this group are panipenem, meropenem etc.

2.14.5 β-Lactamase inhibitor (Clavulanic acid)

Clavulanic acid is a naturally occurring weak antimicrobial agent found initially in cultures of *Streptomyces clavuligerus* (Reading and Cole, 1977; Fu and Neu 1980), It inhibits β-lactamases from Staphylococci and any Gram negative bacteria. This agent acts primarily as a "suicide inhibitor" by forming an irreversible acyl enzyme complex with β-lactamase, leading to loss of activity of the enzyme. Clavulanic acid has been shown to act synergistically with various penicillins and cephalosporins against β-lactamase- producing staphylococci, Klebsiellae, *H. influenzas, Moroxella catarrhalis, N gonorrhoeae, E. coli, Proteus spp* and *Bacteroides* spp (Hunter *et al.,* 1980; Peters *et al.,* 1980; Fuchs *et al.,* 1984; Bensal *et al.,* 1985; Sutherland *et al.,* 1985). Recently discovered plasmid-mediated TEM β- lactamases in ceftazidime-resistant strain of *Klebsiella pneumonias* and *E. coli* are inactivated by this agent (Wiedeman *et al.,* 1989) However, the inducible β-lactamascs of *Enterobacter, Citrobacter, Acinetobacter, Serratia, and Pseudomonas* spp are not inhibited by Clavulanic acid. The combination of clavulanic acid with ampicillin, amoxicillin, or ticarcillin is active *in vitro* against *Mycobacterium tuberculosis* which is known to

produce β-lactamases (Woodley and Kilburu, 1982; Cynamon and Palmer, 1983; Sorg and Cynamon, 1987)

2.14.6 Sulbactam

Sulbactam is a semi-synthetic 6-desaminopcnicillin sulfone with weak antibacterial activity (English *et al.,* 1978). It functions as effective inhibitors of certain plasmid and chromosomally mediated β-laclamases. It inhibits the β-Iatamases of *Staph. aureus* and many Enterobacteriaeceae, *H. influenzae, Moraxella catarrhalis. Neisseria* spp. *Legionella* spp. *Bacteroides* spp., *and Mycobacterium* spp (Greenwood and Eley, 1982) Sulbactam alone is active against *N. gonorrhoeae, N. Meninghides,* some *Acinetobacter* spp. and *P. cepacia* (Retsema *et al.,* 1986; Jacob and Sutton, 1989) it acts synergistically with penicillins and cephalosporins against organism that are otherwise resistant to the β-lactam drugs because of the production of β -lactamases. A combination of sulbactam (8μg/ml) and ampicillm (16ug/ml) inhibits most strains of Staphylococci, *Klebsiella* spp., *E. coli, H. influenzae, Moraxella catarrhaiis, Neisseria* spp., and *Bacteroides* spp that are ampicillin resistant (Retsema *et al.,* 1980; Wise *et al.,* 1980). Sulbactam is inactive against the β -lactamases of Enterobacter, Citrobacter, Providencia spp and indole positive *Proteus* spp and *X. maltophilia.*

2.14.7 Aminoglycosides

Since the first aminoglycoside (amonoglycosidic aminocyclitol) streptomycin, was introduced in 1944, this class of antibiotic has played a vital role in the treatment of serious Gram-negative infection. Among the unique features of the aminoglycosides are the bactericidal activity against aerobic Gram-negative bacilli (including

Pseudomonas spp), activity against *M. tuberculosis,* and relatively low incidence of bacterial resistance. The currently available aminoglycosidcs are derived from *Micromonospora* spp (gentamicin, sisomicin and netilmicin) or from streptomyces spp (streptomycin, neomycin, kanamycin and tobramycin). Isepamicin which is presently undergoing clinical trial in the United States and elsewhere is a semi-synthetic derivative of gentamicin B. Structurally each of these aminoglycosides contains two or more amino sugar linked by glycosidic bonds to an aminocylitol ring nucleus. Aminoglycosides are bactericidal agents that inhibit bacterial protein synthesis by binding irreversible to the bacterial 30s ribosomal subunit. The aminoglycoside bound bacterial ribosomes then become unavailable for translation of mRNA during protein synthesis, thereby leading to cell death (Byan, 1984). They also cause misreading of the genetic code, with the resultant production of nonsense proteins. To reach the intracellular ribosomal binding targets, an aerobic energy dependent process is necessary to enable successful penetration of the aminoglycosides into the bacteria. The bacterial uptake of these agents is facilitated in the presence of inhibitors of bacterial cell wall synthesis such as β -lactams and vancomycin (Giamarellou, 1986).

2.14.8 Quinolones

Quinolones belong to a novel group of potent antibiotics biochemically related to nalidixic acid, which was developed initially as a urinary antiseptic but their use in pregnancy is with caution (Delzell and Lefevre, 2000). Nalidixic acid, and its early analogs, oxolinic acid and cinoxacin, had limited clinical application as a result of widespread emergence of bacterial resistance. Newer quinolones have been synthesized by modifying the original two-ring quinolone nucleus with different side chain substitutions (Wolfson and Hooper, 1985). These new agents also known as fluoroquinolones, contain a fluorine atom

attached to the nucleus, A number of other fluoroquinolones including ofloxacin, enoxacin, lomefloxacin, temafloxacin, fleroxacin have now been released and used clinically. Quinolones possess excellent activity in vivo against enterobacteriaceae:- *Ps. aeruginosa, Citrobacter, Serratia, and Acinetobacter spp,* β-lactamase-postivie and -negative *H.* Influenza, and Gram-negative cocci such as *N.gonorrhoeas, N. Meningitidis,* and *Branhamdla catarrhalii* (Wolfson and Hooper, 1985; Phillips and King, 1988). They are also active against *S. aureus,* including methicillin resistant strains and other staphylococcal species but are less potent against streptococci and enterococci. These agents generally are in active against anaerobic bacteria, with little or no clinically useful activity against *B. fragilis* (Goldstein and Citron, 1985) and *C. difficile* (Chow *et al.,* 1985; Delmee and Avesani, 1986).

2.14.9 Macrolides

Erythromycin is a macrolide antibiotic derived from *Streptomyces erythreus.* Its chemical structure consists of a macrocyclic lactone ring attached to two sugar moieties, desosamine and cladinose. Other marcolides include trioleandomycin and oleandomycin, and those currently under investigation include dirthromycin, clarithromycin, roxithromycin, azithromycin and spiramycin (Hardy *et al.*, 1988; Kirst and Sides, 1989). Erythromycin is a bacteriostatic agent that inhibits bacterial protein biosynthesis. This agent may be bactericidal at high drug concentration and against low inoculums of bacteria. It binds reversible to the 50S ribosomal subunits of susceptible microorganism, thereby blocking the translocation reaction of polypeptide chain elongation (Olenick and Cororan, 1969). Erythromycin is a relatively broad-spectrum antibiotic with activity against Gram positive and some Gram negative

bacteria, mycoplasmas, chlamydiae, treponemas, and rickettsiae (Washington and Wilson, 1985).

2.14.10 Sulfonamides and Trimethoprim

Sulfonamides were the first effective systemic anti microbial agents used in the United States during the 1930s. They are derived from sulfanilamide, which is similar in chemical structure to para-aminobenzoic acid, a factor essential for bacterial folic acid synthesis. Various substitutions at the sulfonyl radical attached to the benzene ring nucleus enhance the antibacterial aclivity and also determine pharmacologic properties of the drug. Trimethoprim (TMP) is a pyrimidine analog that inhibits the enzyme dihydrofolate reductase, interfering with folic acid metabolism and subsequent pyrimidine synthesis and one-carbon fragment metabolism in the bacteria. Since TMP and sulfonamides block the bacterial folic acid metabolic pathway at different sites, they potentiate the antibacterial activity of one another and act synergistically against a wide variety of organisms. Such a combination, Trimethoprim (TMP) sulfamethoxazole (SMX), also called cotrimoxazole was introduced clinically in 1968 and has proven to be very effective treatment of many infections. Sulfonamides competitively inhibit bacterial modification of para-aminobenzoic acid into dihydrofolate, whereas TMP inhibit bacterial dihydrofolate reductase. This sequential inhibition of folate metabolism ultimately prevents bacterial DNA synthesis. Since mammalian cells do not synthesize folic acid, human purine synthesis is not affected significantly by sulfonamides or TMP. The antibacterial effect of these agents may be reduced in patients receiving high doses of folic acid.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Media

Bacteriological media used in this study include Cetrimide Agar, Cystine-Lactose-Electrolyte-Deficient (C.L.E.D) agar, MacConkey Agar, Mannitol Salt Agar, Mueller Hilton Agar, Nutrient agar, Nutrient Broth all from Oxoid Ltd, U.K. Yeast Extract was from Biotech Laboratory Ltd., UK, while Peptone water was product from Fluka, Spain.

3.1.2 Antibiotic Disks

Antibiotic discs were obtained fron Oxoid Ltd, UK. They are:

Ampicillin (AMP) 10µg**,** Amoxicillin-clavulanic acid (AMT) 30µg**,** Cefotaxime (CTX) 30µg, Cefpodoxime (CPD) 10µg, Ceftazidime (CAZ) 30µg**,** Ceftriaxone (CRO) 30µg**,** Ciprofloxacin (CIP) 5µg**,** Co-trimoxazole (SXT) 25µg**,** Erythromycin (E) 15µg**,** Gentamicin (CN) 10µg**,** Methicillin (MET) 5µg**,** Nitrofurantoin (F) 300µg**,** Ofloxacin (OFX) 5µg**,** Streptomycin (S) 10µg**.**

3.1.3 Sugars

Lactose, Sucrose, Glucose were from BDH Chemical LTD, England

3.1.4 API kits

The under listed API kits obtained from BioMerieux Inc, USA were used in the identification of the bacteria isolates.

- API20E for Enterobactericeae

- API20NE, for Non-fastidious, non-enteric Gram negative
- API Staph.

3.1.5 Reagents and Solutions

Solutions of Crystal violet, Lugol's iodine, dilute Carbol-fushin, Kovac's reagent and Koser citrate medium were prepared according to Cheesbough, (2002)

Reagents obtained from Sigma Chemical Ltd were Ethidium bromide dye, Tris-Acetate EDTA (TAE), Tris-HCl (10mM). Lysis solution, Neutralising solution, Re-suspension solution Wash solution and Elution buffer were products of Fermentas Ltd

3.1.6 Equipments

- Autoclave (Certoclav, Model SM280E, SURGIFRIEND MEDICALS, England)
- Oven (Hotbox Size One, Galengkamp, UK)
- Incubator (Model 12-140E, Quincy Lab Inc)
- Refrigerator/Freezer (Model PRN 1313 HCA, BEKO, Germany)
- Oxoid Sensitivity Disc dispenser (900mm for 8 catridges)
- PCR thermocycler (Model TC-312, Techne, England)
- Gel electrophoresis machine (Max Fill Scie-plas. Model HU10 serial no 5237)
- Laminar air flow cabinet (PCR-8 re-circulating laminar flow pre station Labcaire product 220/240v)
- Microscope (Model CME 1349522X, Leica, USA)
- Spectrophotometer (Eppendorf Biophotometer 8.5mm, Lichtstrahihohe)
- UV eliminator (Vilberb Lourmat TFX-35-M seial no N^{o}V02 8104)

- Centrifuge (Model 5417R)
- Microwave oven (HINARI Life Style 800watts model MX310TCSL)
- Electronic weighing balance (Model QT 600)
- Vortex machine (Touch plate Super Mixer, CAT No 1291 Lab-line instrument Inc USA).

3.1.7 Primers

The under listed primers were used to analyse the DNA pattern of the isolates

A) β- lactam Primers

i) TEM with amplicon size 931bp

ii) SHV of amplicon size 868 bp

ii) OXA-2 of amplicon size 478 bp

B) Primers for Aminoglycoside

i) aac(3)-IIa of amplicon size 900 bp

ii) rmta of amplicon size 634bp

C) Primers for Extended Spectrum β-lactamase (ESBLs) Producers

i) gyrA of amplicon size 648 bp

ii) gyrB of amplicon size 448 bp

3.2 METHOD

3.2.1 Selection of Subjects for Sampling

Questionnaires were designed and distributed to pregnant women who attended ante-natal clinics at Sabon-gari Local Government Area, Zaria-Nigeria (Appendix I) to

provide information on their bio-data, stage of pregnancy, records of antibiotic usage etc. From the responses, subjects who were not on any antibiotic therapy and also not exhibiting any symptoms for any ailment were selected for inclusion in the study. Subjects irrespective of the gestational stage who fit into the above criteria were included in the study.

3.2.2 Sample Collection

Sterilized universal bottles were used to collect the urine samples. These were distributed to pregnant women attending Sabon-Gari Local Government Area, Zaria Clinics during their regular ante-natal visits, after educating them on how to collect the mid-stream urine samples. The samples were labelled and transported in ice packs to the laboratory. A total of two hundred and fifty (250) samples were collected from the months of February to April, 2011.

3.2.3 Media Preparations

The dehydrated media were reconstituted in freshly distilled water according to manufacturer's instructions. They were distributed into McCartney bottles and sterilized at 121^{o}C for 15mins. The sterile media were stored in refrigerator until required. Cetrimide agar media was prepared by the addition of 0.3% $^{w}/_{v}$ Cetrimide powder to the reconstituted Nutrient agar and sterilized appropriately.

Luria broth (LB medium) which is an enriched medium is used to grow the isolates to the log phase at the shortest possible time was prepared as follows: Peptone (10g) was weighed into a beaker, 5g each of Sodium chloride, yeast extract and 10ml Sodium hydroxide solution were added and made up to 1000ml with distilled water.

This was thoroughly mixed and 10ml distributed into final containers, sterilized by autoclaving at 121^{o}C for 15mins.

3.2.4 Determination of Bacteria Count

The method of Cheesbough, (2002) was used to determine the bacterial level of the first "clean catch" urine samples collected with clean sterile bottles for the purpose of determining those with significant bacteriuria. Dilutions of urine samples in sterile distilled water were plated on agar surfaces of CLED media, using the spread-plate method. Sterile L-shaped glass rods were used to spread the samples and their dilutions on the media. They were incubated at 37^{o}C for 18-24hrs. Viable colonies were counted and based on the dilution levels; the numbers of organisms in the undiluted samples were computed. Only samples with counts of at least 10^{5}cfu/ml were selected for further analysis.

