

RESEARCH ARTICLE

OPEN ACCESS

Molecular Detection of Key Virulence-associated Genes and Phenotypic Analysis of Virulence Traits of *Klebsiella pneumoniae* Clinical Isolates from Kenya

Fredrick Tiria^{1,2} , Erick Odoyo³ , Martin Georges³ ,
Andrew Nyerere¹ and Lillian Musila^{3*} 

¹Department of Medical Microbiology, Jomo Kenyatta University of Agriculture and Technology, Nairobi P.O. Box 62000-00200, Kenya.

²State Department for the Blue Economy and Maritime Affairs, Kisumu P.O. Box 1084 – 40100, Kenya.

³Kenya Medical Research Institute/United States Army Medical Research Directorate-Africa, Village Market, Nairobi P.O. Box 606-00621, Kenya.

Abstract

Klebsiella pneumoniae is an opportunistic pathogen and a major cause of nosocomial infections. Phenotypic analysis of virulence and molecular typing of virulence-associated genes are powerful approaches to understanding *Klebsiella pneumoniae* infection biology. This study subjected 102 clinical *Klebsiella pneumoniae* isolates to virulence gene screening and phenotypic analysis of serum resistance, biofilm formation, and hypermucoviscosity. The virulence genes *mrkD*, *ybtS*, *wcaJ*, *entD*, and *rmpA* had a prevalence of 95.1%, 30.4%, 27.5%, 22.5%, and 0.98%, respectively. 54.9%, 36.3%, and 8.8% were serum resistant, intermediate, and susceptible, respectively. There was no significant correlation between the presence of *mrkD*, *ybtS*, *wcaJ*, *entD*, and *rmpA* genes and serum non-susceptibility. 53.9%, 22.5%, 6.9%, and 16.7% were strong, moderate, weak, and non-biofilm formers, respectively. The biofilm-forming phenotype was significantly correlated with *mrkD* ($P=0.000098$) and *ybtS* ($P=0.032$) gene presence. In addition, 11.8% of the isolates had the hypermucoviscous phenotype indicating hypervirulence. All of these hypervirulent isolates were positive for the *mrkD* gene and were significantly associated with the presence of the *wcaJ* gene ($P=0.000085$). These results indicate a positive association between virulence genes with biofilm formation and hypervirulence. In conclusion, *Klebsiella pneumoniae* isolates circulating in Kenya are predominantly serum non-susceptible and biofilm formers. *mrkD*, *ybtS*, and *wcaJ* genes were identified as key genes influencing biofilm formation and hypervirulence and would be good targets for vaccine development to reduce the severity of *Klebsiella pneumoniae* infections in Kenya.

Keywords: Hypervirulence, Mucoviscosity, *Klebsiella pneumoniae*, Serum Resistance, Biofilm, Enterobacterales, Vaccine, Phenotype

*Correspondence: lillian.musila@usamru-k.org

Citation: Tiria F, Odoyo E, Georges M, Nyerere A, Musila L. Molecular Detection of Key Virulence-associated Genes and Phenotypic Analysis of Virulence Traits of *Klebsiella pneumoniae* Clinical Isolates from Kenya. J Pure Appl Microbiol. 2023;17(4):2194-2204. doi: 10.22207/JPAM.17.4.16

© The Author(s) 2023. **Open Access.** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License which permits unrestricted use, sharing, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

INTRODUCTION

Klebsiella pneumoniae is a member of the Enterobacteriales order and a major cause of hospital-associated infections (HAI).¹ Healthy individuals can be asymptomatic carriers of *K. pneumoniae* in the gut, nose, and throat.^{2,3} Classical *Klebsiella pneumoniae* (cKP) strains are known to have high levels of antimicrobial resistance (AMR) genes and are the main cause of HAI.¹ In contrast, hypervirulent *Klebsiella pneumoniae* (hvKP), associated with community-acquired infections (CAI) in immunocompetent individuals, have been shown to possess more virulence factor genes and fewer AMR genes.⁴ Hypervirulent *Klebsiella pneumoniae* strains have recently been flagged as an important pathogen causing life-threatening infections worldwide.^{5,6}

However, recent studies have documented the emergence of hypervirulent-antimicrobial resistant strains, which can evade the innate arm of the immunity and antibiotic therapies.^{4,7,8} These strains have acquired multidrug resistance (MDR) genes and potent virulence genes such as *rmpA*, *ybtS*, *wcaJ*, *entD*, *mrkD*, and *mgaA*. These virulence genes have been associated with, but are not limited to, hypermucoviscosity, biofilm formation, serum resistance, and resistance to phage clearance. Scientists have characterized the virulence genes to understand *Klebsiella pneumoniae* infection biology and identified the critical virulence genes responsible for various virulence phenotypes. Results from such studies have led to the formulation of various *Klebsiella pneumoniae* vaccine candidates, although none has been approved for use.⁹ It remains unclear which virulence genes are common in virulent *Klebsiella pneumoniae* strains circulating in Kenya and could be vaccine candidates for research.

In Kenya, MDR and virulent *Klebsiella pneumoniae* have been identified and characterized by Muraya and colleagues in 2022.¹⁰ Findings from this study showed that most *Klebsiella pneumoniae* strains circulating in Kenya harbor numerous virulence and antimicrobial resistance genes.¹⁰ However, limited data exist on hypervirulent *Klebsiella pneumoniae* strains in Kenya. Therefore, this study aimed to screen for five major virulence factor genes, i.e., *rmpA*, *ybtS*, *wcaJ*, *entD*, and *mrkD*, in *Klebsiella pneumoniae*

isolates from Kenya and, in parallel, conduct phenotypic tests of serum resistance, biofilm formation, and string test. The presence of virulence genes was correlated with virulence phenotypes in *Klebsiella pneumoniae* isolates to understand the genetic basis for the phenotypic expressions. This study hypothesized that serum-resistant, biofilm-forming, and hypermucoviscous *Klebsiella pneumoniae* isolates possess these virulence factor genes.

