Summary

Objective 1: Collection, isolation and identification of clinical isolates of pathogenic *Klebsiella* spp.

- Clinical isolates (n=56) of Gram-negative bacteria from patients suffering from UTIs were collected from Surat and Vadodara, Gujarat.
- The collected isolates were cultured on MacConkey agar containing neutral red indicator and MacConkey agar containing Bromocresol purple indicator. Round shaped, Pink coloured, mucoid (glossy) colonies with entire margin were observed on MacConkey agar with neutral red. In case of, MacConkey agar with bromocresol purple two types of colonies (yellow colored pearl-like 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. Further, to identify *Klebsiella* spp. were also grown on *Klebsiella* specific agar medium. Majority of the isolates showed purple-magenta coloured round shaped colonies on *Klebsiella* specific agar medium; n=7 isolates did not grow on this selective agar media.
- Gram staining and capsule staining were performed for the preliminary identification of collected isolates. 6 isolates were found to be Gram-positive. Rest of the isolates were identified as Gram-negative and were proceeded for capsule staining. All isolates were found to be encapsulated except n=3 isolates. Majority of the isolates showed a rod shaped clear halo of capsule around the bacterial cells. But, M-17B and M-15B was observed to have bigger and thicker capsules. Hence, variation in capsule size was observed among the isolates.

  Total n=6 isolates were discarded based on Gram and capsule staining. Rest of the isolates (n=49) were processed further for biochemical tests.
- Citrate test, urease, oxidase and motility tests were performed to evaluate the biochemical parameters of the isolates. 4 isolates were found to be citrate negative; all isolates were urease positive except n=2 isolates. 4 isolates were found to be oxidase positive and 15 were observed to be motile. Bacteria belong to genus *Klebsiella* are citrate positive, oxidase negative and non-

motile. Hence, total 15 isolates were discarded based on biochemical tests. Rest of the 34 isolates were proceed for further molecular analysis.

- Genomic DNA was isolates from all isolates and 16 *rRNA* gene was amplified using PCR. Amplicons of 16s *rRNA* gene were then sent for Sanger's sequencing. N=6 isolates were not belong to the genus *Klebsiella* and were discarded. Rest 28 isolates (identified as *Klebsiella* spp.) and 1 MTCC *Klebsiella pneumoniae 39* strain, n=29 isolates were proceeded for further study.

Objective 2: Study of antibiotic resistance

- Disk diffusion method was performed to evaluate antimicrobial susceptibility of the isolate using commercially available discs of various antimicrobial agents belong to different antimicrobial categories. All (n=29) isolates were resistant against ampicillin, oxacillin and cephalexin. In case of amikacin, n=10 isolates were found to be resistant and 1 was intermediately resistant. n=5, 13 and 12 isolates were resistant to chloramphenicol, ciprofloxacin and co-trimoxazole, respectively. Importantly, n=8 and 7 isolates were found to be resistant to imipenem and meropenem, respectively. Colistin and tigecycline were found to be the most effective antimicrobial agents as all isolates were found to be susceptible against them. However, isolate DJ was found to be resistant against colistin and tigecycline.
- Percentage resistance was calculated against each antimicrobial category. 96% and 89% resistance rate were observed against the penicillins and penicillin + Beta-lactamase inhibitors. Resistance to extended-cephalosporines was the highest after penicillins with resistance rate of 82% followed by folate inhibitors (71%), monobactams (64%), Aminoglycosides (53%), Fluoroquinolones (46%)and Phenicols (42%). Importantly, 39% resistance was observed against carbapenems. Low rate of resistance, only 3% (n=1, DJ) resistance was observed against glycylcyclines (tigecycline) and polymyxin-B (colistin).

