CHAPTER 3:

Collection, isolation, and identification of clinical isolates of UTI pathogenic *Pseudomonas aeruginosa*

3.1 Introduction

P. aeruginosa is a gram-negative opportunistic pathogen infecting immunocompromised patients. It is one of the major causes of nosocomial infections such as UTIs, pneumonia, surgical site infections, pneumonia, bacteremia, and septicemia (Breidenstein et al. 2011; Yayan et al. 2015). The morbidity and mortality rates among elderly hospital patients who develop UTIs from *P. aeruginosa* are substantial. These UTI isolates frequently exhibit higher rates of resistance to antibiotics as compared to *E. coli*- which causes the majority of UTIs (Ironmonger et al. 2015). Further, elderly patients infected with *P. aeruginosa* UTIs frequently have a recurrence of infection following antibiotic treatment (Gomila et al. 2018).

P. aeruginosa infections are difficult to treat owing to resistance to multiple antibiotics due to rapid mutation and adaptions (Blomquist and Nix 2021). In addition, it has been identified as one of the ESKAPE pathogens with medical and epidemiological significance (Tacconelli 2017). It has been linked to severe infections in burn victims and persistent lung infections in cystic fibrosis (CF) patients (Subedi et al. 2018; Rocha et al. 2019). According to recent research, *P. aeruginosa* populations grow and diversify in the CF lung, making it challenging to completely eradicate (Ashish et al. 2013; Evans 2015). Single colonies from urine samples are used in the current procedures for determining a pathogen's susceptibility to antibiotics. This could drastically understate the diversity and, consequently, the resistance of a specific bacteria causing a UTI, resulting in insufficient treatment. Further, *P. aeruginosa* can form biofilm on catheters making it difficult to eradicate as biofilm are more resistant than the planktonic stage. To effectively treat these infections, it is essential to identify clinically isolated *P. aeruginosa.*

3.2 Materials and Methods

3.2.1 Collection of isolates

UTI-causing bacterial isolates (n=22) were collected from Sterling and Toprani Pathology lab, Vadodara, Gujarat, India. Isolates were collected from the patient having UTIs. PAO1 strain was used as a reference strain. Preliminary identification was done on the Vitek system and data was provided by pathology labs.

3.2.2 Isolation and Identification

The collected UTIs-causing isolates were grown on Pseudomonas Isolation Agar (PIA). PIA is a selective and differential medium for primary isolation and identification of *P. aeruginosa*, where triclosan (Irgasan) inhibits Gram-positive and Gram-negative bacteria other than *Pseudomonas spp.* Also, the glycerol present in the medium enhances pyocyanin (blue-green pigment) production. The isolates were inoculated on PIA agar and incubated at 37 °C for 24 hours and isolated colonies obtained were observed on the following day. The isolates (n=22) were further processed for Gram-stain and molecular identification of isolates.

3.2.3 Gram staining

Gram staining was performed for all isolates. On a clean glass slide, a thin smear of cell suspension from an isolated colony was prepared and heat-fixed. Thereafter, the smear was stained with crystal violet (CV) for 5 minutes. The slide was flooded with Gram's iodine and kept for 5 minutes. Acetone or alcohol was used to decolorize the cell. As Gram-negative bacteria bear to have a thin layer of peptidoglycan, they become colourless, while Gram-positive posses thick layer of peptidoglycan layer that traps crystal violet. After the decolorization step, the counter stain (safranin) was applied for 5-7 minutes. Later, slides were gently washed with distilled water (DW). Slides were air-dried and observed under the light microscope at 100X magnification.