MATERIALS AND METHODS

Study isolates

This study utilized archived *Klebsiella pneumoniae* isolates obtained under an approved antimicrobial surveillance protocol (WRAIR#2089/SERU#2767). Standard microbial culture was used to isolate the bacteria causing the infection. The presumptive identity of the bacterial isolates was determined by rapid biochemical tests and gram staining and confirmed on the automated Vitek2® system (bioMérieux, Lyon, France). These isolates were stored at -80 degrees Celsius (°C) in tryptic soy broth (TSB) with 40% glycerol.

In this study, 102 *Klebsiella pneumoniae* isolates from wound swabs, blood, urine, and pus specimens of patients presenting to hospital with signs and symptoms of bacterial infection were used. The isolates were obtained from June 2015 and December 2019 from eight hospitals in Nairobi, Kisii, Kisumu, Kericho, and Kilifi counties and archived.

Determination of the hypermucoviscosity *Klebsiella pneumoniae* (hvKP) phenotype

One hundred and two clinical *Klebsiella pneumoniae* isolates were retrieved from the ultra-low freezer (-80°C), subcultured on blood agar plates, and incubated at 37°C for 24 hours (h). Subsequently, the hypermucoviscosity of the isolates was established by a string test where standard bacteriological loops were used to form a viscous string. A string of ≥ 5mm indicates a positive test.¹¹

Biofilm assay

The 102 *Klebsiella pneumoniae* isolates were evaluated for their ability to form biofilms using an *in vitro* biofilm formation assay. The

bacterial isolates were grown in tryptic soy broth for 24h at 37°C, then 200µl pipetted in a 96-well plate and grown for three days. Each day, planktonic bacteria suspended in the broth were pipetted out, and TSB was added to the individual wells. After three days, planktonic bacteria were washed out using sterile phosphate-buffered saline (PBS), and the well was stained using crystal violet (2% v/v). To release the bound crystal violet dye from the biofilm, 200µl of 33% glacial acetic acid was added to the wells, and the optical density (OD) was read using a plate reader at 630 nm (A_{630}). Three wells with sterile broth only served as a control for contamination. *Klebsiella pneumoniae* strain ATCC 700603 was used as a positive biofilm control, whereas *E. coli* strain K12 was used as the negative control. Each isolate was inoculated in triplicate, and the mean and coefficient of variation (CV) were calculated from the three OD readings. Any experiment with a CV greater than 30% was repeated. Strains were classified using the Donelli formula as strong ($OD > 0.204$), moderate ($0.102 < OD < 0.204$), weak ($0.0551 < OD < 0.102$), and non-biofilm formers ($OD \leq 0.055$).^{12,13}

Serum bactericidal assay

The ability of the various isolates to resist serum killing was evaluated using a modification of the traditional viable count technique.¹⁴ The *Klebsiella pneumoniae* isolates were sub-cultured in an enrichment broth (TSB), harvested early during the mid-log growth phase, and the concentration adjusted to 2×10^6 bacteria/mL of physiological saline. Twenty-five microliters of the bacterial suspension were mixed with 75µl microliters of pooled human serum from healthy blood donors in the USA (Sigma-Aldrich) at 1:3 (v/v) and incubated at 37°C. The serum was harvested. Samples of the incubating cultures were taken at different time points immediately after mixing, i.e., at time zero and after 1, 2, and 3 hours of incubation. Serial dilutions of the samples at each time point were plated on nutrient agar to determine the number of viable bacteria by colony counts. A *Klebsiella pneumoniae* strain ATCC 13883, resistant to serum killing and actively proliferating in non-immune serum, was used as a positive control. ATCC 13883 has also been proved experimentally to be susceptible to serum containing anti-KP antibodies¹⁵ allowing us to

investigate only the innate arm of the immune system. The average survival percentage was plotted against time to generate a killing/survival curve. Strains were classified as serum-sensitive if the viable count dropped to <1% of the initial count and serum-resistant if >90% of organisms survived after 180 minutes. Viable counts between these values were interpreted as intermediate resistance (1-90%).¹⁴

Virulence genes identification by PCR

The *Klebsiella pneumoniae* genomic DNA was extracted using a commercial DNA extraction kit (Zymo Research), and DNA purity and quantity were determined using the Nanodrop (ThermoFisher Scientific, Waltham, MA, USA). The virulence factor genes were detected by conventional uni- and multiplex polymerase chain reaction (PCR) on thermocyclers (SimpliAmp™ Thermal Cycler, applied biosystems). The gene targets, primers, cycle conditions, control strains, and amplicon sizes are listed in Table 1. A multiplex PCR of *wcaJ*, *ybtS*, and *mrkD* and the ATCC 43816 positive control was performed under the following conditions: initial denaturation 95°C for 15 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, elongation at 72°C for 60 s and final elongation at 72°C for 10 min. *entD* was amplified using similar PCR conditions but using *Klebsiella pneumoniae* strain ATCC 13883 strain as the positive control. *rmpA* gene was amplified under the following optimized conditions: initial denaturation at 95°C for 15 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 90 s, elongation at 72°C for 60 s, and final elongation at 72°C for 10 min. *Klebsiella pneumoniae* ATCC 43816 strain was used as the positive control.

The amplicons were detected by electrophoresis on a 1.5% agarose gel alongside a 100bp DNA ladder (Invitrogen TrackIt) and detected using Safe SYBR green on a gel documentation system (Vilber Gel Documentation Systems).

Statistical analysis

The significance of differences between phenotypic characteristics of serum resistance, biofilm formation, hypermucoviscosity, and the presence of virulence genes was evaluated using the chi-square test. Microsoft Excel 2016 was

used to capture raw data and perform basic data analysis. Descriptive analysis and data presentation were done using GraphPad Prism and STATA 16.

RESULTS

Bacterial isolates

Out of the 102 *Klebsiella pneumoniae* isolates, 77 (75.5%), 23 (22.5%), and 2 isolates (2%) had been obtained from patients with community-acquired infection, hospital acquired infections, and unclassified sources, respectively.

