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PLASMID-MEDIATED RESISTANCE IN EXTENDED SPECTRUM BETA–LACTAMASE–PRODUCING *KLEBSIELLA PNEUMONIAE* ISOLATES AT RIVERS STATE UNIVERSITY TEACHING HOSPITAL

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ABSTRACT

Background: The presence of plasmid association with emergence and transmission of antimicrobial–resistant bacteria a serious health issue in developing countries. **Aim**: The aim of this project is to phenotypically and molecularly analyse the antimicrobial resistance induce in *K. pneumoniae*. **Methods**: The clinical isolate was identified both phenotypically and molecularly by amplification and sequencing of the 16S rRNA. The ability of the clinical strain to produce beta–lactamase enzyme was determined by performing antimicrobial sensitivity to Penicillin, Ampicillin, Cefoxitin and Ceftriaxone. Presence of plasmid in the *K. pneumoniae* clinical and laboratory strains were examined. Polymerase chain reaction amplification of the beta–lactamase genes *blaTEM*-1 and *blaSHV* was performed. **Results**: From the 30 isolates investigated, only 7 were positive for *Klebsiella* sp while 23 were negative. This gives a 23% incidence of *Klebsiella* sp in *Enterobacteriaceae* isolates in patients's samples at Rivers State University Teaching Hospital (RSUTH). From the seven confirmed *K. pneumoniae* isolates, only three isolates were positive for the presence beta–lactamase production as they were considered resistant. **Conclusion**: These results show that 10% of *Klebsiella* sp found in RSUTH possess beta–lactamase which is responsible for the resistance to beta–lactam drugs.

KEYWORDS: Plasmid, Resistance, Nosocomial, *Klebsiella pneumonia.*

1. INTRODUCTION

Plasmids are extra-chromosomal, self-replicating, double-stranded DNA elements, harboured by most bacterial cells. They are diverse in size, mode of replication, mode of transmission and host range. Whereas broad–host range plasmids may spread among and be maintained by a wide range of bacterial hosts, narrow–host range plasmids are only maintained by closely related bacterial hosts. The plasmid backbone harbours conserved core genes essential for replication, transfer and maintenance. A plasmid may have one single or several compatible replicons (Helinski, 2004). Plasmids are considered effective vehicles for the spread of antibiotic resistance determinants.

The term antibiotic has been traditionally indicated to natural metabolic products of fungi, actinomycetes, and bacteria that inhibit or kill the growth of microorganisms. Antibiotic production has been particularly linked with soil microorganisms and in the natural environment is believed to provide a selective advantage for organisms in their competition for nutrients and space. While the majority of antimicrobial agents in clinical use today are made from natural products of fermentation, most are then modified chemically (semi-synthetic) to improve their antibacterial or pharmacologic properties. Nonetheless, some agents are totally synthetic such as sulphonamides and quinolones. Therefore the name antibacterial or antimicrobial agent is often used in preference to antibiotic (Bush, 2010).

The β-lactam antibiotics include penicillins, cephalosporins, carbapenems, and monobactams. Their name comes from the presence of a β-lactam ring in their structure; this ring is vital for antimicrobial activity (Sherris, 2004). In 1940, Florey and Heatley travelled to the United States seeking help in mass production of penicillin by fermentation. They were able to isolate Penicillin from *Penicillium chrysogenum*. This work attracted pharmaceutical firms to invest in production of penicillin in enormous amount. Thereafter, different penicillin compounds were introduced by E. R. Squibb & Sons and Florey's group producing benzyl–penicillin (Penicillin G) and 2-pentenylpenicillin respectively (Penicillin F). It was later observed that both of penicillin

forms contained β-lactam rings (Yao & Moellering, 2011).

Beta–lactams target the bacterial cell wall synthesis and act by binding covalently to penicillin binding proteins (PBPs). PBPs are bacterial enzymes involved in the synthesis and crosslinking of peptidoglycan, which is a major component of the bacterial cell wall. PBPs are located in the inner cytoplasmic membrane or in the periplasmic space of Gram-negative bacteria. When PBPs are inactivated by beta–lactams, the peptidoglycan synthesis is inhibited and the bacterial growth is affected. Irregularities in the cell wall synthesis lead to loss of integrity and finally cell lysis (Sherris, 2004).

