Microbial Diversity in Complicated Urinary Tract Infection among Urolithiatic Patients

By

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Department of Microbiology

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By

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DEDICATED

TO LOVING MEMORIES OF MY FATHER
TO MY MOTHER FOR PROVIDING ME WILL
TO ASPIRE
TO THE BEST HUSBAND FOR GIVING
ME COURAGE TO FIGHT AGAINST ODDS
AND
TO THE WORLD' S CUTEST DAUGHTER
HOORAIN FATIMA

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List of Abbreviations

Abbreviations	Description
A	Alpha
ATCC	American Type Culture Collection
API	Analytical profile index
B	Beta
BC	Before Christ
BMI	Body Mass Index
BC	Bray-Curtis
BHI	Brain heart infusion
BMI	Body Mass Index
BP	Blood pressure
BSA	Bovine serum albumin
^{o}C	Degree Celsius
cKP	Classical K. pneumonia
CLSI	Clinical and Laboratory Standards Institute
CRA	Congo red assay
CTX-M	Cefotaximase
cUTI	Complicated urinary tract infection
CV	Crystal violet
fepA	fepA receptor precursor
ESBL	Extended spectrum beta lactamases
entB	Enterobactin biosynthesis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMB	Eosin methylene blue
EPS	Extracellular polymeric matrix/substance
GMM	Glucose Methylene Blue Muller Hinton (GMM)
HIV	Human immunodeficiency virus
HCl	Hydrochloric acid
hlyA	Hemolysin A
HPF	High-power field
hvKP	hypervirulent *K. pneumonia*

Abbreviations	**Description**
I	Intermediate
IL	Interleukin
ITS	Internal transcribed spacer
Kg	Kilogram
L	Liter
M	Molar
MDR	Multidrug resistance
Min	Minutes
mL	Milliliter
MHA	Muller Hinton agar
MSA	Mannitol salt agar
MTP	Microtiter plate
µg	Microgram
µL	Microliter
mM	Millimolar
Mm	Millimeter
NA	Nutrient agar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient broth
NCAC	Non Candida albicans Candida species
NGS	Next generation sequencing
NHAES	National Health and Examination Survey
NMDS	Nonmetric multidimensional
OD	Optical density
ODc	Cut off optical density
OR	Odd Ratio
OUT	Operational taxonomic unit
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCoA	Principal Coordinates analysis

Abbreviations	Description
PIMS	Pakistan Institute of Medical Sciences
PL	Phospholipases
PMS	Phenazine methosulfate
%	Percentage
Psi	Pounds per Square Inch
QAU	Quaid-i-Azam University
QAU-ERC	Quaid-i-Azam University Ethical Review Committee
R	Resistant
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
rUTI	Recurrent Urinary Tract Infection
S	Sensitive
SD	Standard Deviation
SDB	Sabouraud Dextrose Broth
SDS	Sodium Dodecyl sulphate
spp.	Species
TBE	Tris Borate EDTA buffer
TE	Tris EDTA buffer
TSI	Triple sugar iron
UPEC	Uropathogenic *E. coli*
UTI	Urinary Tract Infections
USA	United States of America
UV	Ultraviolet
utA	Aerobactin
WBC	White blood cell
WHO	World Health Organization
XTT	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
w/v	Weight/Volume
ybtS	Yersiniabactin

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Sahar Zafar

Abstract

Urolithiasis is a urological disorder associated with various complications including complicated urinary tract infection which can cause renal failure. Whole microbial diversity associated with complicated UTI (cUTI) in urolithiasis has not been deciphered completely. In present study, culture dependent and independent microbial diversity was investigated in urine specimens of urolithiatic patients with cUTI and compared with the urinary microbial diversity of urolithiatic patients without cUTI and healthy individuals. Standard microbiological procedures were used for culturable microbes and unculturable microbes were identified by targeting V4 region of 16S rRNA for bacterial and *ITS* gene for fungal diversity. Mid-stream urine samples were collected from 164 urolithiatic patients and 40 healthy controls. Overall, among urolithiatic patients 55.5% of the patients were males whereas 44.5% were females with mean age of 43yrs. cUTI was prevalent (64%) condition among these urolithiatic patients. Clinically, catheterized patients having stone disease for extended period were significantly positive for cUTI ($P<0.001$).

By using culture dependent methods, different Gram positive and negative bacterial species were identified. These urolithiatic patients were predominantly (59%) colonized by bacterial species where *K. pneumoniae* (36%) was the most prevalent bacteria followed by *E. coli* (33%), *S. aureus* (9%), *S. saprophyticus* (5%) and *S. epidermidis* (2.4%). But in case of urolithiatic patients with cUTI, *E. coli* was the most prevalent. Female patients were more prone to be colonized with *E. coli* as compared to males ($P=0.02$). Upon analysis and comparison of virulence factors, 87% of *K. pneumoniae* showed strong biofilm formation ability and majority (80%) of these isolates were from urolithiatic patients with cUTI. Although *E. coli* isolates also had strong biofilm forming ability but their number was low. Furthermore, 22/69 *K. pneumoniae* isolates were hypervirulent strains. Majority of these cUTI patients' isolates displayed hemolysis, *K. pneumoniae* isolates were alpha hemolysin producer and *E. coli* isolates had varying degree of hemolysin production. Most of these Gram negative bacterial isolates were

resistant against tested antibiotics. Among *E. coli* isolates, 44 were multi drug resistant (MDR) and 71% of these were isolated from cUTI patients. Thirty seven *E. coli* isolates were phenotypically ESBL positive, and 70% of these were from cUTI cases. In case of *K. pneumoniae,* 21/23 isolates from cUTI subjects were MDR. *E. coli* isolates from urolithiatic patients had 41%, 30% and 9% CTX-M, TEM and SHV genes prevalence respectively. In case of *K. pneumoniae,* 20%, 7% and 1.5% of isolates were positive for CTX-M, TEM and SHV genes respectively. Quinolone resistance genes were less prevalent among *E. coli* and *K. pneumoniae* isolates.

Through culture dependent method, *Candida* was the only fungus which was isolated from the urine samples of urolithiatic patients as well as healthy controls. In these patients, 45% were colonized with three *Candida* species *i.e. C. albicans, C. glabrata* and *C. krusei.* The urolithiatic patients with cUTI had 1.8 fold increased risk for *Candida* colonization as compared to patients without cUTI. A higher isolation of *C. albicans* (66%) was seen than non-albicans (34%) in urolithiatic patients. Overall, in 64 *Candida* isolates, esterase production was in 44/51 *C. albicans*, 17/23 *C. glabrata* and 3/3 *C.krusei.* Of these esterase producers, 80% of *Candida* were from the cUTI patients ($P<0.001$). Phospholipase activity was seen in 38/51 *C. albicans*, 09/23 *C. glabrata* and 3/3 *C. krusei*. A significant esterase and phospholipase activity was detected in *Candida* isolates from urolithiatic patients with cUTI as compared to patients without cUTI. Among all *Candida* isolates, 43% showed strong biofilm forming activity, which was also found to be significantly high in isolates from urolithiatic patients with cUTI ($P<0.05$).

Through culture independent diversity analysis, greater diversity richness and evenness in female urolithiatic patients with cUTI as compared to male urolithiatic patients and the healthy individuals was seen. Highest Bray-Curtis dissimilarity index was seen in male urolithiatic patients with cUTI as compared to healthy females which is an indication of microbial diversity change in number and type of microbes with gender and disease status. In female urolithiatic patients with cUTI, predominant bacterial genera found were *Prevotella* followed by *Enterobacter, Achromobacter, Agrobacterium* and *Alcaligenes.* However, *Acinetobacter* was the most prevalent (84%) among total genera detected in

male urolithiatic patients with cUTI. Female urolithiatic patients with cUTI showed predominance of fungal genera *Paraconiothyrium* followed by *Necteria*, *Cladosporium* and *Cryptococcus.* While in male patients with cUTI *Ustilago* was predominant genera followed by *Aspergillus* and *Necteria.* Known prevalent uropathogens which are thought to be dominant were not predominating microbes in this condition. No *E. coli* and *K. pneumoniae* was observed in the microbiome through culture independent method.

In present work, dual species biofilm assays for different combinations of isolated uropathogens *Candida, E. coli* and *K. pneumoniae* were studied as *in vitro* biofilm development model. A reduction in the biofilm biomass in dual species biofilm of *C. albicans* and *E. coli* isolates from cUTI was observed but, interestingly metabolic activity was enhanced. A possible antagonistic behaviour of *Candida* and *K. pneumoniae* in their dual biofilm was observed as both biofilm biomass formation and metabolic activity was reduced while in dual bacterial consortium of *E. coli* and *K. pneumoniae* a lowered biofilm biomass but enhanced metabolic activity was observed.

Findings from the present work showed there was dysbiosis in urolithiasis where MDR isolates with enhanced virulence expression were present in culturable microbial diversity especially in cUTI cases. Various unculturable microbes were also detected through culture independent method. Data from the present work indicated for the first time, a snapshot of urinary microbial diversity in cUTI among urolithiatic patients. These findings suggest that there should be focus on evidence-based therapy using advanced techniques to determine the real picture of microbial diversity in cUTI patients for proper and targeted eradication of involved microbes in such infections to minimize the chances of antibiotic resistance in other commensal and disease burden.

PUBLICATIONS

- **Zafar S, Hanif S, Akhtar H, Faryal R.** 2019. Emergence of hypervirulent *K. pneumoniae* causing complicated UTI in kidney stone patients. *Microbial pathogenesis.* 103647

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CHAPTER-1 General Introduction

Urolithiasis is the third most common problem of the urinary tract after UTIs and pathologic conditions of the prostate (Moe, 2006). Mankind is being affected by urinary tract stones since centuries dating back to 4000 B.C. (Lopez and Hoppe, 2010). Recurrence of urinary stones has always been a serious issue for human health (Mikawlrawng *et al.,* 2014) and understanding of the mechanisms responsible for urolithiasis is required for the prevention of stone recurrence (Khan *et al.,* 2016). Urolithiasis has been suggested in various studies as a systemic disorder associated with metabolic syndrome. These stones can be responsible for 2 to 3% renal failures if associated with nephrocalcinosis (Courbebaisse *et al.,* 2017). Urinary tract stone symptoms are related to the location of stones whether they are in kidney, bladder or ureters (Kumar *et al.,* 2012). Stone formation at its initial stages, does not show any symptom but with the passage of time different type of symptoms including intense cramping pain, back pain, hematuria, blockage of urine flow, dilation of kidney and especially urinary tract infection are experienced by the urolithiatic patient (Teichman, 2004).

Urolithiasis affects almost 12% of the world population at some stage of their lifetime (Chauhan *et al.,* 2009). It occurs in all sexes, races and ages (Moe, 2006; Romero *et al.,* 2010), but men are more frequently affected than women within the age range of 20-49 yrs (Edvardsson *et al.,* 2013). Prevalence and recurrence rate of stone disease is increasing globally with limited options of effective drugs. Increasing prevalence of urolithiasis in different studies is reported in both developing and developed countries. According to Sofia *et al.,* (2016), this growing trend is believed to be associated with lifestyle changes *i.e.* lack of physical activity and changes in dietary habits. Global warming is also considered to be an important risk factor for increasing prevalence of urolithiasis (Romero *et al.,* 2010). Due to use of advanced technologies like ultrasonography and tomography, it can be quickly and efficiently diagnosed which accounts for higher prevalence rate of urolithiasis being reported (Liu *et al.,* 2018).

Stones are hard masses of crystals, which are formed due to a multifactorial process of supersaturation. Therefore, super-saturation removal is important in eradicating stones and modern strategies for treatment of urolithiasis, work on principle of decreasing super-saturation. There are different pathways which cause urine supersaturation. One of the most common is increased calcium oxalate concentration that causes production of low volume urine and increased calcium oxalate excretion (Singh and Rai, 2014). Therefore, abnormalities in the chemicals of urine are responsible for the composition of urinary tract stones (Chhiber *et al.,* 2014).

On the basis of their composition, stones can be classified into five types *i.e.* calcium stones, uric acid stones, struvite stones, drug induced stones and cystine stones (Alelign and Petros, 2018). Calcium stones are the most common with calcium phosphate and oxalate constituting about 81% followed by struvite 10% and uric acid 8-9% stones (Jan *et al.,* 2008). Different factors contribute to calcium oxalate type of stone formation such as hypercalciuria, hyperoxaluria, hypocitraturia, hyper cystinuria, hyperuricosuria and hypomagnesemia (Dal-Moro *et al.,* 2005). Urinary pH of 5 to 6.5 mostly promotes this type of stone formation (Kishore *et al.,* 2013), while calcium phosphate type stones are formed when pH of urine is greater than 7.5 (Kumar *et al.,* 2012). Struvite stones are also referred to as triple phosphate, which occur in patients with chronic urinary tract infections. Uric acid type of stones occurs mostly as a result of hyperuricosuria and low urinary pH due to diets rich in proteins (Dursun *et al.*, 2015). Uric acid stones are more commonly found in men as compared to women. On the basis of stone location in the urinary tract, they are of two types *i.e.* staghorn and non-staghorn. Staghorn stones obstruct the renal collection system whereas non-staghorn stones can be found at sites other than renal system such as calyx, pelvis and urinary bladder (Evan, 2010). Stones with less than 5mm in size can easily pass through the urinary tract whereas those having diameter of 5-7mm and over 7mm must be removed surgically by using treatment strategies such as ureteroscopy and shock wave lithotripsy (Krambeck and Lieske, 2011).

In adults, the risk of developing urinary stone is high in the USA followed by Europe and Asia. The worldwide prevalence of urinary tract stone disease in the last few decades has been reported to be in the range of 7%-13% in North America, 5% -9% in Europe and 1%-5% in Asia (Sorokin *et al.,* 2017). The Afro-Asian stone-forming belt includes Egypt, Sudan, the United Arab Emirates, Iran, Saudi Arabia, Pakistan, Myanmar, India, Thailand, Philippines and Indonesia (Cook *et al.,* 2016). Pakistan is geographically located in this stone-belt, as there is high incidence of stone disease in the community. The estimated prevalence in our country is 10-15% in the overall population (Iqbal *et al.,* 2017) but only 1-2% symptomatic patients come to the hospitals for treatment (Hussain *et al.,* 2009).

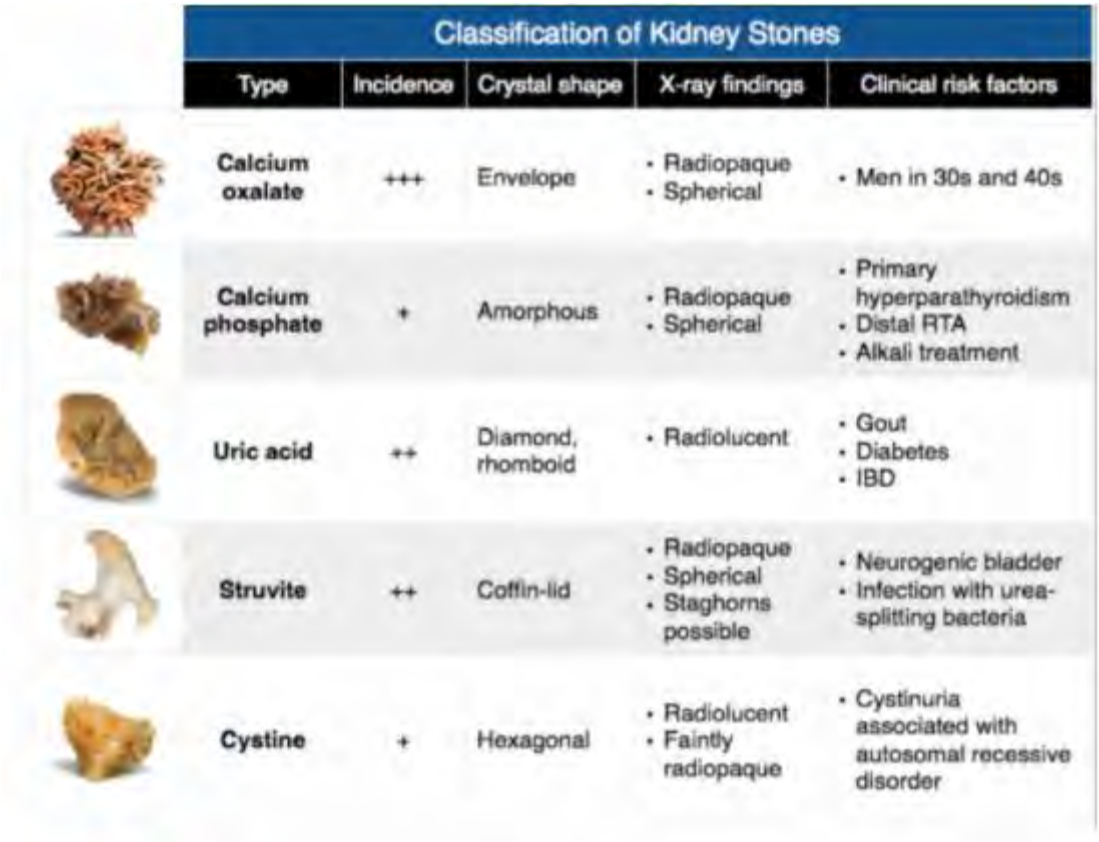
Different risk factors responsible for urolithiasis can be divided into two major categories *i.e.* extrinsic and intrinsic factors. The intrinsic factors consist of age, gender and genetic history while the extrinsic factors include dietary habits, lifestyle, occupation, education and geographical distribution (Liu *et al.,* 2018). The most important factors considered to determine the incidence, prevalence and recurrence rate along with composition of stones are lifestyle, dietary habits and climate change.

Exact data regarding prevalence of urolithiasis in Pakistan is missing due to insufficient centralized epidemiological data and repository. However, different studies reported risk factors of urolithiasis in different areas of Pakistan. Jabbar *et al.,* (2014) conducted a study in Quetta (Pakistan) to determine the urinary risk factors among urolithiatic patients and the most important risk factors associated with urolithiasis was seen to be low intake of water, obesity and family history. Another study was conducted by Ahmad *et al.,* (2016) on prevalence of kidney stone in Southern Punjab Pakistan, where the highest prevalence of stone disease was in males of 40-49 yrs and females of age group 30-39 yrs.

Overall, incidence of urolithiasis increases with age; it is more in the middle age group which decreases in later part of the life. People of the middle age group are more prone to

urinary stone disease due to more laborious and busier lifestyle (Kale *et al.,* 2014) which ultimately results in little fluid intake, hence causing dehydration. Unhealthy lifestyle, occupational stress and endocrine hormonal changes with age can be the reason for the high rate of urolithiasis (Liu *et al.,* 2018). Gender is also an important intrinsic risk factor to be responsible for urolithiasis (Pugliese and Baker, 2009; Huang *et al.,* 2013; Hussein *et al.,* 2013).

Socioeconomic status has also been seen to be a major risk factor in development of this disease. As most of the people belonging to low socioeconomic status have hard life style with lack of proper diet and ignorance of basic knowledge about risks of disease. In developing countries people fed on vegetables and cereals, which are rich in oxalate (Ganesamoni and Singh*,* 2012), being responsible for high rate of urolithiasis. Insufficient fluid consumption is also a major risk factor causing urolithiasis (Chandrajith *et al.,* 2006). Calcium oxalate stones are reported to be associated with high levels of magnesium, sodium, calcium, phosphates and fluoride in drinking water (Scales *et al.,* 2012; Knoll *et al.,* 2011). Main source of drinking water in Pakistan, Sri Lanka and India is underground water, which is undergoing mineralization and this might be responsible for high prevalence of urolithiasis in these regions (Lopez and Hoppe, 2010; Croppi *et al.,* 2012; Osther, 2012). Climate and geographical changes are also related with urolithiasis development (Edvardsson *et al.,* 2013). Education level and type of occupation also affects the incidence of disease (Chhiber *et al.,* 2014).



Classification of Kidney Stones				
Type	Incidence	Crystal shape	X-ray findings	Clinical risk factors
Calcium oxalate	+++	Envelope	• Radiopaque • Spherical	• Men in 30s and 40s
Calcium phosphate	+	Amorphous	• Radiopaque • Spherical	• Primary hyperparathyroidism • Distal RTA • Alkali treatment
Uric acid	++	Diamond, rhomboid	• Radiolucent	• Gout • Diabetes • IBD
Struvite	++	Coffin-lid	• Radiopaque • Spherical • Staghorns possible	• Neurogenic bladder • Infection with urea-splitting bacteria
Cystine	+	Hexagonal	• Radiolucent • Faintly radiopaque	• Cystinuria associated with autosomal recessive disorder

Figure 1.1: Classification of kidney stones on the bases of their composition, crystal shape and clinical risk factors (Tauber D., 2019).

Apart from these factors, some genetic factors are also involved in urolithiasis. Urolithiatic patients commonly suffer from some metabolic diseases such as hypertension and diabetes, which may be another factor for urolithiasis (Khan *et al.,* 2016). Another important risk factor of urolithiasis is urinary tract infection and these types of stones are termed as infection stones. UTI can lead to urolithiasis as causative microbes enhance the crystal aggregation through urease and citrate lyase production. Most common urease producing microbes are *Proteus mirabilis* followed by *K. pneumoniae, P. aeruginosa* and *Enterobacter* (Schwaderer and Wolfe, 2017) while citrate lyase producing microbes are *E. coli, Klebsiella aerogenes* and *Enterococcus faecalis.*

Untreated stones for prolonged periods lead to urinary obstruction, damage to urinary tract epithelium and ultimately complicated UTI. Classification system for UTIs by European Association of Urology categorized UTI as a consequence of urolithiasis as a complicated UTI (Grabe *et al.,* 2013). cUTI is associated with factors responsible for making the urinary tract and host defence system compromised. These factors include immunosuppression, urinary retention, urinary obstruction, renal transplant, urinary catheters, stones and renal failure (Lichtenberger and Hooton, 2008; Levison and Kaye, 2013). In Pakistan many urolithiatic patients suffer from complications of stone disease *i.e.,* pyonephrosis, pyelonephritis and perinephric abscess related to complicated UTI, which can lead to life threatening situations and even death (Hussain *et al.,* 2009).

Different Gram positive and negative bacteria along with fungi are responsible for cUTI. The most prevalent agent for cUTI is uropathogenic *E. coli* (UPEC) (Taylor *et al.,* 2005; Moe, 2006; Chauhan *et al.,* 2008; Romero *et al.,* 2010). Colonization to the host tissues after cell adhesion is required for establishment of infection. Intracellular multiplication, invasion and dissemination to other tissues are also involved in this mechanism of infection (Bien *et al.,* 2012). UPEC encodes virulence factors that help them in colonization, persistence and pathogenesis. Some important virulence factors include biofilm formation, adhesin or fimbriae and toxin such as hemolysin (Dhakal *et al.,* 2008). Similarly to *E. coli*, in *K. pneumoniae* type 1 pili is involved in its colonization and biofilm formation. Pathogenicity of *K. pneumoniae* varies due to a large number of virulence factors that enables it to evade the host immune system and to maintain infection. The most important virulence factors are adhesins, hypermucoviscosity, lipopolysaccharides, iron acquisition system, biofilm formation and serum resistance. Biofilm is also considered to be an important virulence factor in causing UTI among urinary stone patients (El Fertas-Aissani *et al.,* 2013).

Another issue in complication of UTI is emergence of MDR bacterial uropathogens, such as the behaviour of important uropathogenic *K. pneumoniae* towards the antimicrobial

agents, when exposed for a prolonged period. The increased resistance against ciprofloxacin and amikacin was noticed in case of biofilms than planktonic cells (Vuotto *et al.,* 2014). The presence or acquisition of drug resistance plasmids and mutations on chromosomes pose serious threat in treatment of *K. pneumoniae* associated infections (Patel and Bonomo, 2011). Beside bacteria, *Candida* is one of the predominant microbes involved in cUTI (Flores-Mireles *et al.,* 2015). *Candida albicans* and other *Candida* species colonize and propagate a variety of tissues by using their virulence factors (Fisher *et al*., 2011). The expression of these virulence factors occurs at different stages of infection. The pathogenesis of *Candida* species begins when it initially colonized uroepithelium (Hoyer *et al*., 2008). Pathogenesis of *Candida* species includes, morphological shift between hyphal and yeast forms, biofilms formation, release of hydrolytic enzymes and appearance of adhesins and invasions on the cell surface (Mayer *et al*., 2013).

Complicated UTIs can be responsible for prolong treatment and higher probability of treatment failure. cUTI requires intensive and longer antibiotic treatment as compared to the uncomplicated UTIs, which can be treated through brief antibiotic treatment (Li *et al.,* 2017). Initially Khan *et al.,* (1981) reported the frequency of UTIs in urolithiatic patients along with microbial flora associated with UTI. The work was on isolation and antimicrobial resistance, where *E. coli, K. pneumoniae, Pseudomonas, Proteus mirabilis* and *Staphylococci* were not only the major causative agents of UTI in urolithiatic patient but also antimicrobial resistant. Another study conducted in Nawab shah (Pakistan) to analyze the clinical patterns of urolithiatic disease. That study reported complications associated with urolithiasis including pain (67.3%), haematuria (26.7%) and UTI in 15% of patients (Memon *et al.,* 2010). Iqbal *et al.,* (2017) studied the gender differences in microbiology of UTI in urolithiatic patients in Pakistan and *E. coli* was more common in UTI of urolithiatic females as compared to males. *Proteus, Klebsiella, Pseudomonas* and mixed bacterial growth were more common in males than females. High resistance was observed against commonly prescribed antibiotics for UTIs in that study.

Initially, there were limited techniques available for culturing the microorganisms and those traditional methods available favoured only fast-growing aerobic bacteria such as *E. coli.* But the microbes that require special media or anaerobic conditions were usually missed by these methods. The advancements of molecular techniques make it possible for comprehensive microbial assessment in urine (Whiteside *et al.,* 2015; Thomas *et al.,* 2016).

To understand cUTI in urolithiasis, there is a need to identify culturable as well as unculturable microbial diversity by using advanced techniques. Up to now, published studies available are on the microbiome of either uncomplicated or catheter associated UTIs. Still, no work has been carried to determine the complete microbial diversity in urolithiatic patients with cUTI. Previously most of the studies on urolithiasis carried out in Pakistan, only focused on determining the limited demographic risk factors association and selected bacterial species in UTI, rather than deciphering different possible bacterial and fungal species involved in pathogenesis of cUTI. To understand the microbial agents in urolithiasis, there is need to identify the whole microbial diversity in local urolithiatic patients to elucidate main causative agents and their interaction with each other as disease complications are now considered to be due to polymicrobial in nature. The number and type of local circulating strains, their virulence and drug resistance profiles involved in cUTI in renal stone patients is also missing. Such data is needed and will be instrumental for development of guidelines for proper cUTI treatment against uropathogens rather than use of conventional approach of empirical treatment strategies. Therefore, the present study was aimed to identify microbial diversity in cUTI among urolithiatic patients by using culturable and unculturable techniques.

Hypothesis

Urolithiasis is a condition with many complications and one of its consequences is urinary obstruction which can lead to change in the microbial diversity. Such dysbiosis may have role in development of cUTI in urolithiatic patients.

Aim and Objectives

The work was planned to identify and characterize culturable as well unculturable microbial diversity in patients diagnosed with urinary tract stones for their role in complicated urinary tract infection.

Main objectives of this study are:

1) To assess sociodemographic and clinical risk factors of urolithiatic patients for their association with the development of cUTI
2) Identification of culture dependent and independent bacterial diversity from urolithiatic patients with cUTI
3) Identification of culture dependent and independent fungal diversity from urolithiatic patients with cUTI
4) *In vitro* model development of dual microbial species to understand the behaviour of microbial consortia in biofilms.

CHAPTER 2: Literature Review

Urolithiasis is a condition in which there is formation of crystal concretion in the urinary tract. It is a prevalent urological disorder, which has been estimated to affect around 12% of world population and increase risk of renal failure (Alelign and Petros, 2018). Prevalence of urolithiasis is equally increasing in both developed and developing countries (Scales, 2012). This rise in its prevalence is associated with different risk factors which are; changes in BMI and obesity, poor diet, diabetes, work hazards, genetic makeup and climate change (Chen *et al.,* 2019).

Urinary tract stone are composed of salt, minerals and many other constituents present in urine. Urinary stones formation is a slow process, it can take days to months and these stones formed are of different sizes ranging from small sand grain size to size of a golf ball. These stones can be smooth, asymmetric or spiky which are commonly found in three colours yellow, brown and red. During passage from the urinary tract, large sized stones cause discomfort, severe pain or sometimes damage to the urinary tract epithelium due to its sharp and rough edges. In some cases of urolithiasis, stone cannot pass the urinary tract which needs medical intervention. In such conditions, severe complications and substantial damage to renal system if neglected can arise (Moe, 2006).

Prevalence of urolithiasis

Urolithiasis is a problem of all geographical regions around the world with annual prevalence of approximately 3-5% and life time prevalence of 15-25% (Moe, 2006). The recurrence of urinary stones in majority of urolithiatic patients is also an issue. Generally, recurrence rate varies with time with increasing trend, it is considered to be 14% at 1st year of occurrence, 35% in fifth year and 52% in tenth year (Sohgaura and Bigoniya, 2017).

Incidence rate of urolithiasis changes with geographical parameters and conditions of each country. Urinary stone prevalence range is 7% to 13% in North America, 5%-9% in Europe and 1%-5% in Asia (Sorokin *et al.,* 2017). Both incidence and prevalence rate in most of Asian countries is increasing over the last few decades due to genetic factors, dietary habits and climate changes (Ganesamoni *et al.,* 2012; Novikov *et al.,* 2012; Huang *et al.,* 2013; Tae *et al.,* 2017). Prevalence of urolithiasis in China has increased from 4% in 2008 to

6.4% in 2014 (Zeng and He, 2013; Zeng *et al.,* 2017). Prevalence of urolithiasis according to geographical distribution is higher in Southern China as compared to Northern China. As Southern China have warmer climate which was considered to be main factor for higher prevalence of urolithiasis (Chen *et al.,* 2017). In Japanese population, during time period of 1965-2005, urolithiasis prevalence increased from 4.3% to 9% (Yoshida *et al*., 1999; Yasui *et al.,* 2008). A survey conducted in Japan in 2015 reported even more increase in incidence of urinary tract stone compared to previous studies (Sakamoto *et al.,* 2019). Another Asian country South Korea, also have reported an increase in its prevalence from 3.5% to 11.5% in the duration of 1998 to 2013 (Kim *et al.,* 2002; Tae *et al.,* 2018). In Russian population, sharp increase in the incidence of urolithiasis was reported from 53/100,000 in 1980 to 609/100,000 in 2008 with prevalence range of 2-3% (Novikov *et al.,* 2012).

Urology and Andrology Centre of India (2016) reported that almost 2 million people are affected by urolithiasis each year in India. Even from West Asian countries, highest prevalence of urolithiasis is reported, from Saudi Arabia where range is 6.8% to 19.1% in its different areas (Abdel-Halim *et al.,* 1989; Freeg *et al.,* 2012; Ahmad *et al.,* 2015). Prevalence of 5.7% to 8.1% is reported from different areas of Iran (Safarinejad, 2007; Ketabchi *et al.,* 2008; Basiri *et al.,* 2010). High temperature and excessive exposure to sunshine are considered to be the main cause of urolithiasis in South Asian and Southeast Asian countries like Pakistan, Thailand, India and Malaysia (Liu *et al.,* 2018).

There is no central registry in Pakistan, up to now most of the data generated is from different studies conducted in various tertiary care hospitals, hence epidemiological assessment is quite tough in Pakistan (Jan *et al.,* 2008). Only few studies are carried out on Pakistani population, in one such study incident rate in Southern Sindh's population was found to be high compared to other parts of country (Ahmad *et al.,* 1992). Although comprehensive data regarding stone disease in Pakistan is missing but still it roughly estimated to be 40-50% of renal workload in majority of hospitals (Hussain *et al.,* 2009).

Majority of work conducted is on prevalence and association of different risk factors with urolithiasis in particular areas of Pakistan. A silent kidney stones prevalence of 3% was recorded in Radiology Department of Agha Khan University Hospital (Karachi) and interestingly all subjects were males (Buchholz *et al.,* 2003). Jabbar *et al.,* (2015) carried

out a study in Quetta to determine both its prevalence and associated causative risk factors for urinary tract stones in which majority of the patients were of 15-25 yrs age group living in urban areas. Ahmad *et al.,* (2016) conducted a study in Southern Punjab's population where highest prevalence of calcium oxalate stones in the age group of 40-49 yrs of males and 30-39 yrs of females was present.

2.1- Types of urinary tract stones

There are different types of stones found in urolithiasis based on their composition *i.e.,* calcium stones either calcium oxalate or calcium phosphate, uric acid stones, cystine stones, struvite stones and drug induced stones (Alelign and Petros, 2018). Composition of stones is depended upon the mechanisms and etiologic factors responsible for its formation *i.e.,* hyperoxaluria, hyperuricosuria, hypercalciuria and hypomagnesuria *etc* (Dal-Moro *et al.,* 2005).

Calcium stones are considered to be the predominant urinary stones, which accounts for 80% of all urinary tract stones (Coe *et al.,* 2005). Calcium stones are comprised of 50% calcium oxalate, 5% calcium phosphate and 45% mixture of both (Chaudhary *et al.,* 2010). While, main component of this type of stone is always calcium hydrogen phosphate (Skolarikos *et al.,* 2015). Calcium oxalate can be found in the form of calcium oxalate monohydrate, calcium oxalate dihydrate or combination of both (Bensatal and Ouahrani, 2008). There are many factors which contribute in calcium oxalate formation, like, urine pH of 5.0 to 6.5 which usually promotes calcium oxalate stones formation (Kishore *et al.,* 2013), while calcium phosphate stones occur in urinary pH of greater than 7.5 (Kumar *et al.,* 2012). The recurrence rate of calcium stone is overall greater in comparison to other types of urinary stones.

Struvite stones which are also termed as infection stones, most commonly occur in patients with prior UTIs. The most common microbes involved in such type of stones are *Proteus mirabilis* and less common are *K. pneumoniae, Enterobacter* and *P. aeruginosa.* All these microbes have urease, an enzyme which splits urea to ammonia and CO_2 which increases pH of urine (Barbasa *et al.,* 2002; Coe *et al.,* 2005; Giannossi and Summa, 2012). This pH rise is favourable for phosphate precipitation.

Uric acid stones are 3-10% of all stone types (Giannossi and Summa, 2012). Usually, this type of stone is formed as a result of low urine volume and low urinary pH which is caused

by utilizing diet high in animal proteins (Kumar *et al.,* 2012). These stones are more prevalent in males as compared to females. Cystine stones are found in less than 2% of population suffering from urolithiasis.

Drug induced stones account for almost 1% of all stone types formed (Giannossi and Summa, 2012). Different type of drugs such as triamterene, guaifenesin and sulfa drugs induce this type of stones. People taking drugs to treat Human Immunodeficiency Virus are also at high risk of developing kidney stones (Barbasa *et al.,* 2012). These lithogenic drugs can induce the stone formation through its metabolic action (Dursun *et al.,* 2015).

Xu *et al.,* (2017) collected data on type of stones and found calcium stones were more common than other types and also there was an increase in prevalence of uric acid stones from 7% to 14% in urolithiatic patients visiting kidney stone clinic in Texas (USA) from 1980-2015. In another study, calcium stones either oxalate or phosphate were detected in 79% of urolithiatic patients followed by uric acid stones (16.5%) in Argentina (Spivacow *et al.,* 2016). Amir *et al.,* (2018) analyzed the urinary tract stone composition collected from urolithiatic patients visiting Johns Hopkins Aramco Healthcare Saudi Arabia from 1999 to 2013 and reported calcium oxalate as the most common type of stone in both men and women. A recent study on Norwegian population also found calcium oxalate in the 71% of the patients followed by uric acid, struvite and cystine stones (Kravdal *et al.,* 2019).

Stone types and their occurrence in Pakistani patients in different studies showed that calcium oxalate stones are the most prevalent type in urolithiatic patients. From Khyber Pakhtunkhwa (Pakistan), Khan *et al.,* (2013) also found calcium oxalate a predominate type of stone in pure and mixed form followed by uric acid and cystine stones in urolithiatic patients. In a work on patients from Southern Punjab (Pakistan) same trend was seen (Ahmad *et al.,* 2016). Also, another study from Multan (Pakistan) reported different type of stone composition, however again the most prevalent type of stone was calcium oxalate followed by uric acid and mixed stones among urolithiatic patients (Samad *et al.,* 2017).

2.2- cUTI as consequence of urinary tract stones

The association between urolithiasis and UTI is two ways; it can be either UTI leading to kidney stone formation or urolithiasis with subsequent UTIs as its complications (Miano *et al.,* 2007). Whether stones are induced by infections or responsible for subsequent infection, these stones are majorly responsible for secondary infection (Zanetti *et al.,*

2008). The eradication of UTIs associated with urolithiasis can only be possible when stones are removed completely (Dogan *et al.,* 2007; Miano *et al.,* 2007). Lack of proper and timely treatments of urinary tract stones and its subsequent UTI can lead to dreadful complications. There are very few studies published on urinary tract stone as risk factor for infection in patients with urolithiasis.

Urinary tract infections can be categorized as uncomplicated and complicated infections. Healthy individuals with no urinary tract abnormalities are affected by uncomplicated UTIs (Nielubowicz and Mobley 2010; Hooton, 2012). Complicated UTIs are urinary tract infections which are associated with factors including weak host defence system, urinary obstruction, renal transplantation, pregnancy and catheterization (Lichtenberger and Hooton, 2008; Levison and Kaye, 2013). According to the European Association of Urology classification system of UTI, UTIs as a result of urolithiasis fall in the category of complicated UTI (Grabe *et al.,* 2013).

Urolithiasis is a major cause of cUTI and different studies indicated the role of urinary tract stones in development of UTIs in such patients. In Pakistan, 15% of urolithiatic patients admitted in surgical wards of Nawabshah Medical Hospital during 2003 to 2007 were found to be positive for UTI (Memon *et al.,* 2010). Al-Jebouri and Atalah (2012) observed 42% of urolithiatic patients in Iraqi population suffering from UTI and main causative agents found were members of *Enterobacteriaceae*. In China, cUTI was 22% in urolithiatic patients. In this Chinese study, it was observed that age, gender, stone shape, obstruction and multiple sites of stones as potential risk factors for developing cUTI in patients with urolithiasis (Yongzhi *et al*., 2018).

2.3- Risk factors for development of urolithiasis

Urolithiasis is caused by various extrinsic and intrinsic risk factors. Extrinsic factors include dietary habits, lifestyle and climate change while age, gender and genetic variability are intrinsic factors involved in urolithiasis.

2.3.1-Age and gender

The process of urinary tract stone formation and chemical composition is affected by age and gender of the individual (Strope *et al.,* 2010). The risky age for the development of stone disease is from third to fifth decade of life and during the same time period chances of recurrence are also high (Panicker *et al.,* 2010). Urinary tract stones are more prevalent

among males but the distribution of gender varies with age. As different sex hormones play a major role in development of urolithiasis. Oxalate excretion is increased by androgens which subsequently cause calcium oxalate crystals deposition in the kidneys. On contrary, urinary oxalate excretion is reduced by estrogen. Therefore, there are more chances of stone disease in men as compared to women (Fan *et al.,* 1999).

A cross sectional study was carried out in Northern China during the year 2008 showed that the overall incidence rate was 3% in females and 4% in males. Males with urolithiasis were prevalent in all age groups as compared to females. In males, stone incidence increased with aging as prevalence was 2.3% among age group of 20-29 yrs and it increased up to 8% in age group of >70yrs. While females of age group 50-59 yrs were mostly affected compared with younger and older age group females (Zeng and He, 2013).

The gender of patients was assessed as risk factor in multicentre study from Germany where 22 centres during period of 1993-2006 were evaluated. It was reported that calcium stones were more common in both genders. Uric acid stone was present in 11% males and 7% females. Infectious and cystine stones were less common in both gender (Knoll *et al.,* 2011). A cross-sectional analysis carried out in USA during 2007-2010 found rate of stone formation to be higher among men than women (Scales *et al.,* 2012). The ratio of kidney stone formation was 3:1 among men and women in Pakistan in a cross-sectional study of Dera Ismail Khan, where patients' mean age was determined to be 33 yrs (Khan *et al.,* 2013). Iqbal *et al.,* (2017) also reported that more males had urolithiatic disorder as compared to females in their study carried out in Sindh Institute of Urology and Transplantation (Pakistan).

2.3.2- Obesity

The obesity has been declared another important risk factor for development of urolithiasis (Kovesdy *et al.,* 2017). Obesity is defined mostly in terms of BMI. According to World Health Organization, BMI range from 18.5-24.9 kg/m^2 is considered as a normal weight, 25-29.9 kg/m^2 as overweight and >30 kg/m^2 as obesity. Over the past few decades, increase in prevalence obesity has been observed worldwide (Roberto *et al.,* 2015). In USA, the prevalence of obesity in adults was recorded as 40% and 35% among women and men respectively during 2013-2014 (Flegal *et al.,* 2016). The 17% of children in USA have

obesity where highest prevalence was observed among children under the age of 19 years (Olaya *et al.,* 2015).

One of the associated conditions in obesity is hyperinsulinemia, which is known risk for calcium stone formation. Insulin resistance in body enhances the chances of urinary stone development through high calcium excretion, which enhance the probability of calcium deposits in kidney. Urinary pH and body mass are inversely related as with increase in body mass, pH of urine declines due to increase production of uric acid. Men are known to excrete 37% more uric acid if their weight is in range of 100-120 kg or more (Hatiboglu *et al.,* 2011). According to a report by National Health and Examination Survey (NHAES) from USA (2007-2010), urinary tract stones are common in obese (11%) individuals than normal weight (6%) (Scales *et al.,* 2013). An Indian study reported high incidence of uric acid stones in obese urolithiatic patients (Najeeb *et al.,* 2013). The effect of increased BMI on stone formation was greater in women as compared to men, as women have body fat higher than man of same BMI (Nussberger *et al.,* 2017).

2.3.3- Hypertension

Hypertension is also an important risk factor for urolithiasis, which is documented in various studies on hypertensive patients from various countries. Prevalence of urolithiasis in hypertensive patients from Italy showed double chances of having stones (Roudakova and Monga, 2014). In another study on Turkish hypertensive patients, one third such patients (32.8%) were urolithiatic (Polat *et al.,* 2015). In a follow-up study from USA, among symptomatic urinary tract stone patients after a period of 5 years, hypertension was common condition independent of type, severity and treatment of urolithiasis indicating that it is not only a risk factor but is also a consequence of stone formation (Kittanamongkolchai *et al.,* 2017).

2.3.4- Diabetes

Diabetes type 2 mellitus is characterized by resistance to insulin; a metabolic syndrome increases the chances of uric acid type of stone formation (Chu *et al.,* 2017). This is due to increased production of fatty acids, which can interfere with glutamine utilization and production of ammonium. Stone formers diabetic patients form more acidic urine than non-stone formers; therefore, uric acid stones are more prevalent among diabetic patients. Daudon *et al.,* (2006) recorded more uric acid stone (36%) in diabetic patients compared

to non-diabetic patients (11%) in France. A study was carried out in India during period of 2011-2013 to determine the association of diabetes with uric acid stone formation, which was found to be strong. Calcium stones were prevalent among non-diabetic stone patients but the uric acid stones were significantly higher among diabetic patients (Nerli *et al.,* 2015).

2.3.5- Diet

It has been seen that intake of potassium and calcium in diet is linked with reduced risk of urolithiasis whereas vitamins supplements intake was associated with stone formation (Curhan *et al.,* 2004). Effect of different diets on stone formation has been studied in a survey conducted in USA. It was observed that vitamin C supplements enhance the chances of stone formation but intake of magnesium reduced the risk. Animal protein intake increases the risk of urolithiasis through inhibition of citrate excretion (an inhibitor of stone formation) and dietary potassium reduce the chances of having urolithiasis (Taylor *et al.,* 2004). Diet rich in protein decrease the urine pH, which leads to uric acid stone formation. Similarly, effect of metabolic syndrome and diabetes on stone formation are also linked with diet containing carbohydrates and fatty food (Trinchieri and Montanari, 2017). A prospective cohort study was conducted in Italy, which highlighted dietary factors such as calcium, low fat food, vegetables and fruits reduce the risk of urolithiasis. There was also 56% reduction in stone formation by higher intake of fluids. Ferraro *et al.,* (2017) reported up to 45% reduction in risk of urolithiasis associated with consumption of food containing vegetables, fruits and dairy products with low fat.

2.3.6- Fluid intake

Low intake of fluids has been seen to be significantly associated with stone formation. Different studies have shown that high intake of water minimize the chances of urolithiasis. To achieve this, individual's fluid intake recommended by American Urological Association guidelines is 2-2.5 litre per day (Johri *et al.,* 2010; Sayer and Thomas, 2010). This quantity of fluid intake ensures that urine flow is also more than 2.5 litres per days (Pearle *et al.,* 2014). Also, in stone patients, high water intake can result in significant reduction of recurrence chances of urolithiasis (Lotan *et al*., 2013). Therefore, proper hydration should be maintained as it is a simple treatment of urolithiatic patients. The quality of water also affects incidence rate of urolithiasis. Underground water is main

source of drinking water in developing countries and high mineralization of this water can be a major reason for high prevalence of urolithiasis in these countries (Lopez and Hoppe, 2010; Croppi *et al.,* 2012; Osther, 2012).

2.4- Microbial diversity in urine

A variety of microorganisms are hosted by human body even in its healthy state. Approximately, there are ten times more microbial cells present on human body than human cells (Cho and Blaser, 2012). Urine does not allow survival of majority of microorganisms due to its hypertonic nature, low pH, high concentration of urea and some other substances with antimicrobial properties (Ipe *et al.*, 2016). Those microbes that can survive in urine and cause infection are generally termed as uropathogens. These uropathogens possess different virulence factors, which help them to survive and cause infections in urinary tract (Cadieux *et al.,* 2009). Some mechanisms for survival of such organisms in this environment include biofilm forming ability, which enablesnthem to adapt in high urea concentration, low pH and hypertonic environment (Ipe *et al.*, 2016). Initially, there were limited techniques available for culturing the microorganisms and those traditional methods available favoured only fast-growing aerobic bacteria such as *E. coli.* But the microbes that require special media or anaerobic conditions were usually missed by these methods. The advancements of molecular techniques based on 16S ribosomal RNA gene sequencing make it possible for comprehensive microbial assessment in urine (Whiteside *et al.,* 2015; Thomas-White *et al.,* 2016).

Urinary microbiome is distinctive based on different factors such as gender, age, genetics and different diseased conditions. Generally, females possess more heterogeneous microbial population than males (Lewis *et al.,* 2013). In an Australian study, higher number of *E. coli* was cultured from healthy females than males (Ipe *et al.,* 2016). But in females of child bearing age, lactobacilli were found to be the predominant species in their urine (Kok *et al.,* 2016). However, both male and female are known to possess three predominant genera *i.e. Corynebacterium, Lactobacillus and Streptococcus* as their core microbiome throughout their life (Nelson *et al.,* 2012; Fouts *et al.,* 2012).

2.5- Microbes associated with urolithiasis

Even in urolithiasis, there are distinctive microorganisms which exist in association with each other. Increased crystal aggregation has been observed in the presence of

microorganisms, due to reduction in urine citrate level and deposition of calcium oxalate crystals. In case of struvite type stone formation , urease producing bacteria are majorly present (Schwaderer and Wolfe, 2017). Different bacterial species like *E. coli* and *K. pneumoniae* have been identified to adhere to crystals especially crystals of calcium oxalate monohydrate as compared to calcium oxalate dihydrate and silicon dioxide crystals (Chutipongtanate *et al.,* 2013; Barr-Beare *et al.,* 2015).

Microorganisms are also known to induce stone formation through citrate lyase production, which can lead to crystal formation by super saturation of urine through decreased citrate level in urine. Citrate level in urine is lowered in patients with positive bacterial culture as compared to culture negative patients (Wang *et al.,* 2014). Study from Thailand reported that almost all type of stones formation is associated with microorganisms somehow. The most common isolated bacteria in such cases are *Pseudomonas* species and *E. coli* (Tavichakorntrakool *et al.,* 2012). A survey conducted in Russia on microbial diversity in patients suffering from urolithiasis documented that various species of microorganisms were present on stones. The most frequently isolated species from such stones were *E. faecalis*, *E. coli, S. haemolyticus*, *P. aeruginosa*, *K. pneumoniae* and *P. mirabilis* (Romanova *et al.,* 2015).

The most important genera exhibiting urease activity and also involved in urinary tract stone development are *Staphylococcus*, *Proteus*, *Pseudomonas* and *Klebsiella*. Infectious stones are characterized to have a polymicrobial community, which includes urease negative as well as urease positive species. *Proteus mirabilis* is declared as model organism for urease positive pathogens, which is found in stones and also is biofilm developer on catheter which can lead to more infectious stones formation in the bladder (Norsworthy and Pearson, 2017). *Oxalobacter formigenes* is recognized as oxalate degrading microbe, which leaves behind little amount of oxalate for absorption and hence it is involved in reduction of recurrent stone formation (Knight and Holmes, 2015).

