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Research Article

Detection of DHA-1-Producing Strains and other Associated Virulence Factors of Isolates of *Klebsiella pneumoniae* from a Nigerian Teaching Hospital

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ABSTRACT

Klebsiella pneumoniae strains are a major source of horizontal spread of antimicrobial resistance (AMR) using a plasmid coded AmpC β-lactamase Docosahexaenoic acid (DHA-1). DHA-1-producing *K. pneumoniae* were reported in cases of bacteremia from many centers but yet to be reported from our center. We explored the antimicrobial susceptibility profile, significant virulence factors and phenotypic detection of DHA-1-producing strains among *Klebsiella pneumoniae* from Ibadan. Fifty-one non-repetitive isolates identified as Klebsiella pneumonia by Vitek 2 were analysed for antimicrobial susceptibility by disc diffusion on Mueller Hinton agar. The inducibility of the β-lactamases was detected by the blunting of the oxyimino β-lactam zone of inhibition surrounding cefoxitin disc. Klebsiella pneumonia ATCC 700603 control strain was included. Twenty-nine (56.9%) was confirmed as ESBL, while 98% (50/51) were resistant to the β-lactam inhibitor (Clavulanic acid), with 88% (44/50) showing high-level resistance while 31% (16/51) showed high-level resistance to Cefoxitin. Multiple Antibiotic Resistance index ranged from 0.18 – 1.00, with 21 (41.2%) being extensive drug resistant (XDR) and 11 (21.6%) are pan-drug resistant (PDR). Twenty (39.2%) and 16 (31.4%) were associated with bacteremia and UTI respectively. All were resistant to killing by human serum and 15.7% (8/51) were hypermucoviscous. Ten (19.6%) and 5.9% (3/51) from blood and urine respectively produced biofilms. Two (3.9%) demonstrated inducible AmpC β-lactamase. The observed antibiotic susceptibility profiles and presence of isolates with DHA-1, poses a threat to successful antibiotic treatments of infections, with transfer of inducible beta-lactam resistance among strains of K. pneumoniae and possible dissemination within the hospital and its environment.

Keywords: *Klebsiella pneumoniae, DHA-1, Multidrug resistance, hypervirulent KP, Biofilm*

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INTRODUCTION

Klebsiella pneumoniae (KP) in the family Enterobacteriaceae is known to be both a commensal bacterium inhabiting the gastrointestinal tracts of humans, the environment and also an important healthcare-associated pathogen (Marisa *et al*., 2015) involved in a good number of health-care associated infections (HAI) such as bacteriemia, urinary tract infections and respiratory infections such as pneumonia. (Podschun & Ullmann 1998; Hennequin *et al*., 2012). In addition, KP has also being reported as a leading cause of pyogenic liver abscess in some parts of the world. (Siu *et al*., 2012; Zoltan *et al*., 2016) and neonatal sepsis (Verma *et al*., 2015) as it accounts for neonatal sepsis in 4-9% of cases in developed countries, and 16-28% in developing countries. (In Stoll *et al*., 2002; Akindolire *et al*., 2016; Khaertynov *et al*., 2018). The emerging virulence profiles of this bacterium cannot be overlooked, because in Taiwan, hypervirulent strains of *K. pneumoniae* (hv-KP) were recognized and found to cause liver abscess, meningitis and endophthalmitis in previously healthy adult patients (Wang *et al*., 1998; Yan *et al*., 2002). The prevalence of hv-KP in Asia is reported to be as high as 90% while it was estimated to be 5.4% and 8.2% in Spain and Canada respectively (Yan *et al*., 2002; Baekby *et al*., 2018). It produces a number of virulence factors, such as capsule, fimbriae and siderophores (enterobactin), with its capsule considered to be the dominant virulence property mediating resistance to phagocytosis and killing by serum (Podschun and Ulmann, 1998) and also plays a role in biofilm maturation, (Balestrino *et al*., 2008) while organisms within the biofilm are shielded from antibiotics. (Piperaki *et al*., 2017) These virulence mechanisms are known to contribute to pathogenesis and colonization of the gastrointestinal tract, respiratory and urinary tracts, by the bacterium. (Philippon *et al*., 2002; Piperaki *et al*., 2017) Beyond its well-known virulence mechanisms, *Klebsiella pneumoniae* strains are also

a major source of horizontal spread of antimicrobial resistance (AMR) that is currently a global health issue using a plasmid coded AmpC β-lactamase Docosahexaenoic acid (DHA-1). (Zoltan *et al*., 2016).

DHA-1, reported to originate from *Morganella morganii*, ((Philippon *et al*., 2002) is a novel plasmid-mediated and inducible AmpC β-lactamase obtained from *Enterobacteriaceae* and was the first plasmid-encoded β-lactamase found to be inducible and can be expressed in high levels. (Hsieh *et al*., 2015) They are categorized according to their Ambler molecular structure belonging to class C, where AmpC type β-lactamases are cephalosporinases which can hydrolyze broad and extended-spectrum cephalosporins, (Zoltan *et al*., 2016) functionally they are classified into group 1 and are generally known to be uninhibited by β-lactamase inhibitors such as clavulanic acid. (Bush *et al*., 1995; Jacoby, 2009) AmpC β-lactamase is chromosomally encoded (Zoltan *et al*., 2016) but *Klebsiella pneumoniae* is known to lack a chromosomal bla_{AmpC} gene but plasmid-encoded versions occur and several acquired plasmid-encoded AmpC β-lactamases have been characterized from *Klebsiella pneumoniae* and other members of the family *Enterobacteriaceae* lacking inducible chromosomal AmpC enzymes. (Moland *et al*., 2002) Plasmid-mediated AmpCs (pAmpCs) were reported for the first time in 1989 (Bauernfeind *et al*., 1989) and based on differences in the amino acid sequences, various types of plasmid-mediated AmpC β-lactamases described so far include MOX, CMY, MIR, FOX, DHA, ACT, CFE and ACC (Jacoby, 2009) while many of them are constitutively produced, some such as ACT-1, DHA-1, DHA-2 are inducible. (Jacoby, 2009; Zoltan *et al*., 2016). According to Verdet and colleagues, DHA-type (type 1 and 2) enzymes were first characterized in *Salmonella enterica* serovar Enteritidis strain that was isolated in Dhahran, Saudi Arabia in 1992 and in France respectively. Since then, DHA-1 has been detected in two *K. pneumoniae* isolates from California and Florida and some places in Europe. (Verdet *et al*., 2006)