Serum resistance phenotype

Out of the 102 clinical *Klebsiella pneumoniae* isolates, 56 isolates (54.9%) were serum resistant, 37 isolates (36.3%) had intermediate resistance, whereas 9 isolates (8.8%) were serum susceptible. Figure 1 shows a representative growth pattern for each of these resistance profiles. The positive control strain (*Klebsiella pneumoniae* strain ATCC 13883) exhibited exponential growth after 180 minutes reaching up to three-fold the inoculum size. Serum-resistant isolates, after 180 minutes, show more than 90% survival following exposure to

the innate immunity in the blood. Some serum-resistant isolates (e.g., KKP 104 and KKP 187) exhibited an exponential increase of more than two-fold after 180 minutes indicating the complete evasion of the innate immunity that mimics what happens in a natural infection.

Strains KKP 134 and KKP 177 exhibited an intermediate phenotype having fewer CFUs (i.e., between 1% and 90%) after 180 minutes than at the start of the experiment. This intermediate growth indicates partial growth inhibition by innate immunity. Two strains, KKP 108 and KKP 105, represent the serum susceptible phenotype in which <1% of the CFUs are present after 180 minutes. The host's innate immunity quickly eliminates these isolates.

Quantitative biofilm assay

From the 102 clinical *Klebsiella pneumoniae* isolates, 55 isolates (53.9%) were strong biofilm formers (+++), 23 isolates (22.5%) were moderate biofilm formers (++), 7 isolates (6.9%) were weak biofilm formers (+) whereas 17 isolates (16.7%) were negative (-) for the biofilm-forming phenotype. The mean absorbance of strong biofilm formers (+++), moderate biofilm

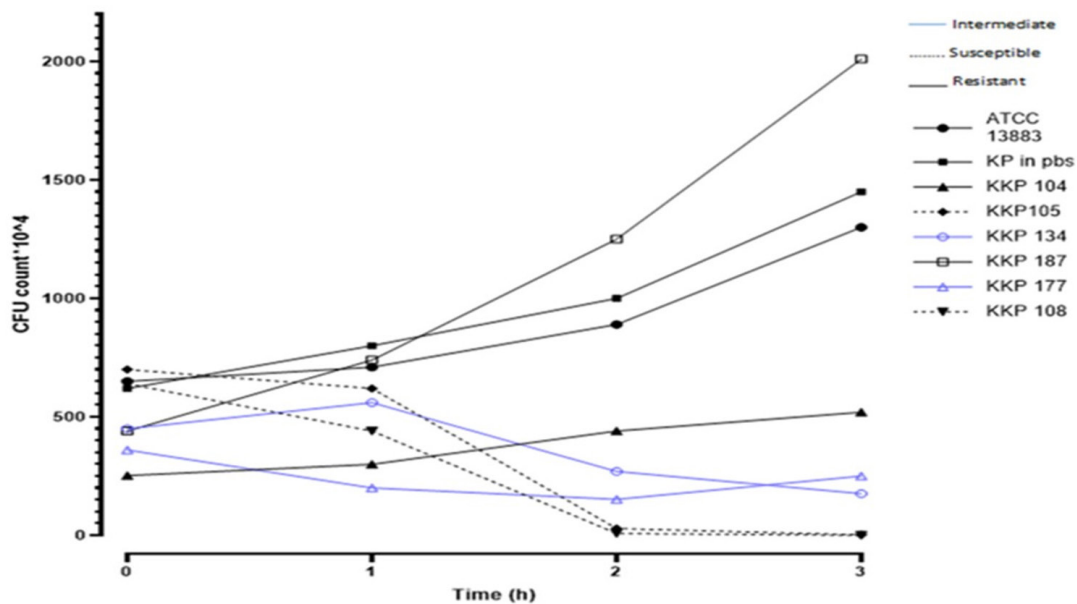


Figure 1. The different killing curves of *K. pneumoniae* isolates exposed to human serum, indicating serum-resistant, intermediate, and susceptible phenotypes

formers (++) and weak biofilm formers (+) was 0.71, 0.16 and 0.08 respectively. Figure 2 below illustrates the phenotypic biofilm results.

Hypermucoviscosity (hv-KP) assay

Of the 102 *Klebsiella pneumoniae* isolates screened for the hypermucoviscosity phenotype, only 12 (11.8%) were positive. Table 2 below shows

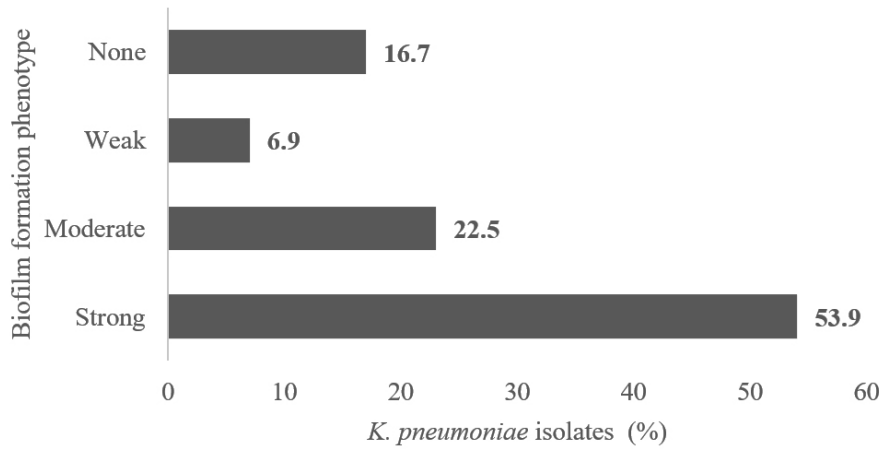


Figure 2. The proportions (%) of *K. pneumoniae* isolates with different biofilm formation characteristics

