Antibiotic resistance and virulence factors of pathogenic *Klebsiella* spp.

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Introduction

Klebsiella* spp.*: Bacteria belonging to this spp. are rod-shaped, Gram-negative, non-motile, encapsulated, lactose fermenting and facultative anaerobes. *Klebsiella pneumoniae (Kp)* ranks 2nd after *E.coli* in Urinary Tract Infection (UTI) and Bacteramia. (Mendelson *et al..*, 2005). Three species in the genus *Klebsiella* are associated with illness in humans: *Klebsiella pneumoniae, Klebsiella oxytoca,* and *Klebsiella granulomatis*. *Klebsiella* spp. is an emerging pathogen and Multidrug-resistant (MDR) *Kp* has become a leading cause of nosocomial infections worldwide. *Klebsiella* is an opportunistic pathogen and majorly infects patients with diabetes, alcoholism, malignancy, liver disease, diseases, renal, certain occupational exposures and in immunocompromised persons. Mortality rates of *Klebsiella* infections are as high as 50% and approach 100% in persons with alcoholism and bacteraemia (Garbati *et al.,* 2013**).** Common infections caused by *Klebsiella* are Urinary Tract Infection (UTI), pyogenic liver abscess (PLA) and pneumonia.

Antibiotic resistance: *Klebsiella spp.* and mainly *Kp* is commonly resistant to multiple antimicrobials and are acknowledged to be a major source of antimicrobial resistance genes that can spread into other Gram-negative pathogens (Karkey *et al.,* 2014). *Klebsiella* infections have become very hard to eradicate because it has developed resistance to a wide range of antibiotics such as beta lactams, aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and trimethoprim/sulfamethoxazole. *Klebsiella* exhibit antibiotic resistance via Extended-Spectrum Beta-Lactamases (ESBL), efflux pumps and plasmid carrying antibiotic resistance genes (Zowawi *et al..*, 2015). Carbapenem resistance is a major issue in clinical field because *Klebsiella* strains were previously sensitive to Carbapenem antibiotics, but in past years a mutant strain, Carbapenem-Resistant *Klebsiella pneumoniae* (CR*KP*) evolved which is able to resist Carbapenems by hyperproduction of ampC beta-lactamase with an outer membrane porin mutation. (Hall JM, 2014).

Virulence factors: Virulence factors are factors which contribute to the pathogenicity of an organism and enable them to achieve the colonization inside the host (this includes attachment to cells). Capsular Polysaccharides (CPS), biofilm, siderophores, fimbriae are the major virulence factors of *Klebsiella* etc. (Podschun *et al.*,1998).

Capsules: Capsule is an acidic polysaccharide generally composed of repeating units of three to six sugars. Capsules protects bacteria from phagocytosis and helps bacteria to survive inside

the host. It also protects bacteria from desiccation, hydrophobic toxic chemicals, detergents and bacteriophages (Domenico *et al.*,1994).

String test: Hypermucoviscosity phenotype of *Kp* is determined by string test. Positive string test is defined as the formation of viscous strings of >5 mm in length when a loop is used to stretch the colony on an agar plate. Strain with positive test is called hypermucoviscous strain of *Kp* and is considered highly virulent (Shon *et al*., 2013).

Exopolysaccharides (EPS): Exopolysaccharides (EPSs) are polymers linked to the cell surface via covalent bond to phospholipid or lipid A molecules (Sathiyanarayanan *et al.*., 2017). EPS appears to be released on the cell surface with no attachment to the cell and they are often sloughed off in form of slime. EPS is one of the components of extra polymeric substances present in the biofilm matrix and helps in biofilm formation.

Biofilm: Biofilm is an aggregate of microorganisms attached to an inert or living surface by a self-produced exo-polymeric matrix, which include polysaccharides, proteins, and extracellular DNA (eDNA) (Bandeira *et al.*, 2014). Biofilms inhibit effective antibiotic penetration, reduce the bacterial growth rate, lead to the development of persister cells, and facilitate genetic exchange (Lewis, 2008). Hence, a detailed understanding of the biofilm may help in developing strategies to combat biofilm formation. *Klebsiella* forms biofilm on instruments and devices like respiratory support equipment, sterile wounds and catheters. Ventilator Associated Pneumonia (VAP) and Catheter associated Urinary Tract Infections (CAUTI) caused by *Klebsiella* has become a severe problem in hospitals (Vuotto *et al.*, 2014).