Klebsiella pneumoniae strains are often multidrug-resistant (Hennequin *et al*., 2011) while the strains with plasmid-mediated AmpC beta-lactamases are consistently resistant to all penicillins and to third generation cephalosporins and the 7-α-methoxy group (Coudron *et al*., 2003; Alvarez *et al*., 2004). The different types of AmpC β-lactamases often occur together with other resistance genes and many other β-lactamases such as the TEMs, PSEs, CTX-Ms, SHVs and VIMs conferring resistance to aminoglycosides, chloramphenicol, quinolones, sulfanomides, tetracyclines. (Coudron *et al*., 2003; Alvarez *et al*., 2004) The bla_{DHA} expression is usually regulated by ampR which is a member of the LysR transcriptional regulator family, (Hennequin *et al*., 2011) AmpR regulator, during normal growth (in the absence of β-lactam as an inducer), binds to the UDP-Mur-NAc pentapeptide in the peptidoglycan of the bacterium thereby repressing the expression of the gene. In the presence of a β-lactam (e.g Cefoxitin) as an inducer, the repression of the gene expression by AmpR is relieved, hence allowing for the transcription of AmpC (Hennequin *et al*., 2011; Hsieh *et al*., 2015). AmpC Beta-lactamase genes are missing from *Klebsiella* species, but it has been shown to be acquired by plasmids in *K. pneumoniae* (Coudron *et al*., 2003). Plasmid-mediated AmpC (pAmpCs) is not as common as the production of Extended-Spectrum β-lactamases (ESBLs), it may be more difficult to detect (Hsieh *et al*., 2015) but are important to recognize since they provide an even broader spectrum of resistance while DHA depresses immune response as it down regulate inflammation. (Philippon *et al*., 2002; Moland *et al*., 2002)

Polymerase chain reaction (PCR) although highly sensitive and specific for the detection of AmpC beta lactamase, it is costly and limited to few reference laboratories and not routine laboratory which, provides services for management of infection. Only phenotypic detection is more readily available and are helpful, but not definitive, for distinguishing R^+AmpC^+ from R^+ESBL^+ strains. (Alvarez *et al*., 2004) Augmentation of the zone diameter by at least 5 mm in the presence of clavulanic acid was observed in 10 of 54 R^+AmpC^+ strains with a ceftazidime disc and in 3 additional strains with a cefotaxime disc, thus yielding an overall positive result for this ESBL confirmatory test of 24%. (Alvarez *et al*., 2004) Therefore, detecting R^+AmpC^+ isolates may be clinically important not only because of their broader cephalosporin resistance but also because carbapenem resistance can arise in such strains by further mutations, resulting in reduced porin expression. (Bradford *et al*., 1997; Martinez *et al*., 1999) However, use of tazobactam may be preferred to Clavulanic acid in detection of co-producers of AmpC and ESBL production as the ability of clavulanic acid to induce AmpC production may interfere with ESBL detection. (Rawat *et al*., 2013) Moreso, the recommended phenotypic confirmation test would fail to detect ESBL in the presence of AmpC, as clavulanic-acid may induce high level of expression of AmpC, thus masking the synergy arising from the inhibition of an ESBL. (Rawat *et al*., 2013). Although, DHA-1 producing *Klebsiella pneumoniae* has been reported from America and Europe, however till date there has not been any reported case from our centre neither was there any from Nigeria as far as we know. This study was therefore designed to explore the antimicrobial susceptibility profile of multiresistant *Klebsiella pneumoniae* isolated from patients and also from the environment of the University College Hospital, Ibadan between 2017 and 2019 for possible phenotypic detection of DHA-1-producing strains and also phenotypically observe for other significant virulence factors such as hypermucoviscosity, biofilm production, haemolysin production and resistance to serum among these *Klebsiella pneumoniae* isolates.

MATERIALS AND METHODS

Study Site: This laboratory based study was carried out in the Medical Microbiology and Parasitology laboratory of University College Hospital (UCH), a tertiary healthcare facility located in Ibadan, Oyo state, southwestern part of Nigeria. It is a 1000 bed space hospital with special care for paediatric, geriatric, cardiac, renal, ophthalmic, surgical, and cancer patients. It has both adult and paediatric intensive care units, and burn unit for high risk patients. The microbiology laboratory processes all types of specimen and has facilities

for bacterial identification to species levels using both Microbact/API systems and VITEK 2.

Study Population: A total of 368 *Klebsiella* species were studied, but following species identification with Vitek 2, only forty-six (46) non-repetitive clinical isolates identified as *Klebsiella pneumoniae* from specimens such as urine, blood, cerebrospinal fluid, wound biopsies, sputum and swabs of patients admitted to the hospital, and five (5) isolates collected during infection control surveillance of ICU, SCBU, Theater and other high risk areas met the inclusion criteria for further analysis.

Bacteria Propagation and Identification: All specimens were cultured on appropriate culture media (Blood agar, nutrient agar, MacConkey agar), and the initial identification was based on substrate utilization on Microbact ™ GNB 24E (OXOID) and only isolates with up to 99% identity were included while further confirmation was based on the VITEK2 system (bioMérieux Vitek Systems Inc. Hazelwood, MO, USA). Thirty-eight of these isolates sent to the US for another molecular study were also confirmed with Kraken ID (Wood and Steven, 2014).

Pure colonies of *Klebsiella pneumoniae* from culture plates were inoculated into Tryptone soy broth, incubated at 37˚C overnight and stored at -80°C. Stored isolates were thawed at room temperature and then streaked on Blood Agar and nutrient agar (non-inhibitory media) and incubated at 37˚C overnight or 24 hours. A sterile wire loop was used to collect/pick 2 colonies from the pure culture on non-inhibitory plate and inoculated into a sterile Bijou bottle containing 5mL peptone water and incubated at 37˚C for 4 hours. This broth was then diluted with sterile normal saline to 10^6 colony forming unit (cfu) per ml by comparing the turbidity with 0.5 McFarland turbidity standard. (Cheesbrough, 2006)

Antibiotic Susceptibility Testing (AST): Resistance phenotypes of isolates were determined by the disc diffusion method on Mueller-Hinton (MH) agar (Bauer *et al*., 1966; CLSI, 2019). Isolates with any zone of inhibition was recorded and interpreted according to CLSI interpretive values.

Quality Control strains: For ESBL test *Klebsiella pneumoniae* ATCC 700603 control strain was included as positive control.