3.2.5 Isolation and Identification Tests

Growth from samples adjudged to be positive for bacteriuria were sub-cultured unto selective media such as Mannitol Salt Agar, McConkey Agar and Cetrimide Agar, for the preliminary detection of Staphylococcus spp, Enterobacteriaceae and Pseudomonas spp isolates respectively.

a) Gram Staining: This was carried out by making smears of the isolates on slides, heat fixed and then covered with crystal violet solution for 30-60seconds, washed with distilled water rapidly. The smears were covered with Lugol's iodine solution for 30-60seconds, drained the iodine and then decolourized with acetone-alcohol mixture. This was immediately washed with distilled water, covered with neutral red stain for 1minutes, washed with distilled water and air dried.

These slides were then viewed under the microscope (x100 magnification) to observe the shapes of the cells whether they are spherical, rod, singles, and clusters or in chain-like forms.

b) Biochemical Tests

i) Catalase Test: This test is used to differentiate the bacteria that produce the enzyme catalase from the non-catalase producing ones. Using sterile glass rod, colonies from overnight cultures of the isolates were picked and immersed into a test tube containing 2ml hydrogen peroxide (3%) solution. The immediate production of gas bubbles indicate a positive result

ii) Citrase Test: The test assists in the identification of enterobacteria, and is based on the ability of an organism to utilise citrate as its only source of carbon. Slopes of the medium were prepared and streaked with the colonies from overnight cultures of the isolates and the bottom part was stabbed with the inoculating wire, and then incubated at 35^{o}C for 48 hours. The presence of bright blue colouration signified positive result.

iii) Coagulase Test: This test is used to identify *S. aureus* which produces the enzyme coagulase. This enzyme causes plasma to clot by converting fibrinogen to fibrin. To prepared smear of the isolates, drops of plasma were added and the presence of immediate agglutination was observed indicating that the enzyme coagulase has been produced.

iv) Indole Test: This test is important in the identification of the Family Enterobacteriaceae. The isolates were cultured in medium (tryptone water) containing

tryptophan. Indole production was detected using Kovac's reagent with the observation of red colouration.

v) Oxidase Test: This test assist in the detection of *Pseudomonas, Neisseria, Vibrio and Brucella* spp. On to filter paper, few drops of oxidase reagent were placed and colonies of the isolates were smeared. The phenylalanine present in the reagent was then oxidised to give deep purple colour indicating the production of oxidase enzyme.

vi) Urease Test: Testing for urease enzyme activity is important especially for *Proteus.* Colonies from overnight cultures of the isolates were inoculated in to 3ml of sterile urea broth and incubated at 35^{o}C (±2) for 5 hours. Pink colouration of the medium indicated positive urease production.

3.2.6 Identification of isolates to specie level (Rapid test using API kits)

The API kits are standardised unit system containing dehydrated substrates prepared for fast and easy biochemical identification of organisms from varieties of samples with high accuracy to the specie levels.

API 20NE is for the identification of non-fastidious, non-enteric Gram-negative rods such as *Pseudomonas.* Colonies from overnight cultures were reconstituted in saline medium from which aliquots were taken and inoculated into tubes as shown in the schematic diagram below. These strips were incubated at stated temperatures overnight and then observed for colour reaction and the readings documented. These reading were interpreted into numbers and the read off from the provided data base.

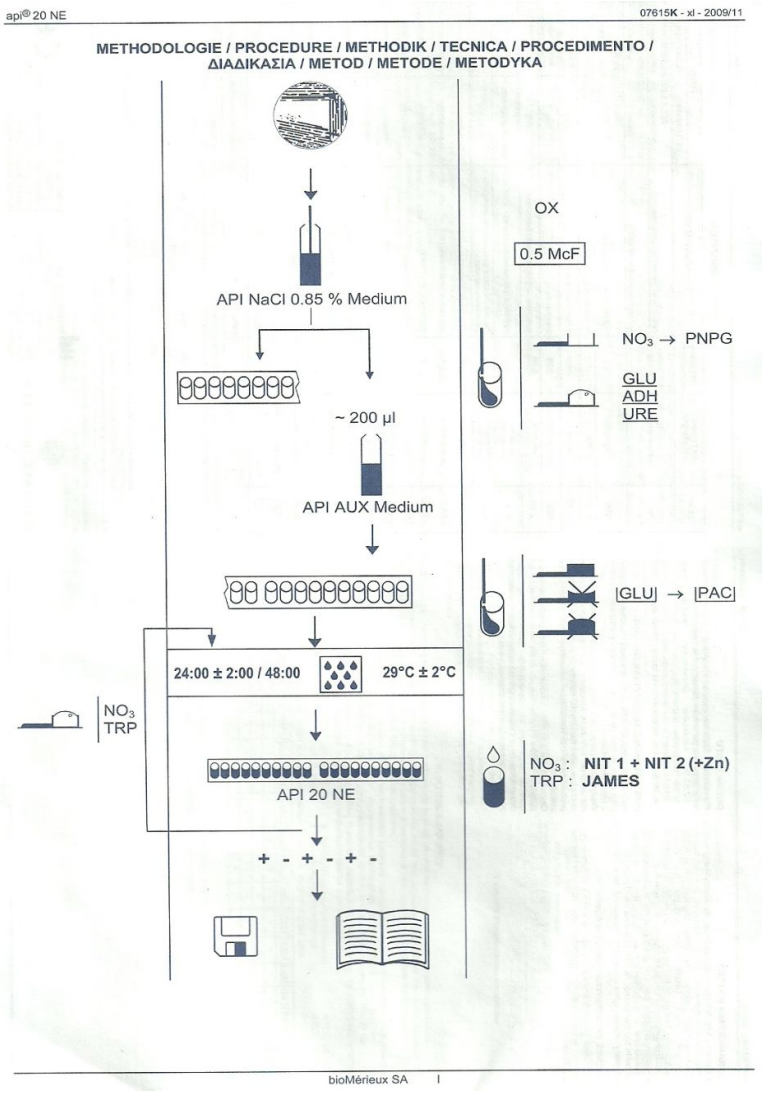


Fig 3.1a: Schematic representation of preparation of API 20NE kit (Source: BioMerieux)

API Staph Kit is used for the identification of the genera *Staphylococcus, Micrococcus* and *Kocuria* species and contains dehydrated substrates to prepared suspensions containing colonies from overnight cultures of the isolates to be tested in the provided API Staph medium provided as shown below. These strips were incubated at stated temperatures overnight and then observed for colour reaction and

the readings documented. These reading were interpreted into numbers and the read off from the provided data base.

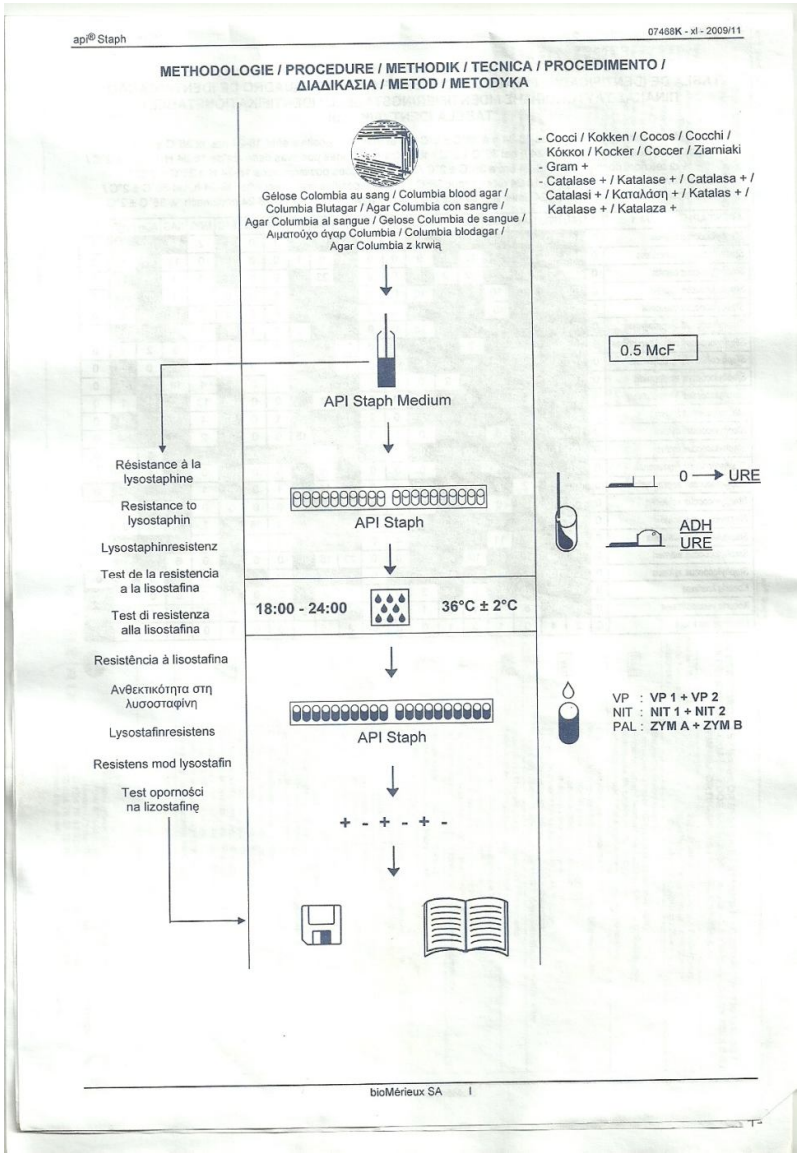


Fig 3.1b: Schematic representation of preparation of API Staph kit

API 20E kit is used for identification of the genera *Enterobacteriaceae* and other non-fastidious Gram-negative rods. Suspensions of the isolates were made in sterile distilled water and inoculated into the strips as shown below and then incubated at the

stated temperature overnight and then observed for colour reaction and the readings documented. These reading were interpreted into numbers and the read off from the provided data base.

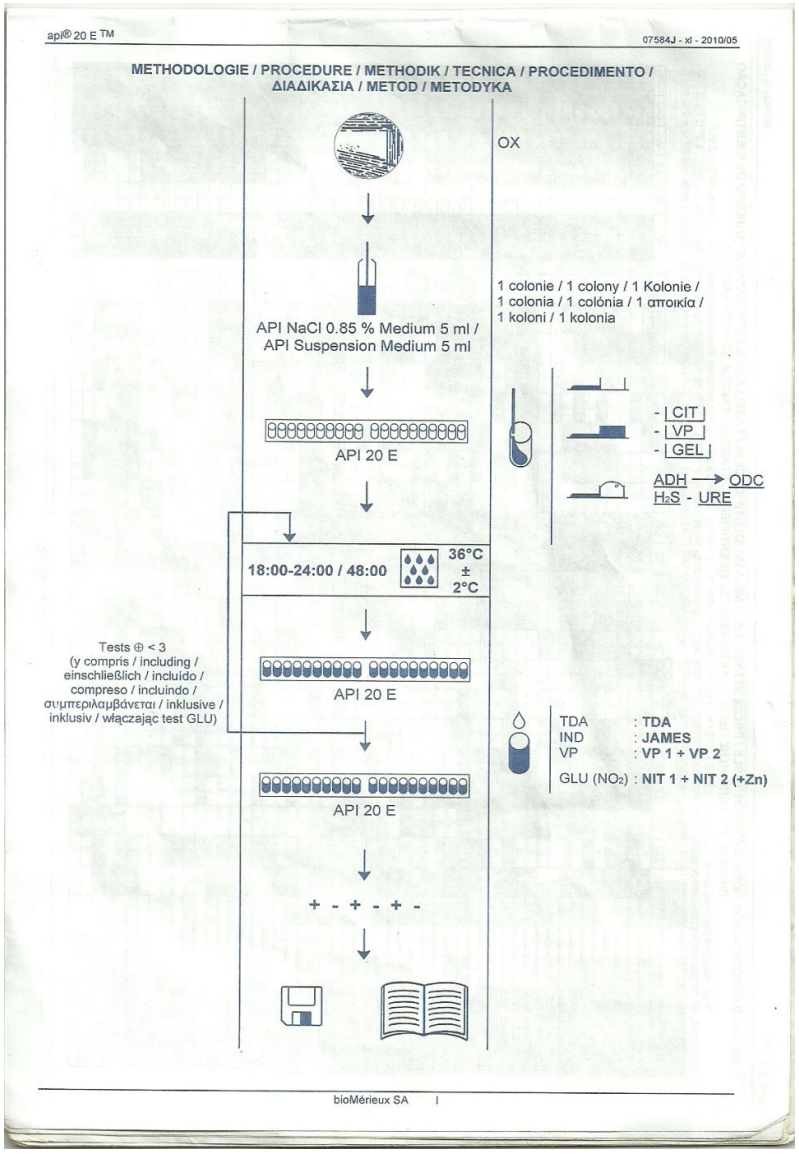


Fig 3.1c: Schematic representation of preparation of API 20E kit

3.2.7 Antibiotic Susceptibility Tests

Mueller Hinton Agar prepared according to manufacturer's manual was melted and poured into petri-dishes. The plates were allowed to solidify and the surfaces dried. The plates were inoculated with suspension of the test organisms, previously standardised to contain 10^5cfu/ml (equivalent to 0.5ml). Using the antibiotic disc dispenser, 8 different antibiotic disks were placed on the surface of the prepared agar, equidistant from each other. The plates were incubated at 37^{o}C for 18-24 hours. Zones of inhibitions were measured and interpreted using the chart from CLSI, (2008).

3.2.8 Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR Index was determined according to the method of Krumperman (1983) and Paul *et al* (1997). From the result of the antibiotic susceptibility test, MARI was calculated using the following formula:

$$\textbf{MAR Index} = \frac{\textbf{Classes of antibiotics to which isolate is resistant}}{\textbf{Total Classes of antibiotics classes tested}}$$

3.2.9 β-Lactamase Production Test

The acidimetric test method as described by Monica Cheesbrough (2002) was adopted. A strip of Whatman No 1 filter paper was placed in a petri dish and a few drops of phosphate buffered crystalline penicillin bromocresol purple solution was added till almost saturated. Colonies of isolates from overnight nutrient agar cultures were smeared on the filter paper. The petri dishes were incubated at room temperature for 30 minutes and then observed for colour change from purple-blue to yellow signifying β-lactamase enzyme production. Only isolates that were resistant to at least 70% of the test antibiotics were selected for use in this test.