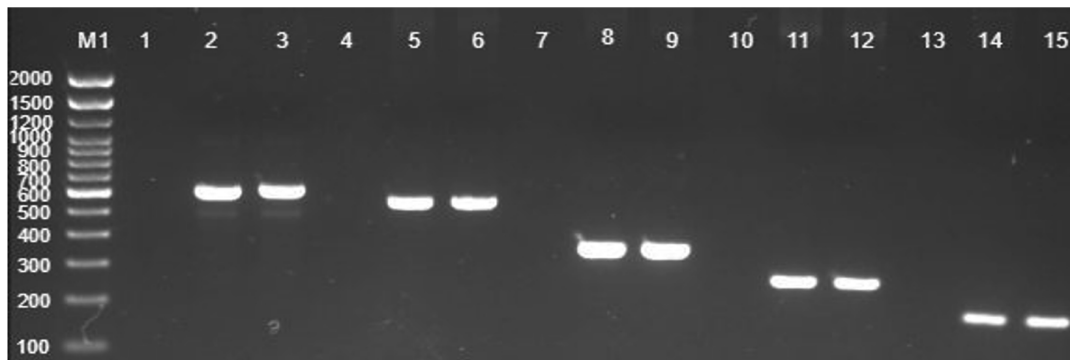


Figure 3. Detection of *K. pneumoniae* virulence genes by PCR in representative isolates.

Table 1. Sequences of the primers used in PCRs

Gene	Primer sequence (5' to 3')	Annealing temp.	Control strain	Amplicon size (BP)
wcaJ FP	AAATGGCGTACCGGTTGTTC	60	ATCC 43816	163
wcaJ RP	CGGCCCTTTCGAGGTAGTTT			
ybtS FP	GACGAAACAGCACGGTAAA	60	ATCC 43816	242
ybtS RP	GAGCATAATAAGGCGAAAGA			
mrkD FP	AAGCTATCGCTGTA CTCCGGCA	60	ATCC 43816	340
mrkD RP	GGCGTTGGCGCTCAGATAGG			
entD FP	CACCATCGACCGTTTTGC	60	ATCC 13883	601
entD RP	TGATGATGATTCCCCTGCCTG			
rmpA FP	ACTGGGCTACCTCTGCTTCA	45	ATCC 43816	536
rmpA RP	CTTGCATGAGCCATCTTTCA			

the hypermucoviscous characteristics of the 12 clinical isolates positive for hypermucoviscosity.

mrkD gene was present in all hvKP isolates, while *wcaJ* was the second most frequently detected gene in hvKP (9/12). A statistically significant correlation was established between the *wcaJ* gene and the hypermucoviscous phenotype (P-value = 0.000085).

Identification of the virulence genes *mrkD*, *ybtS*, *wcaJ*, and *entD*

The results of the screening of the four key virulence genes are summarized in Figure 3. Among all clinical *Klebsiella pneumoniae* isolates, the *mrkD* gene (n=97, 95.1%) was the most prevalent, followed by *ybtS* (n=35, 34.3%), *wcaJ* (n=28, 27.5%), *entD* (n=23, 22.5%). The *rmpA*

gene was the least frequent (n=1, 0.98%). The distribution of these genes amongst the various phenotypes is illustrated in Table 3 and 4.

Agarose gel electrophoresis (1.5%) image of PCR products. Lane M1, DNA ladder (100bp); lane 1, *entD* negative control; lane 2 & 3 *entD* gene, 601 bp; lane 4 *rmpA* negative control; lane 5 & 6 *rmpA* gene, 536 bp; lane 7, *mrkD* negative control; lane 8 & 9 *mrkD* gene, 340 bp; lane 10, *ybtS* negative control; lane 11 & 12 *ybtS* gene, 242 bp; lane 13, *wcaJ* negative control; lane 14 & 15 *wcaJ* gene, 163 bp

The *Klebsiella pneumoniae* isolates classified as skin and soft tissue infection (SSTI) (n=77) or urinary tract infections (UTI) (n=25). The virulence genes (*mrkD*, *entD*, *wcaJ* and *rmpA*) were more prevalent in *Klebsiella pneumoniae*

Table 2. Phenotypic characteristics of hypermucoviscous clinical isolates

Sample No.	Hyper-mucoviscosity	Serum bactericidal (SBA) assay result	Biofilm assay	Virulence genes
KKP 101	Positive	I	+++	<i>mrkD</i> , <i>wcaJ</i> , <i>ybtS</i>
KKP 104	Positive	R	+++	<i>mrkD</i> , <i>rmpA</i> , <i>entD</i>
KKP 138	Positive	I	+++	<i>mrkD</i> , <i>wcaJ</i>
KKP 150	Positive	I	+++	<i>mrkD</i> , <i>ybtS</i>
KKP 152	Positive	R	+++	<i>mrkD</i> , <i>wcaJ</i> , <i>entD</i>
KKP 173	Positive	R	++	<i>mrkD</i> , <i>wcaJ</i>
KKP 179	Positive	S	-	<i>mrkD</i> , <i>wcaJ</i>
KKP 194	Positive	R	+++	<i>mrkD</i> , <i>wcaJ</i>
KKP 197	Positive	R	+++	<i>mrkD</i> , <i>wcaJ</i>
KKP 198	Positive	R	+++	<i>MrkD</i>
KKP 201	Positive	R	++	<i>mrkD</i> , <i>wcaJ</i>
KKP 203	Positive	R	+++	<i>mrkD</i> , <i>wcaJ</i>

R - Serum resistant, I - serum intermediate, S - serum susceptible, +++ - strong biofilm former, ++ - moderate biofilm former, + - weak biofilm former, - non-biofilm former

Table 3. Distribution of virulence genes in serum susceptible and non-susceptible clinical *K. pneumoniae* isolates

Virulence gene	Total isolates positive	Serum resistance phenotype		
		Susceptible (n=9)	Non-susceptible (n=93)	P-value
MrkD	97(95.1%)	8	89	0.37
EntD	35(34.3%)	4	31	0.50
YbtS	28(27.5%)	4	24	0.23
WcaJ	23(22.5%)	1	22	0.39
rmpA	1(0.98%)	0	1	N/A

Table 4. Distribution of virulence genes in biofilm-forming and non-biofilm-forming clinical *K. pneumoniae* isolates

Virulence gene	Total isolates	Biofilm formers (n=85)	Non-biofilm formers (n=17)	p-value
<i>mrkD</i>	97(95.1%)	84	13	0.000098
<i>YbtS</i>	35(34.3%)	33	2	0.032
<i>wcaJ</i>	28(27.5%)	26	2	0.11
<i>EntD</i>	23(22.5%)	19	4	0.92
<i>rmpA</i>	1(0.98%)	1	0	N/A

Table 5. Distribution of virulence genes in relation to the site of infection

Virulence gene	Skin and soft (SSTI) tissue infection	Urinary tract infection (UTI)
<i>MrkD</i>	98.7%	84%
<i>EntD</i>	24.7%	16%
<i>YbtS</i>	27.3%	40%
<i>WcaJ</i>	29.9%	20%
<i>rmpA</i>	1.3%	0%

strains isolated from SSTI than in UTI. Only *ybtS* occurred more in UTI than in SSTI. Table 5 above illustrates the distribution of virulence factor genes in relation to the sample site the clinical *K. pneumoniae* strain was isolated.