Test for Extended-Spectrum β-Lactamase Production

Screening for and Confirmation of ESBL producers: Disc diffusion and DD Synergy method: Ceftazidime, Aztreonam, Cefotaxime and Ceftriaxone were used on MH Agar plates. ESBL production was suspected in isolates either with reduced susceptibility or resistant to Aztreonam, Cefotaxime Ceftazidime and Ceftriaxone and also positive to double disc synergy test (DDST), this is briefly described-Discs containing 30µg of Ceftazidime and 30µg of Cefotaxime, Cefuroxime (30µg), Ceftriaxone (30µg) were placed at 15-20mm apart (center to center) consecutively from a centrally placed disc containing Amoxycillin (20µg) plus Clavulanic acid (10µg) (AMC) and incubated overnight at 37°C. An extension of zone of inhibition around the peripheral discs towards the AMC disc ("Keyhole effect" or "lens of inhibition") indicates ESBL production. This was confirmed with CLSI Phenotypic Confirmatory test (PCT) also known as combined disk method as outlined below but with some modifications as follows: on a plate with Ceftriaxone 30µg and Ceftriaxone/Sulbactam (30µg/10 µg) showing augmentation with the production of an increase of at least 5mm in the zone diameter around Ceftriaxone-Sulbactam disc when compared with Ceftriaxone alone.

Detection of blaDHA-1, using the Disc antagonism test: On Mueller Hinton susceptibility plate, test for antagonism was carried out with antibiotic discs of Ceftazidime (30 µg), Cefotaxime (30 µg), Cefepime (30 µg), and Aztreonam (30 µg). The antibiotic disc containing Cefoxitin (FOX 30 µg) was placed centrally on the plate as inducing agent, to test for the inducibility of the DHA-1 β-lactamases (Bradford, 2001; Moland *et al*., 2002). The antagonism was detected and reported as positive by the blunting of the oxyimino β-lactam zone of inhibition around a cephalosorin (e.g. Cefotaxime, Aztreonam) discs adjacent to the cefoxitin disc as previously described. (Bradford, 2001; Verdet *et al*., 2006).

Screening for other Virulence Factors

String Test for Hypermucoviscosity: Pure colonies cultured on MacConkey agar plates were obtained and studied for their ability to form viscous strings. This phenotype was determined and ascertained by the formation of viscous strings of >5mm length (Baekby *et al*., 2018).

Crystal Violet Assay for Biofilm Quantification (Wilson *et al*., 2017): Bacterial nutrient broth suspension was made in 96-well microtiter-plate and incubated at 37˚C for 24 hours or overnight. The sessile isolates of which biofilms formed on the walls of wells of microtiter-plate were stained with crystal violet for 15 minutes, after planktonic cells in wells of microtiter-plate have been discharged by washing with phosphate buffer saline and biofilms fixed with methanol for 20 minutes. Crystal violet stains were then washed twice with phosphate buffer saline. Based on adherence strength, the biofilm forming abilities were classified into four different categories: non-adherent, weakly adherent, moderately adherent and strongly adherent. The more adherent the higher the biofilm produced.

Serum Bactericidal Assay: The ability of the isolates to resist killing by serum was tested as described previously (Podschun *et al*., 1993). The isolates were grown in nutrient broth at 37˚C overnight. The turbidity of the overnight broth culture was adjusted to 1.0 McFarland standard. Cultures were centrifuged at 1500 rpm for 5 minutes and the deposit re-suspended in 5ml phosphate buffer saline (PBS). Pooled normal serum from healthy individuals who has not been on antibiotics for at least 4 weeks was collected, and 350 µL of the fresh human serum was mixed with 150 µL of bacterial suspension and incubated at 37˚C. Organism growth was determined before the

incubation i.e. within the first 1 hour of mixing, and recorded as zero hour and also at 24 hours. Growth at both times was interpreted as serum resistance.

Hemolysin Production: Hemolysin production was detected using the method described by Martinez. (Martínez- Martínez *et al*., 1999). All bacterial isolates were grown on blood agar (BA) at 37˚C for 18-24 hours. The presence of clear zone around the colonies was taken as positive for hemolysin production.

RESULTS

Sources of isolates: Twenty (39.2%) and 16 (31.4%) of the isolates were associated with blood stream and urinary tract infection respectively, other sources were Sputum (4), amniotic fluid (3), wound (3) and hospital environment (5).

Antibiotic susceptibility and ESBL production: The results of the susceptibility tests and multiple antibiotic resistance index (MAR) are as shown in Table 1 and Fig. 1. Reduction in zone of inhibition around Cephalosporin discs (CAZ, CTX and CRO), implies a possibility of ESBL production while a double-disc synergy test and combined disc tests confirmed the production of ESBL in 74.5 % (38/51) isolates (Table 2 and Plate 1). A total of 98% (50/51) were resistant to the β-lactam inhibitor Amoxicillin-Clavulanic acid while 88% (44/50) of the resistant strains showed high-level resistance (defined as resistance with no zone of inhibition around antibiotic discs) to the same inhibitor. High-level resistance to Cefoxitin was also observed in 31% (16/51) of the isolates. Multiple Antibiotic Resistance index (MAR index) proposed by Krumperman in 1983 was calculated for all the isolates and it ranged from 0.17 – 1.00 with MAR index of 1.00 recorded for 2 isolates, 0.92 - 0.80 for 18 isolates, and 0.81- 0.50 in 17 isolates while 0.49-0.17 was recorded for 14 isolates (Fig. 1)..

Table 1.
Antibiograms of *Klebsiella pneumoniae* isolates

Antibiotic	Total Number Of Organism Tested	Susceptible	Intermediate	Resistant
Ceftriaxone (CRO)	51	21 (41.17%)	1 (1.96%)	29 (56.82%)
Ceftriaxone+Sulbactam (CRS)	51	38 (74.51%)	6 (11.76%)	7 (13.73%)
Ceftazidime (CAZ)	51	12 (23.53%)	4 (7.84%)	35 (68.63%)
Cefotaxime (CTX)	51	11 (21.57%)	0	40 (78.43%)
Cefoxitin (FOX)	51	24 (47.06%)	6 (11.76%)	21 (41.18%)
Cefepime (FEP)	51	14 (27.45%)	9(17.65%)	28 (54.90%)
Aztreonam (ATM)	51	11 (21.57%)	8 (15.69%)	32 (62.74%)
Ampicillin (AMP)	51	1 (1.96%)	1 (1.96%)	49 (96.08%)
Azithromycin (AZM)	51	4 (7.84%)	10 (19.61%)	37 (72.55%)
Amoxicillin-Clavulanic Acid (AMC)	51	1 (1.96%)	0	50 (98.04%)
Amikacin (AK)	51	20 (39.21%)	10 (19.61%)	21 (41.18%)
Erythromycin (E)	51	0	5 (9.80%)	46 (90.20%)
Nitrofurantoin (F)	15	6 (40.0%)	1 (6.67%)	8 (53.33%)
CUMULATIVE %		26.0%	9.73%	64.27%