3.2.10 Extended Spectrum β-Lactamase (ESBLs) Production Test

The two-disc method was employed using standardized disk diffusion assay technique (Jarlier *et al,* 1988) as follows: Amoxicillin-clavulanic acid (30µg) disc was placed in the centre of the plate containing sterilized Mueller Hinton agar (25ml); cefotaxime, cefpodoxime (10µg), ceftaxidime (30µg) and ceftriaxone (30µg) disks were placed 15mm (edge-to-edge) from the centre disc. Enhancement of zone of inhibition in the area between the amoxicillin-clavulanic acid disc and any one of the β-lactam disks in comparism with the zone of inhibition on the far side of the drug disc was interpreted as indicative of the presence of an ESBL in the test strain.

3.2.12 Plasmid DNA Analysis of Isolates

Plasmid DNA analysis was carried out at the Centre for Biotechnology Research and Training (CBRT) Ahmadu Bello University, Zaria-Nigeria.

3.2.11.1 Preparation and purification of total DNA using Spin-Column Protocol

From the overnight cultures, 1.5ml was pipetted into microcentrifuge tubes, centrifuged at 8000 rpm for 30s. The supernatant was discarded and the cell pellets re-dissolved in 180µl of Buffer ATL with the addition of 20µl Proteinase K, mixed thoroughly by vortexing. The tubes were incubated at 56^{o}C for 2hrs to allow lysing of the cells. The tubes were then vortexed for 15s and 200µl Buffer AL and 200µl ethanol (96-100%) were added and mixed by vortexing. This mixture was transferred into Mini-spin columns placed in 2ml collection tubes and centrifuged at 8000 rpm for 1min. The flow through and the collection tubes were discarded. The columns were placed in new sets of 2ml collection tubes, 500µl Buffer AW1 was added into

the column to wash the sediments, centrifuged at 8000 rpm for 1min. The flow through and collection tubes were discarded. The columns were placed in new set of collection tubes and 500µl BufferAW2 was added into the column for a second wash, centrifuged at 14000 rpm for 3mins so as to dry the sediments and to remove residual ethanol. The flow through and collection tubes were discarded. The columns were thereafter placed in 1.5ml collection tubes, 100µl Buffer AE added and incubated at room temperature for 1min and centrifuged at 8000rpm for 1min to elute the DNA. The flow through containing the DNA was collected and used to run the gel electrophoresis and Polymerase Chain Reaction (PCR).

3.2.11.2 Plasmid Extraction

Plasmid extraction was carried out by the alkaline lysis technique. The extracted plasmid was then isolated using horizontal 1% agarose gel electrophoresis. The pelleted bacterial cells were re-suspended and subjected to SDS/alkaline lysis to liberate the plasmid DNA. The resulting lysate was neutralized to create appropriate condition for binding of plasmid DNA on the silica membrane in the spin column. Cell debris and SDS precipitate are pelleted by centrifugation, and the supernatant containing the plasmid DNA is loaded onto the spin column membrane. The absorbed DNA was washed to remove contaminants, and is then eluted with a small volume of the elution buffer (10 mM Tris-HCl, pH 8.5). The purified plasmid DNA was ready for immediate use in all molecular biology procedures (PCR).

3.2.11.3 Detection of Plasmid (Gel Electrophoresis)

The agarose gel was prepared by dissolving 1.0g agarose in 100ml of 1X TBE by heating in a microwave oven. This was cooled to 60^{o}C, few drops of ethidium bromide was added and mixed thoroughly. Combs were inserted into the gel trays and agarose mixture was poured and allowed to solidify. The combs were removed; the trays were placed in the electrophoresis tanks. More TBE solution was poured into the tank until the gel was immersed. The extracted DNA samples were loaded into the wells created by the combs and the tanks were connected to electric leads and allowed to run for 30mins at 7volts. The resulting gels were observed in a UV trans-illuminator.

3.2.11.4 Amplification by Multiplex Polymerase Chain Reaction (PCR)

Using the formula below, the PCR reaction medium was calculated and tabulated as shown in Table 3.1. Example for Primer F1 (100nM in 431.73µl, therefore 0.1nM will be in Xµl)

$$\boldsymbol{X\,\mu l} = \frac{\mathbf{0.1}\,\boldsymbol{x}\,\mathbf{431.73}}{\mathbf{100}} = \mathbf{0.43}\boldsymbol{\mu l}$$

Table 3.1: Calculated PCR reaction medium

	For one reaction	For 20reactions
10XTaqBuffer	5.0	100.0
dNTP Mix 2nM	5.0	100 .0
Primer A1	0.34	6.8
A2	0.41	8.2
B1	0.38	7.6
B2	0.30	6.0
C1	0.31	6.2
C2	0.42	8.4
D1	0.25	5.0
D2	0.30	6.0
E1	0.41	8.2
E2	0.39	7.8
F1	0.43	8.6
F2	0.40	8.0
G1	0.40	8.0
G2	0.365	7.3
Template (DNA)	3.0	60.0
Taq DNA polymerase	0.25	5.0
Water	31.675	632.9
Total	50.0	1000.0

KEY: **Primers used**

A1= TEM (931) forward
A2= TEM (931) reverse
B1= SHV (868) forward
B2= SHV (868) reverse
C1= OXA-2 (478) forward
C2= OXA-2 (478) reverse
D1= aph(3)-lla (532) forward
D2= aph(3)-ll la (532) reverse
E1 = RMTA (635) forward
E2 = RMTA (635) reverse
F1 = gyrB (448) forward
F2 = gyrB (448) reverse
G1 = gyrA (648) forward
G2 = gyrA (648) reverse

Five sets of primers (forward and reverse oligosequences shown in Table 3.1) which target the following gene classes: TEM, SHV, OXA-2a, RMTA, aph (3)-lla, gyrA and gyrB were used to amplify the respective genes from genomic DNA. PCR was performed as shown in Table 3. 1. Thermo cycler (Techne) was used for the DNA amplification following the modified protocol by Vakulenko *et al.,* (2003) as follows:

Bacterial cells were lysed to obtain genomic DNA for PCR as follows: three to five bacterial colonies were emulsified in 25 µl of a 0.25% sodium dodecyl sulfate-0.05 N NaOH solution and boiled for 15 min. Then 200 µl of H_2O was added to the mixture, and 5 µl of the diluted mixture was used in the PCR. PCR experiments were performed in a volume of 50 µl with the following in a reaction tube: 5 µl of DNA template (obtained as described above), 1.5 mM $MgCl_2$, 0.1 mM (each) deoxynucleoside triphosphate, 1× PCR buffer, 2.5 U of *Taq* DNA polymerase, and primer sets for the aminoglycoside resistance genes PCR was performed with an initial lysing step of 5 min at 94°C; 45 cycles of 30 s at 94°C, 1 min at 72°C, and 1 min at 72°C; and a final extension step of 5 min at 72°C. Amplified DNA products were resolved using 1% (w/v) agarose gel electrophoresis.

Table 3.2: Summary of the Amplification processes.

STEP	TEMPERATURE ^{0}C	TIME	NUMBER OF CYCLES
Initial lysing of cells	94	5mins	
Denaturation	94	30 sec	45
Annealing	72	1min	45
Extension	72	1min	45
Final extension	72	5mins	
Final hold	4		
Total time	2h. 35min		

CHAPTER FOUR

RESULTS

4.1 INCIDENCE OF ASYMPTOMATIC BACTERIURIA

Questionnaires that were administered was used to identified Subjects that will be included in the sampling and the outcome was used in Tables 4.1 – 4.4

Out of 250 urine samples examined for asymptomatic bacteriuria, 146 were positive for significant bacteriuria (10^5cfu/ml) giving a prevalence rate of 58.4% among these pregnant women.

Total number of isolates obtained from the urine samples was 197. Most samples had more than one isolates but at most three.

Table 4.1: Incidence of bacteria among Pregnant Women

PARAMETERS	NUMBER
No of Sample screened	250
No with significant bacteriuria	146
No with bacteria organisms	145
No of bacteria Isolates	197

Incidence of asymptomatic bacteriuria according to age distribution showed that majority of the subjects screened were in the age bracket of 21-30 years, with those above 20 years constituting the least population. Women in age bracket 31- ≥40 years showed the highest incidence of asymptomatic bacteriuria (70.2%)

Table 4.2: Incidence of ASB in relation to age distribution of pregnant women

Age group (yrs)	No of Samples	No. Positive (%)
≤ 20	31	21 (67.7)
21-30	172	92 (53.5)
31- ≥ 40	47	33 (70.2)
Total	250	146 (58.4)

Percentage incidence was relatively low in subjects with first pregnancy, which increased as the number of pregnancies increased up to three (3) pregnancies and decreased thereafter but increased again.

Table 4.3: Incidence of ASB by Parity (No. of pregnancy)

Parity	No. Tested	% Positive
1st	34	10 (29.4)
2^{nd}	41	17 (41.5)
3^{rd}	52	41 (78.8)
4^{th}	40	23 (57.5)
$\geq 5^{th}$	83	55 (66.3)
Total	250	146 (58.4)

Table 4.4 shows the the incidence of ASB by trimester. It revealed that incidence of asymptomatic bacteriuria was generally high among the three trimesters, but relatively highest during the second trimester.

Table 4.4: Incidence of ASB by trimester

Trimester	No. Tested	No. Positive (%)
First	25	13 (52.0)
Second	125	77 (61.6)
Third	100	56 (56.0)
Total	250	146 (58.4)

4.2 DISTRIBUTION OF ISOLATES IN THE URINE SAMPLES

Table 4.5 shows the distribution of bacteria species isolated from subjects with asymptomatic bacteriuria. Traditional biochemical tests showed that organisms present in the urine of asymptomatic bacteriuric subjects essentially belong to six (6) genuses, predominantly Gram-negative organisms, constituting 74.1%

Table 4.5: Isolates obtained using standard biochemical tests

ISOLATES	Number (%)
Staphylococcus spp	50 (25.4)
Klebsiella spp	49 (24.9)
Proteus spp	46 (23.4)
Escherichia spp	33 (16.8)
Pseudomonas spp	18 (9.0)
Streptococcus spp	1 (0.5)
Total	197 (100)

API 20E tests kit was able to identify only 33 of the 128 previously identified Enterobacteriaceae organisms to specie level (Table 4.6) Of the 33 presumably identified *Escherichia* spp, only 10 were *E. coli.* Similarly of the 49 presumptive *Klebsiella spp* only 13 could be identified to specie level. None of the *Proteus* spp was identified to specie level. On the other hand, ten of the *Enterobacteriaceae* isolates were identified to belong to *Citrobacter* (4), *Providencia* (1) and *Enterobacter* (5)

Table 4.6: Distribution of Enterobacteriacae Isolates using API kit 20E

ISOLATES	NUMBER	PERCENTAGE (%)
Escherichia coli	10	30.3
Citrobacter freundii	2	6.1
Citrobacter braakii	2	6.1
Providencia retgerii	1	3.0
Enterobacter cloacae	5	15.1
Klebsiella pneumoniae	2	6.1
Other *Klebsiella* spp	11	33.3
Total	33	100

API 20NE tests kit was able to identify only 8 isolates were identified as*Pseudomonas aeruginosa*. Others include *Aeromonas aerohydrophila/caviae* (3), *Ps. leutiola* (1) and *Burkhol pseudomonellei* (1). The remaiming non Enterobacteriaceae isolates could not be identified to specie level as shown in Table 4.7.

Table 4.7: Distribution of Non- Enterobacteriacae Isolates using API kit 20NE

ISOLATES	NUMBER	PERCENTAGE (%)
Pseudomonas aeruginosa	8	6.4
Aeromonas aerohydrophila/caviae	3	2.4
Pseudomonas leutiola	1	0.8
Burkhol deria pseudomallei	1	0.8
Other Non-Enterobacteriacae	112	89.6
Total	125	100

API Staph tests kit was able to identify only 39 of the 50 previously identified *Staphylococcus spp* to specie level (Table 4.8) Of the 39 presumably identified *Staphylococci*, only 7 were *Staph. aureus,* other *Staphylococci* were identified as shown below as well as species of *Micrococcus* (4).

Table 4.8: Distribution of Staphylococcus and Micrococcus spp Isolates using API STAPH.

ISOLATES	NUMBER	PERCENTAGE (%)
Staphylococcus aureus	7	17.9
Staphylococcus lentus	3	7.7
Staphylococcus xylosus	5	12.82
Staphylococcus cohnii	1	2.56
Staphylococcus auriculous	2	5.1
Staphylococcus epidermidis	1	2.56
Staphylococcus heamolyticus	1	2.56
Micrococcus Spp	4	10.3
Other Staphylococcus spp	15	38.5
Total	39	100

4.3 ANTIBIOTIC SUSCEPTIBILITY PROFILES OF ISOLATES

Antibiotic sensitivity profiles of the bacteria isolates from urine of asymptomatic subjects showed that most of the isolates were not susceptible to the inhibitory activities of ampicillin, methicillin and ceftazidime. They were generally sensitive to nitrofurantoin, gentamicin, ciprofloxacin and ofloxacin, and moderately sensitive to co-trimoxazole. *Pseudomonas spp* were resistant to most of the antibiotics and was only sensitive to ciprofloxacin and ofloxacin while slightly sensitive to co-trimoxazole. *Escherichia spp* were sensitive to nitrofurantoin, ofloxacin, and co-trimoxazole but resistant to others while *Staphylococcus spp* were sensitive to ofloxacin, nitrofurantion, gentamicin, ciprofloxacin and co-trimoxazole. *Proteus spp* were sensitive to ciprofloxacin, ofloxacin, nitrofurantoin, and gentamicin but moderately sensitive to erythromycin, streptomycin and co-trimoxazole. *Klebsiella spp* were sensitive to ofloxacin, gentamicin, ciprofloxacin and nitrofurantoin as well as co-trimoxazole as shown in Table 4.9

Table 4.9: **Percentage susceptibility of bacteria isolates from Standard Biochemical tests**

ANTIBIOTICS	% SENSITIVITY of Isolates					
	Staphylococcus n=50	Escherichia n=33	Proteus n=46	Klebsiella n=49	Pseudomonas n=18	Streptococcus n=1
Erythromycin	0.0	60.6	56.5	22.4	22.2	0.0
Nitrofurantoin	86.0	93.9	78.3	69.4	38.9	100.0
GentAamicin	86.0	45.5	78.3	77.6	44.4	100.0
Streptomycin	20.0	33.3	63.0	18.4	16.7	100.0
Methicillin	0.0	0.0	0.0	0.0	0.0	0.0
Ampicillin	0.0	0.0	0.0	2.0	0.0	0.0
Ceftazidime	0.0	6.1	0.0	0.0	0.0	0.0
Ceftriaxone	22.0	18.2	43.5	51.0	16.7	100.0
Ciprofloxacin	84.0	87.9	87.0	77.6	72.2	100.0
Ofloxacin	92.0	75.8	87.0	79.6	77.8	100.0
Cotrimoxazole	72.0	69.7	56.5	65.3	55.6	0.0

All the *Escherichia coli* (10) that were identified using the API 20E kit were sensitive to nitrofurantoin, moderately sensitive to cpirofloxacin, streptomycin and co-trimoxazole but resistant to ampicillin, ceftriaxone, ceftazidime and methicillin (Table 4.10)

Table 4.10: Sensitivity of *E.coli* isolate from API 20E kit to test antibiotics

Antibiotic	**No of Isolates Sensitive (%)** n = 10
Nitrofurantoin (F) 300 µg	10 (100.0)
Gentamicin (CN) 10 µg	1 (10.0)
Streptomycin (S) 10 µg	3 (30.0)
Ciprofloxacin (CIP) 5 µg	4 (40.0)
Ofloxacin (OFX) 5 µg	1 (12.5)
Ampicillin (AMP) 10 µg	0 (0.0)
Methicillin (MET) 5 µg	0 (0.0)
Ceftazidime (CAZ) 30 µg	0 (0.0)
Ceftriaxone (CRO) 30 µg	0 (0.0)
Cotrimoxazole (SXT) 25 µg	3 (37.5)
Erythromycin (E) 15 µg	1 (12.5)

Table 4.11 below shows *Staph. aureus* identified using API Staph kit was sensitive to nitrofurantoin, ciprofloxacin, co-trimoxazole and ofloxacin, gentamicin and resistant to other test antibiotics.

