Biochemical and Molecular Study of Bacteria Causing Urinary Tract Infections

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IN

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In the name of Allah, The Most Gracious, The Most Merciful

Declaration

I hereby declare that the contents of the thesis entitled **"Biochemical and Molecular Study of Bacteria Causing Urinary Tract Infections"** are a creation of my own research and no part has been copied from any published source (except the author's own published work from the current thesis, references, standard mathematical or geometrical models/equations/formulae/protocols etc.). I further declare that this work has not been submitted for award of any other diploma/degree. The university may act if the information provided is found inaccurate at any stage.

Date: 30/05/2022

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CERTIFICATE

It is certified that the research work presented in this thesis titled "Biochemical and Molecular Study of Bacteria Causing Urinary Tract Infections" was conducted by **Mr. Muhammad Haris** under the supervision of **Dr. Muhammad Ali.** This thesis is submitted to the Department of Biotechnology in partial fulfillment of the requirements for the degree of Master of Philosophy (MPhil) in **Biotechnology.**

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Dr. Bilal Haider Abbasi

Dated: 30/05/22

DEDICATION

I dedicate my work to my parents, supervisor, friends and lab members who helped me a lot and motivated me in the entire research work. Specifically, I dedicate this work to my parents because they supported me, motivated me, guided me and remembered me always in their precious prayers.

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TABLE OF CONTENTS

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Muhammad Haris

List of abbreviations

KTH	Khyber Teaching Hospital
MDR	Multi Drug Resistance
XDR	Extensively Drug Resistance
MEGA	Molecular Evolutionary Genetics Analysis
DNA	Deoxyribonucleic Acid
BLAST	Basic Local Alignment Search Tool
AMR	Antimicrobial Resistance
CIP	Ciprofloxacin
CFM	Ceftazidime
CTX	Co-trimoxazole
GN	Gentamicin
SCF	Cefoperazone-sulbactam
ATM	Azithromycin
MEM	Meropenem
IMP	Imipenem
PCR	Polymerase Chain Reaction
UV	Ultra Violet
TSI	Triple Sugar Iron
CTAB	Cetyl Trimethyl Amonium Bromide
S	Sensitive
R	Resistant
IN	Intermediate
ng/µL	Nanogram per Microlitre
CIT	Citrate
rRNA	Ribosomal Ribonucleic Acid
NHSCKS	National Health Service Clinical Knowledge Summaries
UTI	Urinary Tract Infection

List of figures

List of tables

reports for higher than 9.5% of infections delineated by critical-care-health centers (Magill et al. 2018). Nearly all of the UTIs (health center linked) are caused by urinary tract instruments. Relatively, 12 to 16% of the hospitalized patients will possess an indwelling urinary catheter throughout their span of hospitalization, duration of catheter remained for more than 2 days which leads to 3 to 7% risk of catheter acquired urinary tract infection to the patient (CAUTI) (McGuckin 2012; Lo et al. 2014). CAUTI can escort to comparable complications in males like orchitis, prostatitis, and epididymitis, and endocarditis, gram-negative bacteremia, vertebral osteomyelitis, endophthalmitis, pyelonephritis, meningitis and septic arthritis in hospitalized patients. CAUTI related difficulties leads to discomfort, escalated cost, persistent hospital stay, and mortality (Scott 2009). It has been evaluated that more than 13,000 deaths are linked with UTIs every year (Klevens et al. 2007). Around 0.34% of manifestations occur due to contagious agents causing cystitis which proliferates and multiply and further mount to the kidney through the ureters, where these agents infect calices, cortex and pelvis ultimately leading to the symptoms and signs of pyelonephritis (Katchman et al. 2005). Uropathogens can also grow and escalates to the bloodstream via kidney causing bacteremia if remains untreated, while if inflammatory response persist (concurrent) then this can also escort to septicemia (Hsu and Melzer. 2018). Approximately 27% of the clinical manifestations can be allocated to urinary isolates in patients visiting to the emergency department while suffering from sepsis and thus are entitled as urosepsis (Seymour et al. 2017). In clinical settings, the percentage of sepsis as a consequences of UTIs surged to 42% (Hatfield et al. 2018).

Numerous antibiotics have been utilized as a wide range regimens to fight against UTIs i.e. both community and hospital acquired. Although, the persistent resistance of antibiotics, combine with the researchers regards for the function of dependent representative of the host micro-flora, have emphasized crucial demand for the regimens or strategies that can particularly recover UTIs without any modifications and changing the gut structure and vaginal micro-flora. In the digestive tract, utilization of antibiotics escalates inflammation, weakens the immune surrounding of host and further progress pathogen proliferation by escalating substrate accessibility for instance, *E.coli* proliferation by escalated nitrate accessibility while survival in digestive tract is further linked with high risk of recurrent UTI (Spees et al. 2013; Köves et al. 2017; Yamamoto et al. 1997). Recovery with antibacterial regimen may hinder the vaginal

micro-flora (that protect the surface from pathogens) by disrupting colonization with *lactobacillus spp* that produce peroxides which suppress ascension and colonization (Mayer et al. 2015; Macklaim et al. 2015). As a consequences, antibacterial drugs can also be a risk factor for recurrent UTIs due to the contagious effect on vaginal and gut micro-flora (Spees et al. 2013; Hooton et al. 2005; Hooton et al. 2012).

2. Review of Literature

2.1. Clinical history of Urinary Tract Infections (UTIs)

The ninth century Arabic physician, Ar-Rhazi was acknowledged by Asscher in a symposium for the early detection of pyelonephritis with necrotizing papillitis and febrile infection in Baghdad patient (Asscher 1980).

The appearance of bacteria in the urine with the urinary symptoms in patients was observed by Roberts in 1881. Later in 1882, recurrent UTI was delineated by Wagner with focal cytological changes in women. Subsequently, in 1894, "pyelitis" was reported as adolescent disorder when Escherich cultured bacteria "*bacillus coli*" from children's urine effected with UTI (Asscher 1980). Then in 1917, the correspondence between escalating pyelonephritis and recurrent UTI with renal infection at last stage and renal destruction was recognized (Lohlein 1917). Recurrent urinary tract infection and its consequences were characterized and reported in detail in 1930s. In 1933, debilitated pyelonephritis and hypertension were described as the main barriers for repeated renal infection (Longcope and Winkenwerder. 1933). Weiss and Parker, further elucidated pyelonephritis, radiographic substitution along with the acute interstitial infection, focal complexion of the pathology, and ulcer was observed in renal foliage (parenchyma). Moreover, they described "pyelonephritis lenta", a quiescent subclinical pyelonephritis, intensified infection mostly in young adult women often related with hypertension that subsequently escort to end-stage renal infection. These findings revealed that recurrent UTI in patients can leads and escalate the risk of end-stage chronic fatal renal disease. They concluded that persistent pyelonephritis was more perennial as a cause of end-stage renal infection than glomerulonephritis (Weiss and Parker. 1939). Later in 1940, 45 pregnant women introduced with pyelonephritis, five of the patients developed renal stones and three acquired consequential renal impairment (Crabtree and Reid. 1940).

Before the era of antimicrobial regimens, hypothesis revealed that increased destruction of vascular tissues could slowly escort to blood pressure increase and renal damage. In 1940, sulfonamides were introduced and renal infections were treated (Figure 2.1). With sulfonamides, destructive, recurrent and persistent infections became reduced. After 1940s, various studies revealed antimicrobial regimens for the treatment of

asymptomatic and acute infections as new therapeutic agent was established in clinical trials. Sometimes, with asymptomatic infections, natural history of patients with UTIs have to be observed in the inadequacy of the therapeutic arbitration (Ronald and Pattullo. 1991).

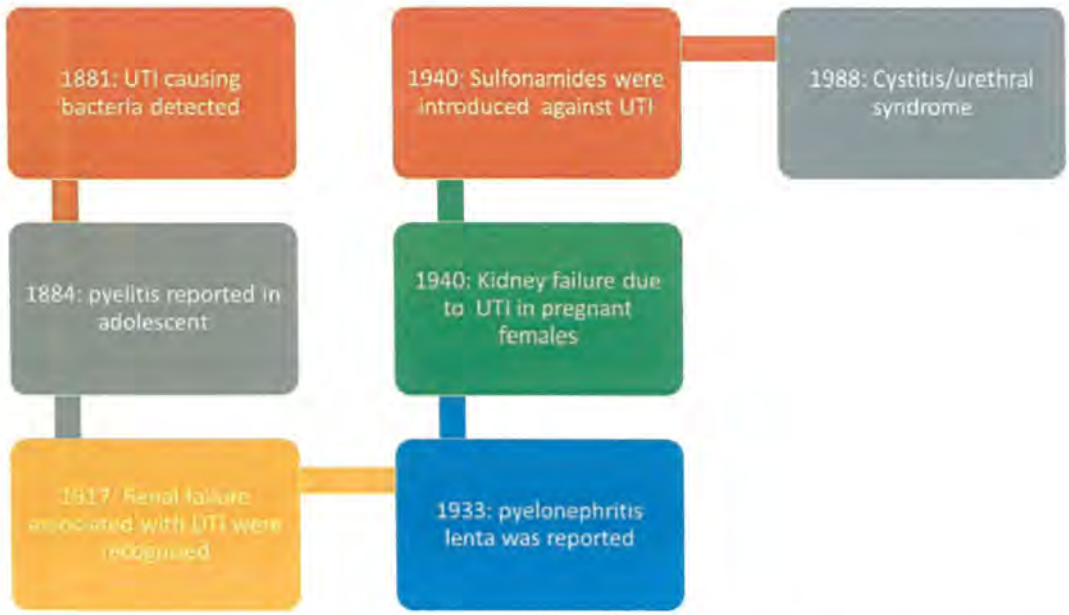


Figure 2.1. Historical perspective and previous clinical history of the urinary tract infections

Uncomplicated UTI in patients with cystitis usually have subclinical pyelonephritis as the prevalence ranges from 8 to 80% based on the studied population and the approach used to detect renal infection (Johnson and Stamm. 1989). Cystitis and pyelonephritis generally develop into asymptomatic if remains untreated, however bacteriuria exist. Uncomplicated UTIs can easily be cured with significant antibiotics. Sometimes may lead to complicating factors if relapse or fails to cure after antibiotics or antimicrobial therapy (Ronald and Pattullo. 1991). Similarly, in a projected investigation, the mean range of pyelonephritis with repeated UTI was 1 of every 18 symptomatic UTIs amid 51 women which accounts for approximately 0.1 incidents of pyelonephritis per patient each year (Stamm et al. 1991). Similarly, an investigation established that 16% of infections (symptomatic) indicated pyelonephritis in women having parenchymal wound with early history of UTIs experienced fever (Jacobson et al. 1987). About 20% of the women face UTI throughout their lifespan and 3% women face single or more

infections every year. The majority of these manifestations are uncomplicated cystitis (Schaeffer 1988). One study revealed that large number of events of the "urethral syndrome" (an infection that impacts the urethra) must be categorized as cystitis (Stamm et al. 1980). Similarly, Kraft and Stamey observed 23 women having repeated infection and established mean of two infections as culture associated inflammation per inpatient each year, on which 95% were clinical and symptomatically coherent via uncomplicated cystitis. *E.coli* reported for 80% (Kraft and Stamey. 1977). Sometimes, asymptomatic bacteriuria can lead to symptomatic infections as well as study revealed in which 90 women with asymptomatic bacteriuria were investigated for about 1 year. Out of 90 patients, 32 established symptomatic infection, generally with the same bacterial strain (Asscher et al. 1969).

Complicated UTI is less understood as the inflammation and infection due to altered host limitations compare to uncomplicated UTI. One study reported the death of 43 patients suffering from spinal cord injury after continuous autopsy in 1953. Among them, 21 died due to other causes while 22 died via renal collapse. Of these 43 patients, thirty two had pyelonephritis while 16 had amyloid disorder. And of these 32, twenty-six had chronic pyelonephritis. Of this concern of the study, half of the patients with amyloid and pyelonephritis established consequential hypertension apparently as a complication; only one-eighth with none necropsy detection had hypertension (Talbot 1966).

2.2. Classification and definitions of terms in UTIs

Urinary tract infections (UTIs) can broadly be divided into two main types i.e. uncomplicated UTI and complicated UTI.

2.2.1. Uncomplicated UTI

Any infection of the kidney or bladder that arises in normal host without any structural change and or functional abnormalities of the urinary tract. These type of infections are sometimes classified as pyelonephritis, cystitis and asymptomatic bacteriuria.

2.2.1.1. Cystitis

A peripheral inflammation of the bladder that frequently occurs in adult women. The most usual cause is infection, however there are non-bacterial causes as well. The symptoms in non-pregnant healthy women are urine recurrence or dysuria and the

condition accounts for about 95% in common practice as a consequence of UTI (Baerheim 2012).

2.2.1.2. Pyelonephritis

The bacterial inflammation or infection of the urinary tract and the kidney which arises in renal parenchyma. The detection is elicited from precise laboratory and on clinical basis that include tenderness, leukocytosis, vomiting and nausea, fever presence, and the examinations like urine cultures or antibody-coated microbes (specifically bacteria). Pyelonephritis may sometimes be chronic as the infection of the kidney persist longer that arises mostly in patients with structural and substantial anatomical or functional abnormalities (Ronald and Patullo. 1991).

2.2.1.3. Asymptomatic bacteriuria

Asymptomatic bacteriuria generally refers to the separation of bacteria in properly collected urine samples with the absence of any symptoms of urinary tract infection (UTI). Throughout the first decade, the frequency of asymptomatic bacteriuria in women was around 1% but thereafter it ascends to approximately 1% per decade (Kass 1978).

Asymptomatic bacteriuria has been frequently recognized throughout the previous 50 years for appropriate treatment strategies in adults as specific target approach named "search and destroy" with one of the following justifications:

- It escorts to repeated acute pyelonephritis and cystitis.
- It can give rise to hypertension.
- It accounts for end-stage renal infection and renal impairment.
- Asymptomatic bacteriuria escorts to enhanced mortality.

2.2.2. Complicated UTI

This type of infection refers to the metabolic, functional or anatomical abnormalities or aberrations of the urinary tract which damage the innate (natural) host defence system including renal collapse, renal graft, neurological disorder due to urine retention, immunosuppression and other exterior sources like indwelling catheters, various drainage instruments or calculi which ultimately escorts to tissue injury (Lichtenberger and Hooton. 2008; Levison and Kaye. 2013). Table 2.1 illustrates various factors that are related to both complicated and uncomplicated urinary tract infections (UTIs).

Natural history of complicated UTI is associated with neurogenic bladder with subsequent neurologic disorder even though the difficulty is apparent in patients having diabetes with spinal cord damage, autonomic neuropathy, various congenital and acquired neurologic disorder and multiple sclerosis (Ronald and Pattullo. 1991).