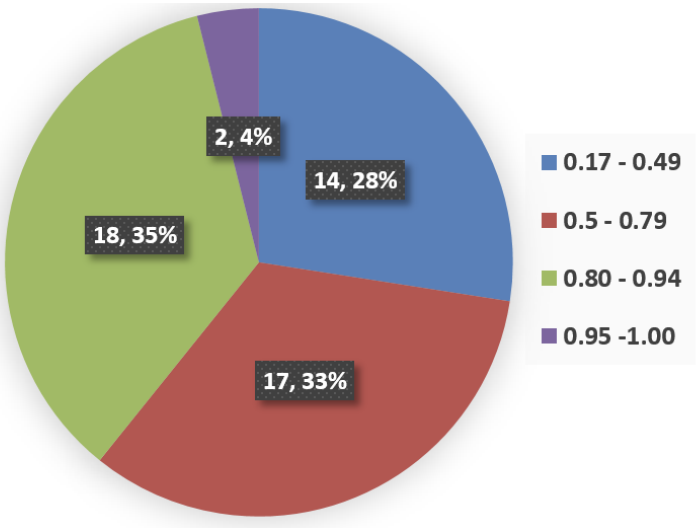

Figure 1:
Multiple Antibiotic Resistance Index

Table 2.
Phenotypic Characterization of Virulence Factors of Isolates

Isolate Source (N)	ESBL		Inducible B-Lactam Resistance		Hmv Test		Serum Resistance Test		Biofilm Production				Hemolysis Test		
	+Ve	-Ve	+Ve	-Ve	+Ve	-Ve	Res	Non-Res.	SA	MA	WA	NA	α	β	NH
Blood (21)	19	2	0	21	3	18	21	0	1	9	1	10	2	3	16
Urine (15)	12	3	0	15	2	13	15	0	1	2	6	6	0	1	14
Wound Biopsy (3)	2	1	0	3	0	3	3	0	0	1	1	1	1	0	2
Sputum (4)	2	2	1	3	0	4	4	0	0	2	1	1	0	0	4
Amniotic Fluid (3)	0	3	1	2	0	3	3	0	0	2	0	1	0	0	3
Environ (5)	3	2	0	5	3	2	5	0	1	1	2	1	0	0	5
Total (%)	**38 (74.5)**	**13 (25.5)**	**2 (3.9)**	**49 (96.1)**	**8 (15.7)**	**43 (84.3)**	**51 (100)**	**0**	**3 (5.9)**	**17 (33.3)**	**11 (21.6**	**20 (39.2**	**3 (5.9)**	**4 (7.8)**	**44 (86.3)**

Key
+VE = Positive result; -VE = Negative result; Res. = Resistant; Non-Res. = Non-resistant; SA = Strongly adherent; MA = Moderately adherent; WA = Weakly adherent; NA = Non-adeherent; NH = Non-haemolytic

Plate 1
A: CLSI Phenotypic Confirmatory test: Combined disc method for Confirmation of Beta lactamase using Ceftriaxone alone and Ceftriaxone/Sulbactam demonstrating an increase of at least 5mm around CRS= Ceftriaxone/Sulbactam disc.
B: Double Disk Synergy Test Positive showing Extension in the zone of inhibition around the peripheral disc towards the centrally placed AMC.

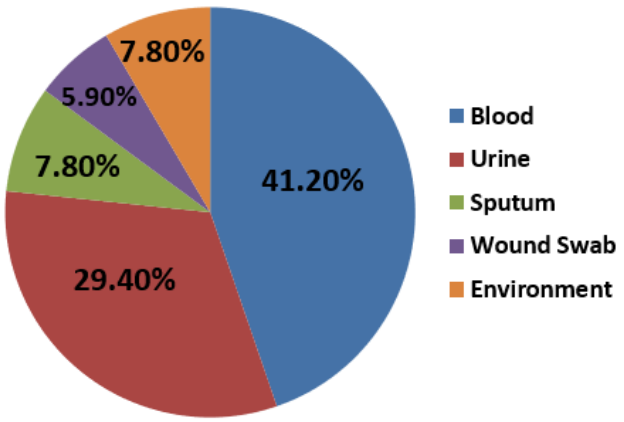

Figure 2.
Pie-Chart showing Distribution of Multiple-Resistance among sources of Isolates

With regards to the sources of isolates and all categories of drug resistance, 41.2% and 29.4% of the multiply resistant isolates were from blood and urine respectively (Fig. 2). In terms of categories of resistance profiles of the isolates, 17 (33.3%) are Multidrug Resistant (MDR), 21 (41.2%) are Extensive Drug Resistant (XDR) strains, 11 (21.6%) are Pandrug Resistant (PDR) while one isolates each were susceptible to Macrolides and Penicillins (Fig. 3).

Inducibility of β-lactamases: Inducibility of the β-lactamases was recognized by the disc antagonism test, which demonstrated blunting of a cephalosporin (Cefotaxime) and Aztreonam discs adjacent to the Cefoxitin disc which was used as an inducer. 3.9% (2/51) of the isolates clearly demonstrated blunting of the cephalosporin and Aztreonam discs, indicating induced resistance to two of the antibiotic drugs in the presence of Cefoxitin (Plate 2)

Other Virulence factors: The virulence factors of all 51 isolates are shown in Table 2. Phenotypically, 15.7% (8/51) showed the hypermucoviscous phenotype (indicated by a 5mm string when an inoculation loop was used to stretch the mucoid colonies) associated with hypervirulent *Klebsiella pneumoniae* (Plate 3). This feature was found in three and two isolates from blood and urine respectively but also in three environmental isolates. All the 51 isolates showed resistance to killing by human serum.

Biofilm formation: Thirty-one isolates tested positive to biofilm formation and the intensity was classified as weak, moderate, and strong but twenty isolates (39.2%) tested strongly positive to biofilm formation. Ten (19.6%) isolates, all from blood produced the highest levels of biofilm formation (Strong and moderate adherence), however, 5.9% (3/51) other isolates from urine also showed strong intensity of biofilm formation and the rest with either moderate or weak intensity were from other specimens (Table 2). This high level of biofilm formation was compared among hypermucoviscous and non-hypermucoviscous isolates and it was observed that 3 (37.5%) of the 8 hypermucoviscous isolates also showed high levels of biofilm formation, compared to 16/43 (37.2%) non-hypermucovisocus isolates.

