" PHENOTYPIC CHARACTERIZATION OF VIRULENCE FACTORS AND ANTIBIOGRAM OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM DIFFERENT CLINICAL SPECIMENS – A CROSS SECTIONAL STUDY "

BY
Dr. MADHAVI -S- HULLUR, MBBS

DISSERTATION SUBMITTED TO
SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH
TAMAKA, KOLAR, KARNATAKA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF MEDICINE
IN
MICROBIOLOGY

UNDER THE GUIDANCE OF

Dr. ARVIND NATARAJAN,
PROFESSOR & HEAD
DEPARTMENT OF MICROBIOLOGY

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IS A BONAFIDE AND GENUINE RESEARCH WORK CARRIED OUT

IN SRI DEVARAJ URS MEDICAL COLLEGE, KOLAR

UNDER THE DIRECT GUIDANCE OF

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LIST OF ABBREVIATIONS

1. WHO : World Health Organization
2. FDA : Food & Drug administration, USA
3. EUCAST : European Committee on Antimicrobial Susceptibility Testing
4. GNB : Gram negative bacilli
5. UTI : urinary tract infection
6. CAUTI : Catheter associated urinary tract infection
7. VAP : Ventilator associated pneumonia
8. CLABSI : Central line associated blood stream infection
9. cKP : Classical *Klebsiella pneumoniae* strains
10. hvKP : Hypervirulent *Klebsiella pneumoniae* strains
11. MDR -hvKP : Multi drug resistant hypervirulent *Klebsiella pneumoniae* strains
12. MS type -1 fimbriae : Mannose sensitive type -1 fimbriae
13. MR type -3 fimbriae : Mannose resistant type – 3 fimbriae
14. MDR : Multidrug resistance
15. XDR – Extensive drug resistance
16. PDR – Pan drug resistance
17. ESBL : Extended spectrum of β - lactamase enzymes
18. TEM :Temoniera type of ESBL
19. SHV -1 : Sulfhydryl variable type of ESBL
20. CTX -M group :Cefotaximase -M group type of ESBL
21. IMP : Imipenem drug
22. MRP : Meropenem drug
23. ETP : Ertapenem drug
24. HMV Test : Hypermucoviscosity test
25. CRKP : Carbapenem resistant *Klebsiella pneumoniae*
26. MIC value : Minimum inhibitory concentration
27. NIH scientists : National Institute of Health scientists
28. CN1, NY9, CR14 : are Genotypic *Klebsiella pneumoniae* strains
29. TLR -4 : Toll like receptor 4
30. PRR :Pattern recognition receptor

ABSTRACT

BACKGROUND : *Klebsiella pneumoniae* is gram negative, non-motile, capsulated organism. It is ubiquitous in nature, which is known to cause both community acquired and hospital acquired infections.

It normally resides on the mucosal surfaces of gastrointestinal tract and occasionally in nasopharynx, thus it can gain entry into circulation and other tissues causing infections. *Klebsiella pneumoniae* produces life threatening infections among the critically ill patients.

K. pneumoniae pathogenicity is mainly dependant on various virulence factors which allows it to overcome the host`s innate immunity and therefore produce infection in a mammalian host. These virulence factors will help in its survival in various environmental conditions.

There are 2 main types of *Klebsiella pneumoniae* strains "classic *Klebsiella pneumoniae [cKp] and hypervirulent Klebsiella pneumoniae [hvKp]*.

The identification of virulence factors and prompt detection of antimicrobial resistance will help the clinicians to treat the cases aggressively resulting in the better management of cases.

MATERIALS & METHODS :

A cross sectional observational study, conducted in Department of Microbiology of R.L. Jalappa Hospital and Research Centre, Tamaka, Kolar, from October 2019 to May 2021. Sample size of 150

RESULTS : The findings of our study on virulence factors are as follows : hemolysis 4.66 %(7 isolates), capsule 100 % (150 isolates), hypermucoviscosity (HMV) formation 44 % (66 isolates), biofilm production 54 % (81 isolates), siderophore production 73.33 % (110 isolates), protease 90 % (135 isolates), gelatinase 84 % (126 isolates), lipase production 79.33 % (119 isolates), lecithinase activity 54.66 % (82 isolates).

The antibiotic resistance pattern as follows : Piperacillin (90.66 %), Piperacillin tazobactum (67.33 %), Ampicillin (100 %), Amoxyclav (90.66 %), Cefotaxime (84 %), Ceftriaxone (82.66 %), Ceftazidime (82.66 %), Ceftazidime-clavulanic acid (71.33 %), Cefoxitin (67.33 %), Cotrimoxazole (71.33 %), Imipenem (63.33 %), Meropenem (70.0 %), Ertapenem (62.66 %), Amikacin (66 %), Gentamicin (65.33 %), Tobramycin (72.0 %), Chloramphenicol (38.66 %), Doxycycline (79.33 %), Ciprofloxacin (75.33 %), Levofloxacin (70. 66 %), Norfloxacin (66.67 %), Nitrofurantoin (77.78 %). ESBL *K. pneumoniae 16.67 % (n = 25 isolates), AmpC producer type 14.67 % (n = 22), multi drug resistance (MDR) – 74.20 % (n = 116), extensive drug resistant (XDR) – 20 % (n = 30), pan drug resistant (PDR) – 28 % (n = 42).* Total carbapenem resistance in our study : average 65.33 % reported.

CONCLUSION : The increasing coexistence of the virulence factors & antimicrobial is of particular concern as it can lead to untreatable and invasive K. pneumoniae infections. Active surveillance to be done not only for antimicrobial resistance, but also for virulence determinants is imperative now to avoid the transmission and spread of multidrug resistant strains.

INTRODUCTION

INTRODUCTION :

Klebsiella pneumoniae is a gram negative, capsulated, non motile bacilli, lactose fermenting, facultative anaerobic bacteria belonging to family Enterobacteriaceae [1, 2].

K. pneumoniae asymptomatically colonizes the skin, mouth, respiratory & gastrointestinal flora, because *Klebsiella pneumoniae* is a well known opportunistic pathogen from the human gut.

K. pneumoniae is one of the known agents of hospital and community acquired infections. "It is usually associated with upper & lower respiratory infections (pneumonia), bacteremia , septicaemia, urinary tract infection, wound infections, intra abdominal infections and neonatal septicemia, liver abscess, pneumonia, meningitis, and endophthalmitis , catheter associated urinary tract infection (CAUTI), ventilator associated pneumonia (VAP) & central line associated bloodstream infection (CLABSI) cases".[3, 4,5]

"The pathogenicity of *K. pneumoniae* mainly arise from various virulence factors which allow it to overcome innate host immunity and to maintain infection in a mammalian host".[5,6, 7]These virulence factors play a role in its survival in different environmental conditions and therefore help in establishing infection in the human body.

"K. pneumoniae pathogenicity is attributed to several virulence factors like fimbrial adhesins, lipopolysaccharides, capsule and siderophores, biofilm formation, lipase, lecithinase, haemagglutination, protease, gelatinase, hemolysis and hypermucoviscosity. All these virulence factors have the potential to produce a wide variety of infectious diseases in hospitalized patients & in the community". [5, 8]

Klebsiella pneumoniae produces hemolysin protein, an exotoxin (cytolyic toxin) which cause lysis of blood cells & therefore facilitate the dissemination of bacteria. Klebsiella pneumoniae being an encapsulated organism, helps in developing resistance to both antibiotics and host defense mechanisms. The capsule is antiphagocytic (prevents the bacteria from killing, by bacterial serum factors), they are associated with hyperviscous (hypervirulent phenotype) in *K. pneumoniae* infections. "There are 3 variants of Klebsiella pneumoniae - Classical K. pneumonia (cKP), Hypervirulent K. pneumoniae (hvKP) & Multidrug-resistant hvKP (MDR-hvKP). *K. pneumoniae* infections are caused by classic *K. pneumoniae* (cKp) type"[8]. These strains persist in hospital environments and cause infections in debilitated patients. (These cKp strains are distinct from hypervirulent *K.pneumoniae*(hvKp) strains).[8]

Klebsiella pneumoniae release many enzymes which have the ability to destroy host tissues. The lipase enzyme digests the host cellular lipid for its nutritional demand.

"*Klebsiella pneumoniae*`s lecithinase production is found to be directly associated with skin, soft tissue and systemic infections"[5, 9].
Klebsiella pneumoniae exhibits haemagglutination phenomenon - fimbrial and nonfimbrial adhesins (hemagglutinins) are mannose sensitive (MS) type 1 and mannose-resistant (MR) type 3. Strains with MS type of fimbriae are known to be more pathogenic and isolates with type 3(MR) are known to bind human endothelial cells, epithelial cells of genitourinary and respiratory system.[18, 21]
K. pneumoniae has the ability to form biofilms, matrix of extracellular polymeric substance adhere to each other and/or to a surface. "*K. pneumoniae* biofilms may also contribute to colonization of the gastrointestinal, respiratory and urinary tract and the development of invasive infections especially in immunocompromised patients. *K. pneumoniae* can adhere to medical devices forming biofilms"[18], avoiding the immune system and favoring the antimicrobial therapy failure.

In the antibiotic era, *K. pneumoniae* is an established cause of healthcare-associated infections (HAI). *K. pneumoniae* strains are naturally resistant to ampicillin, carbenicillin and ticarcillin because of production of a chromosomal penicillinase, sulfhydryl variable (SHV-1). [10] The global rise of multidrug-resistant (MDR) gram-negative bacteria represents an increasing threat to human health.[18,19]

Multidrug-resistant (MDR) *Klebsiella pneumoniae* is an increasing threat to human health & causes difficulty to treat the infections with a high mortality rate. *Klebsiella pneumoniae* is one of the species recognized in *ESKAPE group (Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species),* associated by their characteristic potential to escape or evade the action of antimicrobial agents.

World Health Organization (WHO) lists *K. pneumoniae* as high priority pathogen and promotes the research and development (R & D) for new antibiotics due to the growing global problem of antimicrobial resistance towards this species. (World Health Organization, 2017).

REASON FOR CHOOSING THIS STUDY :
K. pneumoniae is known as the most common causative agent in hospital and community acquired infections. *K. pneumoniae* pathogenicity is mainly due to various virulence factors which allows it to overcome the host`s innate immunity and therefore maintain infection in a mammalian host.[7,8, 36]These virulence factors are important for its survival in diverse environmental conditions and therefore help in establishing infections in the human body. *K. pneumoniae* is associated with high mortality of about 50 % even with antimicrobial therapy. Hence, we conducted research in this area to evaluate the association between virulence factors and antibiotic resistance in *Klebsiella pneumoniae* isolate.

OBJECTIVES OF THE STUDY

- To determine the various virulence factors among *Klebsiella pneumoniae* isolates.
- To determine the antibiotic susceptibility pattern of *Klebsiella pneumoniae* isolates
- To evaluate the association between virulence factors and antibiotic resistance in *Klebsiella pneumoniae* isolates.

REVIEW OF LITERATURE

1) HISTORY OF *KLEBSIELLA PNEUMONIAE :*

a) The genus Klebsiella belongs to the tribe *Klebsiellae*, a member of the family Enterobacteriaceae. This organism is named after Edwin Klebs, a 19th century German & Swiss Microbiologist [14]

Fig : 1 - **EDWIN KLEB**

b) Carl Friedlander, German Microbiologist in 1882 was the 1st to describe this bacteria & isolated from the lungs of patients who had died from pneumonia.[2,3,8]

Fig : 2 - **CARL FRIEDLANDER**

2) **CLASSIFICATION OF KLEBSIELLA GENUS :**

1) Klebsiella pneumoniae

i) Subspecies aerogenes

ii) Subspecies ozaenae

iii) Subspecies pneumoniae

iv) Subspecies rhinoscleromatis

2) Klebsiella ornitholytica

3) Klebsiella oxytoca

4) Klebsiella planticola

5) Klebsiella terrigena

3) STRUCTURE &MORPHOLOGY : This *Klebsiella pneumoniae* is a gram negative bacilli (GNB), short, stout & capsulated bacilli. It is a non- fastidious bacteria & will grow on basal media like Nutrient agar.

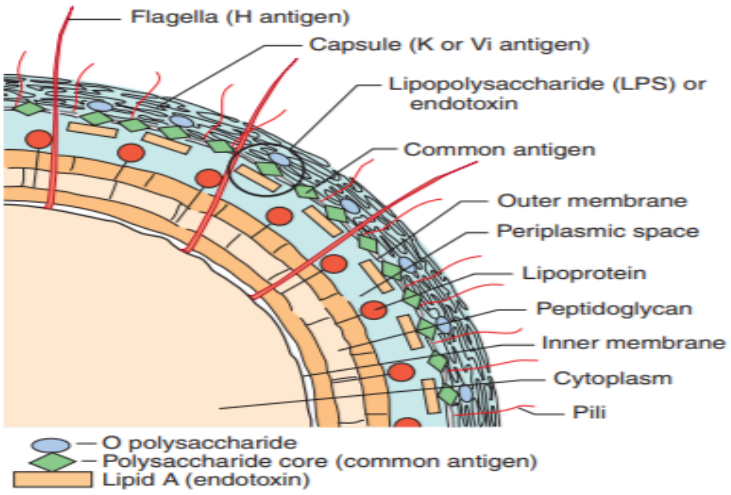
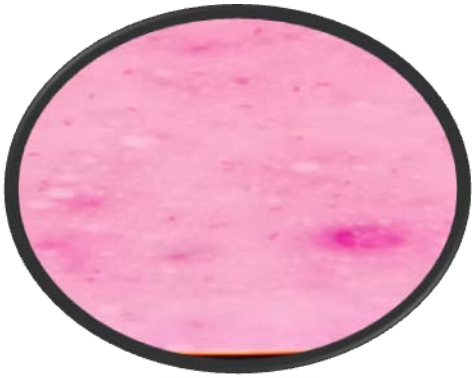
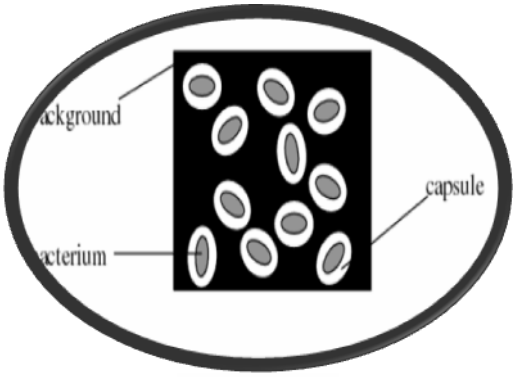


FIG : 3 – Structure Of Gram Negative Bacilli (*Klebsiella Pneumoniae)*

4) MICROSCOPY : Grams stain shows gram negative bacilli, short, stout & capsulated bacilli



5) **CAPSULE STAINING** : India ink staining is a negative stain which demonstrates *Klebsiella pneumoniae* as a capsulated organism. There are different staining methods – like India ink method, Maneval`s method, Hiss method, Anthony`s method.