Table 2.1. Factors related with urinary tract infections (Adopted from Lee and Neild. 2007)

Uncomplicated UTI	**Complicated UTI**
Bacterial virulence	Anatomical and functional abnormalities • Outflow obstruction • Renal scarring • Foreign body (stone, tumour, catheter) • Pregnancy
Host defence • Colonization of vagina and mucosa • Polymorphisms of genes regulating complement, neutrophil biology • Non-secretor status	
Acquired (disruption of host defence) • Antimicrobials (loss of commensal population) • Sexual intercourse • Ageing (post-menopausal oestrogen decrease, loss of detrusor power)	Metabolic • Immunosuppression • Diabetes

An investigation reported in which 10 patients diagnosed with diabetes having complicated UTI, and among them 5 had hypertension with diabetes. Entire group had proteinuria and pyuria, 3 of them had consequential post void-residue and 3 patients had renal calculi as a major factor for complicated UTI. Among them, 2 experienced urological interruption for difficult ureteric calculi suggesting the recurrence UTI (Kotalwar et al. 2021).

2.3. Epidemiology

UTIs are one of the extreme and usual cause due to bacterial infections, influencing approximately 150 million individuals worldwide per year, establishing greater than six billion dollars in immediate medical management cost (Stamm and Norrby. 2001). Alone in the United States in 2007, there were approximately 10.5 million individuals (ambulatory visits) with UTI symptoms, representing around 0.9% of all visits, and

nearly 2 to 3 million hospital catastrophic sector visits (Schappert and Rechtsteiner. 2011). UTIs developed to be the most usual nosocomial septicemia considered enormous as 35% of the nosocomial infections, and examined to be the second most usual base of septicemia in hospitalized patients (Stamm 2002; Weinstein et al. 1997). Study in 2004 revealed in which 49 hospitals in Swiss indicated that approximately 1.5% of patients hospitalized established symptomatic UTIs (Uçkay et al. 2013). There are nearly 50% women which experience UTIs minimum one time during their life span, and although with the antibiotic use, 20 to 30% women with early UTI will confront repeated UTIs in 3 to 4 months of the early inflammation (Foxman 2003). Anatomical characteristics defend apart from that healthy males at nearly all ages and phases of life; although males in their lifetime manifest an escalated occurrence of UTI. Specially, infants (age less than 6) boys with UTI surpass girls (Larcombe 2015), which may linked with circulating androgens, while older men generally speculate urodynamic disorder as a consequences of prostatic hypertrophy. Additionally, several persistent conditions in males such as spinal cord wound, indwelling, or sporadic bladder catheterization and diabetes elevate UTI as well. Similarly, pyelonephritis in males linked with higher rate of morbidity and mortality compared to females (Foxman 2003) recommending variance behind simple physiology can impact the consequences of these additional critical infections. In children, affirmed or suspicious UTIs are amid the commonest cause for the estimation in health care departments. About 1 to 3% of febrile teenage boys and among 3 to 7% of teenage girls (febrile) will confirmed to experience UTI (Figure 2.2) (Habib 2012). Approximately, 7% of UTI (febrile) outcomes results in the establishment of renal damage (Shaikh et al. 2016). Correspondingly, Study illustrates that recurrent UTIs can affect healthy condition of life and embrace health and financial distress (Wagenlehner et al. 2018). In US, around 70 to 80% of the UTIs (complicated) are associated with indwelling catheters which attributes to 1 million cases each year (Foxman 2010).

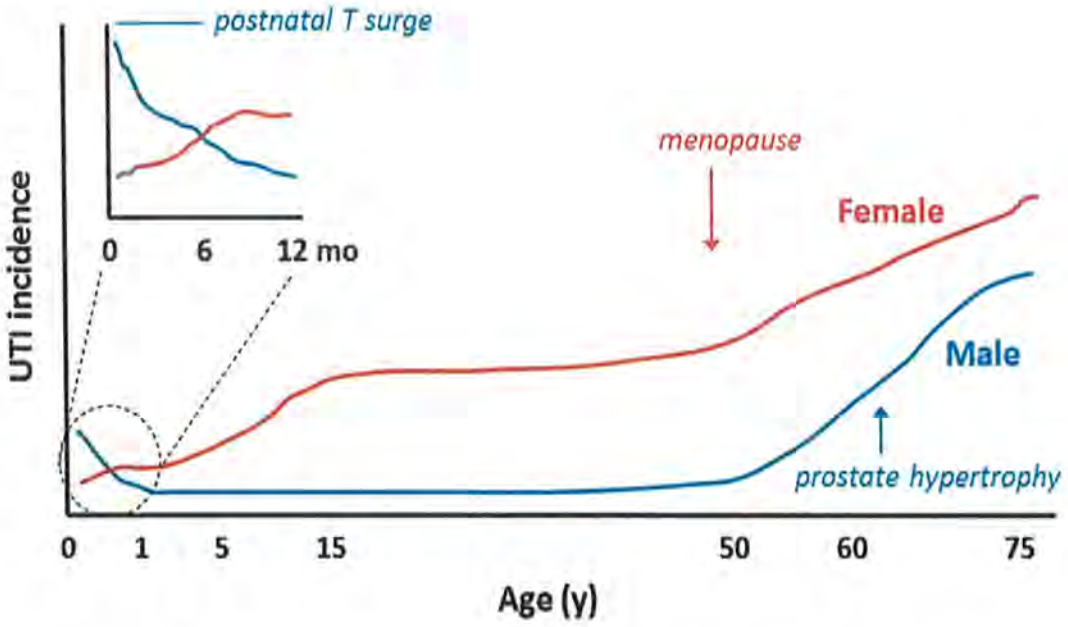


Figure 2.2. Simplified presentation of age linked UTI frequency in both males (blue) and females (red). Boys agonize more at the age of 6 months compared to girls (spotted in small graph); the kinship then overturn by the age of 12 months, following dominancy in females compare to males at much of the life span. Occurrence of UTI increases in postmenopausal women and in older men as well, where it is associated with enlargement of prostate and affiliated with urodynamic abberations (Adopted and modified from Albracht et al. 2021)

2.4. Bacteriology

The UTI's bacteriology is extremely expected and predictable; even though numerous numbers of distinct species can cause UTI. The most usual cause of UTIs are both gram-negative and gram-positive bacteria, although various fungi and some viruses are also responsible for the infection. Uropathogenic *E.coli* (UPEC) is the commonest and extremely studied bacterium, involved in both complicated and uncomplicated UTIs (Flores-Mireles et al. 2015). UPEC are distinct from the *E.coli* specie that persists naturally in the digestive-tract, in such a way that they are better adjusted inside the urinary tract and avoiding the host's immunologic response. There are 2 channels i.e. urothelial cell incursion and biofilm development (Mulvey et al. 2000) that accord triumph to the UPEC in this consideration. In uncomplicated UTI, high prevalence after UPEC is followed by *Klebsiella pneumoniae, Staphylococcus saprophyticus, Enterococcus faecalis, Group B Streptococcus (GBS), Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* and one fungal specie i.e. *Candida spp* (Foxman

2014; Nielubowicz and Mobley. 2010; Kline et al. 2011; Ronald 2002). Similarly for complicated UTI, the grouping of prevalence after UPEC as most usual are *Enterococcus spp., K. pneumoniae,* fungal specie i.e. *Candida spp., S. aureus, P. mirabilis, P. aeruginosa* and *GBS* (Levison and Kaye. 2013; Fisher et al. 2011; Chen et al. 2013; Jacobsen et al. 2008). Figure 2.3 depicted the frequency of both gram-positive and gram-negative bacteria along with fungi.

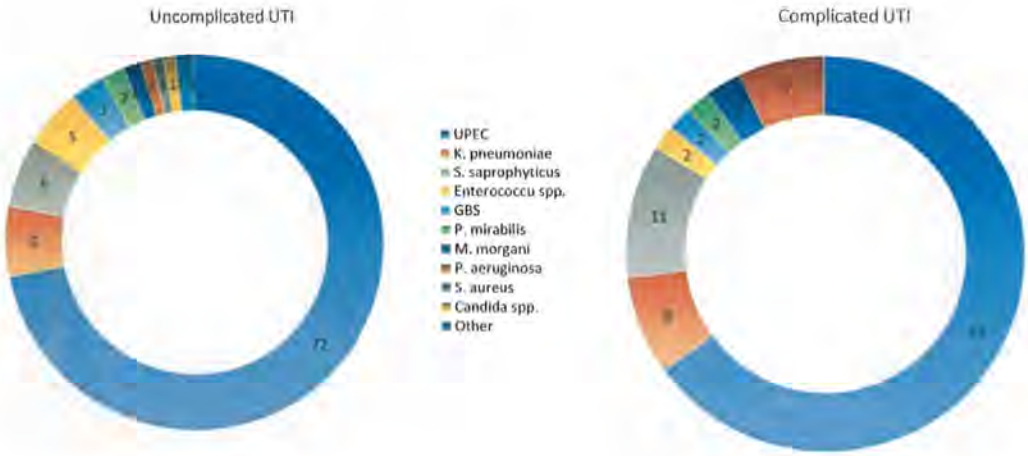


Figure 2.3. Prevalence and percentage of bacteria, fungi and other uropathogens causing urinary tract infections (Adopted and modified from Flores-Mireles et al. 2015)

In a postoperative study of patients studied from 2001 to 2015 for uncomplicated UTIs, *E.coli* was the dominant causative agent accounting for 80% of the cultures, followed by *Staphylococcus saprophyticus (Staph. saprophyticus)* accounting for 6 to 17% of the total cultures (Bollestad et al. 2018). Similarly, another study revealed in which 341 students were examined for UTIs, among them 72 manifest notable bacteriuria accounting for 21.1% of the total population. The extreme bacterial strains found to be *E.coli* (48.6%) followed by *Coagulase-negative staphylococci*, *Staphylococcus aureus* and *Klebsiella spp.* with the total of 23%, 13.5%, and 8.1% respectively (Gebremariam et al. 2019).

2.5. Pathogenesis

Bacterial attachment is a crucial episode commencing individual step in the pathogenesis of UTI. It commonly initiates with the contamination in the periurethra through uropathogen inhabit in the gut, after the event by urethral colonization and

successive shift of the bacterium to the bladder, an episode that need appendages including flagella and pili (Figure 2.4). Subsequently, interactions of fusion host-pathogen in the bladder eventually decides either colonization by uropathogens are successful or eradicated (Flores-Mireles et al. 2015).

Numerous bacterial attachment molecules identify various receptors present on the epithelium of the bladder (uroepithelium) and mediate colonization (Table 2.2). Uropathogenic bacteria including UPEC produce proteases, various toxins and remain in the epithelium of the bladder thereby releasing nutrients from the host cells, and to acquire iron by synthesizing siderophores (Figure 2.4; Table 2.2). Subsequently, after proliferation and overcoming the host immune system, uropathogens mount to the kidneys, and again adhere through adherence molecules or pili to colonize the epithelium of renal parenchyma and later produce toxins that ultimately damage the tissues. As a consequence of tissues damage, the uropathogens are then capable of to travel across the tubular epithelia easily to enter the bloodstream, commencing bacteriemia (Flores-Mireles et al. 2015).

UPEC, *K. pneumoniae* and *S. saprophyticus* are the most common uropathogens causing uncomplicated UTIs, possess the capability to attach to the epithelia of the bladder directly, which consists of umbrella cells (superficial facet cells), transitional cells and basal cells (Table 2.2) (Khandelwal et al. 2009). UPEC and *K. pneumoniae* attach to uroplakins (vital protein element of the apical tissue of the umbrella cell), which establish crystalline assembly defending the bladder tissue of the mammalian from destructive agents in urine (Lee 2011). Additionally, $\alpha_3\beta_1$ integrins, manifest at the exterior of uroepithelial cells, additionally can work as receptors for UPEC (Eto et al. 2007). In contrast to uncomplicated UTIs, complicated UTIs are commenced as soon as the bacteria attach to an indwelling catheter, a vesical calculus, or a renal calculus, or by a physical blockage. Several bacteria, for instance, UPEC can give rise to complicated as well as uncomplicated UTIs. Although, complicated UTIs can be caused by other pathogens including *P. mirabilis, P. aeruginosa* and *Enterococcus spp.* (Figure 2.4). Eventually, these bacteria frequently develop biofilms which are accountable for persistence and colonization (Niveditha et al. 2012; Jacobsen and Shirtliff. 2011).

Table 2.2. Virulence factors utilized by the dominant uropathogens (Adopted from Flores-Mireles et al. 2015)

Pathogenic bacteria	Virulence factors					Reference
	Adherence	Immune evasion	Iron possession	Toxin	Other	
UPEC	F1C pili P pili S pili Type 1 pili Dr adhesins	HlyA Capsular antigens CNF1 Yersiniabactin	Aerobactin Enterobactin Salmochelin Yersiniabactin	HlyA CNF1	Antigen43 Flagella	Nielubowicz and Mobley. 2010; Wright and Hultgren. 2006; Nagamatsu et al. 2015; Garcia et al. 2011; Chaturvedi et al. 2012
Proteus mirabilis	MR/P pili NAFs PMFs AipA adhesin TaaP adhesion	Capsule ZapA	Proteobactin Yersiniabactin-related	Haemolysins (HpmA and HlyA) Pta	Flagella Urease	Nielubowicz and Mobley. 2010; Armbruster and Mobley. 2012; Himpsl et al. 2010; Dumanski et al. 1994
Staphylococcus saprophyticus	Aas adhesion SdrI adhesin Uaf adhesion	ND	ND	Aas	Urease	Gatermann et al. 1989; Hell et al. 1998; Kline et al. 2010
Klebsiella pneumoniae	Type 1 pili Type 3 pili	Capsule	Aerobactin Enterobactin	ND	ND	Rosen et al. 2008; Murphy et al. 2013; Tarkkanen et al. 1992; Podschun et al. 1993
Enterococcus faecium	Ebp pili Esp adhesion	ND	ND	ND	ND	Arias and Murray. 2012
Enterococcus faecalis	Ebp pili Ace adhesin Esp adhesion	Epa	ND	ND	Sortase A SigV MsrA and MsrB	Arias and Murray. 2012; Guiton et al. 2010; Nielsen et al. 2013
Pseudomonas aeruginosa	Extracellular DNA Exopolysaccharides (alginate, PEL and PSL)	Capsule Elastase ExoS Phospholipase Rhamnolipids	Pyochelin Pyoverdin	ND	Quorum sensing	Senturk et al. 2012; Visca et al. 1992; Kumar et al. 2009; Cole et al. 2014

Abbreviations: AipA, adhesion and invasion mediated by the Proteus autotransporter; CNF1, cytotoxic necrotizing factor 1; Ebp, endocarditis- and biofilm-associated; Epa, enterococcal polysaccharide antigen; Esp, enterococcal surface protein; ExoS, exoenzyme S; F1C pili, type 1-like immunological group C pili; HlyA, α-haemolysin; HpmA, haemolysin; MR/P, mannose-resistant Proteus-like; Msr, methionine sulfoxide reductase; NAF, non-agglutinating fimbria; ND, not determined; PMF, P. mirabilis-like fimbria; P pili, pyelonephritis-associated pili; Pta, Proteus toxic agglutinin; TaaP, trimeric autoagglutinin autotransporter of Proteus; UPEC, uropathogenic Escherichia coli.

