Chapter 3

Collection, isolation and identification of clinical isolates of pathogenic *Klebsiella* spp.

Chapter 3

3.1 Introduction

Klebsiella spp. are Gram-negative, rod shape, non-motile, encapsulated, lactose fermenting, facultative anaerobic bacteria. They can be found in the gut flora of animals, healthy individuals and in the natural environments like in soil and water (Wyres and Holt, 2018). However, they are also opportunistic pathogens causing both nosocomial and community infections, mostly targeting the urinary and respiratory tracts. They cause life-threatening infections such as pneumonia, sepsis, meningitis, pyogenic liver abscess (PLA) and complicated Urinary Tract Infections (UTIs) (Podschun and Ullmann, 1998). *Klebsiella* spp. contribute 13-30% and 25-50% in nosocomial and community acquired UTIs (Gajdács et al., 2019, Jean et al., 2016).

In addition to be a significant clinical problem, *Klebsiella pneumoniae* are also resistant to multiple antimicrobials and are acknowledged to be a major source of antimicrobial resistance genes that have spread into other Gram-negative pathogens (Karkey et al., 2015). *Klebsiella pneumoniae* (*Kp*) and *K. oxytoca* are two clinically and epidemiologically important and prevalent species of the genus *Klebsiella* (Paterson et al., 2014). Moreover, resistant and hypervirulent strains of *Klebsiella* act as opportunistic but cause extremely critical infections to treat. Hence, identification of clinically isolated *Klebsiella* spp. is vital to successfully combat these infections.

3.2 Materials and methods

3.2.1 Collection of pathogenic isolates of *Klebsiella*

Clinical isolates (n=55) of pathogenic Gram-negative bacteria were collected from pathology labs in Surat and Vapi, Gujarat, India. All collected isolates were of patients suffering from Urinary Tract Infection (UTI). Majority of the isolates were collected from community-acquired infections. All the patients had mono-infection of *Klebsiella* only and none of the patients have progressed

for Urosepsis. One MTCC *Klebsiella pneumoniae 39* strain was used as reference for the study.

3.2.2 Isolation and identification

All collected isolates were cultured on MacConkey agar containing neutral red and MacConkey agar containing Bromocresol purple (HiMedia Laboratories, Mumbai, India) as indicator dye and incubated overnight at 37 ºC. After the incubation, plates were observed for the isolated colonies and colony morphology. Isolates were also cultured on HiCrome™ *Klebsiella* Selective Agar Base (HiMedia Laboratories, Mumbai, India) to isolate *Klebsiella* spp. selectively among other Gram-negative organisms. Plates were incubated overnight at 37 ºC and the colonies obtained were observed. Isolates were then processed further for biochemical and molecular identification**.**

3.2.3 Biochemical tests

Gram staining and capsule staining were performed for the preliminary screening of collected isolates. Further, biochemical tests such as citrate test, urease test, oxidase test and motility test were performed. All biochemical tests were performed according to the Bergey's Manual of Determinative Bacteriology (Bergey and John, 1994).

(a) Gram staining

Standard procedure for Gram staining was performed. A thin smear of uniform cell suspension of respective isolate was prepared on a clean glass slide and heat fixed. Crystal violet (primary stain) was added to the slide and kept for 1 minute. Slide was rinsed with distilled water for a maximum of 5-10 seconds to remove unbound crystal violet. Slide was flooded with Gram's iodine and kept for 1 minute. Slide was rinsed with alcohol for ~30 seconds and then rinse with a gentle stream of distilled water. Safranin (secondary stain), was added to the slide and incubated for 1 minute. Slide was then washed with a gentle stream of distilled water for a maximum of 5-10 seconds. The slides were airdried and observed under the light microscope at 1000X magnification.

(b) Capsule staining

Capsule staining was performed using Maneval's negative staining technique (Maneval,1941). A drop of 1% Congo red was added to the centre of a glass slide. A thin smear was prepared by taking an inoculum from the bacterial isolate and it was then mixed with the drop of Congo red on slide. The smear was allowed to be airdried. Then it was flooded with Maneval's stain (nigrosine) for at least 1 minute. Excessive stain was removed by tilting a glass slide. The stained area was observed under the light and phase-contrast microscope using a 100X and 40X objective, respectively.

(c) Citrate test

Citrate utilization test was performed using Simmons citrate agar. Slants were prepared using Simmons citrate agar (HiMedia Laboratories, Mumbai, India) in sterile autoclaved test tubes. A light inoculum picked from the centre of a well-isolated colony of respective bacterial culture and streaked on the agar slants. Slants were then incubated at 37 ºC overnight. Next day, change in the colour of the agar was observed. Isolates with the colour changed from green to blue were considered as citrate positive isolates and isolates showed no colour change were identified as citrate negative isolates.

(d) Oxidase test

Oxidase test was carried out using Oxidase disc (HiMedia Laboratories, Mumbai, India) method. A well isolated colony was placed and spread on an oxidase disc using sterile cotton swab. Isolates showed colour change of the disc to deep purplish blue within 10 seconds at room temperature 25-30 ºC were considered as oxidase-positive. No colour change was identified as oxidase-negative isolates.