Rationale:

Kp is a frequent cause of hospital and community-acquired infections and has emerged as an "urgent threat" to public health due to the increasing antibiotic resistance (ABR), including last-resort antibiotics such as carbapenems, colistin and tigecycline (WHO 2014, CDC2013). Treatment of Multidrug-resistant (MDR) *Klebsiella* has become a major issue in clinical field especially when the resistance has been developed for almost all the existing antibiotics and there is no new antibiotic is at horizon. Carbapenem resistant *Klebsiella pneumoniae* (*KP*C-

Kp) infection-related mortality has been rising in recent years and is highly lethal with the mortality rate of 40% (Ramos-Castañeda *et al.*, 2018).

Hence, it is crucial to perform a high-resolution analysis of whole genome of MDR clinical *Kp* strains and compare them with those available in public databases in order to elucidate factors such as phylogeny, CPS (Capsular Polysaccharide) and LPS typing, resistome, virulome, plasmid analysis, etc. These factors might be contributing to the global expansion of this pathogen and its contribution to the global scenario. Whole genome sequencing of Extremely-Drug Resistant (XDR- non-susceptibility to at least one agent in all but two or fewer antimicrobial categories; bacterial isolates in this category remain susceptible to only one or two categories) and Pan Drug-Resistant (PDR -non-susceptibility to all agents in all antimicrobial categories)isolates is crucial because more than 2000 antibiotic resistant genes are reported in *Kp* (e.g. 250 variants of bla_{OXA}, more than 200 variants of bla_{SHV}, it is impossible to detect these variants using the conventional methods. Despite biofilm formation by *Kp* has been extensively studied, the mechanism of strong biofilm formation in *Kp* is underexplored. Moreover, how strong and weak *Kp* biofilm differ from each other is unclear. Study of EPS and CPS has only been done with typed strains and the same has not been reported with the clinical isolates. Besides this, surveillance of highly pathogenic bacteria such as *Klebsiella* is also important.

Objectives:

1. Collection, isolation and identification of clinical isolates of *Klebsiella* spp.
2. To study the mechanism of antibiotic resistance
3. To study the virulence factors involved in the pathogenesis

Results:

56 clinical isolates were collected from pathology labs of Surat and Vadodara, Gujarat, India. All these isolates (n=50) were collected from patients suffering from urinary tract infections (UTIs). Further various biochemical tests (urease, citrate, oxidase, motility test) and using 16s *rRNA* gene sequencing were performed. 3 isolates (M-4, M-11 and M-32) were discarded based on preliminary test, Gram stain and Capsule stain and 13 isolates (M-5,14,16,21,32,38,30,21 and ST-2) were discarded based on biochemical tests. Rest of the 34 isolates were proceed for further molecular analysis. n=28 isolates were identified as *Kp* after 16s *rRNA* gene sequencing.

Disc diffusion was performed using Himedia antibiotic susceptibility discs and isolates were categorized as MDR, XDR and PDR. All the isolates showed resistance against oxacillin, penicillin, vancomycin and cefalexin. 9% isolates showed resistance against 3rd generation carbapenems and 15% showed resistance against 4th generation cephalosporins. Colistin and tigecycline were found to be the most effective against majority of the isolates. However, one isolate (DJ) was found to be resistant against all tested antibiotics of six antibiotic classes including colistin and was identified as PDR. Multiplex PCR was performed for detection of 11 metallo-Beta-lactamase genes: *bla*$_{AIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{DIM}$, *bla*$_{KPC}$, *bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *bla*$_{BIC}$ and *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM}$ in the XDR and PDR isolates. The primers and PCR conditions used for the multiplex PCR were as mentioned by Poirel *et.al.2011*. *bla*$_{NDM}$ was only found to be present in the PDR isolate DJ. Rest of the isolates (XDR and susceptible) isolates showed similar combination of the MBL genes. The summary of beta-lactamase genes present in the selected isolates are shown in table-1.

Table-1 summary of metallo-beta-lactamase genes present in each isolate identified by multiplex PCR.