The microbiome of urinary stones was analyzed by Dornbier *et al.,* (2019) and different genera including *Staphylococcus, Escherichia, Enterobacter, Corynebacterium*, and *Lactobacillus* in urolithiatic patients using enhanced urine culture and 16S rRNA gene sequencing were reported. Another study conducted in USA analyzed microbiome from the gastrointestinal and urinary tracts along with a metabolomic analysis of the urinary

metabolome from urolithiatic patients. The study inferred that higher rates of antibiotic use among urolithiatic patients is associated with an antibiotic driven shift in the microbiome from the one that protects against urolithiasis to one that promotes the disease (Zampini *et al.,* 2019).

2.6- Microbial diversity in complicated UTI

The spectrum of microbial colonization in urinary tract is much broader than considered previously. UTI is commonly associated with *E. coli* in more than 70% of uncomplicated and 25-50% of complicated cases (Cadieux *et al.,* 2009). Although *E. coli* is considered to be the main uropathogens involved in cUTI, a large microbial diversity is present in such patients. The agents involved in cUTI is uropathogenic *E. coli* as the most common followed by *Enterococcus* spp, *K. pneumoniae*, *Candida* spp., *S. aureus, P. aeruginosa* and *P. mirabilis* (Flores-Mireles *et al.,* 2015). Pathogens may remain in urinary system due to frequent failure of antibiotic treatment; such uropathogens later cause the recurrent urinary tract infections. These antibiotics also disrupt the microbial balance residing in urogenital system by decreasing the *Lactobacillus* and increasing *E. coli* which leads to reinfections (Bao *et al.,* 2014).

In an Indian study, *E. coli* (33.81%) was found to be the most common etiological agent for cUTI patients followed by budding yeast (19.78%), *P. aeruginosa* (7.91%) and *S. aureus* (5.93%) (Mohan *et al.,* 2016). Patients with cUTI in China were found to have predominant Gram negative bacteria. These Gram negative bacteria included *E. coli* (48.2%), *K. pneumoniae* (9.5%), *P. aeruginosa* (4.9%), *P. mirabilis* (4.6%) and *Enterococcus* species (Li *et al.,* 2017). In Pakistan, gender based variation in culturable uropathogens were reported by Iqbal *et al.,* (2017). The study reported *E. coli* as the predominant uropathogen in female urolithiatic patients with UTI, whereas large number of *Pseudomonas, Proteus* and *Klebsiella* were found in male patients. Limited data is available for culturable microbial diversity in cUTI of urolithiatic patients while in case of unculturable microbial diversity, no data is available for urolithiatic patients with cUTI. Unculturable microbial diversity in uncomplicated UTI was reported by Moustafa *et al.,* (2018) through metagenome sequencing in USA. The study analyzed urine samples in which a large number of bacterial genera (median 41 genera/sample) and viral sequences (median 3 viruses/sample) were detected.

2.6.1- Urinary tract infection and UPEC

Uropathogenic *E. coli* is the primary cause of UTIs. UPEC is involved in causing the 50% of nosocomial and 70-95% of community acquired infections. Therefore, it is responsible for high morbidity and mortality (Foxman, 2010). *E. coli* was reported to be majorly isolated through culturable method from urine samples (53%) of urolithiatic patients with UTI in China (Yongzhi *et al.,* 2018).

2.6.1.2- Virulence factors of UPEC

UPEC exhibit various virulence mechanisms that add to their pathogenicity. *E. coli* can divert from their commensal attribute, to take on a more pathogenic nature by developing the ability to cause diseases in the host (Yan and Polk, 2004). Different virulence factors of UPEC have combined effect on its pathogenic nature. Some of the common virulence factors include serum resistance, hydrophobicity, hemolysin production, haemagglutination and siderphores (Eto *et al.*, 2007; Hegde *et al.,* 2008; Baby *et al.,* 2016). A prospective study conducted on uropathogenic *E. coli* in India reported that in different pathologies, there are different frequencies of UPEC virulence markers from asymptomatic to acute pyelonephritis (Mittal *et al.,* 2014). The virulence genes in UPEC are present in particular segments of genome with different GC content than other parts and are referred as pathogenicity islands. Phylogenetic studies have revealed that these bacteria fall in four main categories which are A, B1, B2 and D. A group of scientists from Faisalabad (Pakistan) worked on the phylogenetic groups of UPEC isolated from community acquired infections. In that study, it was concluded that, phylogenetic group B2 was more prevalent whereas isolates from group D were more versatile with multiple virulence factors and high level of toxicity (Bashir *et al.,* 2012).

First step in colonization process is the attachment of UPEC to uroepithelial cells. Expression of different fimbrial adhesions decides the ability of UPEC colonization. There are number of adherence factors which are expressed by UPEC and these factors are organized in pili or fimbriae. These fimbriae are oligomeric pilin protein which recognize specific targets of host cells (Volkan *et al.,* 2014). There are different type of fimbriae including type P, type 1 and thin aggregative fimbriae. There are various functions of fimbriae other than adhesion to help microbes for their survival and evasion to the host

immune system (Vigil *et al.,* 2011). Various proteins and toxic substances secreted by UPEC play a major role in progression of infections. Such toxins can alter host cell signalling cascade and inflammatory responses. These toxins cause the cell death and release the nutrients which are required by pathogens to access deeper tissues of urinary tract (Agarwal *et al.,* 2012). Cyclomodulin toxin was first reported in UPEC in 1987 and later on various toxins were reported including cytotoxic necrotizing factor, arginine succinyl transferase, α hemolysin, shigella enterotoxin, plasmid-encoded toxin, secreted auto transporter toxin and cytolysin A (Soltani *et al*., 2018).

α hemolysin (HlyA) is an important toxin among all uropathogenic *E. coli*, which is a lipoprotein and belongs to RTX (repeat in toxin) family. HlyA is a pore forming toxin which causes cell membrane injury and apoptosis (Laura *et al.,* 2012). At high concentrations, it can lyse nucleated host cells and erythrocytes. This enables the UPEC to gain enhanced access to host iron stores and other nutrients by damaging the host immune cells. While in low concentrations, induction of apoptosis of host cells occurs (Russo *et al*., 2005). Alpha hemolysin can also contribute to nephron-pathogenicity and regulates dephosphorylation of Akt, which is multifunctional signaling regulator responsible to control host inflammatory responses. Moreover, it has also role for increased interleukin production by promoting Ca^{2+} oscillations in epithelial cells of kidney (Ristow and Welch, 2016).

Biofilm which is a contributing factor in pathogenicity of UPEC is composed of protein, carbohydrates and glycoprotein. It has been investigated that about 61% of bacterial and 80% of acute infections are due to biofilm development by causative pathogens. In UPEC, biofilm provides an environment that promotes the growth and persistence at the site of infection by protecting the bacteria from hydrodynamic forces and killing activity of host-defence mechanism as well as from antimicrobial substances (Hancock *et al.,* 2010). Maheswari and colleagues while working on virulence markers of UPEC on UTI observed that the matrix of the biofilm contributed to resistance development in pathogenic *E. coli* biofilms and persistent infections (Maheswari *et al.,* 2013). Polymicrobial biofilms have strong ability to withstand harsh environmental conditions encountered, thus is a challenge for clinicians. Type I fimbriae play a major role in development of biofilm in UPEC isolated from kidney stone patients (Shojaeian *et al.,* 2016). Intra and inter species

signalling is also a contributing factor for biofilm development in UTIs (Eberly *et al*., 2017).

Haemagglutination is another virulence factor of UPEC (Mittal *et al.,* 2014). Haemagglutination is caused by the capacity of UPEC adhere to the urinary epithelium and this adhesion is often mediated by fimbriae. The most common fimbriae reported in a study conducted on host pathogen interaction in UTI patients of USA are type1 and P fimbriae. These fimbriae played a major role in colonization and invasion to kidney (pyelonephritis), epithelial cells and bladder (Nielubowicz and Mobley, 2010). Type 1 fimbriae is considered to be a versatile virulence marker of UPEC which help bacteria to stabilize their attachment to different cells in the urinary tract (Vizcarra *et al*., 2016). Type 1 fimbriae are encoded by FimH gene and is important in bladder colonization, its expression is detected by mannose sensitive haemagglutination. Type P fimbriae are polynephritogenic, which attach to carbohydrate structure D-Galp-(1-4)-beta-D-Galp (Hunstad, 2010). Phenotypic expression of P fimbriae in uropathogens isolated from UTI patients can be identified by haemagglutination of human red blood cells (Nachammai *et al.,* 2016).

Serum resistance is a virulence factor as the pathogen gains the ability to resist bactericidal activity of serum due to activation of alternate complement pathway. This characteristic enable pathogenic bacteria to persist in body fluids and internal organs (Baby *et al.,* 2016). Resistance to bactericidal effect of serum is a result of individual or combined effect of capsular polysaccharides, O antigen and surface proteins (Annapurna *et al*., 2014).

Possession of these virulence factors and mechanisms make UPEC more persistence and increases their pathogenicity. Ability of UPEC to colonize and disseminate into the host environment is due to its virulence factors. In addition to these virulence factors, ability of UPEC for utilization of nutritionally rich environment such as blood system, bladder, urine and kidney plays a major role in pathogenesis (Flores-Mireles *et al.,* 2015). Different studies reported *E. coli* colonization in the urinary tract leading towards the bladder to cause infection. In case of untreated infections for prolong period, UPEC can ascend to the kidney and cause secondary infection (Parvez and Rahman, 2018).

2.6.1.3- Antibiotic Resistance in UPEC

The frequency and spectrum of antibiotic resistance in UTI patients has risen, it varies with time and geographical location. UPEC is a major cause of serious bacterial infections such as UTIs and kidney stones. Fluoroquinolones, aminoglycosides, trimethoprim sulfamethoxazole and cephalosporins are commonly used for treatment of infections by *E. coli.* Resistance present in *E. coli* against these antibiotics is responsible for failure of therapies and consequently high morbidity and mortality rate (Foxman, 2010). MDR is alarmingly increasing in *E. coli.* The possible reasons of this antimicrobial resistance include antibiotics sold over the counter, excessive use of antibiotics and non-metabolized antibiotics release along with their residues in the environment through feces (Davies and Davies, 2010).

There are various intrinsic and extrinsic mechanisms adopted by bacteria to develop multidrug resistance including reduced antibiotic permeability by increased efflux pump activity, changes in the antibiotic targets by mutation or target modification, and inactivation of hydrolytic enzymes (Blair *et al.,* 2015). These mechanisms may be located on the bacterial chromosome or transferred horizontally inter or intra species via mobile genetic elements *i.e.* plasmid or transposons (Mehrad *et al.,* 2015). *E. coli* potentially uses all of these mechanisms of drug resistance and are capable of horizontal transfer of resistance elements especially extended spectrum beta lactamase genes (Reygaert, 2016).

2.6.2- Urinary tract infection and *K. pneumoniae*

K. pneumonia is the most common nosocomial pathogen and 3rd most commonly found in cUTI. It has various virulence factors to become a successful pathogen such as capsule, fimbriae, siderophores for iron acquisition, toxins and hypermucoviscosity. *K. pneumonia* associated infections are initiated by attachment of bacteria to host surface through adhesins followed by subsequent colonization by biofilm formation. Bacteria can attach different surfaces either biotic or abiotic such as catheter and medical surfaces to develop

complex biofilm structures. Most commonly encountered adhesins are pilli and fimbrae such as FimH1 (Davies and Davies *et al.,* 2016). Adhesive type3 pilli are also most commonly detected in *K. pneumonia* isolated from UTI patients. Virulent strain of *Klebsiella pneumoniae* use type3 pilli as adhesive factor and mediated attachment of bacteria to host. These pilli were plasmid encoded and can be transferred through the process of conjugation (Sonbol *et al.,* 2013).

Fimbriae are characterized as adhesins and are found in various members of *Enterobacteriaceae*. These are encoded by cluster of genes for complete structure assembly *(Murphy et al., 2013).* Type 1 and type 3 fimbriae has been found in many Gram negative bacteria but most common in *K. pneumoniae*, *Enterococcus* and *Proteus*. Type 3 fimbriae can functionally be characterized by its ability of binding with human erythrocytes and cause haemagglutination. Haemagglutination can occur both in presence and absence of mannose. Therefore, fimbrial adhesin in *K. pneumoniae* strains are referred as mannose-resistant. In addition to binding to erythrocytes, type 3 fimbriae can also bind to epithelial surfaces. The gene regulating the expression of fimbriae is mrkE located upstream of mrkA and have been found on both chromosome and plasmid. It can be transferred to other species through lateral gene transfer (Sonbol *et al.,* 2013). Type 1 fimbriae are another recognized adhesin which play role in biofilm formation by *K. pneumoniae* when developing urinary tract infections. Type3 fimbriae promote biofilm formation by *K. pneumoniae* and are found to be involved in catheter associated infections also (Schroll *et al.,* 2010).

Exotoxins are also produced by *K. pneumoniae* which act as virulence factors. The extracellular toxic complex (ETC) formed by *K. pneumoniae* consists of 30% lipopolysacharides. Although, ETC is produced independently but in absence of capsular polysaccharides it cannot sustain the phagocytic activity of host tissues and cannot release the toxic product (Al-jumaily *et al.,* 2012). Enterotoxins are most commonly secreted by *K. pneumonia* causing diarrhea (Al-salihi *et al.,* 2016). Siderophores are characterized as iron chelators, which are produced and secreted by microorganisms in order to obtain iron from host tissues. *K. pneumoniae* being extracellular pathogen within host body secrete siderophores acquire iron. Most commonly secreted siderophores are enterobactin (entB), aerobactin (utA) and yersiniabactin (ybtS). The presence of entB in all clinical isolates is

an indication that all *K. pneumonia* isolates are producers of siderophores (Fung *et al.,*2012). It has been found that entB is involved in maturation and development of biofilm during infection and fepA an outer membrane receptor of enterobactins is activated during infection. Aerobactin is another iron acquisition protein that was reported in 3-6% clinical isolates of *K. pneumoniae* but it is rarely found in isolates from nosocomial infections. Yersiniabactin is most commonly produced by isolates from blood than from urine cultures. Besides having role in iron acquisition, it can evade innate immune system by blocking the reactive oxygen species of immune system (Lawlor *et al.,* 2007).

Hydrolytic enzymes are produced by many bacteria and are considered to be involved in pathogenicity of bacteria. A study carried out to identify the extracellular enzymes produced by *K. pneumoniae* and it was observed that *K. pneumoniae* produced proteases, urease, lipases, gelatinases and DNases. Among these enzymes most important is the urease which have role in urinary tract stone formation and infection by splitting urea (Egbe and Enabulele, 2014). Urease is an important virulence factor of microorganisms for causing urinary tract infection. It is involved in urinary tract colonization and at the same time it can cause infectious stone formation by breakdown of urea and elevating the urine pH. It was found that most prevailing microorganisms producing urease enzyme are *K. pneumoniae*, *P. mirabilis* and *S. saprophyticus*. Alkaline conditions can lead to infection of urinary epithelium. Highly active urease causes rapid stone formation and can act as site for biofilm development by other microorganisms (Rosen *et al.,* 2008).

K. pneumoniae producing hypermucoviscous phenotype was found in Taiwan in early1990s. Such strains of *K. pneumoniae* were phenotypically hyper-mucoid when grown on selective media MacConkey agar. The genes contributing in hypervirulent *K. pneumoniae* were found to be *rmpA* and *magA*. It has been reported that *rmpA* gene is also associated with serum resistance and hence acts as an important virulence factor of *K. pneumoniae* (Struve *et al.,* 2015). Biofilm formation is a contributing factor in pathogenicity of *K. pneumoniae,* such biofilms are composed of proteins, polysaccharides and glycopeptides. Percival and his colleagues also documented that biofilm formation contribute in development of antimicrobial resistance more prominently than in planktonic cells form (Percival *et al.,* 2015).

The behaviour of *K. pneumoniae* biofilms towards antimicrobial agents is also interesting as biofilms resist killing caused by antimicrobial agents, when exposed for prolong period. The increased resistance against ciprofloxacin and amikacin was noticed in case of biofilms than planktonic cells (Vuotto *et al.,* 2014). Although only moderate amounts of antimicrobial resistance genes are encoded on chromosomes of *K. pneumoniae* but it is now well characterized that multidrug resistance is also encoded on extra chromosomal plasmids that confer resistance to aminoglycosides, encoding Sulfhydryl variables SHVs, Temoniera (TEMs) and extended-spectrum β-lactamases (ESBLs). The presence or acquisition of drug resistance plasmids and mutations on chromosomes pose serious threat in treatment of *K. pneumoniae* associated nosocomial infections (Patel and Bonomo, 2011). UTI due to MDR *K. pneumoniae* is a serious problem in health care facilities due to inappropriate antibiotics usage for treating UTI. The MDR pathogens among UTI patients increase the mortality and morbidity as well as affects economic cost of a country. The high rate of resistance is observed in *K. pneumoniae* isolated from UTIs against ciprofloxacin, gentamycin and trimethoprin-sulfamethoxazole, which leaves only carbapenem as last line of drug of choice. In order to use appropriate antibiotic therapy, there is need to understand the behaviour of pathogens towards antimicrobial agents (El Bouamri *et al.,* 2015).

2.6.3- Urinary tract infections and *Candida*

Candida is the most common fungi isolated from the patients with both complicated and uncomplicated UTIs. However, it is more common in cUTI after *E. coli, Enterococcus* and *K. pneumoniae* (Flores-Mireles *et al.,* 2015). *C. albicans* can be responsible for number of infections which can be from mild superficial to systemic. Different factors are found to be contributing in its pathogenesis (Mayer *et al* 2013). *Candida* species are found to invade urinary tract either via bloodstream, urethra and bladder. Virulence factors of *Candida* are reported to be responsible for invasion and colonization included dimorphism, chemotropism and hydrolytic enzymes.

An Indian study reported the prevalence of *Candida* in UTI patients as 3.5%. *C. albicans* was the most prominent species followed by *Candida tropicalis*, *Candida krusei*, *Candida*

parapsilopsis and *Candida dublinensis* (Agrawal *et al.,* 2017). A retrospective study was conducted to compare the prevalence of UTIs caused by *Candida* spp. among indoor and outdoor patients at a clinical centre in Hungary. The study reported that *Candida* was more prevalent among indoor patients of UTI as compared to outdoor (Gajdacs *et al.,* 2019).

Candida is a dimorphic fungus living in the gastrointestinal and genitourinary tract. Out of all the *Candida* species, *C.albicans* are mostly found in both healthy and diseased conditions (Calderone and Fonzi, 2001). It exists in two forms due to its dimorphism ability. The normal yeast form is non-pathogenic but filamentous hyphal form is invasive, which facilitates *Candida* to invade host tissues. Another significant characteristic of *C. albicans* is ability to form germ tube which differentiates it from other non-albicans species. Germ tube is a long tubular structure protruding from yeast cells which develops at initial stages of morphological transition (Sutton *et al.,* 1998). Emergence of *C. glabrata* as a major opportunistic pathogen is due to common use of immunosuppressive therapies, broad spectrum antibiotics and indwelling medical devices. Ability of *C. glabrata* to form biofilms and its high intrinsic resistance against azoles make infections caused by *C. glabrata* difficult to treat (Rodrigues *et al.,* 2014).

Virulence factors of *Candida*

There are various factors, which help *Candia* spp. in adherence and invasion in to host cells including secretion of hydrolytic enzymes, contact sensing, chemotropism, biofilm formation and phenotypic switching (Mayer *et al.,* 2013). Colonization of *Candida* species to host surfaces is the first step to establish infection of *Candida* which is followed by biofilm development. *Candida* species can adhere to both biotic and abiotic surfaces such as catheters and medical devices to form complex biofilms. This can also lead to resistance towards antifungal drugs (Pfaller and Diekema, 2010). Initially, electrostatic forces and cell surface hydrophobicity helps *Candida* to adhere to host cells then *Candida* adhesion proteins bind to ligands present on the surface of host cell (Aslanimehr *et al.,* 2017). Biofilm formation has been identified to contribute in the pathogenesis of *Candida* infection being responsible for most of the *Candida* associated infections in humans. Biofilms help in the dissemination of infection by conferring resistance to antifungal medications and evasion of host immune responses (Sherry *et al*., 2017).

Silva et al., (2009) studied the biofilm forming ability of non- albicans Candida (NCAC) species in their study. Isolates of *C. parapsilosis, C. tropicalis* and *C. glabrata were* evaluated by using crystal violet staining after being isolated from different sources. Scanning electron microscopy was used to assess morphological characteristics of the biofilms. Protein and carbohydrate contents of biofilm were also analyzed. All NCAC species were capable of forming biofilms although this biofilm forming ability was weak in *C. glabrata* in comparison with *C.parapsilosis* and *C.tropicalis*. Another study conducted by Dabiri *et al.,* (2018) on clinical samples of *Candida* reported that the highest biofilm forming ability in *C. albicans* followed by *C. krusei, C. parapsilosis, C. tropicalis, C. glabrata* and *C. guilliermondii.*

Phenotypic switching is the ability of *Candida* species to switch from yeast to other filamentous forms including germ tube, pseudo-hyphae and hyphae. Numerous environmental and host factors facilitate this switching. After adhesion and hyphal formation, *Candida* species secrete hydrolytic enzymes to disseminate infection. These enzymes include proteinases, phospholipases, hemolysins and esterases (Pandya *et al.,* 2018).

Esterase belongs to lipolytic class of enzyme that hydrolyzes ester bonds of lipid components of host's cell such as monoacylglycerols, di-and tri acylglycerols and phospholipids. This enzyme together with other hydrolytic enzymes destroys different components of membrane of host cell that facilitate pathogens to adhere, penetrate and invade host cell (Lahkar *et al.,* 2017). Phospholipases (PL) are also categorized as extracellular lipolytic enzymes with hydrolytic effect on lipid constituents present in the host cell membrane (Pandya *et al.,* 2018). Phospholipases are secreted by various species of *Candida* which hydrolyze phospholipids of membrane. The four types of phospholipases present in *C.albicans* include PLA, PLB, PLC, and PLD, which impart high virulence to these species. *Candida* species exhibit 90% of phospholipase activity due to PLB. Egg yolk medium is used for examining the activity of phospholipases, since it contains enzyme substrates which are phosphotidylcholine and ethanolamine (Udayalaxmi *et al.*, 2014). Most of the *Candida* spp. produces aspartyle proteinases with other extracellular secreted

hydrolytic enzymes. These enzymes are reported to cleave host proteins and are able to cause damage to host immune cells (Singh *et al.,* 2019).

2.7- Polymicrobial biofilms formation in development of cUTI

Microbes establish the surface associated communities termed as biofilms and these biofilms express high resistance to various antimicrobial agents (Costerton *et al*., 1999). These are involved almost in 65% of infections which can lead to prolong diseased conditions and high morbidity rate (Fuente *et al*., 2014). Predominant form of microbes are polymicrobial biofilms (Harriott and Noverr, 2011; DeLeon *et al*., 2014). These microbial species in biofilms obtain various advantages such as passive resistance, quorum sensing systems, metabolic cooperation and DNA sharing (Wolcott *et al*., 2013).

Complicated urinary tract infections are related to multiple microbial species and their interactions. Polymicrobial biofilms are the major cause of developing complications in urinary tract infection either related to stones, catheters or other urinary obstructions. Polymicrobial biofilms can develop on medical devices such as catheters and up to 86% of catheter associated urinary infections are due to polymicrobial communities. These polymicrobial biofilms include uropathogenic *E. coli, K. pneumoniae, Enterococcus* spp. *P. aeruginosa, P. mirabilis* and *C. albicans* (Hola *et al.,* 2010).

Antimicrobial resistance is one of the most important advantages of polymicrobial biofilms. There are different mechanisms which impart thousand-fold more resistance against antibiotics to these biofilms as compared to their planktonic cells. Matrix of biofilm does not allow some of the antimicrobial agents to diffuse into the biofilm and sometime penetration time required by antibiotic into biofilm is longer than the antibiotic life time. For instance, aminoglycosides penetrate slower than beta lactams through biofilms. Another mechanism to impart resistance is transmission of resistance genes within the biofilms. pH inside the biofilms can also inactivate the diffusion of antibiotic hence antagonize the antibiotic activity (Mack *et al.,* 2004; Lewis, 2005; Costerton *et al.,* 2007; Lewis, 2008; Lewis, 2010)

Different studies observed the interrelationship of different microbes for their biofilm formation to cause infections. Bandara *et al.,* (2009) studied the interaction of different *Candia* species with *E. coli* in their combined *in vitro* biofilms and reported that there are

variations in behaviour of different *Candida* species when co cultured with *E. coli.* Biofilm biomass of *C. albicans and C. glabrata* when co-cultured with *E. coli* was not affected while other species of *Candida* including *C. tropicalis, C. parapsilosis, C. krusei* and *C. dubliniensis* showed antagonistic behaviour for the production of biofilm biomass in the presence of *E. coli.*

In another study, mono and dual species biofilms developed by *S. aureus* and other pathogens *i.e. Salmonella enteric*, *Enteritidi*s, *Raoultella planticola* and non-pathogenic *E. coli* in the time period of 24, 48 and 72 h were analyzed. *E. coli* and *S. aureus* was found to be competitive. The *E. coli* grew better in dual-species biofilms as compared to their mono-species biofilms but reduction in growth of *S. aureus* biofilms was observed in dual-species biofilms (Makovcova *et al.,* 2017). Piva *et al.,* (2011) developed an *in vitro* model of dual species biofilms to assess the interactions of ATCC strains of *C. albicans* and *E. coli.* Biofilm formation of *C. albicans* was not affected by *E. coli.*

Antimicrobial resistance of microbes present in these polymicrobial biofilms is a major health concern (Salisbury *et al.,* 2018). A study was carried out to evaluate the role of biofilms with different antibiotics in Japanese cUTI patients by Tsukamoto *et al.,* (1999). Clarithromycin had an inhibitory effect on biofilm formation in cUTI; hence combination of clarithromycin with some other appropriate antibiotic was suggested for treatment of cUTI. In a recent study, effectiveness of antimicrobial blue light (405nm) was investigated against polymicrobial biofilms of *P. aeruginosa*, *C. albicans* and *S. aureus*. The study reported this antimicrobial blue light was found to be an efficient method to eradicate the infections related to polymicrobial biofilms (Espada *et al.,* 2019).

CHAPTER 3

Sociodemographic and clinical risk factors of urolithiatic patients for their association with the development of cUTI.

3.1- Introduction

Urinary tract stone disease has gained more significance due to its increasing incidence and prevalence throughout the world (Romero *et al.,* 2010; Scales, 2012). These stones can cause recurrent infections, which complicate further. This disease is a multifactorial, which occurs as a result of the combined influence of epidemiological, metabolic, biochemical and genetic factors (Devuyst and Pirson*,* 2007). Epidemiological studies have highlighted that factors like sex, age, socioeconomic status, occupation, water intake, geographical conditions and dietary habits of an individual affect urinary tract stone formation and recurrence (Mitra *et al.,* 2018).

The risk of upper urinary tract stone is constantly increasing in Pakistan and other developing countries. Therefore, Pakistan is included among the countries where the prevalence of this disease is quite high (Iqbal *et al,* 2017). In Pakistan, data regarding incidence of urinary stones and the risk factors causing stone diseases is missing (Ahmad *et al.,* 2016). According to a survey, estimated prevalence of urolithiasis in the country is 10-15% but only 1-2% patients come to the hospitals for treatment. Patients with different complications of stone disease in Pakistan are facing life threatening situations. These complications can be pyonephrosis, pyelonephritis, unilateral nonfunctioning kidneys and cUTI (Hussain *et al.,* 2009).

Urinary tract stones are hard mass of crystal, which are formed in a multifactorial process involving mainly super-saturation of urine as a key step in stone formation. Modern strategies for treating urolithiasis work on principal of decreasing super-saturation. Therefore, pathophysiology of super-saturation is important in eradicating stones. There are different pathways that can cause urine super-saturation. One of the most common is increased calcium oxalate concentration (hyperoxaluria and hypercalciuria) that causes production of low volume urine and increased calcium oxalate excretion (Singh and Rai, 2014). As pathological alteration in metabolism is involved in stone development, diet acts as primary determinant for urolithiasis (Salman *et al.,* 2017).

The mortality rate in stone patients with treatments is 28% without surgery and 5-7% in patients who have surgical removal of stones. Even after removal, remains of stones can obstruct the kidney ducts and ureters in majority of cases resulting in high risk of about 40-85% for stone reoccurrence (Norsworthy and Pearson, 2017). Stones and infection are strongly associated in two ways. Firstly, kidney stone disease can be caused by urea splitting enzyme urease produced by organisms. In second case urolithiasis can be a cause of urinary tract infection commonly known as cUTI (Mendes *et al.,* 2018).

Urolithiasis occurs more commonly in men than in women (Strope *et al.,* 2010). Studies conducted in different areas of Pakistan also reported the male dominance in urolithiatic disease (Rasool *et al.,* 2000; Khan *et al.,* 2013; Ahmed *et al.,* 2016). Also, different studies showed that more susceptible age for this disease is 30-50 yrs (Ahmed *et al.,* 2006; Ahmed *et al.,* 2016).

Socioeconomic status also has a great effect on the incidence of urolithiasis. The people living in poor living conditions with poor quality food and unhygienic lifestyle have more chance to develop disease. Quantity of water intake can also be responsible for stone formation as less water intake can lead to the saturation of urine and ultimately to crystals formation. A study by Mitra *et al.,* (2018) reported that 53.6% of stone patients were consuming less than 3 L of water per day. In a Pakistani study, Jabbar *et al.,* (2015) analyzed different risk factors associated with development of urolithiasis and found that the stone formation was strongly associated with age, high BMI, hypertension and family history. Lack of sufficient amounts of water and consuming hard water was also reported to be the major cause of stone formation in those patients.

Most of the studies carried out previously only focused the risks for development of urolithiasis but not covered the complications occurring as a consequence of stone disease such as cUTI. Gender, age, stone shape, multiple sites of stones and prolong stone disease are also possible risk factors for development of cUTI in patients with urolithiasis. In Pakistan, there is lack of data regarding urolithiasis, its risk factors and consequent complications of this disease. Therefore, the aim of this retrospective study was to analyze the major risk factors involved in development of urolithiasis in Pakistan and its association with complications. This will help in better disease management and infection control.

3.2- Material and Method

3.2.1- Study design

It was a hospital based retrospective study, which was conducted in Department of Microbiology (Quaid-I-Azam University, Islamabad) after approval from ethical review board of Quaid-I-Azam University, Islamabad (QAU-BRC). Mid-stream clean catch urine samples of urinary tract stone patients were collected from Pakistan Institute of Medical Sciences (PIMS) hospital and Services hospital (Islamabad). Data was collected through designed questionnaire and in person interview related to demographic parameters *i.e.,* name, age, sex, area, BMI, source plus quantity of water intake and socioeconomic status of the patients (Appendix AI). Samples were collected after taking consent from patients (Appendix AII). Urolithiasis was confirmed through KUB ultrasound (Cannon Aplio 500) and cUTI was detected through urine analysis report, medical history of patients and culturing.

3.2.2- Study population

Study population included patients with urinary tract stone with and without cUTI and control group comprising of individuals with good health.

Inclusion criteria

In this study, patients having urinary tract stone were included and healthy individuals were considered as controls.

Exclusion criteria

Patients of urinary tract stones with some systemic illness such as hepatitis and allergy were excluded from the study. Patients with some urological disorder other than stone and cUTI were also excluded from the study.

3.2.3- Sample size

Sample size of the study was calculated by using formula previously described by Pourhoseingholi *et al*., (2013) with 95% (1.96) confidence level (Z) and 7% (0.07) effect size (d). The prevalence of disease was 12%.

$$\text{Sample size} = \frac{Z^2(P)(1-P)}{d^2}$$

$$= \frac{(1.96)^2\,(0.12)\,(1-0.12)}{(0.07)^2} = 162$$

On the basis of calculation, urine samples along with their demographic and clinical data from 175 urolithiatic patients were collected. Mid stream urine samples were also taken from 40 healthy individuals.

3.2.4- Data variables of questionnaire

The questionnaire comprised of 2 sections (1) Demographic data of urinary stone patients; including age of the patients suffering from disease, gender, BMI, living environment and area (hilly or plain areas), socio-economic status, source, quality and amount of water intake per day. (2) Clinical data included history of associated diseases like diabetes, hypertension or blood pressure, medication taken, incidence of urinary tract infection, catheterization in case of severe renal infection cases and duration of the stone. Other clinical parameters such as urine pH and white blood cell (WBC) counts in urine were taken from the urine analysis reports. Size and number of stones were confirmed from ultrasound reports. Ultrasound of patients was done by qualified radiologist.

3.2.5- Collection and analysis of water samples

Water used for drinking purpose by patients was collected in 500 mL sterile and clean polyethylene bottles from twelve different locations from where >10 patients were studied. These samples were properly labelled and stored at -4 °C. Five samples of water from each place were collected to get mean values of respective minerals concentrations.

Water becomes hard due to be in contact with soluble, divalent, metallic cations. The most common minerals dissolved in water making water hard are calcium (Ca^{2+}) and magnesium (Mg^{2+}). Each of the samples was analyzed for its Ca^{2+} and Mg^{2+} concentration by atomic absorption spectrometer (VARIAN, England) by using protocol by Alexander (2008).

Hardness is commonly expressed in milligrams of calcium carbonate per liter. Water having concentration of calcium carbonate less than 60mg/L is considered as soft; 60 to120 mg/L as moderately hard, 120 to 160 mg/L as hard and more than 180 mg/L as very hard.

Water hardness was calculated with the formula:

Total hardness = Magnesium hardness + Calcium hardness

Concentration of magnesium and calcium ions referred to be equivalent of calcium carbonate. The molar masses of Mg^{2+}, Ca^{2+} and $CaCO_3$ are 24.3 g/mol, 40.1 g/mol and 100.1 g/mol respectively. Molar masses ratio for Ca^{2+} and Mg^{2+} is 2.5 and 4.1 respectively. Water hardness was calculated by using formula:

$$CaCO_3 = 2.5(Ca^{2+}) + 4.1(Mg^{2+})$$

3.2.6- Statistical Data Analysis

For statistical analysis IBM SPSS (version 21) was used. Mean and standard deviation (SD) were measured for descriptive data, frequencies, and percentages were calculated for categorical data. For risk measurement, odd ratios (OR) were calculated. Chi- square and Fisher's exact test were used to determine significant associations between different variables where $p<0.05$ was considered as level of significance.

3.3- Results

3.3.1- Sampling

A total of 175 mid-stream urine samples of urinary tract stone patients were collected. Eleven samples were excluded from the study on the basis of exclusion criteria and remaining 164 samples were further processed.

3.3.2- Analysis of water

Concentration of calcium and magnesium was determined for all water samples collected from twelve different locations and their hardness was calculated (Table 3.1). Ground water sample taken from the Rawalpindi was found to be very hard. Total seven out of twelve water samples were analyzed as very hard to moderately hard water. Quality of the water taken from the springs of Muzaffarabad, Murree and Gilgit was fine that was found to be the soft water. Five water samples were analyzed as soft water.

3.3.3- Demographic data of urolithiatic patients and healthy controls

According to data collected, 91(55.5%) of the patients were males whereas 73(44.5%) were females with mean age of 43 yrs. It was observed that majority of patients belonged to age group 21-50 yrs. Overall 24% of patients were from age group 41-50 yrs followed by 22% of 21-30 yrs and 20% of 31-40 yrs age group. Almost 34% patients were from age group of more than 50 yrs and less than 20 yrs. Majority of the patients in age group of >50yrs were male. Around 32% of female patients were from 41-50 yrs age group. BMI of the patients was also calculated and 101(62%) patients were healthy with average range BMI 23.4kg, however, 58(35%) patients were overweight. Only 5(3%) of the patients were underweight. In female patients, 28(38%) were overweight while 44(60%) were normal. In case of males, 30(33%) were overweight and 57(63%) were normal.

Among all, 115(70%) of patients were from hilly areas while only 49(30%) were from plain areas. Socioeconomic status was stratified according to monthly income of the patient. Highest percentage of patients were from low socioeconomic status 89(54%) followed by middle class (43%) patients. Most of the patients were drinking underground water 93(56.7%), however, filtered water was used by 42 patients. Remaining patients were utilizing the water from government supply and springs. By analyzing the water hardness, it was observed that more than 70% of patients were utilizing the moderately hard to very hard water. Only 29% of patients were consuming soft water. Daily intake of water varied from less than one to more than three liters a day. Highest number of patients 67(41%) were consuming 1.5-2 liter a day. Patients with low water intake were 48(29%) and with more than 3 liters were 49(30%) (Table 3.2).

Urine samples collected from healthy individuals comprising of 21 males and 19 females. 57.5% of these individuals belonged to low socioeconomic status. Majority of them were of normal BMI and utilizing water from government supply. Almost 58% of healthy controls were living in hilly areas (Table 3.3).

3.3.4- Clinical data of urolithiatic patients

Different clinical attribute of urolithiatic patients were analyzed in this study. Patients having diabetes were 17(10%) from which 08 patients were also suffering from hypertension along with diabetes. Urolithiatic patients with hypertension were 39(24%). Most of the patients had stone duration ranging from 1-3 yrs (42.1%). There were patients 32(19.5%) who were suffering from urolithiatic disease for more than three years. There was significantly high percentage of patients who were taking antibiotics (62%) as medication without any proper diagnosis and treatment. Some patients had herbal and homeopathic treatment before their admission to hospital. Out of all, 105 (64%) were diagnosed with cUTIs. Among these patients 42(40%) were catheterized due to severe renal damage. WBCs in urine were analyzed and it was observed that majority of patients had high level of WBCs. Almost 65% of patients had single stone in their urinary tract while others had two or multiple stones. Most of the stones size ranges from 4-10 mm and 25% of patients had stone of more than 10 mm of size (Table 3.4).

3.3.5- Association of urolithiasis with demographic and clinical attributes as risk factor for cUTI

Different demographic features were analyzed to check their association with cUTI in patients having urinary tract stones. It was observed that socioeconomic status was associated significantly with cUTI cases having urinary stone disease (P=0.03). Almost 72% of patients of age more than 50 yrs were having cUTI. No significant association was observed for source and quantity of water intake with the development of cUTI among urolithiatic patients (Table 3.5).

Contribution of clinical features as risk factors in cUTI among stone patients was also determined. According to data analysis, it was observed that long duration of stone disease was significantly associated with development of cUTI. Majority (86%) of patients suffering from cUTI had stone disease for more than one year (P<0.001). Similarly, significant effect of antibiotic intake was observed in cUTI cases which was in 69.5% of cUTI patients who were taking antibiotics prior to proper treatment (P = 0.02). Catheterization was also found to be significantly associated with cUTI as 40% cUTI patients were catheterized patients (P<0.001). White blood cells level was also found to be associated with cUTI and it was observed that

higher the white blood cell counts in urine more significantly was associated with the development of cUTI in these patients. Majority of patients with urinary pH of more than 6.5 were also suffering from cUTI (Table 3.6).

Table 3.1- Concentration of Ca^{2+}, Mg^{2+} and $CaCO_3$ in various water samples from resident areas of patients to assess role of water hardness in urolithiasis.

Sample no.	Locations	Mean Ca^{2+} Conc. mg/L	Mean Mg^{2+} Conc. mg/L	Mean $CaCO_3$ Conc. mg/L	Water quality
1	Rawalpindi (Supply water)	34.9	19.3	167	Hard
2	Rawalpindi (Underground water)	37.8	27.9	210	V. Hard
3	Islamabad (Supply water)	8.4	16.3	87.8	Moderately Hard
4	Islamabad (Underground water)	05	20.7	97	Moderately Hard
5	Gilgit (Spring)	2.1	0.49	7.3	Soft
6	Gojar khan (Underground water)	28.9	4.3	89.9	Moderately Hard
7	Abotabad (Supply water)	5.6	1.7	21	Soft
8	Murree (Spring)	13.2	2.9	50	Soft
9	Wah (Supply water)	6.8	1.04	21.3	Soft
10	Muzaffarabad (Spring)	8.2	4.1	37	Soft
11	Gujrat (Underground water)	33.7	17.5	156	Hard
12	Sargodha (Underground water)	20.2	6.7	90.5	Moderately hard

Table3.2: Distribution of urolithiatic patients on the basis of demographic data (n = 164).

Category	Variables	Frequency n	Percentage %
Gender	Male	91	55
	Female	73	45
Socioeconomic status	Higher class	05	03
	Middle class	65	40
	Lower class	94	57
Age range(yrs)	11-20	08	05
	21-30	37	22
	31-40	33	20
	41-50	39	24
	51-60	26	16
	61-70	21	13
BMI	Underweight	05	03
	Normal	101	61.6
	Overweight	58	35.4
Source of water	Boring	93	56.7
	Supply	09	5.5
	Filter	42	25.6
	Spring	20	12.2
Quality of water	Soft	47	29
	Moderately hard	74	45
	Hard	31	19
	v.Hard	12	07
Water intake(L)	1-1.5	49	30
	1.5-3	67	41
	>3	48	29
Living area	Hilly	115	70
	Plan	49	30
Living community	Rural	62	38
	Urban	102	62

Table 3.3: Distribution of healthy individuals on the basis of demographic data (n = 40).

Category	Variable	Frequency n	Percentage %
Gender	Male	21	52.5
	Female	19	47.5
Socioeconomic status	Higher class	02	05
	Middle class	15	37.5
	Lower class	23	57.5
Age range(yrs)	11-20	02	05
	21-30	13	32.5
	31-40	07	17.5
	41-50	06	15
	51-60	05	12.5
	61-70	07	17.5
BMI	Underweight	03	7.5
	Normal	28	70
	Overweight	09	22.5
Source of water	Boring	06	15
	Supply	25	62.5
	Filter	09	22.5
	Spring	00	00
Quality of water	Soft	09	22.5
	Moderately hard	16	40
	Hard	10	25
	v.Hard	05	12.5
Water intake(L)	1-1.5	10	25
	1.5-3	23	57.5
	>3	07	17.5
Living area	Hilly	23	57.5
	Plan	17	42.5
Living community	Rural	12	30
	Urban	28	70

Table 3.4: Distribution of urolithiatic patients on the basis of clinical data (n = 164).

Category	Variable	Frequency N	Percentage %
Duration of stone(yrs)	<1	63	38.4
	1-3	69	42.1
	> 3	32	19.5
Associated diseases	Diabetes	09	5.5
	Hypertension	39	24
	Diabetes plus hypertension	08	4.9
Prior Medication	Homeopathic	09	5.5
	Herbal	09	5.5
	Antibiotic	102	62
	Lithotripsy	03	1.8
	None	40	24.2
cUTI	Yes	105	64
	No	59	36
Catheter	Yes	47	28.7
	No	117	71.3
Urine pH	<6	21	12.8
	6-6.5	81	49.4
	>6.5	62	37.8
WBCs in urine/HPF	0-5	32	19.5
	5-100	117	71.3
	>100	15	9.2
Size of stone (mm)	<4	12	7.3
	4-10	111	67.7
	>10	41	25
Number of stones	Single	106	64.6
	Multiple	58	35.4

Table 3.5: Assessment of different sociodemographic attributes as risk factors for development of cUTI among urolithiatic patients

Category	**Variable**	**Urolithiatic patients**		***P-value***
		With cUTI	**Without cUTI**	
Gender	Male	57	34	0.402
	Female	48	25	
Age range(yrs)	11-20	07	01	0.07
	21-30	18	19	
	31-40	21	12	
	41-50	25	14	
	51-60	16	10	
	61-70	18	03	
BMI	Underweight	00	05	0.232
	Normal	64	36	
	Overweight	36	22	
Socioeconomic status	Higher class	03	02	0.03
	Middle class	35	30	
	Lower class	68	26	
Water intake a day (L)	<1.5	32	17	0.608
	1.5-3	45	22	
	>3	28	20	
Source of water	Boring	60	33	0.869
	Supply	06	03	
	Filter	25	17	
	Spring	14	06	
Quality of water	Soft	32	15	0.706
	Moderately hard	45	29	
	Hard	19	12	
	v. hard	09	03	
Living area	Hilly	73	42	0.485
	Plain	32	17	
Living community	Rural	38	24	0.343
	Urban	67	35	

Table 3.6: Assessment of different clinical attributes as risk factors for development of cUTI among urolithiatic patients

Category	**Variable**	**Urolithiatic patients**		***P-value***
		With cUTI	**Without cUTI**	
Duration of stone(yrs)	<1	15	48	<0.001
	1-3	61	08	
	> 3	29	03	
Associated diseases	Diabetes	07	02	0.768
	Hypertension	26	13	
	Diabetes + Hypertension	04	04	
	None	68	40	
Medication	Homeopathic	05	04	0.02
	Herbal	08	02	
	Antibiotics	72	30	
	Lithotripsy	03	00	
	None	17	23	
Catheter	Yes	42	05	<0.001
	No	63	54	
Urine pH	<6	07	14	<0.001
	6-6.5	44	37	
	>6.5	54	08	
WBCs in urine/HPF	0-5	01	31	<0.001
	5-100	91	26	
	>100	13	02	
Size of stone (mm)	<4	07	05	0.286
	4-10	69	42	
	>10	29	12	
Number of stones	Single	71	35	0.566
	Multiple	34	24	

3.4- Discussion

Urolithiasis is a common disease, which is basically formation of stone in ureter (ureterolithiasis), kidney (nephrolithiasis) or urinary bladder (cystolithiasis) by different physicochemical steps including super saturation, aggregation, and retention (Mitra *et al.,* 2018). This part of study was conducted to evaluate different demographic and clinical attributes of urolithiatic patients and their possible role in the development of cUTI. Stone formation is the most prevailing problem worldwide as well as in Pakistan with increasing trend of cUTI as complication among these patients (Ahmad *et al.,* 2016). In the present study, 164 mid-stream urine samples were collected from patients suffering from urolithiasis. Demographic data included age, gender, socio-economic status, quality and source of drinking water and living environment that exert strong effect on prevalence of urinary tract stones and further leading to cUTI. According to data collected, majority of urolithiatic patients were males. Predominance of male patients is in accordance with the study conducted by Zeng and his colleagues on the prevalence of stone disease in which males were more affected than female (Zeng and He, 2013). Some other studies also reported the similar male dominance of renal disease in Pakistan (Memon *et al.,* 2009; Khan *et al.,* 2013; Ahmad *et al.,* 2016).

In this study high prevalence of urolithiasis in males might be due to the effect of sex hormones, like androgen increases the oxalate excretion and calcium oxalate crystals which are deposited in the kidney. These crystals are later the main risk factors for stones formation. Urinary oxalate excretion is decreased by other sex hormone that is estrogen. Therefore, this problem is more prevalent in males as compared to females (Fan *et al.,* 1999; Nussberger *et al*., 2017). There can be some other reasons of this disease to be prevalent among males like greater muscle mass as compared to females. Eventually, due to daily breakdown of the tissue, metabolic waste is increased and hence tendency to form kidney stone (Pandeya *et al.,* 2010). Urolithiasis was detected in 73% of male patients in present study and they were from the age group of >31yrs. The increase in incidence of stones in middle age may be related to diet, work, and lifestyle changes. Age is also a major determinant for type or composition of stones formation. Calcium stones are more common in third and sixth decade of life. However, age for the development of maximum

stone disease is from third to fifth decade and during this period also the recurrence is widespread (Panicker *et al.,* 2010).

Overall urolithiasis was observed to be highest in patients of age group 41-50 yrs in current work. Majority of patients in this age group were females and females of this phase of life are commonly undergoing hormonal changes. One of the most common mechanisms is lowering of estrogen level due to menopause in this age. A study conducted in Germany reported more prevalence of stone formation among patients of lower age group of 30-39 yrs (Knoll *et al*., 2011). Some other risk factors can also be responsible for the development of stone disease in later age such as urinary pH. Increase in stone incidence can be explained on the basis of urine pH as well, which decreases with the older age and provide the opportunity for uric acid stone type to form. Aging is responsible for high incidence of uric acid stones due to low pH and also involved in reduction of phosphate stones which occurs commonly at high pH (Menezes *et al.,* 2019). Urinary pH of urolithiatic patients was also determined in present work and majority of patients had pH ranges from 6-6.5. But urinary pH was not related to stone formation, however, indicated different consequences of disease such as development of cUTI. A majority (87%) of urolithiatic patients with cUTI had urinary pH of more than 6.5.

BMI of most of the patients was normal with an average range of BMI 23.4kg in current work. This could be due to the fact that most of the patients in this study were from hilly areas. Majority of these patients were from low socioeconomic status. Lifestyle of such people from these areas is comparatively hard; therefore they do not gain weight as most of these patients were laborers. There are numerous cases reported in which urolithiasis develops despite a non-obese body type (Takeuchi and Aoyagi, 2019). In contrast to this study, in USA it was observed that BMI is associated with the onset of stone disease in males and females, and stronger association was observed in females (Taylor *et al.,* 2005). Increase in BMI decreases the urine pH, which consequently raises the chance of uric acid stone formation (Najeeb *et al*., 2013). In present work, no significant association of BMI was observed with urinary pH to play role in disease development.