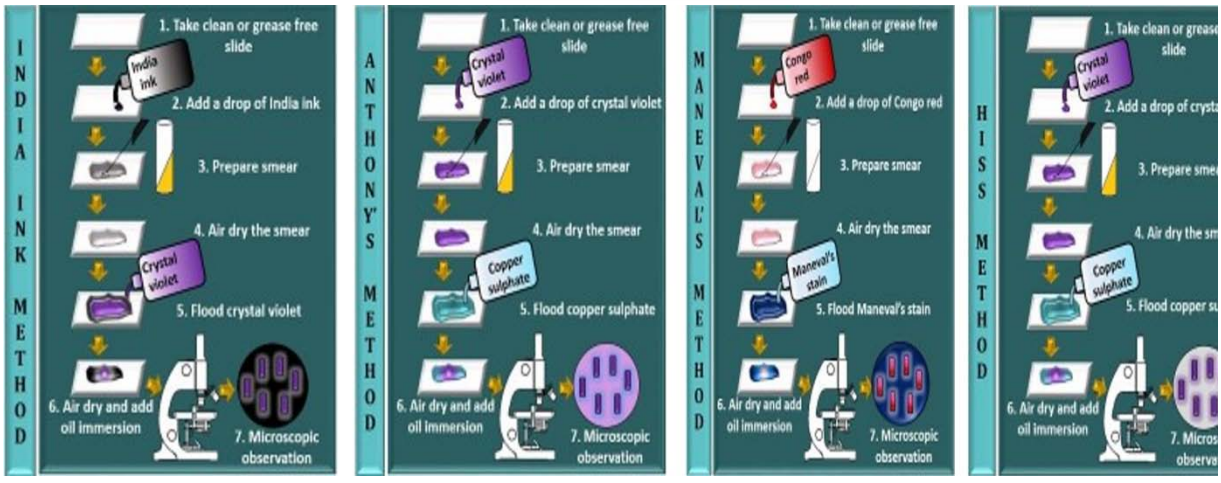
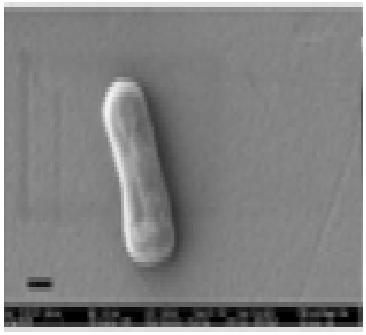
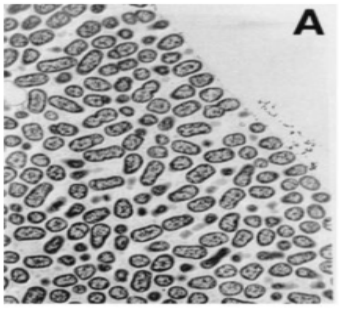


FIG : 5 – Types of Capsule staining methods

FIG : 6 - Pictures On Scanning Electron Microscopy (SEM) / Transmission Electron Microscopy (TEM) :



SEM PICTURE



TEM PICTURE

5) CULTURAL CHARACTERISTICS OF *KLEBSIELLA PNEUMONIAE* [1, 2,4] *:*

On **Nutrient agar** (Non selective media) :*Klebsiella pneumoniae produces* mucoid, round, non- pigmented colonies, no swarming.
On **Blood agar** (Enriched media) :*Klebsiella pneumoniae produces* large, grey, round, opaque, mucoid, non -hemolytic colonies.
On Selective / Differential media : (**Mac conkey agar**) – pink, mucoid, lactose fermenting colonies. (Mucoid nature is due to polysaccharide capsule of this organism).

FIG 7 :Growth on Culture medias

Nutrient agar **Blood agar** **Mac conkey agar**

7) Biochemical Reactions of *Klebsiella pneumoniae* [1,3,4,5]:

Klebsiella pneumoniae produce urease, utilize citrate, produce acidic slant & acidic butt with gas, no H_2S production on Triple sugar iron (TSI) media & Indole test negative, It grows on potassium cyanide medium (KCN), Phenylalanine deaminase

test negative, Voges proskauer test positive (Acetoin production), Methyl red test negative.
*Klebsiella pneumoniae i*sargininedihydrolase negative, Ornithine decarboxylase negative & lysine decarboxylase positive. ONPG (β-galactosidase) positive, and ferments a variety of carbohydrates.

Fermentation of : Glucose, Lactose, Maltose, Mannitol, Mannose, Melibiose, Adonitol, Arabinose, Arabitol, Cellobiose, Glycerol, Inositol, Myo Inositol, Raffinose, Rhamnose, , Sorbitol, Sucrose, Trehalose, Xylose. Fermentation of Salicin& Tartrate – positive.

Table : 1 Distinguishing reactions of *Klebsiella species* (based on Ewing 1986, Barrow & Feltham 1993) :
(+ve) - means > 85 % of strains positive, (-ve) – means > 85 % of strains negative, (+/-) means 16 – 84 % of strains positive after 24 – 48 hours at 37^{0}C

Species :	VP (Voges Proskauer test)	Lactose fermentation	Gas formation	Indole test	Citrate utilization	Malonate test	Urease	Lysine decarboxylation	Growth on KCN media
Klebsiella pneumoniae									
• *Subspecies : aerogenes*	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve
• *Subspecies : ozaenae*	- ve	+ ve /-ve	+ ve/- ve	- ve	+ ve/- ve	- ve	- ve	+ ve /- ve	+ ve
• *Subspecies : pneumoniae*	- ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve
• *Subspecies : rhinoscleromatis*	- ve	- ve	- ve	- ve	- ve	+ ve	- ve	- ve	+ ve/- ve
Klebsiella ornithinolytica	+ ve / -ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Klebsiella oxytoca	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Klebsiella planticola	+ ve	+ ve	+ ve	+ ve /- ve	+ ve	+ ve	+ ve	+ ve	+ ve
Klebsiella terrigena	+ ve	+ ve	+ ve /- ve	- ve	+ ve	+ ve	- ve	+ ve	+ ve

8) COLONIZATION OF *KLEBSIELLA PNEUMONIAE* [14, 15,18]:

K. pneumoniae will asymptomatically colonize the skin, mouth, respiratory & gastrointestinal flora. *Klebsiella pneumoniae* is one of the normal commensal flora of human gut. *K. pneumoniae* is also found in water, sewage, soil, and plant surfaces. The environment acts as a reservoir for human infections. Several studies have shown that *K. pneumoniae* has same biochemical patterns, virulence and bacteriocin susceptibility patterns in the environment as well as their clinical counterparts.

Environmental isolates are easily treatable by antibiotics, than the clinical *Klebsiella pneumoniae* isolates - suggesting that selective pressure exists in a clinical setting.[14, 15]

- Colonization is higher in adults than children,
- Colonization differs at different body sites, community & hospital acquired infections

- In nasopharynx from 3 – 15 %, common in alcoholics), but colonization in hospital acquired nasopharynx is much higher to 19 %.
- Gastrointestinal colonization in HA cases – is 35 %, in CA cases – upto 77 %.
- Colonization also increase following antibiotic treatment.

The wide variation in colonization rates may be due to differences in the patient populations.

9) **SOURCE OF INFECTION** : Infected persons, asymptomatic *Klebsiella pneumoniae* carriers in the community, contaminated surfaces and instrumentation are the potential source.

10) **MODE OF TRANSMISSION** :*K. pneumoniae* will be transmitted from person-to-person, contact between healthcare workers and patients.

Once acquired, *K. pneumoniae* colonizes the mucosal surfaces of nasopharynx and gastrointestinal tract. These bacteria can be found on skin (but transient at this site rather than colonizing).

11) **Host factors that predisposes to colonization and infection are as follow :**

- Admission to an wards & intensive care ward (ICU), ie any hospitalized patients can acquire
- Prolonged use of invasive devices
- Poor infection control practices
- Immunocompromised especially alcoholics and diabetics
- Prolonged use of broad-spectrum antibiotics

Bacteria enter the host either by direct inoculation or by following oropharyngeal aspiration.

12) ROLE OF INNATE IMMUNITY IN *KLEBSIELLA PNEUMONIAE* INFECTIONS :

There are few studies supporting the contribution of Pattern recognition receptor (PRR) governed signalling to control *Klebsiella* infections. Like other bacterial infections TLR4 signalling has an important role in antibacterial defence against *Klebsiella* infection, (that means lack of TLR4 signalling is associated with a decreased activity of IL17 and IL23 in the lungs of infected mice)". [36, 37]

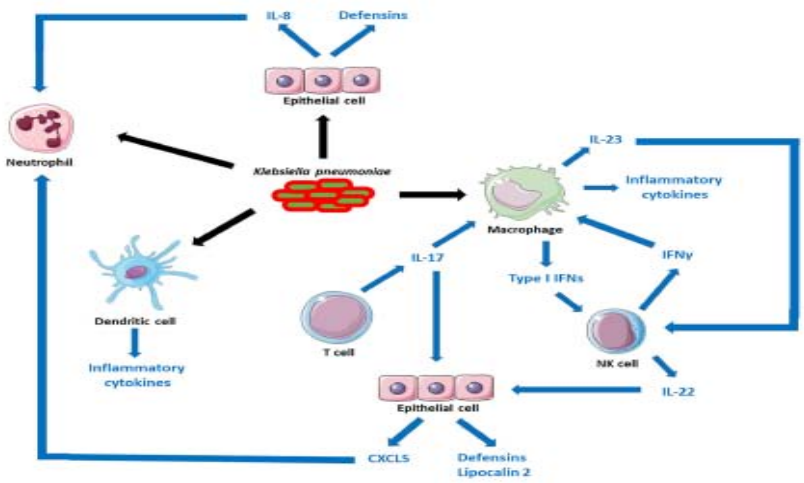


FIG : 8 **-** Mechanisms Of Innate Immunity To *Klebsiella Pneumoniae*

K. pneumoniae remodels its LPS lipid A domain to counteract host cationic antimicrobial peptides (CAMPs) and polymyxins. It has developed strategies to counteract CAMPs & defensins.
CAMPs and antibiotics like quinolones and polymyxins share the same initial target in the outer membrane of Gram-negative bacteria. The lipid A of *K. pneumoniae* shows a remarkable plasticity. [37]OmpA is part of regulatory network controlling systems that is required to upgrade the CAMPs bactericidal action.[36, 37]

It is a widely held belief that *K. pneumoniae* is a pathogen, which fails to stimulate the innate immune responses, but now there is enough evidence to show that even *Klebsiella pneumoniae* can subvert the host defenses.[36,37]

***K. pneumoniae* will resist bacterial killing by one of these following mechanisms :**

a) Counteracting soluble effectors of the immune system,

b) Counteracting immune cells,

c) Ablating host defensesignaling,

d) Subversion of nutritional immunity.

13) VIRULENCE FACTORS

Host protection from bacterial invasion mainly depends on two things:

- polymorphonuclear granulocytes - which phagocytose the bacteria
- serum complement proteins - which are bactericidal.

The alternate pathway of complement activation is more active in *Klebsiella pneumoniae* infection, the capsule protects bacteria from phagocytosis and serum bactericidal proteins. *K. pneumoniae* has many fimbrial and non-fimbrial adhesins which help in host cell adhesion & this is critical for the infectious process". [40, 42]

Some *K. pneumoniae* strains can modify the LPS making it unrecognizable to immune cells, whereas others strains use the capsule to prevent LPS detection by toll-like receptor (TLR4) receptors. *K. pneumoniae* with type 1 and 3 fimbriae responsible for adhesion to biotic and abiotic surfaces & hence facilitate epithelial cell invasion and biofilm formation. The monocyte/macrophage system plays a pivotal role in the innate immune response, through phagocytosis and production of immune mediators such as cytokines and chemokines (IL- 8, 17, 23 & IFN-γ). Other important cytokines are IL-1β (produced via activation of the NOD-like receptor pyrin domain-containing (NLRP3) inflammasome pathway), and other pro-inflammatory cytokines such as (TNF-α) and IL-6. [36,37]

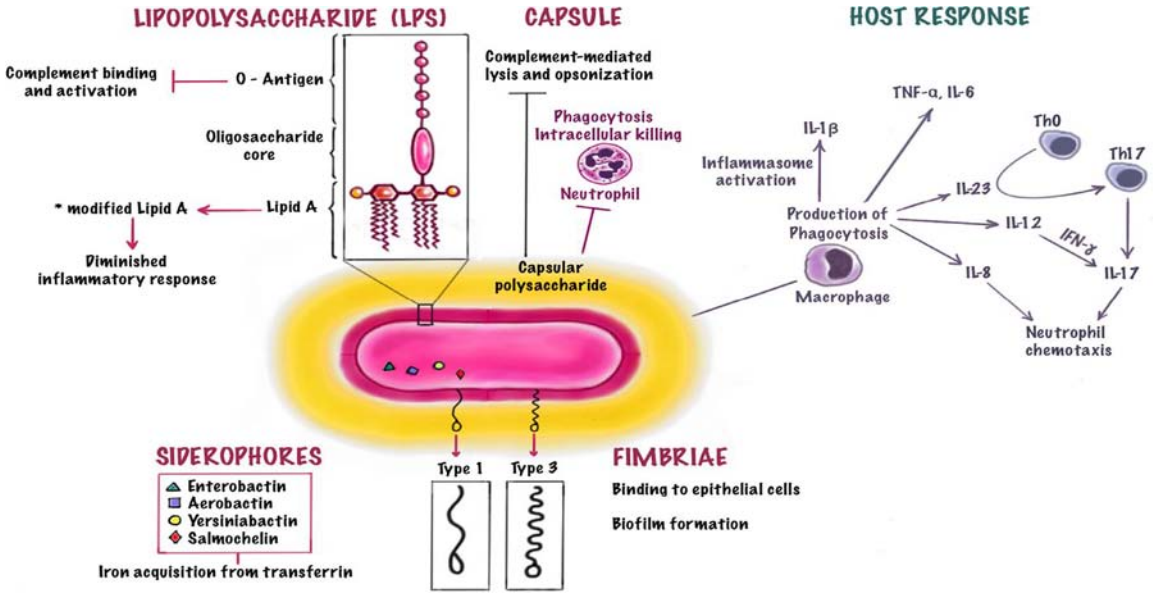


FIG : 9 - Schematic Representation Of *Klebsiella Pneumoniae* Virulence Factors And Of Host Immune Response [7]