Table 4.11: Sensitivity of *Staph aureus* isolates from API kit to test antibiotics

Antibiotic	No of Isolates Sensitive (%) n = 7
Nitrofurantoin (F) 300 µg	7 (100.0)
Gentamicin (CN) 10 µg	5 (71.4)
Streptomycin (S) 10 µg	0 (0.0)
Ciprofloxacin (CIP) 5 µg	6 (85.7)
Ofloxacin (OFX) 5 µg	6 (85.7)
Ampicillin (AMP) 10 µg	0 (0.0)
Methicillin (MET) 5 µg	0 (0.0)
Ceftazidime (CAZ) 30 µg	0 (0.0)
Ceftriaxone (CRO) 30 µg	0 (0.0)
Cotrimoxazole (SXT) 25 µg	6 (85.7)
Erythromycin (E) 15 µg	1 (14.3)

Other identified *Staphylococci spp* were highly sensitive to nitrfurantoin, ofloxacin, ciprofloxacin and cotrimoxazole, moderately sensitive to gentamicin and erythromycin.

Pseudomonas aeruginosa was sensitive to ofloxacin, only moderately sensitive to co-trimoxazole and nitrofurantoin.

Table 4.12: Sensitivity of *Ps. aeruginosa* Isolates from API kit to test antibiotics

Antibiotic	No of Isolates Sensitive (%) n = 8
Nitrofurantoin (F) 300 µg	4 (50.0)
Gentamicin (CN) 10 µg	3 (37.5)
Streptomycin (S) 10 µg	1 (12.5)
Ciprofloxacin (CIP) 5 µg	3 (37.5)
Ofloxacin (OFX) 5 µg	7 (87.5)
Ampicillin (AMP) 10 µg	0 (0.0)
Methicillin (MET) 5 µg	0(0.0)
Ceftazidime (CAZ) 30 µg	0 (0.0)
Ceftriaxone (CRO) 30 µg	2 (25.0)
Cotrimoxazole (SXT) 25 µg	4 (50.0)
Erythromycin (E) 15 µg	1 (12.5)

4. 4 DETERMINATION OF MAR INDEX

The antibiotics used were classified into seven (7) groups based on the CLSI groupings as follows:

- Penicillins:- ampicillin and methicillin
- Aminoglycosides:- gentamicin and streptomycin
- Cephalosoprins:- ceftazidime and cefriaxone
- Quinolones:- ciprofloxacin and ofloxacin
- Macrolides:- erythromycin
- Sulfonamides and Trimthoprim:- co-trimoxazole
- Nitrofurans:- nitrofurantoin

The determination of the MAR indices of the isolates showed that most of isolates had MAR index of ≥ 0.2 and above, being resistant to more than two classes of the tested antimicrobial agents (Table 4.13).

Table 4.13: Multiple Antibiotic Resistance (MAR) Index of Isolates

Classes of antibiotics to which resistant	MAR Index	No. of isolates (%)
0	0.0	22 (11.2)
1	0.14	11 (5.6)
2	0.30	47 (23.9)
3	0.43	54 (27.4)
4	0.57	38 (19.3)
5	0.71	19 (9.6)
6	0.86	4 (2.0)
7	1.0	2 (1.0)

4.5 RESULT OF β-LACTAMASE PRODUCTION

Of the 53 isolates used in this test, 25 were β-Lactamase positive (showed colour change from purple-blue to yellow). Eleven (11) of these were among the isolates that were used in the biotechnology experiments.

Table 4.14: Percentage Distribution of β-lactamase positive isolates

Organism	Number tested	Number (%) β-lactamase positive
Staphylococcus spp	13	4 (30.8)
Escherichia spp	10	7 (70,0)
Proteus spp	10	2 (20.0)
Klebsiella spp	10	8 (80.0)
Pseudomonas spp	10	4 (40.0)

4.6 RESULT OF ESBLS PRODUCTION

Result of the ESBL production test indicated by enhanced zones of inhibition was observed generally between discs containing amoxicillin-clavulanic acid (AMC) at the Center and the cephalosporins (Ceftazidime (CAZ), Cefpodoxime (CPD), Cefotaxime (CTX) and Ceftriaxone (CRO). The isolates used in this test were those identified to specie levels using the API kits

A 

B 

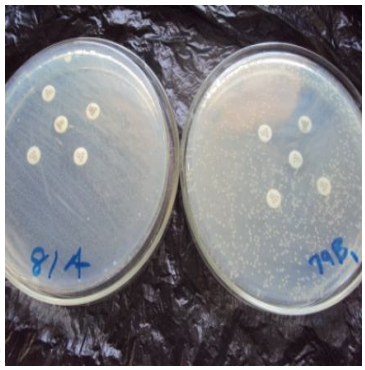
C 

Fig 4.1: Double discs diffusion ESBL tests showing *E. coli,* with positive ESBLs (A and B) production while C shows *Staph. aureus* with negative ESBLs production

The table below (Table 4.15) shows *E. coli* isolates were the highest ESBLs producers while none of the *Ps. aeruginosa* isolates produced ESBLs.

Table 4.15: Percentage distribution of ESBLs producers

Organism	Number tested	Number (%) ESBLs Producers
Escherichia coli	10	8 (80.0)
Staphylococcus aureus	7	3 (42.9)
Pseudomonas aeruginosa	8	0 (0.0)

4.7 RESULT OF DNA EXTRACTION

After the extraction of DNA from the isolates, the resultant products were analysed on gel electrophoresis. Lanes 1, 2, 4, 6 and 10 are *E. coli,* lanes 3, 5 and 8 are *Staph. aureus,* and they all showed genomic DNA bands while 7 and 9 are *Ps. aeruginosa* isolates. The intensity of the bands is indicative of the quantity of DNA extracted. On lane 11, is the standard ladder showing the elution of the different base pairs.

Lanes 1, 2, 4, 6 and 10 were observed to contain plasmids bands, while lanes 3, 5, 7, 8, and 9 did not contain any plasmid bands

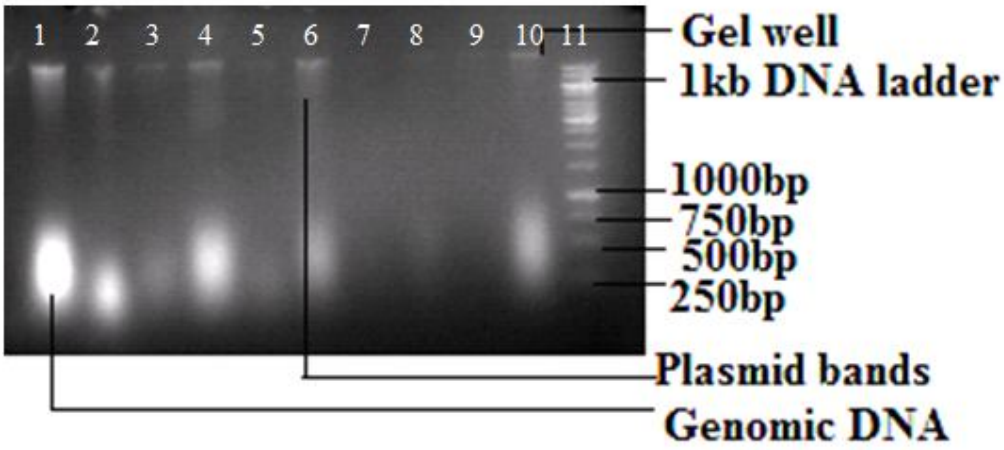


Fig 4.2: Genomics DNA Extraction on Gel Electrophoresis of API Identified *E. coli, Staph. aureus* and *Ps .aeruginosa* Isolates.

4.8 RESULT OF THE MULTIPLEX PCR AMPLIFICATION

The amplification of the total DNA extracts using multiple polymerase chain reactions and the products after analysis on gel electrophoresis gave amplicons as shown in the figures below, the calculated basepairs for each amplicon is also shown in the table that followed.

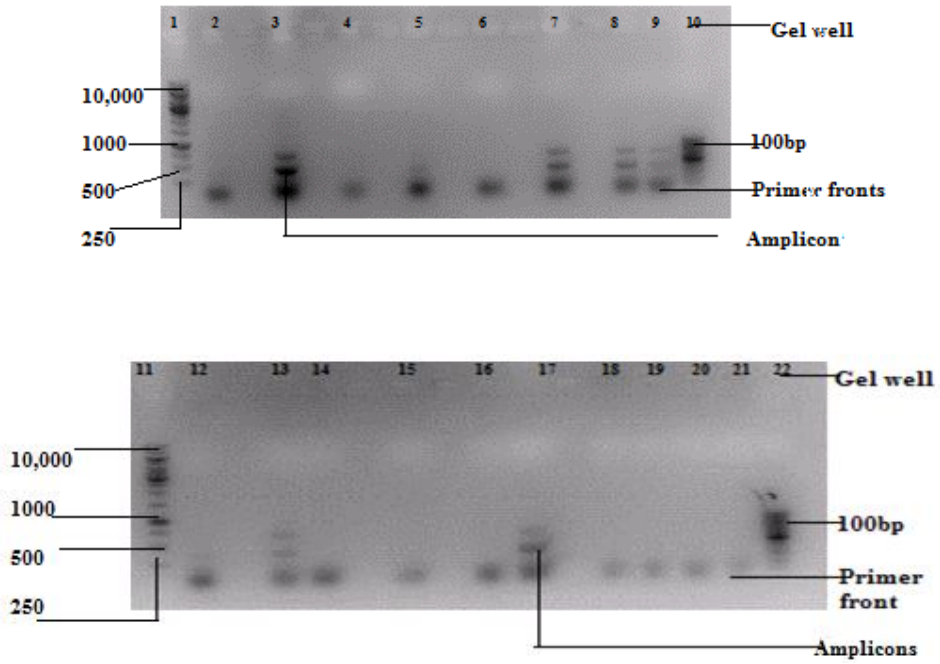


Fig4.3: The result of the gel electrophoresis of the multiplex PCR amplification

KEY:

Lane1: O'GeneRuler1kb DNA Ladder
Lane2: 97A3(*E. coli*)
Lane3: 79B1 (*Staph. aureus*)
Lane4: 6A (*Ps. aeruginosa*)
Lane5: 81B (*Staph. aureus*)
Lane6: 129B (*Staph. aureus*)
Lane7: 37Bsc (*E. coli* small colonies)
Lane8: 37Bbc (*E. coli* big colonies)
Lane9: 72Bsc (*E. coli* small colonies)
Lane10: O'GeneRuler 100bp DNA Ladder
Lane11: O'GeneRuler 1kb DNA Ladder
Lane12: 128BY (*E. coli*)
Lane13: 123B (*E. coli*)
Lane14: 123B2 (*E .coli*)
Lane15: 97A2 (*E. coli*)
Lane16: 72Bbc (*E. coli* big colonies)
Lane17: 53B (*Staph. aureus)*
Lane18: 142A (*Ps. aeruginosa)*
Lane19: 144A (*Ps. aeruginosa)*
Lane20: 128B (*E. coli*)
Lane21: 137A *Ps.aeruginosa*
Lane22: O'GeneRuler 100bp DNA Ladder

Table 4.16 shows the calculated base pair (bp) of each amplicon obtained in the gel electrophoresis analysis of the multiplex polymerase chain reaction. From the table it can be seen that the amplicons obtained could be mutants of the genes coded for by these primers. Other isolates did not show amplification.

Table 4.16: Multiplex PCR products showing the amplicons and their base pair

Gel Lane	Organism	Base pair (bp)
2	*E.coli*	509
3	*Staph. aureus*	1098, 706
5	*Staph. aureus*	706, 509
7	*E. coli* (small colonies)	902, 509
8	*E. coli* (big colonies)	1098, 706
9	*E.coli*(big colonies)	1098, 509
12	*E. coli*	447
13	*E.coli*	921, 447
17	*Staph. aureus*	921, 605

The base pairs of the primers used are TEM (931); SHV (868); OXA-2 (478); aac-(3)-lla (900); rmta (634); gyrB (448) and gyrA (648).

Lane 2 isolate has gene OXA-2; lane 3 isolate has TEM and probably gyrA. Lane 5 isolate has gyrA and OXA-2; lane 7 isolate has aac-(3)-lla andOXA-2. Lane 8 has TEM and gray; lane 9 has TEM andOXA-2; lane 12 has gyrB; lane 13 has probably aac-(3)-lla or TEM and gyrB, while lane 17 haseither TEM or aac-(3)-lla and rmta or gyrA coding for their resistance.