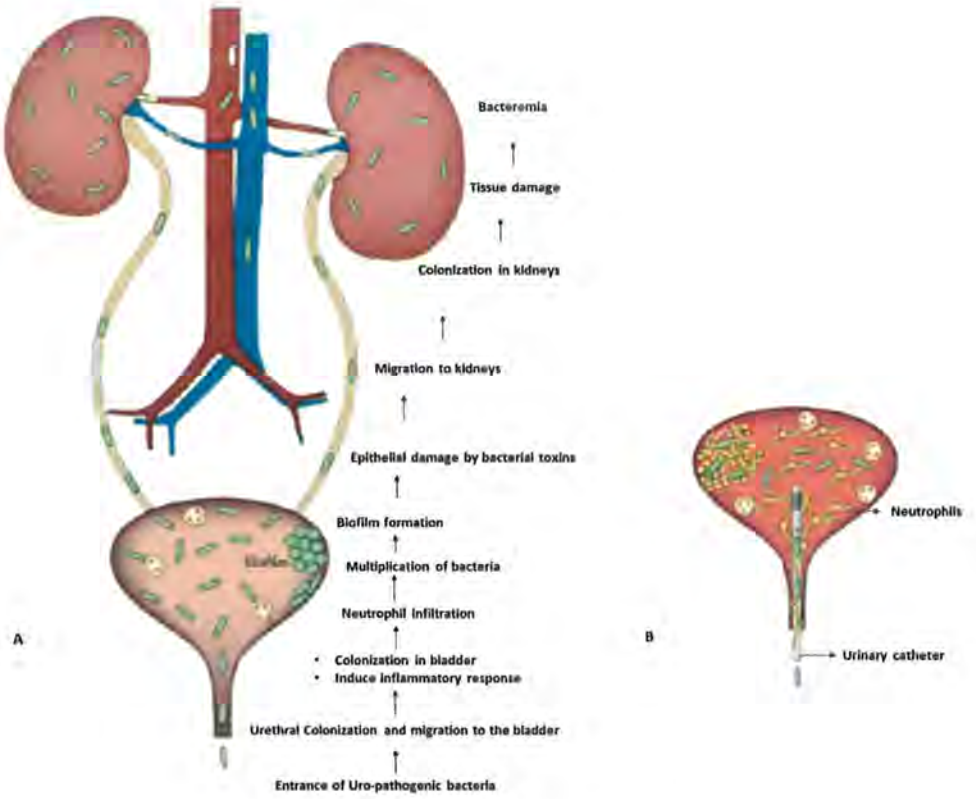


Figure 2.4. Mechanism of pathogenesis of UTI. **A.** Uncomplicated UTIs initiate with the pathogens inhabit in the stomach infect the periurethral environment and are capable of colonization in the urethra. Following transfer to the bladder, pili and adhesions expression leads to the colonization and invasion of the outermost layer of the umbrella cells. Various inflammatory responses of human, such as neutrophil infiltration initiate to eradicate bacteria present on extracellular matrix. Several uropathogens (bacteria) evade the immune system barrier, via invasion of host cells or either through morphological changes that results in resistance to neutrophils, consequently these bacteria go through multiplication and form the biofilms. The bacteria then generate proteases and toxins that ultimately damage the host cell, thereby releasing crucial nutrients which are required for the bacterial survival and ascension to the kidneys. Bacterial toxin production and host tissue damage initiates after bacterial colonization in the kidneys. Pathogens can cross the tubular epithelial barrier in the kidneys and eventually proceed to bacteraemia if remain untreated. **B.** Complicated UTIs causing pathogens follow the same initial pattern as mentioned for uncomplicated inflammations, such as colonization in periurethra, urethral progression and transfer to the bladder. Although, for infection, the bladder have to be compromised for the pathogens to cause infection. Catheterization is the most usual cause of compromised bladder. Due to the robust immune response induced through catheterization, fibrinogen accumulates on the catheter, giving perfect environment to bacteria for the attachment and expression of fibrinogen- binding proteins. After initial attachment to fibrinogen-covered catheters, the bacteria grow, form biofilms, and stimulate the damage of epithelia and can lead to infection of the kidneys, where production of toxins leads to the damage of tissue which ultimately induces neutrophils infiltration. Bacteria causing complicated UTIs can also escort to bacteremia as discussed in uncomplicated UTIs (Modified from Flores-Mireles et al. 2015)

2.6. Host factors

Urinary tract deficiency is sustained by physiological elements such as one-way flux of urine, repeated and entire clearance of the bladder, segregation of the origin of bacteria from the bladder and decreased development of bacteria in urine. Bacterial exposure to epithelium prompt body's defence system, innate immune system activation specifically neutrophils and antibacterial peptides synthesis (Table 2.3) i.e. defensins, cathelicidins. P blood group antigen and uromodulin (TammeHorsfall protein) excretion hinder the pathogen adhesions to the cells of epithelia, resultantly discarded to the urine. Even though, studies suggests that specific immunity can play role in UTI (specific IgA production in urine after inflammation), but it is ambiguous that it play vital role as a defence system in opposed to additional infection (Sheerin and Glover. 2019).

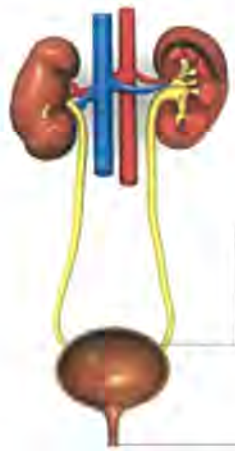
Table 2.3. Host defences and bacterial virulence factors (Adopted from Sheerin 2015)

Host factors	**Bacterial virulence**
Directional flow of urine	Fimbrial adhesions
Complete bladder emptying	Type 1 fimbriae
Normal commensal bacteria	P fimbriae
Neutrophils	Serum resistance
Complement	Toxin production
Antimicrobial peptides • Defensins • Cathelicidins	Haemolysin
	Colony-necrotizing factor
	Iron sequestration
Low urine pH	
TammeHorsfall protein (uromodulin)	
Blood group secretor status	

2.7. Symptoms

In case of acute pyelonephritis, the usual symptoms include vomiting, urgency, urination with burning condition, nausea, fever, and flank pain (Belyayeva and Jeong. 2018), with slight differentiation to cystitis including symptoms mainly of hematuria (Urination with blood) and flank pain with fever, vomiting, septic shock and rigors as systemic manifestation (Figure 2.5). In addition, urethral release and dysuria (urine

difficulty) frequently persists but are asymptomatic. Similarly, prostatitis (redness of prostate gland) persists with the frequent symptoms of pelvic pain, dysuria, urgency and frequency (Sheerin 2011).



Infection	Part of urinary tract affected	Symptoms
Acute pyelonephritis	Kidney	• Back side pain, • High fever, • Shaking and chills, • Nausea, • Vomiting
Cystitis	Urinary bladder	• Pelvic pressure , • Frequent painful urination, • Discomfort in bladder • Blood in urine
Urethritis	Urethra	• Burning with urination, • Discharge
Prostatitis	Prostate gland	• Pelvic pain • Dysuria • Urgency & frequency

Figure 2.5. Symptoms of various infections in UTIs

2.8. Transmission

Pathogenic bacteria frequently linked with UTI, are adjusted to the tract, establishing biofilms and colonize in the epithelium with various virulence or bacterial factors described earlier in table 2.2, thereby multiplying and proliferating in substantial amount in the urine (immensely 100,000 colony forming unit per ml). In addition, if uropathogens progress to digestive system from the vaginal cavity, they might possibly progress in contrary direction as well. With numerous growth in the urine, it is possible that throughout urination the hands develop contamination, resultantly can influence the movement of pathogenic microbes to spouse or partner at the time of sexual intercourse or can impart indirectly through water or food. Sexual intercourse might escort to direct mediation to the urethra via vaginal canal of spouse or partner (sex), or through oral sex indirectly (Foxman 2010).

Uncomplicated UTI has been termed 'honeymoon cystitis' as a consequence that UTI has previously been linked with sexual intercourse. Previous studies in literature revealed the transmission of uropathogens via sexual intercourse (Al-Wali et al. 1989;

Bailey et al. 1986). Vaginal sex activity is a crucial risk factor in the age group of 18 to 29 in women as UTI occurs usually and in addition women begin sexual intercourse in this age (Foxman et al. 2000).

2.9. Treatment

UTI treatment mainly relies on different host factors (including immunodeficiency, urologic aberrations or sex), threat due to multiple drug resistance, and intensity of disease progression. Resistance must be observed and considered as its incidence rate in populations has expanded, unexpectedly in ambulatory or outpatients as well. The Infectious Diseases Society of America (IDSA) has issued recommendations for the cure of uncomplicated cystitis and pyelonephritis, especially in women (non-pregnant and premenopausal) with no investigated urologic aberrations or concurrent circumstances (Gupta et al. 2011).

For first-line treatment, 4 drug regimens are suggested namely, fosfomycin trometamol, piymecillinam, Trimethoprim-Sulfamethoxazole (TMP-SMX), and nitrofurantoin (Gupta et al. 2011). Other regimens include beta lactams and fluroquinolones. Fluroquinolones have been progressed to the ultimate group of regimens after beta lactams and should only be utilized soon as no further oral choices are obtainable due to a threat from the United States of Food and Drug Administration (USFDA) that hazard for consequential injured outweighs welfare (Gupta et al. 2017).

The intrinsic record of cystitis is the determination of symptoms in 4 to 7 days. Time period of symptoms can be decreased with the successful treatment of antibiotics. Empiric treatment of 3 days antibiotics will attain recovery up to 85 to 90% in women having uncomplicated cystitis. Trimethoprim or nitrofurantoin are suggested as a treatment in UK (NHS. 2014). Antibiotic utilization can be decreased with the delay in antibiotics for 48 hours to allow the determination of symptoms without remarkably dragging the symptoms (Little et al. 2009). For men having expanded complicated inflammation and or prostatitis, minimum 7 days treatment is suggested with the antibiotic specifically quinolone due to greater tissue puncture capability in prostate (SIGN. 2006).

Pyelonephritis with mild conditions can be recovered with antibiotics administered orally, initiated temporarily but assessed in culture results. According to National Health Service Clinical Knowledge Summaries (NHSCKS), Co-amoxiclav for 14 days

and ciprofloxacin with 7 days treatment are suggested for non-pregnant women and men, and for preganant women, cefalexin are suggested for 10 to 14 days. Infections with severity need hospitalization for the treatment with antibiotics (oral administration) and fluids administered intravenously, and needs to be carried up to fourteen days (Sheerin 2015).

2.10. Antibiotic resistance

Antimicrobial resistance (AMR) arises as a consequences of various pathogenic organisms including bacteria, fungi, viruses and parasites progress in a way that drugs of various classes does not alter them. As a consequence of resistance, it is complicated or occasionally difficult to cure infections leading to high rate of morbidity and mortality. Presently, there is a global threat due to multi, pan and extensively drug-resistant bacteria. The resistant bacteria are called superbugs due to the nature that they carry resistance to current and previous classes of antibiotics (Mogasale et al. 2021). AMR word was initially invented when *Mycobacterium tuberculosis* revealed resistance to first-line regimens including fluoroquinolones and several second-line regimens such as capreomycin and kanamycin. After that, most of the other bacteria revealed resistance against most antimicrobial regimens. Multidrug resistant (MDR) and pan drug-resistant bacteria are distinct to extensively drug-resistant (XDR) depending on the type of resistance. MDR bacteria are insusceptible to more than one regimens, while pan-drug-resistant bacteria are insusceptible to all previously existing antimicrobial drugs. Similarly, XDR bacteria are resistant to most crucial regimens while sensitive to several regimens (Younas et al. 2021). Resistance in bacteria is a major challenge due to less naturally presenting drugs, while many drugs are synthetic and it is hard to synthesize novel regimens opposed to bacteria that are previously resistant to such antibiotics. This disaster is a consequence of overuse and misuse of these regimens, and the insufficient dose throughout the recovery course of infection (Aslam et al. 2018).

Recent study revealed in which *E.coli* was isolated from urine cultures and assessed for antibiotic resistance. Among extracted bacterial strains, resistance was usual to amoxicillin i.e. 38%, while 18.1% to trimethoprim/sulfmethoxazole. Resistance was approximately 1.9% to cefotaxime and ciprofloxacin which was lesser, 0.4% to nitrofurantoin, while 0% to fosfomycin. Total Extended-spectrum-beta-lactamase

(ESBL) depicted 1.6% of *E.coli* (Rossignol et al. 2017). Similarly, a systematic study in Pakistan revealed that UTIs was the most dominant infections in last 10 years with clinical detection of 16.1%. Of the total studies, 28 (30.1%) of them outlined *E.coli* with excessive resistance to first-line drug regimens. Total investigated cases in *Staphylococcus aureus*, approximately, 49% were found to be resistant to Methicillin. Disk diffusion approach was utilized for the evaluation of phenotypic resistant pattern which was 82.8%. Of these total studies, 28 (30.11%) studies were evaluated using molecular detection of various antibiotic resistance genes in which blaTEM gene was evaluated with 78.94% in *shigella spp*, blaNDM-1 with 32.75% in *klebsiella spp* with dominant genes followed by VanA with 45.53% in *Enterococcus spp*, mcr-1 with the total of 1.61% in *Acinetobacter spp* and blaKPC-2 with 31.67% in *E. coli*. Majority of the investigations were reported from Sindh with 40.86% followed by Punjab with the total investigations of 35.48% (Bilal et al. 2021).

Aims and Objectives

The current study aims:

1. To perform morphological analysis and biochemical testing for the identification of bacterial isolates causing urinary tract infections.
2. To assess the antibiotic resistance profile of the clinical isolates.
3. To identify bacterial isolates using 16S rRNA gene sequencing.

3. Material and Methods

3.1. Research draft

Patients with the urinary tract infections were enrolled at Khyber Teaching Hospital (KTH), Peshawar. Total of 560 urine culture samples from July 2021 to November 2021 were assessed for biochemical, culture sensitivity and molecular study of clinical isolates. The morphological analysis, biochemical testing, gram staining and culture sensitivity were performed at the Microbiology section of KTH, while molecular study was subsequently conducted at the department of Biotechnology Quaid-i-Azam University, Islamabad (Figure 3.1). Selected samples were collected based on the inclusion criteria as described in section 3.3.