Sample ID	AIM set	KPC set	IMP set
M2	bla$_{GIM}$	bla$_{OXA-48}$	bla$_{VIM}$, bla$_{IMP}$
M6	bla$_{GIM}$	bla$_{OXA-48}$	bla$_{VIM}$
M17B	bla$_{GIM}$	bla$_{OXA-48}$	bla$_{VIM}$
M-25	bla$_{GIM}$, bla$_{DIM}$	-	bla$_{VIM}$,bla$_{IMP}$
DJ	bla$_{SIM}$, bla$_{GIM}$,bla$_{DIM}$	bla$_{NDM-1}$, bla$_{OXA-48}$	bla$_{VIM}$
M10	bla$_{GIM}$	bla$_{OXA-48}$	-

Further, the quantification of expression of four beta-lactamase genes bla$_{NDM-1}$, bla$_{TEM-1}$, bla$_{OXA-10}$, bla$_{SHV-1}$ in these isolates (DJ, M17B, M-2, M-6 and M-25) was done using qPCR. *rpoB* was considered as the house-keeping gene and M-25 was used as a reference for the analysis. Expression of *bla*$_{NDM-1}$ was the highest in DJ. Expression of beta-lactamase producing genes such as *bla*$_{OXA-10}$ and *bla*$_{SHV-1}$ were high in XDR isolates (M-2 and M-6). Only expression of *bla*$_{SHV-1}$ was seen in susceptible isolate (M-25).

To investigate the presence of efflux activity, the Ethidium bromide cartwheel assay (Martins *et. al* ,2013) was performed for above selected isolates. *P. aeruginosa* was used as a positive control for the efflux activity. All the 7 *Klebsiella* isolates (M2, M6, M17B, M25,M10, MTCC, DJ) were observed to be negative for efflux activity.

Further, 1 PDR, 5 XDR and 1 susceptible isolate were whole genome sequenced using Illumina NextSeq. Whole-genome sequencing data for 8 strains (M-2, 3, 6, DJ, 17B, 20, 25, 27) was generated using an Illumina NextSeq platform with a 2 x 150 nt paired-end protocol (Illumina, San Diego, CA). Various tools were used to analyse the whole genome data. Genome annotation was done using Prokka (Seemann T, 2014); approximately ~5.4 MB sized genome of *Kp* isolates were obtained with average of 57% GC content. All isolates were identified as *Klebsiella pneumoniae* (*Kp*), phylogroup-1 (*Kp*1).

MLST results showed 7 different Sequence-Types (ST). M2 and M6 belong to the same ST (ST231) with 3.2% of divergence in the cgMLST (20/629 genes different). Among the STs found, ST147-K64 (DJ), ST231-K51(M2, M6) and ST14-K2 (M17B) are considered as high-risk lineages due to their clinical epidemiological relevance (Wyres *et al.*, 2019), and were the main focus of our analysis. Resistance genes and plasmid replicons present in the isolates were detected using Kleborate/BIGSdb and PlasmidFinder, respectively, are shown in table 2 & 3. Carbapenem and colistin resistant genes were only found to be present in XDR and PDR isolates. Table:2 shows antibiotic resistance genes present in each isolate and associated antibiotic classes as well as their resistance profile detected from antibiotic susceptibility testing.

Isolate ID	Resistant genes against class of antibiotics							Mutation in other genes	Resistance profile
	Beta-lacatms	Fluoroquinolone	Aminoglycosides	Sulfonamide	Tetracyclin	Phenicol	Rifampicin		
DJ	NDM-5, OXA-181, CTX-M-15, SHV-11, TEM-1B, RmtF;RmtB	ParC-80I;GyrA-83I	aadA2, strA, strA	sul1, sul2, DfrA12	--	CatA2*	Arr2	mgrB mutation	PDR
M-17B	NDM-1, OXA-232, SHV-28, TEM-1B; OXA-1, Sat-2A	QnrB1, ParC-80I;GyrA-83Y;GyrA-87G	aadA2	Sul1, DfrA1;DfrA12; DfrA14	--	CatB4	-	SAT-2, qacEdelta1	XDR
M-2	OXA-232, CTX-M-15, SHV-1, TEM-1B, RmtF	Qnr-S1,ParC-80I;GyrA-83I	aadA2	Sul1, DfrA12	--	CatA1	Arr2	--	XDR
M-6	OXA-232, CTX-M-15, SHV-11, TEM-1B, RmtF	QnrB4, QepA,ParC-80I;GyrA-83I;GyrA-87G	aadA2	Sul1, DfrA12	TetB	CatA1	Arr2	--	XDR
M-3	SHV-26	ParC-84K;GyrA-83F;GyrA-87N, QnrB1		DfrA14		TetA	--	--	XDR
M-25	SHV-42	--	--	--		--	--	--	Susceptible
M-20	SHV-27	--	--	--		--	--	--	Moderate
M-27	CTX-M-15	--	--	--		--	--	--	Moderate

Table:3 shows the list of plasmids present in *Kp* isolates.