Klebsiella pneumoniae is rod-shaped, Gram-negative, lactose–fermenting bacillus with a prominent capsule. Typical *K. pneumoniae* is an opportunistic pathogen that is widely found in the mouth, skin and intestines, as well as in hospital settings and medical devices. Opportunistic *K. pneumoniae* mostly affects those with compromised immune systems or who are weakened by other infections. Colonization of the gastrointestinal tract by opportunistic *K. pneumoniae* generally occurs prior to the development of nosocomial infections, and *K. pneumoniae* colonization can be further found in the urinary tract, respiratory tract and blood (Schroll *et al.*, 2010). *Klebsiella pneumoniae* biofilms that form on medical devices (e.g., catheters and endotracheal tubes) provide a significant source of infection in catheterized patients. Nosocomial infections caused by *K. pneumoniae* tend to be chronic due to the two major reasons: *K. pneumoniae* biofilms formed *in vivo* protect the pathogen from attacks of the host immune responses and antibiotics; and nosocomial isolates of *K. pneumoniae* often display multidrug–resistance phenotypes that are commonly caused by the presence of extended-spectrum β-lactamases or carbapenemases, making it difficult to choose appropriate antibiotics for treatment (Li *et al.*, 2014). This study aimed at studying the presence plasmid in beta–lactamase producing *K. pneumoniae* in samples obtained from Rivers State University Teaching Hospital.

3. MATERIALS AND METHODS

3.1 Instruments and Reagents

Thermal cycler, Safety cabinet II, Spectrophotometer, phosphate buffered saline (PBS), tryptone soy agar, tryptone soy broth, agarose gel, genomic DNA extraction kits, plasmid extraction kits, *Taq* DNA polymerase (TaKaRa Biotechnology [Dalian] Co. Ltd., China), Penicillin (Oxoid, UK), Ampicillin (Oxoid, UK), Cefoxitin (Oxoid, UK) and Ceftriaxone (Oxoid, UK).

3.3 Study Area and Sample Location

Thirty (30) *Enterobacteriaceae* was collected from Rivers State University Teaching Hospital, Department of Medical Microbiology with the ethical permission from Rivers State Ministry of Health.

3.3 Collection of Organisms

The laboratory strain also known as control strain of *K. pneumoniae* WCDM 0097 (ATCC 13889) was purchased from Sigma United Kingdom.

3.4 Media Preparations

3.4.1 Tryptone Soya Agar (TSA), Tryptone Soya Broth (TSB)

The microbial media used were TSA and TSB. These were prepared according to the manufacturer's instructions and autoclaved for 15 minutes at 121°C. TSA was aseptically poured into sterile Petri dishes.

3.5 Identification of Clinical *Klebsiella pneumoniae* Isolate

Clinical isolates of the *Enterobactericea* family were collected from Rivers State University Teaching Hospital (RSUTH), Port Harcourt, Rivers State Nigeria with ethical approval obtained from the Rivers State Ministry of Health. These samples were further identified using biochemical tests for presumptive confirmation. Confirmation of the *K. pneumoniae* sample was done using specific amplification of the 16s rRNA of the sample described in section 3.6.

3.6 Amplification of 16S rRNA

Specific primers were used to amplify the 16S rRNA gene of the Klebsiella isolates as shown in Table 3.1 on a thermal cyler (ABI 9700 Applied Biosystems) for 35 cycles at volume of 50 µl. the PCR mixture included: the primers at a concentration of 0.4 M, the X2 Dream tag Master mix which contain taq polymerase, buffer, dNTPs, MgCl, and the extracted DNA. The conditions for PCR were 95ºC for 5 minutes for the initial denaturation; 95ºC for 30 seconds for denaturation; 52ºC for 30 seconds for annealing, 72ºC for 30 seconds for extension, and 72ºC for 5 minutes final extension and this was done for 35 cycles. The machine keeps the amplicons cool at 4ºC.

Table 3.1 Primers used for the PCR Amplification of 16S rRNA and Beta-Lactamase Genes'

Gene	Forward Primer	Reverse Primer
blaTEM-1	ATGAGTATTCAACATTTCCG	CTGACAGTTACCAATGCT
blaSHV	CCGCAGCCGCTTGAGCAAA	GCTGGCCGGGGTAGTGGTGTC
16S rRNA	AGAGTTTGATCCTGGCTCAG	GGTTACCTTGTTACGACTT

3.7 Antibiotic Susceptibility and Beta–Lactamase Production

Antibiotic sensitivity pattern to Clinical Laboratory Standard Institute (CLSI) guidelines was determined by the disc diffusion method on Nutrient agar. The beta–lactamase production was determined by application of the Disc method. The bacterial suspension was prepared with 0.5 Mcfarland dilution and was cultured on Muller-Hinton agar plates. Then, four discs containing Penicillin (30 µg), Ampicillin (10 µg), Ceftriaxone (30 µg) and Cefoxitin (30 µg) were used. After incubation at 35±2°C for 24 hrs, beta–lactamase production was evaluated.