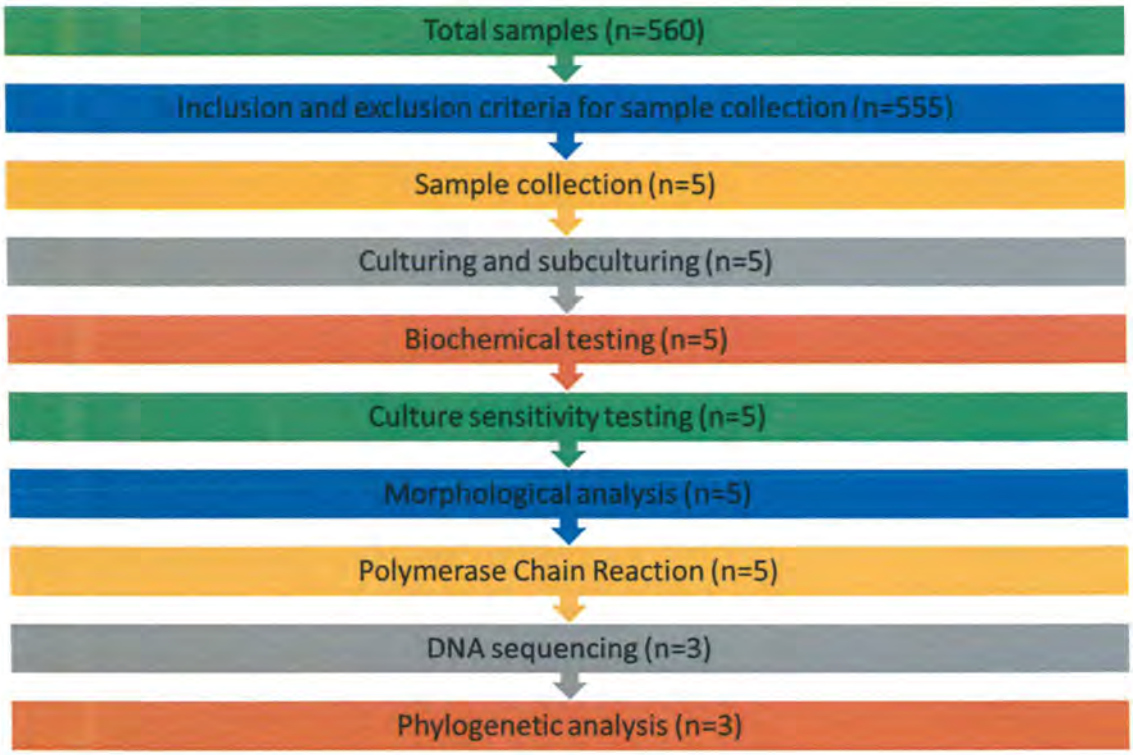


Figure 3.1. Schematic representation of research study

3.2. Set up

Microbiology section of Khyber Teaching Hospital (KTH), Peshawar and Biotechnology department of Quaid-i-Azam University, Islamabad, Pakistan.

3.3. Inclusion criteria

Specimens with only urease positive bacterial isolates (n=5) were included in the study.

3.4. Exclusion criteria

Specimens without urease positive results for bacterial strains were excluded from the study.

3.5. Sample collection and primary screening

Urine samples were collected from various wards of KTH from July, 2021 to November, 2021 and were processed and cultured in Microbiology laboratory. Samples were collected from the patients diagnosed with UTIs and were primarily examined for bacterial infections.

3.6. Media for microbial growth

All microbes needs suitable nutrients, environment, humidity and temperature for their optimum growth. Bacterial pure colonies were obtained in lab under sterilized environment by utilizing agar (polysaccharide extracted from *Rhodophyceae,* a seaweed) plates. As agar is a sticky substance that persists in solid state at high temperature i.e. 37°C. Therefore, it is an appropriate for the morphological examination of microbial isolates. The type of culture medium depends on the type of microbes used for the isolation and identification. Basically, identification of various microbes depends on biochemical reaction i.e. through pH indication and nutrient metabolic process utilized by microbes. Various attributes of solid media such as color and shape are the key elements for the examination of bacterial colonies.

3.6.1. Components

- Prepared media (powdered form already purchased from various companies)
- Sterile Petri plates (100 x 15 mm size)
- Reagent bottles
- Autoclave machine
- 70% ethanol or isopropyl alcohol alone or combine with 10% bleach for the disinfection of working area
- Graduated cylinder for actual measurement
- Weigh balance for proper measurement

- Apparatus for powdered and solid ingredients i.e. weigh boats, spatula, and paper plates)
- 50 ml Falcon tubes for the transportation of broth dispensed with bacterial isolates
- Top-Line Filter-Tips of 100 to 1000 µl for biochemical studies
- Test tubes for agar slants
- Sterile cotton for proper covering of test tubes

3.6.2. Media formation

Powdered agar and broth culture media were prepared. Initially, the powder was weighed on a weighing balance using weigh boat for specific volume of water. Volume of distilled water was measured using graduated cylinder. The powder was mixed with distilled water and transferred to reagent bottle. The bottle was kept at room temperature for 10 to 20 minutes to make sure that powder dissolves completely before autoclaving. Various media including MacConkey agar, Muller Hinton agar, LB broth, TSI agar, Simmon's citrate, urea agar were prepared with various compositions for different analysis as depicted in Table 3.1.

Table 3.1. Composition of prepared culture media

S. No.	Culture media	Total amount	Working amount	Total volume	Required volume	Aim of media
01.	MacConkey agar	51.5 g	10.25 g	1 L	200 ml	Morphology analysis
02.	Nutrient broth	13 g	2.6 g	1 L	200 ml	Morphology analysis
03.	Muller Hinton agar	38 g	7.6 g	1 L	200 ml	Culture sensitivity testing
04.	TSI agar	64.3 g	12.86 g	1 L	200 ml	Biochemical testing
05.	Simmons citrate agar	24 g	4.8 g	1 L	200 ml	Biochemical testing
06.	Urea agar	2.4 g	1.27 g	95 ml	50 ml	Biochemical testing

3.6.3. Autoclaving media and plates

Reagent bottles containing culture media and plates were sterilized to avoid contamination from outer environment. Sterilization was done with the pressure of 15

psi at 121°C for 20 minutes using autoclave machine which eliminates bacteria as bacterial death usually occurs at 15 to 20 minutes.

3.6.4. Media pouring

Media pouring was done in sterilized environment i.e. laminar flow cabinet to avoid contamination of the petri plates. The media was stirred thoroughly, the caps of the reagent bottles were air tight to avoid any contamination, and placed at room temperature to let it cool. After cooling, the plates were properly labeled and media was carefully poured in sterile petri dishes (100 x 15 mm) in a way to avoid bubble formation. The petri plates containing media was then left for 15 to 20 minutes for solidification. The plates were then stored upside down in a refrigerator for future use. Similarly, for broth culturing and biochemical analysis, media (broth and agar slants) was poured in test tubes and was covered with sterile cotton to prevent media from any external contamination. The test tubes were then stored in 4°C refrigerator prior further use. Figure 3.2 illustrates various media utilized for culturing, biochemical testing, and culture sensitivity testing.

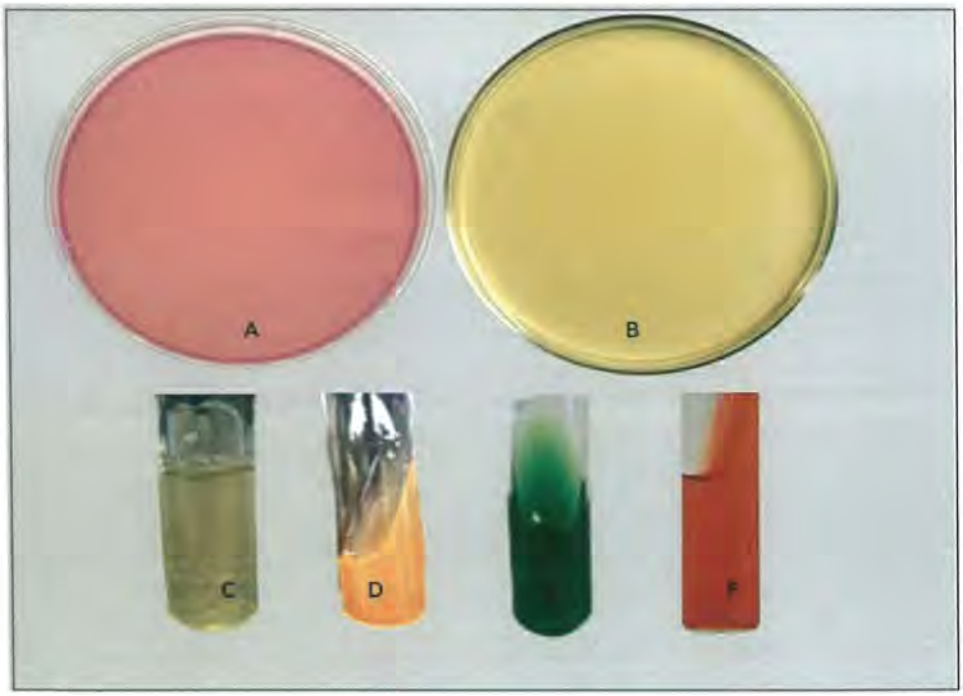


Figure 3.2. Types of media utilized in study. **(A)** MacConkey agar **(B)** Muller Hinton **(C)** Nutrient broth **(D)** Urea agar **(E)** Simmon's citrate **(F)** Triple Sugar Iron (TSI) agar

3.7. Specimen and inoculum processing

Specimens from various wards were brought to microbiology laboratory, labeled appropriately and inoculated on MacConkey agar plates using sterile inoculating loop for initial culturing.

3.8. Bacterial culturing

From collection bottle, a drop of urine was taken using a sterile dropper and placed on to a fresh MacConkey agar media plates. Specimen streaking was done on the quarter of the plates using sterilized inoculating loop. The plates were intersected and the samples were streaked 3 to 4 times to back out a decreased number of bacteria and then spread down the sides of the plates. Again the streaking was done 3 to 4 times by changing the angle of the plates. The plates were labeled and covered entirely to prevent any contamination. The bacterial culture plates were then incubated at 37°C for 24 hours to check and promote the bacterial growth for sub culturing and for future use.

3.9. Sub-culturing of isolates

Isolated bacterial colony was picked from the initial plate using sterile inoculating loop. Fresh media was prepared and poured in new petri plates. Bacterial colony was streaked on the entire plate. The plate was then incubated at 37°C for 24 hours and stored for future use.

3.10. Morphology screening on MacConkey agar plates

Bacterial colonies were observed on MacConkey agar plates to confirm the bacterial isolates. As MacConkey agar differentiates lactose and non-lactose fermenting bacteria therefore, MacConkey agar media was utilized to observe the growth and morphology of gram negative bacteria.

3.11. Gram staining

Gram staining was done to confirm the cell wall and type of bacteria. Initially, clear microscope slides were taken and a drop of normal saline was added to each slide. Then single colony from overnight culture was taken, bacterial smear was made and mixed with the drop of normal saline. The slides were dried in an incubator for 10 to 15 minutes. Heat fixation using spirit lamp was done. A few drops of crystal violet was then added to the slide and left for 2 minutes. The slides were then washed with tap water in such a way that colony does not wash out. Thereafter, a few drops of gram

iodine was added to each slide and left for 2 minutes. Again washing was done with tap water. The slides were then decolorized using ethanol or alcohol and washed with tap water again. Counter staining was done for 1 minute using safranin. The slides were again dried in an incubator for a few minutes. A drop of oil was added to the microscopic slide (Figure 3.3). At last, the slides were observed under microscope at 100 X magnification power.

3.12. Biochemical testing

For further recognition of bacteria at specie level, various biochemical tests were done. Three different agar media were prepared including urea agar, Simmon's citrate agar, and triple sugar iron (TSI) agar. Optimized concentrations of media ingredients (Table 3.1) were used. The prepared media was poured in test tubes and covered with sterile cotton to prevent media from contamination. Using sterilized inoculating loop, single and isolated bacterial colony was taken from overnight culture and suspended in agar slants (Figure 3.2). Agar slants were then incubated at 37°C for 24 hours to examine test results (Table 3.2).

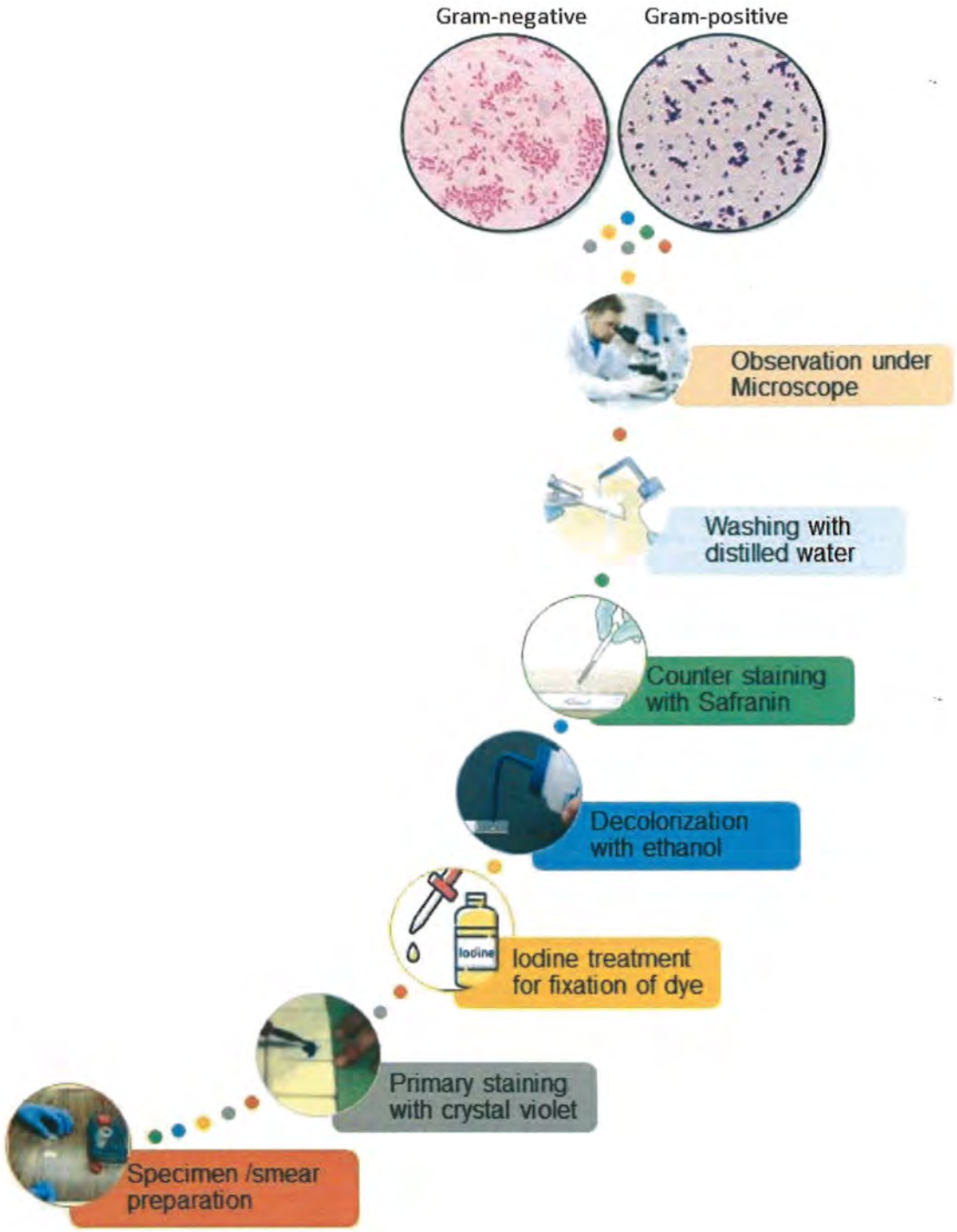


Figure 3.3. Steps involved in gram staining of bacterial isolates

Table 3.2. Bacterial identification through biochemical tests

S. No.	Biochemical tests	Test aim
01.	Triple sugar iron (TSI)	Sugar fermentation and hydrogen sulfide production
02.	Urease	Urea splitting via urease enzyme production
03.	Citrate	Gram negative bacteria separation based on citrate utilization

3.13. Antibiotic sensitivity test

Culture sensitivity testing was done to assess the activity of various antibiotics. For this purpose, Muller Hinton agar media and total of 12 antibiotics were utilized.