(e) Urease test

Christensen's urea agar was used to perform urease test. 24.01 grams of Christensen's urea agar (HiMedia Laboratories, Mumbai, India) was dissolved in 950 ml distilled water and heated to boiling to dissolve the media components completely. The media was sterilized by autoclaving at 10 lbs pressure at 115°C for 20 minutes and cooled to 45-50°C. 50 ml of 40% urea solution was filter-sterilized and added aseptically to the Christensen's urea agar prepared

previously. The mixture was then mixed thoroughly and dispensed into sterile tubes. Slants were prepared by allowing the tubes to set in the slanting position. The surface of the slants was streaked with inoculum of 18-24-hour pure culture of respective isolates. Tubes with loosened caps were incubated at 37°C overnight. Next day, the slants were observed for the colour change. Isolates showed colour-change from orange yellow to bright pink (fuchsia) were considered as urease positive and no colour-change was considered as urease negative isolates.

(f) Motility test

Motility test was done using the hanging drop method. A tiny drop of respective bacterial culture in 0.8% normal saline was placed in the centre of a clean coverslip. Vaseline was applied using toothpick at the corners of the coverslip. A glass concavity slide was turned upside down (concavity down) over the drop on the coverslip so that the vaseline sealed the coverslip to the slide around the concavity. The slide was then turned over, so the coverslip remains on top and the drop can be observed hanging from the coverslip over the concavity. The preparation was placed under the microscope and the edge of the drop was focused carefully with the fine adjustments. Each side of the edge of the drop and movement of bacteria were observed for true motility except the Brownian motion.

3.2.4 16s rRNA gene amplification and analysis

3.2.4.1 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from 16-18 h old culture (grown in Nutrient Broth) by using modified Phenol-chloroform method (Sambrook *et al*., 2001). 5ml of overnight grown culture was centrifuged at 1000 rpm for 10 minutes. Pellet was resuspended in 345µl T.E. buffer and mixed thoroughly. 40µl Lysozyme (10mg/ml) was added and mixed gently by inverting tubes and incubated for 1 hour at 37 ºC. After the incubation, 100µl 1% SDS, 10 µl 0.5 M EDTA (pH=8), 5µl proteinase K (100µg/ml) were added and mixed gently by inverting. Tubes were incubated at 50ºC for 2 hours until the solution becomes clear. Equal volumes of phenol: chloroform: isoamyl alcohol was added and mixed gently by inverting followed by centrifugation at 10000 rpm for 10

minutes. To the upper aqueous phase, equal volume of chloroform: isoamyl alcohol was added and mixed gently by inverting followed by centrifugation at 10000 rpm for 10 minutes. Upper aqueous layer was separated carefully in another tube and 2.5 V of absolute ethanol (2.5X) and V/10 volume of sodium acetate (0.3M) was added. Tubes were stored at -20 ºC overnight for better precipitation of isolated gDNA. Next day, the supernatant was discarded after centrifugation at 10000 rpm 10 minutes. Pellet was washed with 70% ethanol and air dried. 200 µl of sterile nuclease-free molecular grade water was added and DNA stored at 4ºC.

3.2.4.2 16s rDNA gene amplification and analysis

16S *rRNA* gene sequences from 35 isolates and one MTCC strain, *Klebsiella* pneumoniae 39 were amplified using the Polymerase Chain Reaction (PCR) technique in Thermocycler (Bio-Rad Laboratories, CA, USA). 530F Forward and 1492R reverse primers, universal 16S *rRNA* primers were used to amplify the 16S *rRNA* region. (Lillo et al., 2006). The sequence of the primers used are as follows:

530F- 5'GTGCCAGCMGCCGCGG-3'

1492R- 5'TACGGYTACCTTGTTACGACTT-3'

Each reaction system (25µl) contained 12.5µl of SapphireAmp® Fast PCR Master Mix (TaKaRa Bio Inc., Tokyo, Japan) containing dNTPs, MgCl2, Taq polymerase; 3 µl of each primer (10 pmol), 2 µl of properly diluted (80-90 ng/µl) template DNA. The amplification was performed as follows: initial denaturation for 7 min at 95 °C, denaturation for 30 second at 94 °C, annealing for 1 min at 55 °C and primer extension for 1 min at 72 °C, and final extension for 7 min at 72 °C. The presence of 1 kb amplicons was confirmed by electrophoresis of the PCR product along with 100 bp DNA ladder (TaKaRa Bio Inc., Tokyo, Japan) marker on 1% agarose gel containing 0.5 µg/ml of EtBr in 1x TBE buffer, visualized under UV light using Gel-Doc (Bio-Rad Laboratories, CA, USA). The amplicons were sent to Europhins Scientific, Chennai, India for Sanger's sequencing. After sequencing, FASTA files containing the amplified gene sequence were blast against EZ-Taxon Bio-cloud database and National Centre

for Biotechnology Information database (NCBI). Isolates with ≥ 95 % Identity were identified as *Klebsiella* spp.