3.8 Ethical Consideration

Collection of samples of *Enterobacteriaceae* microorganisms were performed in strict accordance with the ethical recommendations of the Ethical Committee under the jurisdiction of the Rivers State Ministry of Health in Port Harcourt of Nigeria.

4. RESULTS

4.1 Conventional Isolation of *K. pneumoniae* Isolates

In order to presumptively identify *K. pneumoniae* isolate, we employed conventional biochemical methods which could differentiate between *Klebsiella* sp. and non–*Klebsiella* sp. Figure 4.1 shows the results of the conventional identification of *K. pneumoniae*. Out of a total of 30 isolates investigated, only 7 were positive for *Klebsiella* sp while 23 were negative. This gives a 23% incidence of *Klebsiella* sp in *Enterobacteriaceae* isolates in patients's samples at RSUTH.

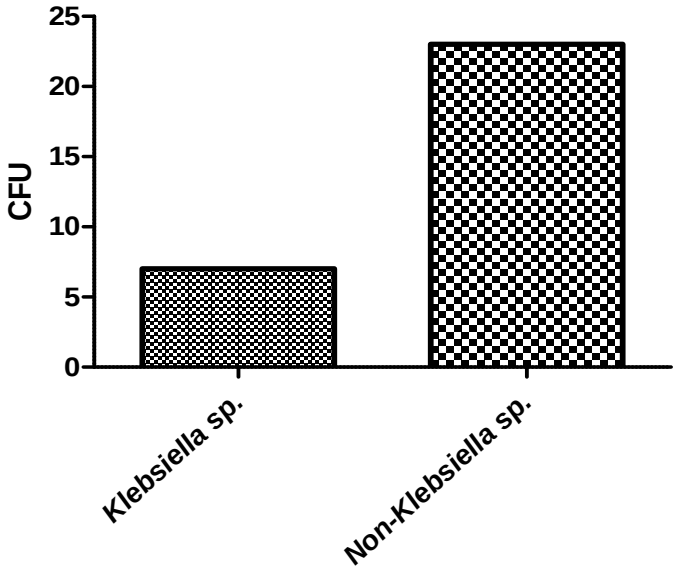

Figure 4.1: Conventional identification of *Klebsiella* sp from the *Enterobacteriaceae* isolates obtained from RSUTH.

4.2 Screening for Beta–Lactamase *K. pneumoniae* Isolates

Beta–lactamase positive isolates were screened based on the ability of *Klebsiella* sp to resist beta–lactam antibiotics. The beta–lactam antibiotics used for this study include: Penicillin, Ampicillin, Cefoxitin and Ceftriaxone. Table 4.1 reveals the antibiotic sensitivity of clinical *Klebsiella* sp isolates. Out of the seven confirmed *K. pneumoniae* isolates, only three isolates were positive for the presence beta–lactamase production as they were considered resistant (Table 1). According to the national committee for clinical laboratory standards (NCCL) (2003) guidelines, zone of inhibition lower than 14 mm is classified as resistant, while 15–24 mm are moderately sensitive and 25–31 mm are considered sensitive.

Table 4.1: *Klebsiella* sp Sensitivity to Beta–Lactam Antibiotics.

Isolates	Zone of Inhibition (mm)			
	Pen	Amp	Cefo	Ceft
1	30	28	29	31
2	14	9	14	13
3	29	33	28	30
4	31	29	29	30
5	12	10	14	9
6	8	9	10	13
7	27	27	30	32

Key: Pen: Penicillin, Amp: Ampicillin, Cefo: Cefoxitin, Ceft: Ceftriaxone.

4.3 Amplification of the 16S rRNA of the Clinical *K. pneumoniae* Strain

Molecular detection of the clinical *K. pneumonaie* isolate used in the study was carried out by the specific amplification of the 16S rRNA gene of the three beta–lactamase positive *K. pneumonaie* isolates. Figure 4.2 shows the agarose gel electrophoresis result from the amplification products of 16S rRNA gene of *K. pneumoniae* clinical isolate. The amplicons sizes correspond to the DNA molecular marker size expected (1500 bp) of 16S rRNA. The isolate 1 amplicon was sequenced and used for further studies. Figure 4.3 represents the phylogenetic analyses of the isolate 1 sequenced 16S rRNA. The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The sequenced clinical isolate has similar sequence with *K. pneumoniae* strain SS4KSU with accession number MH973165. An intriguing observation from the phylogenetic tree is that all the branches showed *K. pneumoniae* with different strains. This confirms that our isolate was *K. pneumoniae*.