13 a) ROLE OF CAPSULE & SIDEROPHORE IN PATHOGENESIS :

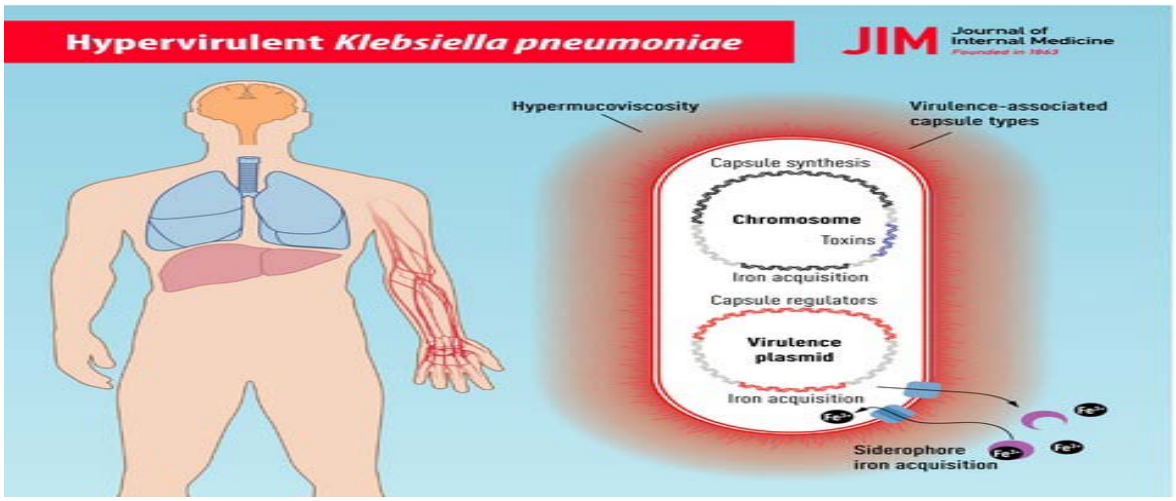


FIG : 10 - Hypervirulent *Klebsiella pneumoniae* – clinical and molecular perspectives.

a) Capsular polysaccharides prevent phagocytosis and block complement-mediated lysis and opsonization. The intact LPS elicits a robust inflammatory response and prevents binding of C1q to bacteria and the subsequent activation of the complement pathway.

b) *Klebsiella pneumoniae* also synthesizes siderophores (enterobactin, aerobactin, yersiniabactin and salmochelin) to acquire iron from the host.

TABLE : 2 - Main characteristic of cKP, hvKP, and MDR-hvKP [17] **:**

Characteristics	Classical *K. pneumoniae* (cKP)	Hypervirulent *K. pneumoniae* (hvKP)	Multidrug-resistant hvKP (MDR-hvKP)	References
Infections	Acquisition: nosocomial Host: immunocompromised patients Geographic region: the whole world Infectious sites: urinary tract infections, pneumonia, bloodstream infections; usually polymicrobial at sites of infection Metastasis: uncommon	Acquisition: community Host: healthy adults Geographic region: Southeast Asia Infectious sites: pyogenic liver abscess, meningitis, endophthalmitis, necrotizing fasciitis; usually monomicrobial at sites of infection Metastasis: Common	Acquisition: nosocomial and community Host: usually immunocompromised patients Geographic region: Asia (especially China) Infection sites: pyogenic liver abscess, bloodstream infections, urinary tract infections	Harada et al., 2019; Russo and Marr, 2019; Liu C. et al., 2020; Tang et al., 2020
Phenotypes	Non-hypermucoviscosity and string <5 mm	Hypermucoviscosity and string ≥5 mm	Hypermucoviscosity or non-hypermucoviscosity	Russo et al., 2018
Common serotypes	K1–K79	K1, K2, K5, K16, K20, K54, K57, KN1	K1, K2, K16, K20, K54, K62, K64, K47	Pan et al., 2008, 2015; Yang et al., 2021
Siderophores	Enterobactin, yersiniabactin	Enterobactin, yersiniabactin, salmochelin, and aerobactin	Enterobactin, yersiniabactin, salmochelin, and aerobactin	Russo et al., 2015; Lam et al., 2018b; Choby et al., 2020

13 b) ROLE OF FIMBRIAE (HEMAGGLUTINATION PHENOMENON) :

Fimbriae mediate stable adherence, a critical step for progression to infection. *Klebsiella pneumoniae* have 2 types of fimbriae / pili (Type -1 & Type – 3). Both types of pili have a role in colonization in urinary catheters / in hospital patients as catheter associated UTI (CAUTI) cases. [41,42]

a) **type 1 (fim) pili** – have ability to adhere to human mucosal & epithelial surfaces,
b) **type -3 (mrk) pili** – adhere to cell surfaces & strong promoters of biofilm (BF) formation.

13 c) ROLE OF BIOFILMS :

K. pneumoniae biofilms are formed on the inner surfaces of catheters and other indwelling devices. *K. pneumoniae* cells within biofilms are partially protected from immune defenses. The matrix blocks the access of antibodies and antibacterial peptides and reduces the efficiency of complement and phagocytosis. K. pneumoniae is able to form biofilms (that is extracellular polymeric substance comprising of polysaccharides, proteins and DNA). *K. pneumoniae* biofilms are those formed on the inner surfaces of catheters and other indwelling devices. *K. pneumoniae* biofilms will colonize the gastrointestinal, respiratory and urinary tract, and promote the development of invasive infections in the immunocompromised patients in hospitals. *K. pneumoniae* biofilms on solid surfaces proceeds from cell adherence to microcolony formation.[7,10,18] Given the dynamic process of biofilm production and the variability of environmental stimuli, embedded cells must be capable of swift and extensive changes in gene expression. Transcriptional regulation is controlled by quorum sensing.[10]

13 d) **ROLE OF LIPASE / PHOSPHOLIPASE** : In hypervirulent strain &carbapenemase producing strains, these enzymes play a role in inter & intra-species killing. Antibiotic usage promotes its secretion. The lipase enzyme digests the host cellular lipid for its nutritional demand, the secretary systems are part of the core & accessory genome which promote competition with other bacteria at sites of colonization & increase the chances of infection. [5, 42]

13 e) **ROLE OF GELATINASE** :*Klebsiella pneumoniae isolates produce* gelatinase enzyme, which hydrolyse gelatinand collagen in subcutaneous tissues during wound infections. [5]

13 f) **ROLE OF PROTEASE PRODUCTION** :*Klebsiella pneumoniae isolates* also produce protease enzyme, which help in breakdown of protein structures in the tissues & organs, which made have a role in invasiveness of the disease process.

Table : 3 - Virulence Factor associated genes

Virulence Factor associated genes	Genes Of Importance
Fimbriae	fim D, fim H, mrk C, mrk D
For fimbrial (Type – 1)	fim H gene
For fimbrial (Type -3)	mrk D
Capsule-associated genes	ycf M, wab G, uge and rmp A
Siderophores	kfu gene
For HMV phenotype	rmp A gene
For non- fimbrial adhesion factor	cf 29A &cf 29K genes.

Table : 4 - Meta analysis report on *Klebsiella pneumoniae* virulence factors from other studies

VIRULENCE FACTORS :	RamaKrishna et al (2019 published)	Imtiaz et al, 2021 published	AL-Ammary et al –2017 published	Aljanaby et al (published in 2016)	Lee et al (study in 2006, only on HMV)	Hassan R et al -2011	Dougnon V et al (2021 article)
Hemolysis	Not done	8 %	No hemolysis			7 %	20.0 %
Capsule Formation	Not done	99 %	100 %	100 %			
HMV Test	7%	22 %	Not done	62 %	14.8%-in hospital infections& 41.5% in community		
Biofilm Formation	79%	94%	75 %	100 %		67 %	3.34 %
Siderophore Production	Not done	Not done	100 %	100 %			
Protease [With Milk Agar]	44%					2.56 % (1 isolate)	0 %
Gelatinase Test	41%	12 %					
Lipase	58%					33 %	23.34 %
Lecithinase Test	55%					0 %	3.34 %
Hemagglutination test	58%	100%				MSHA – 17 %, MRHA – 83 %	31.67 %

- MSHA – Mannose sensitive hemagglutination (absence of hemagglutination)
- MRHA - Mannose resistant hemagglutination (presence of hemagglutination)

14) **EPIDEMIOLOGY [18, 36] :**

Humans are reservoir for *K. pneumoniae,* In the general community – (5% to 38% of individuals carry the organism in their stool) and (1% to 6% in the nasopharynx).

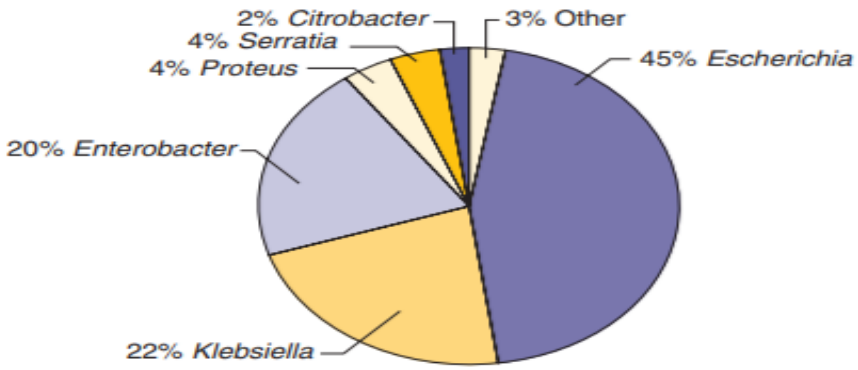


Fig : 11 - Incidence Of Enterobacteriaceae associated with Bacteremia

The main reservoirs of infection are patient's GIT and healthcare workers in hospital, which at times are responsible for nosocomial outbreak. The carrier rate for *K. pneumoniae* in hospitalized patients are much higher than that found in the community. In one-study, carrier rates as high as 77% can be seen in the stool of those hospitalized and are related to the number of antibiotics given.[37]

K. pneumoniae causing pneumonia are of 2 types : community-acquired or hospital-acquired pneumonia. In the western world *K. pneumoniae* accounts for 3% to 5% of all community-acquired pneumonia, but in developing countries *K. pneumoniae* infections accounts to approximately 15% of all cases of pneumonia.

Overall, *K. pneumoniae* accounts for approximately 11.8% of all hospital-acquired pneumonia in the world. (In those who develop pneumonia on a ventilator - between 8% to 12%, while only 7% occur in those patients who are not on ventilator). Mortality ranges from 50% to 100% in patients with alcoholism and septicemia.

15) INFECTIONS CAUSED BY *KLEBSIELLA PNEUMONIAE*[3,5,6] :

K. pneumoniae is a known causative agent of hospital and community acquired infections. It is associated with upper & lower respiratory infections (pneumonia), bacteremia , septicaemia, urinary tract infection, wound infections, intra abdominal infections and neonatal septicemia, liver abscess, “pneumonia, meningitis, and endophthalmitis , catheter associated urinary tract infection (CAUTI), ventilator associated pneumonia (VAP) & central line associated bloodstream infection (CLABSI) cases”.[3,7]

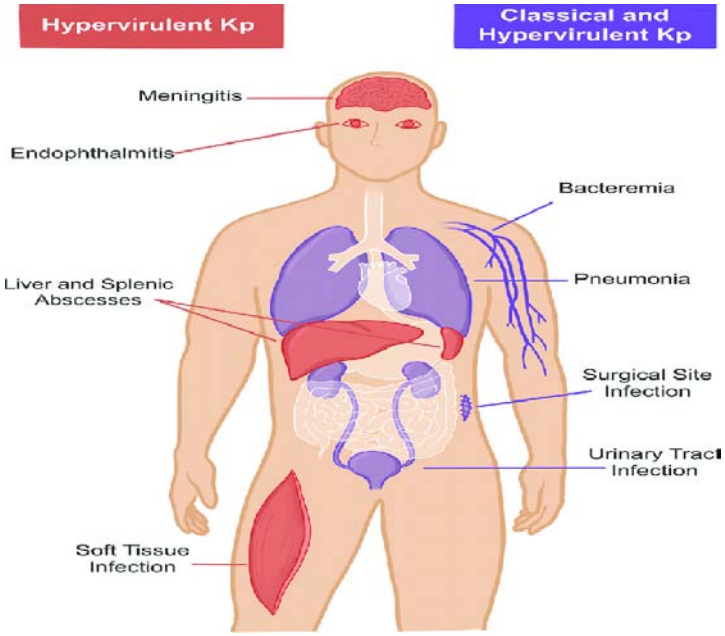


FIG : 12 – Common infections by *Klebsiella Pneumoniae species*

ANTIMICROBIAL RESISTANCE (AMR)

This *Klebsiella pneumoniae* is an opportunistic bacteria, known to cause infections in hospitalized & immunocompromised individuals, *K. pneumoniae* is known to have high resistance to a variety of broad spectrum antibiotics like beta-lactam group, fluoroquinolones and aminoglycosides.

β-lactam group of drugs are commonly prescribed worldwide (which include penicillins, cephalosporins, monobactams, and carbapenems). "The production of β-lactamase enzymes or the active expulsion of β-lactam molecules from *K. pneumoniae* represent the main indications of β-lactam antibiotic resistance" [9, 12,14,15].

Carbapenems are the group of antimicrobials prescribed for the treatment of infections caused by ESBL *K. pneumoniae isolates*. Now carbapenem resistance is on the rise, and a well documented information in other studies now.

"Carbapenemase resistant *K. pneumoniae* (CRKP) have developed due to the production of β-lactamases, efflux pumps, and mutations that alter the expression and/or function of porins and penicillin-binding proteins (PBPs)" [15,19].

