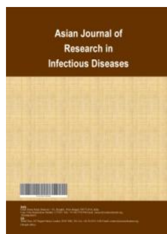

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Molecular Detection of Virulence genes (*rmpA2, iuc & iroB*) of Hypervirulent *Klebsiella pneumoniae* in Clinical Isolates from Patients in Khartoum State, Sudan

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Authors' contributions

This work was carried out in collaboration among all authors. Authors KA, SA, AH were responsible data curation, Methodology and investigation. Authors KA, ST, BM, and AH were responsible for the conceptualization and data curation, Formal analysis and writing, Original Draft Preparation. Authors AH and BM revise the final draft of manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Iron acquisition systems and regulator mucoid phenotype plays an important role in the pathogenesis and virulence of *Klebsiella pneumoniae*. This cross-sectional study aimed to investigate the prevalence of virulence genes *iuc, iroB* and r*mpA2* of hypervirulent *Klebsiella*

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pneumoniae (hykp) in clinical isolates from patients in Khartoum.
Methods: A total of 400 samples were collected from 250 (62.5%) males and 150 (37.5%) females. All samples were cultivated in agar plates to determine the presence of *Klebsiella pneumoniae* isolates. These isolates were screened for hypermucoviscosity phenotypically by string test. Antibiotic susceptibility tests were performed for all isolates to identify their resistance pattern, *iuc*, *rmpA2*, *iroB* genes were investigated by polymerase chain reaction.
Results: Our results demonstrate that the majority of participants 150 (37.5%) are aged 31 – 40 years. Out of 400 samples cultivated, 313 (78.75%) were yield significant pathogenic growth of which 95 (23.75%) are *K.pneumoniae* isolates, and 220 (55.0%) are other pathogenic organisms while the rest of samples 87 (17.25%) were showed no pathogenic growth isolated. The frequency of *iuc*, *iroB, rmpA2* were 74.7%, 57.9%, 2.1% respectively among the clinical isolates. String tests were positive among 30 (31.6%) isolates and the remaining 65 (68.4%) isolates gave negative results.
Conclusion: There was a high prevalence of *iuC* virulence gene of hvkp, high frequency iron acquisition systems coding gene *iuC gene*, *iroB gene* compared to regulator mucoid phenotype A2 (*rmpA2 gene)* among clinical isolates.

Keywords: Klebsiella pneumoniae; rmpA2 gene; iroB gene; iuc gene.

1. INTRODUCTION

Klebsiella pneumoniae is a non-motile, Gram-negative, rod-shaped bacterium with a prominent polysaccharide-based capsule. The organism is facultative anaerobic, catalase positive, oxidase negative, lactose fermenter and reduces nitrate to nitrite. *Klebsiella pneumoniae* is able to grow on non-enriched media such as MacConkey agar and produce pale pink mucoid colonies [1]. It is a common opportunistic pathogen of community – acquired and nosocomial infections. The organism can cause a wide range of infections, with pneumonia coming on top of these infections followed by urinary tract infections. In addition, *Klebsiella pneumoniae* causes septicemia, meningitis, diarrhea, and soft tissue infections [2].

The ability of *K. pneumonia* to acquire new genetic material is an important feature that has enabled its continued evolution. As a consequence, two pathotypes termed classical *K.pneumoniae* (ckp) and hypervirulent *K. pneumoniae* (hvkp) are circulating, each posing unique challenges to the clinician [3,4]. Both pathotypes are global pathogens, but the incidence of disease due to hvkp is steadily increasing, the majority of reported infections due to hvkp have been acquired in the community. Features strongly suggestive of hvkp infection are its ability to infect healthy individuals of any age and the propensity of infected patients to present with multiple sites of infection and/or develop subsequent metastatic spread, a usual occurrence for other members of the family Enterobacteriaceae. Hvkp is best described as a virulent pathogen [5]. Clinical laboratories are unable to differentiate between ckp and hvkp strains, however several biomarkers including peg-344, *iroB*, *iuC*, *rmpA*, *rmpA2* and quantitative siderophore production than 30µg /ml have been found to predict hvkp strains with excellent sensitivity and specificity, these biomarkers could lead to development of a diagnostic fast, and can be utilized by clinical laboratories for patient care [6].