3.3 Results

3.3.1 Isolation and identification

Round shaped, Pink coloured, mucoid colonies with entire margin were observed on MacConkey agar with neutral red indicator after overnight incubation. Majority of the isolates showed morphological characteristics similar to *Klebsiella* spp. Morphological characteristics of the isolated colonies of the clinical isolates are shown in the table 3.1 However, variation in lactose-fermenting ability and mucoidity were observed among the isolates (figure 3.1, 3.2). Plates stored in cold room at 4 ℃ for more than 4 to 6 days showed higher mucoidity than the freshly sub-cultured plate of the same isolate.

Isolates cultured on MacConkey agar containing Bromocresol purple indicator is shown in figure 3.2. Variation in lactose fermenting ability was observed among the isolates (figure 3.2A). Interestingly, two types of colonies (yellow and blueish in colour) were observed to be arose from a single yellow coloured colony of a pure culture, which indicates difference in lactose fermenting ability among the colonies of the same culture. Majority of the isolates showed purple-magenta coloured round shaped colonies on *Klebsiella* specific agar medium (figure 3.3). Isolates M4, M8, M11, M24, M32, M45 and V1 did not grow on this selective agar media.

Table-3.1 Morphological characteristics of collected clinical isolates.

Characteristics	**Observation**
Shape	Round
Size	Varies
Margin	Entire
Opacity	Translucent
Elevation	Convex
Consistency	Mucoid
Appearance	Glossy
Pigmentation	No pigmentation

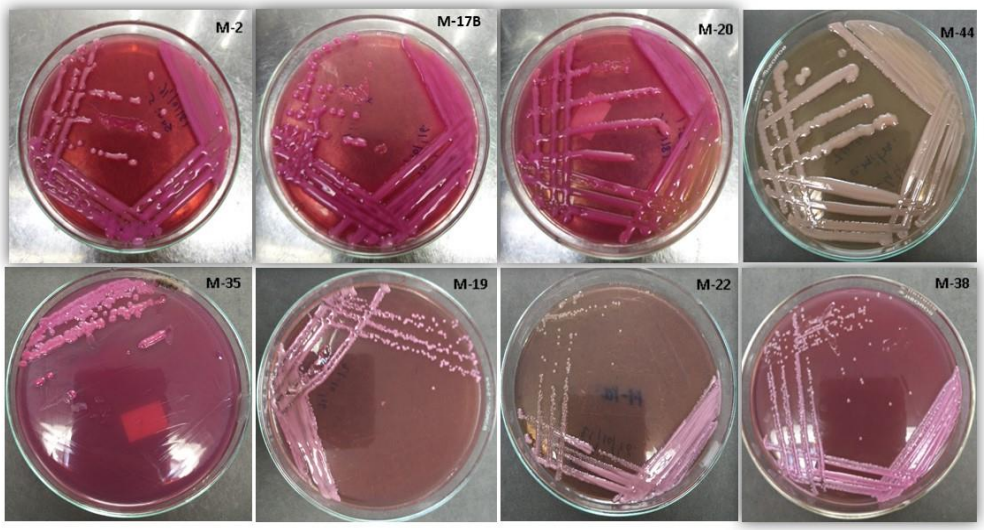

Figure 3.1 Isolates cultured on MacConkey agar (neutral red). Representative image of collected isolates cultured on MacConkey agar containing neutral red. Isolates showed round shaped, pink colored, mucoid colonies. Variation in size and mucoid phenotype was observed among the isolates. Isolates M2 M17B, M20, M44 exhibited more mucoid phenotype compared to less mucoid isolates, M19, M22 and M38. M35 showed moderate mucoid pheonotype compared to the rest of the isolates.