DISCUSSION

This study identified key virulence factor genes (*mrkD*, *ybtS*, *wcaJ*, *entD*, and *rmpA*) and their association with phenotypic expressions in *Klebsiella pneumoniae* of serum resistance, biofilm formation, and hypervirulence. These virulence characteristics are essential in the *Klebsiella pneumoniae* pathogenesis since they determine the bacterium's survival in a host post-infection. Furthermore, virulence genes are important in establishing metastatic, invasive *Klebsiella pneumoniae* infections within the body.^{16,17} *entD* and *ybtS* genes encode products that catalyze the biosynthesis of compounds key in iron acquisition from the host's transferrin and lactoferrin, thereby promoting bacterial survival inside the host.^{18,19} *mrkD* has been linked with biofilm formation, and this is crucial in *Klebsiella pneumoniae* as a cause of catheter urinary tract infections by enhancing the

survival in indwelling catheters.^{20,21} *wcaJ* and *rmpA* have been linked with bacterium mucoviscosity, and these are crucial in bacterium's seroprotection from the hostile humoral and cellular immune components.^{16,20}

We looked at some of the phenotypic indicators of virulence. The first was serum resistance. Serum resistance has been experimentally linked with virulence in many bacterial species.²² The bactericidal activity of serum is a nonspecific response to inhibit the growth and proliferation of infectious microorganisms. From the results, a majority of the isolates, 54.9%, were serum resistant. Serum resistance has been linked to many defense mechanisms, including producing thick capsular material and masking critical antigenic epitopes that are key targets of the host's innate immune cells.^{5,22,23} These defenses make the bacterium invisible to the host immunity and able to establish an infection.²² 8.8% of the isolates were susceptible to the human serum, indicating that the innate immunity in the serum of healthy individuals 11,22 could quickly clear them. Our results suggest that most *Klebsiella pneumoniae* strains circulating in Kenya are resistant to the bactericidal activity of non-immune human serum and can easily establish infections in immunocompetent individuals. These data could account for the high prevalence of *Klebsiella pneumoniae* infections in Kenya, as indicated in a study by Apondi and colleagues in 2016 that found that *Klebsiella pneumoniae* accounted for 23% of bacterial strains isolated in a tertiary teaching and referral hospital in Western Kenya,²⁴ and a second study showing that 20% of total nosocomial bacteremia episodes between 2002 and 2009 at Kilifi County Hospital in Kenya were *Klebsiella pneumoniae* infections.²⁵ These studies implicate *Klebsiella pneumoniae* as a significant cause of community-acquired and nosocomial infections in Kenya. Many studies conducted worldwide show a similar serum resistance profile; for example, Ballen *et al.* found that more than 50% of *Klebsiella pneumoniae* strains isolated from patients in a Spanish hospital were highly resistant to human serum⁸ while Ku and colleagues found that 69.7% *Klebsiella pneumoniae* isolates in a study conducted in China were serum resistant.²⁶

The second phenotypic indicator of virulence was biofilm. Our findings indicated that 83.3% of the *Klebsiella pneumoniae* isolates form bacterial protective aggregates (biofilms), which can enhance virulence and exacerbate antibiotic resistance, contributing to their clinical success. A similar study by Nirwati *et al.*, found that 85.63% of *Klebsiella pneumoniae* strains isolated from a hospital in Indonesia were biofilm formers.¹ Seifi *et al.* reported that most *Klebsiella pneumoniae* strains (93.6%) isolated in Iran were biofilm formers, with only 6.4% negative for the phenotype.²⁷ These results corroborate the findings from this study. Biofilm formers can persist in the environment and these bacterial communities can exchange virulence and antimicrobial resistance genes freely via horizontal gene transfer,^{28,29} and by plasmid conjugation.¹² Biofilm-forming bacteria have been linked with nosocomial infections since they can resist disinfection on hospital surfaces and enhance their transmission. Biofilms can aggregate on tubular structures and tissues such as the urethra and invasive catheters and therefore constitute a significant cause of urinary tract infections and catheter-related infections that can be fatal, especially in immunocompromised hospitalized patients.^{29,30} Bacterial cells within the biofilm's core can survive in a dormant state shutting down metabolism and enhancing their resistance to antimicrobial agents. Furthermore, a biofilm forms a mechanical barrier, protecting bacterial cells within the core from any contact with antibiotics and enhancing their survival in the presence of detergents and disinfectants.²⁹

The third phenotypic indicator of virulence we examined was string formation, an indicator of hypervirulence. 11.8% of the *Klebsiella pneumoniae* clinical isolates were positive for this phenotype. As shown in Table 2, all the hypervirulent isolates were positive for the *mrkD* gene and were positively correlated with the *wcaJ* gene. *wcaJ* functions as an initiator enzyme for the synthesis of colonic acid by adding the first sugar (glucose-1-P) on the lipid carrier undecaprenyl phosphate.³¹ Strains harboring this gene often have a mucoid phenotype, whereas those strains without this glycosyltransferase lack colonic acid rendering them non-mucoid.³² The biosynthesized capsular polysaccharides

lead to the mucoid phenotype, seen in the ability of *Klebsiella pneumoniae* strains harboring the gene to be hypermucoviscous/hypervirulent. A study conducted by Song *et al.* reported that *Klebsiella pneumoniae* isolates mutating in response to evolutionary pressures to resist bacteriophages showed nucleotide deletion mutations of *wcaJ* gene which rendered them dry and non-hypervirulent.¹⁶ This evidence underlines the importance of the *wcaJ* gene in hypervirulence. Other genes linked with the mucoid appearance and positive string test of *Klebsiella pneumoniae* include *rmpA* and *magA* genes.^{20,33}