Table 17: Summary of Multiplex PCR Products

Gel Lane	Organism	Gene type	Antibiotic resistance coded for
2	*E.coli*	OXA-2	β-lactam, Ceph
3	*S. aureus*	TEM, gyrA	β-lactam, Ceph, Quinolone
5	*S. aureus*	gyrA, OXA-2	β-lactam, Ceph, Quinolone
7	*E. coli*	aac-(3)-lla, OXA-2	Aminogly, β-lactam, Ceph
8	*E. coli*	TEM, gyrA	β-lactam, Ceph, Quinolone
9	*E.coli*	TEM, OXA-2	β-lactam
12	*E. coli*	gyrB	Quinolone
13	*E.Coli*	aac-(3)-lla, TEM, gyrB	Aminogly, β-lactam, Ceph, Quinolone
17	*S. aureus*	TEM, rmta, gyrA	β-lactam, Ceph, Aminogly, Quinolone

Key:

Ceph----- cephalosporins

Aminogly-----aminoglycosides

CHAPTER FIVE

DISCUSSION

5.1 DISCUSSION

This study showed asymptomatic bacteriuria prevalence of 58.4% amongst the sampled population of 250 pregnant women attending ante-natal clinic. This level of prevalence is comparable to results of other researchers. Obiogbolu *et al.*, 2009 reported a prevalence of 54% in a study carried out among 100 pregnant women in Akwa metropolice, South-eastern-Nigeria which is similar to the findings of Onifade *et al,* 2005, who found out that asymptomatic bacteriuria was present in 58% of 1,020 pregnant women sampled in Ondo State, South-western-Nigeria. However, these figures are substantially lower than the figure (86.6%) reported by Akerele *et al*, (2001) in a study on 500 pregnant women in Benin City, South-south-Nigeria although the number of samples diferred considerably in each study. The similarity of the result of this study to those of Obiogbolu *et al.,* (2009) and Onifade *et al.,* (2005) even though the studies were not done in the same area, could be due to increase in awareness and improved health facilities as well as the increase in the usage of antimicrobial agents as compared to the time when Akerele *et al.,* (2001) carried out their investigation.

The findings that women in their second trimester had higher incidence of asymptomatic bacteriuria in this study (61.6%), is in agreement with the report of Lindsay (2003), who attributed this high incidence to the increased gestational period. There is usually an increased hormonal activity with increase in gestational age, and this aids the growth of bacteria. It was not surprising that the age group of 31- ≥40 years had the highest incidence of asymptomatic bacteriuria since women in the age

group (25-40) are more sexually active and multiparity is also a risk factor in acquiring asymptomatic bacteriuria (Antoni *et al,* 1997; Kumar *et al*, 2002; Obirikorang *et al,* 2012).

Generally most of the anatomic and physiologic changes in pregnancy influence the urinary tract and can result in urologic diseases and changes in kidney function which are serious threats for both the mother which may result in hypertension, preeclampsia, low birth weight, prematurity, septicaemia, maternal and foetal death (Delzell and Lefevre, 2000; Christensen, 2000; Klein and Gibbs, 2004). High incidence of urinary tract infection has been attributed to hormonal effects observed in pregnancy which reduces tone of the ureteric musculature coupled with mechanical pressure from the gravid uterus leading to urinary stasis, thus encouraging bacterial proliferation in urine (Obiogbolu, 2004).

Screening in pregnancy for asymptomatic bacteriuria, and treatment with antibiotics of positive cases has been recommended in order to prevent complications such as pyelonephritis, premature labour, still birth, hypertension, maternal anaemia, pre-eclampsia and septicaemia (Bachman *et al.*, 1993; Raz, 2003). Also it has been shown that treating asymptomatic bacteriuria in pregnancy decreased the subsequent incidence of symptomatic bacteriuria by 80-90% (Harris, 1979). There is no clear consensus in literature on either the duration of treatment or the choice of antibiotics to be employed in the treatment of asymptomatic bacteriuria as this depends actually on the knowledge of local resistance patterns. Complications of bacteriuria in pregnancy include maternal anaemia, increase in mid trimester abortion, while Asymptomatic bacteriuria can lead to low neonatal birth weight, hypertension, growth retardation and preterm labour.

The findings in this study revealed that *Staphylococcus spp* were the highest (25.40%) isolated organism followed by *Klebsiella spp* (24.90%) and *Proteus spp* (23.35%). Only 16.75% were *Escherichia spp* and 9.10% were *Pseudomonas spp* when the conventional biochemical techniques were employed but the use of API kits identified these genuses to their specie levels as described under the results in chapter four. Although earlier researchers reported that *Escherichia* spp were often the most common isolated organism in asymptomatic bacteriuria, (Onifade *et al.,* 2005; Turpin *et al.,* 2007; Obiogbolu *et al.,* 2009; Andabati and Byamugisha, 2010), recent reports have shown that organisms such as *Staphylococcus spp, Klebiella* spp and *Proteus spp* are overtaking the position of *Escherichia spp* (Ogbolu *et al.,* 2006; Amadi *et al.*, 2007; Ajayi *et al,* 2012). The discrepancies observed in the use of these two methods could be as a result of the inclusion of other test reactions in the API kit systems that enable them to identify these organisms better than the conventional methods as reported by Peuckert and Pelz, (1980); Stanek *et al.,* (1980); Mastroeni *et al.,* (1983); O'hara, (2005). The occurrence of these uropathogenic isolates in pregnant women gives cause for concern as they are prone to several complications as well as foetal risks.

Result of the antibiotic sensitivity tests revealed that many of the isolated bacteria in this study have developed resistance to the commonly prescribed antibiotics. It implies that there is the need to perform culture and sensitivity tests before antibiotics are prescribed in order to achieve complete therapy. This high level of resistance could be attributed to the fact that there has been increase in the use, misuse as well as abuse of antibiotics in recent times by the populace as well as the prescription of especially broad-spectrum antimicrobial agents by physician while awaiting results of laboratory tests where such tests has been requested. It is also possible that emergence

of substandard, adulterated and fake drugs imported or made in the country are contributing to the resistance pattern seen in this work.

Most of the isolates had Multiple Antibiotic Resistance (MAR) index ≥0.2 which is taken as significant, revealing that they were resistant to more than two classes of antimicrobial agents tested. This observation suggests that the isolates in this study may probably have originated from an environment where antibiotics are often used indiscriminately (Paul *et al.*, 1997). Broad-spectrum antibiotics are sometimes reported to be given in place of narrow-spectrum antibiotics as a substitute for culture and sensitivity testing, with the consequent risk of selection of antibiotic-resistant mutants (Krumperman, 1983; Nahum *et al*., 2006; Norwitz and Greenberg, 2009).

The high percentage of isolates with ESBL (Extended-spectrum β-lactamases) production (61%) is indeed of much concern. Extended-spectrum β-lactamases in Gram-negative organisms have been implicated as the enzymes responsible for resistance to β-lactam antibiotics such as cefotaxime, ceftazidime, and aztreonam (Bush *et al.*, 1995; Jacoby and Medeiros, 1991). Since these enzymes were first identified in Western Europe, more than 70 ESBLs have been found worldwide (Jacoby and Medeiros, 1991). ESBLs such as plasmid-encoded class A TEM- and SHV-type enzymes have evolved from successive mutations in their structural genes, resulting in either single or multiple amino acid changes in the encoded enzymes.

As shown by the multiplex PCR, few of the *E. coli* isolates showed the presence of some genes (gyrB and TEM) that code for ESBL production, while only one contained genes that code for aminoglycoside. Most of the genes identified had base pairs different from those of the primers used; these could be due to mutation processes that do occur in bacteria. Some of the isolates did not show amplicons,

indicating that the resistant genes primers used for this study are not contained in these isolates or the amount of gene was too small or the number of cycles required was more than 45 chosen. It could also be due to the fact that the gene is not expressed at the molecular level of the primers used or these isolates might contain other resistant genes, which are also expressed at molecular level. The report of Charlene *et al*, (2004) tends to support these assertions. They, Charlene *et al*, (2004) in their study could not find bands similar to those genes tested and concluded that additional resistance genes may exist but not expressed at molecular level. The findings in this study that some isolates may contain more than one ESBL gene are in agreement with the report of Nadine *et al*, (2012) that of the twenty-seven CTX-M carriers were additionally PCR-positive for bla_{TEM} genes. Thirty-four isolates were CTX-M-1 producers, eight expressed additional TEM-1 and one isolate--from a pig--additionally expressed a TEM-type enzyme, TEM-186 never found before (nucleotide sequence accession number JN227084). Six isolates carried CTX-M-14 with TEM-1 and five isolates specified CTX-M-15, one of which producing additional TEM-1. One isolate from a calf produced TEM-1 in combination with CTX-M-117 a novel CTX-M group 1 ESBL with an amino acid sequence never found before (nucleotide sequence accession number JN227085).

According to Hyunjoo *et al*. (1999) and Ronen *et al*., (2009) analysis using multiplex PCR, *E. coli* isolates which were resistant to carbapenems and cephamycin expressed AmpC gene (CMY-2). It should be noted that plasmid-mediated *AmpC* genes are derived from inducible chromosomal genes that have become mobilized (Pérez-Pérez and Hanson, 2002; Philippon *et al.*, 2002)**.** Hence, the majority of cephamycin resistant *E. coli* strains are attributed to attenuator and/or promotor mutations upstream of the chromosomal AmpC gene (Forward *et al.*, 2001) i.e. resistant strains

emerge as a result of spontaneous mutation of sensitive strains rather than by the spread of strains or plasmids in the hospital setting (Michael *et al.*, 2005). In the formation of small colony variants; when bacteria cells are exposed to environmental stress (antibiotic, acid stress, metals e.g. $CuSO_4$, 2- methyl 1, 4-napthoquinone, etc), bacteria increase the synthesis of general stress proteins and heat shock proteins usually in response to the accumulation of misfolded and denatured proteins (Goff and Goldberg, 1985), which allows them to contend with the adverse environment without changes in their nutrition, fermentation and serology. Many of these stress-induced proteins are chaperones and proteases. Their biological role is to protect cellular proteins against the toxic effects generated by exposure to stress (Laskos *et al.*, 2004). The chaperones function to eliminate misfolded proteins by (i) unfolding these proteins and subsequently promoting proper folding and (ii) targeting unfolded proteins for proteolysis (Gamer *et al.,* 1996). Small colonies variant of *E. coli* has been observed to be resistant to third generation flouroquinolone (Edens *et al*., 1997), aminoglycoside (Balwit *et al*., 1994) antibiotics by changing their biotypes and become resistant after culturing once in their presence. Small colony variants (SCVs) are slow-growing bacteria, which often show increased resistance to antibiotics due to deletion mutation of the hemB gene, and cause latent or recurrent infections (Roggenkamp *et al.*, 1998).

Currently, therapy for strains of *Enterobacteriaceae* that express ESBLs is limited to such broad-spectrum agents as imipenem. However, there have already been reports of therapeutic failures of this drug with strains that produce multiple β-lactamases (Ahmad *et al.,* 1999). There are limited therapeutic options left for some of these organisms. Strains expressing extended-spectrum β-lactamases will present a host of challenges for clinical microbiologists and clinicians alike as we head into the next century.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

This study was designed to determine the incidence of asymptomatic bacteriuria (ASB) among pregnant women attending ante-natal clinics in Sabon-Gari LGA–Zaria, Nigeria. An incidence rate of 58.4% was found. This level of incidence is in consonance with findings from other parts of the country. Higher incidence was recorded among 31- ≥40 years age group and also among those who were in their second trimester.

The most common organisms isolated from asymptomatic bacteriuric pregnant subjects were Gram-negative bacteria, notably *Klebsiella, Proteus, E. coli* and *Pseudomonas* constituting 74.1%. Among the Gram-positive bacteria, *Staph. aureus* and *Staph. xylosus* were the most predominant. Further identification to specie level with API kits revealed the presence of *E. coli* (30.3%), *Ent. cloacae* (15.1%), *Citro. freundii* (6.1%), *Citro. braakii* (6.1%), and *Kleb. pneumoniae* (6.1%). Others are *Staph. aureus* (18%), *Staph. xylosus* (12.82%), *Staph. lentus* (7.7%), *Staph. auriculous* (5.1%), *Staph. cohnii* (2.56%) and *Micrococcus* (10.3%). *Ps. aeruginosa* (6.4%), *Aeromonas aerohydrophila/caviae* (2.4%), *Ps. leutiola* (0.8%), and *Burkhol pseudomallei* (0.8%)

Antimicrobial susceptibility test revealed varying susceptibility profiles for the isolates. Most *Staphylococcus spp* were only susceptible to ciprofloxacin, ofloxacin, nitrofurantoin, gentamicin and cotrimoxazole but resistant to other agents tested. *Escherichia spp* isolates were moderately susceptible to (F), (CIP), (OFX), (SXT) and

erythromycin and were resistant to other agents. Isolates of *Proteus spp* showed good susceptibility to OFX and CIP, F, CN and streptomycin, they were resistant to other agents. Similarly, isolates of *Klebsiella spp* were susceptible to OFX, CN, CIP, F and SXT.

6.2 MULTIPLEX PCR

Multiplex PCR showed that most of the test *E.coli* isolates harbour genes that encode for β-lactamase and ESBL as well as genes that code for resistance to β-lactam antibiotics. The detection of TEM, SHV, OXA-2 encoding genes (though they seem to be mutant strains) for resistance to ESBLs shows that these resistance determinants are found in our environment. These resistant traits call for surveillance in clinical settings because ESBLs gene is a world growing trait for resistance but its epidemiological effect and evaluation is still under estimated with low awareness.

6.3 RECOMMENDATIONS

- It has been reported that approximately 25-30% of asymptomatic bacteriuria in pregnancy will progress to symptomatic infection, which is 3-4 times as in non-pregnant women (Dominic, 2006), hence there is need for early routine screening of all antenatal patients presenting or not presenting with clinical symptoms of urinary tract infection in order to prevent adverse maternal and neonatal outcome.
- Proper routine bacterial culture test should be carried out by clinicians for their antenatal patients. The strip urinalysis method which is been utilized by most clinicians for assessing the urine of these pregnant women can not quantify the

extent of infection as well as provide adequate antimicrobial therapy, so this should be discouraged in antenatal clinics. Asymptomatic bacteriuria occurs without the usual clinical symptoms of UTI, most of these pregnant women might be infected with some uropathogens without their knowledge; and this could lead to adverse maternal and neonatal outcome.

- Asymptomatic bacteriuria must be treated in pregnancy in order to prevent complications such as pyelonephritis, premature labour, still birth, hypertension, preeclampsia and septicemia. (Raz, 2003).
- Rational use of antibiotics should be encouraged in all antenatal clinics to help fight the war against the emergence and transmission of multiple antibiotic resistant strains.
- There is the need to improve patients record keeping in the Primary Health Care System. As at now, virtually no records of patients are being kept making it impossible for follow-up and monitoring of patients that may be placed on antibiotic therapy /management.