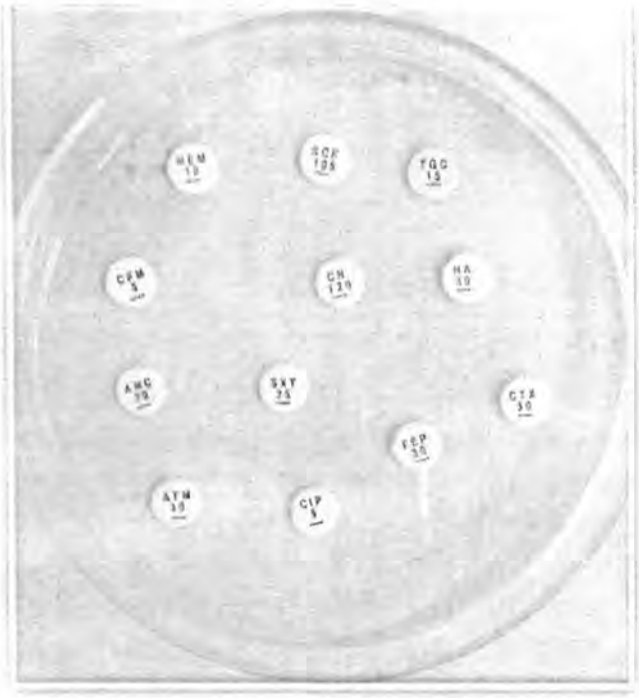


Figure 3.4. A simple illustration of petri plate containing antibiotic discs with various concentrations

3.13.1. Disc diffusion method

Isolated bacterial colony from overnight culture was picked and inoculated in fresh nutrient broth medium. Broth was then incubated at 37°C overnight. Muller Hinton media was prepared and solidified. After solidification, bacterial pure isolate from overnight nutrient broth was taken using sterile cotton swab. Lawn of bacteria was made on solidified Muller Hinton media. Antibiotic discs with various concentrations (Table 3.3) were then placed on inoculated media plates and incubated overnight at 37°C. Next day, the plates were observed and results were recorded accordingly as sensitive, intermediate and resistant.

Table 3.3. Concentrations of various antibiotics utilized for culture sensitivity testing

S. No.	Antibiotics discs	Concentrations (µg)
01.	Meropenem (MEM)	10
02.	Ceftazidime (CFM)	5
03.	Azithromycin (ATM)	30
04.	Ciprofloxacin (CIP)	5
05.	Cefepime (FEP)	30
06.	Co-trimoxazole (CTX)	30
07.	Imipenem (IPM)	25
08.	Gentamicin (GN)	120
09.	Cefoperazone-sulbactam (SCF)	105
10.	Nitrofurantoin (NA)	30
11.	Tigecycline (TGC)	15
12.	Co-amoxiclav	25

3.14. Culture transportation for molecular characterization

After culture sensitivity testing, pure cultures of bacteria (n=5) were transported for molecular identification. For this purpose, isolated bacterial colonies were picked using sterile inoculating loop and suspended in sterilized falcon tubes (50 ml) containing

nutrient broth media. The tubes were then incubated at 37°C overnight at 150 rpm in shaking incubator for significant growth. Next day, tubes were sealed and transported from KTH, Peshawar to Biotechnology's department of Quaid-i-Azam University, Islamabad.

3.15. Procedure of molecular characterization

A generalized strategy for molecular characterization is illustrated in figure 3.5; and each of the method have been discussed separately in the following sections.

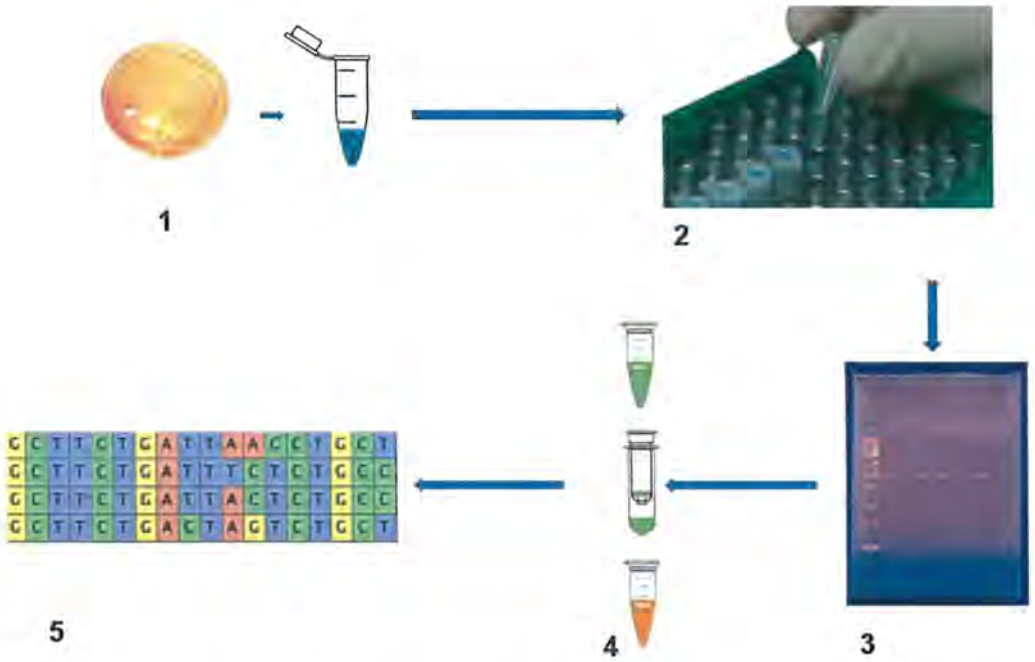


Figure 3.5. Procedure of molecular characterization performed at Biotechnology department, QAU. **1.** DNA extraction of pure bacterial isolates using both plain boiling and CTAB method with slight modifications. **2.** Polymerase Chain Reaction (PCR) using universal primers for the amplification of 16S rRNA gene. **3.** Agarose gel electrophoresis for the confirmation of amplified product. **4.** Purification of amplified product from agarose gel electrophoresis. **5.** Sanger sequencing and post sequence analysis using bioinformatics tools

3.16. DNA extraction by plain boiling method

Plain boiling method was employed as it gives high yield of DNA. For DNA extraction, isolated bacterial colony from overnight culture was picked using micropipette and suspended in 0.2 ml PCR tube containing 50 µl lysis buffer. Bacterial colony was properly mixed with lysis buffer by pipetting 3 to 4 times. Mixing was done in such a

way that bubbles were not formed. Various components including Triton-X 100, 0.5 M EDTA and Tris-HCl with different concentrations were mixed in autoclaved water to make lysis buffer with final pH of 8 (Table 3.4).

Bacterial colony suspended in lysis buffer was denatured at 95°C for 15 minutes using thermal cycler (peqSTAR 96x gradient-VWR). After denaturation, the sample was transferred to microfuge tube. Centrifugation was done at 14000 rpm for 10 minutes. Finally, the supernatant was transferred to fresh microfuge tube and pellet was discarded.

3.16.1. DNA precipitation and purification

Equal amount of isopropanol was added in the supernatant (1:1) and mixed thoroughly. The samples were then again centrifuged at 14000 rpm for 10 minutes. Isopropanol was discarded and 70% ethanol was added in a pellet containing DNA for residual washing. After mixing, ethanol was discarded and the tube was oven dried for 5 to 10 minutes. Finally, 20 to 30 µl elution buffer was added to the pellet.

Table 3.4. Stock solution and composition of lysis buffer

S. No.	Components	Amount
01.	1M Tris HCl (pH 8)	6.304 g
02.	0.5 M EDTA (pH 8)	7.4 g
03.	Triton X 100	4 ml
04.	d.H2O	36 ml
Total		**40 ml**

3.17. DNA extraction by CTAB method

For bacterial isolation, initially the bacterial samples were grown in suspension culture i.e. nutrient broth medium. 2 ml bacterial suspension was added to clean microfuge tubes. The tubes were centrifuged at 12000 rpm for 1 minute and the supernatant was discarded. The pellets were taken in fresh microfuge tubes. The initial step was repeated again for optimum DNA quantity. CTAB was prepared (Table 3.5) and placed in a water bath for 30 minutes at 65°C. Then 500 µl CTAB from stock solution was added in each sample and again placed in water bath at 65°C for 30 minutes. The samples were shaken after each 10 minutes interval. Phenol-chloroform and isoamyl

alcohol was then added with the volume of 500 µl in each sample and vortex for a few seconds. Centrifugation was done again at 13000 rpm for 13 minutes. Approximately, 250 to 300 µl of supernatant was collected and the pellet was discarded this time. Supernatant was added in a fresh microfuge tubes and absolute ethanol was added in the double amount with respect to the supernatant added in a tube i.e. for 300 µl supernatant, 600 µl absolute ethanol was added in each tube. The samples were again centrifuged at 13000 for 13 minutes and this time pellet was taken and supernatant was discarded. Finally, 70% ethanol was then added in double amount as mentioned above and the samples were again centrifuged at 12000 rpm for 10 minutes. Supernatant was discarded and tubes containing pellet were tissue dried overnight. At last, 50 µl of TE buffer was added next day in each sample and vortex for 10 to 15 seconds.

Table 3.5. Preparation of CTAB buffer and quantity of phenol-chloroform and isoamyl alcohol

CTAB preparation
• 1 ml CTAB with 2 µl marceptoethanol
Phenol: Chloroform: Isoamyl alcohol
• 25:24:1

3.18. Confirmation of DNA after extraction

After the extraction using both of the methods, the quantity and bands (quality) of genomic DNA was evaluated using Nanodrop. Nanodrop reading (260nm/280nm) was evaluated to determine the quantity and the purity of the extracted DNA i.e. in ng/µL.

3.19. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction was carried out in order to amplify the selected regions of the extracted DNA. Components including green master mix, nuclease free water, forward primer, reverse primer and bacterial DNA with varying concentrations were added in 0.2 ml PCR tubes (Table 3.6). After the addition of ingredients, the prepared samples were spun at high speed for proper mixing. Final volume for each PCR reaction was kept 20 µl.

Table 3.6. Components with concentrations for optimized PCR reaction

S. No.	PCR components	Concentrations
01.	GoTaq® Green Master Mix, 2X (Thermo scientific)	10 µl
02.	Forward primer (27F)	1.5 µl
03.	Reverse primer (1492R)	1.5 µl
04.	PCR water	04 µl
05.	Template DNA	03 µl
Final volume for reaction		**20 µl**

3.19.1. Primers, procedure and conditions for PCR

Primers utilized for the amplification of 16S rRNA gene are illustrated in table 3.6. All the components were added in 5 different PCR tubes with final reaction volume of 20 µl. The components in the sample tubes were mixed with micropipette and then spun at high speed for appropriate mixing. The PCR tubes were then subjected to thermal cycler with various conditions (Figure 3.6) for approximately 2 hours for 35 cycles. Finally, to examine DNA and size, the amplified products were subjected to gel electrophoresis.

Figure 3.6. Cyclic conditions for optimized PCR procedure for the amplification of the selected region of bacterial genome

Table 3.7. Universal primers utilized in PCR for 16S rRNA gene amplification

S. No.	Primer pairs	5' to 3' Complementary sequence
01.	Forward Primer- 27F	AGAGTTTGATYMTGGCTCAG
02.	Reverse Primer- 1492R	TACGGCTACCTTGTTACGACTT

3.20. Gel electrophoresis

Gel electrophoresis was carried out in order to examine the amplified product.

3.20.1. Gel composition and preparation

Initially, 1 X TAE buffer was prepared from 10 X TAE buffer stock. To make 1 X TAE buffer, about 100 ml of 10 X TAE buffer (stock) was taken in fresh reagent bottle and 900 ml distilled water was added. The gel was then formed by dissolving 0.6 gram agarose in 40 ml of 1 X TAE buffer (Table 3.8). The solution was microwaved for 30 seconds to 1 minute. After microwave heating, the solution was cooled and about 4 µl of ethidium bromide (EtBr) was added when the temperature of the melted gel reaches below 50°C. The gel was poured in casting tray, comb was inserted to make wells and left for 30 to 40 minutes for the solidification of gel. Comb was removed, 1 X TAE buffer was added in the gel tank, gel was positioned in gel tank and at last the samples were loaded in wells (7 to 10 µl). DNA ladder i.e. 1kb GeneRuler^{TM} (2 to 2.5 µl) was added in first well. Subsequently, the gel was run for 30 minutes at 90 volts (V). The results in the form of bands were then analyzed under UV trans-illuminator.

Table 3.8. Components for gel electrophoresis procedure

S. No.	Components (1.5% gel)	Amount
01.	1 X TAE buffer	40 ml
02.	Agarose	0.6 g
03.	Ethidium bromide solution	4 µl

3.21. Gel purification protocol

The bands were excised from gel using surgical blade and transferred to fresh, labelled microfuge tubes. Empty microfuge tube was weighed initially and then tube containing extracted product was weighed and final weight of the tubes were noted. An equal amount of binding buffer (1:1) was added to the tubes containing PCR product. The color of the mixture was yellow which designates an ideal pH for binding DNA. From the above mentioned step, about 800 µl of the mixture was transferred to purification column (GeneJET), centrifuged at 10,000 to 14000 rpm for 1 minute, flow-through was discarded and column was placed back to the collection tube. Then, about 700 µl washing buffer was added to the column, centrifuged again for 14000 rpm for 1 minute and flow through was discarded, then the purification column was placed in the collection tube again. To remove residual washing buffer, the empty column was centrifuged again for 1 minute at 14000 rpm. Fresh microfuge tubes were then properly labeled according to the type sample processed and purification column was transferred to those fresh microfuge tubes (1.5 ml). Consequently, 30 µl of elution buffer was added in each purification column placed in microfuge tube and final centrifugation was done at 14000 rpm for 1 minute. The eluted product was stored at -20°C for future use. Reconfirmation of the DNA was then done by checking the quantity of the DNA using Nanodrop. In addition, 10 µl of the eluted product was confirmed by agarose gel electrophoresis (Figure 3.7).