Highest number of patients in present work belonged to low socioeconomic status 89(54%). The increasing trend of urinary tract stone disease among lower class population

can be due to the lack of awareness regarding risk factors as hygienic conditions, infrequent visits to health care facilities, quality and source of water. Majority of these patients were laborers and belonged to the hilly areas having tough routine and prolonged work under direct sunlight without consumption of sufficient water. Almost 73% of these patients were consuming moderately hard to very hard water. All of these factors might be responsible for high incidence of this disease in such group of people. In current study, most of the patients were having normal BMI and physically tough life style. Diabetes mellitus is a metabolic disorder which can be a risk for urinary stone formation. Diabetes was observed only in few of the urolithiatic patients as diabetes is more common in the people with less physical activity and high BMI but these patients were from active strata of society.

It was observed that 71% of urolithiatic patients were consuming hard or moderately hard water. In this group of patients, 59% belonged to low socioeconomic status. Quality of water in Pakistan is highly compromised and people of lower class cannot afford potable water, this could be the reason for more prevailing urinary tract stone and infections. Calcium oxalate stones are reported to be associated with high levels of magnesium, phosphate, calcium and sodium. Main source of drinking water in Pakistan is underground water which is being mineralized due to different factors (Abdullah *et al.,* 2012). In the patients who were consuming hard and moderately hard water in this study, 71% of the patients were those whose intake of water was less than 3L daily also. So, it can be inferred that low water intake might also be a risk for enhanced tendency of stone formation in these patients.

Different clinical attributes were also studied as risk factors for development of cUTI in urolithiasis. Long duration of stone disease was observed in majority of patients, which was significant for the development of cUTI in such patients as 86% of cUTI patients were having urolithiasis for more than one year. Overall, 64% urolithiatic patients were diagnosed with cUTI in present work. Almost 69% of patients with prolonged urolithiasis belonged to low socioeconomic status. Lack of affordability of health treatments might be the major reason for prolonged stone disease and its complications in this class of patients. These prolonged urinary obstructions might be responsible for development of

cUTI, which provide site for invasion and development of biofilms forming microorganisms in urinary tract that became the major cause of infection. In another survey conducted in Iraq, a lower number (42%) of the renal stone patients were reported to have cUTI (Al-jebouri and Atalah, 2012). This might be due to reason that patients they enrolled in their study were from both out-patient's department and surgical wards of hospital while in current study; all patients were enrolled from surgical wards of hospitals.

Patients with prolonged disease and cUTI in this study had elevated levels of WBCs in their urine, which is a common indication of complications in the urinary tract. cUTI was also observed to be associated with size and number of stones in urinary tract. It was detected that 64% of urolithiatic patients with stone size of more than 4mm were positive for cUTI. Most of the patients in current work had large size of stones which might be due to the fact that this study was carried out in surgical units of hospitals and stones with less than 5mm in size can easily pass-through urinary tract whereas those having diameter of 5-7mm and over 7mm must be removed surgically (Krambeck and Lieske, 2011). These large sized stones if not treated for long duration developed cUTI in these patients.

It was observed that 65% of male patients of >50 yrs of age were suffering from cUTI. cUTI is observed in male patients in later age might be due to weakened immune system. While females of age group 41-50yrs were more prone to cUTI. During this life period, hormonal changes are at their peak in women's life because of pregnancies, menopause and other such factors. This hormonal imbalance can lead to the yeast infections. Majority of male patients (65%) having cUTI were from low socioeconomic status and had hard jobs like laborers and guards. Due to their hard work in open areas and long duration of stay in sun might be the reason of dehydration of their body, urolithiasis and eventually cUTI. High temperature is positively associated with high rates of stone disease. Sunlight enhances the production of 25-hydroxycholecalciferol in skin. This increases the intestinal absorption of calcium, after conversion to 1,25-dihydroxy-vitamin D by the kidneys (Safarinejad, 2007).

This data represents the marked increase in cUTI among urolithiatic patients. Some risk factors were observed to contribute more in urolithiasis and subsequent development of cUTI in patients enrolled in current study. Low socioeconomic status was found to be major risk factor for urolithiasis, which also contributed to further complicate the disease condition. Prolonged disease condition and irrational intake of medicines in low socioeconomic status patients may have led to cUTI in urolithiatic patients. There was a significant association of these risk factors to cause cUTI in urolithiatic patients. Consumption of hard water with low water intake was observed in most of the patients, which depicts unavailability of potable water which aggravates this disease. These lifestyle factors simply play important role in changing epidemiology and complications of this disease in Pakistan. So, there is need to plan a proper awareness programme and better management strategies to control this problem in Pakistan.

CHAPTER 4

Bacterial diversity in urine samples of urolithiatic patients with cUTI by culture dependent and independent method.

4.1-Introduction

Due to unavailability of suitable culturing techniques, identification and characterization of full spectrum of urinary microbiome is still lacking. Also, long ago urine was considered sterile in healthy individuals and presence of bacteria in urinary tract was ambiguously coupled with the UTI, which often led to improper antibiotic treatment (Finucane, 2017). Advances in molecular techniques i.e., 16S rRNA gene sequencing and introduction of new technique called expanded quantitative urine culture make it possible to characterize the diverse urinary microbiome in health and disease (Popovic*et al.,* 2018). Microbiome changes in urinary tract have been observed in various urologic disorders such as urinary incontinence, interstitial cystitis, urologic cancers, sexually transmitted infections, chronic prostatitis and complicated urinary tract infections (Aragon *et al.,* 2018).

Gram positive and negative bacteria and certain fungi are involved in causing the UTIs. UPEC is considered to be the most common causative agents for both uncomplicated and complicated UTIs followed by *Enterococcus* spp*., K. Pneumoniae, Candida* spp*., S. Aureus, P. aeruginosa* and *P. Mirabilis* (Flores-Mireles *et al.,* 2015). This indicates that members of *Enterobacteriaceae* are prevalent in UTIs. A study conducted in USA reported seven bacterial phyla including Firmicutes, Actinobacteria, Fusobacteria, Proteobacteria and Bacteroidetes, Tenericutes and TM7 in male urinary tract having sexually transmitted infections (Nelson *et al.,* 2010).

Antimicrobial resistant and especially ESBL producing *Enterobacteriaceae* have now become a public threat worldwide for being highly prevalent in infections (Mahamat*et al.,* 2019). Most cephalosporins and cephamycins resistant isolates of *E. coli, P. Mirabilis* and *K. pneumoniae* produce ESBLs. Fluoroquinolone and cephamycins resistant *Enterobacteriaceae* are also increasing worldwide in UTI. More than 80% of *Enterobacteriaceae* members are responsible for uncomplicated UTI and 40% for

complicated UTI. Resistance to non beta-lactams such as fosfomycin, fluoroquinolones and co-trimoxazole has also been observed in most of nosocomial ESBL producers, which causes major issues in UTI treatment (Muratani and Matsumoto, 2006).

An Indian study reported *Escherichia coli* as the most common etiological agent followed by budding yeast, *Klebsiella*, *Enterococcus* spp., *P. aeruginosa*, *S. aureus* and coagulase negative Staphylococcal spp. in cUTI patients from different wards of tertiary care hospital. It was also observed that among the isolates from ICU, budding yeast was the most common isolated organism followed by *Enterococci* (Mohan *et al.,* 2016). Another study reported *E. coli* as the leading cause of UTI in catheterized patients followed by *Enterococcus, Enterobacter* spp*, K. pneumoniae,* Group B Streptococcus and some other unidentified bacteria (Manohar *et al.,* 2019).

E. coli are opportunistic pathogens and member of family *Enterobacteriaceae.* The bacterium is an important normal flora of human gastrointestinal tract but also infect millions of people worldwide (Blount, 2015). *E. coli*'s virulence factors help them for their colonization and disease development in urinary tract, such are termed as uropathogenic *E. coli*. UPEC is involved in 50% nosocomial and 85% community acquired UTIs (Davis and Flood, 2011). *K. pneumoniae* is also a major uropathogen being involved in UTIs and its complications. Pathogenicity of *K. pneumoniae* varies due to its various virulence factors enabling it for evasion of host immune system and to maintain infection. The most important virulence factors are adhesins, hypermucoviscosity, iron acquisition system, serum resistance, lipopolysaccharides and biofilm formation. Biofilm of *K. pneumoniae* is considered as an important virulence factor in causing cUTI among urolithiatic patients (El Fertas-Aissani*et al.,* 2013).

Different studies reported incidence of urolithiasis in Pakistani population residing in different areas. However, cUTI and its causative microbes either culturable or unculturable related to urolithiasis are understudied in Pakistan. There are higher chances of developing cUTI in local urolithiatic patients due to several risk factors. There is probability of these bacteria to be also MDR. Therefore, there is need to identify and characterize these causative agents of cUTI carefully for their virulence and drug resistance pattern for better disease management. Conventional microbiological methodsare insufficient for

identification of whole microbial diversity of urine to assess other possible unculturable bacteria and their role in cUTI. The present study was aimed to use culture dependent and independent methods to investigate urinary microbial diversity and its pathogenic potential for causing cUTI among urolithiatic patients.

4.2-Material and Methods

4.2.1- Study Design

Previously described in chapter 3.0 section 3.2.1

4.2.2- Study Population

Previously described in chapter 3.0 section 3.2.2

4.2.3-Part 1: Study of urinary bacteria in cUTI among urolithiatic patients by culture-dependent methods

4.2.3.1-Culturing of urine specimens for bacterial isolation

To isolate the culturable bacterial species, 0.1mL of urine sample was directly inoculated onto petri plates containing Nutrient agar (NA). NA (Oxoid, England) was prepared by adding 28g/L and autoclaved for 15 min at 15psi and 121ºC. Autoclaved media was cooled and poured in sterile petri plates under sterile conditions and allowed to solidify. On the basis of colony morphology, distinctive colonies were picked individually using a sterile loop and streaked separately on NA plates using quadrant streak technique to get pure cultures. These plates were then incubated for 24 h at 37ºC aerobically and growth was observed. For further morphological identification Gram staining was done.

4.2.3.2-Differential Gram staining

To differentiate Gram positive and Gram-negative bacteria, smear was prepared by taking bacterial isolates from culture plate on center of glass slide containing drop of saline water and heat fixed. Glass slide was flooded with crystal violet as primary stain for 60s. Slide was rinsed with water then iodine was added and left for 30s. Decolourization step was performed by adding 95% ethanol for 30 s and rinsed with water. Safranin was added as secondary dye for 45 s and washed with water. Slides were blotted, air dried and observed

under light microscope at 40x and 100x (using immersion oil).**4.2.3.3-Identification of Gram positive cocci**

4.2.3.3.1-Catalase test

Catalase test was used to determine the production of catalase enzyme by bacterial isolates. Catalase enzyme breakdown the hydrogen peroxide H_2O_2 (toxic substance of oxygenated metabolites) into water and oxygen which neutralize the toxic effects of hydrogen peroxide.

$$2H_2O_2 + \text{Catalase} \longrightarrow 2H_2O + O_2$$

Test was performed by placing drop of 3% H_2O_2 on centre of clean microscopic slide and a loop full of fresh bacterial culture was added with the help of wooden applicator stick. Results were observed within 15-20 s.

4.2.3.3.2- Growth on Mannitol Salt Agar (MSA)

MSA was used to differentiate between mannitol fermenting *Staphylococcus aureus* and other *staphylococci.* Acid production in mannitol fermentation by *S. aureus* was detected by phenol red present in the media. The media was prepared by adding 111g of MSA (Oxoid England) in 1000 mL of distilled water and autoclaved for 15 min at 121°C and 15psi pressure. After autoclaving, media was poured into the petri plates under sterilized conditions. Pure colony from media was picked and streaked onto MSA plate and incubated for 24 h at 37°C. Growth of yellow colonies indicated the presence of *S. aureus* and pink colonies showed presence of other *Staphylococcus* species.

4.2.3.3.3- Novobiocin differential test

For identification and differentiation between *S. saprophyticus and S. epidermidis*, novobiocine disc diffusion assay was performed. Sensitive isolates for novobiocin were *S. epidermidis* while resistant against novobiocine are *S. saprophyticus.* Selected isolates were grown on NA for 24 h at 37°C. Suspensions of pure isolates were prepared in normal saline (0.85% NaCl). Novobiocine disc (REMEL^{TM}) was applied on Muller-Hinton agar (MHA) surface after swabbing the lawn of isolate on sterilized condition. Zone of inhibition was interpreted by CLSI -2018-M100 guidelines.

4.2.3.4-Isolation and purification of *Enterobacteriaceae*

Gram negative bacteria identified from Gram staining were further grown on MacConkey agar (selective media for Gram negative microorganisms) based on principle of lactose fermentation by Gram negative bacteria. MacConkey agar contains peptone as source of vitamins and minerals plus lactose monohydrate as source of carbohydrate to differentiate between the lactose fermenters from non-fermenters, neutral red was indicator as at acidic pH upon lactose fermentation, it turns pink or red. Media was autoclaved at 121°C for 15 min and 15psi pressure. After pouring and solidifying the media on plates, Gram negative bacterial isolates were streaked and incubated for 24 h at 37°C. Following incubation period colony morphology was noted to isolate pink mucoid colonies of *Enterobacteriacea*.

4.2.3.5-Biochemical identification of *E. coli* and *K. pneumoniae*

Isolates were identified as *E. coli* and *K. pneumoniae* by observing biochemical profile with a series of tests (Indole, citrate utilization test, TSI, motility test and API kit) used preferably for *Enterobacteriaceae*.

4.2.3.5.1-Indole test

Indole test was performed to determine the ability of bacterial isolates to produce tryptophanase enzyme. The test is based on principle of tryptophan utilization as substrate and production of indole substance, which can be detected by Kovac's reagent (p-dimethyl amino benzaldehyde). Pure isolated bacterial colonies were grown in peptone water for 24 h. Peptone water contains tryptophan, which upon hydrolysis is converted into by products and one of these is indole. After incubation 0.5 mL of Kovac's reagent was added and results were noted to identify indole test positive or negative isolates.

4.2.3.5.2-Citrate utilization test:

Citrate test is also a part of IMViC series, which is used to differentiate between members of *Enterobacteriaceae* family i.e., *E. coli* and *K. pneumoniae.* Simon Citrate medium was used to perform test, containing sodium citrate as a carbon and ammonium salts as nitrogen source. Citrate test is based on principal of citrate utilization and turning ofmedium pH to

alkaline, which can be detected by indicator bromothymol blue which turns from green to Persian blue as an indicator of positive result. Slants were prepared by autoclaving Simon citrate medium and pouring in test tubes placed in inclined position. After solidifying media, slants were inoculated by taking loop full of bacterial culture and streaked on surface of slant under sterilized conditions. Test tubes were capped carefully and incubated for 24 h at 37°C. Results were recorded after incubation.

4.2.3.5.3-Catalase test

Previously described in section **4.2.3.3.1.**

4.2.3.5.4-Motility test:

Motility test was performed by preparing semi solid media. A 0.5% agar was dissolved in nutrient broth and autoclaved for 15 min at 121°C. After cooling, media was poured into test tubes to prepare butt. Loopful of fresh culture picked up with sterile needle and stabbed straight in to the bottom of test tube and removed along the same stabbing line. Test tubes were incubated for 24h at 37°C and growth was observed around stabbing line.

4.2.3.5.5-Triple sugar iron test

Triple Sugar Iron (TSI) is a differential medium commonly used for identifying bacterial isolates having ability of carbohydrate fermentation and gas production. TSI contains 0.1% glucose, 1% lactose and 1% sucrose as sole carbon source and phenol red as indicator. Test is based on principal of carbohydrate fermentation and acid production which can be detected by phenol red indicator. TSI (CM0277, Oxoid) was prepared by dissolving 65 g in 1000 mL of distilled water and autoclaved. Slants were prepared with good butt and short slant. Slants were inoculated by stabbing butt deeply and streaking the surface of slants. Results were observed after incubation period of 24 h at 37°C. Lactose or sucrose fermentation produce large amount of acid which turns indicator yellow both in butt and slant. Those organisms that produce gases generate bubbles or cracks in media. Glucose fermentation causes butt to turn yellow and butt to turn red. In case of H_2S production, black coloration of media occurs due to ferrous sulphide production.

4.2.3.5.6-Urease test

This test is used to differentiate the organism based on its ability to hydrolyze urea. Urea is product of decarboxylation of amino acids. Ammonia and CO_2 is produced as result of urea hydrolysis. Ammonia production alkalinize the media and this pH change is detected through change in colour of phenol red. Light orange colour is turned to magenta with shift in pH from 6.8 to 8.1. Readily urease positive organisms convert colour of media into pink within 24 h

$$(NH_2)_2CO + 2H_2O \longrightarrow CO_2 + H_2O + 2HN_3$$

Urea broth base was added in distilled water and autoclaved at 121°C at 15psi for 15 min. A 5% urea solution was prepared separately. After cooling urea solution was mixed into urea broth base and added into test tubes. Loopful of overnight grown fresh bacterial culture was inoculated and tubes were covered properly. Test tubes were then incubated at 37°C for 24 h and results were observed after incubation.

4.2.3.5.7-Eosin Methylene Blue Agar test

Eosin Methylene Blue (EMB) agar is both selective and differential medium used for identification and differentiating of the Gram negative bacteria. It contains dyes eosin and methylene blue in ratio 6:1 as colour indicators. EMB media inhibits the growth of Gram positive bacteria. Lactose fermenting Gram negative bacteria lower the pH of media by acid production and increase the capacity of colonies to absorb dye and colonies appears purple black in colour. *E. coli* produces purple colonies with green metallic sheen due to the metachromatic properties of fermentation products. EMB was prepared by adding 37.5g/L of medium in distilled water and autoclaved at 121°C at 15psi for 15 min. After pouring in sterile petri plates, all Gram negative rods were cultured on plates and incubated for 24 h at 37°C.

4.2.3.5.8-API kit

Remel RapID ONE system was used to confirm the *Enterobacteriaceae* members according to the manufacturer's guidelines. The system was composed of RapID ONE reagent and RapID ONE panel. RapID ONE panel was a plastic disposable tray

containing different substrates, degradation of which as a result of inoculating organisms was used for identification of specific organism.

4.2.3.6-Screening of virulence factors of *K. pneumoniae* and *E. coli*

4.2.3.6.1-Hypermucoviscosity test for *K. pneumoniae*

Hypermucoviscosity is determined by performing string test. Freshly grown pure isolated colonies of *K. pneumoniae* on Brain heart infusion (BHI) agar plates were touched by sterile inoculating loop and slightly raised to check their ability to form string. The string greater than 5mm in length is an indication of hypermucoviscosity positive isolates.

4.2.3.6.2-Biofilm formation assays

4.2.3.6.2.1-Congo red assay (Qualitative assay)

Congo red assay (CRA) is the most commonly used qualitative method to identify biofilm forming ability of isolates, its composition is given in Table 3.1 (Bellifa *et al.,* 2016)

Table 4.1: Composition for Congo red medium used to determine biofilm forming ability of various isolates from urolithiatic patients

	Ingredients	**Amount/L (g)**
1	Agar	10.5
2	BHI	37
3	Congo red dye	0.8
4	Sucrose	36

The constituents other than Congo red dye (Oxoid) were autoclaved at 121°C at 15psi for 15 min. Congo red stain was autoclaved separately by dissolving 0.8g/L of dye in distilled water. Then it was added to autoclaved BHI agar, when its temperature reached up to 55°C and poured in petri plates. Bacterial isolates were inoculated on CRA plates and incubated at 37°C for 24 h. Results were recorded after incubation.

4.2.3.6.2.2-Microtiter plate assay (Quantitative assay)

Biofilm forming ability of bacterial isolates was quantified by performing microtiter plate (MTP) assay. In this method, optical density (OD) of microtiter plate with biofilms was measured and compared with cut off value to biofilm formers isolate (Saxena *et al.,* 2014). Inoculum was prepared by inoculating BHI broth and incubating overnight at 37°C. Then turbidity of test organism was adjusted with 0.5% McFarland's standard. Overnight grown culture (200 µL) was taken into 96 wells flat bottomed microtiter plate and incubated at 37°C from 24 h. Wells containing sterile BHI broth were taken as negative control whereas wells with biofilm forming ATCC strain were taken as positive control. Media was removed from wells by gentle tapping without disturbing biofilms after incubation. MTP was washed twice with 200µL of phosphate buffer saline (PBS) to remove unattached bacterial components. Addition of 200µL of 95% ethanol was done and incubated at room temperature for about 30 min. Biofilms were stained with 200µL of 1% Crystal Violet and left for 15 min then MTP was rinsed twice with PBS to remove excessive dye. Plate was incubated at room temperature for half an hour to completely dry wells and acetic acid 33% (200µL) was added. After 15 min of incubation, OD was measured at 595nm with ELISA auto reader and compared with cut off value. Cut-off OD (ODc) value was determined by using following formula:

$$ODc = \text{Mean OD of control} + (3x \text{ SD of control})$$

Table 4.2: Classification criteria for biofilm forming ability of bacteria by MTP method

OD values	Biofilm Formers
OD ≤ Odc	Negative
ODc< OD ≤ 2 x Odc	Weak
2 x ODc< OD ≤ 4 x Odc	Moderate
4 x ODc< OD	Strong

4.2.3.6.3-Hemolysin production assay

This test was performed to check the ability of bacterial isolates to produce hemolysin toxin. To carry out this procedure, sheep blood agar base (Oxoide, England) was prepared as per manufacturer's instructions. Media was measured and added in one litre of distilled water, boiled to completely dissolve it and autoclaved at 121ºC for 15 min. Then 7% sheep blood was added in this base aseptically and when cooled to 45°C to 50ºC. Fresh cultures of bacteria from NA were streaked on sheep blood agar plates. Plates were incubated for 24-48 h at 37°C in an incubator and after incubation period results were observed.

4.2.3.6.4-Serum bactericidal assay

Serum activity against various invading microorganisms is first line of defence. Serum mostly affects Gram negative bacteria, whereas Gram positive bacteria are less sensitive to serum activity. This effect diminishes when serum is inactivated by heating at 56°C for 30 min. Serum bactericidal test was performed by using protocol described earlier (Trakulsombon*et al.,* 1989). Serum was collected from healthy individuals in sterile serum tubes and different concentrations were prepared. To prepare 80% and 40% of pooled normal human serum (PNHS), 0.8 and 0.4 mL of serum was added in to 0.1 and 0.2 mL of MH broth respectively. Heat inactivated serum and MH broth were taken as controls.

Freshly grown bacterial culture was inoculated in BHI broth and incubated for 24h and inoculum was adjusted to 10^6 CFU/mL in MH broth after incubation. The 0.1mL of 10^6 CFU/mL was added in to 80% and 40% serum and incubated for 24h at 37°C. After time interval of 2h, 4h, 6h and 24h standard plate count was performed and cells were expressed as $\log_{10}$ CFU/mL. The percentage of bactericidal activity was calculated by using following formula:

% **bactericidal activity** = 100 x number of CFU of experimental tubes / number of CFU of control tubes.

The standard time kill curve was plotted to compare percent bactericidal activity of different concentrations of serum.

4.2.3.6.5-Haemagglutination assay

Haemagglutination is characteristic feature of many bacteria specially members of *Enterobacteriaceae*. Bacteria due to presence of type 1 fimbriae successfully agglutinate RBCs of human blood as described by previous studies. Haemagglutination test was performed using method described earlier (HRV *et al.,* 2016). Blood was collected from healthy individuals in sterile EDTA tubes. RBCs were obtained by centrifugation of blood at 5000rpm for 5min. Supernatant was discarded and RBCs were washed twice with freshly prepared PBS (pH 7.2). Working solution of 3% v/v RBCs was prepared for performing haemagglutination test. Inoculum was prepared by taking loopful of freshly grown bacterial culture in 5.0 mL of BHI broth and incubated overnight. After incubation, culture was centrifuged at 5000rpm for 5min. Supernatant was discarded and bacterial cells were suspended in PBS to obtain the concentration of $5x10^{10}$ per mL. Microtiter plates were taken and 50µL of bacterial suspension was added in to each well with 50µL of 3% v/v blood of different blood groups (blood group A and blood group B) suspension and incubated for 1hour at 4°C. Results were recorded by observing clumping of RBCs.

4.2.3.7-Antibiotic susceptibility test

Antimicrobial susceptibility testing was performed via Kirby-Bauer disc diffusion method, recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines. Bacterial suspension was made by taking isolated colony from NA plate that was 18-24 h old into1.0 mL of saline (0.85% sodium chloride) solution. The saline suspensions of all the samples were compared with 0.5% McFarland standard. MHA media was prepared. Suspensions were independently inoculated on different MHA agar plates and lawn was prepared with the help of sterile swab. After that different antibiotics' discs were placed on the MHA plate. Antibiotic discs were placed at 24 mm from each other on MHA plates. Plates were incubated at 37ºC for 24 h. Results were observed after 24 h according to break point of inhibition recommended by CLSI-2018-M100 for *Enterobacteriaceae* as reference and zones were measured to find out resistant and sensitive isolates (Table 4.3).

Table 4.3: List of antibiotics with break points of inhibition recommended by CLSI-2018-M100

Antibiotics		**Sensitive**	**Intermediate**	**Resistant**
Aminoglycosides	CN50µg	≥15mm	13-14mm	≤12mm
	TOB30µg	≥15mm	13-14mm	≤12mm
	AK30µg	≥17mm	15-16mm	≤14mm
Fluoroquinolones	CP5µg	≥21mm	16-20mm	≤15mm
	OFX5µg	≥16mm	13-15mm	≤12mm
Cephalosporins	KF30µg	≥18mm	15-17mm	≤14mm
	CAZ30µg	≥21mm	18-20mm	≤17mm
	TE30µg	≥23mm	20-22mm	≤19mm
Sulfonamides	TMP/SMX µg	≥16mm	11-15mm	≤10mm
Tetracycline	TE30µg	≥15mm	12-14mm	≤11mm
Penicillin	AML10µg	≥17mm	14-16mm	≤13mm
Lincosamide	DA10µg	≥16mm	11-15mm	≤10mm
Nitrofurantoin	F300µg	≥17mm	15-16mm	≤14mm
Carbapenem	Imipenem 10µg	≥19mm	16-18mm	≤15mm

4.2.3.8-Extended spectrum beta lactamases (ESBL) production by double disc synergy test

A double disc synergy test is performed to detect ESBLs production in the microorganisms. This test was carried out in two steps. First step was screening, which indicated the resistance of organism to cephalosporins, thus identifying isolate likely to have ESBLs. Then in second step confirmatory test was performed by evaluating the synergy between clavulanic acid and cephalosporin to distinguish isolates with ESBL from those which are resistant for some other reasons.

In this test discs of cephalosporins are applied next to the disc with clavulanic acid, amoxicillin+ clavulanic acid or ticarcillin+ clavulanic acid. Positive result was indicated, when the inhibition zone around cephalosporin disc was augmented towards disc with clavulanic acid. Cephalosporin resistant isolates were cultured on MacConkey agar and incubated overnight. Loopful of overnight grown culture was taken and suspended into normal saline to make suspension. A lawn of bacterial suspension was prepared on the plates of MHA with the help of sterile cotton swab. Discs of antibiotics were applied onto the plates at 15-20mm to allow proper measurement of inhibition zone. Plates were incubated for 24 h at 37°C and results were noted by measuring the zone of inhibition according to CLSI guidelines.

4.2.3.9-Molecular identification ESBL and quinolone resistance genes

ESBL genes identification was carried out in steps including plasmid extraction and polymerase chain reaction followed by gel electrophoresis.

4.2.3.9.1-DNA extraction

Boiling method was used to extract whole bacterial DNA. Freshly grown cultures of *E. coli* and *K. pneumoniae* were inoculated with sterile loop in 150mL double distilled water. Bacterial suspension heated in water bath at 95°C for 25min. After incubation the eppendorfs were centrifuged at 10,000rpm for 15min, recovered supernatant in another tube and pellet discarded.

4.2.3.9.1.1- Gel electrophoresis

This protocol is based on principle of fluorescence emission by ethidium bromide upon intercalation with nucleotides under UV light. For visualization of quality of extracted DNA, 1% agarose gel was prepared. For preparation of 1% gel, 0.5 g of agarose was added in 50 mL of 1X TBE. Then it was dissolved in microwave and after cooling 3 µL of ethidium bromide was added into the gel, which stains DNA and help in its visualization. The gel was then poured into the gel tray fixed with combs. After solidification of gel, a 2 µL of DNA was mixed with 2 µL of loading dye (consisting of bromophenol blue solution) and was loaded into the wells next to the DNA marker (thermoscientific 1kb ladder). Gel electrophoresis of extracted DNA was performed at 90 volts for 40 min in TBE buffer (1X). The extracted DNA was visualized on agarose gel by using Gel documentation system.

4.2.3.9.2- PCR amplification for ESBL and quinolone resistance genes of *E. coli* and *K. pneumoniae*

PCR amplification of ESBL genes as well as other antibiotic resistance genes (quinolone resistance) was done by using set of primers designed by Snap Gene® Viewer 4.1.8 software and purchased from Thermo Fischer Scientific. Sequence of primers with melting temperature is given in Table 4.4. Reaction mixture was prepared using volume described in Table 4.5. Among ESBL genes bla*TEM* and bla SHV were done in multiplex PCR because of their same annealing conditions. Similarly, *qnrA*, *qnrB* and *qnrS* were also amplified using multiplex PCR. Conditions used for gene amplification are given in Table 4.6.

Table 4.4: List of primers used for molecular characterization of ESBL and *qnr* genes present in *K. pneumoniae* and *E. coli* isolated from urolithiatic patients.

S. no	Gene	Primer Pair	Sequence (5'-3')	Band size (bp)
1	**ESBL genes**	*CTX-M* F	ATGTGCAGTACCAGTAAGGT	544
		CTX-M R	TGGGTRAARTARGTCACCAG	
2		*TEM* F	CTTCCTGTTTTTGCTCACC	711
		TEM R	AGCAATAAACCAGCCAGC	
3		*SHV* F	TCAGCGAAAAACACCTTG	471
		SHV R	TCCCGCAGATAAATCACC	
4	**Quinolone resistance genes**	*qnrA*- F	AGAGGATTTCTCACGCCAGG	516
		qnrA- R	TGCCAGGCACAGATCTTGAC	
5		*qnrB*- F	GGMATHGAAATTCGCCACTG	526
		qnrB-R	TTTGCYGYYCGCCAGTCGAA	
6		*qnrS*- F	GCAAGTTCATTGAACAGGGT	417
		qnrS- R	TCTAAACCGTCGAGAGTTCGGCC	

Table 4.5: Reaction mixture used for amplification of ESBL and *qnr* genes of *E. coli* and *K. pneumoniae* isolated from urolithiatic patients.

Reaction components	Volume (µL)
2x FIREPOL® master mix	5.0
Forward primer pmol/ µL	0.5
Reverse primer pmol/ µL	0.5
Template DNA	1.5
PCR water	2.5
Total volume	10

Table 4.6: PCR conditions for amplification of ESBL and *qnr* genes of *E. coli* and *K. pneumoniae* isolates

Steps	Temperature °C		Time	Cycles
	CTX-M, TEM, SHV	***qnrA, qnrB, qnrS***		
Initial Denaturation	94	95	5min	1
Final Denaturation	94	94	30sec	40 for *CTX-M*, 35 for *TEM* and *SHV* (Multiplex) 36 for *qnrA*, *qnrB*, *qnrS* (Multiplex)
Annealing	54	53	40sec	
Extension	72	73	50sec	
Final Extension	72	72	10min	1

4.2.3.9.3- Gel electrophoresis

For visualization of PCR products, a 2% gel was prepared. For preparation of 2% gel, 1.0 g of agarose was added in 50 mL of 1X TBE. Then, it was heated in microwave oven to dissolve it. After cooling gel was poured into the gel tray fixed with combs. After solidification of gel, a 2µL of PCR product loaded into the well. Gel electrophoresis of PCR product was performed at 90 volts for 40 min in TBE buffer (1X). The bands of PCR product were visualized on agarose gel by using Gel documentation system (SYGENE).

4.2.3.10- Statistical analysis

Data analysis was performed by using SPSS software version 16 for windows 10. Chi-square (χ^2) and Fisher exact test was performed to determine association between different variables. A p value of < 0.05 was considered as significant.

4.2.4- Part II. Study of bacterial diversity by culture independent method

4.2.4.1-Study population and sample collection

Twenty urine samples from ten urolithiatic patients with cUTI and ten healthy individuals were processed for identification of urinary microbiome by amplification of V4 region of 16S rRNA gene. Patients included five females of 31-40 yrs age and five males of 31-40 yrs age with urolithiasis disease having cUTI. The healthy group included five females and five males of same age group as of patients. Pooled urine samples for each category including female urolithiatic patients with cUTI (SP1), male urolithiatic patients with cUTI (SP2), healthy females (SP3) and healthy males (SP4) were used for microbiome studies.

4.2.4.2-Microbial DNA extraction from urine and sequencing

DNA extraction was carried out by two day protocol as described earlier (https://openwetware.org/wiki/Phenol/chloroform_extraction), with few modifications. The extracted DNA was quantified via A260/A280 ratio by using nanodrop spectrophotometer. PCR amplification of V4 region of 16S rRNA gene was carried out

by using primers 515F/806R (Table 4.7). PCR conditions are mentioned in table 4.8. Product visualization after amplification was done on 2% agarose gel. Equal concentrations of multiple PCR products based on their molecular weights were pooled together and purified by using Ampure, XP beads. From this pooled and purified PCR products, DNA library was prepared by using Illumina TruSeq DNA library preparation protocol. Illumine MiSeq platform was used for sequencing and it was performed at MR DNA (www.mrdnalab.com, shallowater, TX, USA). Raw data sequences have been submitted in Sequence Read Archieve and were assigned an accession number PRJNA579257.

Table 4.7: Primer sequence used for the amplification of V4 region of 16S rRNA gene

Primers	**Sequence 5'→ 3'**
515F	5'GTGYCAGCMGCCGCGGTAA3'
806R	5'GGACTACHVHHHTWTCTAAT3'

Table 4.8: PCR conditions used for the amplification of V4 region of 16S rRNA gene

Steps	**Temperature (°C)**	**Time duration**	**Cycles**
Initial Denaturation	94	03 min	01
Denaturation	94	30s	28
Annealing	53	40s	
Extension	72	01 min	
Final Extension	72	05 min	01

4.2.4.3-16S rRNA gene data processing and analysis

After sequencing data was processed by using MR DNA analysis pipeline. After joining sequences, barcodes were deleted and sequences <150bp were removed. Operational taxonomic units (OTUs) were generated and their clustering was done on 97% sequence similarity. Then taxonomic classification of OTUs was done by using BLASTn against Green Genes, NCBI and RDPII derived databases. Further analysis was performed by using excel and microbiome analyst tool (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/faces/home.xhtml). Alpha diversity was measured by Observed, Simpson's index, Shannon index and Chao 1 with *P-value* by using Mann-Whitney test. Beta diversity was assessed by using Bray-Curtis distance. Statistical significance was done by using Permutational MANOVA tool. Core microbiome analysis was carried out by using software Venni.

4.3 -Results

4.3.1-Part I: Isolation of culturable bacteria from urine samples

4.3.1.1-Isolation of bacterial species

All urine samples were spread on NA plates and incubated for 24 h at 37°C. After incubation, colony morphology was observed. Out of 164 patient samples, 78(47.5%) produced one type of colony, 44 (26.8%) produced two, 16 (9.8%) three whereas 4(2.4%) had four types of colonies. These colonies were sub-cultured and purified on NA by using quadrant streak plate method to get pure bacterial isolates.

4.3.1.2-Gram staining:

Among 230 bacterial isolates, 46 (20%) were Gram positive cocci, rest were Gram negative bacteria. Among these based on shape, 156 (85%) were Gram negative rods and remaining 28(15%) were Gram negative cocci. While in control samples, 44 bacterial isolates were obtained from 40 samples. After Gram staining, 23/44 isolates Gram positive cocci, 02/44 Gram negative cocci, 13/44 Gram negative rods and 06/44 Gram positive rods.

4.3.1.3-Isolation and identification of Gram positive cocci

4.3.1.3.1- Catalase test

Of these all Gram positive cocci, 46 were from urolithiatic patients and 23 from healthy controls which were subjected to catalase test. Out of 69 isolates, 40 (58%) were catalase positive *i.e., Staphylococcus* while 29(42%) were catalase negative i.e. *Streptococcus.* In case of urolithiatic patients, 27 isolates were catalase positive and 19 catalase negative.

4.3.1.3.2-Growth on MSA

All catalase positive Staphylococcal species were cultured on MSA plates. Out of 27 isolates from urolithiatic patients, 15(55.5%) isolates had yellow growth on MSA indicating the possible presence of *S. aureus.* In healthy individuals 13 were catalase positive isolates, in which 5(38%) produced yellow growth while 8(62%) showed pink growth.

4.3.1.3.3- Novobiocin disc sensitivity testing

For identification and differentiation of *S. epidermidis* and *S. saprophyticus* novobiocin disc test was performed. From 12 isolates of urolithiatic patients, 04 were sensitive for novobiocin indicating them as *S. epidermidis* and 08 were resistant against this antibiotic *i.e., S. saprophyticus.* Among healthy individuals, 05 isolates were sensitive to novobiocine while 03 were resistant indicating that these were *S. epidermidis* and *S. saprophyticus* respectively.

4.3.1.4-Isolation and identification of Gram negative rods

After Gram staining, Gram negative rods were selected and streaked on MacConkey agar to differentiate between lactose fermenter and non-fermenters. Following incubation, two types of colonies were observed *i.e.,* pink (lactose fermenters) and pale (non-lactose fermenters). Out of 156 Gram negative, 146 (94%) were lactose fermenters and 10 (8%) were non-lactose fermenters. Among lactose fermenters, isolates showing colony morphology pink, moist, round, regular ends were suspected to be as *Escherichia coli* and *K. pneumoniae* isolates, which were further subjected to biochemical characterization.

4.3.1.4.1-Biochemical characterization

The biochemical characterization of lactose fermenter Gram negative rods was performed using standard protocols (Appendix B-I).

4.3.1.4.1.1- Indole test

The isolates that showed red-violet ring at the surface of tryptophan broth were considered positive based on the production of indole by-product. As *E. coli* produces tryptophan enzyme, it showed positive indole test. While *K. pneumoniae* lacks tryptophanase enzyme, it showed negative indole test. Seventy one (49%) isolates showed indole positive results whereas, 75(51%) were indole negative.

4.3.1.4.1.2- Citrate utilization test

Citrate positive isolates were selected based on the colour change of indicator bromothymol blue from green to Persian blue under acidic conditions. As *K. pneumoniae* ferment the citrate and these isolates showed positive colour change while *E. coli* does not ferment citrate, thus were negative. Out of total, 70(48%) were citrate positive and 76 (52%) were citrate negative.

4.3.1.4.1.3-Catalase test

Both *K. pneumoniae* and *E. coli* were positive for catalase enzyme production. Catalase test was positive for 136 (93%) and 10(7%) isolates were negative.

4.3.1.4.1.4-Urease test

This test was performed to check the presence of urease enzyme in the suspected bacterial isolates. Following incubation, the urease activity of the bacteria was observed. *E. coli* do not produce urease enzyme but *K. pneumoniae* produce this enzyme. Out of all, 75 (51%) isolates were urease negative and 71(49%) were urease positive.

4.3.1.4.1.5-Triple sugar iron test

After incubation results were recorded as A/A slant/butt, K/K slant/butt, A/K slant/butt, gas H_2S production. There were A/A and gas producer 126(86%) isolates, whereas, 20(14%) were negative for TSI.

4.3.1.4.1.6-Motility test

Motility test was performed to check the presence of flagella in bacterial isolates. Cloudy appearance of media and spread of organism over the media indicated motility. As *E. coli* are motile with peritrichous flagella showed positive motility while *K. pneumoniae* are non-motile. Seventy (48%) isolates showed such appearance on media, however, 76(52%) were non-motile.

4.3.1.4.2- Identification of *Enterobacteriaceae* based on EMB agar

Pink mucoid colonies of suspected *E. coli* and *K. pneumoniae* were further confirmed through streaking on EMB agar plates. Bacterial isolates (54) produced green metallic sheen due to chromogenic nature of dyes. While 69 *K. pneumoniae* isolates on EMB showed non-metallic sheen and pink to purple mucoid colonies. Remaining 23 isolates appeared light pink colonies on EMB agar indicating them to be *Enterobacter* spp.

4.3.1.4.3- API kit for *Enterobacteriaceae*

Remel RapIDTMOne system was used for confirmation of suspected *Enterobacteriaceae* members. Among urolithiatic patients out of 146 isolates, 69 were confirmed as *K. pneumoniae* and54 as *E. coli*. While in control group 7 were confirmed as *E. coli* and 4 *K. pneumoniae*.

4.3.1.4.4-Prevalence of *E. coli* and *K. pneumoniae* among urolithiatic patients with cUTI and healthy individuals

It was observed that 35% of urolithiatic patients were detected positive for *E. coli* hence there was 2.5 fold high risk of its infection in patients as compared to healthy individuals. In case of *K. pneumoniae*, significant isolation was observed in urolithiatic patients when compared with the healthy individuals as shown in Table 4.11.

4.3.1.4.5- Association of *E. coli* colonization with various sociodemographic and clinical attributes as risk factors for cUTI among urolithiatic patients

Prevalence of *E. coli* showed significant association with gender of urolithiatic patients (*P* = 0.02) as compared to the healthy controls. However, when comparing of urolithiatic patients with and without cUTI, no significant gender based difference was observed in isolation rate of *E. coli* (Table 4.13). It was observed that female patients were more prone to be infected with *E. coli.* There were 73(44.5%) females and 91(55.5%) males out of which 31 females and 23 males were found to be positive for *E. coli* isolation. About 77% of *E. coli* positive females were also suffering from cUTI. Different clinical risk factors were found to be associated with higher rate of *E. coli* isolation in patients with cUTI. These factors included long duration of stone disease, catheterization and urine pH. Patients with elevated level of white blood cells in the urine of patients had significantly higher *E. coli* isolation (Table 4.13).

4.3.1.4.6- Association of *K. pneumoniae* colonization with various sociodemographic and clinical attributes as risk factors for cUTI among urolithiatic patients

Comparative analysis of *K. pneumoniae* isolation with different sociodemographic features indicated that 63% urolithiatic patients were positive for *K. pneumoniae* isolation who were utilizing very hard and moderately hard water. *K. pneumoniae* isolation was also higher among cUTI patients belonging to low socioeconomic status (Table 4.15). In case of clinical risk factors, it was observed that significant isolation of *K. pneumoniae* was observed in patients with cUTI having long duration of stone disease and inserted catheters. Urinary pH also affected the isolation of *K. pneumoniae* in urolithiatic patients with cUTI (Table 4.16).

4.3.1.4.7- Association of different *Staphylococcus* species isolates with various sociodemographic and clinical attributes as risk factor for cUTI among urolithiatic patients

Low percentage of isolation was observed in case of Gram positive bacteria when compared with the Gram negative bacteria. There was no significant association observed for *Staphylococcus* species isolation with sociodemographic and clinical risk factors of urolithiatic patients with cUTI as shown in Table 4.17 and Table 4.18.

Table 4.9: Prevalence and association of *Staphylococcus* species colonization with urolithiatic patients and healthy controls

Category		**Urolithiatic patients n(%)**	**Controls n (%)**	***P-value***	**OR (95%CI)**
S. aureus	**Present**	15(9)	05(12.5)	0.52	0.71 (0.24-2.06)
	Absent	149(91)	35(87.5)		
S. saprophyticus	**Present**	08(5)	03(7.5)	0.5	0.63 (0.158-2.46)
	Absent	158(95)	37(92.5)		
S. epidermidis	**Present**	04(2)	05(12.5)	0.01	0.175 (0.04-0.68)
	Absent	160(98)	35(87.5)		

Table 4.10: Prevalence and association of *Staphylococcus* species colonization in urolithiatic patients with cUTI

Category		**Urolithiatic patients n(%)**		***P-value***	**OR (95%CI)**
		With cUTI	**Without cUTI**		
S. aureus	**Present**	09(8.6)	06(10)	0.733	0.82 (0.27-2.45)
	Absent	96(91.4)	53(90)		
S. saprophyticus	**Present**	07(6)	01(1.6)	0.156	4.14 (0.49-34.5)
	Absent	98(94)	58(98.4)		
S. epidermidis	**Present**	02(1.9)	02(3.4)	0.554	0.553 (0.07-4.03)
	Absent	103(98.1)	57(96.6)		

Table 4.11: Prevalence and association of *E. coli and K. pneumoniae* colonization with urolithiatic patients and healthy controls

Category		**Urolithiatic patients n(%)**	**Controls n(%)**	***P-value***	**OR (95%CI)**
E. coli	**Present**	54(33)	07(17.5)	0.06	2.3 (1.04-6.03)
	Absent	110(67)	33(82.5)		
K. pneumoniae	**Present**	59(36)	04(10)	0.003	5.1
	Absent	105(64)	36(90)		(1.7-14)

Table 4.12: Prevalence and association of *E. coli and K. pneumoniae* colonization with cUTI among urolithiatic patients

Category		**Urolithiatic patients n(%)**		***P-value***	**OR (95%CI)**
		With cUTI	**Without cUTI**		
E. coli	**Present**	40(38)	14(24)	0.08	1.97
	Absent	65(62)	45(76)		(0.96-4.05)
K. pneumoniae	**Present**	38(36)	21(36)	1	1.02
	Absent	67(64)	38(64)		(0.5-1.99)

Table 4.13: Association of *E. coli* colonization with various sociodemographic attributes as risk factors for cUTI among urolithiatic patients

Category	**Variables**	**Isolation of *E. coli***		***P-value***
		With cUTI	**Without cUTI**	
Socioeconomic status	Higher class	02	01	0.05
	Middle class	12	09	
	Lower class	26	04	
Quality of water	v. hard	11	04	0.879
	Hard	01	01	
	Moderately hard	18	06	
	Soft	10	03	
Water intake daily	Low	12	05	0.832
	Medium	18	05	
	High	10	04	
Location	Hilly	25	10	0.547
	Plan	15	04	
Living environment	Rural	11	08	0.04
	Urban	29	06	
Age range(yrs)	11-20	01	01	0.414
	21-30	09	06	
	31-40	08	03	
	41-50	09	03	
	51-60	07	01	
	61-70	06	0	
Gender	Male	16	07	0.515
	Female	24	07	
Marital status	Married	36	09	0.02
	Unmarried	04	05	

Table 4.14: Association of *E. coli* colonization with various clinical attributes as risk factors for cUTI among urolithiatic patients

Category	**Variables**	**Isolation of *E. coli***		***P-value***
		With cUTI	**Without cUTI**	
Stone duration	<1y	05	13	<0.001
	1-3y	22	01	
	>3y	13	0	
BMI	Underweight	0	0	0.445
	Normal	21	09	
	Overweight	19	05	
Catheterization	Yes	17	0	0.003
	No	33	14	
Urine pH	<6	01	05	<0.001
	6-6.5	18	08	
	>6.5	21	01	
WBCs in urine/HPF	0-5	01	08	<0.001
	5-100	34	06	
	>100	05	0	
Stone size (mm)	<4	04	01	0.557
	4-10	22	10	
	>10	14	03	

Table 4.15: Association of *K. pneumoniae* colonization with various sociodemographic attributes as risk factors for cUTI among urolithiatic patients

Category	**Variables**	**Isolation of *K. pneumoniae***		***P-value***
		With cUTI	**Without cUTI**	
Socioeconomic status	Higher class	0	01	0.08
	Middle class	10	10	
	Lower class	28	10	
Quality of water	v. hard	09	05	0.877
	Hard	0	0	
	Moderately hard	14	09	
	Soft	15	07	
Water intake daily	Low	11	07	0.935
	Medium	13	07	
	High	14	07	
Location	Hilly	26	15	0.810
	Plan	12	06	
Living environment	Rural	17	12	0.361
	Urban	21	09	
Age range(yrs)	11-20	02	01	0.129
	21-30	08	08	
	31-40	04	05	
	41-50	08	05	
	51-60	08	02	
	61-70	08	0	
Gender	Male	22	10	0.448
	Female	16	11	
Marital status	Married	33	15	0.146
	Unmarried	05	06	

Table 4.16: Association of *K. pneumoniae* colonization with various clinical attributes as risk factors for cUTI among urolithiatic patients

Category	**Variables**	**Isolation of *K. pneumoniae***		***P-value***
		With cUTI	**Without cUTI**	
Stone duration (yrs)	<1	04	15	<0.001
	1-3	23	06	
	>3	11	0	
BMI	Underweight	03	0	0.356
	Normal	19	13	
	Overweight	16	08	
Catheterization	Yes	16	01	0.002
	No	22	20	
Urine pH	<6	01	04	0.023
	6-6.5	19	13	
	>6.5	18	04	
WBCs in urine/HPF	0-5	01	09	0.001
	5-100	30	11	
	>100	07	01	
Stone size (mm)	<4	05	02	0.918
	4-10	26	15	
	>10	07	04	

Table 4.17: Association of different *Staphylococcus* species colonization with sociodemographic attributes as risk factors for cUTI among urolithiatic patients

Category	Variables	Isolation of *S. aureus*		Isolation of *S. saprophyticus*		Isolation of *S. epidermidis*	
		With cUTI	Without cUTI	With cUTI	Without cUTI	With cUTI	Without cUTI
Socioeconomic status	Higher class	0	02	0	0	0	0
	Middle class	02	02	01	0	0	01
	Lower class	07	02	06	01	02	01
P-value		0.114		0.686		0.248	
Quality of water	v. hard	03	02	02	01	0	01
	Hard	01	0	01	0	0	0
	Moderately hard	02	01	03	0	0	01
	Soft	03	03	01	0	02	0
P-value		0.808		0.592		0.135	
Water intake daily	Low	04	03	01	0	01	01
	Medium	02	01	02	0	01	01
	High	03	02	04	01	0	0
P-value		0.961		0.710		1	
Location	Hilly	04	04	03	0	01	01
	Plan	05	02	04	01	01	01
P-value		0.398		0.408		1	
Living environment	Rural	02	04	04	0	01	0
	Urban	07	02	03	01	01	02
P-value		0.08		0.285		0.248	
Age range(yrs)	11-20	0	0	01	0	0	0
	21-30	03	03	01	0	01	01
	31-40	02	0	0	0	0	0
	41-50	01	02	02	0	0	01
	51-60	02	01	02	0	01	0
	61-70	01	0	01	01	0	0
P-value		0.526		0.489		0.368	
Gender	Male	03	02	04	0	01	0
	Female	06	04	03	01	01	02
P-value		1		0.285		0.248	
Marital status	Married	09	05	06	01	02	01
	Unmarried	0	01	01	0	0	01
P-value		0.205		0.686		0.248	

Table 4.18: Association of different *Staphylococcus* species colonization with clinical attributes as risk factors for cUTI among urolithiatic patients

Category	**Variables**	**Isolation of *S. aureus***		**Isolation of *S. saprophyticus***		**Isolation of *S. epidermidis***	
		With cUTI	**Without cUTI**	**With cUTI**	**Without cUTI**	**With cUTI**	**Without cUTI**
Stone duration	<1y	02	05	02	01	01	01
	1-3y	04	01	03	0	01	01
	>3y	03	0	02	0	0	0
P-value		0.05		0.386		1	
BMI	Underweight	0	0	0	0	0	0
	Normal	05	05	06	01	02	0
	Overweight	04	01	01	0	0	02
P-value		0.264		0.686		0.04	
Catheterization	Yes	03	0	01	0	01	0
	No	06	06	06	01	01	02
P-value		0.114		0.686		0.248	
Urine pH	<6.5	05	05	02	0	01	02
	6.5-7	02	01	03	0	0	0
	>7	02	0	02	01	01	0
P-value		0.405		0.386		0.248	
WBCs in urine/HPF	0-5	01	02	0	0	0	01
	5-100	08	03	06	01	02	01
	>100	0	01	01	0	0	0
P-value		0.209		0.710		0.248	
Size of stone (mm)	<4	0	0	02	0	0	01
	4-10	07	06	04	01	01	01
	>10	02	0	01	0	01	0
P-value		0.215		0.686		0.368	

4.3.1.5- Phenotypic Screening of virulence factors of *E. coli*

4.3.1.5.1- Hemolysin production by *E. coli*

Hemolysin production assay was performed to check the α-hemolysin production ability of *E. coli*. Isolates that produced clear zone around colonies were considered as positive for β-hemolysin. Those, with partial hemolysis (α-hemolysis) appeared as grey colonies on blood agar. Isolates with ϒ-hemolysis did not change the colour of media. Out of 54 isolates, 40 (74%) showed α-hemolysis, 10 (18.5%) β hemolysis, while 04 (7.5%) were with ϒ-hemolysis activity. While isolates from control samples only two out of seven showed the hemolysis activity. In case of *E. coli* isolated from cUTI patients, it was observed that 92% of isolates showed the hemolysis activity. From beta hemolysin producer *E. coli*, 60% were isolated from urolithiatic patients with cUTI (Table 4.19).