The prevalence of antibiotic resistance in hvkp isolate is rare compared with the high prevalence of antibiotic resistant ckp isolates [7]. A report in 2018 showed that in China 12.6% of hvkp isolates from several infections produced ESBL [8]. *Klebsiella pneumoniae* possesses different virulence factors that contribute to its pathogenicity including lipopoly-saccharide (LPS), O-side chain (endotoxin), capsular polysaccharide, adhesions and siderophores [9-11].The LPS contain lipid A, core and o-polysaccharide antigen [11]. The polysaccharide capsule is a major virulence factor of and based on it the *K.pneumoniae* classified into 77 serological types [11].

Hvkp strains are capable of causing multi-site infections, metastatic spread and lethal disease [12,13], and have the ability to produce 4 different siderophores: Enterobactin, Salmochelin, Yersiniapectin and Erobactin. Molecular epidemiologic studies have shown that siderophores are commonly present in hvkp strains than ckp and shown several virulence factors have been identified to be associated with hvkp, including iron acquisition systems

salmochelin (*iroBCD*) / aerobactin (*iucABCDiutA*) and the regulator of mucoid phenotype A gene (*rmpA/A2*) [8].

Genome of the *K.pneumoniae* capsule comprises gene clusters cps (capsular polysaccharide synthesis), *rmpA2* can magnify the colony mucoidy of different serotypes of *K.pneumoniae* and act as a plasmid-mediated regulator of extra capsular polysaccharide synthesis [14], The primary mechanism of iron acquisition in *K.pneumoniae* is through the production of small molecules called siderophore that are secreted ,bind iron ,and reenter the bacterial cell through specific receptors and the accumulation of siderophore systems in hvkp suggest iron acquisition is a critical component of this pathogen [14,15]. Literature review showed that few studies conduct to determine such gene in our country, therefore, this study was aimed to investigate the prevalence of virulence genes *iuc,iroB* and r*mpA2* of hyper virulent *Klebsiella pneumoniae* obtained from various clinical isolates.

2. METHODS

2.1 Sample Collection

This cross sectional study conducted in Khartoum state, the sample size was involved a total of 400 participants, samples included urine, sputum, wound swabs and vaginal swab, 250 samples were collected from males and the rest from females. Samples were collected from three major hospitals in Khartoum state, namely the Military Hospital, Ibrahim Malik Hospital and Omdurman Teaching Hospital.

2.1.1 Inclusion criteria

As our target is *Klebsiella pneumoniae*, any patients with suspected infection due to *Klebsiella pneumoniae* bacteria (eg, pneumonia, urinary tract infection, wound infection) were included in this study. Patients should not start any antimicrobial therapy for at least 72 hours prior to sample collection.

2.1.2 Exclusion criteria

Any patients not expected to have *Klebsiella pneumoniae* infection (eg, gastritis, enteritis) were excluded from this study. Patients undergoing antibiotic treatment were excluded from this study.

2.1.3 Cultural isolation of the organism

All samples were cultured on blood agar and MacConkey then incubated at 37 °C overnight. Colonies were identified by Gram stain and then followed by biochemical assays such as Kligler's iron agar test, citrate test, urease test and motility test according to Cheesbrough, M. guidelines [16]. A total of 95 *Klebsiella pneumoniae* have been isolated and sub cultured on Mueller Hinton agar to take pure colonies for further analysis, and isolates were obtained from urine (5), sputum (81), wound swabs (8) and one vaginal swab sample.

2.2 Phenotypic Detection of Hyper Mucoviscosity

2.2.1 String test

This was done using a bacteriologic loop to stretch the colonies on the agar plates. Any generated viscous string >5 mm in length was considered as a positive test [17].

2.2.2 Antibiotic susceptibility test

The antibiotic susceptibility of *K.pneumoniae* isolates were determined by the Kirby-Bauer disk diffusion method according to clinical and laboratory standards institute guidelines [18], and the suspension of each isolate was seeded on the surface of the Mueller Hinton agar plate. After antibiotic disks were placed, the plates were incubated at 37°c for 24hrs and susceptibility was determined by measuring the diameter of the inhibition zone. The antibiotics used were Colistin (10 µg), Piperacillin Tazobactam (110 µg), Meropenem (10 µg) and Ceftriaxone (30 µg).

2.3 Genotypic Detection

2.3.1 DNA Extraction

The DNA template was obtained from the isolated colony. Bacterium colonies (2-3 colonies) were picked and suspended in 100µl sterile distilled water, boiled for 20 min in a thermal block at 100 °C, and cooled for 10 min at -20 °C.n the refrigerator, followed by centrifugation at 13,000 rpm for 10 minutes. The supernatant was collected and used as a DNA template [19].