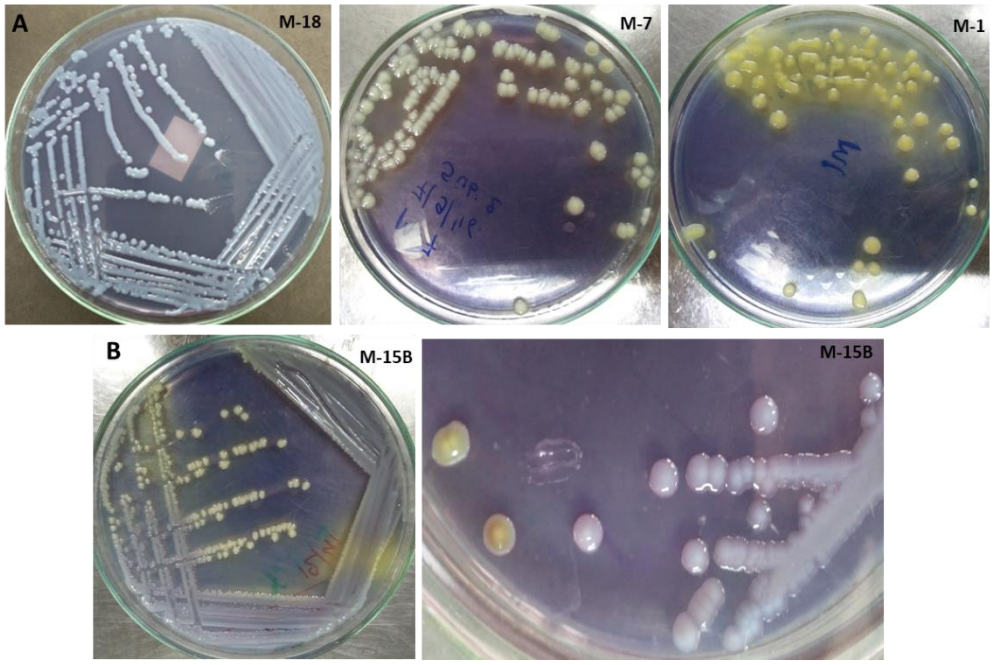

Figure 3.2 Isolates cultured on MacConkey agar (bromocresol purple). Representative images of the isolates cultured on MacConkey agar plate containing bromocresol purple. Variation in size and lactose-fermenting ability among the isolates

was seen. (A) Isolates grown as round, mucoid colonies of Blue-white or yellow in color (M18, M7 and M1). Lactose non-fermenting colonies appeared in Blue-white colour, lactose-fermenting colonies appeared in yellow colour. (B) Isolate M15 showed development of two types of colonies, Blue-White and Yellow in colour from single yellow coloured colony of a pure culture.

Figure 3.3 Isolate cultured on selective medium. Isolates belonged to *Klebsiella* spp. grown as pink coloured round shaped isolated colonies on the selective medium. HiCrome™ *Klebsiella* Selective Agar Base was used as selective medium.

3.3.2 Biochemical tests

(a) Gram's capsule staining

Among all collected isolates, n=53 isolates were observed to be Gram-negative (safranin stained red coloured), short rods during the light microscopy (figure 3.4A). However, 6 isolates, M4, M8, M11, M32, M45 and V1 were discarded as they found to be Gram positive (purple coloured cocci).

(b) Capsule staining

All isolates were found to be encapsulated except M4, M11 and M32. Majority of the isolates showed rod shaped clear halos of capsule around the bacterial cells (figure 3.4B). However, variation in capsule size was observed among the isolates. M17B and M15B was observed to have bigger and thicker capsules compared to rest of the isolates. (figure 3.4B, C, D, E).

Total n=6 isolates were discarded based on Gram and capsule staining. Rest of the isolates (n=49) were processed further for biochemical tests.

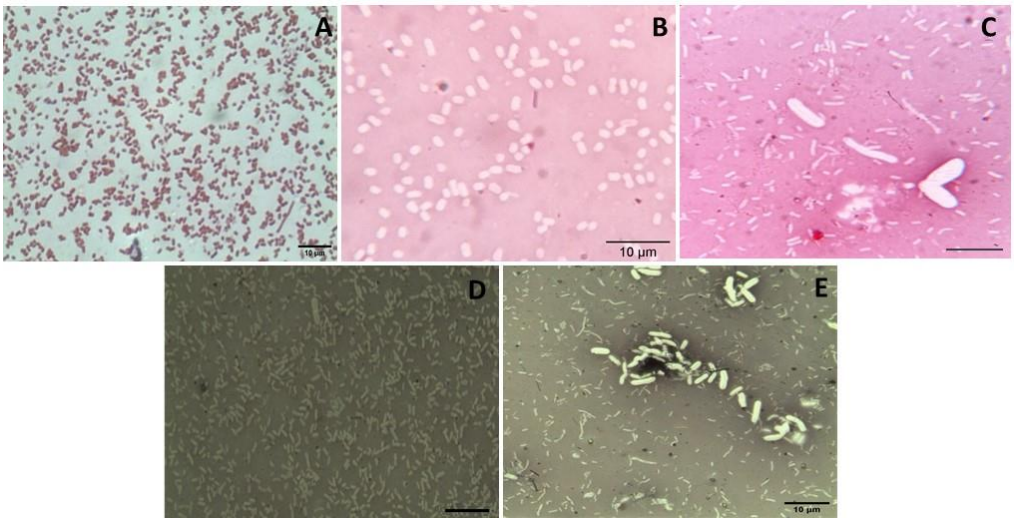

Figure 3.4 Gram and capsule staining. Representative images of Gram-staining and capsule staining are shown. (A)Light microscopy of Gram staining (M2). Safranin stained red coloured short-rods were observed (Gram-negative isolate). (B) Light microscopy of capsule stained using Maneval's negative staining technique. Unstained short-rods shaped capsules were observed against the nigrosine-stained background in isolate M27. (C) Capsules thicker than M27 were observed in isolate M17B along with the regular sized short-rods shaped capsules. (D) Phase-contrast microscopy images of capsule stained using Maneval's negative staining technique. Short rod-shaped capsules were observed against the contrast background (M20) (E) Phase-contrast microscopy images of isolate M17B with thicker capsules. A cluster of large-sized thicker capsules were observed along with the regular sized capsules against the contrast background. Light and phase-contrast microscopy images captured at 1000X and 40X magnification, respectively. Images were analysed using ImageJ software. The scale bars in the images (right bottom) measure 10 µm.

(c) Citrate test

4 isolates (M14, M16, M38 and ST2) were found to be citrate negative as they showed no change in the colour. Rest of the 44 isolates showed colour change from green to blue and were identified as citrate positive isolates (figure 3.5A, A2).