Isolate ID	Taxonomic identification	Phylogroup	Plasmid				
			IncHIB	IncF	IncR	IncN	Col
DJ	Klebsiella pneumoniae	Kp1	--	IncFII, IncFII(pKPX1)	IncR	--	ColpVC
M-17B	Klebsiella pneumoniae	Kp1	IncHI1B + IncFIB(Mar)	IncFII, IncFIB(K)	--	--	ColKP3
M-2	Klebsiella pneumoniae	Kp1	--	IncFIB(pQil), IncFII(K)	--	--	ColKP3, Col440I
M-6	Klebsiella pneumoniae	Kp1	--	IncFIB(pQil), IncFII(K), IncFIB(AP001918), IncFIA, IncFII(pAMA1167-NDM-5)	--	IncN	ColKP3, Col440I, Col(BS512)
M-25	Klebsiella pneumoniae	Kp1	--	--	--	--	--
M-20	Klebsiella pneumoniae	Kp1	--	--	--	--	--
M-3	Klebsiella pneumoniae	Kp1	--	FIA(pBK30683)	--	--	Col440II
M-27	Klebsiella pneumoniae	Kp1	--	IncFIB(K), IncFII(K), IncI2	--	--	--

More number of genes were found to be present in the genomes of XDR and PDR isolates compared to other isolates. Carbapenem resistant genes were only found in XDR and PDR

isolates. In case of virulence factors, type-1 fimbrae (*fim* cluster) type-3 fimbrae (*mrk* cluster) and yersiniabactin siderophore were found to be present in almost all isolates. Other virulence genes reported to be frequently associated with hypervirulent phenotype (such as *rmpA*, *rmpA2*, aerobactin, salmochelin and colibactin) were not detected. Interestingly, in this isolate, blaNDM-5 carbapenemase producing gene was found to be present on the chromosome instead of plasmid, which has not yet been reported in *Klebsiella.* Presence of $bla_{\text{NDM-5}}$, and also of $bla_{\text{OXA-181}}$, on its chromosome was verified with *mplasmid* (https://sarredondo.shinyapps.io/mlplasmids/) and PLACNET (https://doi.org/10.1093/bioinformatics/btx462) tools.

Further, to explore the origin of our PDR isolate (DJ-ST147), we downloaded all the publicly available genomes from the Clonal Group (CG) 147 (n=244) at NCBI (May 2019), and performed a genome-based phylogenetic analysis using Roary v3.12 (Andrew *et al.*, 2015), Gubbins v2.2.0 (Croucher *et al.* 2014) and FastTree v2.1.7 for the construction of a maximum-likelihood tree. Among all CG147 genomes, DJ was the only isolate carrying a *mgrB* mutation responsible for colistin resistance. Mutations in *gyrA* and *parC* were observed in all CG147 isolates.

To study the hypermucoviscous phenotype, all the isolates were grown on blood agar for performing string test. Only M-20 was found to be string positive and rest of all the isolates (n=27) were string negative. A wide range of CPS production among isolates (5.2 µg CPS/mg protein to 116.38 µg CPS/mg protein) was observed. M-34 has the highest CPS production (116.38 ± 0.7 µg CPS /mg protein) while M10 has the lowest amount of CPS production (5.2 ± 0.6 µg CPS/mg protein). In case of EPS also a wide range was observed among the isolates (0 µg EPS/mg protein to 210.79 µg EPS/mg protein). M-20 have no detectable amount of EPS production while M-27 has the highest amount of EPS production (210.79 ±14 µg EPS /mg protein).

Detection of *rmpA* and *rmpA2* genes was done using PCR. *prmpA* was found to be present in M-39, M-41, M-42, M-43 and MTCC strains, while *prmpA2* was found to be present in M-39, M-42, M-43 and M-44. Further, qPCR was done to evaluate the expression of p*rmpA* in p*rmpA* gene carrying isolates. M41 was observed with the highest *prmpA* expression and MTCC has the lowest *prmpA* expression among the four isolates. No correlation between EPS and CPS production is observed.