Table 4.19: Association of hemolysin production by *E. coli* isolates with urolithiatic patients having cUTI and healthy individuals to assess their pathogenic potential

Category		***E. coli* isolates n(%)**			
		Urolithiatic patients	**Controls**	**Urolithiatic patients with cUTI**	**Urolithiatic patients without cUTI**
Hemolysis	**Present**	50(92.5)	02(28.5)	36(92)	14(93)
	Absent	04(7.5)	05(72.5)	03(8)	01(7)
P-value		<0.001		0.89	
OR (95% CI)		31.3 (4.5-215)		0.85 (0.08-8.9)	
Type of Hemolysis	**alpha**	40(74)	02(28.5)	30(76)	10(66)
	beta	10(18.5)	0(0)	06(16)	04(27)
	gama	04(7.5)	05(72.5)	03(8)	01(7)

4.3.1.5.2- Serum bactericidal activity against *E. coli*

There was a slight change in growth with 80% serum as compared to broth. This effect was seen over period of 2-4 h of incubation. During 4-6 h, an increase in growth was observed for isolates in both concentrations of serum, but this growth was low when compared with heated serum and broth. Concentration of serum strongly affected the growth rate. *E. coli* isolates grew rapidly in 40% serum than in 80% serum. *E. coli* isolates showed complete resistance to 40% serum after 24 h of incubation (Appendix B-IV, B-V).

4.3.1.5.3- Haemagglutination assay

It was observed that *E. coli* isolates agglutinated RBCs of blood group B more as compared to RBCs of blood group A. After 2h, slight variation was observed as small number of RBCs was agglutinated in both blood groups (A and B). With the passage of time, haemagglutination was increased for both but more in case of blood group B than blood group A. Only 07 (13%) isolates showed agglutination of blood group A and 12 (22%) showed agglutination of blood group B. There were 05 isolates showed agglutination of both blood groups. Overall, 14 isolates of *E. coli* from which 10 were isolated from urolithiatic patients with cUTI showed the haemagglutination activity (Table 4.20).

Table 4.20: Association of haemagglutination of RBCs by *E. coli* isolates with urolithiatic patients having cUTI and healthy individuals to assess their pathogenic potential

Category		***E. coli* isolates n(%)**			
		Urolithiatic patients	**Healthy Controls**	**Urolithiatic patients with cUTI**	**Urolithiatic patients without cUTI**
Haemagglutination	**Present**	14(26)	02(28.5)	10(26)	04(27)
	Absent	40(74)	05(72.5)	29(74)	11(73)
P-value		0.88		0.93	
OR(95% CI)		0.87 (0.15-5.03)		0.94 (0.24-3.6)	

4.3.1.5.4- Biofilm formation ability of *E. coli*

Biofilm forming ability of *E. coli* was assessed through qualitative and quantitative methods.

4.3.1.5.4.1-Congo red assay

Isolates with pink orange colonies were considered as non-biofilm former and crystal black colonies taken as strong biofilm formers. Out of total isolates, 9 (14%) appeared as strong biofilm former, 37(57%) as moderate, whereas 19 (29%) as non-biofilm former. Among isolates from controls, none appeared to be strong biofilm former; they showed intermediate and weak biofilm forming ability.

4.3.1.5.4.2-Microtiter plate (MTP) assay

In microtiter plate assay, OD and cut off OD was calculated. Among isolates from patients, 02(3%) were strong biofilm formers, 22(41%) moderate and 30 (56%) non-biofilm former were observed. From healthy controls, 04(57%) isolates showed the biofilm forming ability, out of which three weak and one was moderate biofilm formers (Table 4.21). It was observed that majority of *E. coli* isolates from patients with cUTI showed moderate biofilm forming ability.

Table 4.21: Association of biofilm forming ability of *E. coli* isolates with urolithiatic patients having cUTI and healthy controls to assess their pathogenic potential

Category		***E. coli* isolates n(%)**			
		Urolithiatic patients	**Healthy Controls**	**Urolithiatic patients with cUTI**	**Urolithiatic patients without cUTI**
Biofilm activity	**Present**	24(44)	04(57)	17(43.6)	07(47)
	Absent	30(56)	03(43)	22(56.4)	08(53)
P-value		0.53		0.83	
OR(95% CI)		0.6 (0.12-2.94)		0.88 (0.26-2.9)	
Strength of biofilm activity	**Negative**	30(56)	03(43)	22(56.4)	08(53)
	Weak	0	03(43)	0	01(7)
	Moderate	22(41)	01(14	16(41)	05(33)
	Strong	02(3)	0	01(2.6)	01(7)

3.1.5.5-Association of virulence factors of *E. coli* isolates with different sociodemographic and clinical attributes as risk factors for cUTI among urolithiatic patients

Virulence factors of *E. coli* such as biofilm formation, hemolysin production and haemagglutination were assessed as risk factors for cUTI in urolithiasis. There was no significant association observed for virulence factors with any other risk factors as shown in Table 4.22.

Table 4.22: Association of different sociodemographic and clinical attributes with virulence factors of *E. coli* of urolithiatic patients with cUTI

Risk Factors		***E. coli* isolates**								
		Hemolysis activity			Biofilm forming ability			Haemagglutination activity		
		Urolithiatic patients		***P*-value**	Urolithiatic patients		***P*-value**	Urolithiatic patients		***P*-value**
		With cUTI	Without cUTI		With cUTI	Without cUTI		With cUTI	Without cUTI	
Stone duration (yrs)	<1	14	05	0.971	07	03	0.696	03	01	0.511
	1-3	14	06		05	03		02	02	
	>3	08	03		05	01		05	01	
BMI	Underweight	0	0	0.356	0	0	0.476	0	0	0.597
	Normal	19	08		10	03		06	03	
	Overweight	17	06		07	04		04	01	
Catheterization	Yes	14	01	0.111	06	01	0.303	03	0	0.217
	No	22	13		11	06		07	04	
Age (Yrs)	11-20	01	01	0.122	0	01	0.154	0	01	0.248
	21-30	09	04		05	02		02	0	
	31-40	07	04		02	03		0	01	
	41-50	10	01		04	0		05	01	
	51-60	05	02		02	01		02	01	
	61-70	04	02		04	0		01	0	
Gender	Male	15	07	0.346	07	03	0.939	03	03	0.124
	Female	21	07		10	04		07	01	
Urine pH	<6	04	02	0.957	0	01	0.280	01	0	0.680
	6-6.5	14	07		08	03		03	02	
	>6.5	18	05		09	03		06	02	
WBCs in urine/ HPF	0-5	05	04	0.309	0	03	0.013	0	01	0.226
	6-100	26	10		15	04		09	03	
	>100	05	0		02	0		01	0	
Stone size(mm)	<4	04	01	1	01	01	0.739	02	0	0.627
	4-10	22	08		12	04		06	03	
	>10	10	05		04	02		02	01	

4.3.1.6-Phenotypic screening of virulence factors of *K. pneumoniae*

4.3.1.6.1-Hypermucoviscosity test to differentiate Classical and hypervirulent *K. pneumoniae* (*hvKP*) strains

String test was performed to differentiate the isolates as classical vs. hypervirulent. Isolates forming string equal to or greater than 5mm were considered as string test positive. Out of 69 isolates from urolithiatic patients samples, 22 (32%) were string test positive and were assigned as hypervirulent *K. pneumoniae* isolates. Whereas remaining 47(70%) isolates were considered as classical *K. pneumoniae* (*cKP*). No isolate of *K. pneumoniae* was hypervirulent from control samples.

4.3.1.6.2-Hemolysin production by *cKP* and *hvKP*:

Hemolysin production assay was performed to check the α-hemolysin production ability of *K. pneumoniae.* Out of 69 isolates, 51 (74%) showed α-hemolysis and 18(26%) did not show hemolysin production. Among α-hemolysis positive isolates, 19 were *hvKP* and 32 were *cKP* (Table 4.24). While in control samples, no isolate showed the hemolysis. It was observed that majority of isolates from patients with cUTI showed hemolysin production (Table 4.23).

Table 4.23: Association of hemolysin production by *K. pneumoniae* isolates with urolithiatic patients having cUTI and healthy controls to assess their pathogenic potential

Category		***K. pneumoniae* isolates n (%)**			
		Urolithiatic patients	**Controls**	**Urolithiatic patients with cUTI**	**Urolithiatic patients without**
Hemolysis	**Present**	51(74)	0(0)	40(73)	11(77)
	Absent	18(26)	04(100)	15(27)	03(23)
P-value		0.03		0.747	
OR(95% CI)		25 (1.28-488)		0.727 (0.177-2.97)	

Table 4.24: Association of hemolysin production by *cKP* and *hvKP* isolates with cUTI among urolithiatic patients to assess their pathogenic potential

Category		***cK. Pneumoniae* isolates n(%)**		***hvK. Pneumoniae* isolates n(%)**	
		Urolithiatic patients		**Urolithiatic patients**	
		With cUTI	**Without cUTI**	**With cUTI**	**Without cUTI**
Hemolysis	**Present**	28(72)	05(62.5)	12(75)	06(100)
	Absent	11(28)	03(37.5)	04(25)	0(0)
P-value		0.601		0.176	
OR (95% CI)		1.5 (0.3-7.5)		0.2 (0.009-4.6)	

4.3.1.6.3- Haemagglutination activity of *cKP* and *hvKP*

It was observed that *K. pneumoniae* isolates agglutinated RBCs of blood group B as compared to RBCs of blood group A. After 2hrs, slight variation was observed and little agglutination of RBCs from both blood groups A and B was observed. With the passage of time, haemagglutination was increased for both types of RBCs but was more in case of blood group A than blood group B. When haemagglutination activity of *cKP* and *hvKP* was compared, *hvKP* agglutinated RBCs of blood group A, more than *cKP,* whereas *cKP* agglutinated RBCs of blood group B more rapidly than *hvKP.*

Table 4.25: Association of haemagglutination of RBCs by *K. pneumoniae* isolates with urolithiatic patients having cUTI and healthy individuals to assess their pathogenic potential

Category		***K. pneumoniae* isolates n(%)**			
		Urolithiatic patients	**Healthy Controls**	**Urolithiatic patients with cUTI**	**Urolithiatic patients without cUTI**
Haemagglutination	**Present**	39(56.5)	0(0)	28(51)	11(78.5)
	Absent	30(43.5)	04(100)	27(49)	03(21.5)
P-value		0.14		0.07	
OR (95% CI)		5.2 (0.55-48.9)		0.28 (0.07-1.12)	

4.3.1.6.4-Serum bactericidal activity against *cKP* and *hvKP*

Serum bactericidal assay was performed to evaluate the serum resistance characteristics of *cKP* and *hvKP*. Serum resistance was observed in both *cKP* and *hvKP*. *cKP* isolates were more resistant to 80% serum than *hvKP.* This effect was seen during 2h of incubation but with the passage of time *cKP* isolates showed more sensitivity towards 80% serum than *hvKP*. After 24h of incubation, intermediate resistance was shown by both *cKP* and *hvKP.* Overall, there was no complete killing even after 24h of incubation. c*KP* isolates showed complete resistance to 40% serum, when compared with heat inactivated serum. Similar trend was displayed by *hvKP* too. Significant anti bactericidal activity of urolithiatic patients' *K. pneumoniae* isolates was recorded as compared to isolates from control group (Table 4.26) (Appendix B-VI, B-VII).

Table 4.26: Association of serum resistance of *K. pneumoniae* isolates with urolithiatic patients having cUTI and healthy individuals to assess their pathogenic potential

Category		***K. pneumoniae* isolates n(%)**			
		Urolithiatic patients	**Healthy Controls**	**Urolithiatic patients with cUTI**	**Urolithiatic patients without cUTI**
Serum resistance activity	**Present**	64(92.7)	01(0)	51(93)	13(93)
	Absent	5(7.2)	03(75)	04(7)	01(7)
P-value		0.003		0.98	
OR (95% CI)		38.4 (3.3-440)		0.98 (0.1-9.5)	
Strength of serum resistance activity	**Sensitive**	5(7.2)	03(75)	04	01
	Intermediate	5(7.2)	01(25)	05	0
	Resistant	59(85.5)	0(0)	46	13

Table 4.27: Association of serum resistance of c*K.pneumoniae* and *hvK. Pneumoniae* isolates with cUTI in urolithiatic patients to assess their pathogenic potential

Category		***cK. pneumoniae* isolates n(%)**		***hvK. Pneumoniae* isolates n(%)**	
		Urolithiatic patients		**Urolithiatic patients**	
		With cUTI	**Without cUTI**	**With cUTI**	**Without cUTI**
Serum resistance	**Present**	37(95)	08(100)	14(87.5)	05(83)
	Absent	02(5)	0(0)	02(12.5)	01(17)
P-value		0.9		0.8	
OR (95% CI)		0.88 (0.03-20)		1.4 (0.1-19)	
Strength of Serum resistance	**Sensitive**	02(5)	08(100)	02(12.5)	01(17)
	Intermediate	05(13)	0(0)	0(0)	0(0)
	Resistant	32(82)	0(0)	14(87.5)	05(83)

4.3.1.6.5-Biofilm formation characteristics of *cKP* and *hvKP* strains

Biofilm forming ability of *K. pneumoniae* was assessed through qualitative and quantitative methods.

4.3.1.6.5.1- Congo red assay

Congo red assay was performed to identify biofilm forming ability of *K. pneumoniae* based on morphological characteristics shown on Congo red agar plate. Isolates with pink orange colonies were considered as non-biofilm formers and crystal black colonies were taken as strong biofilm formers. Out of total isolates, 38(53.6%) appeared as strong biofilm formers, 15(24.6%) as moderate, 1 weak whereas 15(23%) as non-biofilm formers. Biofilm forming ability was displayed in 23 (53.5%) and 13 (50%) in *cKP* and

hvKP respectively. Among isolates from healthy controls, none of the isolate was strong biofilm formers.

4.3.1.6.5.2- Microtiter Plate Assay

In microtiter plate assay among all *K. pneumoniae* isolates 60(86.9%) isolates were strong biofilm formers, 4(5.7%) moderate biofilm formers, 03(4.3%) weak biofilm former and 03(4.3%) non-biofilm formers were observed. In isolates from healthy control samples, 03 showed the biofilm forming ability where 02(67%) were weak and 01(33%) showed intermediate biofilm forming activity. Strength of biofilm formation activity was observed to be higher in urolithiatic patients with cUTI as compared to healthy individuals. There were more biofilm former *K. pneumoniae* isolates in patients with cUTI as compared to the patients without cUTI (Table 4.28). In case of *hvKP*,20 isolates showed the strong biofilm forming ability (Table 4.29).

Table 4.28. Association of biofilm forming ability of *K. pneumoniae* isolates with urolithiatic patients having cUTI to assess their pathogenic potential

Category		***K. pneumoniae* isolates n(%)**			
		Urolithiatic patients	**Controls**	**Urolithiatic patients With cUTI**	**Urolithiatic patients Without cUTI**
Biofilm activity	**Present**	66(95.6)	03(75)	54(98)	12(86)
	Absent	03(4.4)	01(25)	01(2)	02(14)
P-value		0.12		0.08	
OR(95% CI)		7.3 (0.57- 93.2)		9 (0.75-107.5)	
Strength of biofilm activity	**No**	03(4.4)	01(25)	01(2)	02(14)
	Weak	02(2.8)	02(50)	02(3.7)	0
	Intermediate	04(5.7)	01(25)	04(7.3)	0
	Strong	60(86.9)	0(0)	48(87)	12(86)

Table 4.29. Association of biofilm forming ability of *cK. pneumoniae* and *hvK. pneumoniae* isolates with cUTI among urolithiatic patients to assess their pathogenic potential.

Category		***cK. Pneumoniae* isolates n(%)**		***hvK. Pneumoniae* isolates n(%)**	
		Urolithiatic patients		**Urolithiatic patients**	
		With cUTI	**Without cUTI**	**With cUTI**	**Without cUTI**
Biofilm activity	**Present**	38(97)	07(87.5)	16(100)	05(100)
	Absent	01(3)	01(12.5)	0(0)	0(0)
***P*-value**		0.25		0.5	
OR (95% CI)		5.4 (0.3-97.3)		3 (0.05-169.9)	
Strength of Biofilm activity	**No**	01(3)	01(12.5)	0	0(0)
	Low	01(3)	0(0)	01(6)	0(0)
	Medium	04(10)	0(0)	0(0)	0(0)
	High	33(84)	07(87.5)	15(94)	05(100)

4.3.1.6.6-Association of virulence factors of *K. pneumoniae* isolates with various sociodemographic and clinical risk factors with cUTI in urolithiasis

4.3.1.6.6.1-Hemolysin activity:

Among α-hemolysin positive *K. pneumoniae* isolates, 18/22 were *hvKP* and 33/47 were *cKP* isolates. However, c*KP* isolates with α-hemolysis were more prevalent among urolithiatic patients with cUTI as compared to *hvKP*. More (67%) alpha hemolysin positive *K. pneumoniae* were isolated from patients having urolithiasis for more than one year. Overall *K. pneumoniae* isolates with hemolysin activity were present in cUTI patients with high levels of urinary white blood cells indicating severe infection in these patients (Table 4.30).

4.3.1.6.6.2-Serum bactericidal activity

Majority of serum resistant isolates of *cKP* and *hvKP* were from patients having cUTI. A significantly higher *hvKP* were serum resistant and were isolated from catheterized patients. Majority resistant *cKP* (65%) were isolated from the patients having urolithiasis

for more than one year (P<0.001). Significantly higher *K. pneumoniae* isolated from cUTI patients with elevated white blood cell level and inserted catheters showed serum resistance activity (Table 4.31).

4.3.1.6.6.3-Biofilm formation ability

Most of the *K. pneumoniae* isolates were strong biofilm formers and association of biofilm formation activity with different risk factors was assessed. Of 69 isolates of *K. pneumoniae,* 60 showed biofilm formation ability. *hvKP* isolates were mostly biofilm formers (91%). Comparatively *cKP* showed less biofilm forming activity. Biofilm forming ability of *K. pneumoniae* isolates from urolithiatic patients having cUTI was more pronounced (80%) and had significant association with cUTI (P = 0.02). Isolates from male patients with cUTI were more biofilm former than female patients. When considering age of patients, it was observed that strong biofilm former *K. pneumoniae* isolates were present in all age groups. Significantly higher biofilm formers *K. pneumoniae* were isolated from the patients with prolong disease conditions and catheterization (Table 4.32).

4.3.1.7-Antibiotic susceptibility pattern

4.3.1.7.1-Antibiotics susceptibility pattern of *E. coli*

Kirby-Bauer disk diffusion method was used to determine the antibiotic sensitivity of *E. coli* isolates. In case of aminoglycosides, 83% isolates were susceptible to amikacin, 74% to gentamycin followed by 35% to tobramycin. Ciprofloxacin, ofloxacin and nalidixic acid were tested from fluoroquinolones class of antibiotics. For ciprofloxacin, ofloxacin and nalidixic acid 52%, 35.2% and 11% sensitivity in isolates was recorded respectively. Cephalosporins of first and third generations were used, 30% of isolates were observed to be sensitive for ceftazidime and 31.5% for ceftriaxone. Tetracycline sensitivity was observed in 68% isolates. Only 7.4% amoxicillin sensitive isolates were present. Isolates with susceptibility for nitrofurantoin were 61%. Imipenem was used from class of carbapenem to detect the sensitivity, which was in 66.7% of isolates. Out of 54 isolates, 44 can be considered as multidrug resistant based on CLSI guidelines as resistance was

present to more than two drugs of one class and towards three or more than three classes of antibiotics (Appendix B-II).

4.3.1.7.1.2-Double disc synergy test for ESBL Detection

Double disc synergy test was performed to detect the production of ESBL by *E. coli* isolates. Zone of inhibition was measured after incubation. The extension of zone of inhibition by ceftriaxone toward amoxicillin and clavulanic acid disc was the indication of positive result. Out of 54 *E. coli* isolates, 37 (69%) were ESBL producers.

4.3.1.7.1.3-Detection of ESBL and quinolone resistance genes among *E. coli* isolates from urolithiatic patients

Out of all *E. coli* isolates, bla*CTX-M* was present in 22(41%), bla*TEM* in 16(30%) and bla*SHV* in 5(9%) isolates. Among phenotypic ESBL positive isolates, 19(51%), 15(40%) and 5(13%) had bla*CTX-M*, bla*TEM* and bla*SHV* genes respectively. In total multi drug resistant isolates, 20(39) had bla*CTX-M* gene, 14(29%) bla*TEM* and 5 (10%) had bla*SHV*. Characterization of quinolone resistance genes revealed that, among total *E. coli*, *qnrA* was present in 6 (11%), *qnrB* in 3(6%) and *qnrS* in 10 (19%) isolates. Among ESBL positive isolates, *qnrA*, *qnrB* and *qnrS* positive strains were 6 (15%), 2 (5%) and 09 (24%) respectively (Table 4.33) (Appendix B-VIII, B-IX).

4.3.1.7.2-Antibiotic susceptibility pattern of *K. pneumoniae*

Kirby-Bauer disk diffusion method was used to determine the antibiotic sensitivity in *K. pneumoniae* against different classes of antibiotics. Out of 69 isolates, in case of aminoglycosides 41% were susceptible to gentamycin, 48% to tobramycin and 65.2% sensitivity for amikacin. In case of ciprofloxacin and ofloxacin 38% and 45% sensitive isolates were observed respectively. Cephalosporins of first and third generations were used, 53% sensitive isolates were observed for ceftazidime, 26% for ceftriaxone and 20% for cephalothin. For tetracycline 69.5% of isolates were susceptible. Only 1.4% sensitivity was observed for amoxicillin. Isolates with susceptibility for nitrofurantoin were 39%. Imipenem was used from class of carbapenem for which 48% of isolates were sensitive. Out of 69 isolates, 23 can be considered as multidrug resistant on basis of

guidelines of CLSI as they showed resistance pattern against more than two drugs of one class and towards two or more than two classes of antibiotics. Among them 3 were *hvKP* isolates (Appendix B-III).

4.3.1.7.2.1-Comparison of susceptibility pattern of *hvKP* and *cKP*

Antibiotic susceptibility pattern among *cKP* and *hvKP* was observed. Among aminoglycosides most sensitive drug was amikacin and least sensitive was gentamycin in *cKP* isolates. None of the drug was effective for *cKP* isolates from fluoroquinolones class of antibiotics as 68% resistance was observed for ciprofloxacin followed by 58% for ofloxacin. While in case of *hvKP* most of the drugs were effective except clindamycin, amoxicillin and cephalosporins that showed 100% and 54% resistance respectively.

4.3.1.7.2.2-Double disc synergy test for ESBL Detection

Out of 69 *K. pneumoniae* isolates, 24 (36%) were ESBL producers.

4.3.1.7.2.3-Detection of ESBL and quinolone resistance genes among *K. pneumoniae* isolates

Isolates were tested for antibiotic susceptibility and double disc synergy test for ESBL production. Based on their results, positive isolates were subjected to molecular characterization of ESBL genes (bla*CTX-M*, bla*TEM*, bla*SHV*) along with quinolone resistance genes (*qnrA*, *qnrB*, *qnrS*) to check their prevalence. Out of all *K. pneumoniae* isolates, bla*CTX-M* was present in 14(20%), bla*TEM* in 05(7%) and bla*SHV* in 01(1.5%) isolates. Among ESBL positive isolates, 08(33%), 03(12.5%) and 01(4%) had bla*CTX-M*, bla*TEM* and bla*SHV* genes respectively. In total multi drug resistant isolates, 04(17.4) had bla*CTX-M* gene, 03(13%) had bla*TEM* and 01 (4.3%) had bla*SHV*. Characterization of quinolone resistance genes revealed that, among total *K. pneumoniae* isolates, *qnrA* was present in 02 (3%), *qnrB* in 01 (1.5%) and *qnrS* in 05 (7.2%) isolates. Among ESBL positive isolates, *qnrA*, *qnrB* and *qnrS* positive strains were 02 (8%), 01(4%) and 03(12%) respectively. Also, among multidrug resistant isolates, 02(9%) were positive for *qnrA*, 01(4.5%) for *qnrB* and 05(22%) were positive for *qnrS*. (Table 4.34) (Appendix B-X).

Table 4.30 Association of alpha hemolysin production by *K. pneumoniae* (*hvKP* and *cKP*) with different clinical and sociodemographic attributes as risk factors for cUTI in urolithiatic patients

Risk Factors		Alpha hemolysis activity								
		cK. pneumoniae			*hvK. pneumoniae*			Overall *K. pneumoniae*		
		Urolithiatic patients		*P*-value	Urolithiatic patients		*P*-value	Urolithiatic patients		*P*-value
		With cUTI	Without cUTI		With cUTI	Without cUTI		With cUTI	Without cUTI	
Stone duration (yrs)	<1	05	05	0.001	01	05	0.006	06	10	<0.001
	1-3	17	00		07	01		24	01	
	>3	06	0		04	0		10	0	
BMI	Underweight	02	0	0.804	01	0	0.755	03	0	0.579
	Normal	17	03		06	03		23	06	
	Overweight	09	02		05	03		14	05	
Catheterization	Yes	11	0	0.186	03	0	0.180	14	0	0.054
	No	17	05		09	06		26	11	
Age (Yrs)	11-20	0	0	0.361	02	0	0.252	02	0	0.057
	21-30	07	03		02	04		09	07	
	31-40	05	01		02	0		07	01	
	41-50	03	01		03	02		06	03	
	51-60	09	0		01	0		10	0	
	61-70	04	0		02	0		06	0	
Gender	Male	16	05	0.675	07	03	0.269	23	08	0.360
	Female	12	0		05	03		17	03	
Urine pH	<6	02	0	0.666	01	02	0.325	03	02	0.399
	6-6.5	17	04		06	03		23	07	
	>6.5	09	01		05	01		14	02	
WBCs in urine/ HPF	0-5	03	03	0.028	01	02	0.343	04	05	0.017
	6-100	21	02		10	04		31	06	
	>100	14	0		01	0		05	0	
Stone size(mm)	4	05	0	0.278	0	01	0.325	05	01	0.320
	4-10	18	05		09	05		27	10	
	>10	05	0		03	0		08	0	

Table.4.31 Association of serum resistance of *cKP* and *hvKP* with various clinical and sociodemographic attributes as risk factors for cUTI in urolithiatic patients

Risk Factors		Serum resistance activity								
		cK. pneumoniae			*hvK. pneumoniae*			Overall *K. pneumoniae*		
		Urolithiatic patients		*P*-value	Urolithiatic patients		*P*-value	Urolithiatic patients		*P*-value
		With cUTI	Without cUTI		With cUTI	Without cUTI		With cUTI	Without cUTI	
Stone duration (yrs)	**<1**	07	07	<0.001	02	04	0.023	09	11	<0.001
	1-3	22	01		08	01		30	02	
	>3	08	0		04	0		12	0	
Catheterization	**Yes**	19	0	0.029	03	0	0.259	22	0	0.014
	No	18	08		11	05		29	13	
BMI	**Underweight**	04	05	0.619	01	0	0.827	05	05	0.50
	Normal	20	03		08	03		28	06	
	Overweight	13	0		05	02		18	02	
Age (Yrs)	**11-20**	0	01	0.084	02	0	0.313	02	01	0.08
	21-30	10	04		02	03		12	07	
	31-40	07	01		03	0		10	01	
	41-50	05	02		04	02		09	04	
	51-60	10	0		01	0		11	0	
	61-70	05	0		02	0		07	0	
Gender	**Male**	22	06	0.411	07	03	0.701	29	09	0.418
	Female	15	02		07	02		22	04	
Urine pH	**<6**	03	0	0.55	01	01	0.557	04	01	0.308
	6-6.5	21	07		07	03		28	10	
	>6.5	13	01		06	01		19	02	
WBCs in urine/ HPF	**0-5**	03	04	0.009	01	02	0.203	04	06	0.002
	6-100	28	04		12	03		40	07	
	>100	06	0		01	0		07	0	
Stone size(mm)	**4**	05	01	0.411	0	01	0.701	05	02	0.540
	4-10	25	06		11	04		36	10	
	>10	07	01		03	0		10	01	

Table 4.32: Association of biofilm formation ability of c*KP* and *hvKP* with various clinical and sociodemographic attributes as risk factors for cUTI in urolithiatic patients

Variables		Biofilm formation activity								
		cK. pneumoniae			***hvK. pneumoniae***			**Overall *K. pneumoniae***		
		Urolithiatic patients		***P*-value**	**Urolithiatic patients**		***P*-value**	**Urolithiatic patients**		***P*-value**
		With cUTI	**Without cUTI**		**With cUTI**	**Without cUTI**		**With cUTI**	**Without cUTI**	
Stone duration (yrs)	<1	07	07	<0.001	02	04	0.01	09	11	<0.001
	1-3	23	0		09	01		32	01	
	>3	08	0		05	0		13	0	
Catheterization	Yes	18	0	0.04	05	0	0.15	23	0	0.01
	No	20	07		11	05		31	12	
BMI	Underweight	03	0	0.57	01	0	0.73	04	0	0.61
	Normal	20	05		08	02		28	07	
	Overweight	15	02		07	03		22	05	
Age (Yrs)	11-20	0	01	0.05	0	0	0.26	02	01	0.04
	21-30	09	04		02	0		11	07	
	31-40	07	01		02	03		10	01	
	41-50	05	01		03	0		10	03	
	51-60	15	0		05	02		12	0	
	61-70	06	0		01	0		09	0	
Gender	Male	23	05	0.58	08	02	0.69	31	07	0.95
	Female	15	02		08	03		23	05	
Urine pH	**<6**	03	0	0.3	01	02	0.15	04	02	0.27
	6-6.5	21	06		08	02		29	08	
	>6.5	14	01		07	01		21	02	
WBCs in urine/ HPF	**0-5**	03	04	0.003	01	01	0.57	04	05	0.005
	6-100	28	03		14	04		42	07	
	>100	07	0		01	0		08	0	
Stone size(mm)	**4**	04	0	0.617	01	01	0.42	05	01	0.676
	4-10	27	06		12	04		39	10	
	>10	07	01		03	0		10	01	

Table 4.33: Detection of ESBL and quinolone resistance genes in *E. coli* isolates among phenotypic ESBL producer and multidrug resistant *E. coli*

Genes		**Total *E. coli* isolates n(%)**	**ESBL positive *E. coli* n(%)**	**Multidrug resistant *E. coli* n(%)**
Total isolates		**54**	**37**	**44**
ESBL Genes	bla-*CTX-M*	22 (41)	19 (51)	20 (39)
	bla-*TEM*	16 (30)	15 (40)	14 (29)
	bla-*SHV*	05 (9)	05 (13)	05 (10)
Quinolone Resistance Genes	*qnr-A*	06 (11)	06 (15)	06 (12)
	qnr-B	03 (6)	02 (5)	03 (6)
	qnr-S	10(19)	09 (24)	10 (21)

Table 4.34: Detection of ESBL and quinolone resistance genes among phenotypic ESBL producer and multidrug resistant *K. pneumoniae* isolates

Genes		**Total *K. pneumoniae* n (%)**	**ESBL positive *K. pneumoniae* n (%)**	**Multidrug resistant *K. pneumoniae* n (%)**
Total isolates		**69**	**24**	**23**
ESBL Genes	bla-*CTX-M*	14(20)	08 (33)	04 (17.4)
	bla-*TEM*	05(7)	03 (12.5)	03 (13)
	bla-*SHV*	01(1.5)	01 (4)	01 (4.3)
Quinolone Resistance Genes	*qnr-A*	02(3)	02 (8)	02(9)
	qnr-B	01(1.5)	01 (4)	1 (4.5)
	qnr-S	05(7.2)	03 (12)	5 (22)

4.3.2-Part II: study of Bacterial diversity by culture independent methods

4.3.2.1-Sequence characteristics

From four pooled urine samples, 398 reads were obtained with average of 99 reads per sample. In total detected OTUs were 253. After data trimming, filtering and normalization, predominantly detected OTU with count ≥ 2 were 167.

4.3.2.2-Taxonomic analysis

A total of 13 phyla, 25 classes, 46 orders, 92 families, 158 genera and 223 species were observed in all of these four pooled samples. Subject SP1 had 9 phyla, it has dominated by Proteobacteria (53.7%) followed by Firmicutes (18.8%), Bacteroidetes (18.3%),

Fusobacteria (7.04%), Actinobacteria (2.04%), Verrucomicrobia (0.065%) and Cyanobacteria (0.03%). Urolithiatic patient SP2 had 7 phyla with predominant Proteobacteria (98.3%) followed by Actinobacteria (1.45%), Firmicutes (0.2%) and Bacteriodetes (0.05%). In case of pooled control sample SP3, 11 phyla were present with predominant Firmicutes (73.5%) followed by Proteobacteria (24%), Bacteroidetes (1.3%), Actinobacteria (0.5%), Fusobacteria (0.4%), Verrucomicrobia (0.07%), Cyanobacteria (0.06%) and Ignavibacteriae (0.05%). In SP4 samples 11 phyla were present, it was dominated by Proteobacteria (97%) followed by actinobacteria (1.3%), Bacteriodetes (0.9%) and Firmicutes (0.4%) as shown in Figure 4.1.

In these samples, abundance of major genera was also detected. In SP1 samples major genera were *Prevotella* (15%) followed by *Enterobacter* (10.6%), *Achromobacter* (10%), *Agrobacterium* (8.4%), *Alcaligenes* (7.5%), *Fusobacterium* (7%), *Serratia* (3.8%), *Finegoldia* (3%), *Sporobacterium* (2.9%), *Anaerococcus*(2.8%), *Pseudomonas* (2.7%), *Peptoniphilus* (2.6%), *Streptococcus* (1.8%), *Porphyromonas* (1.5%),*Sphingomonas* (1.5%), *Dialister* (1.4%), *Campylobacter* (1.4%), *Corynebacterium* (1.12%), *Novosphingobium*(1%) and *Granulicatella*(1%). In SP2 samples, the most prominent genus was *Acinetobacter* which covered the 84% bacterial diversity in that patient followed by *Enterobacter* (13.2%) and *Bifidobacterium* (1.4%).

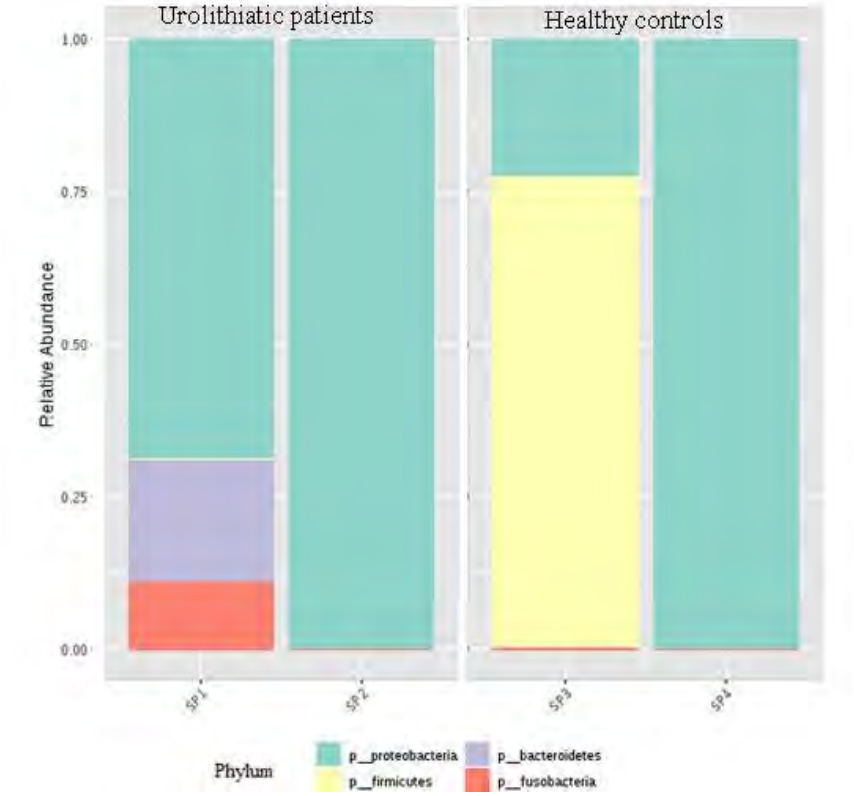


Figure 4.1: Relative abundance of predominant urinary bacterial taxa in urolithiatic patients samples with cUTI(SP1, SP2) and healthy individuals samples (SP3, SP4) at phylum level analyzed by Microbiome analyst tool using cutoff value >10 counts.

In SP3 female healthy individual samples, major genera were Lactobacillus (70.7%) followed by *Enterobacter* (6.1%), *Achromobacter* (5%), *Alcaligenes* (2.5%), *Sphingomonas*(1.2%), *Agrobacterium* (1.2%), *Serratia* (1.16%) and *Pseudomonas* (1.06%). In SP4 sample, predominant genera were *Achromobacter* (42%) followed by*Enterobacter* (13.6%), *Inquilinus* (8.8%), *Sphingomonas* (6.7%), *Alcaligenes* (4.8%), *Bradyrhizobium* (4.5%), *Ochrobactrum* (3.9%) *Ralstonia* (2.5%), *Serratia* (1.7%), *Agrobacterium* (1.6%), *Pseudomonas* (1.6%), *Methylobacterium* (1.6%), *Cryocola* (1.2%) and*Caulobacter* (1.14%).

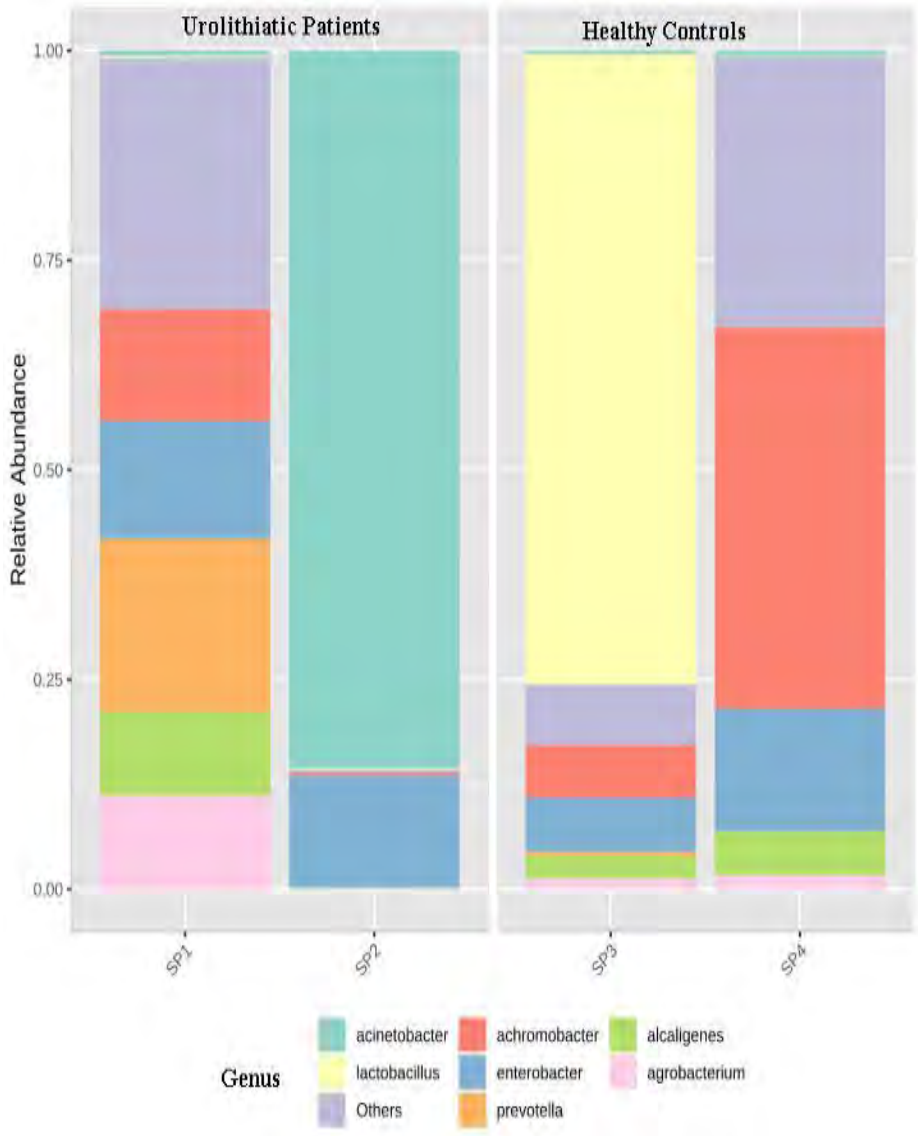


Figure 4.2: Relative abundance of predominant urinary bacterial taxa in urolithiatic patients samples with cUTI (SP1, SP2) and healthy individuals samples (SP3, SP4) at genus level analyzed by Microbiome analyst tool using cutoff value >10 counts.

Abundance of species was observed in all of these samples, where as in SP1 urolithiatic female patients with cUTI, major species found were *Prevotella*spp. (12%) followed by *Enterobacter hormaechei* (10.6%), *Achromobacter xylosoxidans* (10%), *Agrobacterium tumefaciens*(8.3%), *Fusobacterium nucleatum* (7%), *Serratiaquinivorans* (3.8%), *Finegoldia magna* (3%), *Sporobacterium*spp. (2.9%), *Peptoniphilusasaccharolyticus*(2.6%), *Anaerococcushydrogenalis* (1.8%), *Pseudomonas putida* (1.5%), *Sphingomonas caulobacterleidyia* (1.5%), *Porphyromonasuenonis* (1.3%), *Prevotellabivia* (1.3%), *Dialister*spp. (1.3%), *Pseudomonas* spp. (1.2%), *Corynebacterium aurimucosum* (1.08%), *Granulicatellaelegans* (1%) and *Novosphingobium capsulatum* (1%). In SP2 male urolithiatic patients pooled samples with cUTI, major species found were *Acinetobacter genomo specie* (83.2%) followed by *Enterobacter hormaechei* (12.5%) and *Bifidobacterium longum* (1.4%).

In SP3 pooled samples of healthy female, *Lactobacillus crispatus* (41.3%) followed by *Lactobacillus iners* (29%), *Enterobacter hormaechei* (6.1%), *Achromobacter xylosoxidans* (5.9%), *Alcaligenes faecalis* (2.5%), *Agrobacterium tumefaciens* (1.2%), *Serratia quinivorans* (1.2%), *Sphingomonas caulobacterleidyia* (1.2%) and *Pseudomonas* spp. (1%) were found. In SP4 pooled samples of healthy males, *Achromobacter xylosoxidans* (42%) followed by *Enterobacter hormaechei* (13.6%), *Sphingomonas caulobacterleidyia* (6.7%), *Alcaligenes faecalis* (4.8%), *Bradyrhizobium* spp. (4.6%), *Ochrobactrum* spp. (3.9%), *Ralstoniapickettii* (2.5%), *Serratiaquinivorans* (1.7%), *Agrobacterium tumefaciens*(1.6%), *Pseudomonas* spp. (1.6%), *Cryocola*spp. (1.2%), *Methylobacterium organophilum* (1.2%) were found.

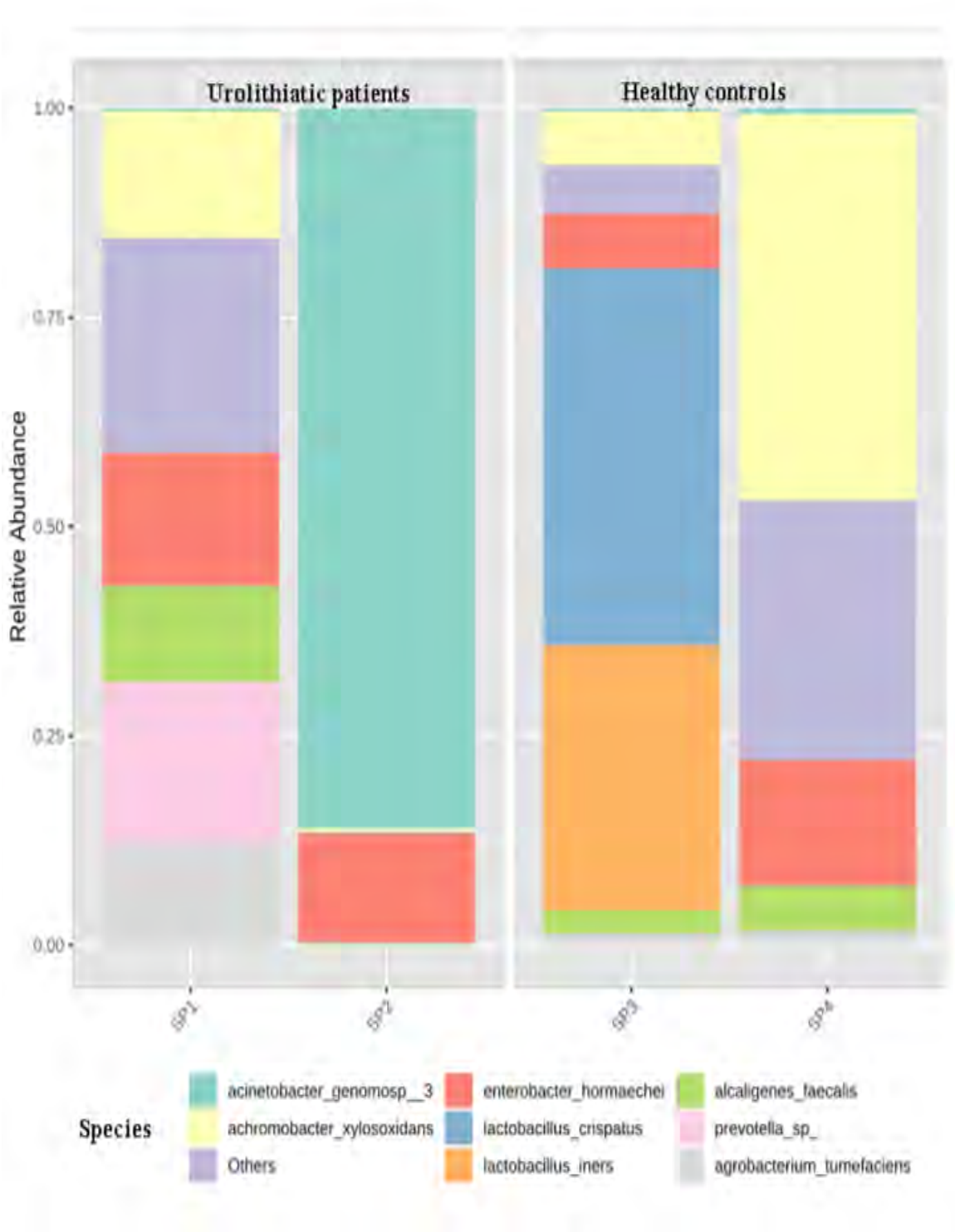


Figure:4.3 Relative abundance of predominant urinary bacterial taxa in urolithiatic patients' samples with cUTI SP1, SP2) and healthy individuals samples (SP3, SP4) at species level analyzed by Microbiome analyst tool using cutoff value >10 counts.