(d) Urease test

All of the isolates showed colour change from yellow to pink (fuchsia) and identified to be urease positive. M42 and M40 were found to be urease negative (yellow in colour) as they did not show any colour change (figure 3.5B).

(e) Oxidase test

Out of all, 4 isolates (M14, 21, 30 and V2B) showed colour change to blue within 10 seconds and were found to be oxidase positive. Rest of all isolates were observed to be oxidase negative as they showed no change in colour of the disc.

(f) Motility test

15 isolates M5, M9, M12 M14, M16, M21, M30, M38, M47, M48, M49, M50, V2B, ST2 and ST3 were observed to be motile during the motility test. Rest of all isolates were observed to be non-motile. Brownian motion of bacteria due to convection was not considered as bacterial motility.

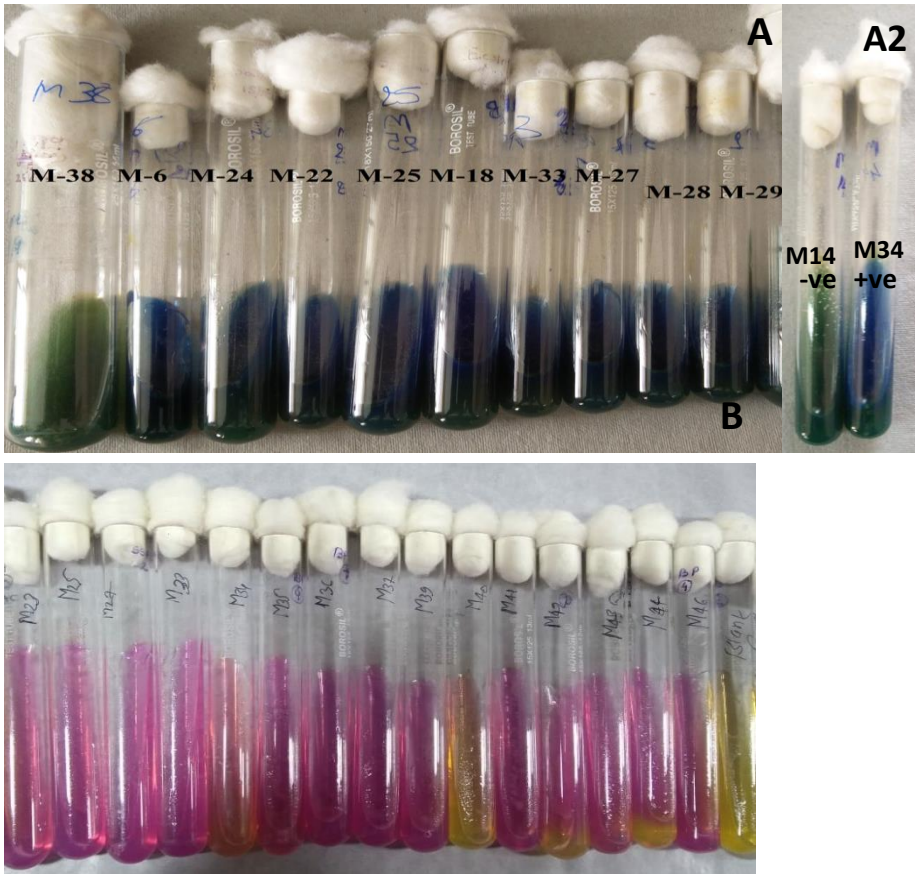

Figure 3.5 Citrate utilization and urease production test. Representative image of citrate utilization test. (A) Majority of the isolates were found to be citrate positive except M38. (A2) Representative image of citrate positive and citrate negative isolates. M14 showed no colour change to green to blue, citrate negative and M34 was citrate

positive isolate, the colour change was observed. (B) Representative image of Urease production test. Majority of the isolates were urease positive, showed colour change from yellow to pink (fuchsia). M42 and M40 were found to be urease negative (yellow in colour) as they did not show any color change.

Summary of the results of biochemical tests is shown in table 3.2.

Table 3.2 Results of biochemical tests of all isolates.

No	Isolate code	Gram stain	Capsule stain	Citrate test	UREASE TEST	Oxidase test	Motility test
1	M1	--	+	+	+	--	--
2	M2	--	+	+	+	--	--
3	M3	--	+	+	+	--	--
4	M4	+	--	NP	NP	NP	NP
5	M5	--	+	--	+	--	+
6	M6	--	+	+	+	--	--
7	M7	--	+	+	+	--	--
8	M8	+	NP	NP	+	NP	NP
9	M9	--	+	+	+	--	+
10	M10	--	+	+	+	--	--
11	M11	+	--	NP	+	NP	NP
12	M12	--	+	+	+	--	+
13	M13	--	+	+	+	--	--
14	M14	--	+	--	+	+	+
15	M15B	--	+	+	+	--	--
16	M16	--	+	--	+	--	+
17	M17B	--	+	+	+	--	--
18	M18	--	+	+	+	--	--