- MIC determination was performed for highly resistant isolates, DJ, M2, M6 and M17B. Only DJ was found to be resistant against colistin (MIC = 4 µg/ml) and tigecycline (MIC= 16 µg/ml); M17B was intermediate against tigecycline (2 µg/ml). Rest of the isolates were susceptible to both the drugs.
- Based on above antimicrobial susceptibility testing, isolates were classified into four categories: Multidrug-resistant (MDR), Extreme drug-resistant (XDR), Pandrug-resistant (PDR) and susceptible. From the collected isolates, 72.4% (n=21) were categorized as MDR, 20.7% (n=6) as XDR, 3.4% (n=1; DJ) as PDR and 3.4% (n=1; M25) as susceptible.
- Phenotypic assays were performed to investigate the presence of Extended spectrum beta-lactamases (ESBL) and metallo-β-Lactamase (MBL) enzymes. Among all, n=12 (41.4%) isolates and n=6 (20.7%) isolates were found to be ESBL-producers and MBL-producers, respectively. n=2 (6.9%) of the isolates were producing ESBL and MBL both; 31% (n=9) isolates were non-ESBL and non-MBL producers.
- Based on the results of antimicrobial susceptibility testing and phenotypic assays, five isolates, 1 PDR (DJ), 3 XDR (M-2, M-6, M-17B) and 1 susceptible (M-25) were selected for investigation of the genotype responsible for different resistance phenotype of these isolates.
- Multiplex PCR was performed to detect n=11 carbapenemase genes present in the selected isolates (n=5). The table below shows the summary of multiplex PCR results.

Isolates	***bla*AIM set**	***bla*KPC set**	***bla*IMP set**
M2	*bla*GIM, *bla*SIM, *blaA*IM	*bla*OXA-48	*bla*VIM
M6	*bla*GIM	*bla*OXA-48	*bla*VIM
M17B	*bla*GIM	*bla*OXA-48	*bla*VIM, *bla*IMP
DJ	*bla*GIM, *bla*SIM, *blaA*IM	*bla*NDM, *bla*OXA-48	*bla*VIM, *bla*IMP
M25	-	-	-

- qRT-PCR was performed to study the expression of *bla*$_{NDM}$, *bla*$_{OXA-48-like}$ (carbapenemases) and *bla*SHV (ESBLs) genes in selected XDR and PDR isolates. Statistically significant difference in the relative expression of *bla*NDM and *bla*OXA-48-like (carbapenemase genes) was observed between PDR/XDR (DJ, M-2, M-6) isolates and susceptible isolate (M-25) (relative p value is shown in figure 4.11). In case of relative expression of, *bla*SHV (ESBL), no significant difference was observed between the isolates, M25 showed no expression of *bla*SHV.
- To investigate the genomic features of PDR, XDR, MDR and susceptible isolates whole genome sequencing (WGS) was performed for 8 selected isolates belonging to each resistance category; namely, M2, M6, M17B (XDR), DJ (PDR), M27, M20, M3 (MDR) and M25 (susceptible).
- MLST was performed to identify the sequence type (ST) of the isolates. All the genomes found to be *K. pneumoniae sensu stricto* (*i.e.,* phylogroup *Kp*1). DJ (PDR) belonged to ST147; M2 and M6 (XDR) belonged to ST231 and M17B (XDR) belonged to ST14. M25 (susceptible) belonged to ST1087. M3, M20 and M27 (MDR) belonged to ST16, ST2943 and ST1715, respectively. K-locus (KL) type of DJ, M2, M6 and M17B was KL-64, KL-51, KL-51 and KL-2, respectively. M25, M20, M3 and M27 possessed KL-132, KL-39, KL-15 and KL-2, respectively.
- The branch length of the phylogenetic tree suggested that M6 (0.385 substitution/site) and M2 (0.384 substitution/site) were the most recently diverged genomes among all 8 genomes.
- Significant difference in number of resistance genes and plasmids were observed between XDR and Susceptible isolates M25 (susceptible) was found to carry only one ESBL producing gene, SHV-42 and no plasmid was found. XDR isolates (M2, M6, M17B) were found to harbour 12 to 15 resistance genes along with presence of n=4 to 9 various plasmids. The PDR isolate (DJ) carried n=8 resistance genes and n=4 plasmids. M20 (susceptible) did not carry genes/mutations except *bla*SHV-42.
- Resistance genes to various classes such as tetracyclines (*tetA, tetB*), sulphonamides (*sul1, sul2*), aminoglycosides (*rmt*) quinolones and fluoroquinolones (FQs), macrolide resistance (*arr2, mphA*) etc. were detected