To determine the major factor involved in resistance of phagocytosis among CPS, EPS, string test and *rmpA*, phagocytosis assay was performed. Phagocytosis assay was performed using neutrophils from human blood. Human blood cells were infected using selected *Kp* isolates based on highest and lowest CPS, EPS and positive string test. Initially we performed the phagocytosis assay for 5, 10, 15, 30, 40 and 60 minutes. Maximum phagocytosis was observed at 15 minutes and no increase or decrease in percentage phagocytosis was observed after 15 minutes. More number of granulocytes engulfed *Klebsiella* than monocytes in phagocytosis assay. Highest resistance against phagocytosis was observed in case of M-20, the isolate with very low EPS, CPS but string positive. Highest phagocytosis was observed in M-43, an isolate with high production of CPS and EPS. Phagocytosis of *Kp* inside lymphocytes was not observed.

Biofilm formation by all the clinical isolates (n = 28) was studied using crystal violet assay in a 96-well polystyrene plate and categorized as per their biofilm forming ability. Majority of the isolates were able to form a strong or moderate type of biofilm. A microbial type culture collection (MTCC) strain *Klebsiella pneumoniae* 39 was used as a standard strain Kp also formed moderate level of biofilm. Among all the collected isolates 43%, 43%, and 14% were strong, moderate, and weak biofilm producers, respectively. From 28 biofilm forming isolates of different categories, three weak (M-20,23,25) and three strong (M-10,27, and 34) biofilm producers were selected randomly for further study. Biofilm formation by these six isolates on various catheters in presence of different media was investigated. In case of weak biofilm, significant difference in biofilm formation between latex and silicone coated latex, as well as silicone catheters was observed ($p < 0.0001$). 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. 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. Hence, biofilm formation was the lowest on silicone catheters followed by silicone-coated latex and latex catheters. In the presence of different media, the biofilm formation was highest in natural urine followed by LB and artificial urine. According to the results of quantification assays, the average amount of eDNA quantified from weak biofilm matrix (344.5 μg/OD600)

was lower compared to eDNA from strong biofilm matrix (1673 μg/OD600), which was significantly higher ($p < 0.01$). The average amount of extracellular protein present in weak and strong biofilm matrix was 197.1 and 584.4 μg/OD600, respectively. Exopolysaccharides (EPS) obtained in weak and strong biofilm matrix was 46.31% and 52.38%, respectively. 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. Significantly less number of live cells were found in strong biofilm than weak biofilm ($p < 0.05$). The number of dead cells present in weak and strong biofilm was evaluated using flowcytometry 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 (105 ± 9 IU) than at 6 h (4.4 ± 0.5 IU) and maximum intensity of PI was measured at 24 h (194 ± 10 IU). Whereas, in case of weak biofilm cell death (intensity of PI) was high at 18 h (37 ± 1 IU) than at 6 h (3.4 ± 0.3). 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, we performed inhibition and addition assays. In case of strong biofilm, significant reduction in biofilm was observed after treating the biofilm with DNase I (46.62%), RNase A (48.12%), and Proteinase K (72.9%). In case of weak biofilm, biofilm was reduced by 26.19%, 0.1%, and 29.4% upon DNase I, RNase A, and Proteinase K treatment, respectively. 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 characterize the weak and strong biofilms, confocal laser scanning microscopy (CLSM), light microscopy, and field emission gun scanning electron microscopy (FEG-SEM) were performed for three weak (M-20,23,25) and three strong (M-10,27,34) isolates. 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 increase, and dead cells decrease with the increase in the depth. In strong biofilms, the number of dead cells increase, and live cells decrease with increase in the depth. The thickness of weak biofilm was observed to be only 19 slices thick

whereas, strong biofilm was observed to be expanded up to 40 slices with the uniform slice interval of 0.36 μm. This also indicates a significant difference in the thickness of weak (7 ± 2 μm) and strong biofilms (14 ± 1 μm). The number of live and dead cells in weak biofilm were 260 ± 33 and 60 ± 11, respectively in the area of 1000 × 1000 pixel (region of interest (ROI)) of the tile image. The number of live and dead cells in strong biofilm are 45 ± 6 and 369 ± 42 cells, respectively. The difference in cell death between FACS and CLSM is due to the difference in the assays. 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, three weak and three strong biofilm producers were subjected to cell adhesion assay followed by light microscopy. Very few Gram-negative rods in light microscopy were observed to be adhered in weak biofilm producers. Conversely, large number of cells were observed to be adhered in strong biofilm during early biofilm stage (4 h). Number of cells adhered to the coverslip after 4 h of biofilm formation were 125 ± 18 and 542 ± 20 in weak and strong biofilms. 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.

