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DETECTION OF VIRULENCE GENES IN HYPERVIRULENT *Klebsiella pneumoniae* FROM CLINICAL SPECIMENS

WADA N. MUHAMMAD[1], UDOSEN I. EDWARD[1], OMAR G. PAN[1], EDIGA B. AGBO[2], AHMED F. UMAR[2],

[1]Department of Science Laboratory Technology, Federal Polytechnic Bauchi

[2]Department of Biological Sciences, Faculty of Sciences, Abubakar Tafawa Balewa University, Bauchi

Corresponding author: wnmuhammad@fptb.edu.ng

Klebsiella pneumoniae is one of the most important pathogenic bacteria, causing many diseases such as pneumonia, urinary tract infections, bacteremia, burns and wounds infections as well as pyogenic liver abscesses. Pathogenicity of *Klebsiella pneumoniae* is due to the presence of many virulence factors. This study was aimed to isolate and characterize *Klebsiella pneumoniae* from clinical specimens of urine and blood. The sample size of 268 were obtained using Cochran's formula. The methods that were used in the study included string test to detect the hypervirulent strains from the *Klebsiella pneumoniae* isolates. The results of this study revealed that 49(18.3%) of females (30-39years) had the highest distribution, while 09(3.4%) of male (>50years) showed lowest distribution in both sexes out of the total sample investigated. Bacterial isolates according to specimens collected during the Study revealed that 152(56.7%) were gram negative having the highest distribution, while 4(35.1%) were gram positive being the lowest distribution. The study also showed that only 19(12.5%) of the total isolates were *Klebsiella pneumonia*. Out of the nineteen (19) sample of *Klebsiella pneumonia*, only 4(21.0%) were hypervirulent. k1k2 genes were detected in hypervirulent isolates from urine, while k1k2 genes were absent in hypervirulent isolates from Blood sample. *Klebsiella pneumoniae* exhibited 100% resistance to Ampicillin and Cephalexin while Streptomycin exhibited 17(89.5%) activity. hvKP in critically ill patients from the ICU might have formed a new threat especially in the presence of antibiotic resistance. It is important to properly investigate for hvKP during routine laboratory analysis.

KEYWORDS: *Klebsiella pneumoniae*, Hypermucoviscosity, Capsular Genes, Antibiotic, Bacteremia

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Introductions

Klebsiella pneumoniae is one of the most important pathogenic bacteria. It is Gram-negative, bacillus, non-motile and causative agent of many diseases, such as pneumonia, urinary tract infections, bacteremia, burns and wounds infections as well as pyogenic liver abscesses [(1)]. The pathogenicity of *Klebsiella pneumoniae* is due to the presence of many virulence genes, which encode virulence factors that allow it to attack the immune system of mammalians and cause many kinds of diseases. Some of these virulence factors are biofilm formation, hypermucoviscosity, capsule synthesis, adhesions, iron uptake and lipopolysaccharides formation [(2;3)]. *Klebsiella pneumoniae* has been found capable to resist many antibiotics, especially the third-generation cephalosporins like cefotaxime, ceftriaxone and ceftazidime [(4)]. Many clinical features of *Klebsiella pneumoniae* infections are related to virulence genes [(5)].

Recently, *Klebsiella pneumoniae* is found causing acute liver abscess as reported in many Asian countries like China, Kuwait and Iraq [(6;3)]. *Klebsiella pneumoniae* mostly contains extended-spectrum beta-lactamase genes (*SHV, TEM* and *C-TXM*) that are encode by the plasmid. These genes have shown resistance to many types of antibiotics (More than three classes), which is considered multidrug-resistant bacterium [(7)]. In spite of that, *Klebsiella pneumoniae* is considered one of the most important opportunistic bacteria. However, knowledge of the mechanism by which this bacterium causes many diseases is still not fully understood.

The majority of infections due to *Klebsiella pneumoniae* in Western countries are due to "Classic" *Klebsiella pneumoniae* strains. This predated the Gram's stain technique, which was developed in 1884. It was initially named Friedlander's bacillus but was changed to Klebsiella in 1886. In the pre-antibiotic era, *Klebsiella pneumoniae* were implicated as a cause of pneumonia, especially in alcoholics and diabetic patients. It will be also an established uropathogen, and a cause of biliary tract infections, osteomyelitis and bacteremia. The epidemiology of *Klebsiella pneumoniae* infections has evolved in the antibiotic era, with most infections, particularly in developed Western countries, occurring in hospitals and long-term care facilities [(8)].