in the genome of the isolates. Resistance to quinolone and FQs was observed in all XDR and PDR isolates because of mutation in *gyrA* and/or *parC* as well as presence of PMQR genes (*qnrA, qnrB*) in some isolates. In case of MDR isolates, only M3 carried mutation in *parC*-84K, *gyrA*-83F and *gyrA*-87N.

- Isolates were found to carry different combinations of plasmids as well as ybt;ICE*Kp* variants. Plasmid content was observed to be associated with different ybt;ICE*Kp* variants.
- Presence of virulence genes was also detected. All the isolates except M3 carried yersiniabactin synthesis genes and were associated with respective ybt,ICE*Kp* variants. None of the isolates were found to carry aerobactin genes (iucABC), colibactin (clb cluster) and salmochelin (iroBCDN) siderophore producing genes. Genes for ferric uptake system (kfuABC) was found to be present in M2, M6, M17B and M27. *rmpA* and/or *rmpA2* were also not found in any isolate. *mrk*ABCD cluster responsible for Type-3 fimbriae expression was present in all 8 isolates.
- Interestingly, in this isolate, *bla*NDM-5 carbapenemase-producing gene was found to be present on the chromosome instead of the plasmid based on Illumina NGS of DJ. The location of *bla*NDM-5 in DJ was further confirmed using long read oxford nanopore NGS platform.
- Oxford nanopore sequencing was performed for PDR isolate DJ. Two copies of each blaNDM-5 and *bla*OXA-181 as well as three copies of *bla*CTX-M_15 were found to be present on chromosome and plasmid. Importantly one copy of blaNDM-5 was found to be located on the chromosome due to insertion of ISEcp1/IS26. Two the copies of *bla*OXA-181 were present adjacent to ISEcp1 on chromosome. In case of *bla*CTX-M-15, one copy was present on IncR plasmid and two chromosomal copies, ISEcp1-*bla*CTX-M-15 were inserted into OmpK35 and MFS-type transporter genes, individually.
- In the genome of DJ 1057 bp insertion of IS5 transposase was found in *mgrB*, which was responsible for colistin resistance in DJ. Further, a premature stop codon caused by a A580T substitution was found in *ramR*, (a negative regulator of RamA), leading to an overexpression of the AcrAB-TolC efflux system was responsible for resistance against Tigecycline.

train DJ also carried *rmtF, rmtB, strA, strB* and *aadA2* genes, responsible for resistance to aminoglycosides, including amikacin. Mutations in quinolone resistance determining regions (QRDRs) of both *gyrA-83I* and *parC-80I* were found.