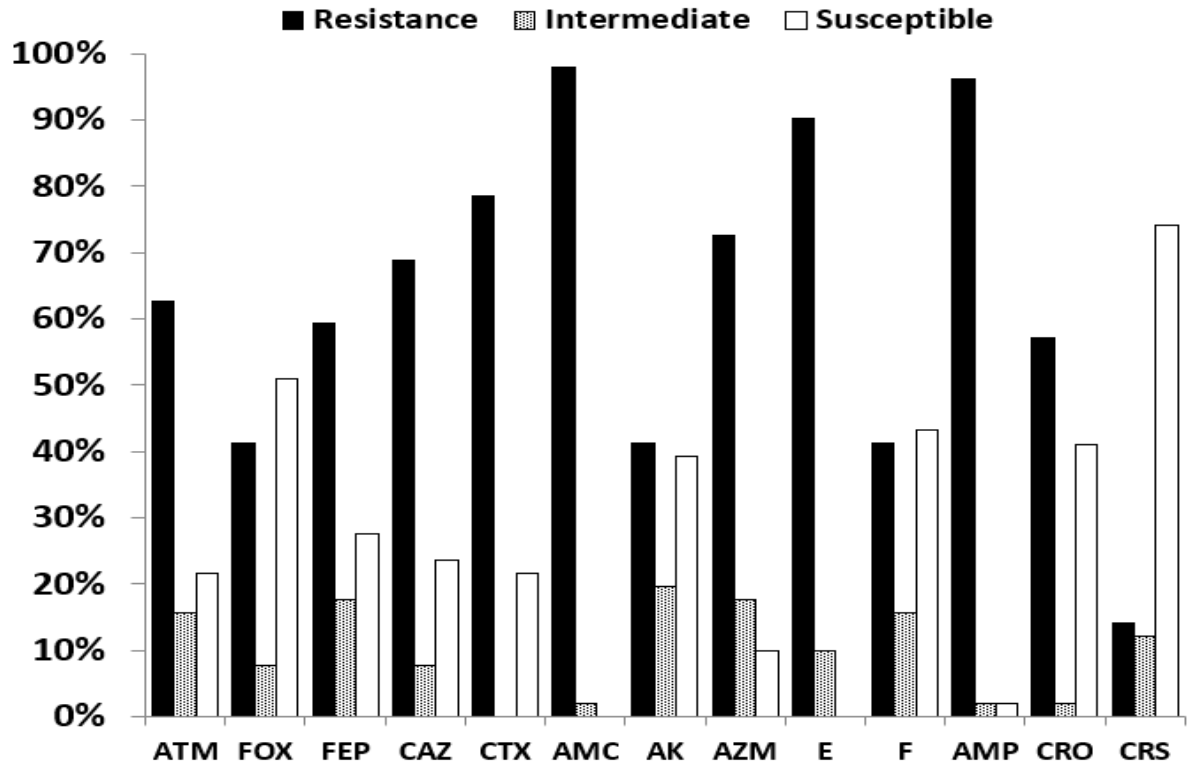

Figure 3.
Antibiotic Susceptibility Distribution for Each Antibiotic Drug Tested
ATM: Aztreonam; **FOX**: Cefoxitin; **FEP**; Cefepime; **CAZ**: Ceftazidime; **CTX**: Cefotaxime; **AMC**: Amoxicillin-Clavulanic Acid; **AK**: Amikacin; **AZM**: Azithromycin; **E**: Erythromycin; **F**: Nitrofurantoin; **AMP**: Ampicillin; **CRO**: Ceftriaxone; **CRS**: Ceftriaxone/Sulbactam

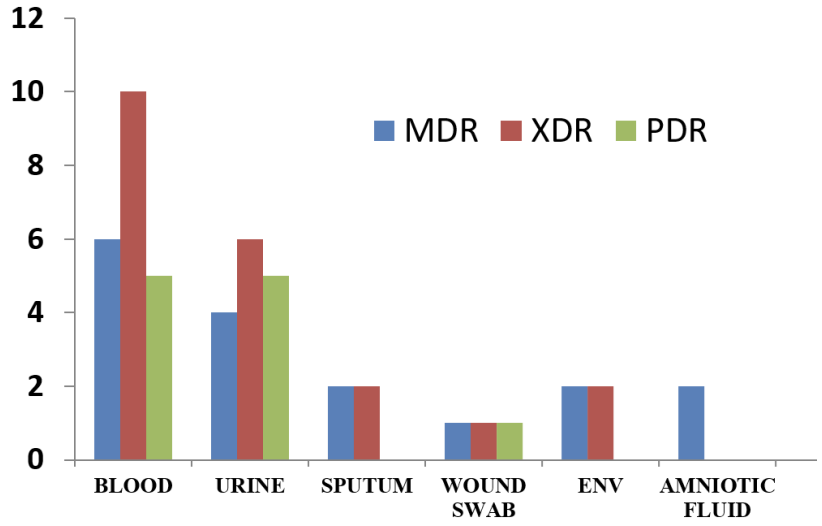

Figure 4.
Chart showing Distribution (in Numbers) of Multiple Resistance Phenotypes among Sources of Isolation

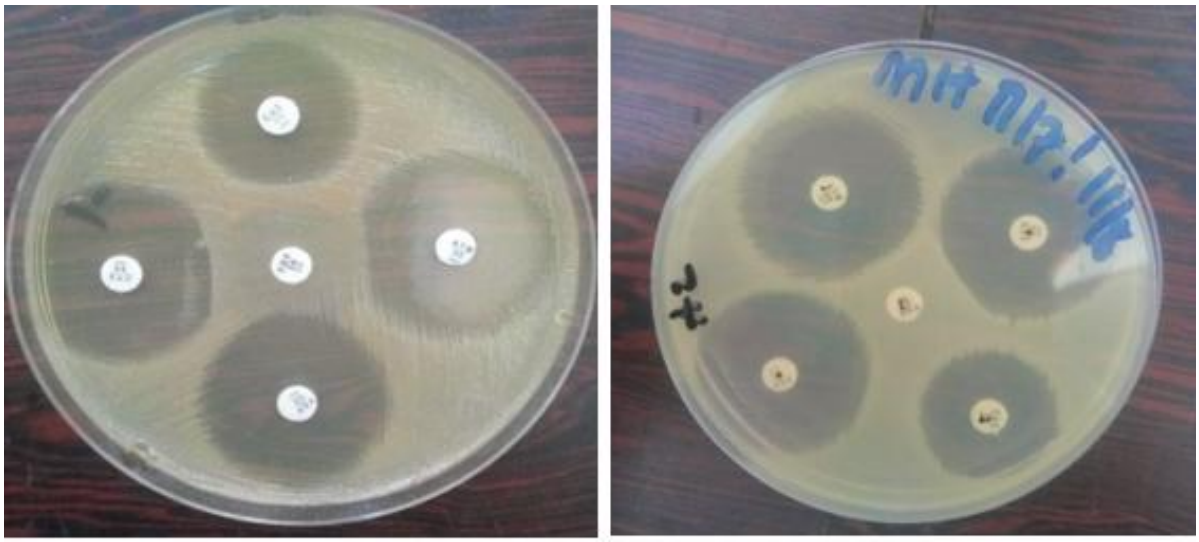

Plate 2.
Mueller Hinton plates showing blunting of a cephalosporin (Cefotaxime) and Aztreonam discs adjacent to the cefoxitin disc, indicating the production of inducible DHA-1 in 2 different *Klebsiella pneumoniae* isolates.