The pathogenic mechanisms of the mucoid phenotype of microbes have been demonstrated for various human pathogen [(8)]. In general, in comparison with non-mucoid isolates, the mucoid phenotypes are more resistant to phagocytosis, less sensitive to serum, and more virulent in animal studies. Phenotypic switching between mucoid and non-mucoid morphology under different *in-vivo* conditions can also influence the host immune response as well as protect microbes from phagocytosis. Clinical *Klebsiella pneumoniae* strains usually form glistening mucoid colonies with viscid consistency on the culture plate. The mucoid phenotype has been associated with virulence in several animal experiments [(9)]. However, the clinical implications of these microbial characters were not clearly defined.

Mucoviscosity-Associated gene A (*magA*) is associated with the hypermucoviscosity phenotype of *Klebsiella pneumoniae* strains that cause *a* liver abscess in Taiwan [(10)]. *Klebsiellae* are gram-negative bacteria with a prominent polysaccharide capsule that produces large, sticky colonies, when plated on an agar plate with nutrient media. The strains with the hypermucoviscosity phenotype demonstrated extremely high viscosity, determined by a string test of the colony cultured in the laboratory [(11)]. Prevalence of hypermucoviscosity-positive strains was higher for cases of *Klebsiella pneumoniae* infection causing liver abscess than from other sites of *Klebsiella pneumoniae* infection 98% vs. 17% [(10)].

The scope of the research work involved the detection of virulence genes in hypervirulent *Klebsiella pneumoniae* from clinical specimens (urine and blood) of patients reporting to Infectious Diseases Hospital, Bayara, Bauchi Local Government Area, Bauchi State.

Klebsiella pneumoniae isolates, like other Enterobacteriaceae, are increasingly becoming resistant to multiple antimicrobial agents, including the aminoglycosides, quinolones and the

third-generation Cephalospsorins such as Ceftazidime. Resistance to the third-generation cephalosporin's is mediated by the production of extended spectrum beta lactamases (ESBLs). Though ESBLs phenotype is widespread in gram-negative bacteria, *Klebsiella pneumoniae* is the most common species producing ESBLs [12]. Most outbreaks of the epidemic strain the major means of spread of ESBLs are limited to areas where high-risk patients are cared for such as neonates, burns patients etc. The mechanism of ESBL resistance has been implicated in the virulence of the organism by decreasing/increasing resistance to phagocytosis and imposing a metabolic fitness cost

Based on the global index on the infection rate of *Klebsiella pneumoniae*, the study by [13] shows that it was first found in the USA in 1996. Since their first description, *Klebsiella pneumoniae* enzymes have spread across countries and continents, although the exact epidemiology of the expansion varies by geographical location. In Nigeria, *Klebsiella pneumoniae* was among the most common causes of lower respiratory tract infections [14]. In view of the fore-mentioned problems, this study became very imperative to detect the virulence genes in hypervirulent *Klebsiella pneumoniae* obtained from clinical specimens in Bauchi, northeastern Nigeria.

The aim of the study was to detect of virulence genes in hypervirulent *Klebsiella pneumoniae* obtained from clinical specimens in patient attending infectious diseases hospital (IDH) Bayara, Bauchi Local Government, Bauchi State, North-eastern Nigeria,

Methodology

Study Area and Population

The study was carried out at the Infectious Diseases Hospital Bayara, Bauchi local government area of Bauchi State. The facility served as a referral hospital for the community as well as others areas beyond the State. The State has an estimated population of about 6.3 million in 2016 [15]

Geographically, Bauchi State occupied a total land area of 49,119 km2 (18,965 sq mi) representing about 5.3% of Nigeria's total land mass and is located between latitudes 9° 3' and 12° 3' north and longitudes 8° 50' and 11° east. The State is bordered by seven States (Kano and Jigawa to the north, Taraba and Plateau to the south, Gombe and Yobe to the east and Kaduna to the west). Bauchi State was until 1976 a province in the then Northeastern State of Nigeria, with a population of 4,6553,066. (Projected populations)

Sampling Technique and Sample Size

The samples size was calculated using Cochran's formula.

$$n_0 = \frac{Z^2 pq}{e^2} \quad \text{..... equation 1}$$

e = is the desired level of precision (i.e the margin of error)

p = is the (Estimated) proportion of the population that has the attribute in questions.

q = 1-P

z-value is found in a Z table.

the formula can also be presented as follows

$$n = \frac{Z^2 p(1-P)}{d^2} \quad \text{..... equation 2}$$

Where n is the sample size, Z is the statistic corresponding to the level of confidence, 95% confidence interval = 1.96, P is expected prevalence (that can be obtained from the same studies or a pilot study conducted by the researchers) 22.5%, and d is precision (corresponding to effect size) 5%

Collection of Specimens

Clinical specimens of urine and blood were collected from patients presenting with cases of respiratory trachea infection (RTI), urinary tract infections (UTI) as well as Septicemia in Infectious Disease Hospital Bayara, Bauchi Local Government Area of Bauchi State. The specimens were collected from both inpatients and outpatients of all age groups and both sexes.