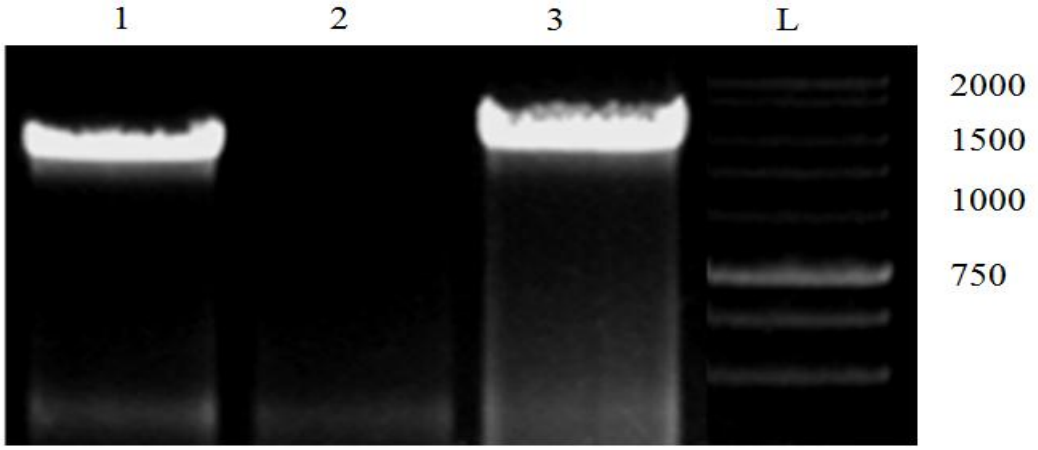

Figure 4.2: PCR products of the 16S rRNA gene amplification. Lane L: molecular standard marker; Lanes 1, 2 and 3: 16S rRNA gene of *K. pneumoniae* of isolates 2, 5, and 6 respectively. The size of the amplicon is 1500 bp.

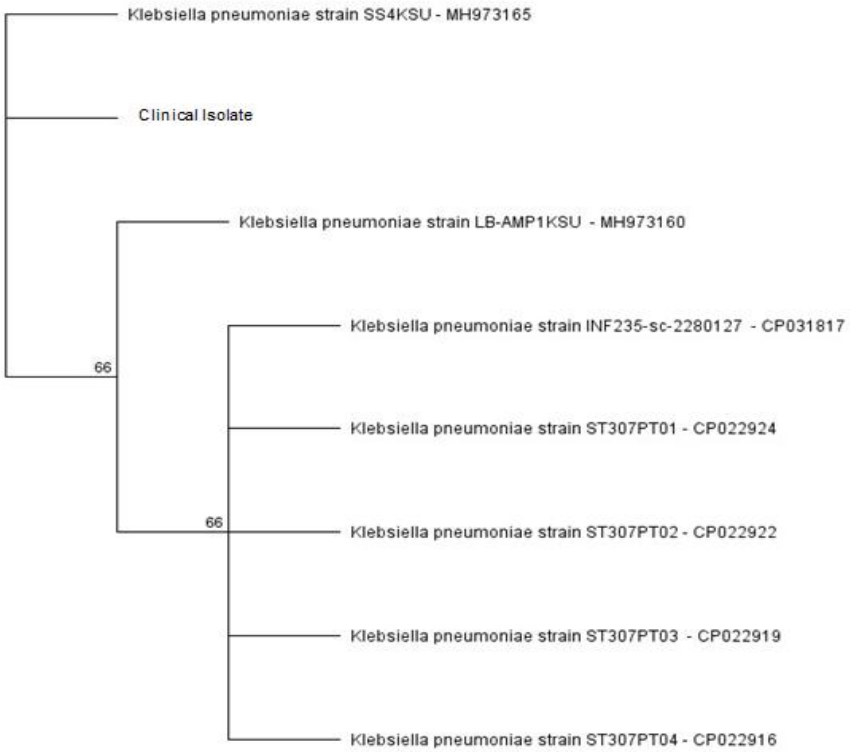

Figure 4.3: Phylogenetic analysis of clinical *K. pneumonaie* of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor–Joining method program in the Geneious package (version 9.0.5).

4.4 Plasmid Profile of the *K. pneumoniae*

The presence of plasmids was investigated in the laboratory and clinical strains of *K. pneumoniae* strain used in the study. Figure 4.4 represents the plasmid profiles of the *K. pneumoniae*. The lane L is the DNA molecular weight ladder, 1 is the no DNA control, 2 and 3 are *K. pneumoniae* ATCC 13883 and 4 and 5 are the clinical *K. pneumoniae* isolate. The *K. pneumoniae* ATCC 13889 did not produce any plasmid. The clinical *K. pneumoniae* isolate in laness 4 and 5 show presence of plasmid DNA of approximately 30 kb.