Antimicrobial resistance (AMR) occur by horizontal gene transfer, for spread of transmissible plasmids, acquisition of resistance genes (which carry virulence factors).

For this bacteria to survive, the acquisition of resistance and virulent traits is necessary. Some reports suggest they have a role in the pathogenesis of *K.pneumoniae* infections.

“Capsule, lipopolysaccharide (LPS), fimbriae (types 1 and 3), and siderophores are virulence factors that mainly contribute to the pathogenicity of *K. pneumoniae”*. [12, 15, 19]

During 1980s *K. pneumoniae* became the index species for plasmids-encoding ESBLs, conferring resistance to expanded-spectrum cephalosporins. Initially, they were Temoniera (TEM)- and SHV type ESBLs and coexisted on the plasmids with elements encoding resistance to aminoglycosides, tetracyclines and trimethoprim-sulfamethoxazole. The 1990s marked the emergence of a new ESBL family, the cefotaximase-M (CTX-M) group, which are currently the dominant ESBLs in *K. pneumoniae*. These enzymes confer resistance to penicillins and expanded spectrum cephalosporins but are ineffective against carbapenems”.[10]

From 2015 carbapenem-resistant *K. pneumoniae* (CRKP) has emerged in several countries.
Many of the antibiotic-resistant genes (mobile pool of virulence and antimicrobial resistance genes) was 1st described in *Klebsiella* as “*K. pneumoniae* mobilome” and they encompass plasmids that harbor antibiotic resistance genes (ARG`s) and transposons.[40]
Very few options are left for treating the patients infected with *MDR K. pneumoniae* - limited to combination therapy / colistin.

Table 5 :**Antibiotic resistance associated genes**

Antibiotic resistance associated genes	**Genes Of Importance**
ESBL-associated genes	blaSHV, blaTEM, blaCTX-M-1, blaOXA 48
Genes conferring resistance to quinolones	aac (6)- Ib-cr, qnrB
For aminoglycosides	aad b
For sulfonamides	sul 1 and sul 2

The history of polymyxin resistance in *K. pneumoniae* is shorter compared to other classes
of antibiotics, owing to restricted use in human medicine between 1980s and 2000s, due to recognized toxicity.[37]

Tigecycline is the first glycylcycline launched, in use against *K. pneumoniae* infections from 2005. Tigecycline was considered a promising drug, with broad-spectrum activity even against ESBL-producing strains, but an MDR *K. pneumonia*

strain possessing decreased tigecycline susceptibility (MIC > 4 *μ*g/mL) was isolated at one of the hospital.[37]
The global emergence of multidrug-resistant (MDR) *Klebsiella* strains significantly reduces the available options to treat the infections.

Whole-genome sequences of *K. pneumoniae* CN1, NY9, and CR14 :

"Three *K. pneumoniae* isolates, CN1, NY9, and CR14, were confirmed to carry both bla_{CTX-M} and bla_{KPC} genes. CN1 and NY9 were recovered from two patients in New York City in 2013, whereas CR14 was cultured from a WMC patient in 2012. To obtain complete genome sequence for structural analysis, they employed both short- and long-read sequencing and conducted *de novo* assembly".[37]

Study Shows Promising Therapy for *Drug-Resistant Klebsiella Pneumoniae* : A promising strategy to tame troublesome drug-resistant bacteria is bacteriophage, or phage therapy, which uses viruses instead of antibiotics. NIH scientists have used two different bacteriophage viruses individually and then together to successfully treat research mice infected with multidrug-resistant Klebsiella pneumoniae sequence type 258 (ST258).

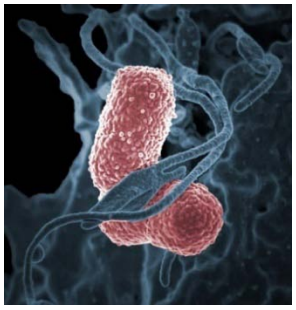


FIG : 13 - Colorized scanning electron micrograph showing carbapenem-resistant Klebsiella pneumoniae interacting with a human neutrophil. (**PHOTO: NIAID – NATIONAL INSTITUTE FOR ALLERGY & INFECTIOUS DISEASES)** K. pneumoniae ST258 is included on a CDC list of biggest antibiotic resistance threats in the U.S (because of high rates of morbidity and mortality).

MATERIALS AND METHODS

Materials and methods:

This was a Cross sectional observational study. Conducted in the Department of Microbiology at Central Diagnostics Laboratory Services (CDLS), R.L. Jalappa Hospital and Research Centre, Tamaka, Kolar. Study done from October 2019 to May 2021, for about 1.6 years.

SAMPLE SIZE: 150 *Klebsiella pneumoniae isolates*

Inclusion criteria:

- All *Klebsiella pneumoniae species* isolated from various clinical samples were included in our study.

Exclusion criteria :

- Patient who refused to give the informed consent for the study.
- *Klebsiella pneumoniae* isolated from stool samples were excluded from the study.

A total of **150 *Klebsiella pneumoniae* species isolated from different clinical samples** :*K. pneumoniae* isolated from Pus sample (n = 51), respiratory samples – sputum (n = 22) & ET samples (n = 43),Blood culture (n = 15), Urine (n = 10) and Body Fluids (n = 5), miscellaneous samples (n = 3) like vaginal sample, central line sample, ear/eye samples etc.

The clinical isolates were determined for the following virulence factors as mentioned below:

- HEMOLYSIS
- CAPSULE FORMATION
- HYPERMUCOVISCOSITY
- BIOFILM FORMATION
- SIDEROPHORE PRODUCTION
- PROTEASE

- GELATINASE
- HAEMAGGLUTINATION ASSAY
- LIPASE
- LECITHINASE ACTIVITY

METHODOLOGY FOR DETECTION OF VIRULENCE FACTORS:

HEMOLYSIS :*Klebsiella pneumoniae* colonies were inoculated on routine sheep blood agar at 37^0 C & then looked for hemolysis around the colonies. [5]

NON –HEMOLYTIC SAMPLES ON BLOOD AGAR

FIG : 14

DETECTION OF CAPSULE : Overnight incubated *Klebsiella pneumoniae* colony was taken on a clean slide, smear stained with methylene blue for 2 mins, then washed with tap water & then a drop of India ink stain was added, with a cover slip on the smear was examined under light microscope.[5]

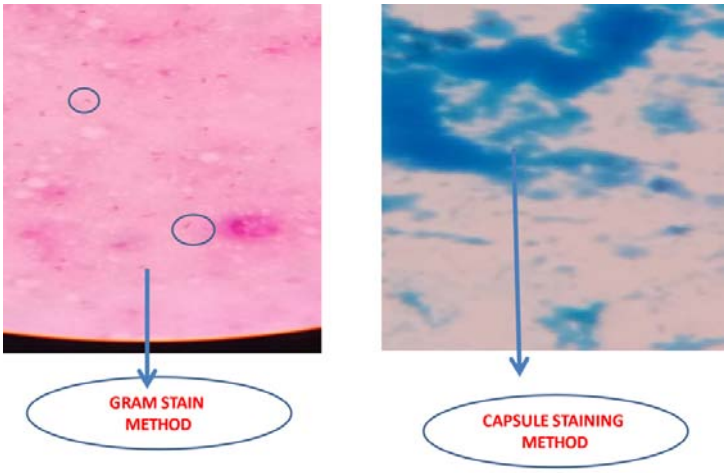


FIG : 15

HYPER MUCOVISCOSITY [HV] : **MODIFIED STRING TEST**

Klebsiella pneumoniae isolate was inoculated on routine sheep blood agar media, incubated at 37^0c for 24 hours. Then a standard inoculation loop was used to demonstrate the string test by touching the colony and gently lifting it. [5,8,20]

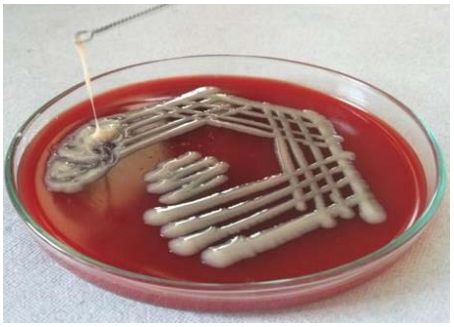


FIG : 16 - An observation of > 5mm length of string formation was taken as positive for hypermucoviscosity. [7,22]

BIOFILM FORMATION :

96 well Microtitre plate method is commonly used to demonstrate the biofilm formation by *Klebsiella pneumoniae isolates* in various samples.

METHOD : 200µl of overnight luria broth (LB) culture was transferred to a 96 well micro titre plate in triplets. Isolates sub cultured on Lysogeny Broth was incubated for 24 hr at 37^0 C. Further 25µl of 1% crystal violet was added to each well and incubated for 15 mins at room temperature. Wells were washed thrice with phosphate buffer solution (PBS) and ethanol was added to dissolve the strain. [8,18, 23, 34,35]

BIO FILM TESTING :

FIG : 17

Interpretation of Biofilm production : After testing the isolates, microtitre plate was assessed by looking at the intensity of coating of the wells with crystal violet stain. As strong positive / negatively stained wells. [34,35]

SIDEROPHORES :

Nutrient agar supplemented with 200mM of 2.2 –dipyridyl is used as Iron – restricted agar medium. *Klebsiella pneumoniae* isolates will be streaked on agar plates & then incubated at 37^{o} C for 24 hrs. Any bacterial growth will be considered positive for Siderophore production.[11, 18]

SIDEROPHORE TEST

CHEMICALS USED FOR THE TEST

POSITIVE & NEGATIVE ISOLATES SHOWN IN THIS PICTURE

FIG : 18

Media preparation for siderophore test :[11]

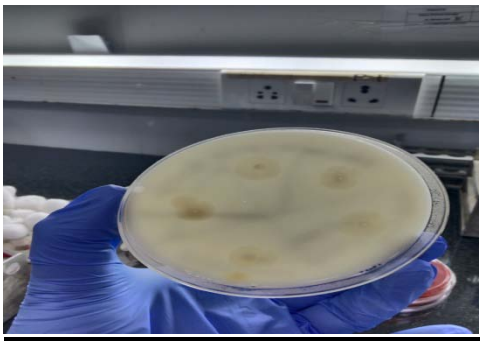
a) Solution-1 (prepared by dissolving 3 g Na2HPO3, 1.5 g KH2PO4, 0.5 g NH4Cl and 10 g agar in 475 ml of distilled water), then sterilized in autoclave at 121°C.

b) Solution- 2 (prepared as followed 2 ml of (1M) MgSO4, 10 ml of (20%) glucose, 0.1 ml of (1M) CaCl2 sterilized by filtration).

Solution 2 was added to solution 1 (after cooling to 50°C), then 0.01562 g of (200) µm of Dipyridyl added. Overnight-activated isolates inoculated to this media and incubated in 37°C for 24 hr, the results based on the presence of growth or not.

PROTEASE :

Klebsiella pneumoniae was inoculated on freshly prepared milk agar and incubated at 37^0 c for 72 hrs, formation of turbidity around the colonies – demonstrate protease production.[7]



Interpretation of the test : Development of any growth at stab inoculated sites were considered as positive & no growth at the inoculation were considered as negative for the test.

GELATINASE :

Nutrient agar plate with 3% gelatin was prepared, colony was inoculated & incubated for 16 hrs at 37^0 C, then extra 5 hrs in refrigeration (at 4^0c). A zone of turbidity around the colonies was taken as positive samples.[7]

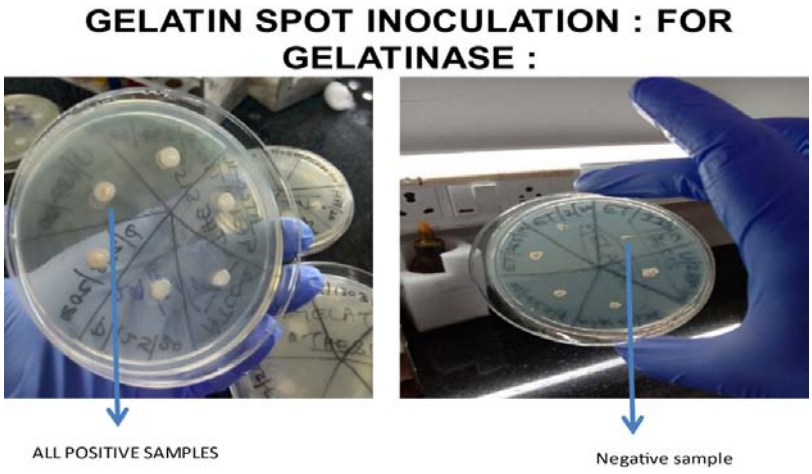


FIG : 20

Interpretation of the test : Development of any growth at stab inoculation sites were considered as positive & no growth at the inoculation were considered as negative for the test.

HAEMAGGLUTINATION ASSAY :

d- MANNOSE by slide METHOD: The isolate of *Klebsiella pneumoniae* was inoculated in nutrient broth and incubated at 37^0C for 48 hours (was allowed for full fimbriation). Then O+ve blood was collected and washed thrice in normal saline and 3% suspension was made in fresh saline. This was used immediately or (within a week, if stored at 3 -5^0C temperature). This test was carried out on a multiple concavity slide.

a) **Mannose sensitive haemagglutination:** was detected by absence of haemagglutination, when a drop of 2% d-mannose was added to red cells and drop of broth culture.

b) **Mannose resistant haemagglutination:** was detected by presence of haemagglutination of 3% O -blood group human RBC in presence of 2% mannose.[6, 13]

Haemagglutination Test : with D- mannose sugar & O + ve pooled blood suspension. RBC`s are crenated, but not hemagglutinated, as there is no clumping

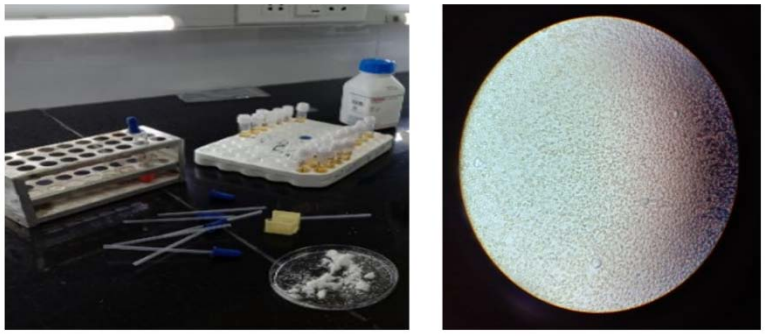


FIG : 21

LIPASE ACTIVITY :

Klebsiella pneumoniae isolate grown on blood agar was re-inoculated on egg-yolk agar & was incubated at 37^0 C for one day. Then plates were flooded with copper sulphate solution for 20 mins, removed the excess solution and plate was allowed to dry. If opalesence of greenish blue colour developed it was taken as positive for lipase activity.[7]

PREPARATION OF MEDIA FOR LIPASE & LECITHINASE



LECITHINASE ACTIVITY :

Klebsiella pneumoniae isolated from nutrient agar was re-inoculated on egg-yolk agar, incubated for 37^0 C for 24 hrs then the plates were sprayed with copper sulphate solution for 20 mins, drain off the excess solution & plate was allowed to dry.