3.2.4 16S rRNA gene amplification and analysis

3.2.4.1 DNA isolation

Genomic DNA (gDNA) extraction was performed using a modified from Sambrook *et al*, 2001. In brief, 5 ml of 16-18 hours old bacterial culture was centrifuged at 8000 rpm for 10 minutes; the pellet was resuspended in 345 µl Tris-EDTA (T. E.) buffer and mixed thoroughly; thereafter, 40 µl of Lysozyme (10 mg/ml) was added to the mixture, gently mixed by inverting tubes and incubated for 1 hour at 37 ºC. After incubation, 100 µl of 1% SDS, 10 µl of 0.5 M EDTA (pH=8), and 5 µl of proteinase K (100 µg/ml) were added to the above mixture and gently

mixed by inverting tubes. The tubes were further incubated at 50 ºC for 2 hours until the solution became clear. To the above mixture equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by inverting the tubes followed by centrifugation at 8000 rpm for 10 minutes. The upper aqueous phase was then transferred to another tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added and gently mixed by inverting followed by centrifugation at 8000 rpm for 10 minutes. After centrifugation, the upper layer was transferred to another tube and 2.5X volume of absolute ethanol and 1/10th volume of sodium acetate (0.3M) was added. Tubes were kept overnight at -20 ºC for precipitation of Genomic DNA (gDNA). The following day, centrifugation was done at 8000 rpm for 10 minutes and the supernatant was discarded. The pellet was washed with 70% ethanol and air dried. The gDNA was dissolved in 20 µl of sterile nuclease-free water and DNA was stored at -20 ºC for a longer period.

3.2.4.2 16S rRNA gene amplification and analysis

The *16S rRNA* gene sequence was amplified from 22 clinical isolates was done using Polymerase Chain Reaction (PCR) technique in a thermocycler (Bio-Rad Laboratories, CA, USA). The universal primers (27F and 1525R) sequence were used for gene amplification listed in table 3.1. The reaction mixture was prepared using SapphireAmp® Fast PCR Master Mix (TaKaRa Bio Inc., Tokyo, Japan) is listed in table 3.2. The SapphireAmp® Fast PCR Master Mix contains dNTPs, MgCl2, and Taq polymerase. PCR reaction conditions are mentioned below table 3.3. Further confirmation of *16S rRNA* gene amplification was done on 1% agarose gel electrophoresis having 0.1% EtBr and visualized under UV light under Gel-Doc (Bio-Rad Laboratories, CA, USA). Thereafter, amplified products were sent to Eurofins Scientific, Chennai, India for Sanger's sequencing. The FASTA files having the gene sequence of the amplified region were aligned using nBLAST (nucleotide-Basic Local Alignment Search Tool) by NCBI (National Centre for Biotechnology Information Database). The isolates with ≥ 99% identity were identified as *Pseudomonas spp.*

Table 3.1: Primer used for *16S rRNA* gene amplification.

Primer	Primer sequence (5'-3')
27F	AGAGTTTGATCMTGGCTCAG
1525R	AAGGAGGTGWTCCARCC

Table 3.2: Reaction mixture for PCR

Reaction mixture	Volume
SapphireAmp® Fast PCR Master Mix	12 µl
Nuclease free water	6.5 µl
Forward primer	2 µl (10pmol)
Reverse primer	2 µl (10pmol)
DNA template	2 µl (100 ng/ml)
Total System	25 µl

Table 3.3: Conditions of PCR

Step	Temperature ºC	Time
Initial denaturation	95	7 minutes
Denaturation	94	30 seconds
Annealing	56	45 seconds
Primer Extension	72	1 minutes
Repeat cycle 35 X		
Final extension	72	5 minutes

3.3 Results

3.3.1 Isolation and identification

As shown in Figure 3.1, the representative images of fully developed colonies were found to round with raised elevation on the Pseudomonas Isolation Agar plate. The green pigment was observed after incubation for 24 hours at 37°C. The green pigment was diffused into the medium surrounding the bacterial colonies which are observed in below shown Figure 3.1. The pyocyanin green pigment was almost observed for all isolates (n=22) on PIA. The selective medium (PIA) used here has glycerol, magnesium chloride, and potassium sulfate which enhance the green pigment. Some isolates also produce blue-green pigment (pyocyanin