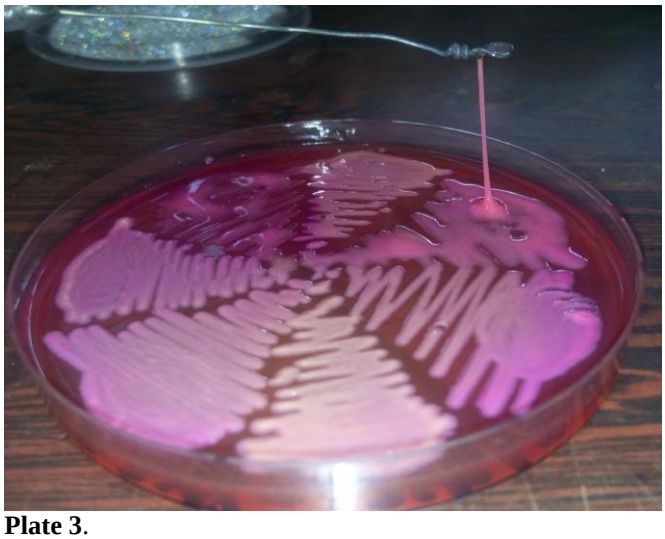

Plate 3.
Positive "**String test**" indicated by formation of a viscous string > 5mm in length when bacterial colony on MacConkey agar plate is picked and stretched by an inoculation wire loop.

Haemolytic activity was found among three isolates from blood and one from urine, which showed ability to lyse red blood cell as was evinced by the clear zone around colonies of the isolate on blood agar, termed beta-hemolysis. Additionally, 2 isolates from blood together with 1 isolate from wound swab showed partial i.e. alpha-hemolysis.

DISCUSSION

In this phenotypic study of the antimicrobial susceptibility patterns of *K. pneumoniae* isolated from patients at the University College Hospital as well as from the hospital environment, more than half of the recovered isolates were resistant to Cephalosporin antibiotics including Cefepime, Ceftazidime, Ceftriaxone and Cefotaxime; and of note is that the majority of isolates showed a very reduced level of susceptibility to all the antibiotics tested. All isolates exhibited high-level resistance to amoxicillin-clavulanic acid, that is suggestive of the β-lactamase class B or D which agrees with the findings of earlier studies conducted from 1999 to 2001 and 1998 to 2003, in which 100% (10/10) and 100% (12/12) respectively of the *K. pneumoniae* isolates collected were amoxicillin-clavulanic acid resistant. (Wood and Steven, 2014; Baekby *et al*., 2018) Majority of the isolates were also found to be ESBL producers in addition to resistance to broad range of antibiotics tested. This feature among *Klebsiella* is suggestive of other mechanism(s) of resistance beyond ESBL production that must be considered in the selection of effective antibiotics for managing serious or systemic infections.

Surprising and worrisome is the observation from the present study, which analyzed a broad range of antimicrobial susceptibility profiles of *K. pneumoniae* isolates from sources including blood, urine, sputum, amniotic fluid, wound swab and the hospital environment, is that 75% of the environmental isolates carried the ESBL enzyme while 50% (19/38) and 31.6% (12/38) of the ESBL producers are from blood and urine respectively. Seven (7) drug groups which include Penicillins, Cephalosporins, Macrolides, Aminoglycosides, Monobactams, Cephamycin and Nitrofuran were tested and out of 51 isolates, 17 (33.3%) are MDR, 21 (41.2%) are XDR, and 11 (21.6%) are PDR, giving a total of 96.1% (49/51) multiple resistance. This finding was also supplemented by the multiple antibiotic resistance (MAR) index which indicated a total of 86.2% occurrence of high index, with thirty-seven (72.5%) of the isolates showing MAR > 0.5 (0.5 – 1.0). A study from Lagos, also in South western Nigeria, reported MAR of 0.4 among Klebsiella species as far back in 2013 (Osundiya *et al*., 2013). Multiple antibiotic resistance (MAR) in bacteria is most commonly associated with the presence of plasmids with one or more resistance genes, but each encoding a single antibiotic resistance phenotype (Daini *et al*., 2005). These results put together could presumably point toward a

dissemination of hyper-resistant *Klebsiella pneumoniae* within the hospital and its environment.

In considering the sources of isolation, isolates recovered from blood infections were found to exhibit the highest level of multiple-resistance, constituting 35.3% (6/17) of MDR isolates, 47.6% (10/21) of XDR isolates and 45.5% of PDR isolates. Isolates recovered from urinary tract infections also exhibited relatively high levels of multiple-resistance, constituting 45.5% (5/11) of PDR isolates, 25.3% (4/17) of MDR isolates, 28.6% (6/21) of XDR isolates. Finding ESBL and MDR isolates from environmental surfaces is an indication that they may serve as reservoirs for multiple-resistant *K. pneumoniae* transmission within the hospital as 4 out of 5 of the environmental isolates were distributed among MDR and XDR phenotypes and 3/5 were ESBL producers.

The presence of the extended-spectrum β-lactamase (ESBL) producers among blood isolates, implies that cases of septicaemia due to *Klebsiella pneumoniae* are likely to be fatal due to failure of therapy and will require combination of expensive and more toxic antibiotic therapy. In addition, 2 isolates showed blunting of the cephalosporin discs adjacent to the Cefoxitin, indicating an unusual inducible β-lactam resistance phenotype (AmpC Beta lactamase i.e.*bla*DHA-1). DHA-1 was the first plasmid-encoded β-lactamase found to be inducible and can be expressed in high levels (Jacoby 2009: Baekby *et al*., 2018). The two isolates showed resistance to Cefoxitin and Amoxycillin-clavulanic acid although, remained susceptible to cephalosporins and cefepime and also exhibited relatively low MAR index (0.25 – 0.42) This is consistent with the finding of a previous study, in which all 10 isolates in the study remained susceptible to cefepime despite being resistant to clavulanic-acid (Wood and Steven, 2014) thus, indicating that fourth-generation cephalosporins could be better choices for treatment of infections caused by DHA-1 producers. According to Alvarez and colleagues in a multicenter study involving 70 sites in 25 States of the United State of America among 752 resistant *Klebsiella pneumoniae* also confirms the presence of AmpC-type from ESBL-determined resistance and found increase resistance to oxyimino-β-lactams in *Klebsiella oxytoca* and *Escherichia coli*, however, DHA-1 was found in only one *Klebsiella pneumoniae* isolate from Los Angelis, California (Alvarez *et al.*, 2004). The report of DHA-1 from the USA, Taiwan, and Seoul South Korea (Verdet *et al*., 2006) and now from Nigeria (this study) is an indication that it is becoming a global occurrence. However, whenever the presence of inducible DHA-type enzymes is detected, clinicians should be informed to avoid the use of strong AmpC-inducing agents such as cephamycins, since AmpC producing phenotype confer resistance to cephamycins and to amoxicillin-clavulanate (Grover *et al*., 2006).