Microbiological Analysis of the Specimens

Macroscopic analysis of specimens

Clean voided midstream urine was collected into screw-top containers. urine specimens were collected in sterile containers while the blood samples were collected using disposable syringes. A loopful of the urine specimens was streaked onto the surface of blood agar, CLED and MacConkey agar and incubated at ambient temperature for 24h [16]
In MacConkey Agar medium, the colonies of *Klebsiella pneumoniae* are usually mucoid (3 to 4 mm in diameter), pink in coloured due to the lactose fermentation which is of great importance in differentiating *Klebsiella pneumoniae* from other Bacteria present in the specimens, especially from gram-positive bacteria and Salmonella species which are non–lactose fermenters and gives colourless colonies on MacConkey agar medium. In Blood Agar medium, the *Klebsiella pneumoniae* colonies are mucoid (3 to 4mm in diameter), non-haemolytic i.e. shows Gamma Haemolysis (γ-haemolysis). *Klebsiella pneumoniae* on Cystine Lactose Electrolyte Deficient Agar (C.L.E.D Agar), the colonies appear Large, yellow or yellowish-white, highly mucoid and elevated. Bromothymol blue indicator in the agar changes to yellow due to acidification of the medium due to lactose fermentation by bacterial growth [16].

Microscopic analysis of specimens

Gram staining

Fixed culture slide was first stain with 5 drops of crystal violet stain and was allowed to stand for 60 seconds. The was Pour off and gently rinse the excess stain with a stream of dH_2O. iodine was added (about 5 drops) on the smear, enough to cover the fixed culture slide, it was allowed to stand for 30 seconds. The iodine solution was poured off and rinsed with running water, the excess water was Shaked off from the surface of the slide. few drops of decolorizer were added so that the solution trickles down the slide, it was than rinsed off with running water for 5 minutes. The slide was counterstain with 5 drops of safranin solution for 20 seconds, it was than wash off and the slide was blotted with bibulous paper to remove any excess water. The slides were than examined under the microscope [17].

Phenotypic Determination of Virulence Factors

Detection of capsule

The presence of a capsule was investigated by staining with Nigrosin. A loopful of the overnight bacterial colony was transferred on a dry and clean slide. Then, it was gently mixed with Nigrosin and allowed to dry in the air, then rinsed with water, the slide was stained with methylene blue for 2 minutes and allowed to air dry. The slide was gently washed with water and view under the light microscope using oil immersion objective. The Nigrosin stain provided a dark background to the unstained capsule while methylene blue stain provided blue colour to the cells [16].

Hypermucoviscosity Test

Single colonies after culturing on Brain Heart Infusion agar plates was obtained and tested for their ability to form viscous strings. A loop was touched onto their surface and slowly raised. The formation of string greater than 5 mm (>5mm) in length was indicative of hypermucoviscosity positive phenotype [18].

Siderophores Production Assay

Nutrient agar (supplemented with 200 mm of 2.2'-dipyridyl) was used as an iron-restricted agar medium. All bacterial isolates were streaked on agar plates and incubated at 37°C for 24h. Any bacterial growth was considered a positive result for the ability of the bacteria to produce siderophores [19].

Determination of Virulence genes in Hypervirulent *Klebsiella pneumoniae*

Detection of the capsular serotypes K1 and K2 using immunochromatographic strip assay (ICS)

The ICS was prepared by KeMyth Biotech, which had been described by [20]. In this study, the isolates (Hypervirulent *Klebsiella pneumoniae*) were added to the sample-loading well of the test cassette. A result obtained after 5 min was considered invalid.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the Kirby-Bauer method according to [21]. A colony from each *Klebsiella pneumoniae* isolate were grown overnight in Mueller Hinton broth at 37°C. Bacterial cultures were adjusted to 0.5 on the McFarland nephelometer scale (1.5×10^8 CFU/ml) and were plated on Mueller Hinton agar by the streaking method using a sterile swab. Antimicrobial susceptibility and resistance were determined by isolate growth zone diameter [21].

Data Presentation

The results obtained from the research was presented using simple descriptive statistics as frequencies and percentages.