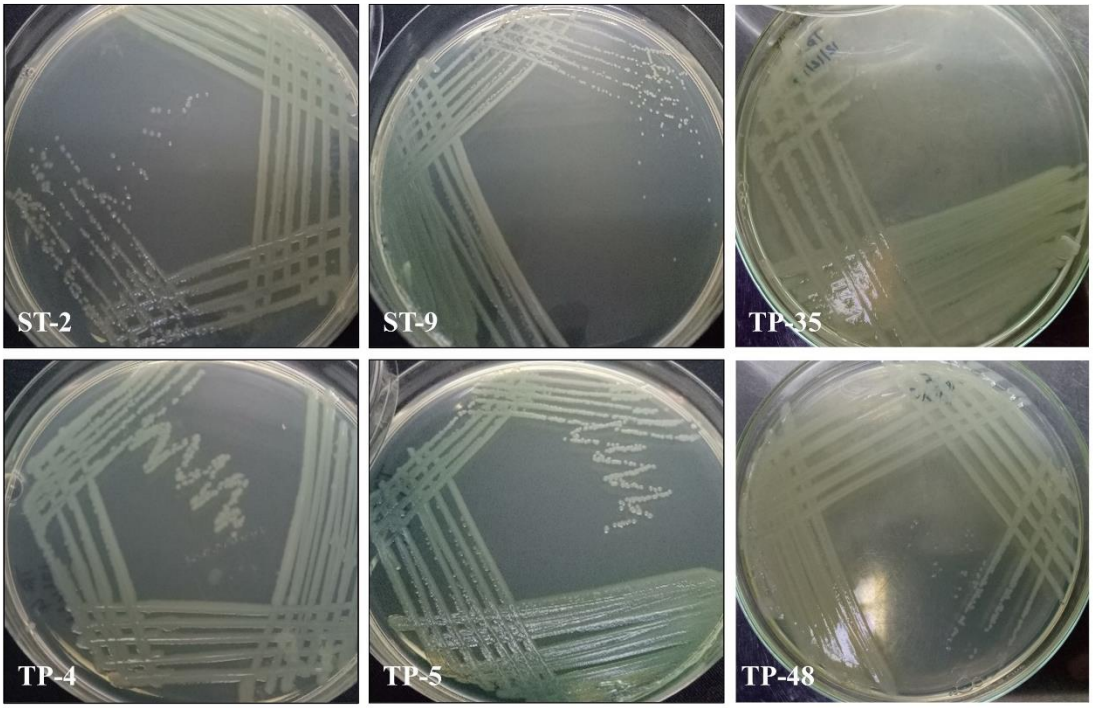

Figure 3.1: Representative images of bacterial isolates on PIA. Representative images of collected isolates cultured on Pseudomonas Isolation Agar. The green pigment was diffused in medium which is particular for *Pseudomonas Spp.*

The isolates colonies were further subjected to Gram staining. Representative images of isolates (TP-48, ST-22, TP-25, and TP-35) that Gram stained are shown in Figure 3.2. All

isolates (n=2) appeared to be pink-colored (due to safranin stain) and rod-shaped which are Gram-negative bacteria.