- Virulence genes were also detected in strain DJ. yersiniabactin gene cluster (*ybt*10), located on an ICE*Kp*4 mobile genetic element was detected. *mrk* and *fim* gene clusters responsible for type-1 and type-III fimbriae, respectively were also found. However, no gene associated with hypervirulence (*rmpA*, aerobactin, salmochelin) was carried by DJ.
- Global analysis of CG147 genomes deposited from worldwide was performed. Majority of the isolates were from human samples and hospital environment. KL64 and KL10 were found to be dominant KL-type in ST147 genomes.
  Regarding antibiotic resistance, 83% of CG147 isolates carried a *bla*CTX-M. 63% of CG147 genomes carried at least one carbapenemase gene; only 37% did not harbour any known carbapenemase gene. Importantly, all CG147 genomes presented QRDR alterations in *gyrA* (83I or 83Y and 87A) and *parC*-80I.
- Prevalence of resistance genes was found to be associated with geographical locations. *bla*NDM was predominant in Southeast Asia and North America, whereas the combination of *bla*NDM and *bla*OXA-48-like was more frequently observed in South and East Asia.
- Few associations between resistance genes and CG147 sublineage ST147-KL10 were observed. ST147-KL10 (*wzi*420, O3a) was almost exclusively found in Asia (85%), mainly in Southeast and South Asia as well as Eurasia; it was associated with *bla*CTX-M, *bla*OXA-48 or *bla*OXA-232, and more occasionally with *bla*NDM-1/-9/-4 and *blaKP*C-2.
- Two types of mutations in OmpK35 were observed in ST147-KL64 genomes with *ybt*10;ICE*Kp*4 (subclade-1) and ybt16/ICE*Kp*12 (subclade-2). (1) n=18 (including strain DJ) ST147-KL64 genomes with *ybt*10;ICE*Kp*4 showed disruption of OmpK35 by IS*Ecp1-bla*CTX-M. **(**2) n=29 ST147-KL64 genomes with ybt16;ICE*Kp*12 exhibited insertion of 2 nucleotide (nt) resulting in a premature stop codon in OmpK35.

- ybt;ICE*Kp* was rarely detected amongst ST392 and ST273 genomes, this virulence factor was observed in 53% ST147 genomes. Despite of a high diversity of ICE observed among ST147 isolates, ybt16;ICE*Kp*12 and ybt10;ICE*Kp*4 were two predominant variants found in ST147 genomes.
- Some networks of acquired resistance genes and plasmids were detected within CG147. For example, Col(BS512), Col*Kp*3, IncFIA (HI1), *cml*, *ereA/B* and *gyrA* 83Y/87A co-occurred frequently and were commonly found within the ST147-KL10 lineage. A negative association between IncR and $IncFII_K$/$IncFIB_K$ was detected, resulting in differences in the content of genes conferring tolerance to copper and silver.
- Regarding the convergence between MDR and hypervirulent genotypes (presence of *rmpA* and/or *rmpA2* and aerobactin), it was observed in two genomes, B-8658 (ST147-KL10, 2014) and *Kp*vST147L (ST147-KL14, 2016), from Russia and United Kingdom, respectively.
- To understand the genomic features of *Kp* ST231 lineage of and compare our isolates M2 and M6 with the ST231 genomes worldwide comparative genomic analysis using n=95 publicly available genomes of ST231 lineage, collected between 2010 to 2018 was performed.

  Regarding carbapenemase resistance in ST231 genomes, *bla*OXA-48-like found to be highly prevalent in ST231 as 77.3% (75/97) genomes were found to harbour *bla*OXA-48-like, mainly *bla*OXA-232. 79.4% (77/97) of the genomes carried at least one of the known carbapenemase genes. Only 18.5% (18/97) of genomes did not carry any known carbapenemase genes.
- Carriage of *bla*NDM by ST231 genomes is lesser compared to ST147 population. It was found to be present in only in 3.1% (3/97) of the ST231 genomes. *blaKP*C was not much prevalent in ST231 genomes and was found only in one isolate from Portugal. *bla*VIM, *bla*IMP were not found in any isolates.
- Geographical distribution of carbapenemase and ESBL genes was also studied. *bla*OXA-48-like genes were observed to be more prevalent in South and Southeast Asia (India, Pakistan & Thailand), whereas *bla*CTX-M disseminated globally with its presence in all 5 continents such as Asia, South America, North America, Africa, Australia and Europe. India was observed to be a

reservoir for MDR genomes belonging to ST231. Highest number of genomes (n=31) carrying *bla*OXA-48-like and *bla*CTX-M-15 (n=26 genomes) were deposited from India; *bla*SHV was observed mainly in Indian genomes (n=12).