4.3.2.3-Community profiling

4.3.2.3.1-Alpha diversity

To measure the alpha diversity Shannon, Simpson and Chao1 diversity index were calculated to determine the richness and evenness of the samples, and statistical analysis were performed through Mann-Whitney method. Diversity analysis showed that there was greater richness in female urolithiatic patients with cUTI as compared to male urolithiatic patients and the healthy individuals. Among healthy individuals female control samples showed the greater richness as compared to males. Among patients, females showed greater diversity. Shannon index showed high richness and evenness in all samples except SP2 which was pooled samples of male urolithiatic patients. Highest richness and evenness were found in female patients.

Table4.35: Alpha diversity indices for urinary microbiome of urolithiatic patients (SP1, SP2) and healthy subjects (SP3, SP4) analyzed by Microbiome analyst tool.

Sample ID	Simpson's index	Shannon index	Chao index
SP1	0.85	1.95	08
SP2	0.23	0.38	02
SP3	0.64	1.18	09
SP4	0.74	1.71	09

4.3.2.3.2-Beta diversity

Bray-Curtis dissimilarity showed highest dissimilarity (94%) between SP2 male urolithiatic patients with cUTI and SP3 female healthy subjects. Lowest dissimilarity (68%) was observed between SP1 female urolithiatic patients with cUTI and SP3 female healthy subjects. It was observed that 92% dissimilarity was present between SP1 female patients and SP2 male patients. Principal Coordinates Analysis is a method to explore and

visualize similarities or dissimilarities of data In principle coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) by using Bray-Curtis, results were insignificant. PERMANOVA statistical analysis detected F-value: 0.84307; R-squared: 0.29653; p-value < 1 and Stress = 0.

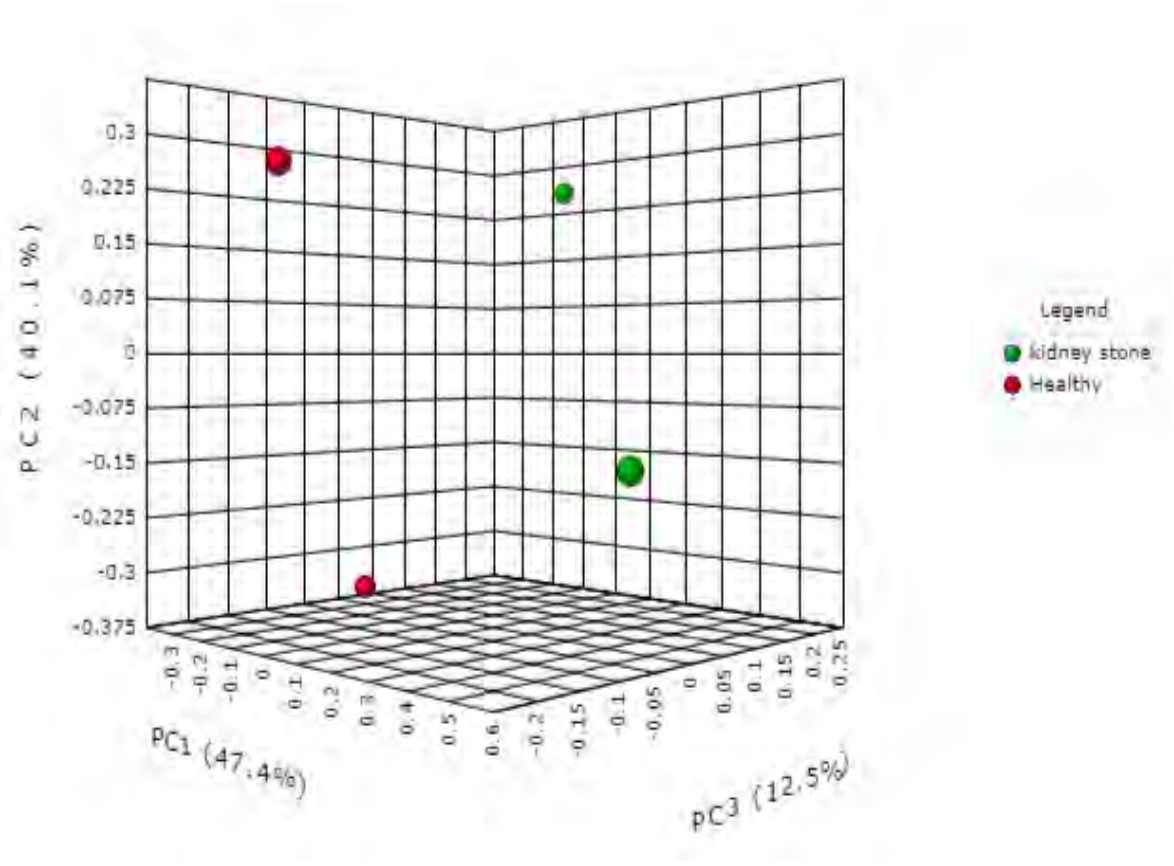


Figure 4.4:- PCoA analysis by Bray-Curtis distance of OTU based clustering of microbial communities in urolithiatic patients(SP1, SP2) and controls(SP3, SP4). PCoA score for Axis 1, Axis 2 and Axis 3 were SP1; -0.077, 0.248, 0.203, SP2; 0.46, -0.148, - 3.36 SP3;-0.30, -0.349, -2.72 and SP4; -0.077, 0.248, -0.203 analyzed by Microbiome analyst with cut off >10% abundance

4.3.2.4-Core microbiome analysis

At genera level, it was observed that 34% genera were shared between all of four urine samples. *Serratia, Pseudomonas, Achromobacter, Agrobacterium* and *Enterobacter* were found to be at top genera present in all samples.

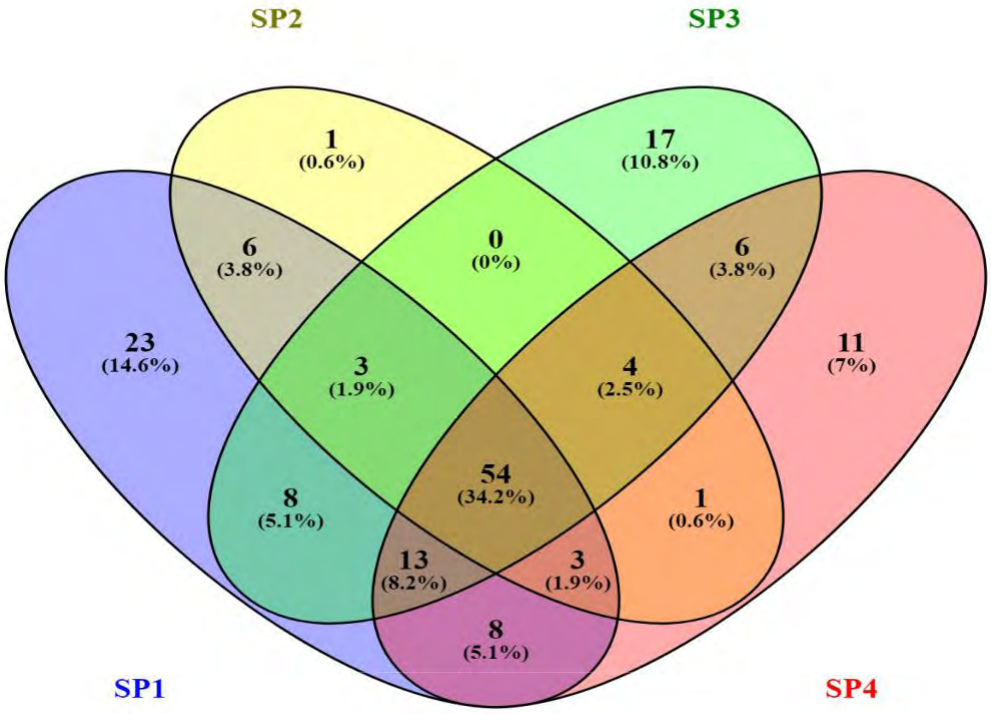


Figure 4.5: Bacteriome analysis on basis of percentage abundance of core genera shared by all urine samples showed 34.2% common core bacteriomeusing software Venny

4.4-Discussion

This part of the study was conducted to detect the culturable and unculturable bacterial diversity in urolithiatic patients with and without cUTI. In present study, 59% of urolithiatic patients were found to be positive for both Gram positive and negative bacterial isolates through culturable method. Overall, Gram negative bacteria were prevalent especially *E. coli* and *K. pneumoniae* followed by the Gram positive bacteria in urolithiatic patients. This work showed similar trends of isolation as reported from Russia by Romanova *et al*., 2015, where the most commonly isolated bacterial isolates from urolithiatic patients were *K. pneumoniae* and *E. coli*.

K. pneumoniae was found to be the most prevalent bacteria followed by *E. coli*, *S. aureus, S. saprophyticus,* and *S. epidermidis* in urolithiatic patients. However, in case of urolithiatic patients with cUTI, *E. coli* was detected as dominant bacteria followed by *K.pneumoniae*, *S. aureus, S. saprophyticus,* and *S. epidermidis.* These results are in accordance with a study conducted in Sindh (Pakistan), which reported *E. coli* followed by *Klebsiella* as most prevalent bacteria in urolithiatic patients with UTI (Iqbal *et al.,* 2017). Another study from Pakistan also detected *E. coli* as the most dominating organism in causing UTI among urolithiatic patients (Mehmood and Zafar, 2008). A study carried out in Turkey reported the same pattern of microbial flora in nephrolithiatic patients with UTI (Cetin *et al.,* 2019). *E. coli* and *K. pneumoniae* were significantly isolated from urine samples of studied urolithiatic patients as compared to the healthy individuals with 2.5- and 5-fold higher chances for their isolation respectively.

In present work, *E. coli* was significantly isolated from the female patients, which is in accordance with earlier study carried out in Pakistan where isolation of *E. coli* in female urolithiatic patients was greater as compared to male patients (Iqbal *et al.,* 2017). This significant association might be due to fact that female patients are more prone to UTI caused by *E. coli* and also a higher proportion (77%) of these female urolithiatic patients had cUTI, out of which 30% of these females were of middle age group (41-50yrs). There is low level of estrogen formation in this age group, which has protective action but it is

reduced especially in menopausal females. It was observed that urolithiatic female patients belonging to low socioeconomic status were significantly more positive for *E. coli* isolation and all of these *E. coli* positive patients had cUTI as well. Therefore, it can be inferred that there is strong effect of socioeconomic status for having the UTI in such group of patients.

All catheterized patients with cUTI were also positive for *E. coli* isolation. In a study conducted in USA, 57% catheterized female patients were diagnosed with *E. coli* (Manohar *et al.,* 2019). High prevalence of *E. coli* in catheterized patients of current study might be due to inserted catheters which act as abiotic surface for microbes to adhere. Duration of urolithiasis was also found to be a significant clinical risk factor for *E. coli* colonization causing cUTI in these patients, as 74% of *E. coli* positive patients with cUTI were suffering from urolithiasis for more than one year. *K. pneumoniae* isolation rate was also higher in same group of patients. The presence of stone for long duration might be a potential factor for development of pathogenic behaviour of *E. coli* and *K. pneumoniae* in such patients.

In present work, 36% of patients had *K. pneumoniae* while among these 13% patients had co presence of *E. coli* and *K. pneumoniae*. Baizet *et al*. (2019) reported that *E. coli* was the most common pathogen followed by *K. Pneumoniae* in pyelonephritis in females and upper UTI in males. Although, overall *K. pneumoniae* was found to be the most prevalent followed by *E. coli* in urolithiasis in current study, but there was difference in both gender with regards to the prevalence rate of these isolates with different pathologies. In case of urolithiatic patients with cUTI, it was detected that male patients were more prone to be infected by *K. pneumoniae*. In the same group of patients, *E. coli* was found to be the more prevalent in female patients. In another study conducted in Korea, males were more infected with *K. pneumoniae* than *E. coli* in hospital acquired urinary tract infections (Hyun *et al.,* 2019).

There was presence of hypervirulent *K. pneumoniae* as well as classical ones. *hvKP* were confirmed from 22 urolithiatic patients. The 73% of hypervirulent *K. pneumoniae* isolates from urolithiatic patients were also suffering from cUTI indicating these isolates might

have triggered cUTI. Lin *et al.,* (2010) also reported hypervirulent *K. pneumoniae* to be more prevalent in UTI patients from Taiwan.

Other virulence factors of *E. coli* and *K. pneumoniae* tested to decipher their possible link to cUTI were biofilm formation, hemolysin production, serum resistance and haemagglutination activity in current work. Biofilm formation either on biotic or abiotic surfaces is a leading step in development of infection by bacterial species. As bacteria in biofilm are less exposed to stresses by antibiotic treatment and can express their other virulence factors more efficiently than in planktonic cells (Bellifa *et al*., 2016). In this study, biofilm forming ability of *E. coli* isolates was determined and it was found that out of 54 isolates only 4% isolates had strong biofilm forming ability and remaining isolates were moderate and half of these were non-biofilm formers. A higher prevalence of biofilm formation was reported in a study conducted in Bulgaria in *E. coli* isolates from different type of UTI patients (Marhova *et al.,* 2010). Another study found the higher biofilm former *E. coli* isolation from different clinical samples in Iran (Zamani and Salehzadeh, 2018). Low biofilm formation ability of *E. coli* isolates in present study indicated that these isolates might not be involved in causing the infection but their isolation is due to their presence as commensal in urinary tract. *K. pneumoniae* isolates behaved differently from *E. coli* in biofilm formation ability. Out of 69 isolates of *K. pneumoniae*, 87% were strong biofilm formers. These results are almost in accordance with study conducted at Algeria in which they reported 75% of *K. pneumoniae* isolates with biofilm formation ability (Bellifa *et al.,* 2016). In current study majority (80%) of strong biofilm formers *K. pneumoniae* were isolated from cUTI patients indicating there might be a strong association of *K. pneumoniae* for development of the infection in such patients. *K. pneumoniae* in biofilm have aggregated lifestyle where these can express their virulence factors for causing the cUTI more efficiently than in planktonic form.

The ability of *K. pneumoniae* and *E. coli* isolates to produce hemolysin production was screened. Majority of *K. pneumoniae* isolates showed alpha hemolysis. Significantly high percentage of *E. coli* isolates from urolithiatic patients was hemolysin producer as compared to isolates from the control group. These results are in accordance with a study conducted in USA where majority of UPEC from pyelonephritis cases showed the

hemolysin production (Ristow and Welch, 2016). Another study conducted in Spain showed that most of the *E. coli* isolates from different urinary tract diseases were positive for hemolysin production (Soto *et al.,* 2007). In a study from Iran, it was observed that 34% of UPEC isolated from UTI patients were positive for hemolysin production (Tabasi *et al.,* 2015). Hemolysin production correlates with infection severity and high rate of alpha hemolysin producer *K. pneumoniae* and *E. coli* in current study might be due to the fact that these patients were from surgical wards and most of them had cUTI.

The activity of normal human serum against bacteria is considered as first line of defense against pathogenic microorganisms. Serum bactericidal activity was analyzed in the current study and it was found that 87% of *K. pneumoniae* isolates were resistant to lower concentration of 40% serum. Most of the *cKP* isolates showed sensitivity to 80% serum compared to *hvKP, however,* majority of both *cKP* and *hvKP* isolates were found to be resistant against 40% serum. Almost similar trend of serum resistance was observed in a study conducted in China which reported 80.5% resistant *K. pneumoniae* isolated from respiratory tract infection against low concentration of serum. Similar to present results, Chinese study also detected that serum resistance was more commonly observed among *hvKP* than *cKP* isolates (Shah *et al.,* 2017). *hvKP* isolates from this work showed that in urolithiasis, *hvKP* can be potentially more virulent and can possibly be involved in causing cUTI in such patients.

E. coli isolates showed more sensitivity to 80% serum than 40%. It was observed that by changing serum concentration, serum activity against *E. coli* isolates was increased with passage of time and these isolates became more susceptible toward serum. The trend of serum resistance, was in accordance to a study from India to current study in which isolates from UTI patients showed 100% resistance to serum during first two hours of incubation and gradually decreased with passage of time (Baby *et al.,* 2014). The mechanism of *E. coli* resistance to bactericidal effect of serum is not clear but some studies showed that, capsular polysaccharide, O antigen and surface proteins play a significant role in resistance to bactericidal activity of serum (Mittal *et al*., 2014; Nachammai *et al*., 2016).

Haemagglutination of RBCs by bacteria was used for identification of type1 fimbriae among these isolates to assess if these were pathogenic (Sahly *et al*., 2008). It was observed

that majority of *K. pneumoniae* isolates showed haemagglutination with blood group B as compared to blood group A. In contrast to present study, HRV *et al.,* (2016) reported that *K. pneumoniae* isolated from clinical samples had more haemagglutination activity against blood group A than blood group B. Comparing the haemagglutination activity of *K. pneumoniae* with *E. coli* isolates there were a smaller number of *E. coli* isolates which possessed this activity. Marhova *et al*., (2010) also observed only 16% of *E. coli* isolates from UTI patients possessing haemagglutination activity.

These isolates were further assessed for resistance to treatment by determining their antibiotic resistance pattern (*K. pneumoniae* and *E. coli)* against different classes of antibiotics. It was observed that both of the bacterial isolates showed higher sensitivity against tetracycline. In case of *E. coli* isolates, the most effective drug observed was imipenem followed by nitrofurantoin and gentamycin. Ciprofloxacin was also effective against 54% of the isolates. A study conducted in Germany reported comparatively lower sensitivity (43%) against gentamycin in UPEC isolated from cUTI patients (Ochoa *et al*., 2016). Almost similar resistance pattern against ciprofloxacin was also reported in a study from Pakistan in uropathogenic *E. coli* (Abdullah *et al.,* 2012). In another Pakistani study, gentamycin was detected to be the effective drug against UPEC isolated from UTI patients (Ali *et al.,* 2017). In addition, nalidixic acid was the least effective antibiotics observed in their UPEC isolates, this trend was same in *E. coli* isolates of present study. In current study, 69% *E. coli* showed sensitivity towards nitrofurantoin and imipenem. All of *E. coli* isolates from UTI patients from Faisalabad were reported to be 100% sensitive to nitrofurantoin and imipenem (Sohail *et al.,*2015). Current work found that majority of the *E. coli* isolates were MDR as they exhibited resistance against more than two antibiotics of a class as well as against more than two classes of antibiotics.

K. pneumoniae isolates showed higher sensitivity towards tetracycline followed by amikacin and ceftazidime. Least effective antibiotics observed in case of *K. pneumoniae* were cephalothin and amoxicilline. In case of fluoroquinolones, 38% sensitive *K. pneumoniae* isolates were observed against ciprofloxacin whereas, 45% of isolates showed susceptibility for ofloxacin. Comparatively high susceptibility pattern was observed in a study conducted in Karachi, in which 64% susceptibility of *K. pneumoniae* was reported

for ciprofloxacin (Abdullah *et al*., 2012). A study conducted by Sohail *et al.,* (2015) reported nitrofurantoin as the most effective drug against all *K. pneumoniae* isolates from UTI patients, however, less susceptibility was observed for this drug in present study. Almost all isolates showed resistance against penicillin, which points towards the fact that against antibiotics earlier used with passage of time there is development of resistance like in *K. pneumoniae* isolates. Although, penicillinases among *K. pneumoniae* were not prevalent earlier, but may be due to presence of plasmid bearing antibiotic resistance genes or their acquisition from surroundings making it MDR pathogen among uropathogens after *E. coli* (Vuotto et al., 2014).

Extended spectrum beta lactams have widely been used to treat severe bacterial infections but emergence of bacterial resistance due to production of extended spectrum beta lactamases has become a serious public theat. These enzymes are involved in hydrolyzing extended spectrum beta lactam antibiotics. *E. coli* and *K. pneumoniae* isolates of present study was screened for their ability to produce beta lactamases through double disc synergy test. It was found that 63% of *E. coli* and 34% *K. pneumoniae* isolates were ESBL positive. Similar results to present study were documented in a research conducted on UPEC from Islamabad where 59% *E. coli* isolates were ESBL producers (Ali *et al*., 2016). According to a work conducted in Khyber Pakhtunkhwa (Pakistan), prevalence of ESBL producers was 33% among UPEC isolates (Jamil *et al.,* 2018).

ESBLs are expressed by the genes commonly encoded by plasmids and these plasmids can readily be exchanged between different bacterial species. Members of *Enterobacteriaceae* such as *E. coli* and *K. Pneumoniae* commonly produce these enzymes. In present study phenotypic ESBL positive isolates of *E. coli* and *K. pneumoniae* were further tested for prevalence of different of ESBL genes among those local bacterial isolates. Among all ESBL positive *E. coli* isolates, majority of isolates possessed different ESBL genes. Less number of *K. pneumoniae* isolates had phenotypic ESBL production as compared to *E. coli* isolates. From these 34% ESBL producer *K. pneumoniae* isolates, only 17% of isolates possessed *CTX-M* gene followed by *TEM* and *SHV*. The *CTX-M* was the most prevalent ESBL gene in both *E. coli* and *K. pneumoniae.* Similar results to present study were observed in Malaysia in which *CTX-M* gene was detected followed by *TEM* and *SHV* in

ESBL producer *E. coli*, 28% of isolates harboured the all three genes (Mahdi *et al.,* 2016). In contrast to present work, study conducted in India by Bajpai *et al.,* (2017) on UTI patients found *TEM* as the most prevalent gene in ESBL producer UPEC isolates. A study conducted in UAE by Khan *et al.,* (2019) reported the high prevalence of ESBL producing *E. coli* isolates as compared to *K. pneumoniae* and the most prevalent ESBL gene as *CTX-M* followed by *SHV* in both type of isolates.

An increased resistance to quinolone through plasmid is found to be associated with *CTX-M* genotype of beta lactamases (Mshana *et al*., 2011). Keeping in mind, *E. coli* and *K. pneumoniae* were characterized for quinolone resistance (*qnr*A*, qnrB, qnrS*) genes as well. Among quinolone resistance, *qnrS* was more prevalent followed by *qnr*A and *qnrB* in both bacterial isolates. Similar results were reported in a study carried out in Mexico where UPEC isolates from UTI patients were detected with *qnrA* followed by *qnrB*(Ramirez-Castillo *et al*., 2018). A study conducted in West Africa reported, 26% of *K. pneumoniae* isolated from different clinical samples as ESBL producers from which 51% were positive for qnr genes (Tahou *et al.,* 2017). It was also observed that the prevalence of quinolone resistance genes was higher among ESBL producer isolates than non-ESBL producers. These results are in accordance with another report in which prevalence of *qnr* genes was 21% among ESBL producers and 5% among ESBL non- producers UPEC isolates (Ramirez-Castillo *et al*., 2018). Different studies conducted in the world have shown the possibility of co-occurrence of plasmid mediated quinolone resistance genes and ESBL genes in uropathogenic *E. coli* and *K. pneumoniae* isolates (Basu and Mukherjee, 2018; Liu *et al*., 2018). The emergence of mechanisms of quinolone plasmid resistance is a real threat to the efficacy of fluoroquinolones (Tahou*et al.,* 2017). The isolates detected positive for *qnr* genes in present study were highly resistant against ofloxacine belonging to the flouroquinolone group of antibiotics. Although, the virulence factors of *E. coli* in present study were not highly expressed, but strains showed much higher levels of MDR and ESBL production. The antimicrobial resistance in these *E. coli* isolates among all pathogens isolated from urolithiatic patients with cUTI makes them a potential threat as treatment strategies are limited. In contrast, *K. pneumoniae* which are worldwide reported to be highly prevailing uropathogens are less resistant to locally administrated antibiotics but were more virulent.

Second part of this study was carried out to identify the urinary bacterial diversity in urolithiatic patients with cUTI and compare it to urinary bacteria of healthy individuals by using next generation sequencing. Proteobacteria was the phylum detected to be shared predominantly in all the urinary samples included in present study. Except female healthy individuals, this phylum was found to be the most prominent in all samples. Proteobacteria is a major phylum of Gram-negative bacteria including *E. coli, Shigella, Salmonella* and other *Enterobacteriaceae.* In a study conducted in USA 10 different bacterial phyla in urine samples of patients with bladder cancer were reported with the most abundant phyla included Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria (Popovic *et al.,* 2018), dysbiosis due to disease pathology might be responsible for this difference. In present work, the general trend observed was a more heterogeneous mix of bacterial genera in the female samples as compared to male samples. In female urolithiatic patients with cUTI, predominant genera found were *Enterobacter*, *Achromobacter*, *Agrobacterium, Alcaligenes* and *Fusobacterium* while in male patients, bacterial diversity was low as compared to female patients with 84% genus *Acinetobacter*. *Acinetobacter* is a genus being responsible for a range of nosocomial infection and commonly presents multidrug resistance to multiple antimicrobial agents (Michalopoulos and Falagas, 2010). In current study, *Acinetobacter* was detected in both male and female patients but in female patients its abundance was low 0.5% as compared to the male urolithiatic patients with cUTI where it was 84%. A study conducted in UK, reported that *Acinetobacter* were absent from microbiome of healthy males (Lewis *et al.,* 2013). In this way, it can be inferred that *Acinetobacter* might be have role in causing the cUTI in urolithiatic male patients in present study. Popovic *et al.,* (2018) worked on bladder cancer patients and detected the most prominent genera as follows: *Prevotella, Streptococcus, Campylobacter, Peptoniphilus, Veillonella*and *Anaerococcus.* In current work *Streptococcus, Campylobacter, Peptoniphilus, Veillonella*and *Anaerococcus*were observed more abundantly only in female urolithiatic patients with cUTI. *Lactobacillus* was the genera found abundantly only in healthy females, as *Lactobacillus* is part of the normal human commensal of gastrointestinal and vaginal flora, but under certain condition is reported to become pathogenic (Goldstein *et al.,* 2015).

In female urolithiatic patients with cUTI, major species found were *Prevotella*spp. followed by *Enterobacter hormaechei*, *Achromobacterxylosoxidans*, *Agrobacterium tumefaciens, Fusobacterium nucleatum* and *Serratiaquinivorans. Prevotella*spp. is commonly found in anaerobic infections. *Enterobacter hormaechei*found in cUTI of female urolithiatic patients, member of the diverse *Enterobacter cloacae* complex and now is reported to be emerging pathogen. *E. hormaechei* can spread in nosocomial environments and is commonly resistant to multiple clinically important antimicrobial drugs (Monahen*et al.,* 2019), its presence in microbiome indicated it as potential uropathogen in urolithiatic patients.

In current study, urinary microbiome of male urolithiatic patients with cUTI included *Acinetobacter genomo*species followed by *Enterobacter hormaechei* and *Bifidobacterium longum.* While in case of healthy males, the most abundant species found were*Achromobacterxylosoxidans* followed by *Enterobacter hormaechei* and *Sphingomonascaulobacterleidyia*. In contrast to present work Nelson *et al.,* (2010) detected *Lactobacillus iners* as the most common bacterial species followed by *Aerococcus, Anaerococcus, Prevotella, Gemella, Veillonella*and *Sneathia* in male urinary microbiome having asymptomatic sexual transmitted infection. Zampini*et al.,* (2019) found *Enterobacteriaceae* as the major pathogens in urine of urolithiatic patients. *Campylobacter hominis, Actinobaculum massiliense,* and *Jonquetella anthropic* were found in urine of bladder cancer patients by using next generation sequencing (Popovic *et al.,* 2018). The finding from various studies along with present work highlights that microbiome varies with different factors and disease conditions.

Diversity analysis showed the greater richness in female urolithiatic patients with cUTI as compared to male urolithiatic patients and the healthy individuals. Overall females in both diseased and healthy group showed the greater richness and these results are aligned with a study conducted in UK on asymptomatic adults where the most heterogeneous mix of bacterial genera were found in females as compared to males (Lewis *et al.,* 2013). In current work, highest dissimilarity was detected between male urolithiatic patients and healthy females. These differences might be due to the reason gender and disease condition, both of these factors play role in changing the micro flora of studied subjects.

The present work contributes to data on the urinary microbiome of urolithiatic patients by both culturable and unculturable techniques. Further shows that bacterial species detection through unculturable method differs from the culturable technique. However, through both of techniques it was evident that most commonly found bacterial types were Gram negatives. Highest percentage of *K. pneumoniae* followed by *E. coli* among bacterial isolates was detected in urolithiatic patients by culturable method. *E. coli* were found to be most prevalent among females while *K. pneumoniae* in male patients. However, urolithiatic patients with cUTI were prevalently colonized with *E. coli*. Many virulence factors were detected in both Gram negative bacteria isolated from patients as compared to the control groups. There was high virulence in *K. pneumoniae* isolates which were also ESBL producer in urolithiatic patients with cUTI, it indicates that these isolates might have role in causing, progression and severity of infection. Less virulent but MDR *E. coli* of this study can also be potential threat as these isolates will have no or reduced efficacy to treatment strategies. These results highlight that there is need to find causative agents and MDR in local circulating strains of *K. pneumoniae* and *E. coli* for proper evidence-based treatments strategies to control their infections in urolithiasis as Pakistan is in stone belt.

CHAPTER 5

Fungal diversity in urine samples of urolithiatic patients with cUTI by culture dependent and independent methods

5.1-Introduction

Fungal microbiota in the host environment is generally termed as mycobiome, which is an important but understudied part of human microbial system. Mycobiome diversity and dynamics are poorly understood especially for their role in infectious diseases. This might be due to the different challenges in characterizing the human mycobiome including low abundance and lack of optimized protocols to isolate quality genetic material to undergo the culture dependent and independent characterization (Ackerman *et al.,* 2019).

Role of different single fungal species has been well studied in disease progression but the diversity of whole mycobiome in relation to the development of disease is not clearly defined (Cui *et al.,* 2013). Bacteria and fungi both are etiologic agents of cUTI resulting from urolithiasis and long-term catheterization. Some studies suggested that there is shift of microbial diversity from *E. coli*, *Proteus*, and *Pseudomonas* species to different fungal species with *Streptococcus agalactiae*, and *K. pneumoniae* to be responsible for the development of UTI (Bongomin *et al.,* 2017; Gharanfoli *et al.,* 2019).

The Saccharomycetes had been reported as the only fungal class in urine for long time but some recent studies using next generation sequencing instead of culture dependent techniques detected a range of fungal taxa from asymptomatic patients (Ackerman and Underhill, 2017). In the upper urinary tract, *Candida* as well as some other invasive fungal species *i.e., Aspergillus* spp*., Histoplasma capsulatum, Cryptococcus neoformans, Blastomyces* spp*., Mucoraceae spp.,* and *Coccidioides immitis* can infect the kidneys as part of mycotic infection (Ho *et al.,* 2019). Various predisposing risk factors of cUTI include age, use of indwelling devices such as catheter and urinary tract stones. The urinary tract stone may cause hindrance and infection in urinary tract when they rise beyond a size of natural clearance from urinary tract (Flores-Mireles *et al.,* 2015).

Candida is the major causative agent for cUTI. *Candida* has become a major opportunistic pathogen among yeast due to its tremendously growing infections (Tasneem *et al.,* 2017). A study conducted on urine sample of UTI cases reported about 50–70% prevalence of *C. albicans* followed by *C. glabrata* and *C. tropicalis* (Alhussaini, 2013). *Candida* species cause UTI by either the ascending or haematogenous routes. The pathogenesis of ascending infection with *Candida* has not been well studied.

Candida species found in urine of the persons have specific factors which allow the organisms to access and colonize the urinary tract mucosa. Candiduria is common finding in hospitalized patients especially in intensive care units. A multicentre study detected *Candida* in urine samples of 861 hospitalized patients. In that study, various risk factors were observed which included catheters in 83%, diabetes in 39% and urinary tract disorders in 37% (Kauffman, 2000). *C. albicans* is the common agent responsible for fungal infections but in the recent years some non-albicans species have also emerged with different virulence and sensitivity patterns (Boekhout *et al.,* 2017). Antifungal resistance of *Candida* has become another serious clinical challenge due to widespread use of the agents like echinocandins and azoles (Goncalves *et al.,* 2016).

Adherence to host tissue, phenotypic variability, enzymes and toxins produced by *Candida* has been listed as virulence factors of *Candida* species. Different hydrolytic enzymes are responsible for pathogenicity of *Candida* (Dabiri *et al.,* 2018). These enzymes facilitate the *Candida* adherence and penetration into the host tissue (Mohandas and ballal, 2009). Secreted aspartyl proteinases and phospholipases are the most important extracellular hydrolytic enzymes of *Candida* (Dabiri *et al.,* 2018).

Biofilm formation also contributes to pathogenesis of *Candida*. Biofilms are complex and interdependent surface associated communities of various microorganisms. These biofilms are enclosed in a matrix of exopolysaccharides and can be formed on any surface such as medical devices. biofilms are difficult to eliminate and can cause the recalcitrant infections, therefore their study has become major concern for public health (Jain *et al.,* 2007). The finding of *Candida* in urine of a patient with or without symptoms should require a careful evaluation, which should proceed in a logical fashion. Li *et al.,* (2017) isolated *Candida* species from 12.5% of cUTI patients in China. In Pakistan, most of the data regarding fungal

infections covered only uncomplicated UTIs as Sohail *et al.,* (2015) reported *Candida* in 14% of culture positive urine samples of uncomplicated UTI patients. In another study of Pakistan by Kausar *et al.,* (2017) reported *Candida* spp. in 5% of uncomplicated UTI patients. Therefore, culturable as well as unculturable fungal diversity in cUTI is an understudied aspect in Pakistan. Local population is at high risk for complicated urinary tract infection with urolithiasis. There is no data on prevalence of fungal infections among local patient to identify their possible role as causative agent of UTI, recurrent UTI and cUTI in urolithiatic patients. Therefore, this part of study was planned to detect fungal species through culture dependent and independent method from patients diagnosed with urolithiasis and cUTI.

`5.2- Material and Methods

5.2.1- Study Design

Previously described in chapter 3.0, section 3.2.1

5.2.2- Study Population

Previously described in chapter 3.0, section 3.2.2

5.2.3- Part 1: Study of urinary mycobiome by culture dependent method

5.2.3.1- Isolation of culturable fungal species from urine samples

To isolate the culturable fungal species, 0.1mL of urine sample was directly inoculated onto petri plates containing Sabouraud dextrose agar (SDA). In order to prepare the media, 65g of SDA (Liofilchem, Italy) was dissolved in 1000 mL distilled water and autoclaved at 121º C and 15 psi for 15 min. After autoclaving, media was cooled and poured into the petri plates under sterile conditions. Then samples were incubated for 48 h at 37ºC in aerobic conditions. On basis of colony morphology, distinctive colonies were picked individually using a sterile loop and were streaked separately on SDA plates using quadrant streak technique to get pure cultures. These plates were then incubated for 48 h at 37ºC aerobically and growth was observed. Further morphological identification was done by Gram staining.

5.2.3.2- Identification of *Candida* species

5.2.3.2.1- Gram staining

Described previously in chapter 4.0, section 4.2.3.2

5.2.3.2.2- Germ tube test

The serum was collected aseptically from fresh human blood. To obtain serum from blood, it was first left to clot for 15-30 min at room temperature, followed by centrifugation at 10,000 rpm for 15 min. After centrifugation, supernatant layer was formed. The isolated *Candida* spp. were inoculated in the serum and incubated at 37ºC for 2 to 3 h. After that, a drop of mixture from the test tube was placed on a clean glass slide and was covered with a cover slip to thoroughly examine under a light microscope (Micros-Austria) using the 10x and 40x objective lenses. Germ tube was observed as elongated tube like structure originating from round mother cell with no septa at point of origin (Jasim *et al*., 2016). *C. albican* are germ tube positive while *C. dubliniensis* can either be positive or negative germ tube test. All other *Candida* species are germ tube negative.

5.2.3.2.3- Growth of *Candida* species at 45°C

All germ tube positive isolates were grown at 45°C to differentiate between *C. albicans* and *C. dubliniensis*, *C. dubliniensis* cannot grow at this temperature while the *C. albicans* can grow (Premkumar *et al*., 2014). In order to perform this test, all germ tube positive freshly cultured *Candida* isolates were inoculated on SDA plate and incubated at 45ºC under aerobic conditions to observe growth for 10 days.

5.2.3.2.4- Characterization of *Candida* species using Chromogenic agar

All *Candida* isolates were separately cultured on SDA at 37°C for 48 h to get fresh cultures. Afterward, colonies from fresh cultures were streaked on Chromogenic agar (48 grams in 1000mL) (Liofilchem, Italy) using sterile loop and incubated at 37°C for 48 h to examine the development of coloured colonies. The Chromogenic agar permits117

differentiation of *Candida* isolates on the basis of colour and morphology of different *Candida* species. Chromogenic agar is selective media as it contains chloramphenicol, which inhibits bacteria and only permits the growth of yeast. Finally, these strains were distinguished by the maker's guidelines. Pale green colonies demonstrate the presence of *C. albicans,* metallic blue colonies demonstrate the occurrence of *C. tropicalis,* pink colonies show the presence of *C. krusei* and *C. glabrata* produces white colonies.

5.2.3.2.5- Molecular identification of *Candida* species

5.2.3.2.5.1- DNA extraction of *Candida*

Extraction of DNA from *Candida* isolates was done by phenol chloroform method with a few modifications (Dhanasekaran *et al.,* 2014). *Candida* colony taken from 24-48 h fresh culture were inoculated in 5mL of Sabouraud dextrose broth and incubated for 24 h at 37°C. After incubation, centrifugation was done at 10,000rpm for 10 min in a micro centrifuge. The culture pellet obtained was mixed with 700µL lysis buffer including10% SDS, 0.5M EDTA and 1M Tris-HCl. Then Protinase K (20mg/mL) and 0.4g of glass beads (0.1 mm) (Sigma-Aldrich) were added. The mixture was vortexed for couple of minutes and placed in incubator at 50°C for 3h. After incubation, 700µL of phenol:chloroformisomyl solution was added and centrifuged at 10,000 rpm for 10 min. Aqueous phase was obtained and phenol:chloroform step was repeated. At that point to the aqueous phase, one-tenth volume of 3M Sodium acetate and 1mL of 99.99% chilled ethanol was added to precipitate DNA. It was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was taken in another tube and washing of pellet was finished with 400µL of 70% ethanol. Again, centrifugation was done at 10,000 rpm at 4°C for 10 min. The pellet obtained after separating supernatant was air dried, mixed with 100µL of TE buffer and stored at -20°C for further analysis.

5.2.3.2.5.2- PCR amplification of *ITS* gene and restriction fragment length polymorphism (RFLP) for identification of *Candida* species

5.2.3.2.5.2.1- Optimization of polymerase chain reaction

After DNA extraction of all *Candida* isolates, PCR amplification of Inter Transcribe Sequences (*ITS*1-*ITS*4) of rDNA regions was accomplished by using a pair of primers (Table 5.1). Conditions were optimized for annealing temperatures, time and concentrations. After amplification, digestion of PCR products was done with 10U *HpaII* enzyme (Fermentas, Germany) for RFLP for 90 min at 37°C.

Table5.1: Primer sequences used for amplification of *ITS* gene of *Candida*

Gene	Primer Sequence 5'→3'
ITS	*ITS*1(forward) 5'GGT GAA CCT GCG G-3' *ITS*4 (Reverse) 5' TCC TCC GCT TAT TGA TATGC-3'

5.2.3.2.5.2.2- Amplification conditions

For amplification, autoclaved PCR tubes were taken and master mix of a final volume of 25 µL was made. Each reaction mixture contained 3 µL of DNA as a template, 0.5 µL of primers, 2.5 µL 10x buffer, 0.4 µL of dNTPs (Solid Biodyne), 0.2 µL Taq DNA polymerase (Roche Diagnostics, Germany), 1.5 µL magnesium chloride and 16.4 µL PCR water.

The amplification process consisted of 36 cycles. Each cycle comprised of primary denaturation step for 5 min at 95ºC, second step of denaturation was completed in 45 s at 95ºC, annealing of primers was done for1 min at 52.5ºC, primary extension for 1 min at 72ºC and last extension was for 5 min at 72ºC.

5.2.3.2.5.2.3- Gel Electrophoresis

Previously described in chapter 4.0, section 4.2.3.9.3

5.2.3.3- Virulence factors of *Candida* isolates

5.2.3.3.1- Yeast suspension

The suspensions of identified *Candida* isolates were prepared to determine their esterase and phospholipase activity. Initially, fresh cultures were made by inoculating on SDA

media and plates were incubated at 37 °C for 48 h. At that point the yeast cells were added in PBS. The density of 1.0×10^7 cells/mL was adjusted by comparing with 0.5 McFarland standards and furthermore adjusting the optical density (OD) of 0.38 at 520nm by spectrophotometer (Deepa *et al*., 2015).

5.2.3.3.1.1- Esterase assay

Esterase activity of *Candida* isolates was determined utilizing Tween-80 media. The media was prepared by adding 10g peptone, 5g NaCl, 0.1g $CaCl_2$ and 15g agar in 1L distilled water and pH was adjusted to 6.8. Tween 80 (5mL) was added in the autoclaved, cooled media and dissolved properly. Inoculation of yeast suspension was done by adding ten microliters of it on tween 80 media in triplicate and incubated for 10 days aerobically at 37°C. Precipitation halo were observed around the inoculation site and outcome interpreted to be positive in this case (Slifkin, 2000). Esterase activity of each isolate was measured utilizing a standard formula (Aktas *et al*., 2002).

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All measurements were taken in millimeter. Results were interpreted according to the criteria described in Table 5.2. (Tsuboi *et al*., 1996; Yu *et al*., 2016).

Table 5.2: Classification of Esterase activity of *Candida* according to Ez index

Ez Index	Esterase activity
1	Negative
<0.81-0.99	Very low
0.61-0.80	Low
0.41-0.60	Moderate
<0.40	High

5.2.3.3.1.2- Phospholipase assay

Candida isolates were evaluated for their phospholipase activity on egg yolk agar media as described by Sachin *et al.,* (2013). The test medium comprised of 65g SDA, 58.4g NaCl, 5.5g $CaCl_2$ with 10% sterile egg yolk. In order to make test medium sterile, all the ingredients except egg yolk were blended in distilled water (980mL) and autoclaved at 121°C for 15 min. Sterile egg yolk was centrifuged for 10 min at 5000g and 10% of this was added to autoclaved media after cooling. The subsequent egg yolk media was mixed and poured in plates. Ten micro litres of yeast suspensions for each test isolate were inoculated in triplicates onto egg yolk medium and then was left to dry at room temperature.

These plates were incubated for four days at 37°C. Then phospholipase enzyme activity (Pz) was detected utilizing a formula which is a ratio of colony diameter and colony diameter plus precipitation zone formed around colony. Phospholipase action was categorized according to Pz values as shown in Table 5.3 (Shirkhani *et al*., 2016).

Table 5.3- Classification of Phospholipase activity of *Candida* according to Pz index.

Pz index	Phospholipase activity
1	Negative
<0.90- 0.99	Very Low
0.80-0.89	Low
0.70-0.79	Moderate
<0.70	Strong

5.2.3.3.2- Screening assays for biofilm formation

5.2.3.3.2.1- Congo red assay (Qualitative assay)

Previously described in chapter 4.0, section 4.2.3.6.2.1

5.2.3.3.2.2- Microtiter plate assay (Quantitative assay)

Previously described in chapter 4.0, section 4.2.3.6.2.2

5.2.3.4- Antifungal susceptibility test

Antifungal susceptibility testing was done via disc diffusion method, as recommended by CLSI. Inoculum of each isolate was first prepared by adding freshly cultured isolates in 1.0 mL of saline (0.85% sodium chloride) solution. The saline suspensions of each isolate were set up as per the 0.5% McFarland standard. Glucose Methylene Blue Muller Hinton (GMM) media was then prepared supplemented with glucose (2%) and methylene blue (0.5µg/mL). Suspensions were independently inoculated on different GMM agar plates and lawn was prepared with the help of cotton swab and plates were air dried. Later, two antifungal discs including 01µg Voriconazole and 25µg Fluconazole (Liofilchem, Italy), were placed on the GMM agar plate. Both antifungal discs were placed at a distance of 24 mm on prepared lawn on plates. All plates were incubated at 37ºC for 24-48 h.

5.2.3.5- Statistical analysis

Data analysis was performed by using SPSS software version 16 for windows 10. Chi-square (χ^2) and Fisher exact test was performed to determine association between different variables. A p value of <0.05 was considered as significant.

5.2.4- Part II: study of urinary fungal diversity by using culture independent method

5.2.4.1-Study population and sample collection

Described previously in chapter 4, section-4.2.4.1

5.2.4.2-DNA Extraction, sequencing, processing and analysis

Described previously in chapter 4 (section-4.2.4.2), however *ITS* region from extracted DNA sample was amplified by using primers *ITS1F* (CTTGGTCATTTAGAGGAAGTAA) and *ITS2*R (GCTGCGTTCTTCATCGATGC) with forward primers having barcode in 30 cycle PCR instead of primers used for bacterial sequencing. Raw data sequences have been submitted in Sequence Read Archieve and were assigned an accession number PRJNA603419

5.3- Results

5.3.1- Part 1- Isolation of culturable fungi from urine samples

Only fungi isolated was *Candida* species by culture dependent methods from urine samples of urolithiatic patients. These isolates were confirmed through different biochemical and molecular techniques.

5.3.1.1- Identification of *Candida* species

5.3.1.1.1- Growth of *Candida* on SDA

Candida isolates produced smooth, creamy and convex colonies with characteristic yeast odor on SDA. Among 164 urinary tract stone patients, 74 urine samples were suspected to have 77 *Candida* colonies on SDA. In case of controls, 19 (47.5%) were positive for suspected *Candida* species while 21 (52.5%) did not show any growth on SDA plate.

5.3.1.1.2- Gram Staining

On the basis of colony morphology on SDA, isolates were Gram stained in order to check the morphological features of suspected *Candida* species. *Candida* species appeared as Gram positive oval shaped cells.

5.3.1.1.3- Germ tube Test

Out of them, 48(62%) were positive for germ tube formation and 29 (38%) were negative. The positive isolates were suspected to be *C.albicans* and *C. dubliniensis,* were further distinguished by growing on SDA at a temperature of 45ºC. Among healthy controls, 10 (56%) out of 18 isolates were germ tube positive while 8(44%) negative for germ tube formation.

5.5.1.1.4- Characterization of *Candida* species using Chromogenic agar

The growth of *Candida albicans was* indicated by green coloured colonies, *Candida krusei* pink coloured colonies and *Candida glabrata* with white coloured colonies on Chrome (Figure 5.1). On the basis of growth and colour on Chrome agar media, *C. albicans* were 52, *C.glabrata* 21 and *C.krusei* 04 while in controls, *C.albicans* were 12 and *C.glabrata* 6.

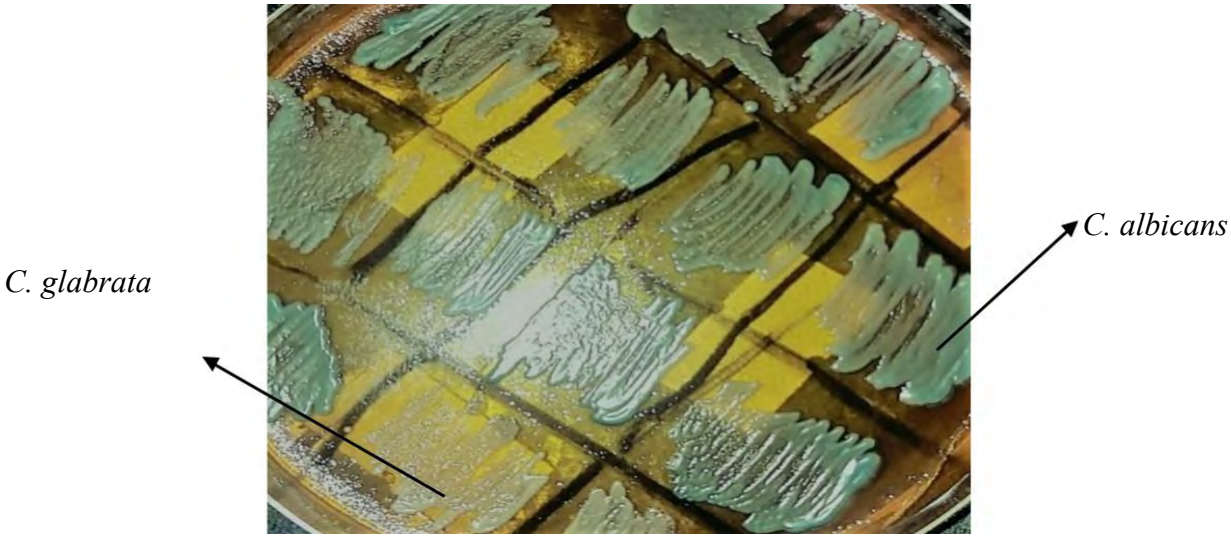

Figure 5.1: Growth of different *Candida* Species on Chrome agar after incubation period of 48 h at 37°C. Green colour indicated *C.albicans* (SF-56) and white colour indicated *C.glabrata* (SF-97).