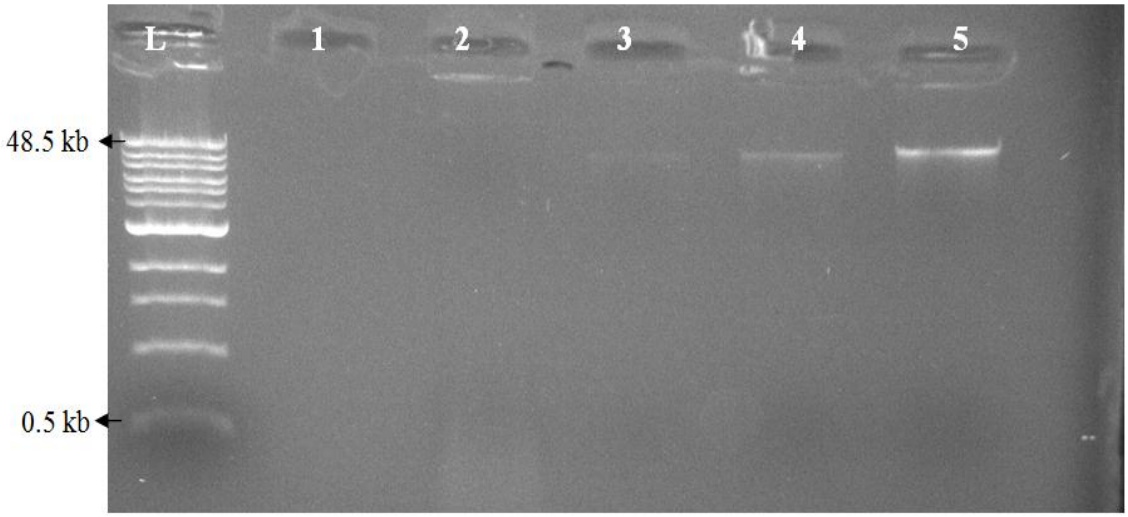

Figure 4.4: Plasmid Profiles of *K. Pneumoniae* Isolates Analyzed With 0.8% Agarose Gel Electrophoresis Stained With Ethidium Bromide. L is 0.5–48.5 kb DNA ladder (molecular marker), 2 and 3: *K. pneumoniae* ATCC 13889, 4 and 5: clinical *K. pneumoniae* isolate. The bacterial samples were seeded in duplicate wells.

5. DISCUSSION

5.1 Conventional Identification of *K. pneumoniae* Isolates

The first goal of our study was to identify the clinical strain of *K. pneumoniae* for the exposure studies. To achieve this, *Enterobacteriaceae* isolates were subjected to some conventional and biochemical tests which include: presence of gas production in lactose at 44.5ºC, citrate utilization, negative motility, lactose fermentation on MacConkey agar, indole negative and absence of growth at 10ºC. The results from the conventional detection showed only 7 out of the 30 isolates were identified as *Klebsiella* sp. This translates to 23% incidence of *Klebsiella* sp in the *Enterobacteriaceae* isolates. This presumptive confirmation of *Klebsiella* sp was as a result of the morphological and biochemical tests reactions as previously reported in Alves *et al.*, (2006). Their phenotypes were consistent with the results obtained by Jesumirhewe *et al.*, (2016) on the identification of *K. pneumoniae*. As the use of phenotypic methods is not accurate enough to confirm the species of an organism (Alves *et al.*, 2006; Hansen *et al.*, 2004), it was therefore imperative to identify the clinical isolate to be used for further studies using a suitable molecular technique.

Conversely, our incidence rate differs from those obtained by previous studies (Alves *et al.*, 2006; Haeggman *et al.*, 2004) which showed an 84% incidence for *K. pneumoniae*, 15% *K. oxytoc*a and 1% *R. planticola*. This is much higher than the 23% incidence obtained in this current study. These discrepancies in the incidence rates do not depict a decline in the prevalence of *Klebsiella* sp in the hospital settings but depends on the source of our samples. These previous studies identified different isolates of *Klebsiella* sp obtained from a hospital into different species, while our study identified *Klebsiella* sp from *Enterobacteriaceae* samples from hospital. *Enterobacteriaceae* is a much larger family of gram–negative bacteria that encompass *Salmonella*, *Klebsiella*, *Yersinia pestis*, *E. coli* and *Shigella* (Boye & Hansen, 2003); hence *Klebsiella* is only a component of this larger family.