RESULTS

Table 1: Distribution of Specimens According to Age and Gender of the Participants in the Study Populations

Interval (Years)	Male (%)	Female (%)	Total (%)
0-9	12 (4.5%)	23 (8.6%)	35 (13.1%)
10-19	19 (7.1%)	18 (6.7%)	37 (13.8%)
20-29	20 (7.5%)	37 (13.8%)	57 (21.3%)
30-39	36 (13.4%)	49 (18.3%)	85 (31.7%)
40-49	22 (8.2%)	10 (3.7%)	32 (11.9%)
≥50	09 (3.4%)	13 (4.9%)	22 (8.3%)
Total	118 (44%)	150 (56%)	268 (100%)

Table 2: Distribution of Bacterial Isolates According to Specimens Collected During the Study

Sample	G+ (%)	G- (%)	Others (%)	Total (%)
Urine	38 (14.2%)	121 (45.1%)	07 (2.6%)	166 (61.9%)
Blood	56 (20.9%)	31 (11.6%)	15 (5.6%)	102 (38.1%)
Total	**4 (35.1%)**	**152 (56.7%)**	**22 (8.2%)**	**268 (100%)**

Key: G+= Gram-positive
G-= Gram-negative

Table 3: Distribution of Gram-Negative Bacteria According to Specimens

Sample	*Escherichia coli* (%)	*Pseudomonas aeruginosa* (%)	*Klebsiella pneumonia* (%)	Others (%)	Total (%)
Urine	76 (50)	07 (4.6)	16 (10.5)	22(14.5)	121 (79.6)
Blood	07 (4.6)	04 (2.6)	3 (2.0)	17 (11.2)	31 (20.4)
Total	83 (54.6)	11 (7.2)	19 (12.5)	39 (25.7)	152 (100)

Determination of Capsules, Siderophores and Hypervirulence Antigens of the Klebsiella pneumoniae Isolate

S/N	Age/sex	Samples tested/ Code	Capsule detections	Siderophores production	String test for HVKP
1	21/F	U/701	+	+	-
2	46/F	U/702	+	+	-
3	48/F	U/703	+	+	-
4	33/F	U/704	+	+	+
5	36/F	U/705	+	+	-
6	32/F	U/706	+	+	-
7	16/M	U/707	+	+	-
8	51/F	U/708	+	+	+
9	68/F	U/709	-	+	-
10	36/F	U/710	+	-	-
11	59/F	U/711	+	+	+
12	52/M	U/712	+	+	-
13	44/F	U/713	+	+	-
14	47/F	U/714	+	+	-
15	49/F	B/715	-	+	-
16	49/F	B716	+	+	+
17	40/F	U/717	+	+	-
18	51/M	U/718	+	+	-
19	47/F	B/719	+	+	-

KEY: F=Female, M=Male, U=Urine, B=blood, +=positive, -=negative

Table 5: Distribution of hvKp According to Bacterial Isolates Obtained from the Samples

Sample	**hvKp *(%)***	**Non-hvKp *(%)***	***Klebsiella pneumoniae (%)***
Urine (n=16)	3 (15.8)	13(68.4)	16 (84.2)
Blood (n=3)	1(5.2)	2(10.6)	3 (15.8)
Total (n=19)	4 (21.0)	15 (79.0)	19 (100)

Table 6: Distribution of occurrence of K1K2 Serotype in hvKp Bacterial Isolates Obtained from the Samples

Sample	**k1k2**	**Non-k1k2**	**hvKp**
Urine (n=3)	3	0	3
Blood (n=1)	0	1	1
Total (n=4)	3	1	4

Table 7: Antimicrobial Susceptibility Pattern of Klebsiella pneumoniae

Antibiotics	***Klebsiella pneumoniae* (n=19)**	
	S (%)	**R (%)**
Ampicillin (30µg)	0 (00)	19 (100)
Augmentin (30µg)	3 (15.8)	16 (84.2)
Cefalexin (10µg)	0 (00)	19 (100)
Ciprofloxacin (5µg)	2 (10.5)	17 (89.5)
Co-trimoxazole (30µg)	1 (5.2)	18 (94.7)
Gentamicin (15µg)	5 (26.3)	14 (73.7)
Nalidixin acid (30µg)	1 (5.2)	18 (94.7)
Ofloxacin (10µg)	4 (21.1)	15 (78.9)
Reflacine (10µg)	6 (31.6)	13 (68.4)
Streptomycin (30µg)	17 (89.5)	2 (10.5)