5.3.1.1.5- Identification of *Candida* species by PCR-RFLP

Further confirmation for the species level identification of *Candida* was done by amplification of *ITS* gene followed by RFLP using 10U of *HpaII* enzyme. Size of fragments used for identification of various species and number of isolates of each species are shown in Table 5.4. Among urolithiatic patients 74 including 50 with cUTI were positive for *Candida. C. albicans* was the predominant species as 51 isolates out of total 77 isolates were C. *albicans* followed by *C. glabrata* 23 (29.8%) and *C. krusei* 3 (4%). Co-existence of *C. albicans* and *C. glabrata* was found in three patients.

5.3.1.2- Comparison of *Candida* colonization among urolithiatic patients with cUTI and healthy controls

It was observed that 45% of the urolithiatic patients were colonized by *Candida* species. High number of *C. albicans* was isolated as compared to other species. There was no difference in colonization of *Candida* or any of its species in urolithiatic patients and

controls. About 32% of patients with cUTI were positive for *Candida* isolation and 31.7% were not infected by *Candida* species. It was observed that there were 1.8-fold higher chances of *Candida* colonization in urolithiatic patients suffering from cUTI (Table 5.5-5.6).

5.3.1.3- Virulence factors of *Candida* isolated from urolithiatic patients with cUTI and healthy controls

5.3.1.3.1- Esterase assay

It was observed that 64(83%) of *Candida* isolates showed the esterase production which were 44/51 *C. albicans*, 17/23 *C. glabrata* and 3/3 *C.krusei.* Overall, isolates with high activity were 26(41%), 34(53%) showed medium whereas 04(6%) isolates had low esterase activity (Table 5.6). Of these esterase producers, 78% of *Candida* were isolated from the patients having cUTI (P=0.03). Among isolates possessing high activity of esterase ,16/51 were *C. albicans*, 07/23 *C. glabrata* and 3/3 were *C.krusei,* while moderate esterase producers included 26/51 *C. albicans*, 08/23 *C.glabrata* and 0/3 *C.krusei*. In controls, 03(25%) *C. albicans* isolates were positive, while 09(75%) isolates were negative for esterase activity. Among 06(33%) *C.glabrata*, 01(17%) isolate exhibited high and 03(50%) showed low esterase activity. Chi square analysis showed the significantly high esterase activity among overall *Candida* isolates from urolithiatic patients with P <0.01 and around 8 fold increased activity as compared to control group isolates. A significant raised esterase activity was also observed in *C. albicans* isolated from urolithiatic patients (P <0.01) as compared to healthy controls (Table 5.8). It was also observed that *Candida* isolated from urolithiatic patients with cUTI had significant raised esterase activity as compared to the *Candida* from patients without cUTI (Table 5.7). There were higher chances of isolation of esterase producer *C. albicans* and *C. glabrata* in urolithiatic patients with cUTI as compared to patients without cUTI (Table 5.9).

5.3.1.3.1- Phospholipase assay

From 77 isolates, 50 (64.9%) *Candida* isolates showed phospholipase production indicating the presence of phospholipase as virulence factor in these *Candida* species. Among phospholipase positive *Candida* isolates, 38/51 were *C. albicans*, 09/23 were *C.glabrata* and

3/3 were *C.krusei*. Among *C.albicans*, 35 isolates showed high, 03 medium while 13 showed no phospholipase activity. Out of 23 *C. glabrata* isolates, 04 possessed high, 05 medium and 14 did not possess any phospholipase activity. In controls, 02(17%) *C.albicans* showed high and 1(8%) showed moderate phospholipase activity. Chi square analysis showed the significantly high phospholipase activity in overall *Candida* isolates from urolithiatic patients with $P = 0.001$ and around 9-fold increased activity as compared to control group. *Candida* isolated from urolithiatic patients with cUTI showed higher phospholipase activity as compared to the isolates from patients without cUTI (Table 5.10). A Significant association was observed for the phospholipase activity in urolithiatic patients with cUTI in *C. albicans* isolated from urolithiatic patients as compared *C. albicans* from patient without cUTI and healthy controls (Table 5.11- 5.12).

5.3.1.3.3 Biofilm formation ability

Of 77 isolates of *Candida,* 33(43%) isolates showed strong and 31(40.2%) moderate biofilm formation ability while 9(11.6%) showed weak and 4(5.2%) did not show any biofilm formation activity. Out of 33 strong biofilm formers, 20 were *C. albicans,* 11 *C.glabrata and* 02 were *C. krusei.* While 25 *C. albicans,* 05 *C.glabrata and* 01 *C. krusei* were moderate biofilm former. Among controls, 2(11%) isolates showed moderate, while all others were weak biofilm former. Chi square analysis showed the significantly high biofilm forming ability in overall *Candida* isolates from urolithiatic patients with $P<0.01$ and around 15-fold increased activity as compared to control group (Table 5.13). A significantly high biofilm forming ability was observed in *C. albicans* isolated from urolithiatic patients ($P <0.01$) (Table 5.14). Chances of biofilm former *Candida* isolation was 2.8-fold higher in urolithiatic patients with cUTI as compared to patients without cUTI. Furthermore, stronger biofilm forming activity was seen in *Candida* isolates from patients with cUTI as compared to patients without cUTI where majority of *Candida* isolates showed moderate and weak biofilm forming activity (Table 5.15).

Table 5.4: Product sizes and number of isolates of different species of *Candida* identified through PCR-RFLP.

***Candida* species**	**PCR amplification product size (bp) of *ITS* gene**	**RFLP product sizes (bp)**	**Number of isolates**
C. albicans	535	338,297	51
C. glabrata	871	557,314	23
C. krusei	510	261,249	03

Table 5.5: Association of *Candida* colonization with urolithiatic patients having cUTI and healthy controls

Category		**Urolithiatic Patients n(%)**	**Healthy controls n(%)**	**Urolithiatic patients with cUTI n(%)**	**Urolithiatic patients without cUTI n(%)**
Candida	Positive	74(45)	18(45)	53(32.3)	21(13)
	Negative	90(55)	22(55)	52(31.7)	38(23)
P-value		0.9		0.07	
OR (95%CI)		1 (0.5-2)		1.8 (0.95 -3.55)	

Table 5.6: Association of different *Candida* species colonization with urolithiatic patients having cUTI and healthy controls

Category		**Urolithiatic patients n(%)**	**Healthy controls n(%)**	**Urolithiatic patients with cUTI n(%)**	**Urolithiatic patients without cUTI n(%)**
C. albicans	**Yes**	51(31)	12(30)	35(33)	16 (27)
	No	113(69)	28(70)	70(67)	43 (73)
P-value		0.8		0.4	
OR (95%CI)		01 (0.4-2.2)		1.34 (0.6-2.7)	
C. glabrata	**Yes**	23(14)	6(15)	19(18)	04 (7)
	No	141(86)	34(85)	86 (82)	55 (93)
P-value		0.87		0.05	
OR (95%CI)		0.9 (0.3-2.4)		3.03 (0.9-9.4)	
C. krusei	**Yes**	3(2)	0(0)	02(1.9)	01(1.7)
	No	161(98)	40(100)	103 (98.1)	58 (98.3)
P-value		0.7		0.9	
OR (95%CI)		1.75 (0.02-0.3)		1.1 (0.09-12.6)	

Table 5.7: Association of esterase activity of *Candida* with urolithiatic patients having cUTI and healthy controls to assess their pathogenic potential

Category		***Candida* species n(%)**			
		Urolithiatic Patients	**Healthy controls**	**Urolithiatic patients with cUTI**	**Urolithiatic patients without cUTI**
Esterase activity	**Present**	64(83)	7(39)	50(89)	14(67)
	Absent	13(17)	11(61)	06(11)	07(33)
P-value		<0.001		0.03	
OR (95% CI)		7.7 (2.5-23.7)		5.9 (1.85-19.2)	
Strength of esterase activity	**No**	13(17)	11(61)	06(11)	07(34)
	Low	04(5)	03(17)	01(2)	0(0)
	Medium	34(44)	0(0)	25(45)	12(57)
	High	26(34)	04(22)	24(43)	02(9)

Table 5.8: Association of esterase activity by different *Candida* species with urolithiatic patients having cUTI and healthy individuals to assess their pathogenic potential

Category		***C. albicans* n(%)**		***C. glabrata* n(%)**		***C. krusei* n(%)**	
		Urolithiatic patients	**Controls**	**Urolithiatic patients**	**Controls**	**Urolithiatic patients**	**Controls**
Esterase activity	**Present**	44 (86)	03 (25)	17 (74)	04 (67)	03 (100)	0 (0)
	Absent	07 (14)	09 (75)	06 (26)	02 (33)	0 (0)	0 (0)
***P*-value**		0.01		0.7		0.4	
OR (95% CI)		18.8 (4.0-87.1)		1.4 (0.2-9.8)		-	
Strength of esterase activity	**No**	07 (14)	09 (75)	06 (26)	02 (33)	0 (0)	0 (0)
	Low	02 (4)	0 (0)	02 (9)	03 (50)	0 (0)	0 (0)
	Medium	26 (51)	0 (0)	08 (35)	0 (0)	0 (0)	0 (0)
	High	16 (31)	03 (25)	07 (30)	01 (17)	03 (100)	0 (0)

Table 5.9: Association of esterase activity by different *Candida* species with cUTI among urolithiatic patients to assess their pathogenic potential

Category		***C. albicans* n(%)**		***C. glabrata* n(%)**		***C. krusei* n(%)**	
		Urolithiatic Patients		**Urolithiatic patients**		**Urolithiatic patients**	
		With cUTI	**Without cUTI**	**With cUTI**	**Without cUTI**	**With cUTI**	**Without cUTI**
Esterase activity	**Present**	32(91)	12(75)	16(84)	01(25)	02(100)	01(100)
	Absent	03(9)	04(25)	03(16)	03(75)	0(0)	0(0)
P-value		0.13		0.03		0.82	
OR (95% CI)		3.5 (0.6-18.3)		16(1.2-210)		-	
Strength of esterase activity	**No**	03(9)	04(25)	03(16)	03(75)	0(0)	0(0)
	Low	01(3)	01(6.2)	02(10)	0(0)	0(0)	0(0)
	Medium	16(45)	10(62.6)	07(37)	01(25)	0(0)	0(0)
	High	15(43)	01(6.2)	07(37)	0(0)	02(100)	01(100)

Table 5.10: Association of phospholipase activity of *Candida* with urolithiatic patients having cUTI and healthy controls to assess their pathogenic potential

Category		***Candida* species n(%)**			
		Urolithiatic patients	**Healthy controls**	**Urolithiatic patients with cUTI**	**Urolithiatic patients without cUTI**
Phospholipase activity	**Present**	50 (64.9)	03 (17)	42 (75)	08 (38)
	Absent	27 (35.1)	15 (83)	14 (25)	13 (62)
***P*-value**		0.001		0.006	
OR (95% CI)		9.1 (2.5-38.4)		4.8 (1.7-14.2)	
Strength of phospholipase activity	**No**	27(35.1)	15(83)	14(25)	13(62)
	Low	0(0)	0(0)	0(0)	0(0)
	Medium	08(10.4)	01(6)	07(12.5)	01(5)
	High	42(54.5)	02(11)	35(62.5)	07(33)

Table 5.11: Association of phospholipase activity of different *Candida* species with urolithiatic patients having cUTI and healthy controls to assess their pathogenic potential

Category		***C. albicans* n(%)**		***C. glabrata* n(%)**		***C. krusei* n(%)**	
		Urolithiatic patients	**Controls**	**Urolithia-tic patients**	**Controls**	**Urolithiatic patients**	**Controls**
Phospholip-ase activity	**PresenT**	38(74.5)	02 (17)	09 (39)	01 (17)	03 (100)	0 (0)
	Absent	13 (24.5)	10 (83)	14 (61)	05 (83)	0 (0)	0 (0)
***P*-value**		0.001		0.3		0.4	
OR (95% CI)		14.6 (2.8-75.6)		3.2 (0.3-32.2)			
Strength of phospholipa-se activity	**No**	13 (25)	10(83)	14(61)	05(83)	0(0)	0(0)
	Low	0 (0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Medium	03(6)	0(0)	05 (22)	01(17)	0(0)	0(0)
	High	35 (69)	02 (17)	04(17)	0(0)	03(100)	0(0)

Table 5.12: Association of phospholipase activity by different *Candida* species with cUTI among urolithiatic patients to assess their pathogenic potential

Category		***C. albicans* n(%)**		***C. glabrata* n(%)**		***C. krusei* n(%)**	
		Urolithiatic Patients		**Urolithiatic patients**		**Urolithiatic patients**	
		With cUTI	**Without cUTI**	**With cUTI**	**Without cUTI**	**With cUTI**	**Without cUTI**
Phospholipase activity	**Present**	31(89)	07(44)	09(47)	0(0)	02(100)	01(100)
	Absent	04(11)	09(56)	10(53)	04(100)	0(0)	00(0)
***P*-value**		0.001		0.17		0.82	
OR (95% CI)		9.96 (2.4-41.8)		8.1 (0.38-172)		-	
Strength of phospholipase activity	**No**	04(11)	09(56)	10(53)	04(100)	0(0)	0(0)
	Low	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Medium	02(6)	01(6)	05(26)	0(0)	0(0)	0(0)
	High	29(83)	06(38)	04(21)	0(0)	02(100)	01(100)

Table 5.13: Association of biofilm forming activity by *Candida* with urolithiatic patients having cUTI and healthy controls to assess their pathogenic potential

Category		***Candida* species n(%)**			
		Urolithiatic patients	**Healthy Controls**	**Urolithiatic patients with cUTI**	**Urolithiatic patients without cUTI**
Biofilm activity	**Present**	73(95)	10(55.5)	54(96)	19 (90.5)
	Absent	04(5)	08(44.5)	02(4)	02 (9.5)
***P*-value**		0.01		0.3	
OR (95% CI)		14.6 (3.7-57.4)		2.8 (0.3-21.6)	
Strength of biofilm forming activity	**No**	04(5)	08(44.5)	02(4)	02(9.5)
	Weak	09(12)	08(44.5)	04(7)	05(24)
	Moderate	31(40)	02(11)	19(34)	12(57)
	Strong	33(43)	0(0)	31(55)	02(9.5)

Table 5.14: Association of biofilm formation ability by different *Candida* species with urolithiatic patients having cUTI and healthy controls to assess their pathogenic potential

Category		***C. albicans* n(%)**		***C. glabrata* n(%)**		***C. krusei* n(%)**	
		Urolithiatic patients	**Controls**	**Urolithiatic patients**	**Controls**	**Urolithiatic patients**	**Controls**
Biofilm activity	**Present**	50 (98)	06(50)	20 (87)	04 (67)	03 (100)	-
	Absent	01 (2)	06 (50)	03 (13)	02 (33)	0 (0)	-
***P*-value**		0.01		0.2		0.4	
OR (95% CI)		50 (5.1-488)		3.3 (0.4-26.8)		-	
Strength of biofilm activity	**Absent**	01 (2)	06(50)	03 (13)	02 (33)	0(0)	-
	Low	05 (10)	04(33)	04 (17)	04 (67)	0(0)	-
	Medium	25 (49)	02(17)	05 (22)	0(0)	01(33)	-
	High	20 (39)	0(0)	11 (48)	0(0)	02(67)	-

Table 5.15: Association of biofilm formation ability by different *Candida* species with cUTI among urolithiatic patients to assess their pathogenic potential

Category		***C. albicans* n(%)**		***C. glabrata* n(%)**		***C. krusei* n(%)**	
		Urolithiatic patients		**Urolithiatic patients**		**Urolithiatic patients**	
		With cUTI	**Without cUTI**	**With cUTI**	**Without cUTI**	**With cUTI**	**Without cUTI**
Biofilm activity	**Present**	35(100)	15(94)	17(90)	03(75)	02(100)	01(100)
	Absent	0(0)	01(6)	02(10)	01(25)	0(0)	0(0)
***P*-value**		0.24		0.4		0.8	
OR (95% CI)		6.9 (0.26-178)		2.8 (0.19-41.9)		-	
Strength of biofilm activity	**Absent**	0(0)	01(6)	02(10)	01(25)	0(0)	0(0)
	Low	02(6)	03(19)	02(10)	02(50)	0(0)	0(0)
	Medium	14(40)	11(69)	05(27)	0(0)	0(0)	01(100)
	High	19(54)	01(6)	10(53)	01(25)	02(100)	0(0)

5.3.1.4- Antifungal Sensitivity Test

It was observed that out of 77 isolated *Candida* spp. 16.9% isolates (*C.albicans* 11, *C. glabrata* 02) showed the resistance against fluconazole and 19.5% isolates (*C.albicans*11, *C. glabrata* 04) were resistant against voriconazole. Of total resistant isolates, 11 isolates were resistant against both tested antifungals. Among isolates from controls 07(39%) *Candida* isolates (*C.albicans* 05, *C.glabrata* 02) showed resistance against fluconazole while 6 isolates (*C.albicans* 05*, C.glabrata* 01) were resistant against voriconazole (Table 5.16). Co-existence of different virulence factors and antibiotic resistance was determined. All exhibiting resistance to either fluconazole or voriconazole were also esterase enzyme producers. Biofilm formers were also voriconazole resistant isolates as shown in Table 5.17.

5.3.1.5- Association of *Candida* colonization with different sociodemographic and clinical risk factors among urolithiatic patients

Candida colonization was significantly associated with age of urolithiatic patients with cUTI (P = 0.006) as compared to the patients without cUTI (Table 5.18). There were 73 (44.5%) females and 91(55.5%) males out of which 39 females and 35 males were found to be positive for *Candida* isolation. From these *Candida* positive females, 27(69%) were suffering from cUTI while in case of males 26(72%) also had cUTI. Irrespective of age, males were equally susceptible to candiduria. High prevalence of *Candida* was found in female patients of less than 50 year (31, 79%) in which 21 female patients were having cUTI. Majority of cUTI patients suffering from urolithiatic disease for more than one year were positive for *Candida* isolation. Catheterized patients having cUTI were more colonized by *Candida* (Table 5.19).

Table 5.16: Antifungal susceptibility pattern of different *Candida* species isolated from urolithiatic patients with cUTI against fluconazole and voriconazole

Samples	***Candida* Species**	**Total number of isolates**	**Fluconazole n(%)**		**Voriconazole n (%)**	
			Resistant	Sensitive	Resistant	Sensitive
Urolithiatic patients	*C.albicans*	51	11(21.6)	40(78.4)	11(21.6)	40(78.4)
	C.glabrata	23	02(8.6)	21(91.3)	04(17.4)	19(82.6)
	C.krusei	03	0(0)	03(100)	0(0)	03(100)
Healthy control	*C.albicans*	12	05(42)	07(58)	05(42)	07(58)
	C.glabrata	06	02(33)	04(67)	01(17)	05 (83)

Table 5.17: Association of different virulence factors with fluconazole and voriconazole resistant *Candida* isolates from urolithiatic patients

Antifungals		**Phospholipase activity**		**Esterase activity**		**Biofilm formation**	
		Positive	**negative**	**positive**	**negative**	**Positive**	**negative**
Fluconazole	**Resistant**	11	02	13	0	13	0
	Sensitive	39	25	47	17	51	13
P-value		0.13		0.06		0.109	
Voriconazole	**Resistant**	12	03	15	0	15	0
	Sensitive	38	24	45	17	49	13
P-value		0.23		0.032		0.06	

Table 5.18: Association of *Candida* colonization with different sociodemographic risk factors among urolithiatic patients with cUTI

Category	**Variable**	**Isolation of *Candida***		***P-value***
		Urolithiatic patients		
		With UTI	**Without UTI**	
Socioeconomic status	Higher class	01	03	0.006
	Middle class	15	11	
	Lower class	37	07	
Quality of water	v. hard	10	06	0.652
	Hard	05	01	
	Moderately Hard	23	10	
	Soft	15	04	
Water intake daily	Low	10	09	0.03
	Medium	29	05	
	High	14	07	
Location	Hilly	28	15	0.982
	Plan	15	06	
Living environment	Rural	21	09	0.798
	Urban	32	12	
Age range(yrs)	11-20	05	01	0.04
	21-30	11	11	
	31-40	09	02	
	41-50	12	04	
	51-60	04	03	
	61-70	12	0	
Gender	Male	26	10	0.911
	Female	27	11	
Marital status	Married	45	12	0.01
	Unmarried	08	09	

Table 5.19: Association of *Candida* colonization with different clinical risk factors among urolithiatic patients with cUTI

Category	**Variable**	**Isolation of *Candida* from urolithiatic patients**		***P-value***
		With cUTI	**Without cUTI**	
Stone duration	<1y	09	18	<0.001
	1-3y	29	02	
	>3y	15	01	
BMI	Underweight	03	0	0.449
	Normal	30	11	
	Overweight	20	10	
Catheterization	Yes	32	01	<0.001
	No	21	20	
Urine pH	<6	18	17	0.001
	6-6.5	15	03	
	>6.5	20	01	
WBCs in urine/HPF	0-5	0	11	<0.001
	5-100	48	10	
	>100	05	0	
Stone size (mm)	<4	05	02	0.796
	4-10	34	15	
	>10	14	04	

5.3.2- Part II: Study of urinary mycobiome by culture-independent method

5.3.2.1- Sequence characteristics:

A total of 399 reads were obtained from four types of pooled urine samples. Each sample contained 99 reads. A total of 150 OTUs were detected in all of these samples. After data trimming, filtering and normalization, detected OTUs with count $\geq$ 2 were 115.

5.3.2.2- Taxonomic analysis

5.3.2.2.1- Predominant fungal taxa in urine samples:

Ascomycota and Bisidiomycota were the predominant phyla found in all pooled urine samples. A total of 18 classes, 30 orders, 41 families, 49 genera and 70 species were detected in all of these samples. Sample SP1 had dominating Ascomycota phylum 84.4% followed by Bisidiomycota 15.6%. SP2 had Ascomycota phylum 51.1% followed by Bisidiomycota 48.9%. In contrast to these pooled patients' samples, control group sample was dominated by Bisdiomycota phylum as in SP3 69.65% was Bisidiomycota phylum followed by 30.3% Ascomycota phylum. In case of control sample SP4, Bisidiomycota was the predominant phyla with 78.2% followed by Ascomycota 21.8%. So, it can be concluded the kidney stone patients with cUTI were predominant with Ascomycota phylum while Bisidiomycota phylum was found to be prominent in healthy controls.

Abundance of major genera was also observed in all of these four pooled samples. In SP1, major genera were *Paraconiothyrium* (44%) followed by *Necteria* (22.4%), *Cladosporium* (12.2%), *Cryptococcus* (8.6%), *Malassezia* (4.4%), *Septoria* (3%), *Ustilago* (2.6%), *Fusarium* (2.1%), *Aspergillus* (0.13%), *Aureobasidium* (0.06%), *Candida* (0.06%), *Permelia* (0.06%) and *Eurotium* (0.05%). SP2 included the predominant genera *Ustilago* (42%) followed by *Aspergillus* (13.5%), *Necteria* (10.5%), *Aureobasidium* (6.7%), *Hanseniaspora* (6.2%), *Malassezia* (3.6%), *Fusarium* (3.5%), *Blastobotrys* (3.13%), *Omphalina* (3%), *Cladosporium* (1.5%), *Candida* (1.5%) and *Leptosphaerulina* (1.2%).

SP3 had major genera including *Malassezia* (55.5%), *Dioscorea* (18%), *Eurotium* (8.6%), *Fusarium* (4.8%), *Cladosporium* (4.13%), *Necteria* (3.4%), *Neurospora* (1.4%),

Candida (1.4%), *Ustilago* (0.6%), *Liposcelis* (0.3%), *Aspergillus* (0.2%), *Aureobasidium* (0.08%) and *Paraconiothyrium* (0.05%). SP4 sample included major genera *Ustillago* (77%), *Necteria* (14%), *Fusarium* (2.3%) *Aureobasidium* (2.1%) and *Alterneria* (1.1%).

Penicilliume, Curvulaia, Prostelium, Parmelia, Menegzzia, Septoria and Geomyces genera were detected in patients with cUTI having kidney stone but were not found in healthy individuals. Abundance of *Paraconiothyrium* was strikingly high (44%) in female patients of cUTI with stone as compared to the male patients with stone and cUTI (0.12%). *Cryptococcus, Cladosporium* and *Septoria* were also the genera found abundantly in female patients as compared to male patients. In male patients with cUTI and stone, more abundant genera were *Ustilago* (42%) and *Apergillus* (13.5%) as compared to female patients. In female controls *Malassezia* (55.5%), *Dioscorea*(18%) and *Eurotium* (8.6%) are more abundant as compared to male controls. *Ustillago* (77%) and *Necteria* (14%) were the genera found as major genera in male controls. *Phoma* is the genera found in males but was not found in female samples.

At species level, in SP1 major species found were *Paraconiothyrium brasilience* (44%) followed by Necteria *mauritiicola* (22.4%), *Cladosporium cladosporiodes* (12%), *Cryptococcus heimaeyensis* (9%), *Septoria arundinacea* (3.02%), *Ustilago sparsa* (2.5%), *Malassezia globsa* (2.2%), *Fusarium gibberella fujikuroi* (2.09%), *Aspergillus sydowii* (0.1%), *Dioscoreaalata*(0.08%), *Parmelia saxatilis* (0.07%) and *Aureobasidium pullulans* (0.06%).
In SP2, predominant species were *Ustilago sparsa* (42%) followed by *Aspergillus sydowii* (11%), *Necteria mauritiicola* (10.6%), *Aureobasidium pullulans* (6.6%), *Hansenia sporauvarum* (6.2%), *Fusarium gibberella fujikuroi* (3.6%), *Blastobotrys proliferans* (3.13%), *Omphalina pyxidata* (3.02%), *Malassezia restricta* (2.01%), *Candida albicans* (1.5%), *Aspergillus penicillioides* (1.3%), *Leptosphaerulina sp.* (1.2%), *Malasseziaglobsa* (1%), *Neurospora* spp. (0.8%), *Srelitziana Africana* (0.7%), *Alternaria alternate* (0.7%), *brasilience* (0.12%), *Curvularia cochlioboluslunatus* (0.1%), *Aspergillus oryzae* (0.1%), *Penicillium glabrum* (0.1%) and *Paraconiothyrium dioscoreaalata* (0.09%).

In SP3, major species found were *Malasseziaglobsa* (36%) followed by *Malassezia restricta* (19%), *Dioscoreaalata* (18%), *Eurotium halophilicum* (8.6%), *Fusarium gibberella fujikuroi* (4.8%), *Cladosporium cladosporioides* (4%), *Necteria mauritiicola* (3.4%) and *Candida tropicalis* (0.9%). In SP4, predominant species found were *Ustilago sparsa* (77%) followed by *Necteria mauritiicola* (14%), *Fusarium gibberella fujikuroi* (2.3%), *Aureobasidium pullulans* (2.06%), *Alternariaalternate* (1%) *Cladosporium cladosporioides* (0.5%), *Malassezia globsa* (0.4%) and *Malassezia restricta* (0.4%).

5.3.2.3- Community profiling

5.3.2.3.1- Alpha diversity

To measure the alpha diversity, Shannon and Simpson diversity index were calculated for determination of the richness and evenness of the samples, along with statistical analysis through Mann-Whitney method. Diversity analysis showed the greater richness among the patients with kidney stone and cUTI as compared to the healthy individuals. Among healthy individuals' female controls showed the greater richness as compared to males. Among patients, males showed greater diversity. Shannon index showed high richness and evenness in all samples except SP4 which was healthy male controls pooled sample. Highest richness and evenness were found in male patients (Table 5.20).

5.3.2.3.2- Beta diversity

Bray-Curtis dissimilarity showed highest dissimilarity (90%) between SP1 female urolithiatic patients with cUTI and SP4 male healthy subjects. Lowest dissimilarity (54%) was observed between SP2 male urolithiatic patients with cUTI and SP4 male healthy subjects. It was observed that 84% dissimilarity was present between SP1 female patients and SP2 male patients. In principle coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) by using Bray-Curtis, results were insignificant. PERMANOVA statistical analysis detected F-value = 0.49389, R^2= 0.19804, p-value < 1 and Stress = 0.

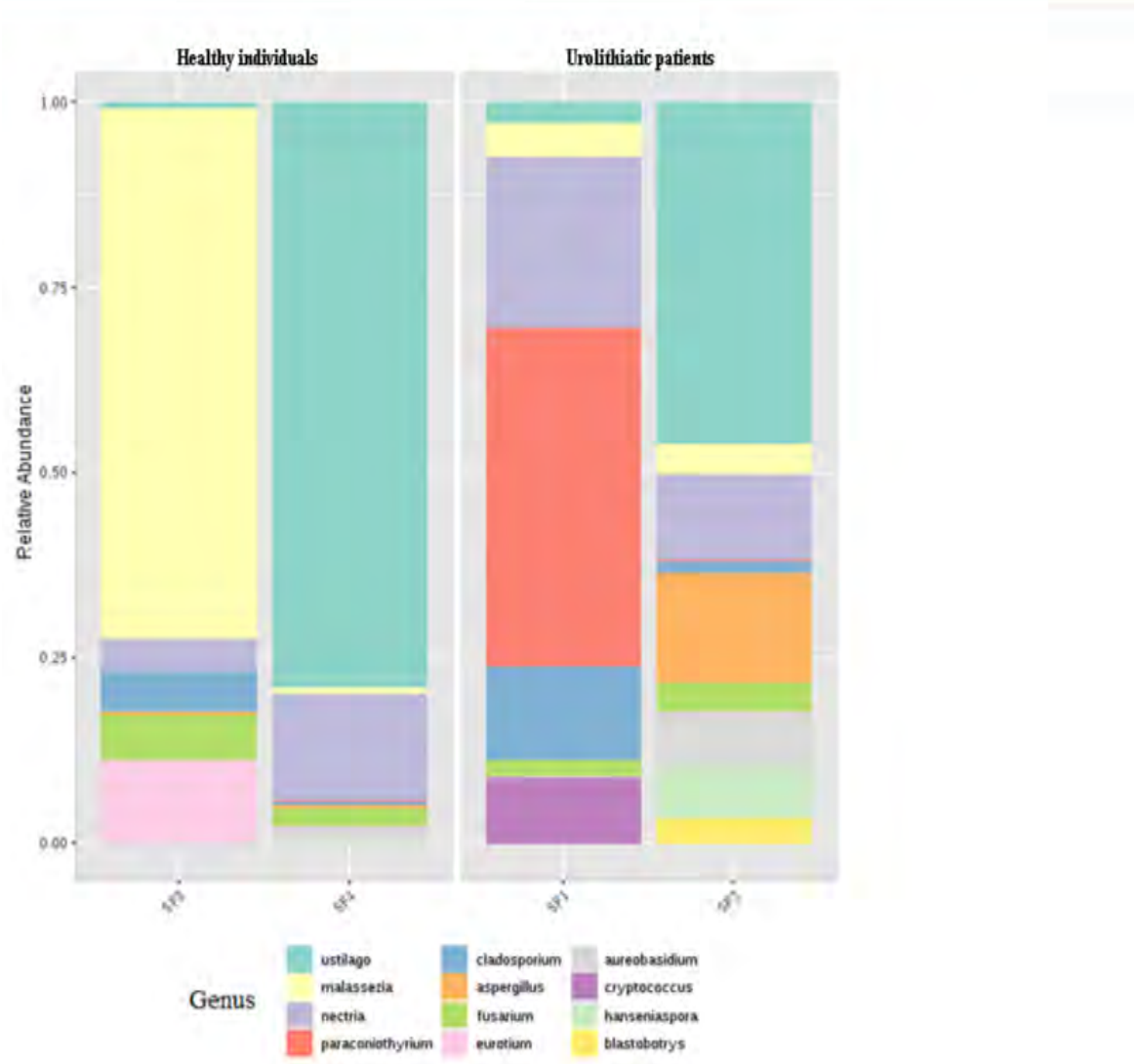

Figure 5.2: Relative abundance of predominant fungal taxa in urolithiatic patients with cUTI (SP1 and SP2) and healthy subjects (SP3 and SP4) at genus level analyzed by Microbiome analyst tool using cut off value >10 counts.

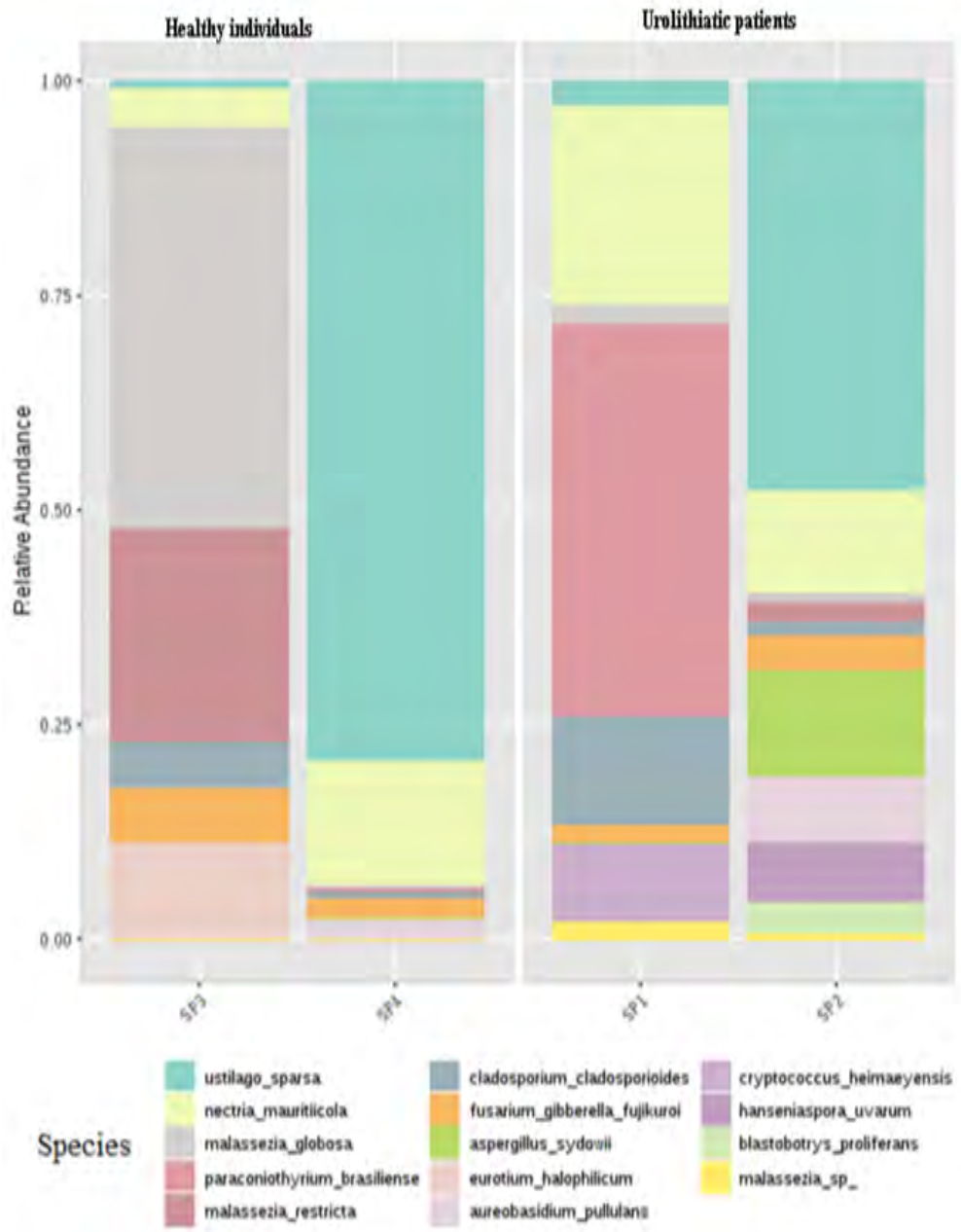

Figure 5.3: Relative abundance of predominant fungal taxa in urine samples of urolithiatic patients with cUTI(SP1 and SP2) and healthy subjects(SP3 and SP4) at species level analyzed by Microbiome analyst tool using cutoff value >10 counts.

Table 5.20: Alpha diversity indices for urinary mycobiome of urolthiatic patients (SP1, SP2) and healthy subjects (SP3, SP4) analyzed by using Microbiome analyst tool.

Sample ID	Shannon index	Simpson index
SP1	1.57	0.73
SP2	1.64	0.71
SP3	1.53	0.73
SP4	0.66	0.31

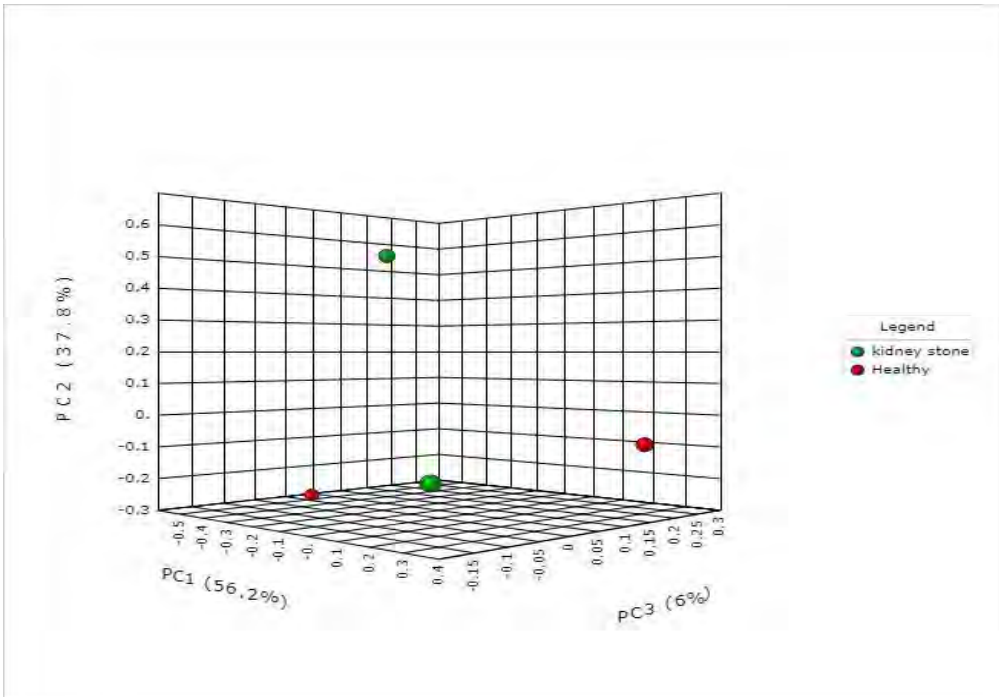

Figure 5.4- PCoA analysis by Bray-Curtis distance of OTU based clustering of microbial communities in urolithiatic patients with cUTI (SP1 and SP2) and controls (SP3 and SP4). PCoA score for Axis 1, Axis 2 and Axis 3 were SP1; -0.13722, 0.52017 and 0.00325, SP2; 0.32422, -0.13332 and -0.12882, SP3; 0.55081, 0.27918 and 0.007358, SP4; 0.3638, 0.10767 and 0.12472 analyzed by Microbiome analyst with cut off >10% abundance

5.3.2.3.3- Core mycobiome analysis

At genera level 29 out of 49 genera were shared between all pooled urine samples (Figure 5.5). At genera level *Paraconiothyrium, Aureobasidium, Alternaria, Omphalina, Cladosporium, Fusarium, Wallemia* and *Neurospora* were found at top to be present in all samples.

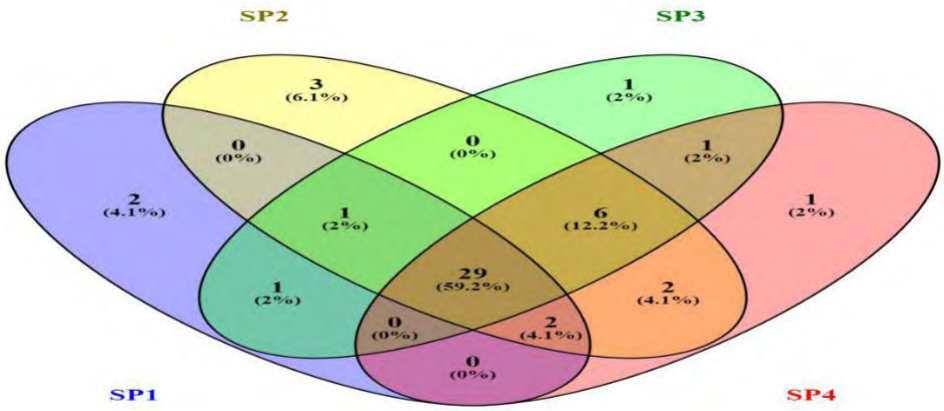

Figure 5.5: Core mycobiome analysis; percentage abundance of core genera with all urine samples sharing 59.2% of detected genera performed by using Venny.

5.4- Discussion

This part of the study was conducted to detect the culturable and unculturable fungal diversity in urolithiatic patients with and without cUTI. *Candida* was the only fungus observed using culturable techniques from urine samples of patients. The urolithiatic patients with cUTI had 1.8 fold increased risk for *Candida* colonization as compared to patients without cUTI. cUTI is a condition, which is a consequence of number of factors that promote infection and type of infecting organisms. The recovery rate of *Candida* species from urine samples of complicated urinary tract infection patients varies in different studies as there are multiple factors contributing in the development of complications in urinary tract, hence due to variation in risk factors *Candida* species isolation rate was different in present work. In this study comparatively higher (45%) isolation of *Candida* spp. was seen compared to an Indian study (Dalal *et al.,* 2016) in which less number of isolates of *Candida* were reported. That Indian study included patients visiting various departments like nephrology, urology and endocrinology with cUTI as compared to the present work in which only urolithiatic patients with cUTI admitted in surgical urology wards were included. Likewise, a study conducted in Karachi, Pakistan (Lewis, 2018) reported low prevalence of 12.5% *Candida* spp. from cases of cUTI but these patients were visiting different wards. As their patients inclusion criteria was presence of cUTI irrespective of type of disease, therefore patients with different pathologies such as upper and lower urinary tract obstruction, diabetes mellitus, renal insufficiency, renal stones, immune suppression and surgery of the urinary tract were assessed. Higher prevalence of *Candida* isolates in the present study might be due to stringent inclusion criteria of only urolithiatic patients from surgical wards, as these patients might have cUTI caused by the urinary obstruction with stones or inserted catheters in urolithiatic patients.

C. albicans is normally the predominant species as compared to non-albicans isolated from different type of urinary tract infections, which is in line with the current work as higher prevalence of *C. albicans* was seen compared to non-albicans. These results are also in agreement with other studies conducted in Malaysian Medical Centre (Ding *et al.,* 2014) and Egypt (Alhussaini, 2013), where urinary tract infection patients had prevalence

rate of 59.4% and 54% of *C. albicans* respectively. In contrast to these studies, Rishpana and Kabbin, (2015) reported non-albicans to be more prominent in urine samples of catheterized patients in India which might be due to the fact that they observed only catheterized patients irrespective of their disease.

It was observed that 46% of male urolithiatic patients with cUTI were positive for *Candida* isolation while in case of female urolithiatic patients with cUTI, 56% were detected positive for *Candida* colonization. In overall urolithiatic patients, majority of female patients were positive for *Candida* isolation. This high prevalence might be due to shorter urethra and common colonization of *Candida* in the urogenital tract of females (Colodner *et al*., 2008). A significant numbers of *Candida* species were isolated from urolithiatic patients of age group 21-30yrs. High isolation of *Candida* colonization in this age group can be related to their life style and eating habits. Majority of patients in this age group were students. Interestingly 80% of female urolithiatic patients in age group of 21-30yrs were also *Candida* positive. High glucose level due to eating habits in this age group can be one of the reasons of high *Candida* colonization. Almost half of the *Candida* positive patients of younger age group were not suffering from cUTI, but in case of older age group (61-70 yrs) all *Candida* positive urolithiatic patients were also significantly positive for cUTI. This can be due to age, which plays an important role in lowering the immunity of a person and raising the chances to be infected by different types of pathogens. A study conducted in Iran on UTI patients concluded that age was significantly associated with the isolation rate of *Candida* (Gharanfoli *et al.,* 2019). Glucose concentration is enhanced in the urine with aging and elevation of glucose to more than 150mg/dL is suitable environment for *Candida* to grow (Emami *et al.,* 2016).

In this study catheterized patients were found to be significantly positive for *Candida* isolation ($P<0.01$). A significant *Candida* isolation was seen among urolithiatic patients with catheterization, which could be due to the fact that catheters are physical obstruction in the urinary tract and can serve as site for colonization of biofilm forming microbes. Infection is the largest concern with catheter use in both short term and long term cases. *Candida* was detected more (68%) among catheterized patients, who had cUTI as compared to patients without cUTI. In a study conducted in India, 26% of people using

catheter developed cUTI due to *Candida* species (Rishpana and Kabbin, 2015). A significant association was observed for *Candida* isolation in catheterized patients with their socio-economic status. It was observed that majority (59%) of catheterized patients positive for *Candida* isolation belonged to low socioeconomic status. Further, such *Candida* positive patients also had urolithiasis for long duration. Interestingly, when socioeconomic status was assessed as risk factor alone, there was insignificant association with *Candida* colonization. However, *Candida* infections when analyzed in catheterized patients, a significant association to socioeconomic status transpired. Individuals from lower socioeconomic strata live in poor hygienic conditions and also have less access to proper health care system.

Candida species colonizes the urinary tract as commensal, but under certain conditions they express their virulence factors which help them to transit from commensal to pathogen and cause cUTI. In case of disturbed microbial balance, commensal *Candida* may become pathogenic. This development of pathogenicity depends upon various factors such as parasitic load, host immunosuppression and fungal virulence facilitating the tissue invasion and evasion of host defence system (Ying and Chunyang, 2012). Epidemiological studies showed that pathogenic *Candida* species are generally responsible for causing superficial to systemic infections (Jamil *et al.,*2017).

To detect the presence of virulent strains screening of various virulence factors including biofilm, esterase and phospholipase activity of *Candida* species were carried out. In this study, 80% of *Candida* isolates exhibited esterase activity, out of which 86% were *C. albicans*. This enzyme has the ability to digest surface membrane of host and contribute in tissue invasion. A study from Turkey reported esterase activity in 80% of *Candida* isolates and 98% of *C.albicans* from different clinical samples (Aktas *et al.,* 2002). In current work, a significant high esterase enzyme activity was observed in the *Candida* isolated from urolithiatic patients as compared to the healthy individuals. This enzymatic activity was significant in *Candida* isolates from urolithiatic patients who had cUTI. This point towards pathogenic potential of the *Candida* isolated from cUTI. Majority of isolates with esterase enzyme production were isolated from the cUTI patients who were

suffering from urolithiasis for more than one year. The long duration of disease provided the opportunities to commensal *Candida* to be pathogenic.

Phospholipase production by *Candida* species facilitates in the adhesion and invasion of host cells (Ghannoum, 2000). In current work, 65% of *Candida* isolates with 75% of *C. albicans* were observed to possess phospholipase enzyme activity. These results are consistent with previous Brazilian study in which high phospholipase activity in *Candida* species was observed from different clinical isolates and all *C. albicans* were positive for this activity (Riceto *et al.,* 2014). In contrast to present work, another study conducted in Brazil reported only 8.7% of *C. albicans* with moderate phospholipase activity isolated from hospitalized patients (Wiebusch *et al.,* 2017). This low rate of phospholipase activity in that study might be due to the fact that patients were hospitalized but were not analyzed for having cUTI specifically. In present work, this enzyme activity was high in isolates as these were from urolithiatic patients. Furthermore, *Candida* isolates from cUTI patients had significantly more phospholipase activity. An Iranian study reported 66.7% of *Candida* positive for phospholipase enzyme activity isolated from viginitis patients (Seifi *et al.,* 2015). Catheterized patients with cUTI had more *Candida* with strong phospholipase activity in current work.