5.2 Molecular Identification of *K. pneumoniae* Isolates Producing Beta–Lactamase

The current study used PCR amplification of the 16S rRNA genes. The amplification result (Figure 4.2) of the 16S rRNA gene from the *K. pneumoniae* isolates, using a primer set that corresponds to highly conserved regions, produced a band which shows that the bacteria isolate was *K. pneumoniae*. The 16S rRNA gene was used as it is highly conserved in microorganism. However, Gutell *et al.*, (1999) and Palys *et al.*, (1997) have shown that although the 16S rRNA amplification is a good detection method of microorganism, it is not sensitive enough to allow for clear differentiation of closely related microorganism. This is consistent with the observations noted by Wang *et al.*, 2008. The band of DNA amplified is 1500 bp which corresponds to the gene of the 16S rRNA of *K. pneumoniae*.

Furthermore, to confirm the identity of the organism the 16S rRNA was sequenced and analysed using bioinformatics software to generate a phylogenetic tree. The tree (Figure 4.3) shows that the clinical isolate was *K. pneumoniae* as the closest branch on the tree is *K. pneumoniae* strain SS4KSU–MH973165. This is similar to the method used by Wang *et al.,* 2008 to differentiate the species of *K. pneumoniae*.

5.3 Antimicrobial Screening and Beta–Lactamase Production in *K. pneumoniae* Isolates

The presence of beta–lactamase in the *Klebsiella* sp is a serious health concern as it enables bacteria to develop resistance to several antimicrobial agents (Magdy, 2013). Diseases caused by beta–lactamase producing *Enterobacteriaceae* are classified as high risk infections as they are associated with treatment failure (Livermore & Brown, 2001). The resistance to beta–lactam antibiotics is usually mediated by *blaTEM*-1 and *blaSHV*. Hence, *Klebsiella* sp possessing these enzymes was screened from the seven conventionally identified isolates. This detection was performed with beta–lactam antibiotics, Cefoxitin, Penicillin, Ampicillin, and Ceftriaxone, using the disc diffusion method (Table 4.1) previously used in other studies (Jayapradha *et al.,* 2007; Mansouri *et al.,* 2011). These drugs have been previously used to determine both antimicrobial sensitivity of bacteria and the production of beta–lactamase in *Enterobacteriaceae* (Ahmed *et al.,* 2009). Our results show that only three isolates of *Klebsiella* sp were resistance to all the beta–lactam drugs. This implies an incidence of 42.9% of beta–lactamase producing *Klebsiella* sp from the conventionally identified isolates. This is similar to the observation seen in a study which reported resistance in beta–lactamase producing–bacteria to the Penicillin, Cephalosporin and monobactam class of drugs. Our incidence is higher than the 33.33% reported by Magdy in 2013. This result suggests possible patient–to–patient transmission of ESBL–producing *Klebsiella* sp within the hospital setting. Nosocomial outbreaks of ESBL–producing bacteria have been previously reported worldwide (Van't–Veen *et al.,* 2005). The current study points to the critical role of health facility in the spread and transmission of beta–lactamase–producing *Klebsiella* isolates. The three isolates that were positive for beta–lactamase production showed resistance across all the beta–lactam drugs, hence demonstrating multidrug resistance. Magdy, 2013 has reported that beta–lactamase producing bacteria showed higher levels of multidrug resistance than non–beta–lactamase producers.

5.4 Plasmid–Mediated Resistance in *K. pneumoniae*

Plasmid is an extrachromosomal DNA encoded by bacteria. This is usually responsible for the resistance phenotypes in bacteria. The presence of plasmid in bacteria depicts a form of adaptational mechanism against antimicrobials and harsh environmental factors. A lot of antimicrobial resistance genes in *K. pneumoniae* are plasmid–mediated (Navon–Venezia *et al.,* 2017). From the results obtained, two clinical isolates of *K. pneumoniae* possess plasmid while *K. pneumoniae* ATCC 13889 did not produce plasmid (Figure 4.4). The resistance experiments in this study reveal a reduced sensitivity in the *K. pneumoniae* isolates previously exposed to herbal drugs. The presence of plasmid and the lowered sensitivity observed in clinical *K. pneumoniae* isolate signifies that the resistance is probably plasmid–borne.

The role of plasmids in the development of antimicrobial resistance was first observed in Japan when multi–resistant and susceptible strains were isolated from a single patient (Ramirez *et al.,* 2014). *K. pneumoniae* have been previously reported to possess multiple plasmids (Holt *et al.,* 2015; Navon–Venezia *et al.,* 2017; Rozwandowicz *et al.,* 2018). This is in contrast to the findings in our current study as only one plasmid was detected in the clinical isolate. The existence of multiple plasmids in bacteria usually causes higher levels of resistance and in most cases no zone of clearance.