The therapeutic implication of the clavulanic-acid resistant *Klebsiella pneumoniae* is the fact that such isolates most likely carried plasmid mediated enzymes (R^+AmpC^+ or transmissible resistance strains) conferring resistance to extended spectrum lactams, therefore reducing further the available choice of antibiotic therapy. Also, the clinical implication of detecting R^+AmpC^+ isolates, apart from being resistant to a broader range of cephalosporins is that carbapenem resistance can arise in such strains by further mutations from reduced porin expressions according to some previous reports (Martínez- Martínez *et al*., 1999, Bradford *et al.*, 2001). Another important implication is diagnostic, as ESBL-mediated resistance is not routinely tested for, neither is it easily detected during routine susceptibility testing, the susceptibility report may indicate false susceptibility to some β-lactam antibiotics. Moreso, the recommended phenotypic confirmation test would fail to detect ESBL in the presence of AmpC, as clavulanic-acid may induce high level of expression of AmpC, thus making the synergy arising from the inhibition of an ESBL (Rawat *et al.*, 2013). False negative results may occur in isolates that produce high levels of AmpC enzymes, therefore misleading the clinicians into ineffective treatment of the infected patients, which may increase morbidity or even mortality.

The relationship between antibiotic resistance and virulence is still a question of debate. It has been reported overtime that bacterial virulence is indeed difficult to reconcile with antibiotic resistance. (Hennequin *et al*., 2012). The present study observed that, despite the very high level of multiple-resistance, exhibition of virulence factors by the isolates was not directly proportional. Out of the 51 isolates, only 13.7% (7/51) showed production of either β- or α-hemolysin, however, 20 (39.2%) of the isolates exhibited high levels of biofilm formation, while all 51 isolates were resistant to killing by normal human serum. The major significance of *K. pneumoniae* biofilm production is in the inner surfaces of indwelling devices especially urinary catheters and shunts, such organisms are shielded from the activities of the immune system and antibiotics which most often act on rapidly dividing organisms and could form a source of high-level resistance to antibiotics. In addition it may contribute to colonization of the other systems such as GIT, Urinary tract and invasion of the blood stream especially in immunocompromised patients. Phenotypically, using the hypermucoviscosity string test, 8 (15.7%) isolates were found to be positive for hypermucoviscosity, and this could be indicative of hyperproduction of capsule. Although these findings showed little to no direct relationship to antimicrobial resistance and virulence, it is worth noting that these isolates exhibited high levels of capsule production, meanwhile, *K. pneumoniae* capsule has been linked to resistance to killing by serum (Podschun and Ulmann, 1998) and biofilm maturation, while the hypermucoviscous phenotype has been linked with hypervirulence (Nassif *et al*., 1989). It has also been observed that bacteria in biofilms showed increase resistance to antibiotics, disinfectants, as well as to host immune system clearance (Donlan and Costerton 2002).

Although the positive string test does not automatically translate to hypervirulence, hypermucoviscosity in *K. pneumoniae* is a typical characteristic of hypervirulent *K. pneumoniae* (Akindolire *et al*., 2016). In the current study 37.5% and 25% of the hypermucoviscous isolates were from cases of septicaemia and UTI respectively, and also 37.5% were found in the environment. Therefore, detection of this phenotype among isolates in Ibadan should raise concern, as it may indicate circulation of a population of hypervirulent strain. This is presumably the first report of the detection of *K. pneumoniae* with this phenotypic trait in Nigeria. Reports from several parts of the world such as Taiwan, France and in

North America, indicated that hypervirulent *K. pneumoniae* is a known cause of serious infections such as necrotizing fasciitis, primary endophthalmitis, meningitis, non-hepatic abscesses and infectious syndromes of pyogenic liver abscesses with or without metastatic spread. (Wang *et al*., 1998; Siu *et al*., 2012; Baekby *et al*., 2018). Therefore, finding them in patients environment in the present study could be an indication of the emergence and spread of this trait in the hospital and a wake-up call for improved AMR surveillance and active infection control and prevention practices, as poor IPC practice can influence the spread of the hypervirulent strain. The observed resistance and virulence profiles among these isolates may not be unconnected with the multiple antibiotic treatments used in the hospital which may have influenced the expression of unknown genes that allow these strains to resist the hostile antibiotic drug environment and expression of virulence factors.

In conclusion, the observed antibiotic susceptibility profiles pose a threat to antibiotic treatments of infections caused by *Klebsiella pneumoniae* in our hospital and possibly in Nigeria. It also suggests a possible transfer of inducible beta-lactam resistance among strains of *K. pneumoniae* in the hospital which could hinder successful treatment of *Klebsiella pneumoniae* infections with cephalosporins and cephamycins. This calls for more awareness, a rethink in the formulation and application/implementation of antibiotic policy and strict adherence to rational use of antibiotics. Further extensive and explorative study into the genetic basis of hypervirulence and ways to tackle antimicrobial resistance in the country is recommended.

REFERENCES

Akindolire E. A., Tongo O., Dada-Adegbola, H., Akinyinka O. (2016): Etiology of early onset septicemia among neonates at the University College Hospital, Ibadan, Nigeria. *J Infect Dev Ctries*. 10 (12), 1338-1344.

Alvarez M., Tran J. H., Chow N. and Jacoby G. A. (2004): Epidemiology of Conjugative Plasmid-Mediated AmpC β-Lactamases in the United States. *Antimicrob. Agents Chemother*. 48, 2; 533–537.

Baekby M, Nicolas H, Thomas D. S., Karen A. K., Carsten S. (2018): Hypervirulent *Klebsiella pneumoniae* K1 liver abscess and endogenous endophthalmitis in a Caucasian man. *Clin Case Rep*. 6, 1618–1623. doi: 10.1002/ccr3.1696

Balestrino D, Ghigo J. M., Charbonnel N, Haagensen J. A., Forestier C. (2008): The characterization of functions involved in the establishment and maturation of *Klebsiella pneumoniae* in vitro biofilm reveals dual roles for surface exopolysaccharides. Environ. Microbiol. 10, 685-701.

Bauernfeind, A., Chong, Y. & Schweighart, S. (1989): Extended broad spectrum beta-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. *Infection*. 17, 316-321

Bradford, P. A. (2001): Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev*. 14, 933-951.

Bradford, P. A., Urban C., Mariano N., Projan S. J., Rahal J. J. and Bush K. (1997): Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase, and the loss of an outer membrane protein. *Antimicrob. Agents Chemother*. 41, 563– 569.