2.3.2 Polymerase chain reaction (PCR) for amplification of virulence genes:

Amplification was performed using (Techne prime Thermal Cycler, UK). Multiplex PCR was performed with a total volume of 25 µl using a multiplex PCR kit (iNtRON Biotechnology, Korea), according to the manufacturer's instructions. Primer used in this study were (for rmpA2 gene F=AAGGGAGAAAGGCGGGAACAG, R=CTTGTAGGTGCCGGGGAAAT; for iucABCD gene F= TCGGTCTGATTTTGGTCGCA, R= ACCAATTCTGCGCGTTTTCC; for iroBCD gene F=GCTACTGGCGTAACCCTTCC, R=GGCAATGACGTTTGGATCG) [18]. The reaction mixture contained 5µl master mix ready to used solution (which consists of pre-optimized concentrations of hot start DNA polymerase, $MgCl_2$, di-nucleoside triphosphate [dNTP], and PCR buffer), 2µl from each primer, 5µl template DNA and 13µl sterile distilled water.

2.3.3 PCR cycling conditions

The DNA was amplified using the specific PCR cycling conditions including initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72 °C for 30 sec, followed by final extension at 72°C for 5 min. PCR products (for rmpA2 gene 369 bp; for iucABCD gene 309 bp; for iroBCD gene 670 bp) were separated in 2% agarose gel for 120 V at 60 min, stained with ethidium bromide added at 3µl and detected by U.V transilluminator (Uvite-UK). The gel results were photographed by Polaroid film.

2.4 Data Collection

Structural questionnaires have been utilized to gather data from each participant including age and gender. Laboratory data have been collected after analyzing samples.

2.5 Data Analysis

Data were entered and analyzed using the Statistical Package for the Social Sciences version 20 (SPSS 20). Descriptive statistics were used to determine the frequency and distribution of demographic characteristics of the participants as well distributions of isolates and the studied genes. Chi-square cross tabulation analysis was used to determine the presence of any significant difference between studied genes regarding participant's samples as well as presence of any correlation between studied genes and antimicrobial sensitivity test results. The P-value was significant at level of 0.05 and less.

3. RESULTS

Samples of this study were collected from 250 (62.5%) males and 150 (37.5%) females (Table 1). Our results demonstrate that the majority of participants 150 (37.5%) are aged 31 – 40 years, followed by age group of 50 years and above with percentage of (100/25%), followed by 20 – 30 age group (80/20%) and finally the age group 14 – 50 years (70/17.5%) (Table 2). Out of 400 samples cultivated, 313 (78.75%) were yield significant pathogenic growth of which 95 (23.75%) are *K. pneumoniae* isolates, 220 (55.0%) are other pathogenic pathogens, while the rest of samples 87 (17.25%) were showed no pathogenic growth isolated (Table 3).

String test and molecular study have been done to a total of 95 *K.pneumoniae* which was obtained from urine (5), sputum (81), wound swabs (8), and one sample from vaginal swab. Results of the string test showed that only 30 (31.6%) isolates were string test positive, the remaining 65(68.4%) isolates were string test negative (Table 4). The result of susceptibility test revealed that all isolates were resistant to Ceftriaxone, 36 isolates (37.9%) were resistant to Meropenem, only 5 isolates (5.3%) were found resistant to Colistin and 64 isolates (67.4%) were resistant to Piperacillin Tazobactam (Table 5).

PCR amplification for the presence of three genes, showed that 81 isolates were positive for virulence genes as follows; 71 isolates (74.7%) were positive for *iuc gene,* 55 isolates (57.9%) were positive for *iroB gene* (Table 6) (Fig. 1). Only two (2.1%) isolates were positive for *rmpA2 gene* (Table 6). One isolate possessed three genes, forty-six isolates possessed two genes, thirty-four isolates possessed only one type gene and the remaining fourteen isolates were free from these genes. Our results demonstrated a significant correlation between iuc gene and iroB gene and the samples from which the *K.pneumoniae* isolated with p-value of 0.02 and 0.01 respectively. However, rmpA2 gene showed no correlation with p-value of 0.10. Most of the iuc gene (85.9%) and iroB gene (94.6%) are detected in sputum samples *K.pneumoniae* isolates (Table 7). Our result demonstrates a significant difference between studied genes in the results of antimicrobial sensitivity test with p-value of 0.40, 0.69 and 0.14 for iuc gene, iuc gene and rmpA2 gene respectively (Table 8).