Interpretation of Lipase & Lecithinase test : If there is zone of clearance around the colonies it was considered as positive for this test. For lipase production – development of bluish green type of colonies even after removing excess copper sulphate were considered as positive test results. [7]

METHODOLOGY FOR DETECTION OF ANTIMICROBIAL RESISTANCE:

According to **Kirby Bauer Disk diffusion method**, a lawn culture of *Klebsiella pneumonia* will be done on Mueller Hinton Agar [MHA] and antibiotic discs will be placed and incubated for 18- 24 hours. Based on the zone size, the isolates will be classified as sensitive, moderately sensitive and resistance as per the CLSI [Clinical and Laboratory Standards Institute] guidelines.[22]

The gram negative antibiotic panel for routine antibiotic susceptible testing will be as follows:

1. Piperacillin, Piperacillin –Tazobactam, Ampicillin, Amoxicillin- Clavulanate, Gentamicin, Amikacin, Tobramycin, Cefotaxime, Ceftriaxone, Ceftazidime, Ceftazidime -Clavulanic Acid, Cefoxitin, Ciprofloxacin, Levofloxacin, Imipenem, Meropenem, Ertapenem, Trimethoprim –Sulfamethoxazole (COT), Chloramphenicol, Doxycycline, Nitrofurantoin & Norfloxacin [For Urine Sample Only]

KPC positive samples: detected by **KPC Hi Media agar**, when blue- green colonies are produced on culture plate it will be considered as positive for Carbapenem resistance.[8]

***Klebsiella* pneumoniae on KPC plate :**

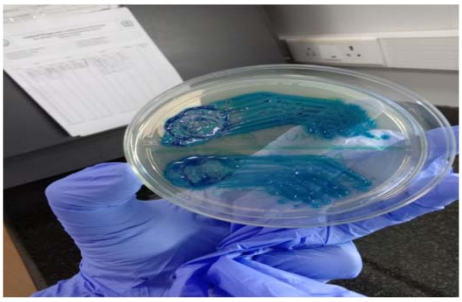


FIG : 23

STATISTICAL INPUTS : In our study only descriptive statistics was used, Pearson Chi square tried for p-value with type of samples to virulence factors – nothing significant, but based on gender association with virulence factors – HMV (Chi square = 0.488, p = 0.485), siderophore (Chi square = 1.220, p = 0.543), milk agar for protease (Chi square = 0.033, p = 0.857), for gelatinase test (Chi square = 0.188, p = 0.665), for lipase test (Chi square = 1.657, p = 0.198), for lecithinase test (Chi square = 0.299, p = 0.585), for biofilm production (Chi square = 7.944, p = 0.005).

RESULTS

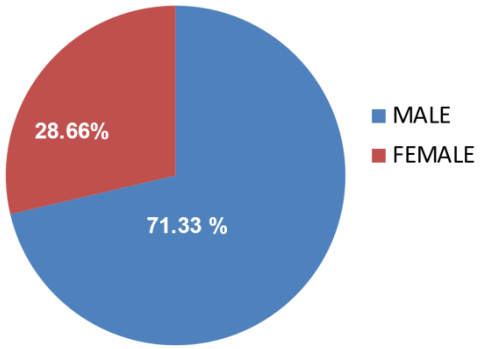


Table 6: Gender wise distribution

Gender	No: of patients (%), n = 150
Male	107 (71.33 %)
Female	43 (28.67 %)

Table 7: Age wise distribution of *Klebsiella pneumoniae* isolates in the study

Age distribution	Distribution of isolates based on age of the patients (n=150), (%)
0 - 10 yrs	12 (8 %)
11 – 20 yrs	8 (5.33 %)
21 - 30 yrs	16 (10.66 %)
31 - 40 yrs	18 (12.0 %)
41 - 50 yrs	24 (16.0 %)
51 – 60 yrs	25 (16.66 %)
61 – 70 yrs	28 (18.67 %)
71 – 80 yrs	16 (10.66 %)
More than 81 yrs	3 (2.0 %)

Table 8: **Type of samples collected in our study**

Type of samples	**No: of isolates (%)**
Pus sample	51 (34 %)
Endotracheal tube (ET) sample	43 (28.67 %)
Sputum	22 (14.66 %)
Blood culture	15 (10.0 %)
Urine	10 (6.67 %)
Fluids (CSF, pleural, Peritoneal, Synovial fluids)	5 (3.33 %)
Miscellaneous (Ear, eye swab, vaginal sample)	4 (2.67 %)

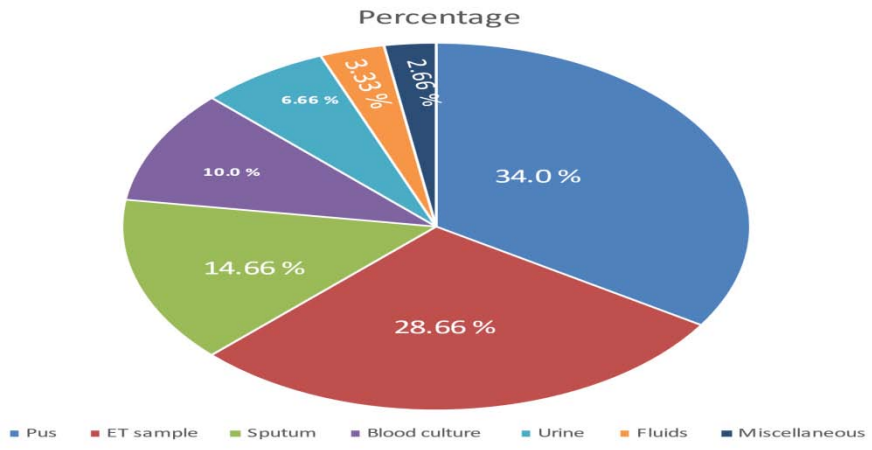


Table 9 :Distribution of *Klebsiella pneumoniae* samples positive for virulence factors

S. No	Detection of various virulence factors by phenotypic methods	*Klebsiella pneumoniae* samples positive for virulence factors (%)
1	Hemolysis	7 (4.66 %)
2	Capsule	150 (100.0 %)
3	Hypermucoviscosity (HMV)	66 (44.0 %)
4	Biofilm Formation	81 (54.0 %)
5	Siderophore Production	110 (73.33 %)
6	Protease	135 (90.0 %)
7	Gelatinase Test	126 (84.0 %)
8	Lipase	119 (79.33 %)
9	Lecithinase Activity	82 (54.66 %)

Table 10 : Antibiogram pattern of *Klebsiella pneumoniae* samples

Drug Name	No: of Resistant Isolates (%)
β- lactam group of drugs	
Piperacillin (PI)	136 [90.66 %]
Piperacillin & Tazobactam (PIT)	101 [67.33 %]
Ampicillin (AMP)	150 [100.0 %]
Amoxicillin & clavulanic acid (AMC)	136 [90.66 %]
Cefotaxime (CTX)	126 [84.0 %]
Ceftriaxone (CTR)	124 [82.66 %]
Ceftazidime (CAZ)	124 [82.66 %]
Ceftazidime with clavulanic acid (CAC)	107 [71.33 %]
Cefoxitin (CX)	101 [67.33 %]
Aminoglycosides	
Gentamicin (GEN)	98 [65. 33 %]
Tobramycin (TOB)	108 [72. 0 %]
Amikacin (AK)	99 [66. 0 %]
Fluoroquinolones	
Ciprofloxacin (CIP)	113 [75. 33 %]
Levofloxacin (LE)	106 [70. 66 %]
Carbapenem group	
Imipenem (IMP)	95 [63.33 %]
Meropenem (MRP)	105 [70.0 %]
Ertapenem (ETP)	94 [62.66 %]
Others	
Cotrimoxazole (COT)	107 [71.33 %]
Chloramphenicol (C)	58 [38.66 %]
Doxycycline (DOX)	119 [79. 33 %]

Table 11 : Types of antimicrobial pattern in our study

Types of antimicrobial pattern in our study	No: of isolates (%) , n = 150
ESBL type	25 (16.67 %)
AmpC producers	22 (14.67 %)
MDR	116 (74.20 %)
XDR	30 (20.0 %)
PDR	42 (28.0 %)
Imipenem (IMP) resistance	95 (63.33 %)
Meropenem (MRP) resistance	105 (70.0 %)
Ertapenem (ETP) resistance	94 (62.66 %)

Note : "Lists of antimicrobial categories proposed for antimicrobial susceptibility testing were created using documents and breakpoints from (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Food & Drug administration, USA (FDA).

1. MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories,

2. XDR was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and

3. PDR was defined as non-susceptibility to all agents in all antimicrobial categories". [50]

ASSOCIATION BETWEEN EACH VIRULENCE FACTORS & ANTIMICROBIAL RESISTANCE OBSERVED (Tables : 12 – 20)

Table : 12 Association between antibiotic resistance & Hemolysis (n = 7)

DRUG NAME	DRUG RESISTANCE (NO: OF ISOLATES), (%)
PI (Piperacillin)	5 (71.42 %)
PIT (Piperacillin & Tazobactam)	2 (28.57%)
AMP (Ampicillin)	7 (All resistant)
AMC (Amoxicillin & clavulanic acid)	5 (71.42%)
CTX (Cefotaxime)	4 (57.14%)
CX (Cefoxitin)	1 (14.28 %)
CTR (Ceftriaxone)	3 (42.85 %)
CAC (Ceftazidime with clavulanic acid)	3 (42.85 %)
CAZ (Ceftazidime)	3 (42.85 %)
IMP (Imipenem)	1 (14.28 %)
MRP (Meropenem)	2 (28.57 %)
ETP (Ertapenem)	1 (14.28 %)
AK (Amikacin)	2 (28.57 %)
GEN (Gentamicin)	3 (42.85 %)
TOB (Tobramycin)	3 (42.85 %)
CIP (ciprofloxacin)	3 (42.85 %)
LE (levofloxacin)	3 (42.85 %)
DOX (Doxycycline)	3 (42.85 %)
C (Chloramphenicol)	2 (28.57 %)
COT (Cotrimoxazole)	3 (42.85 %)

Table : 13 Association between Capsule production & antibiotic resistance (n = 150)

DRUG NAME	DRUG RESISTANCE (NO: OF ISOLATES), (%)
PI (Piperacillin)	136 [90.66 %]
PIT (Piperacillin & Tazobactam)	101 [67.33 %]
AMP (Ampicillin)	150 [100.0 %]
AMC (Amoxicillin & clavulanic acid)	136 [90.66 %]
CTX (Cefotaxime)	126 [84.0 %]
CX (Cefoxitin)	101 [67.33 %]
CTR (Ceftriaxone)	124 [82.66 %]
CAC (Ceftazidime with clavulanic acid)	107 [71.33 %]
CAZ (Ceftazidime)	124 [82.66 %]
IMP (Imipenem)	95 [63.33 %]
MRP (Meropenem)	105 [70.0 %]
ETP (Ertapenem)	94 [62.66 %]
AK (Amikacin)	99 [66. 0 %]
GEN (Gentamicin)	98 [65. 33 %]
TOB (Tobramycin)	108 [72. 0 %]
CIP (ciprofloxacin)	113 [75. 33 %]
LE (levofloxacin)	106 [70. 66 %]
DOX (Doxycycline)	119 [79. 33 %]
C (Chloramphenicol)	58 [38.66 %]
COT (Cotrimoxazole)	107 [71.33 %]

Table : 14 Association between siderophore production & antibiotic resistance (n = 110)

DRUG NAME	DRUG RESISTANCE (NO: OF ISOLATES), (%)
PI (Piperacillin)	100 (90.91 %)
PIT (Piperacillin & Tazobactam)	76 (69.09 %)
AMP (Ampicillin)	100 %
AMC (Amoxicillin & clavulanic acid)	102 (92.72 %)
CTX (Cefotaxime)	93 (84.54 %)
CX (Cefoxitin)	75 (68.18 %)
CTR (Ceftriaxone)	92 (83.63 %)
CAC (Ceftazidime with clavulanic acid)	80 (72.73 %)
CAZ (Ceftazidime)	91 (82.72 %)
IMP (Imipenem)	68 (61.81 %)
MRP (Meropenem)	78 (70.91 %)
ETP (Ertapenem)	71 (64.54 %)
AK (Amikacin)	76 (69.09 %)
GEN (Gentamicin)	74 (67.27 %)
TOB (Tobramycin)	80 (72.73 %)
CIP (ciprofloxacin)	84 (76.36 %)
LE (levofloxacin)	80 (72.73 %)
DOX (Doxycycline)	86 (78. 18 %)
C (Chloramphenicol)	42 (38. 18 %)
COT (Cotrimoxazole)	80 (72.73 %)

Table : 15 Association between hyper mucoviscosity & antibiotic resistance (n = 66)

DRUG NAME	DRUG RESISTANCE (NO: OF ISOLATES), (%)
PI (Piperacillin)	59 (89.39 %)
PIT (Piperacillin & Tazobactam)	43 (65.15 %)
AMP (Ampicillin)	100 %
AMC (Amoxicillin & clavulanic acid)	59 (89.39 %)
CTX (Cefotaxime)	55 (83.33 %)
CX (Cefoxitin)	41 (62.12 %)
CTR (Ceftriaxone)	55 (83.33 %)
CAC (Ceftazidime with clavulanic acid)	44 (66.67 %)
CAZ (Ceftazidime)	54 (81.81 %)
IMP (Imipenem)	38 (57. 57%)
MRP (Meropenem)	43 (65. 15 %)
ETP (Ertapenem)	39 (59. 09 %)
AK (Amikacin)	42 (63. 63 %)
GEN (Gentamicin)	41 (62. 12 %)
TOB (Tobramycin)	46 (69. 69 %)
CIP (ciprofloxacin)	50 (75. 76 %)
LE (levofloxacin)	46 (69.70 %)
DOX (Doxycycline)	47 (71.21 %)
C (Chloramphenicol)	22 (33.33 %)
COT (Cotrimoxazole)	44 (66. 67 %)