Bush, K., Jacoby, G. A. & Medeiros A. A. (1995): A functional classification scheme for beta-lactamase and its correlation with molecular structure. *Antimicrob Agents Chemother*.39, 1211-1233

Cheesebrough M. (2000): Medical laboratory manual for tropical countries. vol II. Microbiology. Cambridge University Press

Clinical and Laboratory Standards Institute (CLSI). (2019): Performance standards for Antimicrobial Susceptibility Testing. 29th ed. CLSI Supplement M 100 (ISBN 978-1-68440-032-4. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA.

Coudron, P. E., Hanson N. D. and Climo M. W. (2003): Occurrence of extended-spectrum and AmpC β-lactamases in blood stream isolates of *Klebsiella pneumoniae*: isolates harbor plasmid-mediated FOX-5 and ACT-1 AmpC β-lactamases. *J. Clin. Microbiol*. 41, 772–777.

Grover S. S., Sharma M, Chattopadhya D, Kapoor H, Pasha S. T., Singh G. (2006): Phenotypic and genotypic detection of ESBL mediated cephalosporin resistance in *Klebsiella pneumoniae:* emergence of high resistance against cefepime, the fourth generation cephalosporin. *J Infect*. 53 (4), 279-288. doi:10.1016/j.jinf.2005.12.001

Hennequin C, Robin F, Cabrolier N, Bonnet R, Forestier C. (2011): Characterisation of a DHA-1-Producing *Klebsiella pneumoniae* Strain Involved in an Outbreak and Role of the AmpR Regulator in Virulence. Antimicrobial Agents and Chemotherapy. 288-294 doi:10.1128/AAC.00164-11

Hsieh W. S., Wang N. Y., Feng J. A., Weng L. C., Wu H. H. (2015): Identification of DHA-23, a novel plasmid-mediated and inducible AmpC beta-lactamase from *Enterobacteriaceae* in Northern Taiwan. *Front. Microbiol*. 6, 436. doi: 10.3389/fmicb.2015.00436

Jacoby G. A. (2009): AmpC beta-lactamases. *Clin. Microbiol. Rev.* 22, 161-182. doi: 10.1128/CMR.00036-08

Khaertynov K. S., Vladimir A. A., Albert A. R., Yuriy N. D., **Dina R. S., Sergey A. L., Natalia N. S. (2018):** Virulence Factors and Antibiotic Resistance of *Klebsiella pneumoniae* Strains Isolated from Neonates with Sepsis. *Front. Med.* 5, 225. doi: 10.3389/fmed.2018.00225

Krumperman, P. H. (1983): Multiple Antibiotic Resistance Indexing of Escherichia coli to Identify High-Risk Sources of Fecal Contamination of Foods. *Appl. Environ. Microbiol*. 46 (1), 165-170.

Marisa B, Marthie M, Ricardo F, Marleen M. (2015): Review-understanding beta-lactamase producing *Klebsiella pneumonia*. http://dx.doi.org/10.5772/61852

Martínez-Martínez, L., Pascual A., Hernandez-Alles S., Alvarez-Díaz D., Suarez A. I., Tran J., Benedí V. J., *et al.* (1999): Roles of β-lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae. Antimicrob. Agents Chemother*. 43,1669–1673.

Moland E.S., Black J.A., Ourada J., Reisbig M. D., Hanson N. D., and Thomson K.S. (2002): Occurrence of newer Beta-lactamases in *Klebsiella pneumoniae* isolates from 24 U.S. hospitals. *Antimicrob. Agents Chemother*. 46,3837– 3842.

Nassif X, Fournier J. M., Arondel J, Sansonetti P. J. (1989): Mucoid phenotype of *Klebsiella pneumoniae* is a plasmid-encoded virulence factor. *Infect. Immun.* 57,546–552

Philippon A., Arlet G., and Jacoby G. A. (2002): Plasmid-determined AmpC type Beta-lactamases. *Antimicrob. Agents Chemother*. 46, 1–11.

Piperaki E. T., Syrogiannopoulos G. A., Tzouvelekis L. S., Daikos G. L. (2017): *Klebsiella pneumoniae*: Virulence, Biofilm and Antimicrobial Resistance. *The Pediatric Infectious Disease Journal*. 36 (10), 1002-1004.

Podschun R, Ullmann U. (1998): *Klebsiella spp*. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. 1, 589-603; PMID:9767057.

Rawat V, Singhai M, Verma P. K. (2013): Detection of different b-lactamases and their co-existence by using various discs combination methods in clinical isolates of Enterobacteriaceae and Pseudomonas spp. *J Lab Physicians*. 5, 21-5.

Siu L. K., Yeh K. M., Lin J. C. (2012): *Klebsiella pneumoniae* liver abscess; a new invasive syndrome. Lancert Infect. Dis.12, 881-7

Stoll Barbara J., Nellie Hansen, Avroy A. Fanaroff, Linda L. W., Waldemar A .C. (2002): Late-Onset Sepsis in Very Low Birth Weight Neonates: The Experience of the NICHD Neonatal Research Network. *Pediatrics*.110;285 DOI: 10.1542/peds.110.2.285

Verdet Charlotte, Yahia Benzerara, Valerie Gautier, Oliver Adam, Zahia Ould-Hocine, and Guillaume Arlet. (2006): Emergence of DHA-1-Producing *Klebsiella spp*. in the Parisian Region: Genetic Organization of the ampR Genes Originating from Morganella morganii. *Antimicrobial Agents and Chemotherapy*. 607-617. doi:10.1128/AAC.50.2.607-617.2006

Verma P, Berwal P. K., Nagaraj N, Swami S, Jivaji P, Narayan S. (2015): Neonatal sepsis: epidemiology, clinical spectrum, recent antimicrobial agents and their antibiotic susceptibility pattern. *Int. J. Contemp. Pediatr*. 2, 176-80 doi: 10.18203/2349-3291.ijcp20150523

Wang J. H., Liu Y. C., Lee S. S., Yen M. *Y., Chen Y. S. (1998):* *Primary liver abscess due to Klebsiella pneumoniae in Taiwan. Clin. Infect. Dis.* 26, 1434-8; PMID: 9636876;

Wood D. E. and Steven L. S. (2014): Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biology. 15: R46.

Yan J. J., Ko W. C., Jung Y. C., Chuang C. L., and Wu J.J. (2002): Emergence of *Klebsiella pneumoniae* isolates producing inducible DHA-1 beta-lactamase in a university hospital in Taiwan. *J. Clin. Microbiol*. 40, 3121-3126.

Zoltan K, Akos T, Laura J and Ivelina D. (2016): Country wide dissemination of DHA-1-type plasmid-mediated AmpC beta-lactamase-producing *Klebsiella pneumonaie* ST11 international high-risk clone in Hungary, 2009-2013. *J*. Med. Microbiol. 65, 1020-1027. doi:10.1099/jmm.0.000302