Among all the isolates, 83% of *Candida* isolates showed biofilm forming ability. A study conducted in India reported 65% of *Candida* isolates from different clinical samples positive for biofilm formation (Golia *et al.,* 2012). Biofilm formation is involved to impart several advantages to the microbes such as nutrient availability, protection from environment, acquisition of new traits and metabolic cooperation (Mohandas and Ballal, 2011). Biofilms are of particular significance due to their major involvement in human infections. According to estimation, biofilms are involved in almost 65% of these infections (Golia *et al.,* 2012). In current study, the isolates from cUTI patients with long duration of stone disease were significantly biofilm formers. Based on the statistical analysis of different virulence factors tested for isolated *Candida* spp., it can be inferred that the patients with cUTI, catheters and long duration of stone disease are colonized with more virulent isolates of *Candida* spp. It was seen that 57% of isolates were positive for all the virulence factors in current work, which indicate they might have

pathogenic role in causing cUTI of urolithiatic patients. Long term catheterization or urinary tract stones can cause damage to the urinary tract providing opportunity to the microbes to develop infection. Such infections are result of microbial succession where initially some bacterial strains and then commensal *Candida* turn into pathogens play role in pathogenesis.

Out of 77 isolated *Candida* spp., 16.9% isolates were resistant to fluconazole and 19.5% isolates to voriconazole in this study. Overall sensitivity rate against fluconazole in this study was 83.1%, which is in agreement with the study conducted in Pakistan by Aslam *et al*., (2015). That study also observed 87.4% of sensitivity against fluconazole in *Candida* isolated from different clinical samples. Similar to present findings, another study conducted in Pakistan showed that 14.6% isolates of *Candida* were resistant to fluconazole while 18.26% were resistant to voriconazole (Tasneem *et al.,* 2017). Both studies were from Islamabad, which showed antifungal resistance trend similar to present work, which may be due to lack of prescription of antifungal therapy in local hospitals, a general trend in local hospitals of Islamabad even in whole Pakistan. An important finding of by Tasneem *et al.,* and present work was an increased voriconazole resistance in *Candida* isolates from urolithiatic patients as compared to fluconazole resistance. This voriconazole resistance emergence in isolates might be related to cross resistance, due to similarity in chemical structure of fluconazole and voriconazole.

On the basis of these findings, it can be inferred that there is significant prevalence of virulent *Candida* spp. in local urolithiatic patients suffering from cUTI is present. Isolated *Candida* species exhibited multiple virulence factors and antibiotic resistance. This work highlights the possible role of *Candida* spp. in cUTI which is less investigated for the treatment of cUTI in urolithiatic patients. *E. coli* and *K. pneumonia* are considered to be prime pathogens involve in complications of UTI; therefore antibacterial therapy is prescribed against such pathogens. The detection of virulent *Candida* spp. more in cUTI in this work compared to *E. coli* and *K. pneumonia* indicates that *Candida* spp. might also have role in complicated and recurrent UTI of urolithiatic patients but not focused in hospital settings. *Candida* is normally overlooked in the local tertiary care hospitals and no antifungal therapy is given; therefore it can be concluded that a detailed evaluation of

cUTI in urolithiatic patients with respect to *Candida* spp. and their virulence factors can be useful for improving clinical management and to reduce the disease burden due to missed antifungal therapy.

In second part of the study, sequencing technique was used to explore the mycobiome involved in cUTI among urolithiatic patients. The majority of fungal diversity is unculturable; to investigate the whole mycobiome NGS was used. Urinary mycobiome is a complex and divers' community but there are only a few fungal species which are known to be involved in different human infections (Iliev and Underhil, 2013). In current study by using the MiSeq illumine platform, urinary mycobiome of urolithiatic patients with cUTI and healthy controls was identified. It is the first study, which was conducted to determine the mycobiome associated with development of cUTI among patients with urinary obstructions. In this study, it was observed that phylum Ascomycota was strikingly predominant in patients as compared to controls where Basidiomycota was prominent. Ascomycota is known to have lot of pathogens and there are numerous studies on the fungal pathogens in the Ascomycota but less data on the frequency with which they cause human diseases (Heitman, 2011). In a study conducted in China on pregnant diabetic women detected predominant Ascomycota in their vaginal mycobiome (Zheng *et al.,* 2013). Another study conducted in USA identified fungal population in urinary samples of asymptomatic patients by using NGS. The study reported that each individual had a diverse population of fungi (Ackerman and Underhill, 2017).

In present work, there was major difference found between the predominant genera in diseased and healthy subjects. In urolithiatic female patient with cUTI, *Paraconiothyrium* followed by *Necteria*, *Cladosporium* and *Cryptococcus were* detected. They all belonged to Ascomycota phylum. *Paraconiothyrium brasiliense* was the predominant species found in female urolithiatic patients. This is coelomycetous fungi. Coelomycetous fungal infections are rare and these fungi cannot be identified by using only phenotypic tools, therefore these infections are poorly characterized (Hermoso *et al.,* 2019). A study indicated that *Paraconiothyrium* infection was present in a kidney transplant recipient which is also predominate genus in current work (Colombier *et al.,* 2015). Also study conducted in Europian reference centres reported *Medicopsisromeroi* followed by

Paraconiothyrium spp. in cutaneous lesions infection, although site is different but shows that these are pathogenic fungi (Hermoso *et al.,* 2018). *Cladosporium* species are dematiaceous and ubiquitous fungi which is rarely associated with human infections. Denis *et al.,* (2015) identified large number of *Cladosporium* isolates from clinical samples in USA.

Male urolithiatic patients with cUTI had *Ustilago* as the predominant genera followed by *Aspergillus*, *Necteria* and *Aureobasidium*. Pathogenic role of Ustilago is unclear in literature. The most dominant species *Utilago sparsa* was detected in male urolithiatic patients of cUTI. *Aureobasidium pullulans* is a dematiaceous saprophytic fungus which is widely distributed in environment. It can be isolated from decaying plant debris, soil, rock, wood as well as human skin, nails and hair (Hawkes *et al.,* 2005). *Aureobasidium* was found as a major causative agent in nosocomial fungal infection in a case study of a severely traumatized patient in Italy (Bolignano1 and Criseo, 2003). Another case study reported subcutaneous mycosis due to *Aureobasidium*. This is a rare infection with only a few cases reported so far (Eswarappa *et al.,* 2015). *Aspergillus* had been identified to be involved in UTIs (Hedayati *et al*., 2007). *Aspergillus* is not common involved in fungal UTIs but detected in current study as a major participant in urinary mycobiome of male patients. Bibler *et al*., (1987) reviewed 11 patients with aspergillomas of the renal system and ten of these were men. All detected fungal genera by NGS, indicated that these genera might not be detected through culturable techniques due to their special nutritional and environmental requirement but might be present as potential pathogens in urinary mycobiome.

There was major difference in abundance of different organisms detected in patients with cUTI having urinary tract stone as compared to healthy individuals indicating the role of fungi in cUTI. A striking difference in mycobiome of urolithiatic patients on the basis of gender was detected in this study. This might be due to the difference in anatomy and physiology of urinary tract of man and woman (Abelson *et al.,* 2018).

Data from the present work indicated for the first time, a snapshot of urinary mycobiome in cUTI among urolithiatic patients with both culturable and unculturable methods. There

was a major difference observed in culturable and unculturable fungal community. Only *Candida* was the fungus obtained from culturable technique. *Candida* isolates from urolithiatic patients expressed enhanced virulence characteristics as compared to the isolates from healthy individuals. Through sequencing, a number of unculturable fungi were detected and most interestingly, more species of *Candida* but with low abundance as compared to other fungi was detected. Comparatively greater diversity (genera) was observed in patients as compared to healthy controls. Overall fungal communities detected along with their virulence suggested that there is a major role of fungi in progression of cUTI in urolithiatic patients after *Candida* colonization. Despite small size of study, mycobiome provided basic knowledge of fungal population and genera in urolithiasis. These results only provide baseline information but there are intra population and individual variabilities in diseased conditions which needs to be deciphered with larger sample size.

CHAPTER 6

In vitro model to understand the development of poly-microbial biofilms

6.1-Introduction

Poly-microbial biofilms play a vital role in UTIs where complex communities of different species adhere to either biotic or abiotic surfaces, such interaction with each other develop into infections (Donlan and Costerton, 2002). There are three distinct steps in biofilm development starting from attachment of microbial cells to the surface, later growth into a sessile biofilm colony and finally detachment of these cells into the surrounding medium from colony. Every stage can be shaped by interspecies interactions from synergistic relationships to competition (Burmolle *et al.,* 2014).

UTI is an important cause of morbidity due to which health care spending increases in a country (Soto, 2014). UTI related to urolithiasis or due to any structural abnormality of urinary tract is considered to be the complicated UTI. cUTI is hard to treat as it is thought to be due to diverse and highly pathogenic microbes involved in its pathogenesis. According to the National Institutes of Health (USA), biofilms are involved in more than 60% of microbial infections (Sevanan *et al.,* 2011). Major pathogens involved in cUTI are *E. coli* followed by *P. aeruginosa, Acinetobacter baumannii, Enterococcus* spp*., K. pneumoniae* and *C. albicans* (Lee *et al.,* 2018). However, organisms commonly involved in biofilm formation are poly-microbial communities of *Enterococcus* species*, K. pneumoniae, C. albicans, P. mirabilis* and *P. aeruginosa* (Hola *et al.,* 2010; Galvan *et al.,* 2016). The microbes in biofilm communities have lower growth rates but higher resistance to antimicrobial treatment. With regard to biofilm formation, UTI is becoming a major health problem especially in MDR *E. coli* as these form intracellular poly-microbial communities in bladder epithelium (Sevanan *et al.,* 2011). These biofilms can inhibit the diffusion of different substances and antimicrobials bindings like antimicrobial protein lysozyme (Karigoudar *et al.,* 2019).

The interaction between different fungal and bacterial species is of great importance in urinary tract related poly-microbial biofilms. *Candida* is the most common fungal species being interacting with different bacterial pathogens in uncomplicated and complicated UTI. Biofilms enhance the establishment of infections in the human host. Biofilms formed by different *Candida* species are responsible for both superficial and systemic infections (Bandara *et al.,* 2009).

As 80% of microorganisms are involved in biofilm formation, this is an irrevocable field to explore for their role in poly-microbial pathogenesis. A study was conducted in Hong Kong by Bandara *et al.,* (2009) in which, *in vitro* interactions of *E. coli* with different *Candida* species were evaluated. It was observed that dual species biofilm biomass remained unaffected in combination biofilms of *C. Albicans* - *E. coli* and *C. glabrata – E. coli.* On the contrary, Thein *et al.,* (2006) reported a significant inhibition of *C. albicans* in dual species biofilm when co-cultured with *E. coli* at 24 h of incubation. Dual-species biofilms formed by different uropathogens and their interactions were studied *in vitro* by Galvan *et al.,* (2016) on catheter associated UTI. Their study included four different dual-species interactions of prevalent bacteria in catheter-associated poly-microbial biofilms, namely: *E. coli–E. faecalis, K. pneumoniae–E. coli, K. pneumoniae– P. mirabilis* and *K. pneumoniae–E. faecalis*. Comparison between the mono and dual species biofilm in that study showed a significant reduction in the number of *E. coli* cells in dual species biofilms after 48 h of incubation.

Limited data is available in literature regarding the interspecies interactions of *Candida* species with other Gram negative uropathogens such as *E. coli and K. pneumoniae* in developing poly-microbial biofilms in urinary tract. There is no work on isolates of cUTI from urolithiatic patients and their *in vitro* mono and dual species interaction for biofilm formation. Therefore, there is need to explore these interactions for understanding poly-microbial behaviours towards urinary tract related infections. In this context, current study was aimed to develop and assess interactions of *Candida* species with *E. coli* and *K. pneumoniae* in dual species biofilm model.

6.2-Materials and methods

6.2.1-Isolates used for mono and dual species biofilms assays

Single and dual species biofilms were assessed by using 96 well microtiter plates and spectrophotometric techniques. For these assays *K. pneumoniae, E. coli* and *Candida* isolates from urine samples of urolithiatic patients were analyzed for mono and different combinations of dual species biofilms (Table 6.1). These mono and dual combinations are referred as groups in statistical analysis. *Candida* isolates were inoculated in test tube containing SDB with 10% glucose and bacterial isolates were inoculated in NB with 10% glucose and incubated at 37°C for 24 h to assess biofilm formation and metabolic activity, XTT reduction and CV assays were used. Centrifugation of selected uropathogens for 10 min at 5000 rpm (4°C) was done to get pellet after incubation and pellet was washed twice with sterile PBS. These cells were resuspended in 1.0 mL of RPMI 1640 media. Inoculum was prepared with final cell density of 1.0×10^7 cells/mL using 0.5 McFarland and OD was adjusted to 0.38 at 520 nm wavelength.

Table 6.1: Mono and dual species combinations of ATCC and cUTI isolates (*C. albicans, C. glabrata, E. coli* and *K. pneumoniae* strains) used to study biofilm development

Mono-species biofilms	**Dual-species biofilms**
ATCC *C. albicans* (90029)	ATCC *C. albicans* (90029)+ ATCC *K. pneumoniae* (700603)
ATCC *K. pneumoniae* (700603)	ATCC *C. albicans* (90029)+ ATCC *E. coli* (25922)
ATCC *E. coli* (25922)	ATCC *K. pneumoniae* (700603)+ ATCC *E. coli* (25922)
C. albicans SF27	*C. albicans* SF27 + *K. pneumoniae* SF63
C. glabrata SF6	*C. glabrata* SF6 + *K. pneumoniae* SF63
K. pneumoniae SF63	*C. glabrata* SF6 + *E. coli* SF45
E. coli SF45	*C. albicans* SF27 + *E. coli* SF45
	K. pneumoniae SF63 + *E. coli* SF45

6.2.2-Crystal violet (CV) assay for analysis of biofilm biomass

Previously described in chapter 4.0, section 4.2.3.6.2.2

6.2.3-XTT reduction assay

XTT reduction assay is based on reduction of tetrazolium dyes e.g., 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilite inner salt (XTT). It is an effective method to assess the metabolic activity of adherent cells. XTT is a colourless compound that is reduced by oxidoreductases of microorganisms to brightly orange substance in the presence of Phenazine metho-sulfate (PMS) as electron transporter (Klancnik *et al*., 2014). XTT (sigma) reagent was prepared (1mg/mL) in ultrapure water and filtered. Solution of PMS stock was prepared freshly by adding 0.32mg/mL in deionized water and was stored in dark. This assay was carried out in 96 wells microtiter plate for 48 h. After incubation media was removed and wells were washed thrice by using 150 µL of 0.1M PBS (pH 7). Ninety µL of XTT solution and 10 µL of PMS were added. Incubation was done for 1 h at 37°C in dark and absorbance was measured at 492nm. All isolates were analyzed for XTT reduction assay in triplicate for both 24 and 48 h and average absorbance was used for further analysis.

6.2.4-Total protein estimation assay

The total protein formed by *Candida* and bacterial isolates in 5 mL of BHI broth for 24 h and 48 h at 37°C was estimated. Centrifugation of culture medium at 5000rpm for 5min was done after incubation. Biuret reagent was prepared by adding 1.5g of cupric sulfate pentahydrate and 6g of sodium potassium tartrate tetrahydrate in 500mL of distilled water into which 300mL of 10% NaOH solution was added and final volume was made up to 1000 mL with distilled water. Culture supernatant of 100µL was pipetted in each well along with 200µL of Biurette reagent, mixed thoroughly with pipetting and incubated in dark at room temperature for 15min. After incubation, 20µL of each Folin phenol (1N)

and Ciacalteu's reagent was added in each well and colour change was observed after incubation in dark for 30min. Absorbance was measured at 650nm by using multi-scan go microtiter plate reader. Protein concentration was measured in µg/mL by using equation Y = 0.5034x+0.1246, derived from BSA curve. Here Y = absorbance of unknown sample-absorbance of control sample and x = concentration of unknown sample (Appendix C-I).

6.2.5-Statistical analysis

Standard deviation and means were calculated for descriptive and categorical data by using IBM SPSS version 21. ANOVA analysis was carried out to assess, if there was a significant change in biofilm formation, metabolic activity and protein estimation in these different mono and dual biofilms using significance level of $P<0.05$.

6.3-Results

6.3.1-*Candida* spp. interaction with *K. pneumoniae* in dual species biofilms

6.3.1.1-CV biofilm biomass assays for *Candida* spp. *and K. pneumoniae* biofilms

CV assay was used to assess the biofilm formation by ATCC strains of *C. albicans* and *K. pneumoniae* which showed reduction in biomass production in their dual biofilm assay after an interval of 24 h and 48 h of incubation. In case of cUTI isolates, combination of *C. albicans* SF27 and *K. pneumoniae* SF63 showed reduced biofilm biomass as compared to ATCC strains dual biofilm. In case of cUTI isolate *C. glabrata* SF6 when co-cultured with *K. pneumoniae* SF63, same result that is reduction in biofilm biomass production was observed (Table 6.2, Figure 6.1). Significant difference was detected in biomass of dual species biofilms by using one way ANOVA analysis (Table 6.5).

6.3.1.2-XTT reduction assays for *Candida* spp. and *K. pneumoniae* biofilms

Through XTT reduction assay, enhanced metabolic activity was observed in dual species biofilm of ATCC *K. pneumoniae* and ATCC *C. albicans* up to 48 h of incubation. In contrast to ATCC strains, cUTI isolates of *K. pneumoniae* SF63 and *C. albican* SF27

when co-cultured with each other showed reduced metabolic activity. Same reduced metabolic activity was observed in case of dual species biofilm assay of *K. pneumoniae* SF63 and *C. glabrata* SF6 (Table 6.3, Figure 6.2). A change in metabolic activity was observed in dual species biofilms after 48 h and was statistically significant by one way ANOVA analysis (Table 6.5).

6.3.1.3-Total protein estimation of *Candida* spp. and *K. pneumoniae* biofilms

In dual species biofilms of ATCC *K. pneumoniae* and ATCC *C. albicans,* increase in the total protein concentration was observed as compared to the mono species biofilms of these strains up to 48 h of incubation. Dual biofilms of ATCC strains showed greater protein concentration as compared to dual biofilm of cUTI isolates of *C. albicans* and *K. pneumoniae.* When *K. pneumoniae* SF63 was co-cultured with *C. glabrata* SF6, same trend of enhanced protein concentration was observed after 24 h of incubation. However, dual biofilm of *K. pneumoniae* SF63 with *C. albicans* SF27 showed greater protein concentration as compared to the dual biofilm of *K. pneumoniae* SF63 and *C. glabrata* SF6. Highest protein concentration was observed in dual biofilm of ATCC strains followed by cUTI isolates of *K. pneumoniae* + *C. albicans* and *K. pneumoniae* + *C. glabrata* (Table 6.4, Figure 6.3). This difference was significant even statistically by using one way ANOVA (Table 6.5).

Table 6.2: Quantification of biofilm biomass formation by mono and dual species biofilm combinations of ATCC *C. albicans* (90029) with ATCC *K. pneumoniae* (700603) and cUTI isolates of *K. pneumoniae* SF63 with *C. albicans* SF27 and *C. glabrata* SF6 using CV assay at 492nm

***Candida- K. pneumoniae* combinations**	**ODs after 24 h** Mean±SD	**ODs after 48 h** Mean±SD
ATCC *K. pneumoniae* (700603)	0.132 ± 0.007	0.476 ±0.02
ATCC *C. albicans* (90029)	0.27± 0.01	0.78 ±0.03
ATCC *C. albicans* (90029) + ATCC *K. pneumoniae* (700603)	0.181 ± 0.01	0.452 ± 0.02
K. pneumoniae SF63	0.13 ±0.002	0.355 ± 0.005
C. albicans SF27	0.237 ± 0.002	0.32 ± 0.002
C. glabrata SF6	0.167 ± 0.002	0.39 ± 0.01
C. albicans SF27 + *K. pneumoniae*SF63	0.147 ±0.003	0.359 ± 0.002
C. glabrata SF6 + *K. pneumoniae* SF63	0.165 ± 0.003	0.27 ± 0.007
Blank	0.08 ± 0.01	0.15±0.01

Table 6.3: XTT reduction assay of mono and dual species biofilms of ATCC *C. albicans* (90029) with ATCC *K. pneumoniae* (700603) and cUTI isolates of *K. pneumoniae* SF63 with *C. albicans* SF27 and *C. glabrata* SF6 to check their metabolic activity

***Candida- K. pneumoniae* combinations**	**ODs after 24 h** Mean±SD	**ODs after 48 h** Mean±SD
ATCC *K. pneumoniae* (700603)	0.548 + 0.06	2.343+0.25
ATCC *C. albicans* (90029)	1.645+0.131	1.17+0.10
ATCC *C. albicans* (90029) + ATCC *K. pneumoniae* (700603)	2.45+0.16	2.58+0.29
K. pneumoniae	0.51+0.112	1.52+0.11
C. albicans	1.48+ 1.6	1.49+0.17
C. glabrata	1.24+0.1	1.7+0.05
C. albicans + K. pneumoniae	0.87+0.01	0.94+0.005
C. glabrata + K. pneumoniae	0.67+0.05	0.85+0.014
Blank	0.05+ 0.01	0.1 + 0.01

Table 6.4: Total protein estimation of Mono and dual species biofilm of ATCC *C. albicans* (90029) with ATCC *K. pneumoniae* (700603) and cUTI isolates of *K. pneumoniae* SF63 with *C. albicans* SF27 and *C. glabrata* SF6

***Candida- K. pneumoniae* combinations**	**Protein Concentration after 24 h (µg/mL)** Mean±SD	**Protein Concentration after 48 h (µg/mL)** Mean±SD
ATCC *K. pneumoniae* (700603)	2.64 ± 0.02	3.29 ±0.16
ATCC *C. albicans* (90029)	3.47 ±0.08	2.40 ±0.02
ATCC *C. albicans* (90029) + ATCC *K. pneumoniae* (700603)	4.63 ±0.08	3.25 ±0.1
K. pneumoniae	1.27 ±0.09	2.24 ±0.15
C. albicans	2.09±0.05	2.65 ±0.05
C. glabrata	1.47±0.2	4.49 ±0.13
C. albicans + K. pneumoniae	2.65 ±0.1	3.19 ±0.07
C. glabrata + K. pneumoniae	2.06 ±0.09	2.63 ±0.14

Table 6.5: Comparison of biofilm formation, metabolic activity and total protein estimation of ATCC and cUTI isolates of *C. albicans, C. glabrata and K. pneumoniae* using (*P<0.05*)

Assays		**DF***	***P value***
CV assay ODs after 24 h (492nm)	Between groups Within groups	8 18	<0.05
CV assay ODs after 48 h (492nm)	Between groups Within groups	8 18	<0.05
XTT reduction assay ODs after 24 h (492nm)	Between groups Within groups	8 18	<0.05
XTT reduction assay ODs after 48 h (492nm)	Between groups Within groups	8 18	<0.05
Total protein concentration after 24 h (μg/mL)	Between groups Within groups	7 16	<0.05
Total protein concentration after 48 h (μg/mL)	Between groups Within groups	7 16	<0.05

DF* = **Degree of freedom**

Groups- 1-ATCC *K. pneumoniae*
2- ATCC *C.albicans*
3- ATCC *C.albicans*+ATCC *K. pneumoniae*
4- cUTI *K. pneumoniae*
5- cUTI *C. albicans*
6- cUTI *C. glabrata*
7- cUTI *K. pneumoniae* + cUTI *C. albicans*
8- cUTI *K. pneumoniae* + cUTI *C. glabrata*

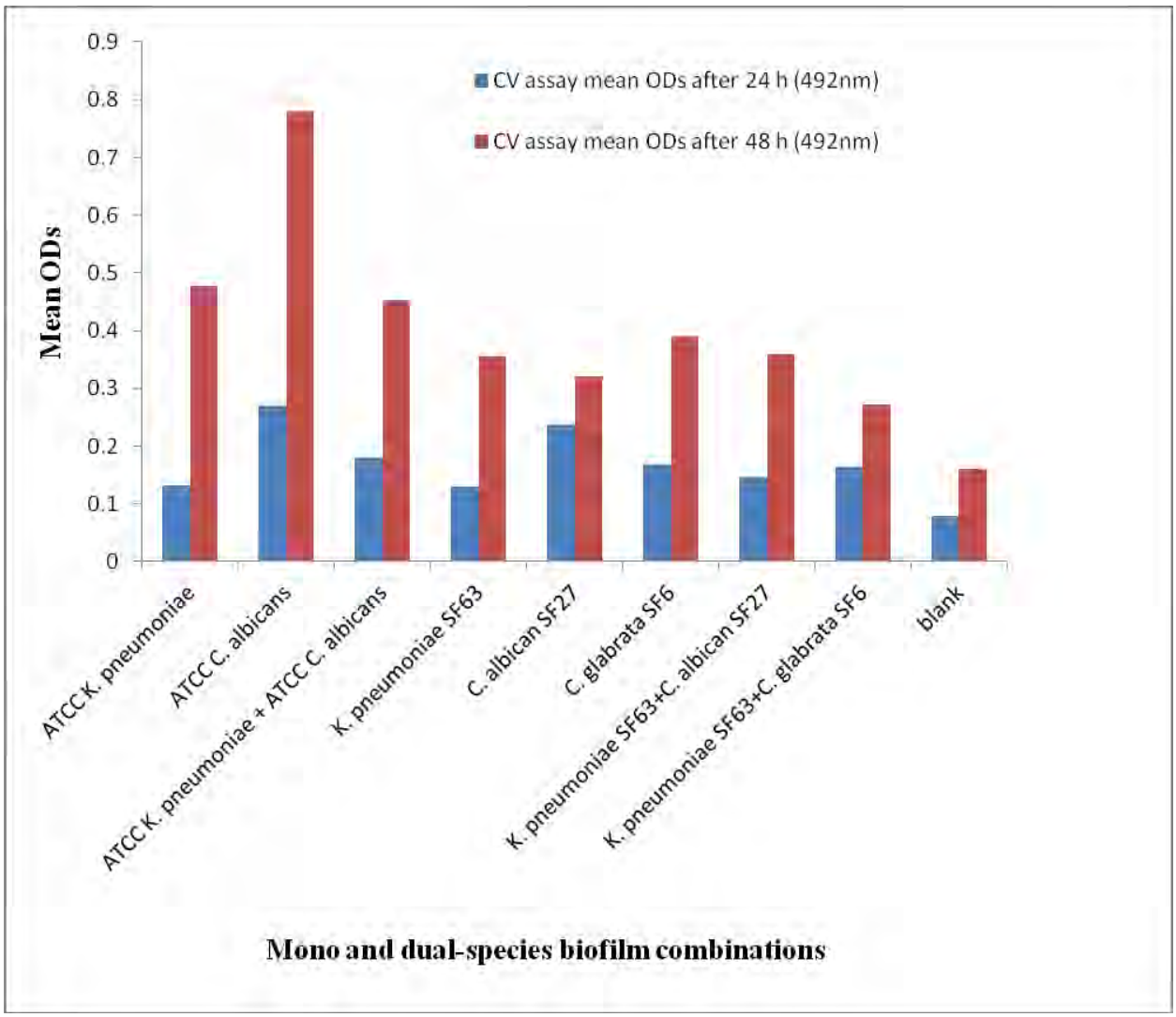

Figure 6.1: CV assay of ATCC *C. albican,* ATCC *K. pneumoniae*, urinary strains of *C. albicans* SF27, *C. glabrata* SF6 and *K. pneumoniae* SF63 in their mono and dual biofilms after 24h and 48 h of incubation to see their biofilm biomass difference

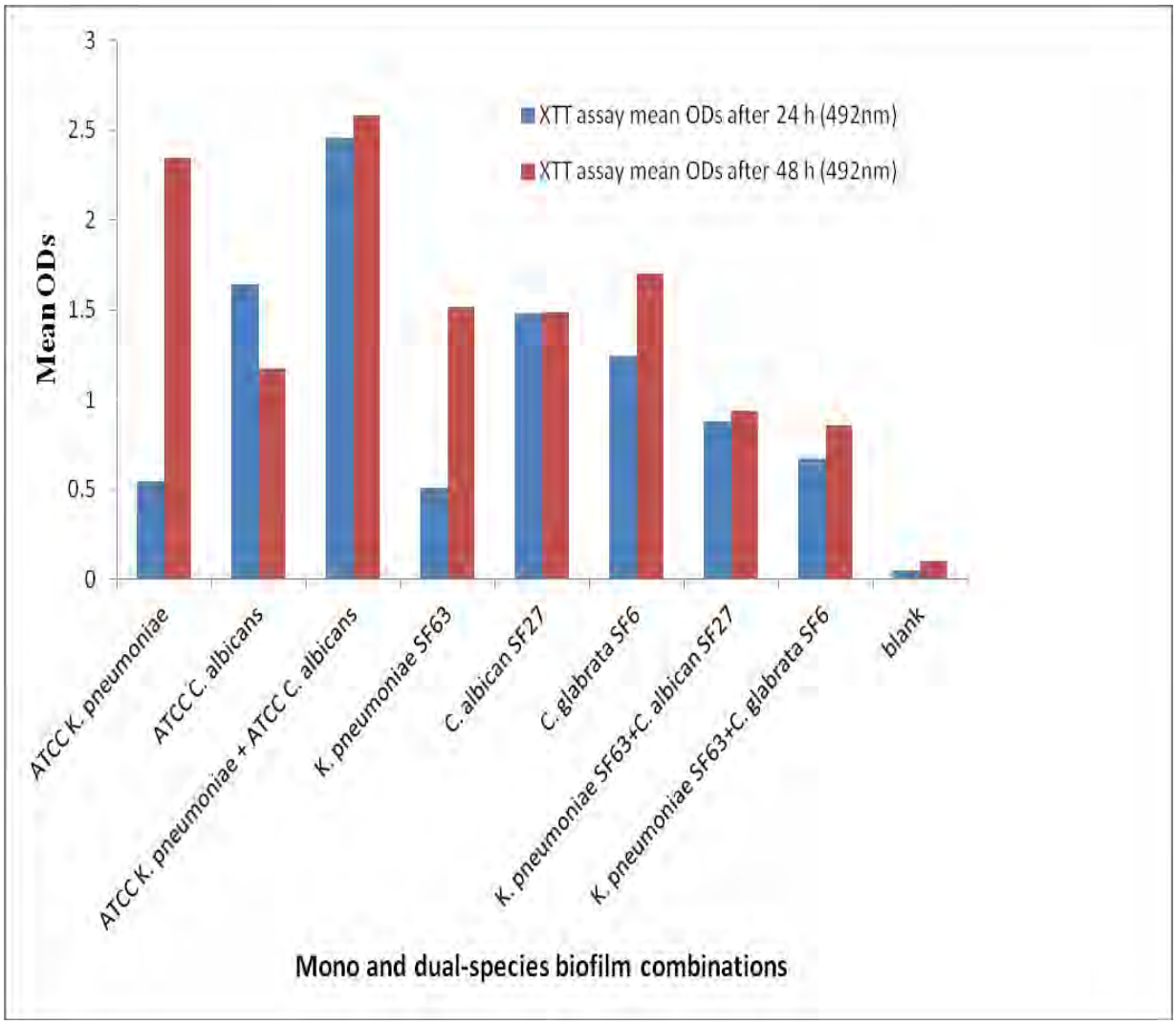

Figure 6.2: XTT reduction assay of ATCC *C. albican,* ATCC *K. pneumoniae*, urinary strains of *C. albicans*SF27, *C. glabrata* SF6 and *K. pneumoniae* SF63 in their mono and dual biofilms after 24 h and 48 h of incubation to see their difference in metabolic activity.

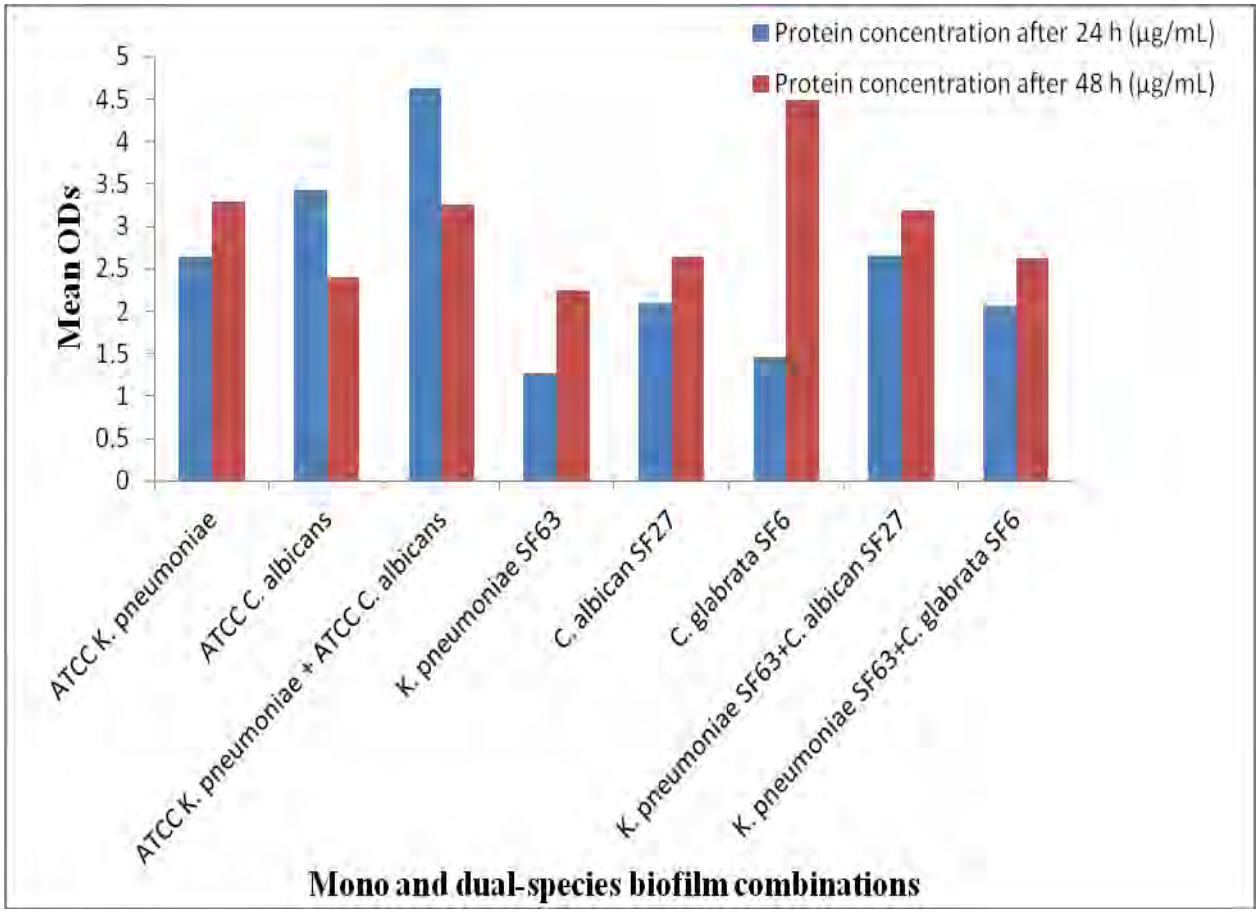

Figure 6.3: Total protein estimation assay of ATCC *C. albican,* ATCC *K. pneumoniae*, urinary strains of *C. albicans*SF27, *C. glabrata* SF6 and *K. pneumoniae* SF63 in their mono and dual biofilms after 24 h and 48 h of incubation to see their difference in protein concentration.

6.3.2-*Candida* spp. interaction with *E. coli* in dual species biofilms

6.3.2.1-CV biofilm biomass assays for *Candida* spp. with *E. coli* biofilms

It was observed that dual biofilm of ATCC *C. albicans* and ATCC *E. coli* produced reduced biofilm biomass. Same trend of reduction in biomass was observed in cUTI isolates of *E. coli* SF45 and *C. albicans* SF27. When compared these dual biofilms of ATCC and cUTI isolates, cUTI strains produced greater biomass in their dual form after 24 h of incubation. But after 48 h of incubation, greater biomass was observed in ATCC strains. Lowest biofilm biomass was observed in dual species biofilm of *E. coli* SF45 and *C. glabrata* SF6 as compared to other dual species biofilms (Table 6.6, Figure 6.4). A significant difference in biofilm biomass production was analyzed in dual species biofilms by using one way ANOVA (Table 6.9).

6.3.2.2-XTT reduction assay for *Candida* spp. with *E. coli* biofilms

Metabolic activity of ATCC strains of *E. coli* and *C. albicans* was observed to be enhanced up to 48 h of incubation in dual species biofilm. In case of cUTI isolates *E. coli* SF45 and *C. albicans* SF27, huge increase in metabolic activity was observed in their combined biofilm after 48 h of incubation. In contrast to these results, cUTI isolate *C. glabrata* SF6 when co-cultured with *E. coli* SF45, reduction in metabolic activity was observed. Highest metabolic activity was observed in dual biofilm of cUTI isolates *E.coli* SF45 and *C. albicans* SF27 (Table 6.7, Figure 6.5). This difference was found to be the significant in dual biofilms by using one way ANOVA (Table 6.9).

6.3.2.3-Total protein estimation of *Candida* spp. with *E. coli* biofilms

In case of ATCC strains of *E. coli* and *C. albicans,* protein concentration was observed to be remained unaffected after 24 h of incubation of their dual biofilm. Protein concentration in dual biofilms of cUTI isolates of *C. albican* SF27 and *E. coli* SF45 was lower as compared to protein concentration in dual biofilm of ATCC strains. In case of

cUTI isolates dual species biofilm of *E. coli* SF45 with *C. glabrata* SF6, enhanced protein concentration was observed when compared with dual species biofilm of *C.albicans* SF27(Table 6.8, Figure 6.6). Significant difference in protein concentration was observed in these dual species biofilms of ATCC and cUTI isolates (Table 6.9).

Table 6.6: Quantification of biofilm biomass formation of mono and dual species biofilm combinations of ATCC *C. albicans* (90029) with ATCC *E. coli* (25922) and cUTI isolates of *E. coli* SF45 with *C. albicans* SF27 and *C. glabrata* SF6 using CV assay at 492 nm.

***Candida spp- E.coli* combinations**	**ODs after 24 h** Mean±SD	**ODs after 48 h** Mean±SD
ATCC *E. coli* (25922)	0.15±0.002	0.78±0.02
ATCC *C. albicans* (90029) + ATCC *E. coli* (25922)	0.17±0.005	0.45±0.003
E. coli SF45	0.11±0.003	0.21±0.001
C. albicans SF27 + *E. coli* SF45	0.18±0.005	0.28±0.003
C. glabrata SF6 + *E. coli* SF6	0.15±0.002	0.24±0.004

Table 6.7: XTT reduction assay of mono and dual species biofilms of ATCC *C. albicans* (90029) with ATCC *E. coli* (25922) and cUTI isolates of *E. coli* SF45 with *C. albicans* SF27 and *C. glabrata* SF6 to check their metabolic activity.

***Candida- E.coli* combinations**	**ODs after 24 h** Mean±SD	**ODs after 48 h** Mean±SD
ATCC *E. coli* (25922)	0.879±0.01	1.17±0.01
ATCC *C. albicans* (90029) + ATCC *E. coli* (25922)	1.03±0.62	1.54±0.01
E. coli SF45	0.31±0.005	1.34±0.004
C. albicans SF27 + *E. coli* SF45	1.48±0.004	2.67±0.02
C. glabrata SF6 + *E. coli* SF45	0.44±0.009	1.43±0.001

Table 6.8: Total protein estimation of Mono and dual species biofilm of ATCC *C. albicans* (90029) with ATCC *E. coli* (25922) and cUTI isolates of *E. coli* SF45 with *C. albicans* SF27 and *C. glabrata* SF6.

***Candida species-E.coli* combinations**	**Protein Concentration after 24 h (µg/mL)** Mean±SD	**Protein Concentration after 48 h (µg/mL)** Mean±SD
ATCC *E. coli* (25922)	3.32±0.01	2.11±0.1
ATCC *C. albicans* (90029) + ATCC *E. coli* (25922)	3.52±0.24	2.99±0.08
E. coli SF45	2.79±0.16	2.39±0.08
C. albicans SF27 + *E. coli* SF45	1.97±0.03	2.82±0.14
C. glabrata SF6+ *E. coli* SF45	2.27±0.08	3.06±0.07

Table 6.9: Comparison of biofilm formation, metabolic activity and total protein estimation of ATCC and cUTI isolates of *C. albicans, C. glabrata and E. coli* using ANOVA (*P<0.05*)

Assays		**DF***	***P value***
CV assay ODs after 24 h (492nm)	Between groups Within groups	8 18	<0.05
CV assay ODs after 48 h (492nm)	Between groups Within groups	8 18	<0.05
XTT reduction assay ODs after 24 h (492nm)	Between groups Within groups	8 18	<0.05
XTT reduction assay ODs after 48 h (492nm)	Between groups Within groups	8 18	<0.05
Total protein concentration after 24 h (μg/mL)	Between groups Within groups	7 16	<0.05
Total protein concentration after 48 h (μg/mL)	Between groups Within groups	7 16	<0.05

DF*= Degree of freedom

Groups = 1-ATCC *E.coli*
2- ATCC *C.albicans*
3- ATCC *C.albicans*+ATCC *E.coli*
4- cUTI *E.coli*
5- cUTI *C. albicans*
6- cUTI *C. glabrata*
7- cUTI *E.coli* + cUTI *C. albicans*
8- cUTI *E.coli* + cUTI *C. glabrata*

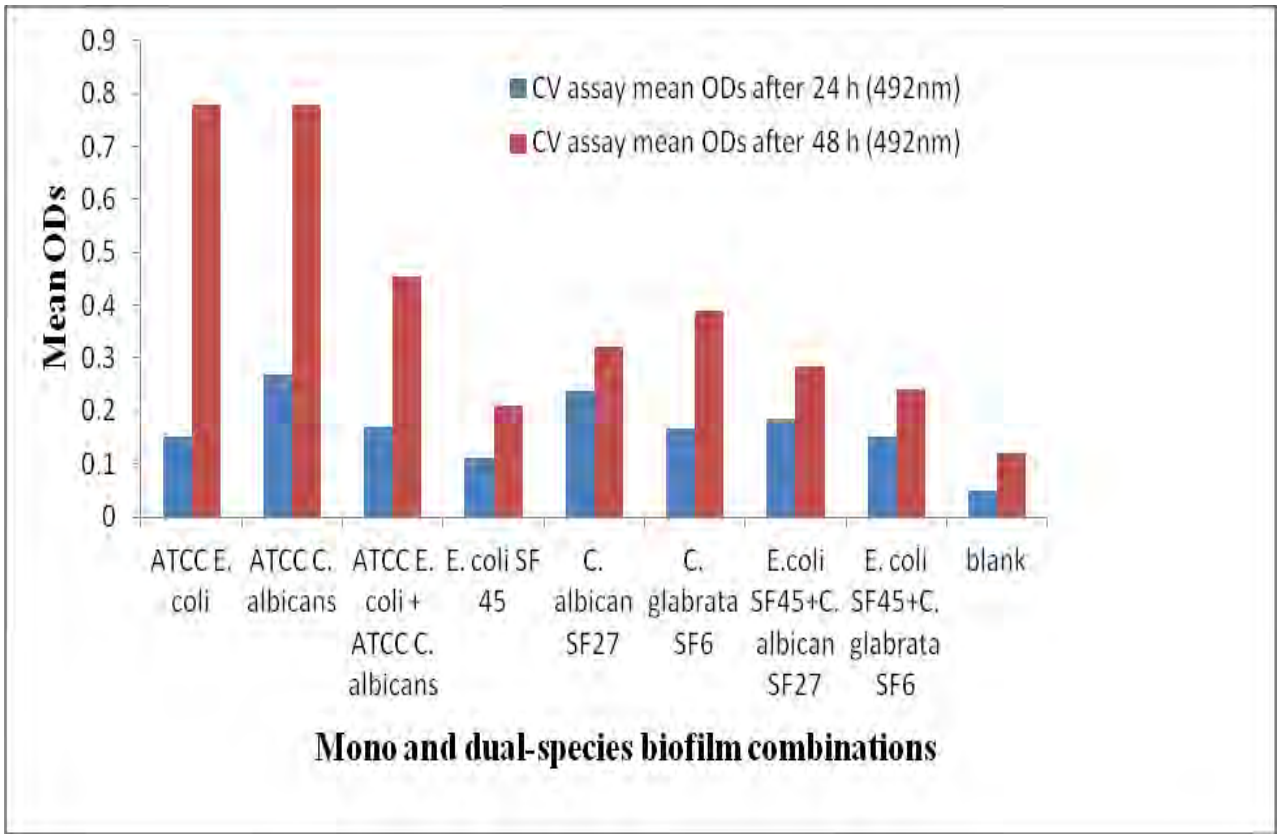

Figure 6.4: CV assay of ATCC *C. albican,* ATCC *E. coli*, urinary strains of *C. albicans*SF27, *C. glabrata* SF6 and *E. coli* SF45 in their mono and dual biofilms after 24h and 48 h of incubation to see their biofilm biomass difference.

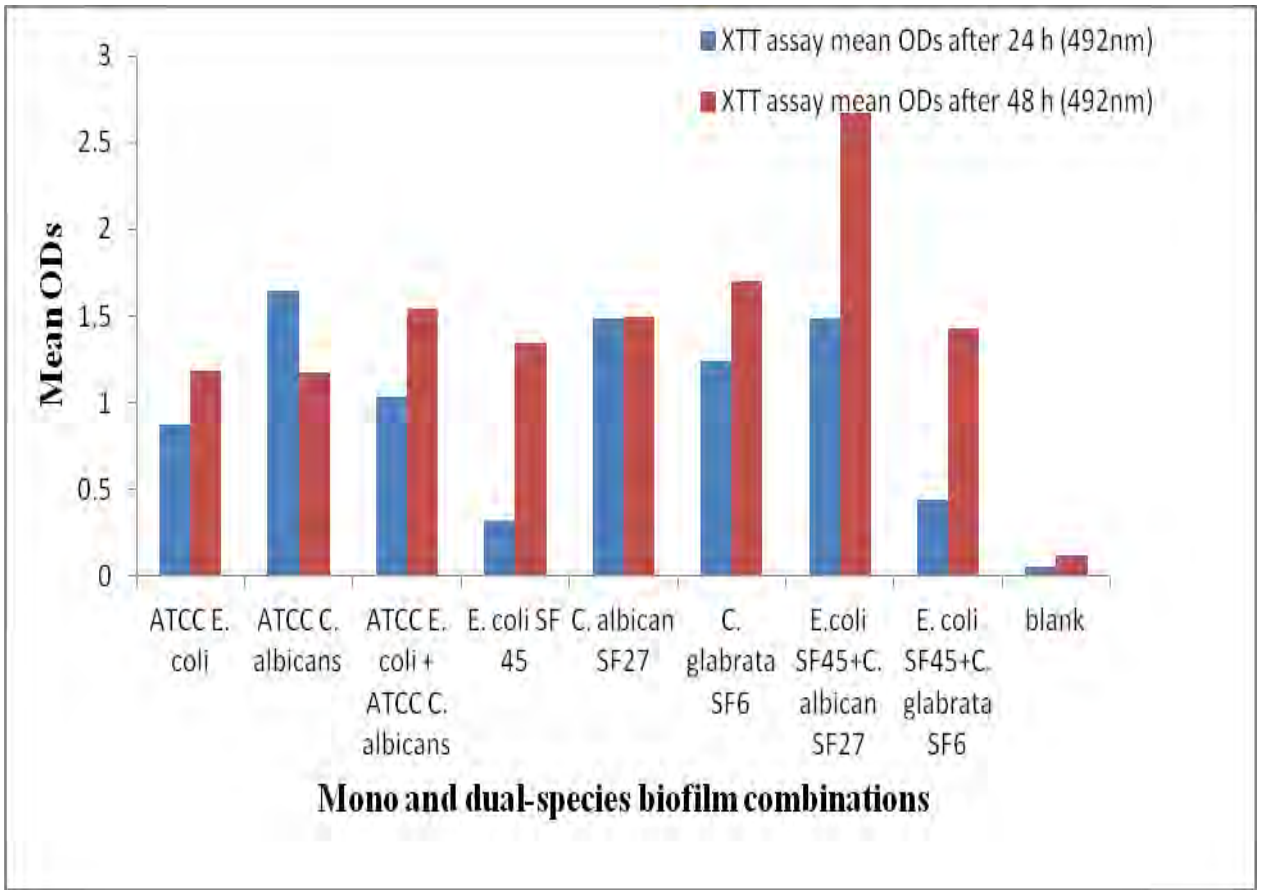

Figure 6.5: XTT reduction assay of ATCC *C. albican,* ATCC *E. coli*, urinary strains of *C. albicans*SF27, *C. glabrata* SF6 and *E. coli* SF45 in their mono and dual biofilms after 24h and 48 h of incubation to see their metabolic activity difference.