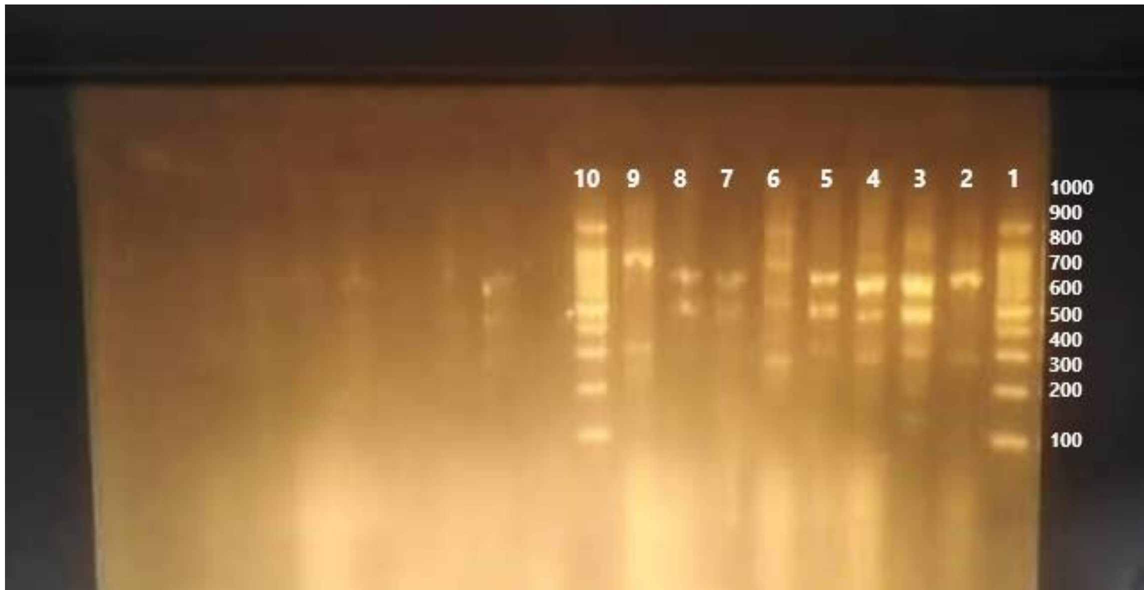

Fig. 1. The multiplex PCR performed in the study. *Lane 1, 10 are DNA ladders of 100bp. Lane 2 is positive control for iro BCD with band size 670 bp. Lanes 3,4,8,7,8 are typical positive isolates for iro BCD with band size 670 bp. Lane 3 is positive control* for iucABCD **with band size of 309 bp. *Lane 4,6 are typical positive isolates* for** iucABCD **with band size of 309 bp**

Table 1. Distribution of participants according to their gender

Gender	Frequency	Percentage%
Male	250	62.5
Female	150	37.5
Total	400	100

Table 2. Distribution of participants according to their age

Age group/ years	Frequency	Percentage%
20 – 30	80	20
31 – 40	150	37.5
41- 50	70	17.5
50 years and above	100	25
Total	400	100

Table 3. Distribution of participants according to result of Cultures

Result of culture	Frequency	Percentage%
K.pneumoniae	95	23.75
Other pathogenic growth	220	55.0
No pathogenic growth	87	17.75
Total	400	100

Table 4. Result of String test

Result of String test	Frequency	Percentage %
Positive	30	31.6%
Negative	65	68.4%

Table 5. Result of Antimicrobial Sensitivity test

Antibiotic	Sensitive	Resistant
Ceftriaxone	0 (0%)	95 (100%)
Meropenem	49 (62.1%)	36 (37.9%)
Colistin	90 (94.7%)	5 (5.3%)
Piperacillin Tazobactam	31 (32.6%)	64 (67.4%)

Table 6. Frequency and percentage of isolated genes

Name of gene	Frequency	Percentage
iuc gene	71	74.7%
iroB gene	**55**	**57.9%**
rmpA2 gene	**2**	**2.1**

Table 7.Cross tabulation analysis between studied genes and participants samples

Name of gene	Samples				P-value
	Urine	Sputum	wound swabs	vaginal swab	
iuc gene	4 (5.6%)	61 (85.9%)	6 (8.5%)	0 (0%)	0.02
iroB gene	0(0%)	52 (94.6%)	2(3.6%)	1 (1.8%)	0.01
rmpA2 gen	1 (50%)	1 (50%)	0 (0%)	0 (0%)	0.10
Total	5	113	8	1	