Discussion:

Study of antibiotic resistance and high-resolution analysis of pathogenic isolates are important to study because of increasing number of hospital and community acquired infections, especially those associated with high resistance rates. qPCR results indicate that the presence of beta-lactamase genes such as bla_{SHV} and bla_{TEM} do not contribute to XDR or PDR status of an isolate. However, presence of more number of carbapenemase producing genes like bla_{NDM} along with colistin resistant genes contribute to XDR or PDR phenotype of a strain.

The MDR, XDR and PDR isolates were found to carry different combinations of genes. Integration of $bla_{\text{NDM-5}}$ in chromosome of the PDR isolate is of a particular concern because it

is responsible for carbapenem resistance, which could transfer laterally across the generations. Moreover, co-existance of *rmt-F* and *bla*$_{\text{-NDM-5}}$ was observed in the PDR isolate. This co-existance was reported to be responsible for higher level of resistance against carbapenems and aminoglycosides (Shen *et al.*,2019). *blaOXA-181* producing DA48896 strain of *Kp* was reported from Pakistan and was highly similar to *Kp* isolate MS6671 from the United Arab Emirates by Sonnevend *et al.*, 2017. DA48896 did not carry mutation in *mgrB* and was susceptible to colistin. Here, we report the colistin resistant PDR isolate from India with insertional inactivation of *mgrB* and it also carries OXA-181 on chromosome. According to the analysis of CG147 tree it was apparent that majority of the CG-147 genomes carrying carbapenem resistance genes such as *bla*$_{\text{NDM}}$ and *bla*$_{\text{OXA-48}}$ were isolated between 2014 to 2016 and most of them were isolated from Asian countries. Later they spread to other continents and became globally dominant. Mutation in *gyrA* and *parC* are reported to be very rare in *Klebsiella* (Chen *et al.*, 2003), however in our study of CG147 genomes of *Kp*, we found *gyrA* and *parC* mutations in all the members of CG147 *Kp*, which is worrisome as this results in resistance to Fluoroquinolones.

rmpA is reported to be a positive transcriptional regulator of capsular polysaccharide synthesis and codes for the counterpart of RcsA protein, which leads to hyperproduction of capsular polysaccharide and hypermucoviscosity. (Cheng *et al.*, 2010, Hsu *et al.*, 2014). Contrarily, in our results we observed that the isolates with very high amount of CPS (M-34, M-10) did not carry *rmpA* or *rmpA2* and isolates carrying these genes (M-41,42,39) did not show very high production of CPS. Moreover, *rmpA* or *rmpA2* was not detected in the isolate with positive string test. Hence, no association between rmpA/ *rmpA2* and hypermucoviscosity or hyperproduction of CPS was observed. Infect, the amount of CPS and EPS production was very low in a string positive isolate compared to other string negative isolates. Hence, no association between amount of CPS and string test (hypermucoviscous phenotype) was observed. Association between K1-K2 serotype and string test is reported by Remya *et al.*, 2018. On other hand, Catalán-Nájera *et al.* observed 93% K1 isolates and 46% non-K1K2 isolates with string positive phenotype. In our results, we found isolates with K2 serotype (n=3) were negative for string test and one isolate belong to K39 and ST2943 showed positive string test. However, what regulates string is still unclear. According to the results of phagocytosis assay, higher CPS and EPS production does not seem to play a role in phagocytosis because the isolates with very high CPS and EPS did not resist phagocytosis more compared to other isolates. However, string test could be associated with the resistance to phagocytosis as the

percentage phagocytosis was very low in the string positive isolate compared to rest of the isolates.