In contrast to the low hvKP prevalence in our study, other studies, especially in Taiwan and Asian countries, have reported a higher occurrence of hvKP. For example, findings from a study by Liu and Guo reported a hvKP prevalence of 45.7% (80/175) in China.³⁴ In another study in 2017, Guo *et al.* reported a hvKP prevalence of 22.8% (84/369) in *Klebsiella pneumoniae* strains isolated from patients with invasive infections.³⁵ Finally, Hao and colleagues reported a 68.8% prevalence rate of hvKP in a 2020 study conducted in China.³⁶

Hypermucoviscous strains form a viscous string due to a mucus-like matrix produced by the bacterial cells. Results from this study show that hypermucoviscosity influences biofilm formation and serum resistance because out of the 12 hypervirulent strains, 11 of them were also serum non-susceptible and biofilm formers. The mucoid capsule of these hypervirulent strains facilitates the aggregation of bacterial cells into microcolonies that help form biofilms.^{12,37} Hypervirulence can also influence serum resistance since the mucoid slime layer masks key antigenic sites (Pathogen associated molecular patterns) that are recognizable by the immune system, especially the PRRs (Pathogen recognition receptors) that are found on immune cells patrolling the bloodstream and those stationed in sentinel sites mopping up any pathogens.^{8,20} Consequently, the bacterial cells are unrecognized by the host and can establish an infection.⁸

From the molecular screening of virulence factor genes, the *mrkD* gene was predominated at 95.1%, followed by *ybtS*, *wcaJ*, *entD*, and *rmpA* at 34.3%, 27.5%, 22.5%, and 0.98% respectively. Contrary to studies worldwide which indicate

rmpA as a major virulence factor gene, it was rare in *Klebsiella pneumoniae* isolates from Kenya. When we examined which genes were more strongly associated with the virulence phenotypes, our study found that *mrkD* and *ybtS* had a strong statistical correlation with biofilm formation. *mrkD* is a type 3 fimbrial adhesin gene reported to facilitate biofilm formation in *Klebsiella pneumoniae*.^{19,38} A similar study in Egypt reported a strong statistical correlation between the *mrkD* gene and biofilm formation in *Klebsiella pneumoniae*.³⁸ Another study conducted in Mosul also reported a strong statistical correlation between the presence of *mrkD* and biofilm formation phenotype.³⁹ These studies validate the findings of this study. *ybtS* gene is crucial in the growth of *Klebsiella pneumoniae* since its translation product, *ybtS* enzyme, catalyzes the biosynthesis of yersiniabactin, facilitating iron acquisition from the environment or in vivo from the host's transferrin and lactoferrin.¹⁹ A study by Han *et al.*, reported that the *ybtS* gene had no significant effect on colony morphology and biofilm formation in *Klebsiella pneumoniae*, but it can significantly increase the virulence of *Klebsiella pneumoniae* strains.⁴⁰ There are no published reports of the relationship between the presence/expression of the *ybtS* gene and biofilm formation. The strong correlation between the presence of *mrkD*, *ybtS*, and *wcaJ* genes with virulence traits suggests that they can be good vaccine targets to reduce the clinical impact of *Klebsiella pneumoniae* in Kenya.

The study had a few limitations. First, we only focused on a few virulence factor genes in a relatively small collection of *Klebsiella pneumoniae* isolates. These factors could have led to excluding or misrepresenting clinically relevant virulence genes in Kenyan isolates. The causal relationship of the genes with the phenotype was not examined using techniques such as gene knock-out or cloning to study the individual contributions of the genes to each phenotype. More virulence studies, including a more comprehensive panel of virulence genes and clinical isolates, should be conducted to understand the landscape of virulence factor genes among *Klebsiella pneumoniae* found in Kenya.

CONCLUSION

This study has shown that hypermucoviscous *Klebsiella pneumoniae* strains are circulating in Kenya, which is clinically significant as these hypervirulent *Klebsiella pneumoniae* strains are usually fatal.^{4,35} With most *Klebsiella pneumoniae* isolates circulating in Kenya being serum-resistant and biofilm formers, strong intervention measures like proper disinfection of hospital surfaces, management of catheter-associated infections, and environmental monitoring to eliminate biofilms need to be implemented to curb the spread of this bacteria. Studies like this that indicate the main virulence factor genes provide crucial data on the critical antigenic epitopes that should be targeted in vaccines for use in Kenya. Vaccine development to prevent severe infections and alternate approaches for biofilm control, like bacteriophages, should be emphasized to reduce the morbidity and mortality associated with hypervirulent *Klebsiella pneumoniae*.

ACKNOWLEDGMENTS

The authors would like to thank the interns attached to the Kenya Medical Research Institute's Center for Microbiology and the United States Army Medical Research Directorate – Africa (USAMRD-A), Felix Korir, Paul Kimani, and Moses Gachoya, for their technical support in sample analysis and Nicholas Lokomwa of State Department for the Blue Economy and Maritime Affairs for his help in data analysis. The authors also acknowledge USAMRD-A staff stationed in hospitals countrywide who collected the samples and the various study participants at the participating hospitals who consented to the bacterial pathogens isolated from their specimens to be used for further studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

FT, LM and AN conceptualized the study. FT, EO and MG applied methodology. FT and EO performed data analysis. FT and LM wrote original draft. LM and AN supervised the study. All authors read and approved the final manuscript for publication.

FUNDING

This research was partly funded by the Armed Forces Health Surveillance Division (AFHSD), Global Emerging Infections Surveillance (GEIS) Branch, ProMIS ID P0115_22_KY.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (KEMRI/SERU/CCR/176/4100), Jomo Kenyatta University of Agriculture and Technology (JKU/2/11/HSB325-1879/2017) and the National Commission for Science, Technology, and Innovation (NACOSTI/P/21/8450).