CLSI, (2014) Key: S= sensitive, R= Resistance

Table 8: Antimicrobial Susceptibility Pattern of hvKp

Antimicrobial	hvKp **(n=4)**	
	S (%) K1/k2	**R (%) K1/k2**

Ampicillin (30µg)	0 (00)	4 (100)
Augmentin (30µg)	0 (00)	4 (100)
Cefalexin (10µg)	0 (00)	4 (100)
Ciprofloxacin (5µg)	0 (00)	4 (100)
Co-trimoxazole (30µg)	0 (00)	4 (100)
Gentamicin (15µg)	1 (25)	3 (75)
Nalidixin acid (30µg)	1 (25)	3 (75)
Ofloxacin (10µg)	1 (25)	3 (75)
Reflacine (10µg)	1 (25)	3 (75)
Streptomycin (30µg)	4 (100)	0 (00)

CLSI, (2014)
Key; S= sensitive, R= Resistance

Discussions

Klebsiella pneumoniae has been recognized as an urgent threat in hospital settings due to the emergence of multidrug-resistant and hypervirulent strains. Genomic analyses of *Klebsiella pneumoniae* isolates showed a wide spectrum of genetic variation, which includes multidrug resistance and hypervirulence. In this study, the ability of *Klebsiella pneumoniae* to form a mixed species biofilm with *Pseudomonas aeruginosa* posed further challenges in the management of infections, particularly chronic wound and mechanical ventilation-associated infections.

Moreover, extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriages of *Klebsiella pneumoniae* and the development of multidrug-resistant strains like those of extended spectrum beta lactamases (ESBLs). Antibiotic resistance of bacteria is the main cause of non-effective therapy of nosocomial infections and sepsis. *Klebsiella* species have been associated with different types of infections. However, the main importance of *Klebsiella* as a pathogen is in causing infections in hospitalized patients, the strains responsible are nearly always biochemically typical members of *Klebsiella pneumoniae*

Conclusion

The emergence of these high-risk isolates and the global spread of these isolates have left clinicians with very few therapeutic options. Hence, it is critical to have protocols in place for antimicrobial stewardship and enhanced surveillance control efforts to limit the spread of multidrug-resistant and biofilm-forming *K. pneumoniae* strains. hvKP in critically ill patients from the intensive care unit (ICU) may form a new threat, especially in antibiotic resistance. Although the validity of the string test in detecting metastatic *Klebsiella* is questionable, it is a simple and easy test that can be done in any laboratory, indicating the presence of this organism. Serotypes and genomic background may provide helpful and confirmatory tools to diagnose hvKP.

Pathogenic *Klebsiella pneumoniae* can harbour single to multiple virulence genes. Invasive infection with even a single virulence gene-harbouring *Klebsiella pneumoniae* can lead to poor outcomes. Both multidrug-resistant (MDR) and non-MDR *Klebsiella pneumoniae* can harbour a variety of virulence genes. None of the virulence genes have a significant association with mortality. The distribution of virulence genes according to the clinical origin suggests a role of enterobactin in urinary infections. The capsule and biofilm formation were commonly found in isolates, they seem to be at the basis of classic pathogenicity of *Klebsiella pneumoniae*. The

mucoid factor and hypermucoviscosity detected concomitantly in some isolates, constitute a threat for vulnerable populations, even more if they are in combination with antibiotic resistance.

Recommendations

i. It is recommended that molecular kits, such as Immunochromotagraphic strips, should be used in sero-epidemiological studies and early-tracing of contingent metastatic infections in patients with urinary tract infection (UTI). This may provide a new way to "genotype", an unknown capsular type strain, but will require validation with several strains of each serotype to determine specificity and sensitivity.
ii. Knowledge about the common organisms associated with infections and the resistance patterns of these bacterial strains in geographical areas have helped to guide in the used of antimicrobial appropriately and judiciously.
iii. It is recommended that proper investigation should be done as a routine laboratory analysis for hvKP.

Contributions to Knowledge

Though *Klebsiella pneumoniae* was once thought to be an opportunistic, hospital-acquired infection that primarily infects immunocompromised individuals, two new strains have emerged: carbapenem-resistant and hypervirulent. Intestinal colonization rates are high in all three categories, and they act as a reservoir for isolates that might cause illness. The link between colonization and subsequent infection in hospital-acquired illnesses is well-established and significant. The relationship between colonization and subsequent infection in hvKP and CR-KP is unknown. Furthermore, the risk factors for infection in colonized individuals with all forms of Klebsiella pneumoniae are poorly recognized. Understanding colonization and infection as two independent phases with possibly differing risk factors would help researchers better understand Klebsiella pneumoniae pathophysiology.

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