Table : 16 Association between Biofilm formation & antibiotic resistance (n = 81)

DRUG NAME	DRUG RESISTANCE (NO: OF ISOLATES), (%)
PI (Piperacillin)	75 (92. 59 %)
PIT (Piperacillin & Tazobactam)	54 (66. 66 %)
AMP (Ampicillin)	100 %
AMC (Amoxicillin & clavulanic acid)	76 (93. 82 %)
CTX (Cefotaxime)	68 (83. 95 %)
CX (Cefoxitin)	53 (65. 43 %)
CTR (Ceftriaxone)	67 (82. 71 %)
CAC (Ceftazidime with clavulanic acid)	56 (69. 13 %)
CAZ (Ceftazidime)	67 (82. 71 %)
IMP (Imipenem)	48 (59. 25 %)
MRP (Meropenem)	55 (67. 90 %)
ETP (Ertapenem)	50 (61. 72 %)
AK (Amikacin)	56 (69. 13 %)
GEN (Gentamicin)	54 (66. 67 %)
TOB (Tobramycin)	58 (71. 60 %)
CIP (ciprofloxacin)	59 (72. 83 %)
LE (levofloxacin)	53 (65. 43 %)
DOX (Doxycycline)	63 (77. 78 %)
C (Chloramphenicol)	28 (34. 56 %)
COT (Cotrimoxazole)	59 (72. 83 %)

Table : 17 Association between Protease & antibiotic resistance (n = 135)

DRUG NAME	DRUG RESISTANCE (NO: OF ISOLATES), (%)
PI (Piperacillin)	121 (89.62 %)
PIT (Piperacillin & Tazobactam)	90 (66.67 %)
AMP (Ampicillin)	100 %
AMC (Amoxicillin & clavulanic acid)	122 (90.37 %)
CTX (Cefotaxime)	111 (82.22 %)
CX (Cefoxitin)	91 (67.40 %)
CTR (Ceftriaxone)	110 (81.48 %)
CAC (Ceftazidime with clavulanic acid)	94 (69. 62 %)
CAZ (Ceftazidime)	111 (82.22 %)
IMP (Imipenem)	87 (64.44 %)
MRP (Meropenem)	94 (69.62 %)
ETP (Ertapenem)	84 (62. 22 %)
AK (Amikacin)	87 (64.44 %)
GEN (Gentamicin)	85 (62.96 %)
TOB (Tobramycin)	95 (70.37 %)
CIP (ciprofloxacin)	100 (74.07 %)
LE (levofloxacin)	94 (69.62 %)
DOX (Doxycycline)	105 (77.78 %)
C (Chloramphenicol)	51 (37.78 %)
COT (Cotrimoxazole)	97 (71.85%)

Table : 18 Association between Lipase & antibiotic resistance(n = 119)

DRUG NAME	DRUG RESISTANCE (NO: OF ISOLATES), (%)
PI (Piperacillin)	108 (90.75 %)
PIT (Piperacillin & Tazobactam)	82 (68. 90 %)
AMP (Ampicillin)	100 %
AMC (Amoxicillin & clavulanic acid)	106 (89. 07 %)
CTX (Cefotaxime)	99 (83. 19 %)
CX (Cefoxitin)	82 (68. 90 %)
CTR (Ceftriaxone)	98 (82. 35 %)
CAC (Ceftazidime with clavulanic acid)	85 (71. 42 %)
CAZ (Ceftazidime)	98 (82. 35 %)
IMP (Imipenem)	76 (63. 86 %)
MRP (Meropenem)	81 (68. 06 %)
ETP (Ertapenem)	73 (61. 34 %)
AK (Amikacin)	79 (66. 38 %)
GEN (Gentamicin)	77 (64. 70 %)
TOB (Tobramycin)	85 (71. 42 %)
CIP (ciprofloxacin)	87 (73. 10 %)
LE (levofloxacin)	80 (67. 22 %)
DOX (Doxycycline)	93 (78. 15 %)
C (Chloramphenicol)	45 (37. 81 %)
COT (Cotrimoxazole)	84 (70. 58 %)

Table : 19 Association between Lecithinase & antibiotic resistance (n = 82)

DRUG NAME	DRUG RESISTANCE (NO: OF ISOLATES), (%)
PI (Piperacillin)	74 (90. 24 %)
PIT (Piperacillin & Tazobactam)	58 (70. 73 %)
AMP (Ampicillin)	
AMC (Amoxicillin & clavulanic acid)	75 (91. 46 %)
CTX (Cefotaxime)	70 (85. 36 %)
CX (Cefoxitin)	54 (65. 85 %)
CTR (Ceftriaxone)	70 (85. 36 %)
CAC (Ceftazidime with clavulanic acid)	59 (71. 95 %)
CAZ (Ceftazidime)	69 (84. 14 %)
IMP (Imipenem)	55 (67. 07 %)
MRP (Meropenem)	55 (67. 07 %)
ETP (Ertapenem)	50 (60. 97 %)
AK (Amikacin)	57 (69. 51 %)
GEN (Gentamicin)	56 (68. 29 %)
TOB (Tobramycin)	62 (75. 60 %)
CIP (ciprofloxacin)	60 (73. 17 %)
LE (levofloxacin)	59 (71. 95 %)
DOX (Doxycycline)	63 (76. 82 %)
C (Chloramphenicol)	33 (40. 24 %)
COT (Cotrimoxazole)	60 (73. 17 %)

Table : 20 Association between Gelatinase & antibiotic resistance (n = 126)

DRUG NAME	DRUG RESISTANCE (NO: OF ISOLATES), (%)
PI (Piperacillin)	115 (91.26 %)
PIT (Piperacillin & Tazobactam)	83 (65.87 %)
AMP (Ampicillin)	150 (100 %)
AMC (Amoxicillin & clavulanic acid)	113 (89.68 %)
CTX (Cefotaxime)	105 (83.33 %)
CX (Cefoxitin)	83 (65.87 %)
CTR (Ceftriaxone)	103 (81.74 %)
CAC (Ceftazidime with clavulanic acid)	88 (69.84 %)
CAZ (Ceftazidime)	104 (82.53 %)
IMP (Imipenem)	81 (64.28 %)
MRP (Meropenem)	88 (69.84 %)
ETP (Ertapenem)	77 (61.11 %)
AK (Amikacin)	81 (64.28 %)
GEN (Gentamicin)	81 (64.28 %)
TOB (Tobramycin)	89 (70.63 %)
CIP (ciprofloxacin)	95 (75.39 %)
LE (levofloxacin)	87 (69.05 %)
DOX (Doxycycline)	98 (77.78 %)
C (Chloramphenicol)	52 (41.26 %)
COT (Cotrimoxazole)	89 (70.63 %)

DISCUSSION

DISCUSSION :

K. pneumoniae strains produce a variety of infections and employs various virulence factors to colonize and spread in the human body. The exploding antimicrobial resistance of this bacteria in recent years is of great concern to the scientific world.

Pathogenicity of *K. pneumoniae* is the result of production of many virulence factors that help these bacteria overcome the immune system and cause various diseases.

In our study, we collected 150 clinical *K. pneumoniae strains* from Pus sample, respiratory samples, Blood culture, Urine and Body Fluids.

The following 10 virulence factors were analysed in our study – Hemolysis, Capsule Formation, Hypermucoviscosity, Biofilm Formation, Siderophore Production, Protease, Gelatinase, Haemagglutination Assay, Lipase & Lecithinase Activity.

The *Klebsiella pneumoniae* isolates from our study producing capsule was 100%, this is in concordance with other study findings – Aljanaby et al, Imtiaz et al & AL -A amary et al. *Klebsiella pneumoniae has* thick polysaccharide capsule (this provides protection to bacteria against opsonization and phagocytosis by macrophages and neutrophils), due to blockade of Toll like receptors (TLR-4) & inhibit the expression of IL -8. [18,32,40]

High virulence is typical for *K. pneumoniae strains* that cause community-acquired infections, LPS protects against humoral defences and activate immune system.

The *Klebsiella pneumoniae* isolates producing siderophore was 73.33% is our study results, (Siderophore production is more from endo tracheal & pus samples in our study). This is in contrast to other studies which reported 100% in – Aljanaby et al & 100% siderophore from AL -A amary et al. [18]

Klebsiella pneumoniae strains produce siderophores (are iron chelating molecules from host cells). Types of siderophores—aerobactin, enterobactin, yersiniabactin, and colibactin for growth and survival in an infected host. Siderophoresare critical for virulence, Iron being an essential component for bacterial growth and replication, this indicates invasive properties of *K. pneumoniae* in an infection in immunocompetent people. It has been shown that the development of invasive forms of the disease is associated with the production of several siderophores by K. pneumoniae strains. (Hv-KP strains produce more siderophore molecules and in their more active form, than c-KP).

The *Klebsiella pneumoniae* isolates producing Hypermucoviscosity (HMV) detected by positive string test was 44 % in our study. The study conducted by other investigators on HMV produced from *Klebsiella pneumoniae* varies from 7 – 62 %, Aljanaby et al reported 62 %, Lee et al studied extensively on HMV – showed 14.8 % from hospital acquired *Klebsiella pneumoniae* infections & 41.5% from community acquired *Klebsiella pneumoniae* infections. But in contrast Rama Krishnan et al study done in 2019 reported only 7% and Imtiaz et al reported 22%. [5, 18, 20,32]

Detection of the rmp A gene in *K. pneumoniae isolates*. Hv-KP causes the most severe, invasive forms of infection in previously healthy adults (liver abscesses, meningitis, and endophthalmitis). hv-KP strains are more resistant to the complement and phagocytosis.

The *Klebsiella pneumoniae* isolates from our study producing lipase was 79.33%, this is in contrast to Rama Krishnan et al study showed 58 %, Hassan. R et al reported 33 % of their isolates positive for lipase. [5]

In our study the *Klebsiella pneumoniae* isolates producing biofilm are 54 % (n = 81), our study findings are in contrast to Aljanaby et al reported 100%, Imtiaz et al – showed 94% results, AL -A amary et al showed 75% & Rama Krishnan et al study showed 79 %. Our study findings are more consistent to Hassan et al findings of 67 %. In contrast a study Dougnan et al (2021) reported with only 3.34 % of biofilm formation in *Klebsiella pneumoniae isolates.* [5,18, 32, 42]

Our findings on hemolysis was 4.66 % this is in concordance with other findings of Imtiaz et al – showed 8 % & Hassan R et al with 7 %. Our findings are in contrast to Dougnan et al which reports 20 % for hemolysis. [32]
In our study the *Klebsiella pneumoniae* isolates producing lecithinase enzyme as virulence factor 54.66 % (n =82), this is in concordance with Rama Krishnan et al study showed 55 %. There are very few studies done on this virulence factor, our study is in contrast to Dougnan et al (2021) showed 3.34 %. [5, 18,42]
In our study the *Klebsiella pneumoniae* isolates producing protease enzyme was 90% (n =135), this is in contrast to earlier studies like Rama Krishnan et al study showed 44% & Hassan R et al which reported only 2.56 % (with only 1 isolate). [5]

In our study the *Klebsiella pneumoniae* isolates producing gelatinase enzyme on gelatin media was 84 % (n = 126), this findings are in contrast to Rama Krishnan et al study showed 41% & Imtiaz et al – showed 12 % only. In contrast we had higher % of gelatinase reported with *K.pneumoniae* isolates. [5, 32]

The antibiotic resistance of *K. pneumoniae* strains is associated mainly with the production of ESBL. In 2017 the World Health Organization included ESBL-

producing *K. pneumoniae* in the list of the most dangerous superbugs along with *Acinetobacter baumanii* and *Pseudomonas aeruginosa.*

This is of great concern because some antibiotic resistance are carried by mobile genetic elements that facilitate horizontal genetic exchange and promote the spread of antimicrobial resistance within and between species.

The information from the 2017 European Antimicrobial Resistance Surveillance Network report showed more than one third of K. pneumoniae isolates were resistant to at least one of the antimicrobial groups under regular surveillance. The report showed resistance percentages for fluoroquinolones (31.5%), followed by third-generation cephalosporins (31.2%), aminoglycosides (24.1%), and carbapenems (7.2%).

Compared to the multicenter study carried out in Garcia Fernandez et al, our study findings out of 150 *K. pneumoniae isolates* showed : ESBL *K. pneumoniae 16.67 % (n = 25 isolates), AmpC producer type 14.67 % (n = 22), multi drug resistance (MDR) – 74.20 % (n = 116), extensive drug resistant (XDR) – 20 % (n = 30), pan drug resistant (PDR) – 28 % (n = 42).* [47,50]

Zhang et al reported *ESBL- K.pneumoniae* of 31.8 %, (range of 10.2 – 50.3 %) from different geographic locations of China. Abera et al reported *ESBL- K.pneumoniae*as 44.0 % in their study. A cohort study done by Xercavins et al in 2019 *showed* 52 % of ESBL hospital acquired infections. Majority of them were CTX-M 15 type reported. According to a meta analysis (Kahraman EP et al with 25 studies) conducted between 2000 – 2015 reported 39.66% ±12.46. [41]

In our study MDR isolates reported was 74.20 %, this is in contrast to a meta analysis done by Asri et al on *MDR K. pneumoniae* isolates was 32.8%, Our study is in concordance with Ferreira et al 2019 study based on genotypic analysis reported 84 % of *MDR Klebsiella pneumoniae* in their study. [43, 49]

In our study the XDR isolates were 20.0 %. Our findings are in concordance with Hassuna NA et al in 2020 showed 15.3 % of XDR isolates , and Wenzi Bi et al in 2017 showed 26.71. In their study XDR was resistant to all antibiotics except Tigecycline & Polymyxin B. In our study we have reported as resistance to all antimicrobials except chloramphenicol. [44, 45]

Our study findings on CRKP isolates were as follows : Imipenem resistance 63.33%, meropenem resistance 70% & ertapenem resistance 62.66%, Our study results are in contrast to studies Lombardi et al which reported 35% of CRKP isolates & Kahraman EP et al showed Imipenem resistance of 5.1% & meropenem resistance of 3.4 % respectively. [41]

The reasons for antimicrobial drug resistance in *Klebsiella pneumoniae* could be due to diminished expression / loss of porin channels, alteration in outer membrane proteins (OMP) permeability, or efflux pump overexpression & carbapenem hydrolysis.