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APPENDIX

APPENDIX I

PROJECT QUESTIONNAIRE

1. How many pregnancies have you had to date? _____

2. How many children alive? _____
   No of boys _____ No of girls _____

3. Have you ever had any miscarriages? _____
   If yes how many _____
   Is this your first visit to the clinic _____ if no state number _____

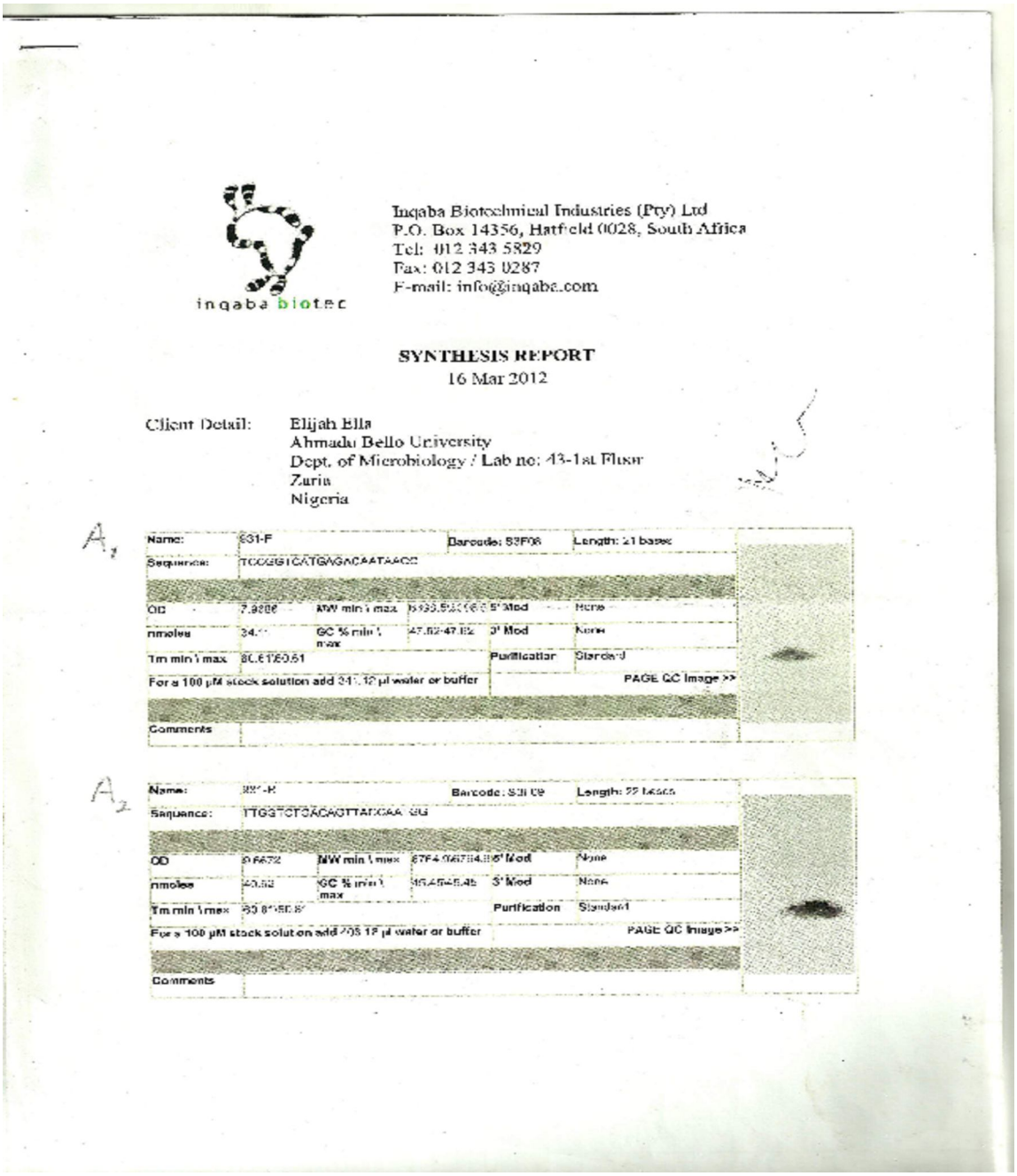
4. Age: a. Below 20 [ ] b. 21-30 [ ] c. 31-40 [ ]
   d. Above 40 [ ]
5. Level of education? a. None (0) [ ] b. Primary (1) [ ]
   c. Secondary (2) [ ] d. FCE [ ] e. Polytechnic [ ]
   f. University [ ]
6. What do you do for a living?
   Nil [ ]
   Others (specify) _____
7. Age of this pregnancy a. 1-3 months [ ] b. 4-6 months [ ]
   c. 7-10 months [ ]
8. Have you been falling sick during this pregnancy?
   a. How many times?
   b. UTI (painful urination, burning sensation etc)
   c. What treatment did you receive?
9. What drugs are you on?
   a. Anti-malarial; (specify) _____
   b. Heamatinics; (specify) _____
   c. Antibiotics; (specify) _____
   d. Herbal products;
   e. Others (specify) _____
10. How long have you been on them? _____
11. How many wives does your husband have? _____

APPENDIX II

Clinical Laboratory Standards Institute (CLSI Formerly NCCLS**) Interpretative Chart for Antimicrobial Sensitivity Testing (Eighteenth Information Supplement,** January, 2008)

Antibiotics	Disc Potency	Diameter of zone of growth inhibition		
		Resistant	Intermediate	Sensitive
Ampicillin	10µg	≤13	14-16	≥17
Methicillin	5 µg	≤9	10-13	≥14
Ceftrazidime	30µg	≤14	15-17	≥18
Ceftriaxone	30µg	≤13	14-20	≥21
Gentamicin	10µg	≤12	13-14	≥15
Erythromycin	15µg	≤13	14-22	≥23
Ciprofloxacin	5µg	≤15	16-20	≥21
Ofloxacin	5µg	≤14	15-17	≥18
Nitrfurantoin	300µg	≤14	15-16	≥17
Co-trimoxazole	25µg	≤10	11-15	≥16
Streptomycin	10µg	≤11	12-14	≥15

APPENDIX III



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A_1

Name:	63f-F		Barcode: 63F08		Length: 21 bases
Sequence:	TCCGGTCATGAGACAATAACC				
OD	7.9806	MW min \ max	6433.5\6516.3	5' Mod	None
nmoles	34.[illegible]	GC % min \ max	47.62\47.62	3' Mod	None
Tm min \ max	60.6\60.61			Purification	Standard
For a 100 µM stock solution add 341.12 µl water or buffer				PAGE QC Image >>	
Comments					

A_2

Name:	8[illegible]-R		Barcode: 83R09		Length: 22 bases
Sequence:	TTGGTCTGACAGTTACCAA GG				
OD	9.6672	MW min \ max	6764.0\6784.8	5' Mod	None
nmoles	40.52	GC % min \ max	36.4\45.45	3' Mod	None
Tm min \ max	59.8\60.8			Purification	Standard
For a 100 µM stock solution add 405.18 µl water or buffer				PAGE QC Image >>	
Comments					

B1

Name:	968-F			Barcode: S3F0A	Length: 21 bases
Sequence:	TGGTTATGGTTA ATTCGCG				
OD	8.0976	MW min \ max	6715.4	5' Mod	None
nmoles	35	GC % min \ max	42.85 47.62	3' Mod	None
Tm min \ max	58.86 66.66			Purification	Standard
For a 100 µM stock solution add 350 µl water or buffer					PAGE QC Image >>
Comments					



B2

Name:	968-R			Barcode: S3F0B	Length: 19 bases
Sequence:	GGTTAGCGTTGCCAGTGCT				
OD	6.6253	MW min \ max	5850.8 5850.8	5' Mod	None
nmoles	45.03	GC % min \ max	47.37 57.89	3' Mod	None
Tm min \ max	62.32 62.32			Purification	Standard
For a 100 µM stock solution add 340.29 µl water or buffer					PAGE QC Image >>
Comments					



C1

Name:	970-F			Barcode: S3F0C	Length: 20 bases
Sequence:	AACAAACCCTACTCGCCTGC				
OD	8.848	MW min \ max	6070.0 6070.0	5' Mod	None
nmoles	30.38	GC % min \ max	55.55	3' Mod	None
Tm min \ max	62.16 62.16			Purification	Standard
For a 100 µM stock solution add 303.75 µl water or buffer					PAGE QC Image >>
Comments					

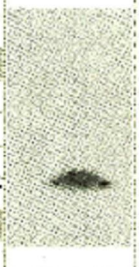


Name:	478-R			Barcode: 53F00	Length: 20 bases
Sequence:	CTACTTAACCGATCGTACCC				
OD	7.3458	MW min \ max	5886.3 5886.5	5' Mod	None
nmoles	41.6	GC % min \ max	47.40	3' Mod	None
Tm min \ max	54.0/64.0			Purification	Standard
For a 100 µM stock solution add 416.01 µl water or buffer					PAGE QC Image >>
Comments					

Name:	338fF			Barcode: 53F0F	Length: 32 bases
Sequence:	CCGAATTCAGAGGAGATATCCGCGATGCAAGG				
OD	8.1622	MW min \ max	9860.6/9901.8	5' Mod	None
nmoles	24.71	GC % min \ max	50.00	3' Mod	None
Tm min \ max	69.7/69.7			Purification	Standard
For a 100 µM stock solution add 247.1 µl water or buffer					PAGE QC Image >>
Comments					

Name:	338fR			Barcode: 53F0F	Length: 21 bases
Sequence:	CATTGTCGACGGCCTCTAAGC				
OD	6.2776	MW min \ max	6541.6/6581.8	5' Mod	None
nmoles	29.78	GC % min \ max	57.14/57.14	3' Mod	None
Tm min \ max	64.0/64.0			Purification	Standard
For a 100 µM stock solution add 297.8 µl water or buffer					PAGE QC Image >>
Comments					

Name:	RMTA-F		Barcode: 33[illegible]0		Length: 20 bases
Sequence:	CTAGCGTCCATCCTTGCTC				
OD	7.34[illegible]6	MW min \ max	995[illegible]7.6	5' Mod	None
nmoles	40.4[illegible]	GC % min \ max	55.00	3' Mod	None
Tm min \ max	62.4962.46			Purification	Standard
For a 100 µM stock solution add 4[illegible] µl water or buffer					PAGE QC Image >>
Comments					



Name:	RMTA-R		Barcode: 33[illegible]1		Length: 20 bases
Sequence:	TTTGCTTCCATGCCCTTGCC				
OD	6.[illegible]1	MW min \ max	6035.5-6886.6	5' Mod	None
nmoles	38.38	GC % min \ max	47.37	3' Mod	None
Tm min \ max	62.4962.46			Purification	Standard
For a 100 µM stock solution add 309.35 µl water or buffer					PAGE QC Image >>
Comments					



RECOMMENDATIONS FOR HANDLING AND STORAGE OF OLIGOS

- Lyophilized oligo pellets might become displaced from the bottom of the tube during shipment. Briefly centrifuge each tube before opening to prevent loss of the pellet.
- Prepare stock solution of oligos (e.g. 100 U/ml = 100 pmole per µl) preferably with a sterile buffered solution such as TE (10 mM Tris, pH 7.5 to 8.0, 1 mM EDTA). If sterile distilled water is used, make sure that the pH is above 7.0 since acidic solutions will cause oligo depurination and subsequent loss of activity.
- Working solutions might be diluted from the stock solution with sterile, nuclease-free water to prevent inhibition of enzymatic reactions (e.g. PCR) by EDTA.
- Store the oligos as concentrated stock solution or lyophilized at -20° C.
- Avoid frequent freeze-thaw cycles by dividing the stock solution into single aliquots for long term storage and to prevent accidental contamination.
- Dye-modified oligos are light sensitive and should always be stored in the dark.
- Re-suspend modified oligos preferably in a slightly basic solution (i.e. TE at pH 8.0). However, Cy dye modified oligos are best kept at pH 7.0 or -20° C.
- Preferably store the modified oligos as dried aliquots at -20° C.

APPENDIX IV

PRIMERS USED

Beta- lactams and Cephalosporin: e.g Ampicillin, Ceftazidime

Resistance gene	Amplicon size (bp)	Oligosequence	Target Source
(A) TEM TEM190	931	F = 5′TCCGGTCATGAGACAATAACC3′.	TEM1-
		R = 5′TTGGTCTGACAGTTACCAATGG3′	
(B) SHV SHV63	868	F = 5′TGGTTATGCGTTATATTCGCC3′	SHV1-
		R = 5′GGTTAGCGTTGCCAGTGCT3′	
(C) OXA-2 OXA15	478	F = 5′AAGAAACGCTACTCGCCTGC3′	OXA1-
		R = 5′CCACTCAACCCATCCTACCC3′	

Aminoglycoside: e.g Gentamicin

PCR targe	**Primer**	**Amplicon size (bp)**	**Sequence**
(D) *aac(3)-IIa*	aacIIaF	900	GGG AAT TCA GAG GAG ATA TCG CGA TGC ATA CG.
	aacIIaR		CAT TGT CGA CGG CCT CTA ACC

Accession no. (Reference or Source): X13543

Quinolones: e.g Ciprofloxacin

(E) *rmta*	RMTA-F	634	CTA GCG TCC ATC CTT TCC TC
	RMTA-R		TTT GCT TCC ATG CCC TTG CC

Accession no.(Reference or Source): AB083212 (46)

(F) *gyrB*	448	F= GCGCGTGAGATGACCCGCCGT
		R= CTGGCGGTAGAAGAAGGTCAG
(G) *gyrA*	648	F= TACACCGGTCAACATTGAGG
		R= CCGGATCGGTAAGCTTCTTCAAT

