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ORIGINAL ARTICLE

Low prevalence of hypervirulent *Klebsiella pneumoniae* in Anatolia, screened via phenotypic and genotypic testing

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ABSTRACT

Hypervirulent *Klebsiella pneumoniae* (hvKP) strains are associated with vigorous clinical presentation and relapses. Initially reported from Asia, these variants have spread globally and become an emerging agent of significant health threat. This study was carried out to identify hvKP strains in a previously uninvestigated region and to evaluate the impact of commonly-employed phenotypic and genotypic markers as diagnostic assays. A total of 111 blood culture isolates, collected at a tertiary care center was investigated. The hvKP strains were sought by a string test and the amplification of partial *magA*, *rmpA*, *iucA* and *peg344*. All products were characterized via sequencing. Evidence for hvKP was observed in 10.8% via *iucA* amplification (7.2%), string test (2.7%) and *magA* amplification (0.9%). Specific products were not produced by assays targeting *rmpA* and *peg344* genes. Antibiotic susceptibility patterns compatible with possible extensive or pan-antimicrobial resistance was noted in 66.7% of the hvKP candidate strains. Capsule type in the *magA* positive strain was characterized as K5. We have detected hvKP in low prevalence at a region with no prior documentation. Targetting the aerobactin gene via *iucA* amplification provided the most accurate detection in this setting. The epidemiology of hvKP in Anatolia requires elucidation for effective control and management.

KEYWORDS

Klebsiella pneumoniae, hypervirulent, hypermucoviscous, capsule

INTRODUCTION

Klebsiella pneumoniae is a global agent of clinically relevant bacterial infections with significant health impact [1]. The majority of *K. pneumoniae* infections are hospital or institution-acquired, with a propensity for antimicrobial resistance obtained via plasmid-based determinants, resulting in serious challenges for control via antibiotics [2]. In addition to the strains referred as the "classical" *K. pneumoniae* (cKP), infections caused by particular bacterial variants with different biological and epidemiological properties have been documented [2]. Originally reported from Taiwan, they were generally community-acquired and frequently presented with clinical features involving tissue invasion in otherwise healthy, immunocompetent individuals. Intraabdominal abscesses in multiple sites with metastatic spread are common, as well as endophthalmitis, necrotizing fasciitis, pneumonia and meningitis occuring in individual cases [3–6]. These variants are frequently designated as "hypervirulent" *K. pneumoniae* (hvKP) and despite vigorous clinical presentation and relapses, they often lack the antimicrobial resistance patterns observed in cKP [6]. These strains also frequently demonstrate increased production of capsule polysaccharide, leading to a

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hypermucoviscous phenotype and biofilm formation [7]. Following initial description, hvKP strains have been subsequently reported from many Asian countries, as well as in South Africa, Australia, America, and Europe [8]. However, as the worldwide spread of hvKP becomes evident, so does the prevalence of antimicrobial resistance [9, 10], making hvKP an emerging infectious agent of significant concern.

The capacity to identify and differentiate hvKP is, therefore, likely to impact outcome and overall patient care enormously. It would not only enable rapid diagnosis of possible unrecognized sites of infection but facilitate optimal surgical or antimicrobial therapy as well. Unfortunately, a diagnostic assay for accurate identification of hvKP is currently lacking [11]. Clinical and epidemiological features, as well as formation of hypermucoviscous colonies in agar plate, due to prominent polysaccharide capsule formation, have been used to identify hvKP stains [5, 12]. Since the biological behaviour of the hvKp strains is partly mediated by genes on a large virulence plasmid or within chromosomal islands [13–17], some of these regions including mucoviscosity-associated gene A (*magA*), regulator of mucoid phenotype (*rmp*) A and A2 (A), iron-acquisition systems aerobactin biosynthetic gene (*iucABCD*), and putative transporter *peg344* have been suggested as markers [2, 11, 18, 19]. However, no specific assay that can be employed for the definitive identification of hvKP could be established. In this study, we aimed to investigate the occurence and prevalance of hvKP strains and evaluate the impact of commonly-employed phenotypic and genotypic markers as potential diagnostic assays in a microbiology laboratory setting.

METHODS

Bacterial strains

Blood culture isolates of *K. pneumoniae*, identified from January, 2015 to March, 2018 at Hacettepe University Hospital, Ankara, Turkey were included in the study. The strains were characterized using VITEK2 ID/AST system and MALDI-TOF MS (Biomerieux, France). Baseline antibiotic susceptibility patterns were evaluated by VITEK2 ID/AST and/or BD Phoenix NMIC-400/ID panel (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md, USA) when required. The tested antibiotics were Amoxicillin/Clavulanate, Amikacin, Aztreonam, Cefepime, Ceftazidime, Ceftriaxone, Cefuroxime, Ciprofloxacin, Ertapenem, Gentamicin, Imipenem, Meropenem, Piperacillin, Piperacillin/Tazobactam, Tigecycline, Trimethoprim/Sulfamethoxazole. Bacterial isolates were stored at −80 °C until use. Different isolates from the same individual obtained during one round of admission were omitted. Further information and patient medical histories were retrieved from hospital records. As the study involved evaluation of bacterial strains exclusively and no identifying information of the infected individuals were disclosed, an institutional/regional ethics approval was not sought.