- Regarding other acquired resistance genes, average 13 resistance genes were found to be present in ST231 genomes ranging from 6 to 15 resistance genes in some genomes. Interestingly, mutations in *parC*80I and *gyrA*83I were found in all genomes of ST231 sublineage along with the presence of *qnrS1* and *qnrB* in 50.5% (49/97) and 10.3% (10/97) of the genomes, respectively.

- Concerning colistin resistance, only one isolate from Thailand in 2015 was found to carry *mcr* gene; however, it did not carry any carbapenemase genes. *mcr* did not found in ST231 lineage after 2015. Several other genes responsible for resistance to macrolids, tetracycline and sulphonamides were also found in ST231 genomes.
- Virulence genes were also detected. Aerobactin genes(*iuc*ABC) were found to be present in 73.2% (71/97) and yersiniabactin (ybt) genes were present in 92.8% (90/97) genomes. *rmpA* and *rmpA2* were not found in any genome of ST231 lineage.
- Some important networks of plasmids and resistance genes were observed. In 69.1% (67/97) genomes co-occurrence of Col*KP*3 and *bla*OXA-48 like was observed. A negative association between QRDR mutations (*gyrA*83I and *parC*80I) and Col(BS512) plasmid was observed.
- Analysis of the plasmidome also exhibited coexistence of IncFIA, IncFIB (pQil) and InFII(K) in 76.2% (74/97) of the genomes. Coexistence of IncFIA and Col*Kp*3 was observed in 93.8% (91/97) of genomes. A negative association between IncR and IncFII(K)/IncFIB(K) was observed.
- Convergence between antibiotic resistance and hypervirulence was observed in ST231 genomes. 57.7% (56/97) of *bla*OXA-232 and Col*Kp*3 carrying genomes were also harbouring aerobactin genes (iucABC); all belong to ybt14;ICE*Kp*5 variant. These convergent genomes were mainly from India (21.6%, 21/97) and Thailand (24.7%, 24/97).
- Different combinations of plasmids were observed to be present in different ybt;ICE*Kp* variants. ybt14;ICE*Kp*5 was the most prevalent (79.4%; 77/97) in

ST231 lineage. Moreover, all the convergent genomes possessed ybt14;ICE*Kp*5. The genomes carrying Col*KP*3 or MDR plasmid IncFII(PMA1167-NDM-5) also possessed ybt14;ICE*Kp*5 variant; all genomes containing ybt14;ICE*Kp*5 carried a specific set of plasmids namely, IncFIA, IncFIB(pQil), IncFII(K) and Col440I. Further, co-occurrence of FIB(K), FIB(p*KP*HS1), IncFII(p*Kp*91) and ColRNAI was only found in genomes with ybt15;ICE*Kp*11 (n=2, 2/2).

Objective 3: Study of virulence factors involved in pathogenesis of *Klebsiella* spp.

- CPS and EPS were extracted from the collected isolates and quantification of CPS and EPS was done. Wide range of CPS production among isolates was observed ranging from 5.2 µg CPS/mg protein to 116.38 µg CPS/mg protein. M34 was found to produce the highest amount of CPS, 116.38 µg CPS /mg protein; production of CPS was the lowest in M10, 5.2 µg CPS/mg protein.

  A wide range of EPS production was observed among the isolates from no EPS production (0 µg EPS/mg protein)) in M20 to 210.79 µg EPS /mg protein in M27.
- Correlation between CPS and EPS production by isolates was investigated. No correlation between CPS and EPS production by isolates was found. Value of Pearson's correlation co-efficient (r) was found to be 0.20.
- The frequency distribution of CPS and EPS produced by isolates was analysed. The analysis suggested that though the isolates showed a wide range of CPS production and the maximum CPS production found was 116.38 µg CPS /mg protein, majority of the isolates have produced CPS between the range of 10 to 40 µg/mg protein (median 23.052) as the frequency distribution of CPS production showed right skewed histogram**.** In case of EPS, gaussian distribution with a bell-shaped curve was found, which indicates that production of EPS in majority of the isolates was similar with average EPS production of 84 µg CPS /mg protein.