Accession no. (Reference or Source): D87842

APPENDIX V

ANTIBIOTIC SUSCEPTIBILITY TEST

Isolate	**ANTIBIOTICS**										
No	E	CIP	CN	F	CAZ	OFX	CRO	AMP	S	SXT	MET
1A	I	S	R	S	R	S	R	R	R	S	R
2A	R	R	S	R	R	R	R	R	S	S	R
3A	I	S	S	S	R	S	S	R	R	R	R
4A	R	S	S	R	R	S	R	R	R	R	R
5A	R	S	S	R	R	S	S	R	R	R	R
6A	R	S	I	R	R	S	R	R	R	R	R
7A	R	S	S	S	R	S	R	R	R	S	R
9A	S	S	S	S	R	S	R	R	S	S	R
10A	I	S	R	S	R	S	R	R	R	S	R
11A	R	S	S	R	R	S	R	R	R	R	R
12A	S	S	S	S	R	S	R	R	S	R	R
13B	R	S	S	S	R	S	R	R	S	S	R
14B	R	S	S	S	R	S	I	R	R	S	R
15BC	R	S	S	S	R	S	S	R	R	R	R
16BW	R	I	R	S	R	I	R	R	R	R	R
16BY	R	I	R	S	R	I	R	R	R	R	R
17B	I	I	R	S	R	I	R	R	R	S	R
18B	S	S	R	R	R	S	R	R	S	R	R
19B	S	S	S	S	R	S	R	R	R	S	R
20B	R	S	S	I	R	S	R	R	S	S	R

21B	R	R	R	R	R	R	R	R	R	R	R
23B	R	I	S	S	R	S	R	R	R	S	R
24B	S	I	R	S	R	S	R	R	R	R	R
25B	R	S	S	S	R	S	R	R	R	R	R
26BY	R	R	R	S	R	S	R	R	S	S	R
26BOR	I	S	S	S	R	S	S	R	R	S	R
27BC	I	S	S	S	R	S	R	R	R	S	R
27BB	I	I	R	S	R	R	R	R	R	S	R
28B	R	S	S	R	R	S	S	R	I	S	R
29B	R	S	S	S	R	S	S	R	S	S	R
30B	R	I	S	S	R	I	S	R	S	R	R
31BY	R	S	I	S	R	I	S	R	R	S	R
31BB	R	S	S	S	R	S	S	R	I	S	R
32B	R	S	I	S	R	I	R	R	R	S	R
33B	S	I	S	S	R	R	R	R	R	S	R
34B	S	S	S	S	R	S	R	R	S	S	R
35B	S	S	S	S	R	S	R	R	S	S	R
36B	R	S	S	S	R	S	R	R	R	I	R
37BBC	R	S	R	S	R	S	R	R	R	R	R
37BSC	R	S	I	S	R	S	R	R	R	R	R
37B Blue	R	I	I	S	R	I	I	R	R	S	R
38B	S	R	R	S	R	R	R	R	R	R	R
39B	R	S	S	S	R	S	R	R	S	S	R

40BW	R	I	S	R	R	S	S	R	R	I	R
40BB	I	S	S	S	R	S	S	R	S	S	R
41B	I	S	S	R	R	S	R	R	R	S	S
42B	R	S	S	S	R	S	S	R	I	S	R
$44B^{B}$	R	I	R	S	R	S	R	R	R	R	
44BY	R	I	R	S	R	R	R	R	R	R	R
45B	R	S	S	S	R	S	R	R	R	R	R
47BY	S	S	S	R	R	S	R	R	S	R	R
47BB	I	S	S	R	R	S	S	R	S	S	R
48B	I	S	S	R	R	S	S	R	S	S	R
48B	I	S	S	I	R	S	R	R	S	R	R
49B	R	S	R	S	R	S	R	R	R	S	R
50B	S	S	S	S	R	S	R	R	S	R	R
51BC	I	S	S	S	R	S	R	R	I	R	R
$51B^{B}_{1}$	S	S	S	I	R	S	R	R	R	S	R
52B	S	S	S	S	R	S	R	R	I	I	R
53B	R	S	R	S	R	S	R	R	R	S	R
54B	R	S	S	S	R	S	R	R	R	S	R
55B	R	S	S	R	R	S	R	R	R	S	R
56B	I	I	R	S	R	S	R	R	R	S	R
B1	S	S	S	S	R	S	R	R	S	S	R
57BB2	R	S	S	S	R	S	S	R	S	S	R
58B	R	S	S	S	R	S	R	R	R	S	R
59B	R	S	R	S	R	S	R	R	R	R	R

60B	R	S	S	S	R	S	R	R	R	R	R
61B	R	S	S	S	R	S	R	R	R	R	R
63B	R	S	S	S	R	S	I	R	R	R	R
64B	R	S	S	I	R	S	S	R	S	S	R
65B	R	S	S	R	R	S	R	R	S	S	R
67B	R	I	R	S	R	R	R	R	R	I	R
$69B^{P}$	R	S	S	S	R	S	S	R	R	S	R
69BY	R	S	S	S	R	S	S	R	I	S	R
70B1	R	S	S	S	R	S	R	R	R	I	R
70B2	R	S	S	S	R	S	I	R	R	R	R
71B	R	S	S	S	R	S	I	R	R	R	R
$72B^{BC}$	R	I	S	I	R	I	I	R	R	S	R
72BSC	R	S	S	S	R	S	R	R	R	I	R
73B	R	S	S	S	R	S	S	R	R	S	R
74B	R	R	R	S	R	R	R	R	R	R	R
76B	R	R	R	S	R	R	R	R	R	R	R
77B	A	S	S	S	R	S	R	R	S	R	R
78B	R	S	S	S	R	S	R	R	S	S	R
$79B^{B}{}_{1}$	I	S	S	S	R	S	R	R	S	R	R
$79BB_{2}$	R	I	R	S	R	I	R	R	R	S	R
80B	R	S	S	S	R	S	R	R	R	R	R
81B	I	R	R	S	R	R	R	R	I	S	R
82A	R	I	S	S	R	S	R	R	R	S	R
84A	R	S	S	S	R	S	I	R	R	S	R

85A	S	S	S	S	R	S	R	R	I	S	R
86A	R	S	S	R	R	S	R	R	R	R	R
87A	R	S	S	S	R	S	I	R	R	S	R
88A	R	S	S	S	R	S	I	R	R	R	R
98A	R	I	S	S	R	I	R	R	R	S	R
90A	I	S	R	S	R	S	R	R	R	S	R
91A	R	S	S	R	R	S	R	R	R	I	R
92A	R	S	R	S	R	S	I	R	R	R	R
93A	R	S	S	S	R	S	R	R	I	S	R
94A	I	I	S	S	R	S	R	R	S	R	R
95A	R	I	R	S	R	R	R	R	R	R	R
96A1	I	S	S	S	R	S	R	R	S	R	R
96A2	S	I	R	S	R	I	R	R	R	S	R
$97A_2$	R	S	I	S	R	S	I	R	I	S	R
97A3	I	S	S	S	R	S	I	R	S	S	R
98A	R	I	S	S	R	S	R	R	R	R	R
99A	R	S	S	S	R	I	R	R	R	R	R
100A	R	S	S	S	R	S	R	R	R	R	R
101BY	R	R	R	S	R	S	R	R	R	R	S
$101B^{C}$	S	S	S	S	R	S	R	R	R	I	R
$102B^{C}$	I	S	S	S	R	S	S	R	S	S	R
102BW	R	S	S	S	R	S	S	R	R	S	R
$103B^{B}$	R	S	S	S	R	S	R	R	R	R	R
103BW	S	S	R	S	R	S	R	R	R	S	R

104B	I	S	S	S	R	S	S	R	I	S	R
105B	R	S	S	R	R	S	R	R	R	R	R
106B	R	S	S	S	R	S	I	R	R	R	R
107B[B]	R	I	S	S	R	S	S	R	R	S	R
109BW	R	S	S	S	R	S	I	R	S	S	R
109B[B]	I	S	S	S	R	S	I	R	-	S	R
110B	S	S	S	S	R	S	R	R	S	R	R
111B	R	I	S	R	R	S	R	R	R	S	R
112B[B]	S	S	S	S	R	S	S	R	S	S	R
112BW	R	S	S	S	R	S	S	R	R	I	R
113B[B]	R	S	S	S	R	S	S	R	I	S	R
113BW	S	S	S	S	R	S	R	R	S	R	R
114B	S	I	S	S	R	S	R	R	I	R	R
115B[DB]	R	I	R	S	R	S	R	R	R	S	R
115BW	R	I	R	S	R	I	R	R	R	S	R
116B	S	S	S	S	R	S	S	R	I	S	R
117B[W]	I	S	I	S	R	S	I	R	S	S	R
117BB	I	S	S	S	R	S	S	R	R	S	R
118B[W]	S	S	S	S	R	S	R	R	S	R	R
118BB	I	S	S	S	R	S	R	R	S	R	R
119B	S	I	R	S	R	I	R	R	R	S	R
121B[B]	R	I	S	S	R	S	S	R	S	I	R
121BY	R	S	S	S	R	S	I	R	S	S	R
122B	R	S	S	S	R	S	I	R	S	S	R

123B	R	R	S	S	R	S	I	R	R	R	R
124B	R	I	S	S	R	S	R	R	R	R	R
125B[B]	R	S	S	S	R	S	I	R	S	I	R
125BW	R	S	S	S	R	S	S	R	R	S	R
126B	R	S	S	S	R	S	I	R	S	S	R
127BBC	I	S	S	S	R	S	R	R	I	S	R
128B[B]	R	S	S	S	R	S	R	R	R	R	R
128BY	I	I	R	S	R	I	R	R	R	R	R
129B[B]	R	S	S	S	R	S	R	R	S	S	R
129BW	R	S	R	S	R	S	R	R		S	R
130B	-	S	R	S	R	I	R	R	R	S	R
131B	R	I	S	S	R	S	S	R	R	S	R
132B[B]	R	S	R	R	R	S	R	R	-	S	R
132B[W]	R	S	I	S	R	S	R	R	-	S	R
133A	R	I	S	S	R	S	S	R	R	S	R
134A[W]	R	S	S	S	R	S	S	R	R	S	R
134AY	I	S	S	I	R	S	S	R	R	S	R
135AB[C]	I	S	R	R	R	S	I	R	S	I	R
135A	I	I	R	S	R	S	R	R	R	S	R
136AB	R	R	S	S	R	S	R	R	R	S	R
136AY	R	I	S	R	R	S	R	R	R	S	R
136AW	-	S	S	S	R	S	I	R	R	S	R
137A	R	I	S	R	R	S	R	R	R	S	R
138A	R	S	S	S	R	S	I	R	R	S	R

139A	R	S	S	S	R	S	S	R	I	S	R
140A	R	I	S	R	R	S	S	R	R	S	R
142AW	R	S	S	S	R	S	R	R	R	R	R
$142A^{B}$	I	R	S	S	R	R	R	R	I	R	R
143A	R	S	S	S	R	S	R	R	R	R	R
144A	R	I	S	R	R	S	R	R	R	I	R
145A	R	R	R	S	R	R	R	R	R	S	R
$146A^{B}$	R	R	R	S	R	R	S	R	R	R	R
146AY	R	R	R	I	R	R	R	R	R	I	R
$146A^{W}$	R	S	S	I	R	S	I	R	R	S	R

APPENDIX VI

PHOSPHATE BUFFERED SOLUTION (PBS)

a. Stock phosphate solution A

Sodium dihyderogen phosphate - - - - -	27.6g
Distilled water - - - - - - - -	to 1000m

b. Stock phosphate solution B

di-sodium hydrogen phosphate - - - -	28.39g
Distilled water - - - - - - - -	1000ml

For pH 8.0

Solution A - - - - - - -	26.5ml
Solution B - - - - - - -	473.5ml
Distilled water - - - - - - - -	500ml

CRYSTALLINE PENICILLIN BROMOCRESOL PURPLE SOLUTION

Benzylpenicillin - - - - - -	2g
Phosphate buffer pH 8.0 - - - - -	40ml
Bromocresol purple (0.2%$^{w}/_{v}$ - - - - -	0.08ml

To make 40ml solution

APPENDIX VII

RESULT OF BIOCHEMICAL TESTS

Isolate No	1 ONPG	2 ADH	4 LDC	1 ODC	2 CIT	4 H_2S	1 UPE	2 TDA	4 IND	1 VP	2 GEL	4 GLU	1 MAN	2 INO	4 SOR	1 RHA	2 SAC	4 MEL	1 AMY	2 ARA	4 OX
1A	-	+	-	-	+	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-
2A	-	+	-	-	+	-	+	-	-	+	+	+	+	-	-	-	+	-	-	-	-
3A	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
4A	-	+	-	-	-	-	-	-	-	+	+	+/ -	+/ -	+/ -	+/ +	+/ -	+/ -	-	+/ +	-	-
5A	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+/-	-	-	-
6A	-	+	+	-	+	-	+	+	-	-	-	+	-	+	+/-	-	-	-	+	-	-
7A	-	+	+	+	+	+	+	+	-	-/-	+	+	-	-	-	-	-	+	-	+	+
8A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9A	-	+	-	-	+	-	+	-	+	-	+	+	+	+	+	-	-	-	+	+	-
10A	-	+	-	-	+/-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-
11A	-	+	+	+	+	-	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+
12A`	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	-
13B	-	-	-	-	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
14B	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+	-	+/+	+/+	-
15B	+	+	+	+	+	+	-	-	+	-	-	+	+	-/-	+	+	-	-	-/-	+	=
16B	-	+	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	-
17B	-	+	+	+	+	-	-	-	-	+	-	+	+	-	+/-	-	-	-	+	-	-
18B	+	+	-	-	+	-	+	-	+	-	-	-	+	-	+	-	-	-	+	-	-
19B	+	+	-	-	+	-	-	-	+	-	-	+	+	+	-	-	+	-	+	-	-
20B	+/-	+	+	-	+	+	+	+	+/-	+	+	+	+	-	-	-	-	-	-	+/-	+
21B	+/-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
22B	+/-	+	+	+	+	-	+	-	+	+	+	-	+	-	-	+	+	+	-	+	-
23B	+/-	+	+	+	+	-	+	-	+	+	+	+	-	-	-	-	+	-	-	+	-
24B	+	+	+	+	+	-	-	-	+	-	-	+	+	-	+	+	-	+	+	+	-
25B	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
26B	+/-	-	-	-	+	-	+	-	+	+	+	+	+	-	-	-	+	+	-	+	-
27B	+	+	-	-	+	-	-	-	+	+	-	+	+	-	-	-	-	+	+	-	-
28B	-	+	-	-	+	-	-	-	+	+	-	+	+	-	-	+	+	-	-	-	-
29B	+/-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30B	+/-	+	+	+	+	-	+	-	+	+	+	+	-	-	+	-	+	-	+	-	-
31B	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+	-	+	+	-
32B	+	+	+	+	+	+/-	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-