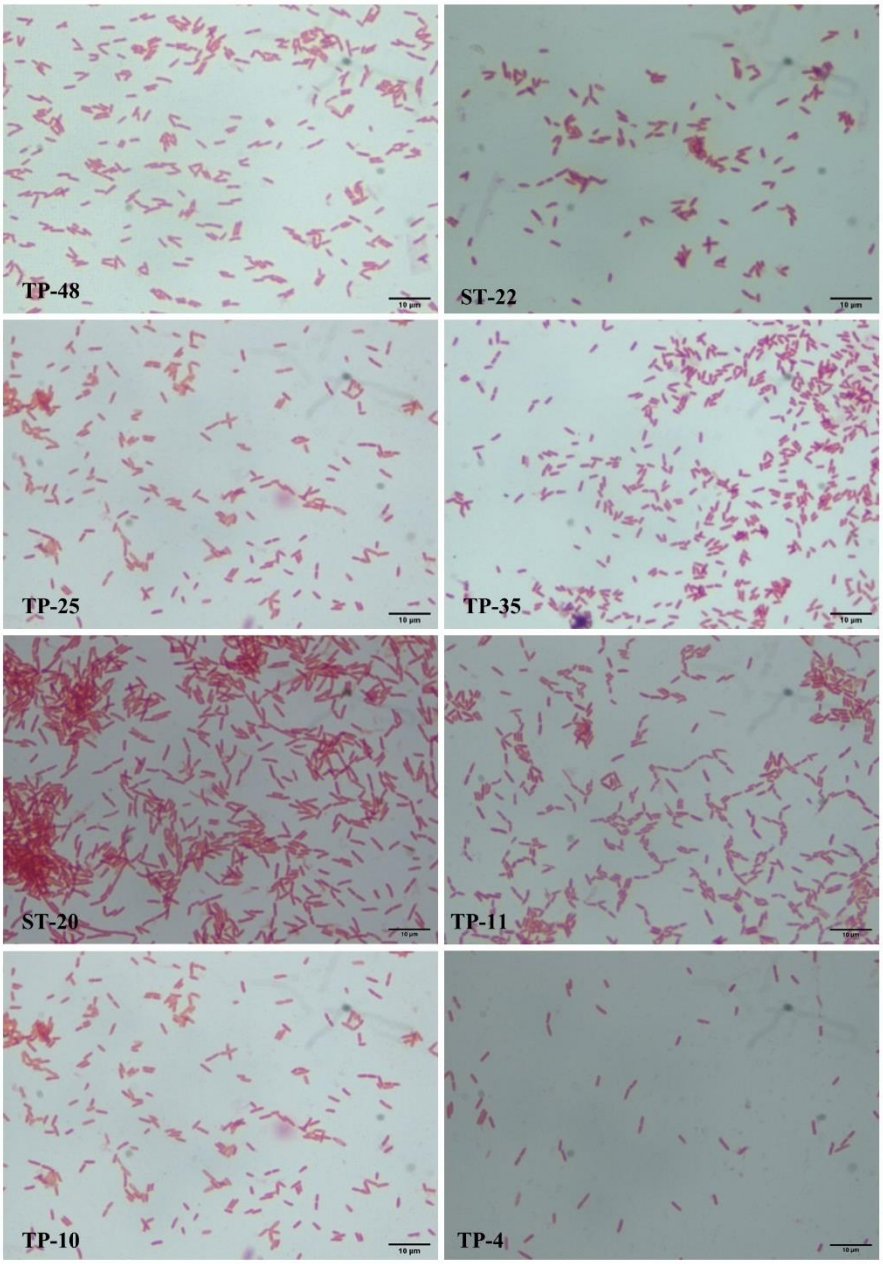

Figure 3.2: Gram staining. Representative images of Gram staining observed under 100X oil immersion microscope (TP-48, ST-22, TP-25, TP-35, ST-20, TP-10 and TP-4)

3.3.2 16S rRNA gene amplification and analysis

3.3.2.1 Genomic DNA extraction

DNA extraction was done for all n=22 isolates. Representative images of genomic DNA were observed after the gel electrophoresis (Figure 3.3).

3.3.2.2 PCR amplification and Sanger's sequencing of 16s rRNA gene

The PCR amplification was done for the 16S rRNA gene and a 1.5 kb sized amplicon product was obtained (Figure 3.4).