REFERENCES

- Nirwati H, Sinanjung K, Fahrnunissa F, et al. Biofilm formation and antibiotic resistance of *Klebsiella pneumoniae* isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. *BMC Proc.* 2019;13. doi: 10.1186/s12919-019-0176-7
- Brogden KA, Guthmiller JM, Taylor CE. Human polymicrobial infections. *Lancet.* 2005;365(9455):253-255. doi: 10.1016/S0140-6736(05)17745-9
- Gorrie CL, Mirc Eta M, Wick RR, et al. Gastrointestinal Carriage Is a Major Reservoir of *Klebsiella pneumoniae* Infection in Intensive Care Patients. *Clin Infect Dis.* 2017;65(2):208-215. doi: 10.1093/cid/cix270
- Sanchez-Lopez J, Garcia-Caballero A, Navarro-San Francisco C, et al. Hypermucoviscous *Klebsiella pneumoniae*: A challenge in community-acquired infection. *IDCases.* 2019;17:e00547. doi: 10.1016/j.idcr.2019.e00547
- Phan MD, Peters KM, Sarkar S, et al. The Serum Resistome of a Globally Disseminated Multidrug Resistant Uropathogenic *Escherichia coli* Clone. *PLoS Genet.* 2013;9(10):e1003834. doi: 10.1371/journal.pgen.1003834
- Zhang S, Zhang X, Wu Q, et al. Clinical, microbiological, and molecular epidemiological characteristics of *Klebsiella pneumoniae*-induced pyogenic liver abscess in southeastern China. *Antimicrob Resist Infect Control.* 2019;8(1):166. doi: 10.1186/s13756-019-0615-2
- Vuotto C, Longo F, Balice MP, Donelli G, Varaldo PE. Antibiotic resistance related to biofilm formation in *Klebsiella pneumoniae*. *Pathogens.* 2014;3(3):743-758. doi: 10.3390/pathogens3030743
- Ballen V, Gabasa Y, Ratia C, Ortega R, Tejero M, Soto S. Antibiotic Resistance and Virulence Profiles of *Klebsiella pneumoniae* Strains Isolated From Different Clinical Sources. *Front Cell Infect Microbiol.* 2021;11:738223. doi: 10.3389/fcimb.2021.738223
- Feldman MF, Mayer Bridwell AE, Scott NE, et al. A promising bioconjugate vaccine against hypervirulent *Klebsiella pneumoniae*. *Proc Natl Acad Sci U S A.* 2019;116(37):18655-18663. doi: 10.1073/pnas.1907833116
- Muraya A, Kyanya C, Kiyaga S, et al. Antimicrobial Resistance and Virulence Characteristics of *Klebsiella pneumoniae* Isolates in Kenya by Whole Genome Sequencing. *Pathogens.* 2022;11(5):545. doi: 10.3390/pathogens11050545
- Fang CT, Chuang YP, Shun CT, Chang SC, Wang JT. A Novel Virulence Gene in *Klebsiella pneumoniae* Strains Causing Primary Liver Abscess and Septic Metastatic Complications. *J Exp Med.* 2004;199(5):697-705. doi: 10.1084/jem.20030857
- Mirzaie A, Ranjbar R. Antibiotic resistance, virulence-associated genes analysis and molecular typing of *Klebsiella pneumoniae* strains recovered from clinical samples. *AMB Express.* 2021;11(1):122. doi: 10.1186/s13568-021-01282-w
- Donelli G, Vuotto C, Cardines R, Mastrantonio P. Biofilm-growing intestinal anaerobic bacteria. *FEMS Immunol Med Microbiol.* 2012;65(2):318-325. doi: 10.1111/j.1574-695X.2012.00962.x
- Podschun R, Sievers D, Fischer A, Ullmann U. Serotypes, Hemagglutinins, Siderophore Synthesis, and Serum Resistance of *Klebsiella* Isolates Causing Human Urinary Tract Infections. *J Infect Dis.* 1993;168(6):1415-1421. doi: 10.1093/infdis/168.6.1415
- Babu L, Uppalapati SR, Sripathy MH, Reddy PN. Evaluation of recombinant multi-epitope outer membrane protein-based *Klebsiella pneumoniae* subunit vaccine in mouse model. *Front Microbiol.* 2017;8:1805. doi: 10.3389/fmicb.2017.01805
- Song L, Yang X, Huang J, et al. Phage Selective Pressure Reduces Virulence of Hypervirulent *Klebsiella pneumoniae* Through Mutation of the *wzc* Gene. *Front Microbiol.* 2021;12:39319. doi: 10.3389/fmicb.2021.739319
- Siu LK, Yeh KM, Lin JC, Fung CP, Chang FY. *Klebsiella pneumoniae* liver abscess: A new invasive syndrome. *Lancet Infect Dis.* 2012;12(11):881-887. doi: 10.1016/S1473-3099(12)70205-0
- Chen Y, Brook TC, Soe CZ, et al. Preterm infants harbour diverse *Klebsiella* populations, including atypical species that encode and produce an array of antimicrobial resistance-and virulence-associated factors. *Microb Genomics.* 2020;6(6):e000377. doi: 10.1099/mgen.0.000377
- Amaretti A, Righini L, Candelieri F, et al. Antibiotic resistance, virulence factors, phenotyping, and genotyping of non-*Escherichia coli* enterobacteriales