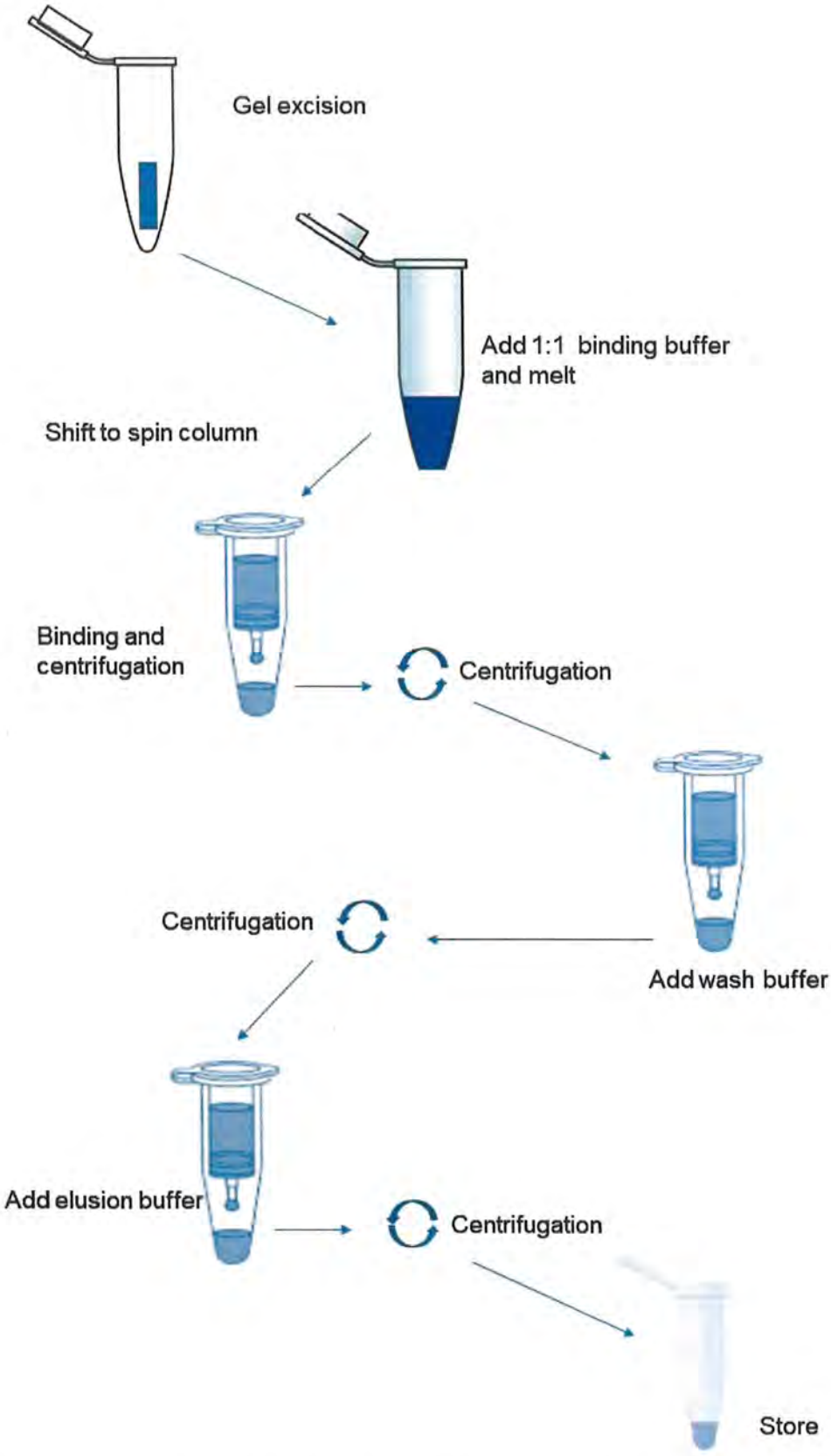


Figure 3.7. Gel purification of the amplified PCR product

3.22. DNA Sequencing

Following purification protocol, about 10 µl of the mixture was prepared in which 8 µl was the amplified product and 2 µl was forward primer i.e. 27F; AGAGTTTGATYMTGGCTCAG. The suspension was prepared in 0.2 ml PCR tubes for each sample and were sent for Sanger sequencing to Macrogen, a public biotechnological Company situated in South Korea.

3.23. Bioinformatics Analysis

Consequently, the sequences obtained after Sanger sequencing were analyzed using BLAST (Basic Local Alignment Search Tool) from National Centre for Biotechnology Institute (NCBI) (www.ncbi.nlm.nih.gov/BLAST), a database utilized for phylogenetic and evolutionary analysis. After BLAST, sequences of 16S rRNA were observed and reference sequences were obtained from NCBI based on highest similarity index. Later, sequence alignment with similar sequences was done using MEGA-X software. Similarly, for phylogenetic analysis and tree construction, Neighbor-Joining method of the same software was used that usually helps out inferring the evolutionary relatedness of the isolates to the closely related isolates (Saitou and Nei 1987).

4. Results

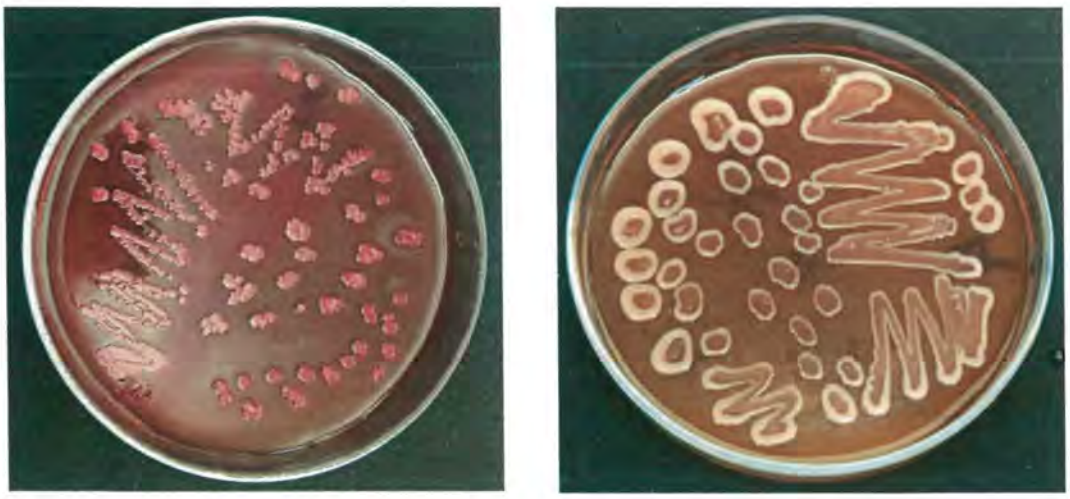
In this research study, five samples of urine positive culture specimens were collected from various care units of KTH hospital Peshawar, based on several gender distribution. Of total 5, 3 were females and 2 samples were from males (Table 4.1). The samples in the urine culture bottle depicted positive as per doctor prescriptions and urine culture reports.

Table 4.1. Patient's Attributions

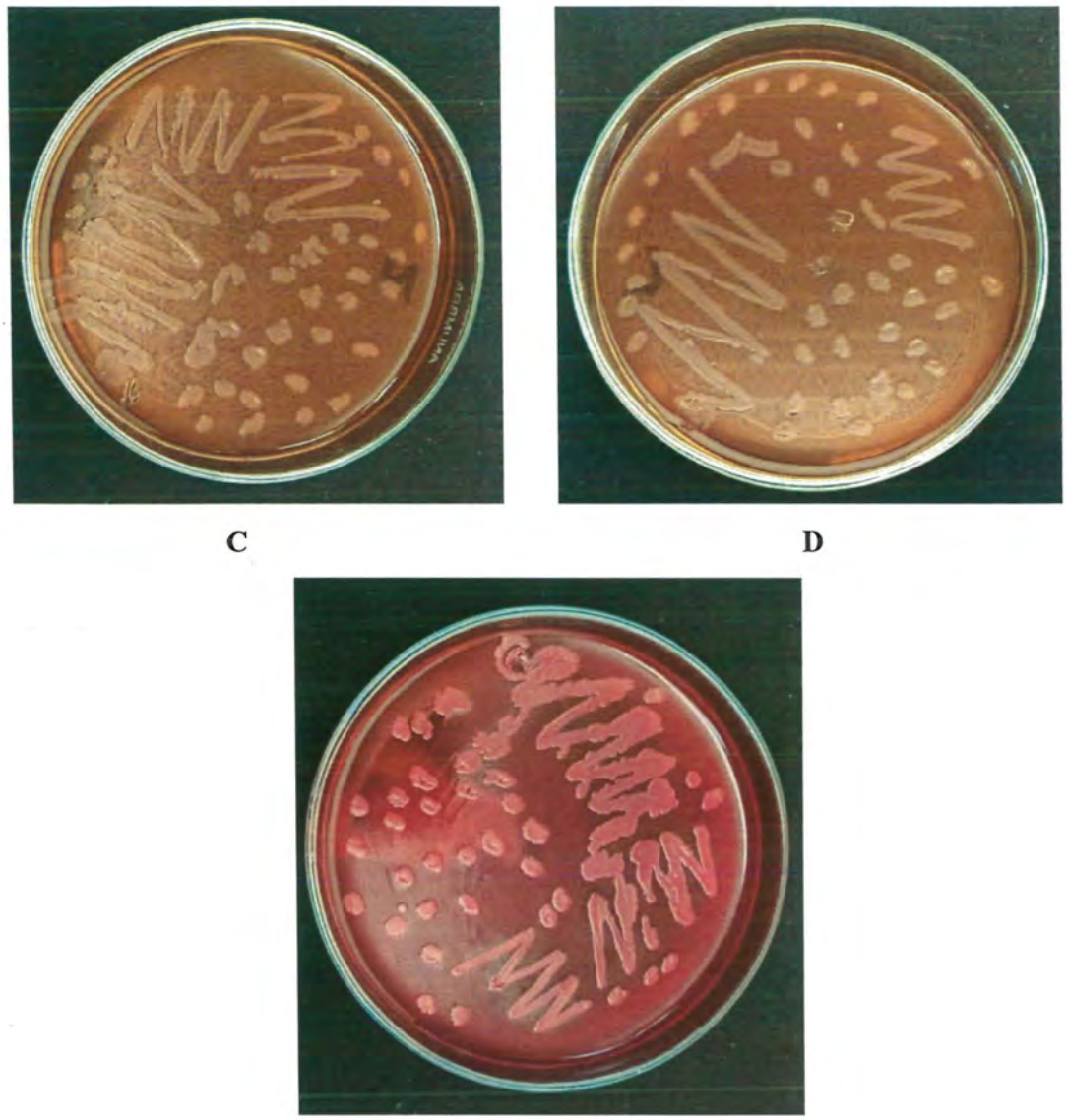
S. No.	Isolate name	Age	Gender
01.	M1	45 years	Female
02.	M2	19 years	Male
03.	M3	8 months	Female (child)
04.	M4	10 months	Female (child)
05.	M5	23 years	Male

4.1. Observation of strains on MacConkey Agar Plates

MacConkey agar is a differential and chromogenic media which differentiates lactose and non-lactose fermenting bacterial isolates. Three of the bacterial isolates were colorless indicating non-lactose fermenting bacteria while two specimens were appeared red/pink depicted as lactose-fermenting bacteria (Figure 4.1).



A **B**



C D

E

Figure 4.1. Observation of pure bacterial isolates on MacConkey agar plates indicating 3 non-lactose and 2 lactose fermenting bacteria. **A.** Lactose fermenting bacteria. **B. C. D.** Non-lactose fermenting bacteria. **E.** Lactose fermenting bacterial isolate

4.2. Gram staining

Gram staining results of isolated bacterial strains from petri plates depicted that all of the isolates were gram-negative bacteria (Figure 4.2).

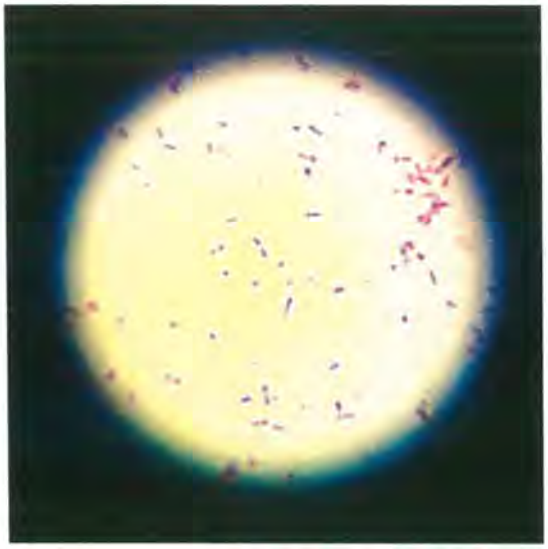


Figure 4.2. Gram negative rods of Urease positive bacteria under 100X resolution

4.3. Biochemical testing

After gram staining, morphology observation on MacConkey agar plates, bacterial identification was done through various biochemical tests to differentiate desired bacteria from other various gram negative rods and bacilli bacteria's. Test screen of the bacteria was done within 24 hours after incubation (Figure 4.3). Table 4.2 illustrates the results of biochemical tests performed in Microbiology section of KTH hospital Peshawar.