Table 8. Cross tabulation analysis between studied genes and result of sensitivity test

Name of gene	Antimicrobial sensitivity test								P-value
	Ceftriaxone		Meropenem		Colistin		Piperacillin Tazobactam		
	S	R	S	R	S	R	S	R	**0.40**
iuc gene	0	71	35	36	69	2	23	48	
iroB gene	**0**	**55**	**22**	**23**	**53**	**2**	**19**	**36**	**0.69**
rmpA2 gene	**0**	**2**	**1**	**1**	**0**	**2**	**0**	**0**	**0.14**
Total	**0**	**128**	**58**	**60**	**122**	**6**	**42**	**84**	

4. DISCUSSION

In recent years, there have been reports of the occurrence of community acquired liver abscesses in people with a healthy immune system by pathogenic subspecies of *K.pneumoniae* in Taiwan and some other Asian countries. This syndrome is associated with dissemination of the infectious strain to multiple organs. This is a theory that the presence of iron acquisition related genes is effective in increasing the pathogen city of *K.pneumoniae.* The most critical factors identified to date, which are encoded on a large virulence plasmid, are increased production of capsules and molecules called siderophores that enable iron acquisition needed for infection [20].

This study showed that most common gene among the 95 isolates were *iuc gene* at 74.7% which agreement with that previous study from China by Ouyang et al, (64.5%) [22], while differ from previous study in Iran by Tahereh et al. (16.7%) [23]. the second most prevalent gene in this study was *iroB* gene (57.9%) and this finding was agreed with previous study from China [22]. Moreover, two other studies are totally and dramatically disagreed with our finding, the first study determined that *iuc* was detected in 8.7% ($n = 217$) and *iro* in 7.2% of their isolates and this was dramatically lower than our finding [23] the other study found that *iuc* associated with hypervirulence, was detected in 28% of isolates [24]. The least detected gene in this study was *rmpA2* (2.1%) which differs with previous study from China by (30.6%) [20].In this study given results, three genes positive in one isolate, forty-six isolates possessed two genes (salmochelin and aerobactin). Genes for the hvkp hypervirulent phenotype are clustered on mobile genetic elements, thus facilitating transfer of virulence factors to other strains [25]. In a study from Iran conducted by Tahereh et al. [23] only one isolate possessed two genes (salmochelin and aerobactin).

This study determined that Hyper mucoviscosity by string test positive in this is study 30 isolates (31%) which was differ from previous studies from China they found 4.9%, 45.7% and 33% of *K. pneumonia* were identified as hypermucoviscous through a positive string test [22,28,29] and also differ from previous study from China by [22] show string test not proper method to assess hvkp , (64.3%) positive string test with (100%) were regulated mucoid gene (*rmpA*),where in non-hypermucoviscosity isolates (95%)were *rmpA* positive. Marr and Russo show this hyper mucoviscous phenotype has been shown not to be optimally accurate. The hypermucoviscosity string test is widely used as a phenotypic test to detect hypervirulent *Klebsiella pneumoniae* but this study of some organisms contains its negative string test and at the same time contains the genes. The majority of research showed that only one bacterium contains one HV gene but this study revealed that forty-six organisms contain two genes for the three genes used in this study.

This results demonstrate that most of iuc gene (85.9%) and iroB gene (94.6%) are detected in *K.pneumoniae* isolated from sputum samples, this is not surprising because most of the isolates are obtained from sputum samples and we include only few isolates from other specimens. Our result demonstrates a significant difference between studied genes in the results of antimicrobial sensitivity test and this point needs further studies to roll out the underlying causes which lead to such finding.

5. CONCLUSION AND RECOMMENDATION

In conclusion, there was a high prevalence of *iucA*gene (aerobactin) and high frequency of iron acquisition systems (aerobactin and salmochelin), this system might play an important role in the growth and survival of bacteria against its host**.** Control of this organism in the hospital environment and the general community is an important concern.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

DISCLAIMER

The products used for this research are commonly and predominantly used in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT AND ETHICS CONSIDERATIONS

The study received approval from the ethics commission of University of Al Butana (Number 2019/12MLS) and Khartoum hospital, and was conducted in accordance with the Declaration of Helsinki. Informed consent was taken from each participant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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