However, our results showed zones of clearance but not sufficient to be classified as sensitive according to NCCLS, (2003).The presence of plasmids in *K. pneumoniae* usually depends on the fitness level of the bacterium to harbour plasmid (Wyres & Holt, 2018). *In vivo*, *K. pneumoniae* has been shown to demonstrate long–term plasmid maintenance (Lohr *et al.,* 2015). This ability to retain plasmids depends on complex plasmid–bacterium interactions that consist of specific adaptations of hosts to plasmid and vice versa. A recent study showed that helicase and RNA polymerase mutations are chromosomal adaptations that increase the ability of bacteria to retain plasmids (Loftie–Eaton *et al.,* 2017).

The results from the amplification of the beta–lactamase genes, *blaSHV* and *blaTEM*-1 suggested that *K. pneumoniae* possess *blaTEM*-1 on plasmid in some herbal drug treatment conditions but absent in others. The same observation was noted for the *blaSHV* gene. This implies that depending on the treatment conditions, some of the resistance gene could be induced while some. Another reason for this scenario is the possibility of plasmid loss. Loss of plasmids from bacteria has been shown to be responsible for the increased susceptibility trend seen in microorganisms (Hammer *et al*., 2012).

6. CONCLUSION

The study reveals a 23% incidence of *Klebsiella* sp in *Enterobacteriaceae* isolates in patients's samples at Rivers State University Teaching Hospital (RSUTH). Three isolates were positive for the presence beta–lactamase production as they were considered resistant. This implies that the resistance observed in the *Klebsiella* sp was plasmid–borne.

REFERENCES

1. Ahmed, S. H., Daef, E. A., Badary, M. S., Mahmoud, M. A. & Abd-Elsayed, A. A. (2009). Nosocomial blood stream infection in intensive care units at Assiut University Hospitals (Upper Egypt) with special reference to extended spectrum β-lactamase producing organisms. *Bio Med Central Research Notes*, 2: 1186–1756.
2. Alves, M. S., Dias, R. C. D. S., Castro, A. C. D. D., Riley, L. W. & Moreira, B. M. (2006). Identification of clinical isolates of indole–positive and indole–negative *Klebsiella* spp. *Journal of Clinical Microbiology*, 44(10): 3640–3646.
3. Boye, K., & Hansen, D. S. (2003). Sequencing of 16S rDNA of *Klebsiella*: taxonomic relations within the genus and to other *Enterobacteriaceae*. *International Journal of Medical Microbiology*, 292: 495–503.
4. Bush, K. (2010). Bench – to – bedside review: the role of β-lactamases in antibiotic-resistant gram–negative infections. *Critical Care*, 14: 224.
5. Gutell, R. R., Larsen, N. & Woese, C. R. (1994). Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiology Review*, 58(1): 10–26.
6. Haeggman, S., Lofdahl, S., Paauw, A., Verhoef, J. & Brisse. S. (2004). Diversity and evolution of the class A chromosomal beta–lactamase gene in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*, 48: 2400–2408.
7. Hammer, J. A., Freytag, B., Lanka, E., Appel, B. & Hertwig, S. (2012). The pYV virulence plasmids of *Yersinia pseudotuberculosis* and *Y. pestis* contain a conserved DNA region responsible for the mobilization by the self-transmissible plasmid pYE854. *Environmental Microbiology and Reproduction*, 4(4): 433–438.
8. Hansen, D. S., Aucken, H. M., Abiola, T. & Podschun, R. (2004). Recommended test panel for differentiation of *Klebsiella* species on the basis of a trilateral interlaboratory evaluation of 18 biochemical tests. *Journal of Clinical Microbiology*, 42(8): 3665–3669.
9. Helinski D. In: Phillips G, Funnell B, Plasmid biology. Washington DC, ASM Press, 2004; 1-16.
10. Holt, K. E., Wertheim, H., Zadoks, R. N., Baker, S., Whitehouse, C. A., Dance, D., Jenney, A., Connor, T. R., Hsu, L. Y. & Severin, J. (2015). Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proceedings of the National Academy of Sciences*, 112: 3574–3581.
11. Jayapradha, R., Murugesh, S., Mahesh, N. & Brahatheeswaran, D. (2007). Prevalence of ESBL producing strains in tuberculosis patients. *Research Journal of Microbiology*, 2: 491–495.
12. Jesumirhewe, C., Ogunlowo, P. O., Olley, M., Springer, B., Allerberger, F. & Ruppitsch, W. (2016). Accuracy of conventional identification methods used for *Enterobacteriaceae* isolates in three Nigerian hospitals. *Peer Journal*, 4: e2511.
13. Li, B., Zhao, Y., Liu, C., Chen, Z. & Zhou, D. (2014). Molecular pathogenesis of *Klebsiella pneumoniae*. *Future Microbiology*, 9(9): 1071–1081.
14. Livermore, D. M. & Brown, D. F. J. (2001). Detection of β-lactamase-mediated resistance. *Journal of Antimicrobial and Chemotherapy*, 48: 59–64.
15. Loftie–Eaton, W., Bashford, K., Quinn, H., Dong, K., Millstein, J., Hunter, S., Thomason, M. K., Merrikh, H., Ponciano, J. M. & Top, E. M. (2017). Compensatory mutations improve general permissiveness to antibiotic resistance plasmids. *Nature, Ecology and Evolution*, 1(9): 1354–1363.
16. Lohr, I. H., Hulter, N., Bernhoff, E., Johnsen, P. J., Sundsfjord, A. & Naseer, U. (2015). Persistence of a pKPN3–like CTX–M–15–encoding IncFIIK plasmid in a *Klebsiella pneumoniae* ST17 host during two years of intestinal colonization. *Public Library of Science One*, 10(3): e0116516.
17. Magdy, M. A. (2013). Detection of Extended Spectrum Beta–Lactamase Producing *Klebsiella pneumoniae* and *Escherichia coli* of Environmental Surfaces at Upper Egypt. *International Journal of Biological Chemistry*, 7: 58–68.
18. Mansouri, S., Norouzi, F., Moradi M. & Nakhaee, N. (2011). Comparison of virulence factors among clinical isolates of *Pseudomonas aeruginosa* producing and non-producing extended spectrum β – lactamases. *Current Research in Bacteriology*, 4: 85–93.
19. Navon–Venezia, S., Kondratyeva, K. & Carattoli, A. (2017). *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *Federation of European Microbiological Societies Microbiology Review*, 41: 252–275.
20. NCCLS, (2003). Performance Standards for Antimicrobial Susceptibility Tests: 13th informational supplement. National committee for clinical laboratory standards, M100–S12, Wayne P. A. USA.
21. Palys, T., Nakamura, L. K. & Cohan, F. M. (1997). Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. *International Journal of Systematic and Evolutionary Microbiology*, 47(4): 1145–1156.
22. Ramirez, M. S., Traglia, G. M., Lin, D. L., Tran, T. & Tolmasky, M. E. (2014). Plasmid– Mediated Antibiotic Resistance and Virulence in Gram-negatives: the *Klebsiella pneumoniae* Paradigm. *Microbiology Spectrum*, 2(5): 1–15.
23. Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez–Zorn, B., Guerra, B., Mevius, D. J. & Hordijk, J. (2018). Plasmids carrying antimicrobial resistance genes in *Enterobacteriaceae*. *Journal of Antimicrobial and Chemotherapy*, 73(5): 1121–1137.