KPCs also inactivate all beta-lactam antibiotics and are only partially inhibited by beta-lactamase inhibitors like clavulanic acid, tazobactam and boronic acid.

The association between virulence factors & antimicrobial resistance was not significant in our study, based on our observations.

SUMMARY :

Our study was done with 150 clinical samples of *Klebsiella pneumoniae isolated* during the period of Oct 2019 to May 2021 for a period of 1 year and 6 months received at the Dept of Microbiology, R. L. Jalappa hospital, Kolar, India.

The findings of our study on virulence factors are as follows :hemolysis 4.66 %(7 isolates), capsule 100 % (150 isolates), hypermucoviscosity (HMV) formation 44 % (66 isolates), biofilm production 54 % (81 isolates), siderophore production 73.33 % (110 isolates), protease 90 % (135 isolates), gelatinase 84 % (126 isolates), lipase production 79.33 % (119 isolates), lecithinase activity 54.66 % (82 isolates).

The antimicrobial resistance pattern as follows : Piperacillin (90.66 %), Piperacillin tazobactum (67.33 %), Ampicillin (100 %), Amoxyclav (90.66 %), Cefotaxime (84 %), Ceftriaxone (82.66 %), Ceftazidime (82.66 %), Ceftazidime-clavulanic acid (71.33 %), Cefoxitin (67.33 %), Cotrimoxazole (71.33 %), Imipenem (63.33 %), Meropenem (70.0 %), Ertapenem (62.66 %), Amikacin (66 %), Gentamicin (65.33 %), Tobramycin (72.0 %), Chloramphenicol (38.66 %), Doxycycline (79.33 %), Ciprofloxacin (75.33 %), Levofloxacin (70. 66 %), Norfloxacin (66.67 %), Nitrofurantoin (77.78 %).

ESBL *K. pneumoniae 16.67 % (n = 25 isolates), AmpC producer type 14.67 % (n = 22), multi drug resistance (MDR) – 74.20 % (n = 116), extensive drug resistant (XDR) – 20 % (n = 30), pan drug resistant (PDR) – 28 % (n = 42).* Total carbapenem resistance in our study : average 65.33 % reported.

CONCLUSION :

The presence of several virulence factors accompanied by antimicrobial resistance had made *Klebsiella pneumoniae* an important infectious agent of global concern and lead to treatment failure. The increasing coexistence of the virulence factors & antimicrobial is of particular concern as it can lead to untreatable and invasive *K. pneumoniae* infections. Active surveillance to be done not only for antimicrobial resistance, but also for virulence determinants to avoid the transmission and spread of multidrug resistant strains. In our study a lot of *MDR K. pneumoniae* strains were observed which warrants the role of antimicrobial stewardship programme, as It is vital & plays an important role in prevention & control of *MDR K. pneumoniae* strains.

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ANNEXURES

INFORMED CONSENT TO PARTICIPATE IN THE STUDY

DATE : **SERIAL NO :**

LAB NO:

TITLE :PHENOTYPIC CHARACTERIZATION OF VIRULENCE FACTORS AND ANTIBIOGRAM OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM DIFFERENT CLINICAL SPECIMENS – A CROSS SECTIONAL STUDY.

I have read / the investigator has read the purpose of this study. I understand that my clinical sample sent to CDLS lab, Sri Devaraj Urs Medical College, which yields *Klebsiella pneumonia*, will be analysed for virulence factors and antimicrobial resistance.

I am aware that there is no benefit to me, apart from the treatment I receive at the hospital, there is no compensation involved.

I have been informed this study is done for the betterment of mankind in future. Hence, I agree to participate in this study by my free will.

NAME [PATIENT]: **SIGNATURE / THUMB IMPRESSION**

NAME

[WITNESS / RELATIVE]: **SIGNATURE / THUMB IMPRESSION**

For any further clarification you can contact the study investigator:

Dr. Madhavi S Hullur

Mobile no: 9844746666.

Email:dr.msh@yahoo.com

PATIENT INFORMATION SHEET

SERIAL NO :

TITLE :PHENOTYPIC CHARACTERIZATION OF VIRULENCE FACTORS AND ANTIBIOGRAM OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM DIFFERENT CLINICAL SPECIMENS – A CROSS SECTIONAL STUDY.

PURPOSE OF THE STUDY: *Klebsiella pneumoniae* is a gram negative, non-motile, capsulated organism. It is ubiquitous in nature, known to cause community acquired and hospital acquired infection.

Nosocomial infections are caused by a variety of organisms. *Klebsiella pneumoniae* produces life threatening nosocomial infections.

With the emergence of multi drug resistance [MDR] clones and global dissemination of hypervirulent strains have renewed the interest in *Klebsiella pneumoniae.* The interplay between virulence factors and antibiotic resistance of *Klebsiella pneumoniae* strains will be studied further in our attempt.

PROCEDURE FOLLOWED: When *Klebsiella pneumoniae* is isolated from your clinical sample, patient demographic & clinical details will be collected as per a pre-designed proforma.

The *Klebsiella pneumoniae* isolates will be analysed for virulence factors and antimicrobial resistance.

RISKS AND BENEFITS: Participation in this study is purely voluntary. There is no risk involved in the study. You will be given appropriate treatment for your disease as per hospital norms, but you will not be given any compensation for participation in this study.

CONFIDENTIALITY: All information that you provide will be kept confidential, no mention of your name or other information will appear on samples or in any publication in connection to this study.

PROFORMA

Serial no: **LAB NO:**

TITLE: PHENOTYPIC CHARACTERIZATION OF VIRULENCE FACTORS AND ANTIBIOGRAM OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM DIFFERENT CLINICAL SPECIMENS - A CROSS SECTIONAL STUDY.

NAME:
AGE
SEX :
ADDRESS:
PHONE NO:
UHID NO:

CLINICAL DIAGNOSIS [As mentioned on Lab request form] :

SAMPLE TYPE COLLECTED :

CLINICAL ISOLATE COLLECTED :*KLEBSIELLA PNEUMONIAE*

The clinical isolates will be determined for the following virulence factors as mentioned below:

VIRULENCE FACTORS :		
• HEMOLYSIS		
• CAPSULE FORMATION		
• HYPERMUCOVISCOSITY		
• BIOFILM FORMATION		
• SIDEROPHORE PRODUCTION		
• PROTEASE		
• GELATINASE		
• HAEMAGGLUTINATION ASSAY		
• LIPASE		
• LECITHINASE ACTIVITY		
ANTIBIOTIC RESISTANCE PATTERN: • **<u>20 - disk plate</u>:** 1. AMPICILLIN [AMP] 2. GENTAMICIN [GEN] 3. AMIKACIN [AK] 4. AMOXICILLIN-CLAVULANATE [AMC] 5. PIPERACILLIN [PI] 6. PIPERACILLIN–TAZOBACTAM [PIT] 7. CEFOTAXIME [CTX]		

8. CEFTRIAXONE [CTR] 9. CEFTAZIDIME [CAZ] 10. CEFTAZIDIME –CLAVULANIC ACID [CAC] 11. CIPROFLOXACIN [CIP] 12. LEVOFLOXACIN [LE] 13. IMIPENEM [IMP] 14. MEROPENEM [MRP] 15. ERTAPENEM [ETP] 16. TRIMETHOPRIM – SULFAMETHOXAZOLE 17. CHLORAMPHENICOL [C] 18. TETRACYCLINE [TE] 19. TOBRAMICIN [TOB] 20. DOXYCYCLINE [DO] **<u>URINE</u> :** 1. NITROFURANTOIN 2. NORFLOXACIN • COLISTIN strip [EM -020] • Himedia –KPC agar plate		

MASTER CHARTS

1) VIRULENCE FACTORS :

Sl. no	GENDER	AGE GROUP	SAMPLE TYPE	HEMOLYSIS	CAPSULE STAIN	HMV Test	SIDEROPHORE	Milk agar	Gelatinase	Lipase	Lecithinase	Hemagglutination	Biofilm formation
1	M	64	PUS	no	YES	No	SP	P	P	NO	NO	NO	NO
2	M	50	PUS	no	YES	yes	SP	P	P	NO	NO	NO	NO
3	F	59	PUS	no	YES	yes	SP	P	P	NO	NO	NO	NO
4	M	45	PUS	no	YES	No	SP	P	P	NO	NO	NO	NO
5	F	86	SPUTUM	no	YES	yes	SP	P	P	P	NO	NO	POSITIVE
6	F	55	PUS	no	YES	yes	N	P	P	P	POSITIVE	NO	NO
7	M	71	ET-SAMPLE	no	YES	No	SP	P	P	P	POSITIVE	NO	POSITIVE
8	M	27	SPUTUM	no	YES	yes	SP	P	P	P	NO	NO	NO
9	M	44	PUS	no	YES	yes	SP	P	P	P	POSITIVE	NO	POSITIVE
10	M	55	BLOOD CULTURE	no	YES	No	SP	P	P	P	POSITIVE	NO	POSITIVE
11	M	48	BLOOD CULTURE	no	YES	No	P	P	P	NO	POSITIVE	NO	NO
12	M	82	ET-SAMPLE	no	YES	yes	SP	P	P	NO	POSITIVE	NO	POSITIVE
13	F	32	URINE	no	YES	No	SP	P	P	P	POSITIVE	NO	POSITIVE
14	M	65	SPUTUM	no	YES	yes	SP	P	P	NO	POSITIVE	NO	POSITIVE
15	M	62	ET-SAMPLE	no	YES	yes	SP	P	P	P	NO	NO	NO
16	M	56	ET-SAMPLE	no	YES	yes	SP	P	P	P	NO	NO	NO
17	F	60	PUS	no	YES	No	SP	N	NO	NO	NO	NO	NO
18	F	55	ET-SAMPLE	no	YES	No	SP	N	P	P	POSITIVE	positive	POSITIVE
19	M	79	BLOOD CULTURE	no	YES	No	SP	N	NO	P	NO	NO	POSITIVE
20	M	DAY - 6	ET-SAMPLE	no	YES	yes	SP	N	P	P	NO	NO	POSITIVE
21	M	70	ET-SAMPLE	no	YES	No	SP	N	P	P	NO	NO	POSITIVE
22	F	25	ET-SAMPLE	no	YES	yes	SP	P	P	P	POSITIVE	NO	POSITIVE
23	M	75	PUS	no	YES	No	SP	P	P	P	POSITIVE	NO	POSITIVE
24	M	27	PUS	no	YES	No	SP	P	P	P	POSITIVE	NO	NO
25	M	40	SPUTUM	no	YES	yes	N	P	P	NO	POSITIVE	NO	NO
26	M	DAY - 7	BLOOD CULTURE	positive	YES	No	SP	N	P	NO	NO	NO	POSITIVE
27	M	70	PUS	no	YES	No	SP	P	P	NO	NO	NO	POSITIVE
28	M	45	PUS	no	YES	No	SP	P	NO	NO	NO	NO	NO
29	M	56	PUS	positive	YES	yes	SP	P	P	NO	POSITIVE	NO	NO
30	M	70	ET-SAMPLE	no	YES	yes	SP	P	P	P	NO	NO	POSITIVE
31	M	DAY 6	BLOOD CULTURE	no	YES	No	SP	P	P	P	POSITIVE	NO	POSITIVE
32	M	38	SPUTUM	no	YES	yes	SP	P	NO	NO	POSITIVE	NO	NO
33	M	29	ET-SAMPLE	no	YES	No	SP	N	NO	NO	NO	NO	POSITIVE
34	M	50	PUS	no	YES	yes	SP	P	NO	NO	NO	NO	POSITIVE
35	M	60	BLOOD CULTURE	no	YES	No	SP	P	P	NO	NO	NO	NO
36	M	55	SPUTUM	no	YES	yes	SP	N	P	NO	NO	NO	NO

37	M	75	PUS	no	YES	yes	SP	P	NO	NO	NO	NO	NO
38	M	55	URINE	positive	YES	No	N	N	P	P	POSITIVE	NO	POSITIVE
39	F	57	ET-SAMPLE	no	YES	No	SP	N	NO	P	POSITIVE	NO	POSITIVE
40	F	48	ET-SAMPLE	no	YES	No	SP	N	NO	NO	NO	NO	NO
41	M	30	ET-SAMPLE	no	YES	yes	SP	P	NO	P	NO	NO	POSITIVE
42	M	70	SPUTUM	no	YES	No	SP	P	P	P	POSITIVE	NO	NO
43	F	60	PUS	no	YES	yes	SP	P	P	NO	NO	NO	NO
44	M	70	URINE	no	YES	yes	SP	P	P	P	NO	NO	POSITIVE
45	M	72	SPUTUM	no	YES	yes	SP	P	P	P	NO	NO	POSITIVE
46	M	32	SPUTUM	no	YES	yes	SP	P	P	NO	NO	NO	NO
47	M	10	PUS	no	YES	yes	P	P	P	NO	NO	NO	NO
48	F	65	PUS	no	YES	No	SP	P	NO	P	POSITIVE	NO	POSITIVE
49	M	67	BLOOD CULTURE	no	YES	yes	SP	P	NO	P	POSITIVE	NO	NO
50	F	62	SPUTUM	no	YES	yes	SP	P	NO	P	POSITIVE	NO	POSITIVE
51	M	77	PUS	no	YES	No	SP	P	NO	P	POSITIVE	NO	NO
52	M	38	ET-SAMPLE	no	YES	yes	SP	P	NO	P	NO	NO	POSITIVE
53	M	65	ET-SAMPLE	no	YES	yes	SP	P	NO	P	POSITIVE	NO	NO
54	M	65	PUS	no	YES	No	SP	P	NO	NO	NO	NO	POSITIVE
55	M	52	SPUTUM	no	YES	No	SP	P	NO	P	NO	NO	POSITIVE
56	F	64	PUS	no	YES	No	SP	P	P	NO	NO	NO	POSITIVE
57	M	67	SPUTUM	no	YES	No	N	P	P	P	POSITIVE	NO	NO
58	M	64	PERITONEAL	no	YES	yes	P	N	P	P	POSITIVE	NO	NO
59	M	61	ET-SAMPLE	positive	YES	yes	SP	N	P	P	POSITIVE	NO	NO
60	M	48	ET-SAMPLE	no	YES	No	SP	P	P	P	POSITIVE	NO	POSITIVE
61	F	17	PUS	no	YES	yes	P	P	P	P	POSITIVE	NO	POSITIVE
62	M	35	PUS	no	YES	No	P	P	P	P	POSITIVE	NO	POSITIVE
63	M	78	PUS	no	YES	yes	P	N	P	P	POSITIVE	NO	NO
64	M	76	SPUTUM	no	YES	yes	SP	P	P	P	POSITIVE	NO	POSITIVE
65	M	18	PERITONEAL FLUID	no	YES	No	P	P	P	P	NO	NO	POSITIVE
66	F	19	Mis - vaginal	no	YES	No	SP	P	P	P	NO	NO	NO
67	M	75	SPUTUM	no	YES	No	SP	P	P	P	POSITIVE	NO	POSITIVE
68	M	55	ET-SAMPLE	no	YES	yes	SP	P	P	P	NO	NO	NO
69	F	50	PUS	no	YES	yes	N	P	P	P	POSITIVE	NO	NO
70	M	38	PUS	no	YES	yes	N	P	P	P	POSITIVE	NO	NO
71	F	38	ET-SAMPLE	no	YES	yes	SP	P	P	P	NO	NO	POSITIVE
72	M	67	ET-SAMPLE	no	YES	yes	SP	P	P	P	NO	NO	NO
73	F	day - 5	BLOOD CULTURE	no	YES	No	N	P	P	P	POSITIVE	NO	POSITIVE
74	M	60	PUS	no	YES	No	N	P	P	NO	NO	NO	NO
75	M	22	Mis -Drain tip	no	YES	No	N	P	P	P	POSITIVE	NO	NO