- String test was performed using sheep-blood agar. Only M20, the isolate with low CPS was found to be string positive; rest of the isolates were found to be string negative.
- *RmpA* gene was amplified using PCR and later, the expression of *rmpA* was studied in *rmpA* positive isolates. Only in five isolates M39, M41, M42, M43, MTCC were found to carry *rmpA*.
- M39 showed highest overexpression of *rmpA* (3315 folds) compared to MTCC strain. Whereas, M41, M42 and M43 showed 4.8 folds 4.05 and 1.72 folds overexpression of *rmpA* compared to MTCC strain, respectively.
- Based on CPS, EPS production, *rmpA*, and K-type of the isolates, the following isolates were selected to perform phagocytosis assay to determine the factors responsible for resistance to phagocytosis (shown in the table below).

Isolate	**Amount of CPS**	**Amount of EPS**	**String test**	***rmpA* gene**	**K-type**
M-10	+	++	--	--	K23
M-20	+	--	+	--	K39
M-27	++	+++	--	--	K2
M-34	+++	++	--	--	K2
M-43	++	+	--	+	K10

+++ Highest; ++ high-moderate; + Very low; -- Not present/not

- K-type of the selected isolates was determined using *wzi* sequencing. K-type of M10, M20, M27, M34 and M43 were found to be K23, K39, K2, K2 and K10, respectively. FITC-labelling of the isolates was also performed.
- During a pilot experiment of phagocytosis assay, the tubes were incubated at different time points (0', 10', 15', 30' and 60'). However, maximum phagocytosis was observed at 15 minutes and after 15 minutes (in tubes with 30 and 60 minutes) no further increase or change in amount of phagocytosis was observed.

- Phagocytosis assay was performed for 15 minutes and the total amount of phagocytosis was analysed using flow-cytometry (FACS). Among all the isolates, the least amount of phagocytosis was observed in case of the only string positive isolate, M20 (59.7%) followed by M10 (70.9%) and M27 (74.6%). Highest phagocytosis (87.3%) was observed in M43 (high CPS, string negative) followed by M34 (highest CPS, string negative) (82.5%).
- Visualization of phagocytosis assay was done using fluroscent microscope and confocal laser scanning microscopy (CLSM). During fluorescence microscopy, FITC-labelled bacteria localized inside neutrophils/PMN were observed in green color. Bacteria not localized inside neutrophils/phagocytosed were observed in orange color. CLSM further confirmed the occurrence of phagocytosis event and location of FITC-labelled *Kp* inside neutrophils.). FITC-labelled *Kp* bacterial cells (green) were clearly observed to be phagocytosed and localized inside polymorphonuclear neutrophils (red ,EtBr stained).

- Biofilm formation ability of the isolates was investigated by performing biofilm assay in 96-well microtiter plate, and isolates were categorized into strong, moderate and weak biofilm producers. Among all the collected isolates 43%, 43%, and 14% were strong, moderate, and weak biofilm producers, respectively. From 28 biofilm producer isolates of different categories, three weak, M-20,23, and 25 and three strong (M-10,27, and 34) biofilm producers were selected randomly for further study.
- Comparative analysis of biofilm formation was performed on different types of catheters and media conditions. In case of weak biofilm, significant difference in biofilm formation between latex and silicone coated latex, as well as silicone catheters was observed ($p < 0.0001$) (figure 5.12C). The difference in biofilm formation between silicone coated latex and silicone catheters was also significant with $p < 0.001$ in weak biofilms. In case of strong biofilm, significant difference between latex and silicone ($p < 0.01$); latex and silicone-coated latex ($p < 0.05$) was observed. No significant difference between silicone-coated latex and silicone was observed in case of strong biofilm. the

biofilm formation was lowest on silicone catheters followed by silicone-coated latex and latex catheters.