19	M19	--	+	+	+	--	--
20	M20	--	+	+	+	--	--
21	M21	--	+	+	+	+	+
22	M22	--	+	+	+	--	--
23	M23	--	+	+	+	--	--
24	M24	--	+	+	+	--	--
25	M25	--	+	+	+	--	--
26	M26	--	+	+	+	--	--
27	M27	--	+	+	+	--	--
28	M28	--	+	+	+	--	--
29	M29	--	+	+	+	--	--
30	M30	--	+	+	+	+	+
31	M31	--	+	+	+	--	--
32	M32	+	--	NP	+	NP	NP
33	M33	--	+	+	+	--	--
34	M34	--	+	+	+	--	--
35	M35	--	+	+	+	--	--
36	M36	--	+	+	+	--	--
37	M37	--	+	+	+	--	--
38	M38	--	+	--	+	--	+
39	M39	--	+	+	+	--	--
40	M40	--	+	+	--	--	--
41	M41	--	+	+	+	--	--
42	M42	--	+	+	--	--	--

43	M43	--	+	+	+	--	--
44	M44	--	+	+	+	--	--
45	M45	+	NP	NP	+	NP	NP
46	M46	--	+	+	+	--	--
47	M47	--	+	+	+	--	+
48	M48	--	+	+	+	--	+
49	M49	--	+	+	+	--	+
50	M50	--	+	+	+	--	+
51	V1	+	NP	NP	+	NP	NP
52	V2B	--	+	+	+	+	+
53	ST1	--	+	+	+	--	--
54	ST2	--	+	--	+	--	+
55	ST3	--	+	+	+	--	+

NP: Respective test was not performed. Test results in red colour are the biochemical characteristics do not belong to *Klebsiella* spp.

6 isolates were discarded based on preliminary screening by Gram staining and Capsule staining. 15 isolates were discarded based on biochemical tests (Red coloured test results table 3.2). Rest of the 34 isolates were proceed for further molecular analysis.

3.3.3 16s rRNA gene amplification and analysis

3.3.3.1 Genomic DNA extraction

The band of genomic DNA was obtained after the gel electrophoresis (figure 3.6).

3.3.3.2 PCR amplification and Sanger's sequencing of 16s rRNA

1kb sized amplicons were obtained from PCR of *16s rRNA* gene (figure 3.7). Table 3.3 shows NCBI BLAST results of the Sanger sequenced amplicons of *16s rDNA.*

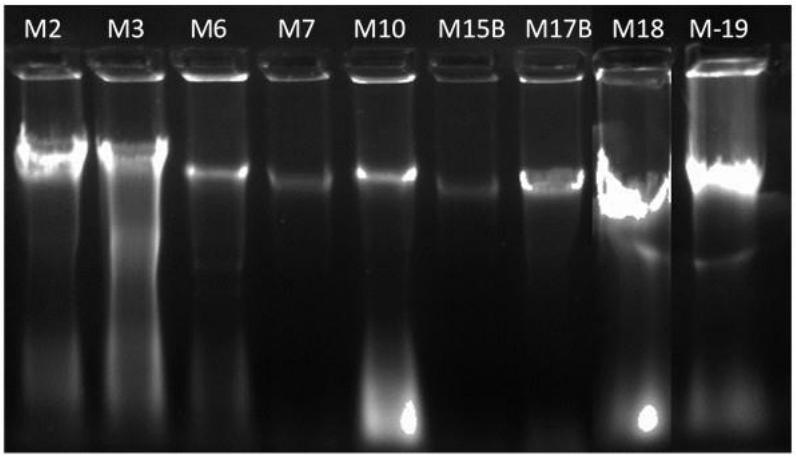

Figure 3.6 Agarose gel analysis of gDNA. 0.8% agarose gel stained with ethidium bromide was used for analysis of genomic DNA isolated from collected clinical isolates. DNA samples used to load the gel are indicated on top of each lane. The lanes from left are as follows: Lane 1: M2; lane-2:M3; lane3:M6; lane-4:M7; lane-5:M10; lane-6:M15B; lane-7:M17B; lane-8:M18; lane-9:M19.

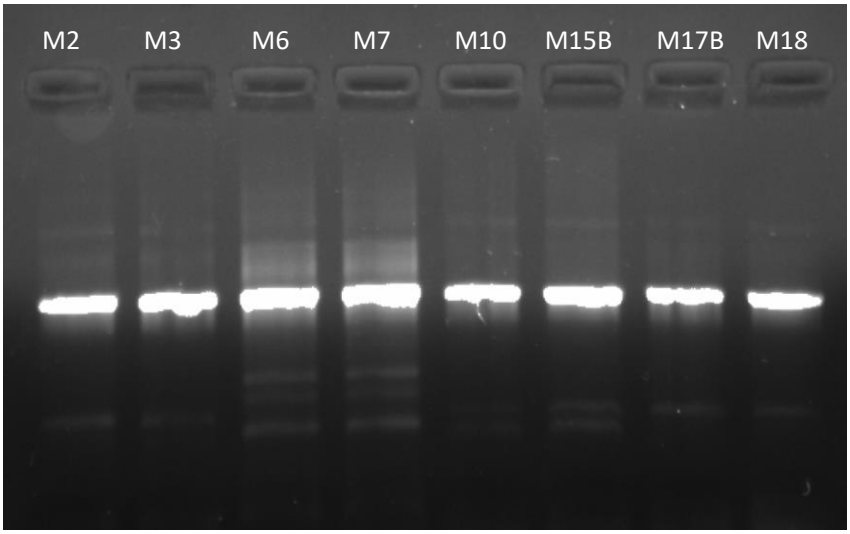

Figure 3.7 Molecular identification using 16s*rRNA* gene amplification. Representative image of 1% agarose gel stained with ethidium bromide showing PCR