Phenotypic testing

String test was employed for the phenotypic evaluation of hypermucoviscosity associated with hvKP. For this purpose, each *K. pneumoniae* isolate was grown overnight at 37 °C on blood agar plates. A positive assay result was defined as the formation of a viscous string > 5 mm in length while taking a colony with a loop wire, as described previously [12]. Three colonies from individual plates were at least tested.

Genotypic testing

Previously-described markers associated with hvKP were screened in the isolates by polymerase chain reaction (PCR). Bacteria were cultured overnight, 300 μl nuclease-free water were added on single colonies and boiled for 15 min to release DNA template. The supernatant was, used for downstream amplification steps, following centrifugation at 4,000 rpm for 5 min. Partial *magA* (mucoviscosity-associated gene A), *rmpA* (regulator of mucoid phenotype A), *iucA* (aerobactin siderophore biosynthesis) and *peg344* (putative transporter) genes were amplified, as previously reported [1, 11, 12]. Standard precautions to prevent carry-over contamination were strictly followed. Amplified PCR products were visualized under ultraviolet light via ethidium bromide staining after electrophoresis in 1.0–1.7% agarose gels, depending on the amplicon size. The expected products of the assays were 1,282 (*magA*), 535 (*rmpA*), 583 (*iucA*) and 411 (*peg344*) basepairs (bp). The hvKP strain DNAs, kindly provided by Thomas Russo and Stephen Libby, were used for optimization and as positive controls.

All amplicons produced by individual PCR assays were characterized by sequencing. The products were cleaned up using PureLink PCR Purification Kit (Thermo Fisher Scientific, Hennigsdorf, Germany). The forward and reverse primers of the particular assay and the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) were employed for sequencing via an ABI PRISM 3500xL Dx genetic analyzer (Thermo Fisher Scientific). Obtained sequences were handled using Geneious software v11.1.5 (Biomatters Ltd, Auckland, New Zealand). BLASTn and BLASTp algorithms were employed for similarity searches in the NCBI website (www.ncbi.nlm.nih.gov/blast/) [20]. Nucleotide and putative amino acid alignments and pairwise sequence comparisons were generated via the CLUSTAL W program, implemented in the Geneious software [21]. Evolutionary history was inferred via the maximum-likelihood method based on the estimated optimal substitution model according to the Bayesian information criterion and conducted using MEGA-X [22].

RESULTS

Clinical isolates

A total of 111 *K. pneumoniae* isolates were screened, which originated from pediatric (33/111, 29.7%) and adult patients (78/111, 70.3%). No repeat isolate from the same individual

was identified. Thirty-eight (38/78, 48.7%) isolates were from adults admitted at internal medicine departments (including oncology, gastroenterology, nephrology, neurology, infectious diseases, rheumatology, hematology and emergency medicine clinics) whereas 23 (23/78, 29.5%) individuals were located at intensive case units (ICU). Ten (12.8%) and 7 (8.9%) adults with *K. pneumoniae* infection were hospitalized at surgery wards (including general, gynecologic, cardiovascular and neurosurgery) and associated ICUs, respectively. The patients of the pediatric age group originated from internal medicine (22/33, 66.7%), surgery (3/33, 9.1%) and ICUs (8/33, 24.2%).

Amplification of the virulence-associated genes

The *iucA* PCR produced detectable products in 8 isolates (8/111, 7.2%), which yielded identical 510–553 bp segments of the aerobactin synthase gene via sequencing. The sequences were aligned to homologous sequences from various strains of *Klebsiella*, *Citrobacter*, *Escherichia* and *Salmonella* species, that formed well-demarcated clusters in the maximum likelihood analysis (Fig. 1). The characterized sequences grouped with several *K. pneumoniae* strains and pairwise nucleotide comparisons demonstrated a maximum of 0.5% divergence. The 553 bp sequence was submitted to GenBank (GenBank accession: MK714037).

Amplification of the partial *magA* region produced products of the expected range in 8 (8/111, 7.2%) isolates. In one strain originating from a pediatric case with intestinal obstruction and subsequent bacteremia, sequencing provided a section of the *wzc* gene encoding the inner membrane tyrosine autokinase, within the capsule polysaccharide synthesis gene cluster (GenBank accession: MK695698). The capsule type was identified as K5 and pairwise alignments showed 19.3%–10.3% nucleotide and deduced amino acid divergence, respectively, when compared to the available complete sequence data (AB371292 and LT174568). However, sequences obtained from remaining strains were unrelated to the expected target and comprised highly-similar sequences in the *K. pneumoniae* chromosome (located at 4119593–4120709 and 4281574–4282595 positions on the strain AR0158, CP021696). The virulence-associated genes *peg344* and *rmpA*, were negative in all isolates.