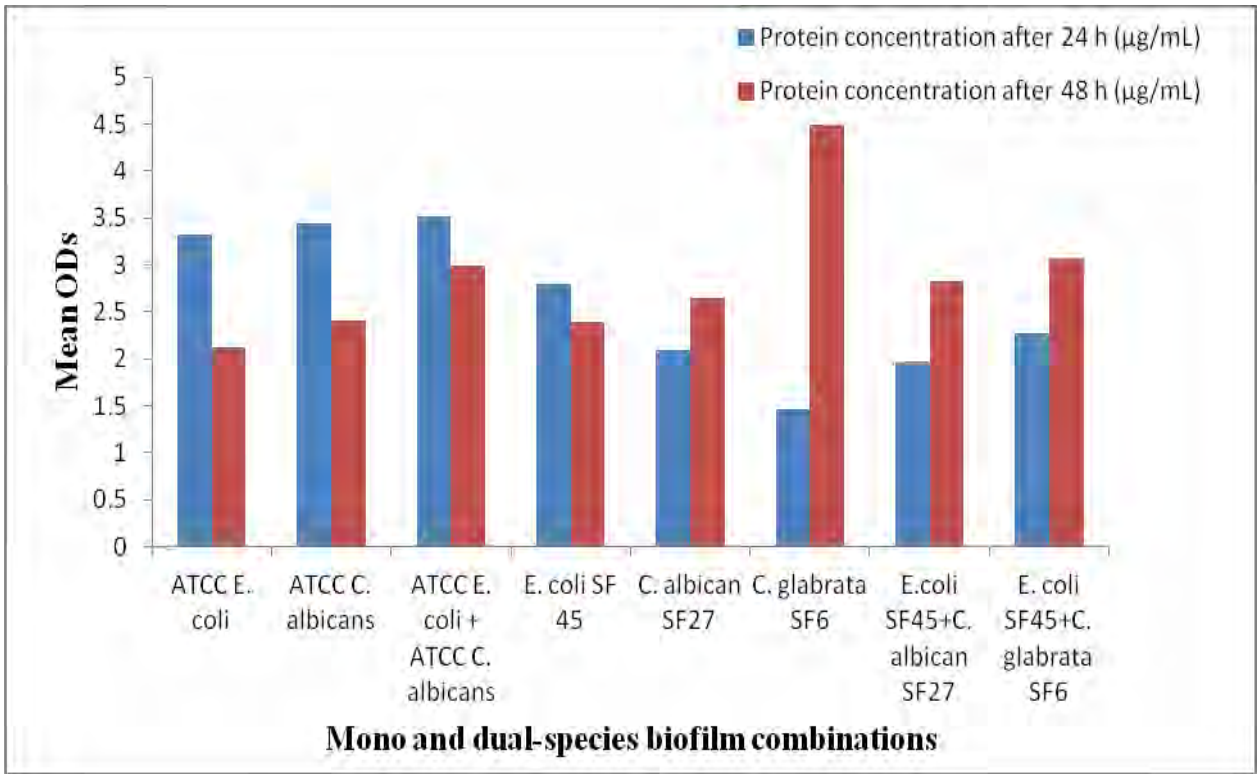

Figure 6.6: Total protein estimation assay of ATCC *C. albican,* ATCC *E. coli*, urinary strains of *C. Albicans* SF27, *C. glabrata* SF6 and *E. coli* SF45 in their mono and dual biofilms after 24h and 48 h of incubation to check their protein concentration difference.

6.3.3-*E. coli- K. pneumoniae* interaction in dual species biofilms

6.3.3.1-CV biofilm biomass assays for *E. coli- K. pneumoniae* biofilms

Reduction in biofilm biomass was observed in dual species biofilm of ATCC *K. pneumoniae* and ATCC *E. coli*. Same decreasing trend of biomass in dual species biofilm of cUTI isolates of *K. pneumoniae* SF63 and *E. coli* SF45 was observed (Table 6.10, Figure 6.7). Dual species biofilm of ATCC strains produced higher biomass than dual species biofilm of cUTI isolates of *K. pneumoniae* and *E. coli* (Table 6.11).

6.3.3.2-XTT reduction assays for *E. coli- K. pneumoniae* biofilms

In XTT reduction assay, overall increase in metabolic activity was observed in all dual species biofilms. An increase in metabolic activity in dual species biofilm of ATCC *E. coli* and ATCC *K. pneumoniae* was observed throughout its incubation. Almost same trend was observed in case of isolates *E. coli* SF45 and *K. pneumoniae* SF63 as increase in metabolic activity was observed in their dual species biofilms up to 48 h (Table 6.10, Figure 6.8). This activity was higher in case of dual biofilm of ATCC strains as compared to combined biofilm of their cUTI isolates.

6.3.3.3-Total protein estimation of *E. coli- K. pneumoniae* biofilms

ATCC *K. pneumoniae* and ATCC *E. coli* dual species biofilm showed enhanced protein concentration. Enhanced protein concentration was also observed in dual species biofilm of isolates *K. pneumoniae* SF63 and *E. coli* SF45. Protein concentration was higher in dual biofilm of ATCC strains as compared to the dual biofilm of their cUTI isolates (Table 6.10, Figure 6.9). Through one way ANOVA analysis, a significant change in protein concentration in dual biofilms of ATCC and cUTI isolates was seen (Table 6.11).

Table 6.10: Quantification of biofilm biomass, metabolic activity and total protein concentration in mono and dual biofilms of ATCC *K. pneumoniae* (700603) with ATCC *E. coli* (25922) and cUTI isolates *E. coli* SF45 with *K. pneumoniae* SF63.

		***K. pneumoniae- E.coli* combinations**	
		ATCC *K. pneumoniae* (700603) + ATCC *E. coli* (25922)	*K. pneumoniae* + *E. coli*
CV assay ODs	After 24 h Mean±SD	0.147 ± 0.02	0.127 ±0.005
	After 48 h Mean±SD	0.453 ± 0.1	0.27 ± 0.05
XTT reduction assay ODs	After 24 h Mean±SD	1.23±0.004	0.61 ±0.01
	After 48 h Mean±SD	3.15±0.03	1.77 ±0.01
Protein Concentration (µg/mL)	After 24 h Mean±SD	3.56 ± 0.12	2.32 ± 0.09
	After 48 h Mean±SD	3.56±0.08	2.32 ±0.05

Table 6.11: Comparison of biofilm biomass, metabolic activity and total protein estimation of ATCC and cUTI isolates of *E. coli and K. pneumoniae* using one way ANOVA (*P<0.05*)

Assays		**DF***	***P value***
CV assay ODs after 24 h (492nm)	Between groups Within groups	6 14	<0.05
CV assay ODs after 48 h (492nm)	Between groups Within groups	6 14	<0.05
XTT reduction assay ODs after 24 h (492nm)	Between groups Within groups	6 14	<0.05
XTT reduction assay ODs after 48 h (492nm)	Between groups Within groups	6 14	<0.05
Total protein concentration after 24 h (μg/mL)	Between groups Within groups	5 12	<0.05
Total protein concentration after 48 h (μg/mL)	Between groups Within groups	5 12	<0.05

DF*= Degree of freedom

Groups= 1-ATCC *K. pneumoniae*
2- ATCC *E. coli*
3- ATCC *E. coli* +ATCC *K. pneumoniae*
4- cUTI *K. pneumoniae*
5- cUTI *E. coli*
6- cUTI *K. pneumoniae* + cUTI *E. coli*

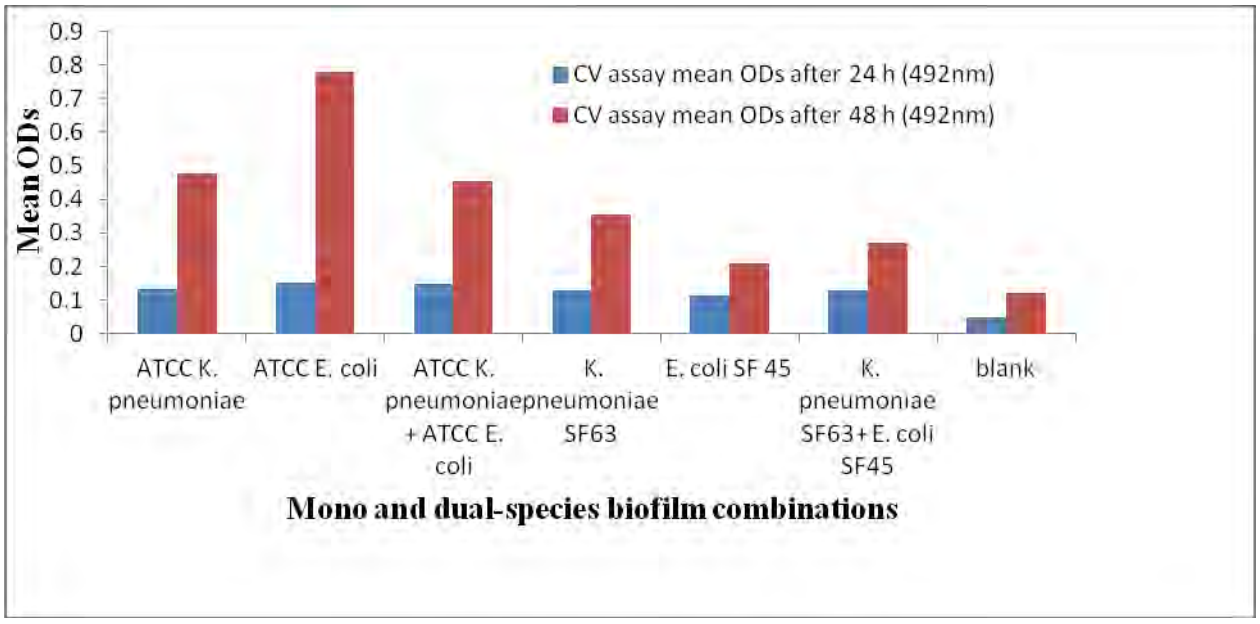

Figure 6.7: CV assay of ATCC *K. pneumoniae,* ATCC *E. coli*, urinary strains of *K. pneumoniae* SF63 and *E. coli* SF45 in their mono and dual biofilms after 24h and 48 h of incubation to check their biofilm biomass difference.

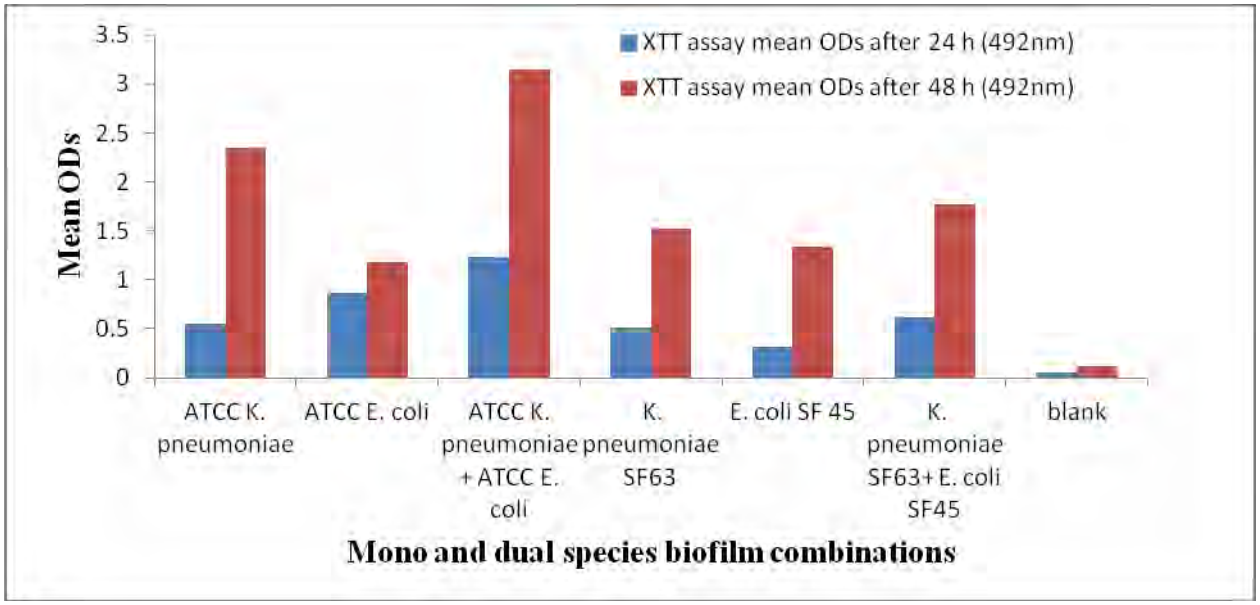

Figure 6.8: XTT assay of ATCC *K. pneumoniae,* ATCC *E. coli*, urinary strains of *K. pneumoniae* SF63 and *E. coli* SF45 in their mono and dual biofilms after 24h and 48 h of incubation to check their metabolic activity difference

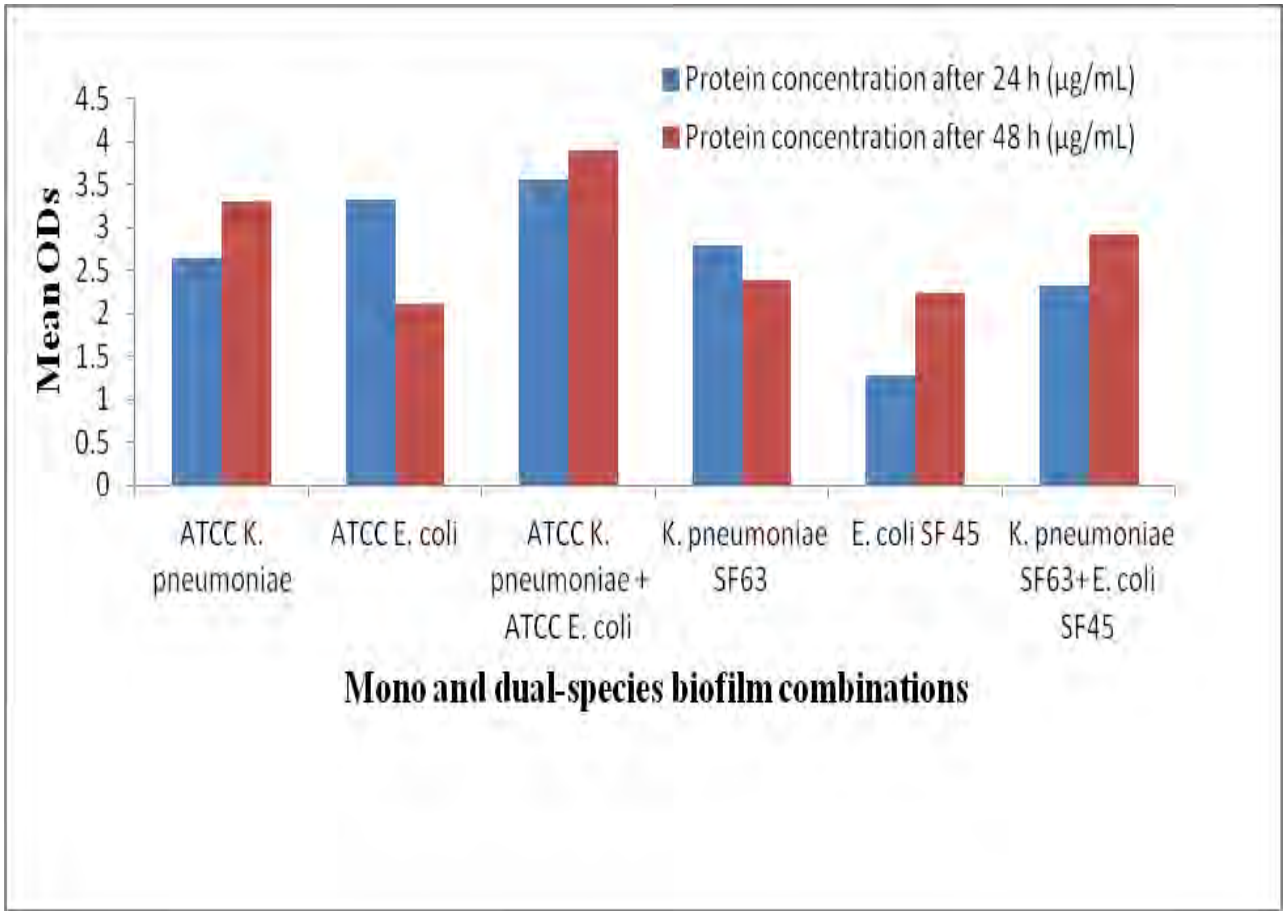

Figure 6.9: Total protein estimation assay of ATCC *K. pneumoniae,* ATCC *E. coli*, urinary strains of *K. pneumoniae* SF63 and *E. coli* SF45 in their mono and dual biofilms after 24h and 48 h of incubation to check their protein concentration difference.

6.4-Discussion

In this part of study, potential of different bacterial and *Candida* species isolated from the urolithiatic patients with cUTI to develop and survive in dual species biofilms was analyzed through *in vitro* biofilm model using CV biofilm biomass, XTT reduction and total protein estimation assays. These dual species biofilms between different bacterial and *Candida* species were developed on polystyrene surface and investigated along with their ATCC strains as control. Dual- species biofilms of bacterial and *Candida* species can provide protection to one or both the species by synergistic interactions. Synergistic as well as antagonistic affect among different microbial interactions in biofilms were reported in different studies (Forster *et al.,* 2016; Lohse *et al*., 2018).

In present study, *Candida* species (*C. albicans, C. glabrata*) and *E. coli* were studied as dual species for biofilm formation. Reduction in the biofilm biomass was observed in dual species biofilm of both ATCC and cUTI isolates of *C. albicans* and *E. coli.* In agreement to these results, Thein *et al.* (2006) reported a significant inhibition of biofilm biomass of *C. albicans* in dual species biofilm with *E. coli*. This decrease in biofilm biomass might be due to microbial competitive behaviour for limited supply of nutrients in microtiter plates. *In vitro* interactions of *Candida* species and *E. coli*, in dual-species biofilms were studied in Hong Kong but on ATCC strains only. That study reported

biofilm biomass unaffected in dual species biofilms of *C. albicans*-*E. coli* and *C. glabrata – E. coli* (Bandara *et al.,* 2009). In contrast to present study, Nair and Samaranayake (1996) reported enhanced biofilm biomass production in dual biofilm of *Candida* with *E.coli.* Acrylic surface was used in that study for development of biofilm. Therefore, these variable results might be due to different type of strains, media and surfaces for biofilms development used in these studies.

In contrast to reduced biofilm biomass production, metabolic activity was observed to be enhanced in dual biofilms of both ATCC and cUTI isolates of *C. albicans* and *E. coli.* This synergistic relationship for survival was contrary to previous study where reduction in cell viability was reported for dual biofilm of ATCC strains of *C. albicans* and *E. coli*

(Piva *et al.,* 2011). Bandara *et al.,* (2009) reported no effect on metabolic activity of *C. albicans* in the presence of *E. coli* in dual species biofilms. High metabolic activity observed in dual biofilms of cUTI strains in current study might be due to the reason that isolates were taken from urolithiatic patients with cUTI in which these two bacteria might be interacting in the actual infection in these studied patients. Such interactions of *E. coli* and *C. albicans* could be responsible for enhanced survival of *C. albicans* and *E. coli* in their dual species biofilm and further complications. The ability of these two organisms to enhance the metabolic activity when they were grown together might have potential clinical implications for poly-microbial cUTI.

Candida-K .pneumoniae dual species biofilm assays were also established in current study to quantify the biofilm biomass production, metabolic activity and total protein estimation of these isolates. Here reduction in biofilm biomass was observed in case of both ATCC and cUTI isolates of *Candida* and *K. pneumoniae* in their dual biofilms with more reduced metabolic activity in cUTI isolates. This points to a possible antagonistic relationship of cUTI isolates in dual biofilms. Decrease in biofilm biomass and metabolic activity might be due to limited nutrients and sites for colonization available to grow on microtiter plate. To the author's knowledge, there is no data available to study the interactions of *K. pneumoniae* and *Candida* species in dual biofilms related to cUTI isolates.

E. coli and *K. pneumoniae* are considered to be the major microbes responsible for urinary tract infections (Hanna-Wakim *et al.,* 2015). Interaction of *E. coli* and *K. pneumoniae* was also studied in dual species biofilms for their biomass production, metabolic activity and total protein concentrations in present study. Similar to the other combinations studied in current work, reduction in biofilm biomass was observed in dual species biofilms of ATCC as well as cUTI isolates of *E. coli* and *K. pneumoniae* depicting their competitive behaviour. With lowering of biomass, however enhanced metabolic activity was observed in dual biofilm of these isolates after 48 h of incubation. Galvan *et al.,* (2016) studied four different dual-species combinations: *E. coli–E. faecalis,*

K. pneumoniae–E. coli, K. pneumoniae–P. mirabilis and *K. pneumoniae–E. faecalis*. Dual biofilms were developed in artificial urine on siliconized surfaces. That study reported significant reduction in the number of *E. coli* cells in dual species biofilms with *K. pneumoniae* after 48 h of incubation. This reduction might be due to the detachment of cells from the biofilms and their dispersion into the medium as a part of the biofilm life cycle (Boles *et al*., 2005).

Overall, there was a competitive behaviour observed in case of biofilm formers for all the combinations studied in present work. Highest biomass was observed in dual biofilm of ATCC *Candida* and *K. pneumoniae* followed by their cUTI isolates when compared with the other micro organisms studied. In case of metabolic activity and their growth, most of the microbial combinations showed synergistic behaviour in their dual biofilms. Highest metabolic activity was observed in case of dual biofilm of cUTI isolates of *C. albicans* with *E. coli* as compared to other cUTI isolates studied. This indicated that cUTI C. *albicans* in combination with *E. coli* was metabolically more active and might have role in poly-microbial UTI. In case of protein estimation, higher concentrations of proteins were observed in case of ATCC strains of all microbes. However, in case of cUTI isolates, highest protein concentration was observed in dual biofilm of *K. pneumoniae* with *C. albicans* suggesting their higher growth in combination as compared to other studied microbial combinations.

There was poly-microbial biofilms development by cUTI isolates. This was an attempt to develop a model to analyze the role and interactions of local isolates involved in biofilm development in cUTI patients. These findings suggested that in poly-microbial biofilms, microbial consortia can behave synergistically as well as antagonistically according to the microbes for their survival and disease stage. There is need to understand these microbial interactions more deeply by using *in vivo* animal models coupled with some advanced techniques to combat these poly-microbial biofilms and to develop therapies to eradicate such complex consortium of microbes in cUTI after urolithiasis.

Conclusion

- Urolithiasis was more prevalent in males as compared to females.
- More than 70% of urolithiatic patients were consuming hard or moderately hard water and were from low socioeconomic status.
- Complicated UTI was prevalent in 64% of urolithiatic patients, where female patients were more positive for cUTI as compared to male urolithiatic patients.
- Clinically, patients having stone disease for extended period and inserted catheters were significantly positive for cUTI.
- Highest prevalence of *Candida* was observed among all microbes detected through culturable method followed by *K. pneumoniae, E. coli, S. aureus, S. saprophyticus,* and *S. epidermidis* in urolithiatic patients.
- *Candida* was also prevalent in urolithiatic patients with cUTI followed by *E. coli, K. pneumoniae, S. aureus, S. saprophyticus* and *S. epidermidis.*
- Three different species of *Candida* (*C. albicans, C. glabrata* and *C. krusei*) were detected in these patients through culture dependent method.
- A higher isolation of *C. albicans* was seen than non-albicans in urolithiatic patients.
- Different virulence factors (esterase, phospholipase and biofilm forming activity) tested were significantly higher in *Candida* isolates from urolithiatic patients as compared to healthy controls especially in urolithiatic patients with cUTI.
- Comparatively female patients were more colonized with *Candida.*
- Significant isolation of *Candida* was observed in catheterized patients as compared to non-catheterized patients.
- A low antifungal resistance was observed in *Candida* isolates against fluconazole and voriconazole.
- Gram negative bacteria were isolated more prevalently as compared to Gram positive bacteria from urolithiatic patients; among this isolation of *K. pneumoniae* was significantly higher from urolithiatic patients as compared to healthy controls.

- However, higher numbers of *E. coli* and *K. pneumoniae* were isolated from the patients with cUTI as compared to patients without cUTI.
- *K. pneumoniae* were more prevalent in male patients while isolation of *E. coli* was more from female urolithiatic patients.
- Out of 69 isolates of *K. pneumonia*, 22 were detected to be hypervirulent on the basis of hypermucoviscosity and most of these isolates were also positive for other virulence factors tested. Majority of hypervirulent *K. pneumoniae* (72%) isolated were from the patients with cUTI.
- Different virulence factors tested for these Gram negatives indicated that there was enhanced expression of these virulence factors from isolates of urolithiatic patients with cUTI as compared to patients without cUTI and healthy subjects.
- In case of *E. coli,* strong biofilm forming ability was observed but was in less number of isolates, however, these isolates had high expression of other virulence factors tested.
- Majority of these bacterial isolates were phenotypically MDR and ESBL positive.
- Molecular identification of ESBL genes (*CTX-M, TEM* and *SHV*) indicated that there was less number of bacterial isolates genotypic positive compared with the ESBL positive isolates by phenotypic identification.
- There was difference in microbial diversity and abundance by culture dependent and independent method. Although, *C. albicans* was observed by both methods in these urolithiatic patients with cUTI but there was difference in its abundance.
- In addition, many bacterial and fungal species were detected by culture independent method, which were unknown previously in urolithiasis by culturing method and also already known prevalent uropathogens were not the predominating microbes in this condition.
- Through culture independent method diversity analysis showed the greater diversity richness and evenness was in female urolithiatic patient with cUTI as compared to male urolithiatic patient and the healthy individuals.

- Dual species biofilms model of isolates from urolithiatic patients with cUTI showed that different species change their behaviour in consortium and can be antagonistic or synergistic with other species.

Future prospects

- There is need to investigate the virulence characteristics of microbes detected by culture independent method in these patients to specify their role in the development of disease.
- Virulence factors of culture dependent microbes can further be studied by using different *in vivo* conditions to check their expression under specific conditions.
- Molecular identification of virulence genes involved in enhanced virulence nature of these isolates from cUTI patients is required.
- There is need to identify the molecular mechanisms of MDR isolates, as ESBL producers were harbouring only few known resistant genes.
- There is need to investigate the poly-microbial consortia for biofilms development more extensively to understand the behaviour of these microbes at molecular level in such infections.
- Although the sample size for culture dependent microbial study was enough but for culture independent study, larger sample size is required to get more authenticated results of microbial diversity involved in urolithiasis and cUTI.

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APPENDIX AI

STUDY PROFORMA
DEPARTMENT OF MICROBIOLOGY
QUAID-I- AZAM UNIVERSITY ISLAMABAD

Date _____ File No. _____

Name _____ SO/DO _____

Age _____ years Gender M ☐ F ☐

Marital status ☐ Single ☐ Married Occupation:

Monthly income:

City:

Location: ☐ Hilly ☐ Plan

Living environment: ☐ Rural ☐ Urban

Disease: ☐ cUTI ☐ kidney Stone ☐ Bladder stone

Duration of stone Months ____ Year ____

Catheterized yes☐ No ☐

Medication prior to hospitalization Allopathic☐ antibiotics ☐ ☐ Herbal

Associated disease ☐ Diabetes ☐ Hypertension ☐ Heart

Quantity of daily water (glasses) intake ☐ 2-3 3-7 ☐ 7-10 ☐

Quality of water intake ☐ Boring ☐ Supply ☐ Boiled ☐ Spring

BASELINE DATA

Weight _____ Height _____ BMI _____

Blood Pressure _____ Systolic _____ Diastolic _____ mm Hg

Urolithiasis test:

APPENDIX AII

RESEARCH PARTICIPANT CONSENT FORM
FOR
MICROBIAL DIVERSITY IN COMPLICATED URINARY TRACT INFECTION AMONG UROLITHIATIC PATIENTS
AT
DEPARTMENT OF MICROBIOLOGY
QUAID-i- AZAM UNIVERSITY ISLAMABAD

Investigator	Sahar Zafar
Location	Molecular Microbiology Lab Department of Microbiology Quaid-I- Azam University Islamabad
Contact No.	051- 9064 3065

Purpose and Background

Urolithiasis is a worldwide prevailing disease and Pakistan is at high risk for this disease and its complications. This study will provide baseline data to prepare guidelines management strategies to avoid this disease and its possible complications.

Methodology: This is a retrospective study; participants need to provide urine samples for microbiological and other analysis.

Potential Risks of Study: It is minimally invasive study and no risks are involved in this study.

Financial Consideration: There will be no financial compensation for participation in the study.

Confidentiality: All the records will be kept confidential. The result of the study including laboratory or any other data may be published for scientific purpose.

Termination from Participation: you have all the right not to participate in the study even if the consent form is signed by you.

I_____ S/D/O _____ have been informed about the details and applications of the study. I understand that this study will not involve any hazardous effects on my health. I have gone through the contents of informed consent, and I am willing to provide urine sample for this study. I also understand that the data provided will be kept confidential and will only be used for research outcome. I voluntarily choose to participate but I understand that my consent does not take away any legal rights in case of negligence or other legal fault or anyone who is involved in this study. I further understand that nothing in this consent form is intended to replace my applicable, federal state or local laws.

Signature of participant _____

Signature of person obtaining consent _____

Name of the person obtaining consent _____

Date _____

APPENDICES B

BI: Biochemical profile of bacterial isolates from urine samples of urolithiatic patients

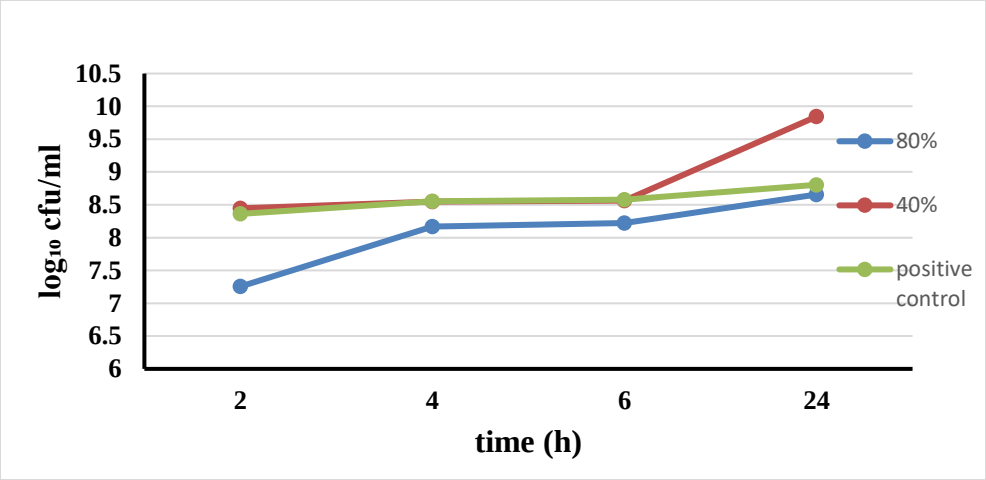
Biochemical Test		**Isolates n(%)**
Indole	**Positive**	71(49)
	Negative	75(51)
Citrate	**Positive**	70(48)
	Negative	76(52)
Urease	**Positive**	71(49)
	Negative	75(51)
TSI	**A/A**	126(86)
	Gas	126(86)
	H_2S	00 (0)
Catalase	**Positive**	136(93)
	Negative	10(7)
Motility	**Positive**	70(48)
	Negative	76(52)

B-II: Antibiotic resistant pattern of *E. coli* isolates from urolithiatic patients

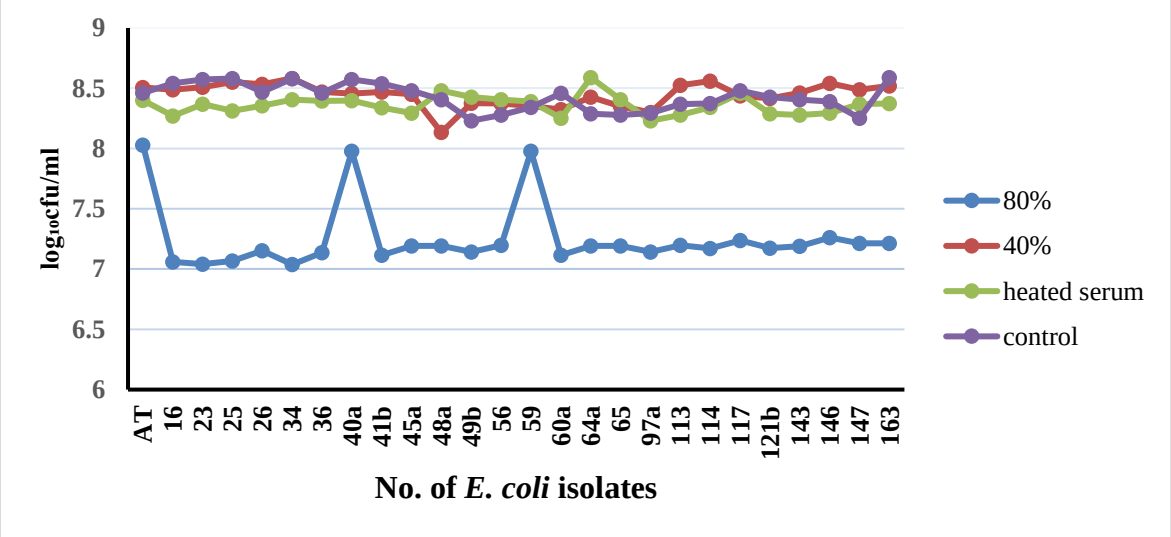
Classes	**Antibiotics**	***E. coli*** **n(%)**		
		Sensitive	**Intermediate**	**Resistant**
Aminoglycosides	CN 50µg	40(74)	1(2)	13(24)
	TOB 30µg	19(35)	13(24)	22(41)
	AK 30µg	45(83)	7(13)	2(4)
Fluoroquinolones	CP 5µg	28(52)	3(5.5)	23(42.5)
	OFX 5µg	19(35.2)	3(5.5)	32(59.3)
	NA 10ug	6(11)	7(13)	41(76)
Cephalosporins	CRO 30µg	17(31.5)	8(14.8)	29(53.7)
	CAZ 30µg	16(30)	8(14.8)	30(55.6)
Sulfonamides	TMP/SMX	22(40.7)	9(16.7)	23(42.6)
Tetracycline	TE 30µg	34(68)	8(14.8)	12(22.2)
Penicillin	AML 10µg	4(7.4)	4(7.4)	46(85.2)
Lincosamides	DA 10µg	10(18.5)	3(5.6)	41(76)
Nitrofurantoin	F 300µg	33(61)	11(20.4)	10(18.6)
Imipenem	IMP 10µg	36(66.7)	7(13)	11(20.3)

B-III: Antibiotic resistant pattern of *K. pneumoniae* isolates from urolithiatic patients

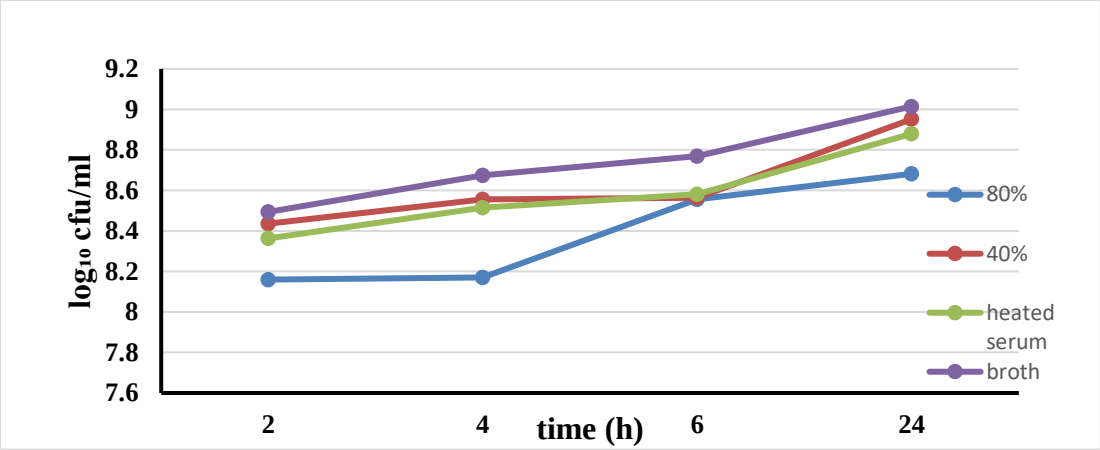
Classes	**Antibiotics**	***K. pneumoniae*** **n(%)**		
		Sensitive	**Intermediate**	**Resistant**
Aminoglycosides	CN 50µg	28(41)	7(10)	34(49)
	TOB 30µg	33(48)	11(16)	25(36)
	AK 30µg	45(65.2)	21(30.4)	3(4.3)
Fluoroquinolones	CP 5µg	26(38)	0(0)	43(62)
	OFX 5µg	31(45)	5(7.2)	33(47.8)
Cephalosporins	KF 30µg	14(20)	6(9)	49(71)
	CAZ 30µg	37(53)	8(12)	24(35)
	CRO 30µg	18(26)	1(1.4)	50(72.6)
Sulfonamides	TMP/SMX	6(8.7)	3(4.3)	60(87)
Tetracyclines	TE 30µg	48(69.5)	2(3)	19(27.5)
Penicillin	AML 10µg	1(1.4)	3(4.3)	65(94.3)
Lincosamide	DA 10µg	1(1.4)	3(4.3)	65(94.3)
Nitrofurantoin	F 300µg	27(39)	27(39)	15(22)
Imipenem	IMP 10µg	33(48)	26(38)	10(14)



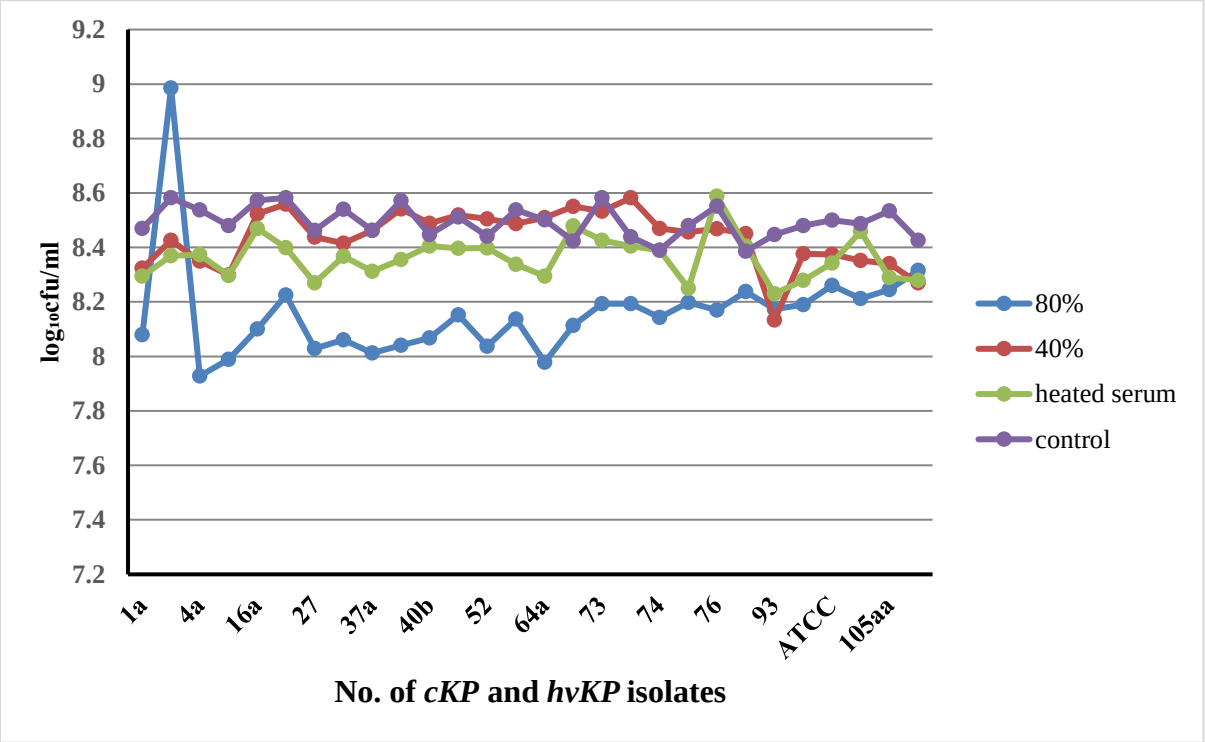
B-IV: Average serum anti-bactericidal activity of *E. coli* isolates after incubation period of 2 h, 4 h, 6 h and 24 h



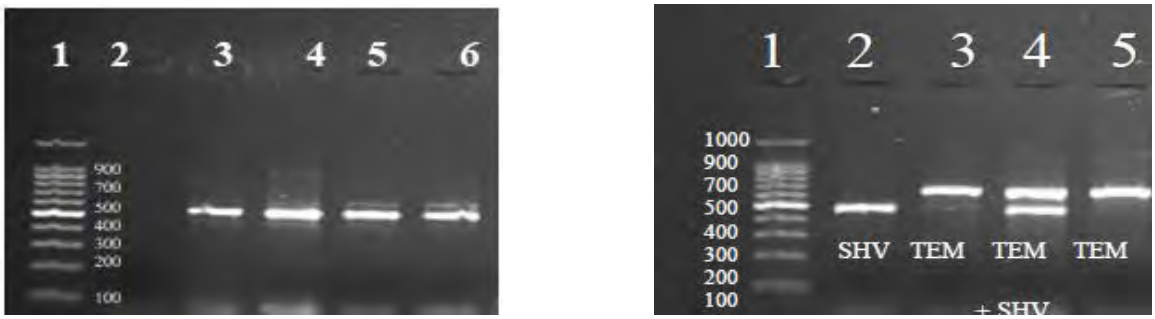
B-V: Comparison of serum anti-bactericidal activity of ATCC and highly resistant *E. coli* isolates SF-12, SF-23, SF-25, SF-26, SF-34, SF-36, SF-40a, SF-41b, SF-45a, SF-48a, SF-49b, SF-56, SF-59, SF-60a, SF-64a, SF-65, SF-97a, SF-113, SF-114, SF-117, SF-121b, SF-143, SF-146, SF-147, SF-163 respectively in 40% 80% and heat inactivated serum.



B-VI: Average serum anti-bactericidal activity of *K. pneumoniae* isolates after incubation period of 2 h, 4 h, 6 h and 24 h

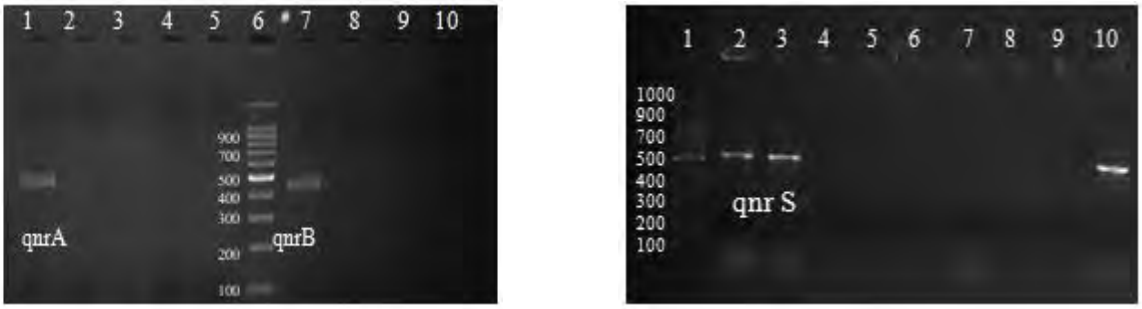


B-VII: Comparison of serum anti-bactericidal activity of highly resistant *cKP* isolates SF-1, SF-4, SF-52, SF-64, SF-93, SF- 105a, *hvKP* isolates SF-17, SF-27. SF-37a, SF-40, SF-73, SF-74, SF-76 and ATCC strains against different concentrations of PNHS



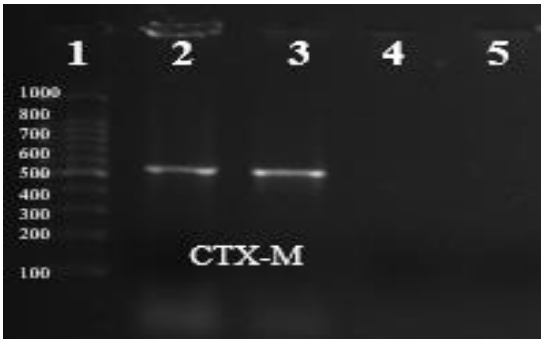
A **B**

B-VIII: PCR results to confirm ESBL genes of *E. coli* isolates, A) showing band of bla *CTX-M* gene in lane no 3, 4, 5, and 6 of *E. coli* isolates SF-16, SF-27, SF-34 and SF-36 respectively. B) Multiplex PCR result showing bands of *TEM* and *SHV* genes. Lane no 2 showing band of bla-*SHV* (491bp) of isolate SF-25, lane no 3 and 5 are showing band of bla-*TEM* (623bp) of isolate SF-26 and SF-41 respectively. Lane 4 showing band of bla *TEM* and *SHV* multiplex of isolate SF-26. Lane no 1 showing 1Kb ladder.

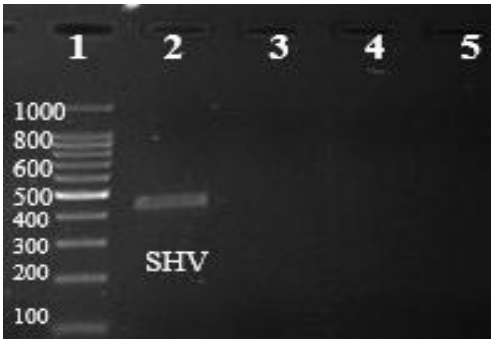


A B

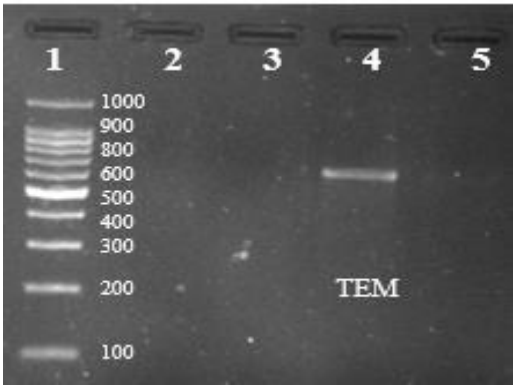
B-IX: PCR results of Quinolone resistance genes A, B, S of *E. coli* isolates. A) showing the bands of *qnr*A (516bp) in Lane no 1 of isolate SF-34 and *qnr*B (427bp) in Lane no 7 of isolate SF-68. B) Lane no 2,3 and 10 showing the band of *qnr*S (526bp) of *E. coli* isolate SF-34, SF-36 and SF-68 respectively. Lane no 6 in A) and lane no 1 in B) showing 1Kb ladder



A



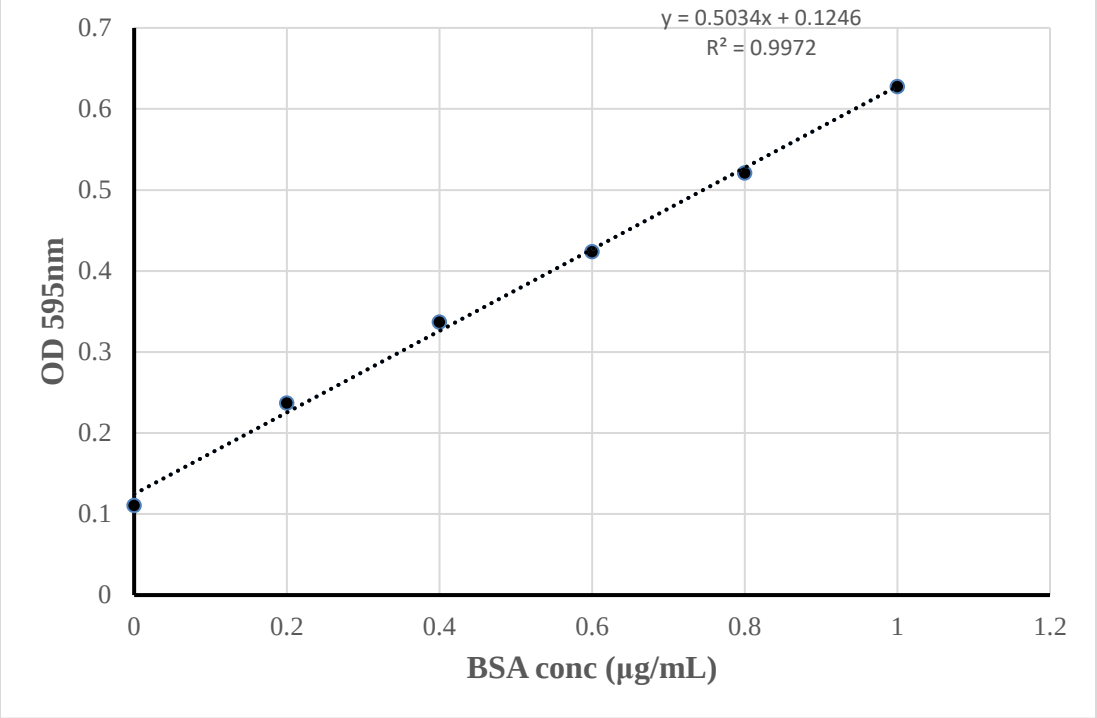
B



C

B-X: PCR results to confirm ESBL genes of *K. pneumoniae* isolates, A) showing band of bla *CTX-M* gene in lane no 2 and 3 of *K. pneumoniae* isolates SF-15a and SF-39a respectively. B) Showing bands of *SHV* gene. Lane no 2 showing band of bla-*SHV* (491bp) of isolate SF-117a. C) Showing *TEM* gene in lane no. 4 of *K. pneumoniae* isolate SF-101. Lane no 1 in all showing 1Kb ladder.

APPENDICES C



C-I: BSA standard curve for total protein estimation of ATCC and cUTI isolates *E. coli, K. pneumoniae* and *Candida spp.* after 24 h and 48 h of incubation.