How strong and weak *Kp* biofilm differ from each other is unclear. Results of biofilm formation on latex, silicone-coated latex and silicone catheters show high, moderate, and low biofilm formation, respectively. Another important observation was that *Kp* isolates with weak biofilm forming capacity formed a strong biofilm on latex urinary catheters. The issue with latex is its cytotoxicity in addition to increased biofilm formation. Silicone catheters are not only hypoallergenic, but they also have shown reduced biofilm formation compared to latex (Dolan, 2001). Lee *et al.*. have reported that the rough surface of latex catheters makes the microbial attachment easy and an additional amount of biofilm formation occurs, whereas smooth surface and less hydrophobicity of silicone catheters are responsible for reduced biofilm formation. Our results corroborate with these findings in favour of silicone catheters to be preferred over latex with respect to biofilm formation. Composition of growth medium and substratum are known to have influence on the production of extracellular components and biofilm density (Bandeira *et al.*, 2017) and our findings further validate these reports. Results of quantification study showed high eDNA, protein, EPS, cell adhesion, and unusual cell death in strong biofilms. Our results with *Kp* biofilms corroborate with these reports, that in *Kp* biofilms, significant reduction was observed upon treatment with DNase I, as well as no significant increase was seen upon the addition of *Kp* genomic DNA. In addition to DNase I, no significant effect in the presence of RNase A and Proteinase K was reported in case of *Listeria monocytogenes* (Harmsen *et al.*, 2010). However, we have observed significant reduction in both weak and strong biofilms upon treatment with Proteinase K. Moreover, biofilm was significantly reduced after treatment of RNaseA in case of strong biofilms. The multicellular structure of biofilm provides a selective pressure for programmed cell death which eliminates damaged cells and enhances nutrient availability for the healthy cells in the biofilm matrix (Lewis, 2009). Cell death is caused by self-destruction of individual cells and lysis of dead bacteria releases genomic DNA (Claverys *et al.*, 2007). Except programmed cell death, several other mechanisms are reported for eDNA release such as membrane vesicle formation (Renelli *et al.*, 2004), prophage-mediated cell death in *Pseudomonas aeruginosa* (Webb *et al.*, 2003), and specialized secretion in *Neisseria gonorrhoeae* (Salgado-Pabón *et al.*, 2010). However, in *Kp* the mechanism for the release of eDNA is unclear. We hypothesize that cell death could be the cause of increased eDNA, protein, and EPS in strong biofilms. Bandeira *et al.*. had

categorized the biofilm forming strains into most and the least efficient, as well as intermediately efficient biofilm assembler (Bandeira *et al.*, 2014). Singla *et al.*. have shown 3D structure of *Kp* biofilms with enhanced exopolysaccharide production and water channel formation (Singla *et al.*, 2014). Here, we report SEM of weak and strong *Kp* biofilms grown on silicone-coated latex catheters with more number of bacteria and increased EPS in strong biofilm compared to weak.

Summary:

The present work was done to understand the antibiotic resistance and virulence of Klebsiella spp. For the study of antibiotic resistance, the isolates were categorized in three categories: PDR, XDR and MDR. Among these, the PDR isolate was found to possess distinguishing characteristics. In the PDR isolate $bla_{\text{NDM-5}}$ was integrated in chromosome, which is worrisome because it's a carbapenemase gene, which could be laterally transferred across the generations. Insertional inactivation in *mgrB* gene is responsible for colistin resistance of the PDR isolate. To prevent the global spread of such threatening genetic combinations, continuous surveillance of pathogenic genomes is warranted. Moreover, emergence of such clones of CG147 from multiple geographical locations is of concern. For the study of virulence factors, properties such as EPS, CPS, *rmpA* and biofilm were evaluated. Clinical isolates show a wide range of variation in amount of CPS and EPS production. No association between CPS and EPS produced by an isolate was observed. String is not associated with the amount of CPS produced by the isolate. *rmpA* and *rmpA2* are not solely responsible for capsular polysaccharide synthesis. Hyperproduction of CPS is not associated with hypermucoviscosity. Amount of CPS and EPS does not contribute to the resistance to phagocytosis. String positive phenotype/hypermucoviscosity seem to be associated with the resistance to phagocytosis. We found heterogeneity in biofilm formation by clinical isolates, increased biofilms on latex compared to silicone catheters and increased biofilms in the presence of natural urine. Latex catheters used in healthcare settings of developing countries due to its cost effectiveness should be avoided to reduce the high prevalence of biofilm associated infections. High eDNA, protein, EPS, cell adhesion, and unusual cell death were found to be associated with the strong biofilms. It is evident that increased eDNA, protein, and RNA in strong biofilm matrix is a consequence of cell death. Virulence of an isolate appears to be a result of multiple attributes and a single feature may not be solely responsible for high virulence. In our study we observed that factors responsible for resistance and virulence are not found in the same isolates. Study of above

parameters suggested that fortunately the convergence of resistant and hypervirulent isolates is quite rare and has not been observed in our isolates.

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