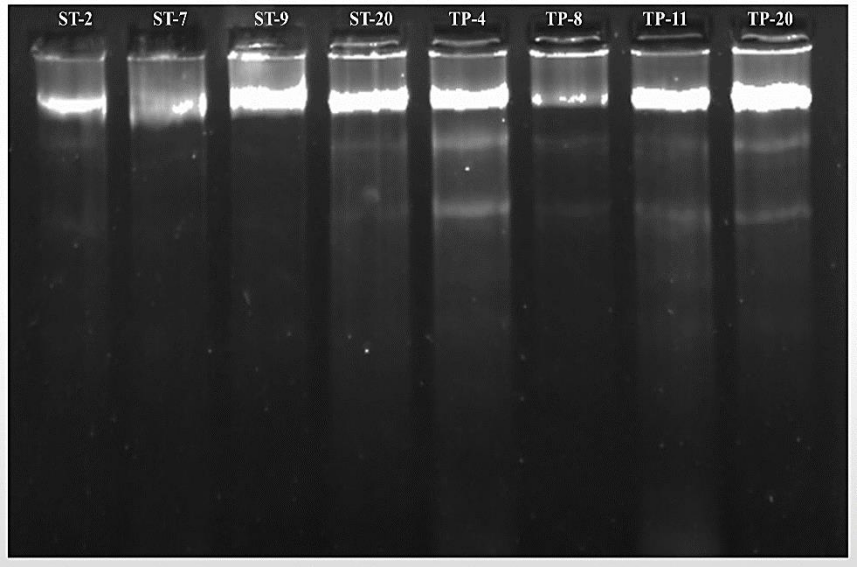

Figure 3.3: A 0.8% agarose gel electrophoresis of extracted DNA. The DNA was isolated from each clinical isolates collected. Further confirmation was done by running DNA on 0.8% gel agarose with ethidium bromide. DNA sample was loaded at top of each well. The lane from left is as follows: lane1: ST-2, lane2: ST-7, lane 3: ST-9, lane 4: ST-20, lane 5: TP-4, lane 6: TP-8, lane 7: TP-11, and lane 8: TP-20.

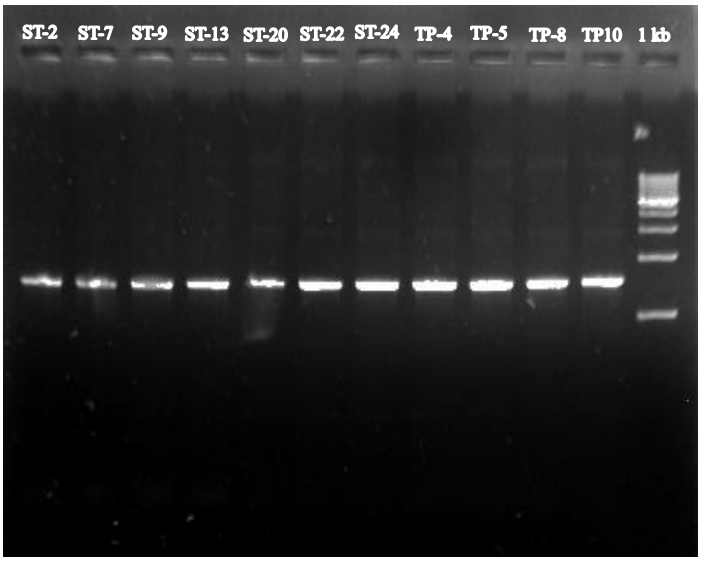

Figure 3.5: A 0.8% agarose gel electrophoresis of 16S rRNA gene amplification. Representative image of PCR product (1.5 kb) of 16S rRNA bands on 1% agarose gel with ethidium bromide. The gDNA template was used for amplification are indicated on the top of the each well. The well from left is as follows: lane 1:ST-2, lane 2: ST-7, lane 3: ST-9, lane 3: ST-13, lane 3: ST-20, lane 3: ST-20, lane 3: ST-22, lane 3: ST-24, lane 3: TP-4, lane 3: TP-5, lane 3: TP-8, lane 3: TP-10, lane 3: 1 kb ladder. The 27F and 1525R primers were used for PCR amplifications

Figure 3.4: **A 0.8% agarose gel electrophoresis of 16S rRNA gene amplification.** Representative image of PCR product (1.5 kb) of 16S rRNA bands on 1% agarose gel with ethidium bromide. The gDNA template was used for amplification are indicated on the top of the each well. The well from left is as follows: lane 1:ST-2, lane 2: ST-7, lane 3: ST-9, lane 3: ST-13, lane 3: ST-20, lane 3: ST-20, lane 3: ST-22, lane 3: ST-24, lane 3: TP-4, lane 3: TP-5, lane 3: TP-8, lane 3: TP-10, lane 3: 1 kb ladder. The 27F and 1525R primers were used for PCR amplifications

Table 3.4: NCBI BLAST result of 16S rRNA gene amplicon product through Sanger Sequencing.