76	M	73	ET-SAMPLE	no	YES	yes	P	P	P	P	POSITIVE	NO	POSITIVE
77	F	47	PUS	no	YES	yes	N	P	P	P	POSITIVE	NO	POSITIVE
78	M	18	PUS	no	YES	yes	N	P	P	NO	NO	NO	NO
79	M	40	PUS	positive	YES	No	N	P	P	P	POSITIVE	NO	NO
80	F	61	Mis - Central IV line	no	YES	yes	P	P	P	P	NO	NO	POSITIVE
81	M	60	pleural fluid	positive	YES	yes	P	P	P	P	POSITIVE	NO	POSITIVE
82	F	32	PUS	no	YES	yes	N	P	P	P	POSITIVE	NO	POSITIVE
83	M	66	SPUTUM	no	YES	yes	SP	P	P	P	NO	NO	POSITIVE
84	F	30	ET-SAMPLE	no	YES	yes	SP	P	P	P	POSITIVE	NO	POSITIVE
85	F	23	URINE	no	YES	No	N	P	P	P	NO	NO	NO
86	M	29	ET-SAMPLE	no	YES	yes	SP	P	P	P	POSITIVE	NO	POSITIVE
87	M	day - 1	BLOOD CULTURE	no	YES	No	N	P	P	P	NO	NO	POSITIVE
88	F	58	PUS	no	YES	No	N	P	P	P	NO	NO	POSITIVE
89	F	day - 16	BLOOD CULTURE	no	YES	No	N	P	P	P	NO	NO	POSITIVE
90	M	45	ET-SAMPLE	no	YES	yes	SP	P	NO	P	NO	NO	NO
91	M	50	SPUTUM	no	YES	yes	N	P	P	P	NO	NO	POSITIVE
92	F	50	PUS	no	YES	yes	SP	P	P	P	NO	NO	POSITIVE
93	M	34	PUS	no	YES	yes	N	P	NO	P	NO	NO	NO
94	M	39	PUS	no	YES	No	N	P	P	P	POSITIVE	NO	NO
95	M	58	ET-SAMPLE	no	YES	No	SP	P	P	P	POSITIVE	NO	NO
96	M	46	PUS	no	YES	No	SP	P	P	P	NO	NO	POSITIVE
97	M	19	PUS	no	YES	No	SP	P	P	P	NO	NO	POSITIVE
98	F	40	URINE	no	YES	No	N	P	P	P	NO	NO	POSITIVE
99	F	42	ET-SAMPLE	no	YES	No	P	P	P	P	POSITIVE	NO	POSITIVE
100	M	60	SPUTUM	no	YES	yes	P	P	P	P	POSITIVE	NO	NO
101	M	58	PUS	no	YES	yes	N	P	P	P	POSITIVE	NO	NO
102	M	40	URINE	no	YES	No	SP	P	P	P	NO	NO	POSITIVE
103	M	65	PUS	no	YES	No	SP	P	P	P	POSITIVE	NO	NO
104	M	48	ET-SAMPLE	no	YES	yes	N	P	P	P	NO	NO	POSITIVE
105	M	30	PUS	no	YES	No	N	P	P	P	POSITIVE	NO	POSITIVE
106	M	25	ET-SAMPLE	no	YES	yes	SP	P	P	P	POSITIVE	NO	POSITIVE
107	M	40	PERITONEAL	no	YES	yes	P	P	P	P	POSITIVE	NO	POSITIVE
108	F	43	PUS	no	YES	No	N	P	P	P	NO	NO	POSITIVE
109	F	50	ET-SAMPLE	no	YES	yes	N	P	P	P	POSITIVE	NO	POSITIVE
110	M	80	PUS	no	YES	No	N	P	P	P	POSITIVE	NO	NO
111	M	72	PUS	no	YES	No	N	P	P	P	POSITIVE	NO	NO
112	M	40	PERITONEAL	no	YES	No	N	P	P	P	NO	NO	NO
113	F	61	PUS	no	YES	yes	N	P	P	P	POSITIVE	NO	POSITIVE
114	M	26	ET-SAMPLE	no	YES	No	N	P	P	P	POSITIVE	NO	POSITIVE

115	M	60	SPUTUM	no	YES	No	N	P	P	P	NO	NO	NO
116	M	75	SPUTUM	no	YES	yes	P	P	P	P	NO	NO	NO
117	M	47	ET-SAMPLE	no	YES	No	N	P	P	P	POSITIVE	NO	POSITIVE
118	F	18	ET-SAMPLE	no	YES	No	SP	P	P	P	NO	NO	POSITIVE
119	F	45	PUS	no	YES	No	SP	P	P	P	NO	NO	NO
120	F	24	Mis - Central IV line	no	YES	No	P	P	NO	P	NO	NO	POSITIVE
121	M	54	PUS	no	YES	No	SP	P	P	P	NO	NO	NO
122	M	80	ET-SAMPLE	no	YES	No	SP	P	NO	P	POSITIVE	NO	POSITIVE
123	M	day - 5	BLOOD CULTURE	no	YES	No	P	N	NO	P	NO	NO	NO
124	M	day - 3	BLOOD CULTURE	no	YES	No	SP	P	P	P	P	NO	NO
125	M	64	ET-SAMPLE	no	YES	No	P	P	P	P	P	NO	NO
126	M	1.5	BLOOD CULTURE	no	YES	No	P	P	P	P	P	NO	NO
127	F	41	PUS	no	YES	No	P	P	P	P	P	NO	NO
128	M	63	ET-SAMPLE	no	YES	No	N	P	P	P	P	NO	NO
129	M	62	ET-SAMPLE	no	YES	No	N	P	NO	NO	NO	NO	POSITIVE
130	M	27	SPUTUM	no	YES	No	N	P	P	P	P	NO	POSITIVE
131	M	79	ET-SAMPLE	no	YES	No	P	P	P	P	P	NO	NO
132	M	40	ET-SAMPLE	no	YES	No	P	P	P	P	P	NO	NO
133	F	22	ET-SAMPLE	no	YES	No	P	P	P	P	P	NO	POSITIVE
134	M	55	ET-SAMPLE	no	YES	No	P	P	P	P	P	NO	POSITIVE
135	F	17	URINE	no	YES	No	N	P	P	NO	NO	NO	NO
136	M	95	URINE	no	YES	yes	P	P	P	P	P	NO	NO
137	M	57	ET-SAMPLE	no	YES	No	P	P	P	P	P	NO	POSITIVE
138	M	65	BLOOD CULTURE	no	YES	No	P	P	P	P	P	NO	NO
139	M	80	ET-SAMPLE	no	YES	No	P	P	P	NO	NO	NO	NO
140	F	1.5 year	URINE	no	YES	No	P	P	P	P	NO	NO	POSITIVE
141	M	55	PUS	no	YES	No	N	P	P	P	P	NO	NO
142	M	65	PUS	no	YES	yes	SP	P	P	P	P	NO	POSITIVE
143	F	25	URINE	no	YES	yes	SP	P	P	P	P	NO	POSITIVE
144	F	17	PUS	no	YES	No	SP	P	P	P	P	NO	POSITIVE
145	M	42	SPUTUM	positive	YES	yes	SP	P	P	P	P	NO	POSITIVE
146	M	65	SPUTUM	no	YES	No	P	P	P	P	P	NO	POSITIVE
147	M	31	PUS	no	YES	No	N	P	P	P	P	NO	NO
148	F	48	ET-SAMPLE	no	YES	No	N	P	P	P	P	NO	POSITIVE
149	F	42	BLOOD CULTURE	no	YES	No	P	P	P	P	NO	NO	NO
150	F	14 day female	PUS	no	YES	No	P	P	P	P	P	NO	POSITIVE

2) ANTIMICROBIAL PATTERN CHART :

AMC	CTX	CX	CTR	CAC	CAZ	COT	IMP	MRP	ETP	AK	GEN	TOB	CIP	LE	DO	C	NX	NIT	COLISTIN
R	S	S	S	S	S	R	S	R	S	S	S	S	S	S	R	S			
R	R	S	R	S	R	R	S	R	R	S	R	R	R	R	R	R			
R	R	S	R	S	R	R	S	S	S	S	R	R	R	S	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			S
R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S			
R	R	R	R	R	R	R	S	S	S	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	S			S
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	S	S	S	S	S	S	R	S	S	S	S	S	S	R	S			
R	R	S	R	S	R	S	S	S	S	S	S	S	R	S	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	S	S	S	S	S	S	R	R	S	S	S	S	S	S	R	S			
R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	S	R	S	R	S	R	R	R	R	S	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	S	R	R	R	R	S	S	S	S	R	R	R	S	R	R	R	R	
R	R	S	R	R	R	R	S	S	S	R	R	R	R	R	R	S			
R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	S	R	R	R	S	S	S	S	S	S	R	S	S	S	S			

R	R	S	R	S	R	R	R	R	R	S	S	S	R	S	R	R	R	S	
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	S	R	R	R	R	S	S	S	S	S	S	R	R	S	S			
R	R	S	R	R	R	R	S	S	S	S	S	S	R	R	R	S			
R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S			
R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S	0	0	
R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	0	0	
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	S	R	S	S	R	S	S	S	R	R	R	S	S	R	S	0	0	
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	R	S	0	0	0
S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			S
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	S	R	R	R	S	S	S	S	R	R	R	R	R	S	S	0	0	
R	R	S	R	R	R	S	S	S	S	R	R	R	R	R	R	S	0	0	
R	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R	R	0	0	
R	R	R	R	R	R	R	S	S	S	S	R	R	R	R	S	S	0	0	
R	R	R	R	R	R	S	R	R	R	R	S	R	R	S	R	R	0	0	
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	S	R	R	S	S	S	R	R	R	R	S	0	0	
R	R	R	R	R	R	R	R	S	R	S	S	S	R	S	R	R	0	0	
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	S	S	R	S	S	S	S	R	R	S	S	0	0	
R	R	S	R	S	R	R	R	R	S	S	R	R	R	S	R	R	0	0	
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	0	0	
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	0	0	
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
S	S	S	S	S	S	S	S	R	S	S	S	S	R	R	S	S	0	0	
S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	S	0	0	
R	R	R	R	R	R	S	R	R	R	S	S	S	R	S	R	R			
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	0	0
R	R	S	R	S	R	S	S	S	S	R	R	R	R	R	S	S	0	0	0
R	R	S	R	S	R	R	R	R	R	R	R	R	R	R	S	S	0	0	0
R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	S	0	0	0
R	R	R	S	R	R	R	S	S	S	S	S	S	R	R	R	R	R	R	0
R	R	S	R	S	R	R	S	S	S	S	S	S	S	S	R	S	0	0	0
R	R	S	R	S	R	R	S	R	R	S	S	R	R	R	R	S			
R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S			
R	R	R	R	R	R	S	R	R	S	S	S	S	S	S	R	S	0	0	0

R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	0		
R	R	R	R	R	R	S	R	R	S	S	S	S	S	S	R	S	0	0	0
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	0		
R	R	S	R	S	R	R	R	S	S	S	S	R	R	S	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	0	0	
R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	0	0	0
S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	S	R	R	S	R	R	R	S	S	R	S	0	0	0
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S			
R	R	R	R	R	R	R	S	R	R	R	S	R	R	R	R	S	R	R	
R	R	S	R	S	R	R	S	S	S	S	S	S	R	R	R	R	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	S	0	0	0
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S			
R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	R	S	0	0	0
R	R	R	R	S	R	R	R	R	R	R	S	R	R	R	R	R	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	S	R	S	R	R	S	S	S	R	R	R	R	R	S	S	0	0	0
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	0	0
R	R	S	R	S	R	R	S	S	S	R	R	R	S	S	S	S	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	0		
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	0		
R	R	S	R	S	R	R	R	R	R	R	R	R	R	R	R	S	0	0	
R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	R	S	0	0	0
S	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
S	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	

R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	S	R	R	S	R	R	R	S	R	R	S	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	0
R	R	R	R	R	R	S	R	R	R	S	R	R	S	R	R	S	0	0	0
R	R	S	R	S	R	R	S	S	S	R	R	R	R	R	S	S	0	0	0
R	S	S	S	S	S	S	S	S	S	R	R	R	R	S	S	R	S	R	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	S	R	R	R	R	S	S	S	R	R	R	S	S	S	S	0	0	0
R	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	0	0	0
R	R	S	R	R	R	S	R	R	R	R	S	R	S	S	R	S	0	0	0
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R			
R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S	0	0	0

THANK YOU