24. Schroll, C., Barken, K. B., Krogfelt, K. A. & Struve, C. (2010). Role of type 1 and type 3 fimbriae in *Klebsiella pneumoniae* biofilm formation. *Bio Med Central Microbiology*, 10: 179.
25. Sherris, C. J., Ryan, K. J., Ray, C. G., Champoux, J. J., Neidhardt, F. C., Drew, W. L., Plorde, J. J., Marchalonis, J. J., Falkow, S. & Robinovitch, M. R. (2004). Sherris Medical Microbiology, An Introduction To Infectious Diseases, Gourth Edition, McGRAW–HILL Medical Publishing Division, 224–234.
26. Van't–Veen, A., Zee, A. V. D., Nelson, J., Speelberg, B., Kluytmans, J. A. & Buiting, A. G. (2005). Outbreak of infection with a multiresistant *Klebsiella pneumoniae* strain associated with contaminated roll boards in operating rooms. *Journal of Clinical Microbiology*, 43: 4961–4967.
27. Yao, J, Moellering, R. (2011). Antibacterial Agents. In: Versalovic, J., Carroll, K., Funke, G., eds, Manual of clinical microbiology. 10th Editon. Washington DC, ASM Press, p. 1043–1081.
28. Wang, M., Cao, B., Yu, Q., Liu, L., Gao, Q., Wang, L., & Feng, L. (2008). Analysis of the 16S–23S rRNA gene internal transcribed spacer region in *Klebsiella* species. *Journal of Clinical Microbiology*, 46(11): 3555–3563.
29. Wyres, K. L., & Holt, K. E. (2018b). *Klebsiella pneumoniae* as a key trafficker of drug resistance genes from environmental to clinically important bacteria. *Current Opinions in Microbiology*, 45: 131–139.