When biofilm formation was studied on two types of catheters, in the presence of different media, significant increase in biofilm formation was observed in case of natural urine as compared to Luria-Bertani (LB) broth and artificial urine on silicone-coated latex catheter and silicone catheter. In the presence of different media, the biofilm formation was highest in natural urine followed by LB and artificial urine.

- Components of biofilm matrix were quantified in strong and weak biofilms. Significantly higher amount of eDNA ($p < 0.001$), protein ($p < 0.001$), EPS ($p < 0.05$), and dead cells ($p < 0.05$) were observed in strong biofilms than in weak biofilms.
- The measure of live cells in biofilm was obtained using resazurin assay. Average fluorescence units (FU) obtained in weak and strong biofilms were 2658 and 1381 FU, respectively.
- The number of dead cells present in weak and strong biofilm was evaluated using flow- cytometry analysis after 48 h. 23% of dead cells (Propidium iodide (PI) positive cells) were observed in weak biofilm as compared to 65% in strong biofilm. This indicates that more number of dead cells were present in strong biofilm than weak biofilm with $p < 0.01$
- Time bound live dead assay was done at 6, 18, and 24 h to see the live dead ratio in weak and strong biofilms. In case of strong biofilm, cell death (intensity of PI) was observed to be increased at 18 h than at 6 h and maximum intensity of PI was measured at 24 h. Whereas, in case of weak biofilm cell death (intensity of PI) was high at 18 h than at 6 h. However, in 24 h, intensity of PI was significantly lesser (47 ± 5 IU) compared to that of the strong biofilm (194 ± 10 IU). Though the growth rate of all the isolates are similar, cell death was found to be increased with the time only in strong biofilms.
- To further validate the role of different matrix components in biofilm formation, inhibition and addition assays were performed. In case of strong biofilm, significant reduction in biofilm was observed after treating the biofilm with DNase I, RNase A and Proteinase K. In case of weak biofilm also biofilm was reduced after treatment with the respective enzymes. However, exogenous

addition of *Kp* cell extracted DNA and protein to both weak and strong biofilms did not show any significant change in biofilm formation.

- To visualize 3D structure of live and dead cells embedded inside the biofilm matrix and to further evaluate the huge amount of cell death observed in strong biofilms, CLSM was performed. Weak and strong biofilms showed differences in thickness and arrangement of live and dead cells in the biofilm matrix. Weak biofilm was observed to be sparsely packed with more numbers of live cells whereas strong biofilm was densely populated with more numbers of dead cells compared to live cells.

  It was observed that in weak biofilm, the number of live cells increased, and dead cells decreased with the increase in the depth. In strong biofilms, the number of dead cells increased, and live cells decreased with increase in the depth. A significant difference in the thickness of weak (7 ± 2 μm) and strong biofilms (14 ± 1 μm) was observed.

  More number of dead cells were observed in strong biofilms compared to weak, whereas, more number of live cells were observed in weak biofilms compared to strong biofilms. Interestingly, 3D structure of strong biofilm showed pockets of live cells embedded within the thick layers of dead cells.
- To study the differences in adhesion capacity, adhesion assay was performed. Number of cells adhered to the coverslip after 4 h of biofilm formation were very few in weak (125 ± 18) biofilms; conversely, large number of cells were observed to be adhered in strong biofilms (542 ± 20). This indicates that adhesion capacity of strong biofilm producers is higher than the weak biofilm producers.
- FEG-SEM was performed to investigate the differences in the structure of weak and strong biofilms formed on silicone-coated latex catheters. FEG-SEM micrographs of weak biofilms showed very less number of cells embedded in cloud like EPS. It also suggests the presence of micro-channel like structures in the network of exo-polymeric matrix. On other hand, the strong biofilm micrographs showed higher number of interconnected cells embedded in densely populated and abundant extracellular matrix.