A B C

Figure 4.3. Results of biochemical testing. Triple sugar iron (TSI) and citrate were negative while only urease was positive for all the bacterial samples. **A.** Negative triple sugar iron (TSI). **B.** Positive urease. **C.** Negative citrate

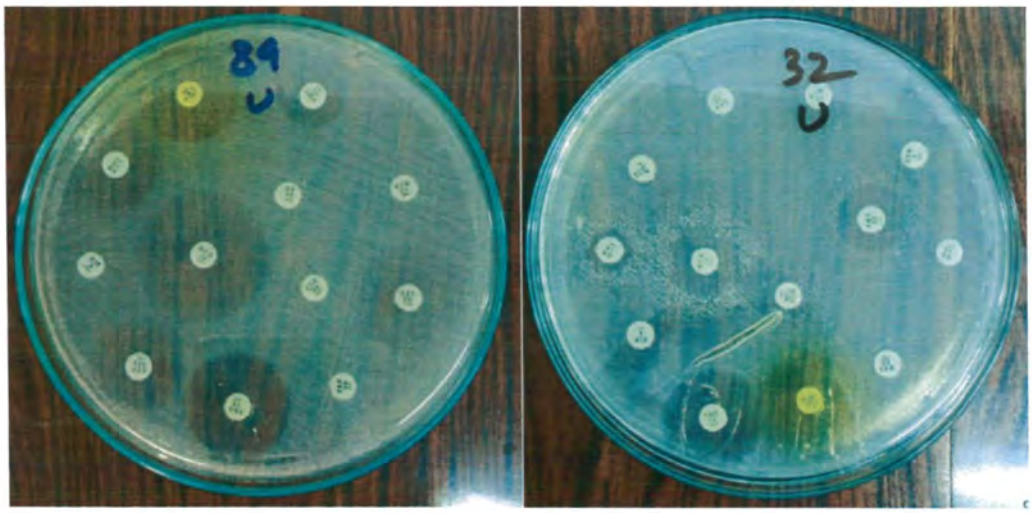
Table 4.2. Biochemical tests: The bacterial isolates were positive for urease while negative for citrate and TSI

S. No.	Biochemical tests	Results
01.	Triple sugar iron (TSI)	Negative
02.	Urease	Positive
03.	Citrate	Negative

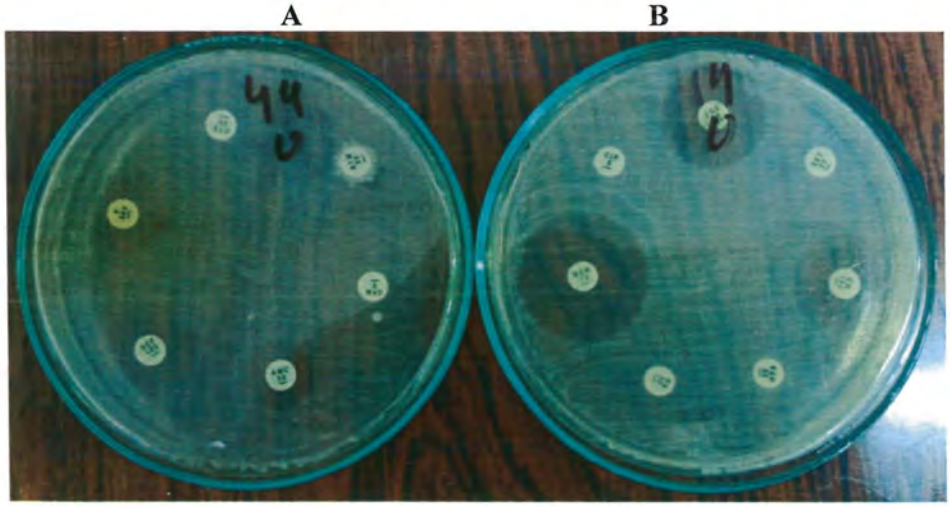
4.4. Antibiotic sensitivity results

Out of total 5 specimens, 3 were found to be multidrug resistant (MDR) while 2 of the samples were extensively drug resistant (XDR) bacteria. Of the 3 MDR strains, first showed sensitivity to meropenem, nitrofurantoin, tigecycline and gentamycin while resistant to all other regimens utilized in the study. Similarly, second revealed sensitivity to meropenem, tigecycline, nitrofurantoin, co-amoxiclave and third showed

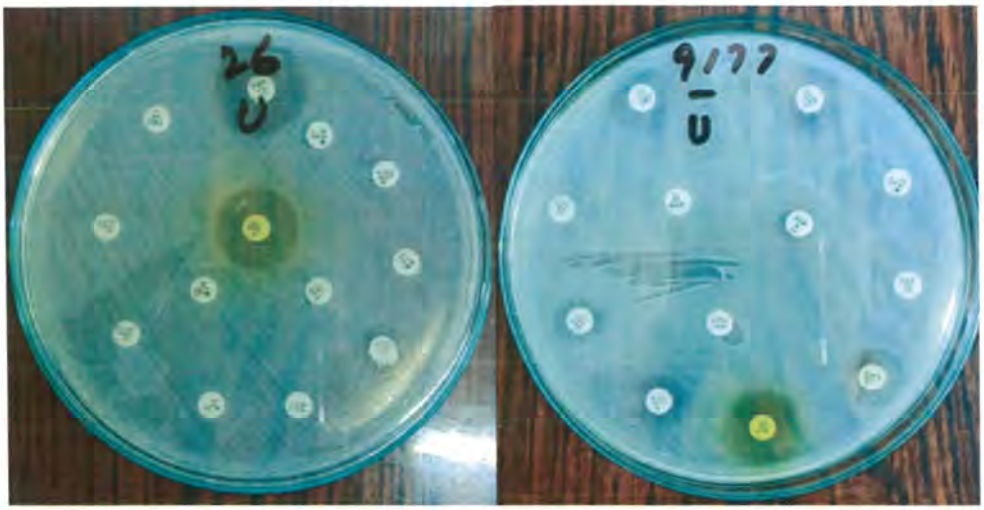
sensitivity to meropenem, tigecycline, gentamicin, nitrofurantoin and cefoperazone-sulbactam respectively (Figure 4.4; Table 4.3). Of the two XDR bacteria, one showed sensitivity pattern just to nitrofurantoin and tigecycline while second showed sensitivity to tigecycline and cefepime while resistant to all other 10 drug regimens respectively (Table 4.3).



A B



C



D E

Figure 4.4. **A. B.** and **C.** Culture sensitivity test result of patient MDR bacteria of patient 1,2, and 3 depicting sensitivity and resistance to various antibiotics. **D** and **E.** Antibiotic sensitivity test results of XDR bacteria of specimen 4 and 5

Table 4.3. Drug profile of isolates showing resistance and sensitivity pattern

S. No.	Antibiotics	Isolates				
		M1	M2	M3	M4	M5
01.	Meropenem (MEM)	S	S	S	R	IN
02.	Ceftazidime (CFM)	R	R	R	R	S
03.	Azithromycin (ATM)	R	R	R	R	S
04.	Ciprofloxacin (CIP)	R	R	R	R	IN
05.	Cefepime (FEP)	R	R	R	R	R
06.	Co-trimoxazole (CTX)	R	R	R	R	S
07.	Gentamicin (GN)	S	S	R	R	S
08.	Cefoperazone-sulbactam (SCF)	S	S	R	R	IN
09.	Nitrofurantoin (NA)	S	S	S	S	S
10.	Tigecycline (TGC)	S	S	S	S	R
11.	Co-amoxiclav	IN	R	S	R	S
12.	Imipenem (IPM)	S	S	S	R	S

Abbreviations: S: Sensitive, R: Resistant, IN: Intermediate

4.5. Results of comparative analysis of DNA

Total number of bacterial samples (n=5) were processed and DNA was extracted using both plain boiling and CTAB method with slight modifcations as mentioned above. Using plain boiling method, the nanodrop results revealed the quantity of approximately 300.52 ng/µL, 450 ng/µL, 300.98 ng/µL, 375.76 ng/µL and 276.88 ng/µL respectively with slightly deficient purity. Treatment with isopropanol, and ethanol reduces the quantity up to 40 ng/µL and increases the purity of isolated DNA (Table 4.4). Similarly, CTAB method utilized in the study displayed the quantity of 40 ng/µL, 45 ng/µL, 53 ng/µL, 60 ng/µL and 47 ng/µL respectively. Quality and genomic DNA further confirmed from gel revealed sharp bands.

Table 4.4. Nanodrop results of plain boiling method and CTAB method

S. No.	Isolates	Plain boiling method		CTAB method	
		ng/µL	260/280	ng/µL	260/280
01.	M1	60.59	1.38	40.64	1.77
02.	M2	67.88	1.76	45.77	1.10
03.	M3	56.70	1.45	53.87	1.65
04.	M4	40.43	1.90	60.55	1.25
05.	M5	46.66	2.06	47.86	1.80

4.6. Results of PCR amplified product

After PCR reaction, the amplified DNA product was tested on 1.5% gel. The samples were labeled as M1, M2, M3, M4, and M5 respectively. A ladder of 1kb was utilized to compare the product size (GeneRuler DNA ladder 1kb, Fermentas). The band size of the amplified product was approximately 1500bp for all the samples respectively (Figure 4.5).

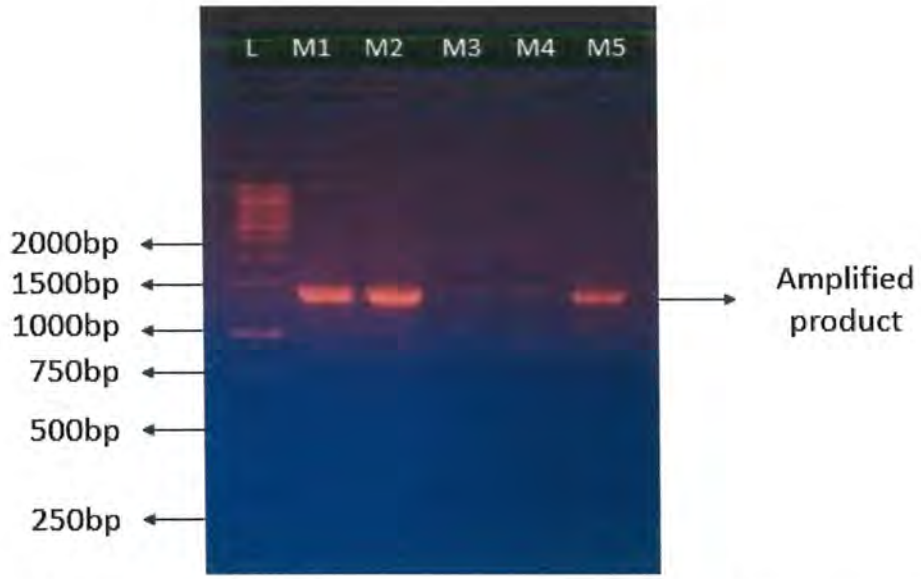


Figure 4.5. Amplicon size of approximately 1500bp as indicated in samples **M1, M2, M3, M4** and **M5. "L"** represents DNA ladder of 1 kb

4.7. Sequencing of PCR product

PCR product was purified and sequenced using single universal primer (27F: AGAGTTTGAT[Y]MTGGCTCAG) which is specific to the 16S rRNA gene. Then DNA sequences of 3 samples were used for molecular typing and post sequencing analysis.

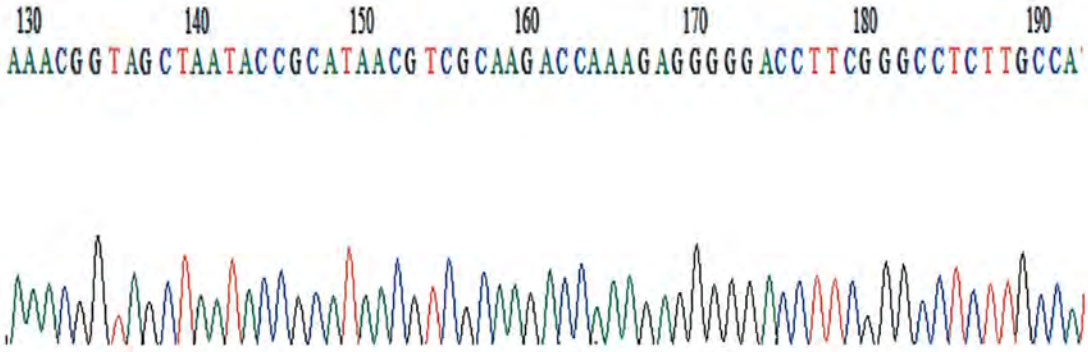


Figure 4.6. Representative chromatogram of isolate M3 (BioEdit)

4.8. Phylogenetic analysis

After sequencing the 16S rRNA gene for 3 samples, the sequences were compared to reference sequences collected from National Centre for Bioinformatics database for phylogenetic analysis. For similarity comparison BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST) was used. After obtaining sequences that showed the most similarity to the sequences understudy, multiple sequence alignment (MSA) of all the sequences were done using ClutalW (multiple alignment tool) of BioEdit software. MEGA (version X) was then used to construct a phylogenetic tree using the Neighbor-Joining method which helps infer the evolutionary relationship of the identified strain to their closest reference strains. Sequencing results of 2 isolates indicated the strains of *Escherichia fergusonii*, while 1 indicated *Morganella morganii* (Figure 4.7).

4.9. Phylogenetic tree construction

MEGA version X was used to analyze the nucleotide sequences from the isolates included in this study in comparison with 49 and 36 various reference nucleotide sequences of variable urease and non-urease positive bacteria from different regions of the world including Pakistan. The evolutionary history of the strain was observed using the Neighbor-Joining and Jukes-Cantor model (Jukes and Cantor 1969; Kumar et al. 2018). The associated taxa clustered together is shown next to the branches in the tree. The initial tree was generated automatically by applying Neighbor-Joining algorithms. The results revealed 2 strains of *Escherichia fergusonii* with the similarity index of 99.93% and 1 strain of *Morganella morganii* with the similarity index of 97.80% after BLAST. The isolate QAU 1011 and 1012 were phylogenetically related to *Escherichia fergusonii* strain of Canada (Accession number: NR 027549). The clade represents an evolving bacteria from both methods which were evolutionary distinct from *enterobacter, cronobacter, citrobacter, atlantibacter, shimwellia, salmonella, klebsiella, E. coli* and *shigella* respectively (Figure 4.7). Similarly, QAU 103 was phylogenetically related to *Morganella morganii* strain of Pakistan (Accession number: OL831045). The clade depicts the bacteria which was evolutionary distinct from *provedencia rettgerii, provedencia staurtii* while related to the strains of *morganella morganii*.

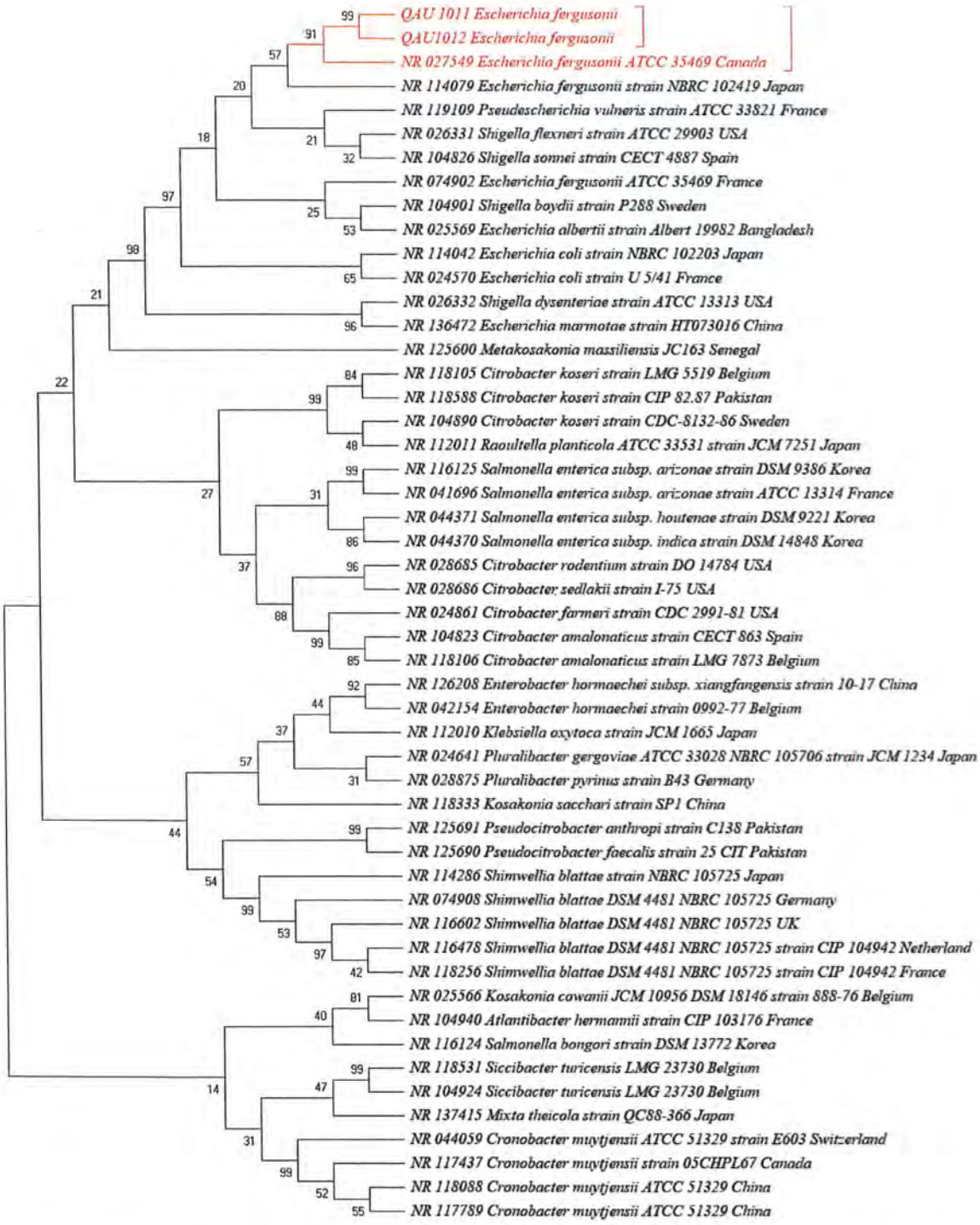


Figure 4.7. Phylogenetic tree illustration of QAU 1011 and 1012 sequenced isolates. Reference sequences (n=49) were obtained from GenBank database to assess the phylogenetic analysis of the bacterial isolates using Neighbor joining method