amplified 16 rRNA bands. The source of the templet gDNA used for the amplification is indicated on top of each lane. The lanes from left are as follows: lane-1:M2; lane-2: M3, lane-3: M6; lane-4: M7, lane-5: M10 (lane-5), lane-6: M15B; M17B (lane-7). The amplicon length is approximately 1000 bp. Universal primers for 16s rRNA amplification used were 530F and 1492R.

Table 3.3 describes the NCBI BLAST results of the Sanger sequenced amplicons of *16s rRNA*. E-value represents the error value, query coverage dictates the percentage of the query matched with the hit, and % identity shows percentage of identical to the hit, identified genus/species is the genus/species of the top hit matched to the query sequence on local alignment using NCBI BLAST.

Table 3.3 NCBI BLAST results of the Sanger sequenced amplicons of 16s rRNA.

No.	**Sample Code**	**Query coverage**	**E-value**	**% Identity**	**Identified Genus/species**
1	M1	90%	0	99%	*Klebsiella* spp.
2	M2	100%	0	100%	*Klebsiella pneumoniae*
3	M3	99%	0	99%	*Klebsiella pneumoniae*
4	M6	100%	0	99%	*Klebsiella varicola*
5	M7	95%	0	99%	*Klebsiella* pneumoniae
6	M10	99%	0	99%	*Klebsiella* spp.
7	M13	94%	0	98%	*Enterobacter cloacae*
8	M15B	90%	0	99%	*Klebsiella pneumoniae*
9	M17B	99%	0	100%	*Klebsiella pneumoniae*
10	M18	90%	0	99%	*Klebsiella quasipnuemoniae*
11	M19	93%	0	94%	*Klebsiella* spp.

12	M20	100%	0	99%	*Klebsiella pneumoniae*
13	M22	100%	0	99%	*Klebsiella pneumoniae*
14	M23	96%	0	99%	*Klebsiella pneumoniae*
15	M24	85%	0	93%	*Pseudomonas stutzeri*
16	M25	95%	0	98%	Uncultured *Klebsiella* spp.
17	M26	96%	0	99%	*Enterobacter aerogens*
18	M27	94%	0	95%	*Klebsiella pneumoniae*
19	M28	91%	0	87%	*Enterobacter* spp.
20	M29	91%	0	99%	*Enterobacter* spp.
21	M31	99%	0	97%	Uncultured *Enterobacter spp.*
22	M33	91%	0	99%	Uncultured *Klebsiella* spp.
23	M34	98%	0	99%	*Klebsiella pneumoniae*
24	M35	99%	0	99%	*Klebsiella pneumoniae*
25	M36	100%	0	97%	*Klebsiella pneumoniae*
26	M37	98%	0	99%	*Klebsiella pneumoniae*
27	M39	87%	0	96%	*Klebsiella pneumoniae*
28	M40	94%	0	97%	Uncultured *Klebsiella* spp.
29	M41	91%	0	98%	*Klebsiella pneumoniae*
30	M42	99%	0	99%	*Klebsiella spp.*
31	M43	92%	0	98%	*Klebsiella pneumoniae*
32	M44	93%	0	99%	*Klebsiella pneumoniae ozanae*

33	M46	99%	0	99%	*Klebsiella pneumoniae*
34	ST1	88%	0	99%	*Klebsiella pneumoniae*
35	DJ	100%	0	100%	*Klebsiella pneumoniae*

Based on *16s rDNA* identification, 6 isolates (M13, M24, M26, M28, M29, M31) were discarded based molecular identification as they were not belonging to the genus *Klebsiella*. Rest 28 isolates and 1 MTCC *Klebsiella pneumoniae 39* strain, n=29 isolates were proceeded for further study.

3.4 Discussion

Prevalence of UTIs in nosocomial and community settings is 10–30% and 25–50%, respectively, which are an important factor of morbidity and a serious public health issue. *Klebsiella* is reported to be a major etiological agent of 3-20% of UTIs, the second highest after *E. coli* (Gajdács et al., 2019; Jean et al., 2016). Hence, diagnosis/identification of pathogenic isolates of *Klebsiella* spp. is crucial for the timely treatment of the infections.