Sample name	Identified on basis of NCBI BLAST	Query cover %	Identity %	Query length
ST-2	*P. aeruginosa* isolate ID40 genome assembly, chromosome: ID40-chromosome	100	99.39	974
ST-7	*P. aeruginosa* (P.A strain JBL-5 16s ribosomal RNA gene)	99	97.26	844

ST-9	*P. aeruginosa* (P.A strain DN9 16s ribosomal RNA gene)	98	97.59	841
ST-13	*P. aeruginosa*	94	95.62	1157
ST-20	*P. aeruginosa* (P.A strain R-57 16s ribosomal RNA gene)	100	99.52	1123
ST-22	*P. aeruginosa* (P.A strain C116s ribosomal RNA gene)	97	98.49	1488
ST-24	*P. aeruginosa*	99	98.52	1495
TP-4	*P. aeruginosa (P.A strain P1B30 16s ribosomal RNA gene), PARTIAL SEQ*	98	98.51	1492
TP-5	*P. aeruginosa (P.A strain XJU-2 16s ribosomal RNA gene)*	99	98.97	1468
TP-8	*P. aeruginosa* (P.A. E80 chromosome)	99	98.99	1491
TP 10	*P. aeruginosa* (P.A strain P1B30 16s ribosomal RNA gene)	98	99.66	1485
TP-11	*P. aeruginosa* PA 16s RNA gene	99	97.26	1459

TP-18	*P. aeruginosa* (P.A strain PsADMC06 16s ribosomal RNA gene)	100	99.42	1026
TP-20	*P. aeruginosa* (P. A isolate paerg012 genome assembly, chromosome=0)	99	98.72	1495
TP-21	*P. aeruginosa* (P.A strain PA-VAP-2 chromosome	99	98.92	1485
TP-22	*P. aeruginosa* (P. A strain Partial 16s ribosomal RNA gene, isolate L1)	99	97.42	999
TP-25	*P.aeruginosa* (P.A strain CL8 16s ribosomal RNA gene)	99	95.04	950
TP-26	*P. aeruginosa*	99	98.05	1484
TP-29	*P. aeruginosa* (P.A strain PA-VAP-2 chromosome)	99	99.73	1487
TP-35	*P. aeruginosa* (P. A strain YU-V28 16s ribosomal RNA gene) partial sequence	99	99.40	1493
TP-36	*P. aeruginosa* (P.A strain P1B30 16s ribosomal RNA gene)	99	99.12	1484
TP-48	*P. aeruginosa* (P.A. E80 chromosome)	99	98.99	1491