33B	+/-	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
34B	+	+	+	+	+	+/-	+	+	+	+	+	+	+	-	+	+	+	+	=	+	-
35B	+	+	+	+/+	+	+/-	-	+	+	-	-	+	+	-	+	-	-	+	-	-	-
36B	-	+	-	-	+	-	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-
37B	+	+	-	-	+	-	-	-	+	+	-	+	+	-	-	-	-	-	+	-	-
38B`	+/-	+	-	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-
39B	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	+
40B	+	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-
41B	-	+	-	-	+	-	-	-	+	+	+	+	+	-	+	-	-	-	+	-	-
42B	-	+	+	-	+	+/-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+
43B	+/-	+	-	-	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
44B	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-
45B	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-		--	-	+
46B	+/-	+	+	+	-/+	+/-	-	+	-	-	+	+	-	-	-	-/-	-	-	+	-	-
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48B	-	+	+	+	+	-	+	+	-	-	+	-	-	-	-	-	-	-	_	-	+
49B	+	+	+	+	+	+	-	+	+	-	+	+	+	-	+	-	+/-	+	+	+	-
50B	-	+	+	+	+	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-
512B	+	+	+	+	+	+/-	+	-	+	+	-	+	+	+/-	+	+	+	+	+	+	-
52B	+/-	+	+	-	+	-	+	-	-	+	+	+	-	-	+/-	-	+	-	-	-	-
53B	-	+	-	-	+	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	-
54B	-	+	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-
55B	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
56B	-	+	-	-	+	-	-	-	-	+	+	+	+	-	+	-	-	+	+	-	+
57B	-	+	+	-	+	-	-	-	-	+	+	+	+	-	+	-	+	-	+	-	+
58B	+	+	-	-	+	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	-
59B	-	+	-	-	+	-	+	-	-	-	+	-	-	-	-	-	+/-	-	-	-	-
60B	-	+	-	-	+	+/-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+
61B	-	+	-	-	+	-	-	-	-	-	+	+/-	-	-	-	-	+/-	-	+	-	-
62B	+	+	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-
63B	+	+	+	+	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+	+	-
64B	+/-	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-
65B	-	+	+	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-
66B	+/-	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-
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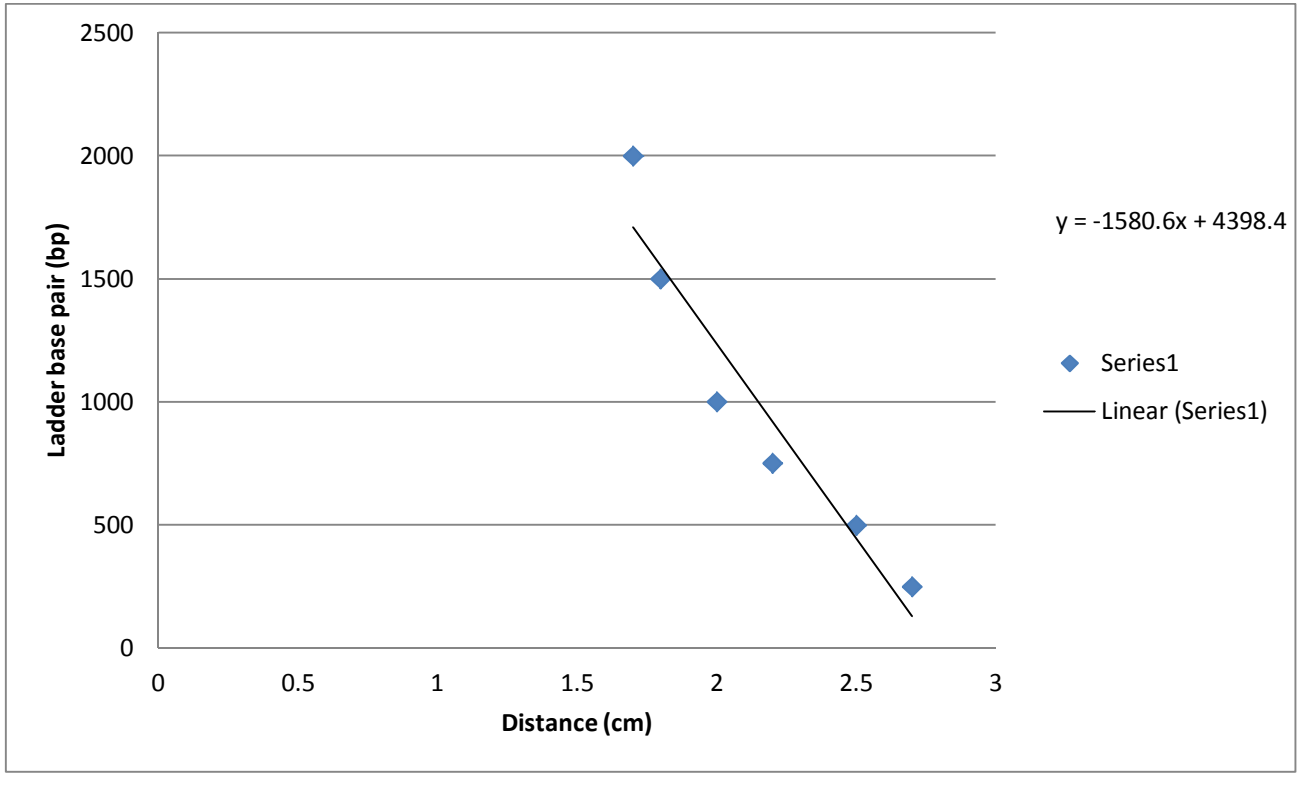
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69B	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	+	-	+
70B	+	+	-	-	+	-	-	-	-	+	-	+	+	-	-	-	+	-	-	-	-
71B	+	+	-	-	+	-	-	-	+	-	-	+/-	-	-	-	-	+	-	-	-	-
72B	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	+	-	+	-
73B	-	+	-	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-
74B	+	+	+	+	+	-	-	+	+	+	-	+	+	-	+	+	+	+	+	+	-
75B	+	+	-	-	+	-	-	-	+	-	-	+	+	+	-	-	+	-	-	+	-
76B	+	+	+	-	+	-	-	+	+	+	+	+	+	-	+	-	+	-	+	-	+
77B	-	+	+	+	+	-	-	-	-	-	+	+/-	-	-	-	-	-	-	+	-	-
78B	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-
79B	+	+	-	-	+	-	+	-	+	+	+	+	+	-	-	-	+	-	-	-	-
80B	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	-
81B	+/-	+	+	-	+	-	-	-	-	-	-	+	+	+	+	+/-	+	-	+	-	-
82A	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
83A	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
84A	+	+	-	-	+	-	-	-	+	-	+	+	+	-	-	-	+	-	+	+	-
85A	+	+	+	-	+	-	+	-	-	+	+	+	+	-	-	-	+	-	-	-	-
86A	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
87A	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-
88A	+	+	+	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
89A	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
90A	-	+	-	-	+	-	-	-	-	+	+	+	-	-	+	=	+/-	-	+	-	-
91A	-	+	+	+	+	-	+	-	-	-	+	+	-	-	-	-	-	+	-	+	+
92A	+	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+
93A	+/-	+	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	-
94A	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95A	-	+	-	-	+	-	-	-	-	+	-	+	+	+	+	-	+	-	+	-	-
96A	-	+	-	+	+	-	+	+	-	-	+	+	+	+	+	-	+	-	+	-	+
97A	+	+	+	+	+	-	-	+	+	-	-	+	+	-	+	+	-	+	+	+	-
98A	-	+	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-
99A	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	-
100A	-	+	+	-	+	-	+	+	-	-	+	-	-	-	-	-	+	-	+	-	+
101B	+	+	-	-	+	-	+	-	+	-	+	+	+	+/-	-	-	+	-	-	-	-
102B	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-

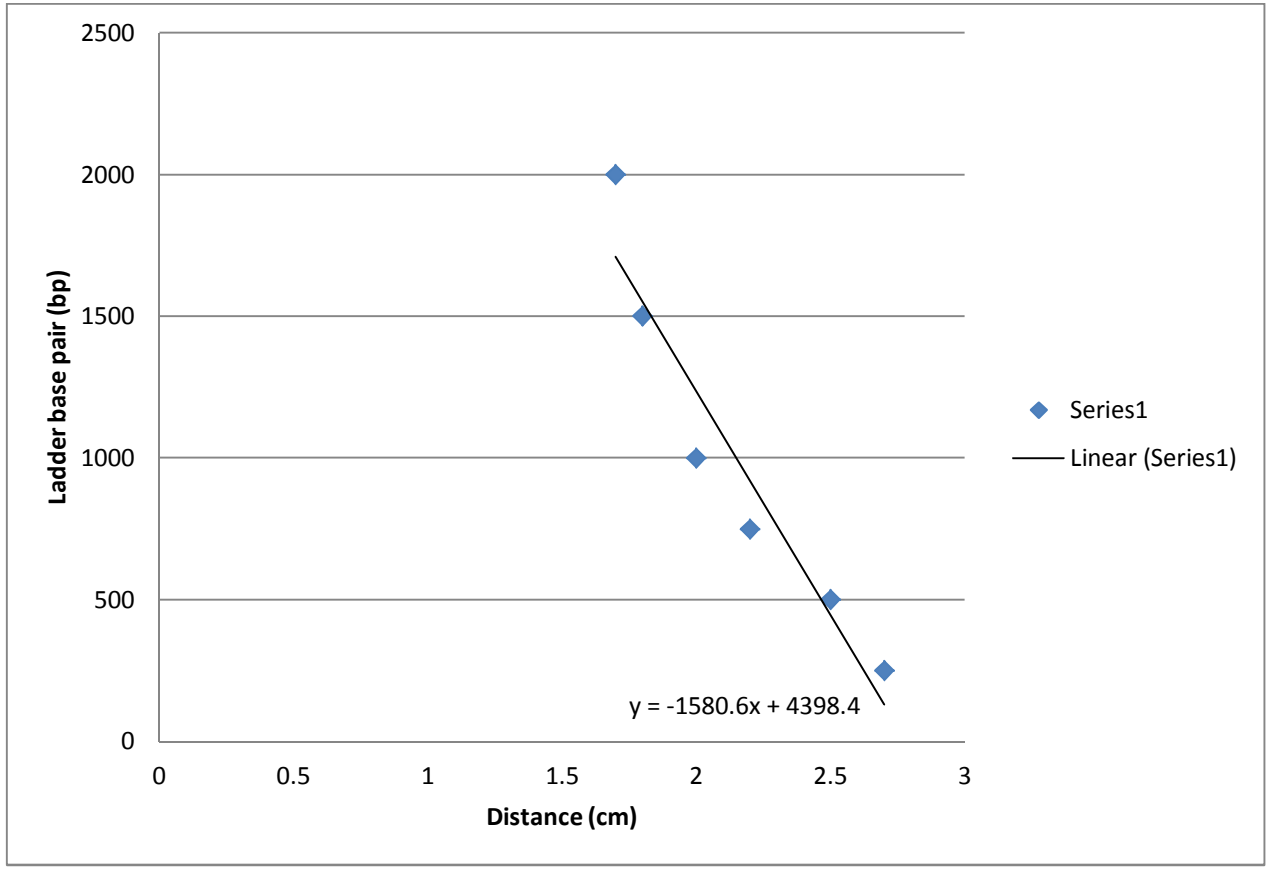
103B	+	+	-	-	+	-	+	-	-	+	+	+/+	+	-	+	+	+	+	-	-	-
104B	+	+	+	-	+	-	-	+/-	-	+	+	+	+	-	+	-	+	+	+	+	+
105B	+	+	+	+	+	-	-	+	+	-	-	+	+	-	+	+	+	+/-	+	+	-
106B	+	+	+	+	+	-	-	+	+	-	-	+	+	-	+	+	-	+	+	+	-
107B	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
108B	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+/-	-	-	-	-
109B	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+
110B	-	+	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-
111B	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
112B	+	+	-	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-
113B	+	-	-	-	+	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	-
114B	-	+	+	+	+	+/-	+	+	-	-	+	+	-	-	-	-	+	-	+	-	-
115B	+	+	-	-	+	-	+	+	+/-	-	+	+	+	+	+	+	+	-	+	+	-
116B	+	+	+	_	+	_	_	_	_	+	+	+	+	_	_	_	_	_	_	_	+
117B	+	-	-	-	+	-	-	+	-	=	-	-	-	-	-	-	-	+	-	-	+
118B	+	+	+	-	+	+	+	+	+/-	-	+	+	+	-	-	-	+	-	+	+	+
119B	+	+	+	+	+	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	-
120B	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
121B	+	+	+	+	+	+/-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-
122B	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
123B	+	+	-	-	+	-	+	-	-	+	+	+	+	-	+	+/-	+	-	+	-	-
124B	-	+	-	-	+	+	-	+	-	-	+	+	+	+	+	+/-	+	+/-	-	+/-	+
125B	+	+	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	+	--	-
126B	+	+	-	-	+/-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
127B	+	+	-	-	+	-	-	-	-	+	-	+	-	-	-	-	+	-	+	-	+
128B	-	+	+	+	+	-	+	+	-	-	+	+	-	-	-	-	+	-	+	-	+
129B	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
130B	+/-	+	-	-	+	-	-	-	-	+	+	+	+	-	+	-	+	-	+	-/-	-
131B	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+/-	-
132B	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	+	+	+	+	+	-
133A	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
134A	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-
135A	+	+	-	-	+	-	-	+	-	+	+	+	-	-	-	-	+	-	+	-	-
136A	-	+	-	-	+	-	-	+/-	-	+	+	+	+	-	+	-	-	-	+	+/-	-
137A	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

138B	-	+	-	-	+	-	+	-	-	+	+	+/-	-	-	-	-	+	-	+	-	+
139A	-	+	-	+	+	-	-	+	-	+	+	+/-	-	-	-	-	-	-	-	-	+
140A	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+	+/-	+	+/-	+	+/-	-
141A	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
142A	+	+	-	-	+	-	-	+/+	-	-	+	+	+	-	-	+/-	+	+	+	+	-
143A	-	+	+	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-
144A	-	+	+	+	+	-	+	-	-	-	+	+	+/-	-	+	+	-	-	+	+	
145A	-	+	-	-	+	-	-	-	-	+	+	+	+	-	+	-	+	-	+	-	-
146A	+	+	+	-	+	-	-	+	+	-	+	+	+	-	+	+	+	+	-	+	-

APPENDIX VIII

Graph of Multiplex PCR amplification products




2500
2000
1500
1000
500
0
Ladder base pair (bp)
0
0.5
1
1.5
2
2.5
3
Distance (cm)
y = -1580.6x + 4398.4
Series1
Linear (Series1)