Despite of belonging to the same genus, the collected clinical isolates of *Klebsiella* demonstrated variations in their phenotypic characteristics. Exhibition of two types of colonies by a single isolate (yellow and blue-white) (figure 2B) could be the results of the mutative fermentation. Mutative fermentation is a phenomenon where the pure culture in which fermentation (lactose to lactic acid) takes place has two types of forms: lactose-fermenting and non-fermenting. The fermenting ones develop yellow colonies because the acid production changes the colour of indicator (bromocresol purple) present in the media and the non-fermenting forms appear in blue color because of no acid production, which results in no change of color. The observer could misunderstand it as a mixed culture. But actually, they are colonies of a pure culture exhibiting mutative fermentation phenomenon (Kristensen, 1948). It is reported by Henriksen and Kristensen in *K. ozanae* and Salmonella, respectively (Henriksen, 1950; Kristensen, 1948). Here, we observed the same phenomenon

in the clinical isolates of *Klebsiella* spp. Mucoid phenotype of the isolates seem to be associated with the temperature as the mucoidity of the isolates was observed to be increased after 4 to 6 days of cold storage at 4 ºC. Difference in mucoid phenotype with the temperature incubation was reported by Tipton et al in *Acinetobacter baumannii*; they reported that heterogenous colony appearance could be due to changes in capsule production within a clonal bacterial population. The appearance of colony variants *in vitro* also depends on the medium, time and temperature incubation (Tipton et al., 2015). Our observations are in agreement with them, but in contrary to the reports that described low prevalence of mucoidity in *Kp* clinical isolates (Cavalcanti et al., 2019; Vernet et al., 1995) as majority of the collected clinical isolates showed mucoid phenotype.

Citrate, oxidase, urease and motility tests were performed to eliminate species other than *Klebsiella* grown on MacConkey agar. *Klebsiella* is oxidase negative, citrate positive and non-motile. Citrate and oxidase was performed to eliminate *E. coli* (citrate positive), *Pseudomonas aeruginosa* (oxidase positive), and *Enterobacter* spp. (Motility positive), respectively. After elimination based on citrate and oxidase tests, urease test was performed to observe the diversity of *Klebsiella* spp. in the collected isolates as *K. pneumoniae* (*Kp*) and *K. oxitoca* are urease positive but two subspecies of *Kp*, *K. rhinoscleromatis* and *K. ozaenae* are urease negative (Bascomb et al., 1970; Farmer et al., 1985).

Identification of *Klebsiella* spp. is tricky as few species of genus *Klebsiella* and *Enterobacter* share similar colony morphology and biochemical parameters except ornithine decarboxylase and motility test. *Klebsiella* is non-motile, and more than 98% of the strains are ornithine decarboxylase-negative, whereas, more than 95% of *Enterobacter* strains are ornithine decarboxylase-positive and motile. But organisms which are ornithine decarboxylase-positive and non-motile create a problem in classification (Edward and Ewing, 1962; Ewing and Jhonson, 1960; Fife et al., 1965; Matsen and Blazevic,1969). Moreover, *Enterobacter aerogens* which was renamed as *Klebsiella aerogens* (Bascomb et al., 1970) can be motile (possess peritrichous flagella) or non-motile **(**Davin-Regli, 2015; Brenner et al., 1972). *K. aerogens* and *Enterobacter cloacae* complex are very closely related, and it is not clear how their infections, clinical

characteristics and virulence factors differ from each other; because of this difficulty, clinicians also identify them as '*Klebsiella-Entero* group' (Wesevich et al., 2019). Osman et al., tested the accuracy of identification of *Kp* using conventional phenotypic and biochemical methods as well as by amplification of 16S-23S *rDNA* and sequencing of *rpoB*, *gapA* and *pgi*; they demonstrated that identification using phenotypic and biochemical methods only is not enough for accurate identification of *Kp* and use of molecular methods such as 16s-23s *rDNA* sequencing is crucial. This study further suggested that hospitals of Sudan were inaccurately identifying *Kp* and led to overestimation of the prevalence of this organism (Osman et al., 2020). He et al., reported that 16S rRNA and *khe* gene could discriminate the clinical isolates at the genus level, whereas, *rpoB* sequencing could discriminate *Klebsiella* at the species and even subspecies level (He et al., 2016). Caputo et al., demonstrated that genome and pan-genome analysis coupled with a method of rapid identification by MALDI-TOF, or 16S rRNA is also very important for classification of bacterial strains. With the help of pan-genomic analyses, bacterial taxonomy is undergoing many changes (Caputo et al., 2019). Furthermore, recent advances in molecular capabilities have also shown that a portion of clinical isolates identified as *Kp* are in fact other *Klebsiella* species (Brisse and Verhoef, 2001; Brisse et al., 2004; Maatallah et al., 2014; Berry et al., 2015; Long et al., 2017a) and MLST based identification is more reliable for identifying phylogroup of *Kp* (Diancourt et al., 2005).We also recommend molecular identification such as 16s *rRNA/ rDNA or rpoB* sequencing for identification of the genus *Klebsiella.* However, for accurate identification of Klebsiella species and subspecies, MALD-TOF, MLST, cg-MLST or other whole genome sequencing based approaches are more appropriate and accurate.

Limitation of the study presented is that identification of isolates done was only up to genus level using Sanger's sequencing. Whole Genome Sequencing (WGS) based Multi-locus-sequence typing (MLST) was not performed for all isolates for species level identification because of the limitation of funds.