- from the gut microbiota of healthy subjects. *Int J Mol Sci.* 2020;21(5):1847. doi: 10.3390/ijms21051847
20. Rastegar S, Moradi M, Kalantar-Neyestanaki D, Dehdasht AG, Hosseini-Nave H. Virulence factors, capsular serotypes and antimicrobial resistance of hypervirulent *Klebsiella pneumoniae* and classical *Klebsiella pneumoniae* in Southeast Iran. *Infect Chemother.* 2021;53(1):e39. doi: 10.3947/ic.2019.0027
21. Nahar N, Rashid RB. Phylogenetic analysis of antibiotic resistance genes and virulence genes of *Klebsiella* species *in silico*. *Dhaka Univ J Pharm Sci.* 2017;16(1):119-127. doi: 10.3329/dujps.v16i1.33389
22. Jensen TS, Opstrup K V., Christiansen G, et al. Complement-mediated *Klebsiella pneumoniae* capsule changes. *Microbes Infect.* 2020;22(1):19-30. doi: 10.1016/j.micinf.2019.08.003
23. Tomas JM, Benedi VJ, Ciurana B, Jofre J. Role of capsule and O antigen in resistance of *Klebsiella pneumoniae* to serum bactericidal activity. *Infect Immun.* 1986;54(1):85-89. doi: 10.1128/iai.54.1.85-89.1986
24. Apondi OE, Oduor OC, Gye BK, Kipkoech MK. High prevalence of multidrug resistant *Klebsiella pneumoniae* in a tertiary teaching hospital in Western Kenya. *Afr J Infect Dis.* 2016;10(2):85-89. doi: 10.21010/ajid.v10i2.3
25. Henson SP, Boinett CJ, Ellington MJ, et al. Molecular epidemiology of *Klebsiella pneumoniae* invasive infections over a decade at Kilifi County Hospital in Kenya. *Int J Med Microbiol.* 2017;307(7):422-429. doi: 10.1016/j.ijmm.2017.07.006
26. Ku YH, Chuang YC, Chen CC, et al. *Klebsiella pneumoniae* Isolates from Meningitis: Epidemiology, Virulence and Antibiotic Resistance. *Sci Rep.* 2017;7(1):6634. doi: 10.1038/s41598-017-06878-6
27. Seifi K, Kazemian H, Heidari H, et al. Evaluation of biofilm formation among *Klebsiella pneumoniae* isolates and molecular characterization by ERIC-PCR. *Jundishapur J Microbiol.* 2016;9(1):e30682. doi: 10.5812/jjm.30682
28. De Araujo C, Balestrino D, Roth L, Charbonnel N, Forestier C. Quorum sensing affects biofilm formation through lipopolysaccharide synthesis in *Klebsiella pneumoniae*. *Res Microbiol.* 2010;161(7):595-603. doi: 10.1016/j.resmic.2010.05.014
29. Vuotto C, Longo F, Pascolini C, et al. Biofilm formation and antibiotic resistance in *Klebsiella pneumoniae* urinary strains. *J Appl Microbiol.* 2017;123(4):1003-1018. doi: 10.1111/jam.13533
30. Rendueles O. Deciphering the role of the capsule of *Klebsiella pneumoniae* during pathogenesis: A cautionary tale. *Mol Microbiol.* 2020;113(5):883-888. doi: 10.1111/mmi.14474
31. Cai R, Wang G, Le S, et al. Three capsular polysaccharide synthesis-related glucosyltransferases, GT-1, GT-2 and WcaJ, are associated with virulence and phage sensitivity of *Klebsiella pneumoniae*. *Front Microbiol.* 2019;10:1189. doi: 10.3389/fmicb.2019.01189
32. Pal S, Verma J, Mallick S, Rastogi SK, Kumar A, Ghosh AS. Absence of the glucosyltransferase wcaJ in *Klebsiella pneumoniae* ATCC13883 affects biofilm formation, increases polymyxin resistance and reduces murine macrophage activation. *Microbiol (United Kingdom).* 2019;165(8):891-904. doi: 10.1099/mic.0.000827
33. Cubero M, Grau I, Tubau F, et al. Hypervirulent *Klebsiella pneumoniae* clones causing bacteraemia in adults in a teaching hospital in Barcelona, Spain (2007-2013). *Clin Microbiol Infect.* 2016;22(2):154-160. doi: 10.1016/j.cmi.2015.09.025
34. Liu C, Guo J. Hypervirulent *Klebsiella pneumoniae* (hypermucoviscous and aerobactin positive) infection over 6 years in the elderly in China: Antimicrobial resistance patterns, molecular epidemiology and risk factor. *Ann Clin Microbiol Antimicrob.* 2019;18(1):4. doi: 10.1186/s12941-018-0302-9
35. Guo Y, Wang S, Zhan L, et al. Microbiological and clinical characteristics of hypermucoviscous *Klebsiella pneumoniae* isolates associated with invasive infections in China. *Front Cell Infect Microbiol.* 2017;7:24. doi: 10.3389/fcimb.2017.00024
36. Hao Z, Duan J, Liu L, et al. Prevalence of Community-Acquired, Hypervirulent *Klebsiella pneumoniae* Isolates in Wenzhou, China. *Microb Drug Resist.* 2020;26(1):21-27. doi: 10.1089/mdr.2019.0096
37. Ye M, Tu J, Jiang J, et al. Clinical and genomic analysis of liver abscess-causing *Klebsiella pneumoniae* identifies new liver abscess-associated virulence genes. *Front Cell Infect Microbiol.* 2016;6:165. doi: 10.3389/fcimb.2016.00165
38. Elbrolosy AM, Eissa NA, Al-Rajhy NA, El-Mahdy EE-SA, Mostafa RG. *MrkD* Gene as a Regulator of Biofilm Formation with Correlation to Antibiotic Resistance among Clinical *Klebsiella pneumoniae* Isolates from Menoufia University Hospitals. *Egypt J Med Microbiol.* 2020;29(3):137-144. doi: 10.51429/EJMM29318
39. Mahmood MT, Abdullah BA. The relationship between biofilm formation and presence of fimH and mrkD genes among *E. coli* and *K. pneumoniae* isolated from patients in Mosul. *Mosul J Nurs.* 2015;3(1):34-42. doi: 10.33899/mjn.2015.160018
40. Han R, Niu M, Liu S, Mao J, Yu Y, Du Y. The effect of siderophore virulence genes entB and ybtS on the virulence of Carbapenem-resistant *Klebsiella pneumoniae*. *Microb Pathog.* 2022;171:105746. doi: 10.1016/j.micpath.2022.105746