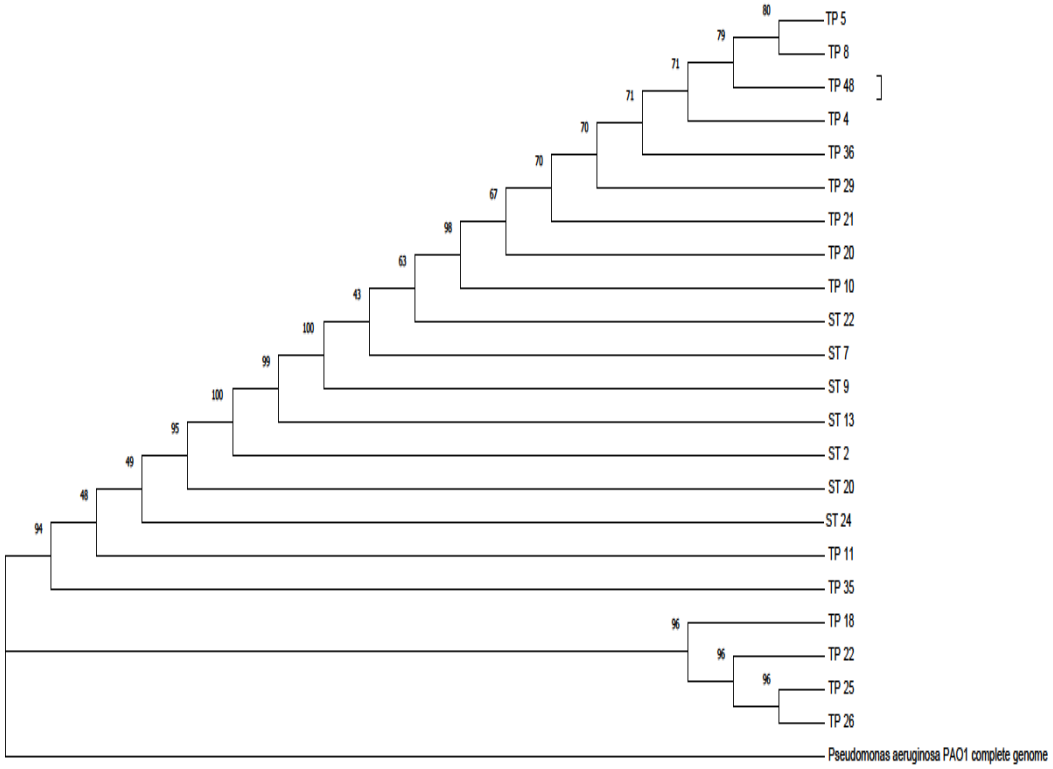

Figure 3.6: Phylogenetic tree of pathogenic *Pseudomonas aeruginosa* isolates (n=22). The phylogenetic tree of pathogenic *Pseudomonas aeruginosa* isolates (n=22) using the neighbour-joining method (MEGA-X software) based on its 16S rRNA gene sequences.

3.4 Discussion

UTIs are most prevalent in hospital-acquired infections and account for 30% of all nosocomial infections (Klevens et al. 2007). *P. aeruginosa* is responsible for 7–10% of UTIS in hospitals (Bouza et al. 2001; Djordjevic et al. 2013). In addition, *P. aeruginosa* is often resistant to multiple antibiotics. There are many reports regarding *P. aeruginosa* causing infection in CF patients. Reports regarding UTIs causing *P. aeruginosa* are less especially from India.

Most conventional diagnostic procedures for *P. aeruginosa* depend on culture-dependent techniques, and accurate identification of *P. aeruginosa* is crucial (Deschaght et al. 2011). For the isolation of *P. aeruginosa*, a variety of selective agars are available, including those that contain inhibitory selective agents such as cetrimide, Fucidin, cephaloridine, phenanthroline, nalidixic acid, and irgasan (Lilly and Lowbury 1972; Fonseca et al. 1986; Weiser et al. 2014). In the present study, Pseudomonas Isolation Agar containing irgasan was used for the isolation of *P. aeruginosa* as it inhibits other gram-negative bacteria and other *Pseudomonas sp*. Further, in the present study, all isolates (n=22) were grown on Pseudomonas Isolation Agar and almost all isolates produced green pigmentation at 37 °C for 24 hours of incubation (Figure 3.1). Apart from this grape-like odor was detected in many isolates which have been earlier reported due to the production of 2-aminoacetophenone from tryptophan present in the media and useful in confirmation (Senthil et al. 2011; Hossain et al. 2013). The pigmentation observed on a selective medium makes easy detection of *P. aeruginosa* from other bacteria. While for some isolates no pigmentation was observed (ST-22 data not shown). Similar findings were earlier reported in strains isolated from CF patients (Reyes et al. 1981; Daly et al. 1984; Vos et al. 2001; Lee et al. 2005). The phenotypic identification may give false/negative results so other methods such as genotypic may be useful for the identification of *P. aeruginosa*

Due to several difficulties in the accurate identification of *P. aeruginosa* more accurate genotypic identification is used. The hypervariable, highly conserved portions of the *16S rRNA* gene sequences can distinguish between species-specific signature sequences that are useful for classifying bacteria (Guasp et al. 2000; Adekambi et al. 2003). For instance, the DNA sequence typing of the 16S rRNA gene has shown to be 100% specific and sensitive for *P. aeruginosa* identification (Spilker et al. 2004). Further, Didelot et al 2016 reported that 16S rRNA gene sequencing is common in medical microbiology as a quick and inexpensive substitute for phenotypic identification (Didelot et al. 2016). In the present study, we identified all collected isolates (n=22) as *P. aeruginosa.* Many studies have documented the *P. aeruginosa* identification using *oprI*, *oprL, algD* gene in CF patients' sputum samples (Vicente et al. 1999;

Vos et al. 2001; Spilker et al. 2004; Xu et al. 2004). Due to the limitations of the above gene used did not achieve 100% sensitivity and specificity, *16S rRNA* gene sequencing is preferred for genus/species identification of *P. aeruginosa.*

For accurate identification of ST type or clonal type, Next Generation Sequencing (NGS) may help to better know the clonal expansion of *P. aeruginosa.* Also, the detection of virulence genes and antibiotic resistance can be carried out.