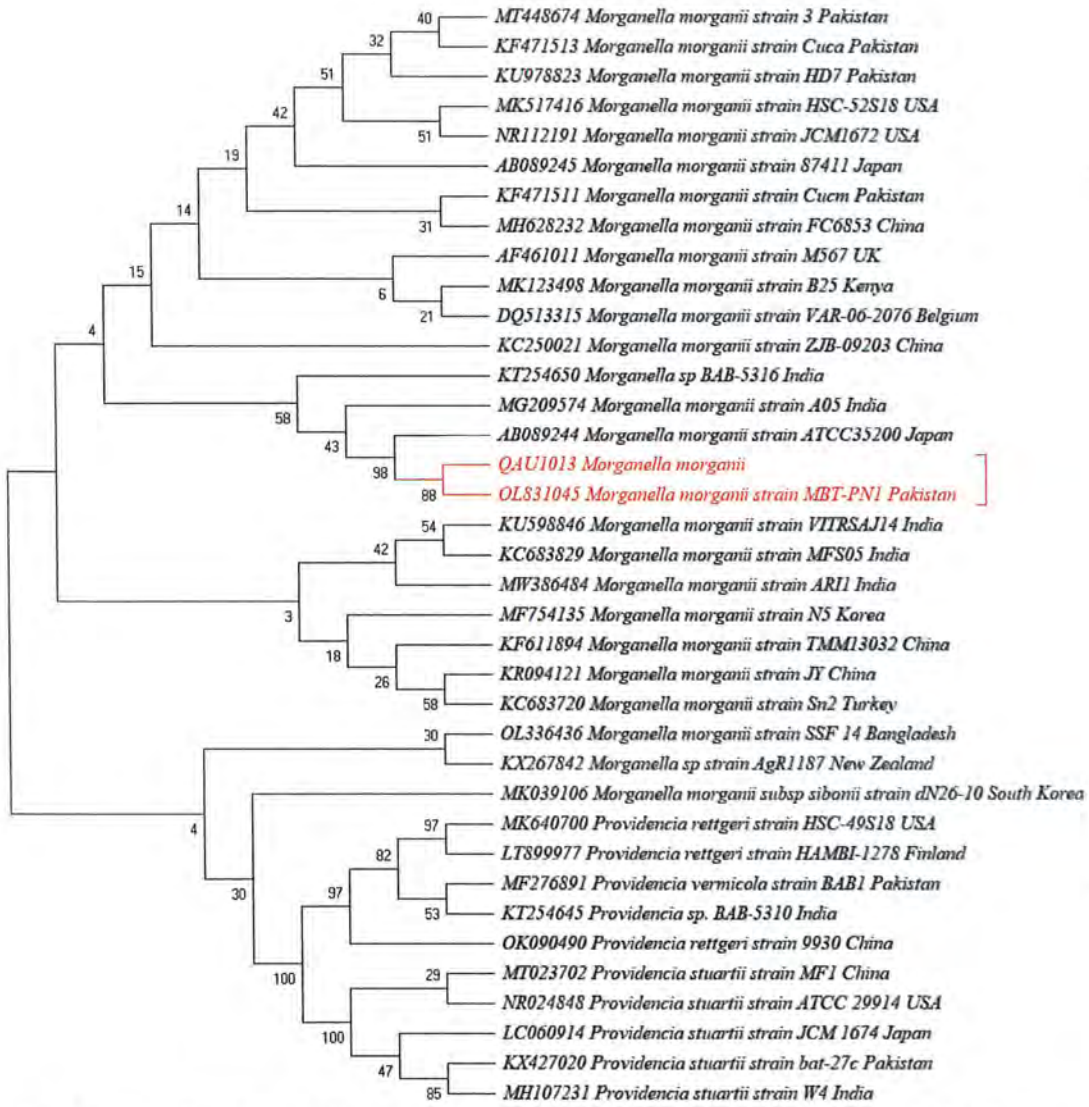


Figure 4.8. Phylogenetic tree illustration of QAU 1013 sequenced isolate. Reference sequences (n=36) were obtained from GenBank database to assess the phylogenetic analysis of the representative sequenced bacterial isolate using Neighbor joining method

5. Discussion

Urinary tract infections (UTIs) are usual, repeated infections which can be sympathetic to mortal. The fifth persistent infection associated with health-care hospitals are UTIs, with approximately 62,700 of total UTIs in critical-care-hospitals in 2015. In addition, infections associated with health-care centers report for higher than 9.5% of the total infections (Magill et al. 2018). The resistance against antibiotics has emerged with destructive effects on useful microflora of the host via antibiotic use revealing deficiency in the treatment for UTIs (Klein and Hultgren. 2020). The existing effect of bacteria that cause urinary tract infections is a major challenge to evaluate due to misdiagnosis of bacteria with other gram-negative bacterial isolates, while identification can be done via biochemical and molecular detection using PCR assay (Lehman et al. 2010).

PCR based assay is highly specific and sensitive for the detection of isolates compared to biochemical testing as PCR based detection does not require any tests for gram-positive and gram-negative bacteria. An experimental study revealed that multidrug resistant bacteria causing urinary tract infections can be precisely detected via PCR because of higher specificity and sensitivity compare to other approaches (Pirkani et al. 2020). Similarly, for subsequent PCR amplification, extraction of DNA must be appropriate and high yielded DNA must be obtained for efficient amplification (Sintsova et al. 2019).

In an experimental study, uropathogenic *E. coli* obtained from urine cultures were brought to lab and DNA was extracted using CTAB method with slight modification and the results yielded accurate and sufficient amount of DNA for the amplification of different genes (Sintsova et al. 2019). Dilhari et al. investigated various DNA extraction methods to assess the quantity and quality of DNA. Extraction boiling method was utilized with slight modifications opted from Asadzaheh et al. 2010 revealed high amount of DNA with optimum purity (Dilhari et al. 2017). In our experiment, comparative analysis of both plain boiling method and CTAB method was done to assess the quantity of total genomic DNA of bacteria. Plain boiling method was utilized from Asadzaheh et al. 2010 with slight modifications provides high amount of genomic DNA with less purity compared to CTAB method opted from Doyle and Doyle. 1987 with slight modifications. CTAB method is laborious therefore

contamination in genomic DNA was observed with initial extraction but consequently provides accurate results with high purity. In addition, plain boiling method utilized in the study also provide accurate results but the quantity of the genomic DNA observed was high and for this reason precipitation and purification was done to obtain purity of the extracted DNA. Both of the methods employed for DNA extraction yielded sufficient quantity for PCR amplification.

Resistance to antibiotics and drug regimens is progressive and emerging constantly in health-care settings (Sharma 2011). Unreasonable utilization of drugs also leads to progressive resistance rate (Cars et al. 2008). Antibiotic resistance can be reduced due to less prescription rate but it is difficult to execute changes (Arason and Sigurdsson. 2010). UTI causing bacterial isolates specifically *E. coli* contributes to total infections of 80% with resistance to first-line antibiotics and other regimens such as nitrofurantoin, ciprofloxacin, trimethoprim and trimethoprim-sulfamethoxazole (TMP-SMX) in both community and hospital acquired clinical-care settings (Sanchez et al. 2016). Similarly, urease positive bacterial isolates previously showed resistance against nitrofurantoin, nalidixic acid, ceftriaxone and cefotaxime while revealed sensitivity to gentamycin, norfloxacin, ciprofloxacin, vancomycin, amikacin, ciprofloxacin, and imipenem (Dheyab et al. 2018).

Another study revealed susceptibility pattern of XDR and MDR gram-negative isolates in kidney transplant patients with UTI. Results revealed total of 88 events of gram-negative XDR and MDR UTIs with 5 patients i.e. 6.2%. *E. coli* was frequent cause with the total of 62.5% leading to hospital mortality. All of the MDR and XDR isolated bacteria were resistant to first-generation and second-generation beta-lactam (monocyclic) and cephalosporin. Sensitivity pattern of all isolated bacteria was observed to amikacin, tigecycline and meropenem. Among them, 12 XDR bacteria showed resistant to meropenem, while tigecycline resistancy was upto 25%. Among all of the XDR isolated strains *E. coli* and *Acinetobacter baumannii* were tigecycline sensitive (Yuan et al. 2018). In contrast to these studies, our results of biochemical study revealed all of the isolates as urease positive bacterial isolates in which 3 of them were MDR strains while 2 isolates were XDR. MDR revealed sensitivity to meropenem, nitrofurantoin, tigecycline, gentamycin, co-amoxiclav and cefoperazone-sulbactam. Similarly, XDR strains showed sensitivity to nitrofurantoin, tigecycline and

cefepime respectively. While all of the other antibiotics utilized for culture sensitivity were resistant to both MDR and XDR bacteria as described previously.

Biochemical testing for bacteria is not an optimum and suitable approach for the identification of bacteria causing UTIs as most of the bacterial strains show resemblance and similar pattern particularly bacterial representatives of the same family including enterobacteriaceae, bacilliacea, morganelliaceae etc. Molecular method for the identification of bacteria remains the best and appropriate approach. *E. coli* with respected representatives i.e. *E. albertii* and *E. fergusonii* were accurately distinguished from *E. coli* based on specific primers to certain genes present in both of the strains. PCR results with specific primers revealed accurate identification of both the strains (Maheux et al. 2014). Similarly, beta-lactam genes i.e. antibiotic resistance strains and identification can be done accurately as results revealed in which various isolates of bacteria producing extended spectrum beta-lactamase were detected using 16S rRNA gene sequencing (Adesina et al. 2019). In current work, antibiotic resistant bacterial isolates were biochemically identified revealing all the strains as *Morganella morganii* using various tests but due to inaccuracy of these tests, 16S rRNA sequencing was done to precisely detect the bacteria causing urinary tract infections. Results of 16S rRNA gene sequencing and phylogenetic analysis of 3 isolates revealed the strains as *Escherichia fergusonii* and *Morganella morganii* (Table 5.1) as sequence similarity was up to 99.98% and 99.80% after BLAST. Phylogenetic tree illustrates both as evolutionary bacteria which were distinct from other gram-negative rods such as *salmonella, shigella, E. coli, providencia, enterobacter, citrobacter* etc. isolated from various regions of the world.

Table 5.1. Contradictory results of biochemical and 16S rRNA sequencing

S. No.	Isolates	Biochemical tests	16S rRNA sequencing
01.	M1	*Morganella morganii*	*Escherichia fergusonii*
02.	M2	*Morganella morganii*	Not determined
03.	M3	*Morganella morganii*	*Escherichia fergusonii*
04.	M4	*Morganella morganii*	Not determined
05.	M5	*Morganella morganii*	*Morganella morganii*

Conclusion

Escherichia fergusonii and *Morganella morganii* are opportunistic and rare bacteria causing hospital acquired urinary tract infections with high resistant to present antibiotics and drug regimens has emerged worldwide even in children. The resistant pattern has progressed as a consequence of MDR and XDR isolates indicating that the accessibility of medicines for the recovery has weaken. The diagnosis methods specifically biochemical testing leads to misdiagnosis of bacterial isolates. To overcome the problem of misdiagnosis, molecular approaches needs to be adopted to specify the type of bacteria. Moreover, quick diagnosis of resistant bacterial isolates is required for the purpose of suitable treatments and to block the escalated resistance. In future, genes responsible for the antibiotic resistance can be identified via molecular approaches using specific primers (i.e. mutation detection in various genes). Further, homology modeling and drug docking studies through bioinformatics tools can be utilized to target the antibiotic resistant genes and to predict new antibiotics that can overcome the problem of resistance.

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Appendix

MacConkey Agar media

Ingredients	Quantity (g/L)
MacConkey Agar	51g

Mueller Hinton Agar media

Ingredients	Quantity (g/L)
Mueller Hinton Agar	38g

TSI agar media

Components	Quantity (g/L)
Peptone	20
Meat extract	3
Yeast extract	3
Lactose	10
Sucrose	10
Dextrose	1
Sodium chloride	5
Ferric ammonium citrate	0.3
Sodium thiosulfate	0.3
Phenol red	0.025
Agar	12
Final pH	7.4 to 7.6

Simmons citrate agar media

Components	Quantity (g/L)
Magnesium sulfate	0.2
Monoamonium phosphate	1
Dipotassium phosphate	1

Sodium citrate	2
Sodium chloride	5
Bromothymol blue	0.08
Agar	15
Final pH	6.8 to 7

1M Tris-HCl

Ingredients	**Concentration (g/L)**
Tris-HCl	157.64
Distilled water	1L
pH	8.0

Lysis buffer

Ingredients	**Concentration**
Autoclave distilled Water	1ml
Tri-X 100	200µl
Tris-HCL	40µl
EDTA 0.5 molar	8µl
PCR Water	752 µl
pH	8

10X TAE (Tris-acetate-EDTA) buffer

Ingredients	**Concentration**
Tris- base	108g
Acetate Acid	55g
0.5M EDTA	40ml
pH	8.0

1.5% Agarose gel

Ingredients	Quantity in 40ml
1X TAE	40ml
Agarose	0.6g

0.5 molar EDTA

Ingredients	Concentration
EDTA	186.18g
dH_2O	1000ml

CTAB buffer

Ingredients	Concentration
CTAB	1ml
Marceptoethanol	2 µl

Phenol-chloroform and isoamyl alcohol

Ingredients	Concentration
Phenol	25ml
Choloroform	24ml
Isoamyl alcohol	1ml

thesis

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