Patient features and assay evalution

Pheotypic or genotypic evidence for hvKP could be identified in 12 isolates (12/111, 10.8%) (Table 1). The isolates originated from individuals from a diverse age range (1–77 years, female/male ratio: 7/5) and from outpatient clinics as well as internal medicine or surgery wards. Various underlying conditions were noted in each patient (Table 1). All hvKP isolates were tested against the following antibiotics; Amoxicillin/Clavulanate, Amikacin, Aztreonam, Cefepime, Ceftazidime, Ceftriaxone, Cefuroxime, Ciprofloxacin, Ertapenem, Gentamicin, Imipenem, Meropenem, Piperacillin, Piperacillin/Tazobactam, Tigecycline, Trimethoprim/Sulfamethoxazole. In 8 isolates (8/12, 66.7%), antibiotic

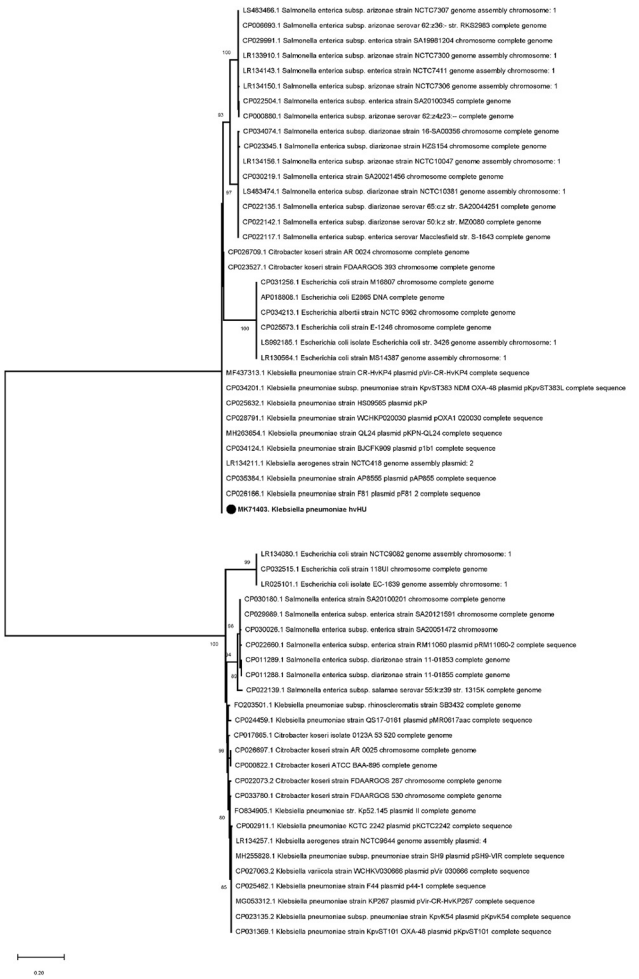

Fig. 1. Evolutionary history of the partial aerobactin operon (*iucA*) sequences, inferred by maximum likelihood method and Kimura 2-parameter model. The tree with the highest log likelihood (−2,460.71) is shown. Bootstrap values lower than 60 are omitted. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 3.8621)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The sequences are identified wth GenBank accession number, strain name and identifier. The sequence characterized in the study is given in bold

susceptibility patterns compatible with possible extensive drug resistance (XDR) or possible pan-antimicrobial drug resistance (PDR) was observed [23], leaving only 4 isolates (33.3%) to be susceptible to all tested antibiotics. Intra-abdominal or metastatic abscesses frequently noted in initial hvKP reports were observed in 3 individuals (3/12, 25%), with all isolates were found to be positive *iucA* PCR with being possible PDR. String test was reactive in 3 isolates (25%). Among four virulence-associated genes screened, *iucA* and *magA* PCRs yielded positive results, confirmed via product sequencing. No agreement among phenotypic or genotypic tests were observed and all isolates were reactive via a single assay only, however clinical findings and iucA results of 3 isolates were consistent with the hvKP phenotype (Table 1). The *iucA* PCR provided the majority of the positive results, producing detectable

Table 1. Features of the individuals with hypervirulent *Klebsiella pneumoniae* (hvKP) detection

						Assays for hvKP		
						Phenotypic	Genotypic	
No.	Age/ Gender	Clinic	Underlying condition	Isolation site	Antibiotic resistance	String test	PCR-magA	PCR-iucA
1	51/ Male	Emergency medicine	Bladder cancer	Blood	n.s.	+	−	−
2	66/ Male	Cardiovascular surgery	Graft infection	Blood	n.s.	+	−	−
3	1/Male	Pediatrics	Retinoblastoma, septic arthritis, osteomyelitis	Blood	XDR	+	−	−
4	2/ Female	Pediatric surgery	Intestinal obstruction	Blood	n.s.	−	+	−
5	84/ Female	ICU‡-Neurosurgery	Subdural hemorrhage	Blood+Abscess	PDR	−	−	+
6	62/ Male	ICU-Internal medicine	Acute myeloid leukemia	Blood	PDR	−	−	+
7	20/ Male	Neurology	Subacute sclerosing panencephalitis, hypogammaglobulinemia	Blood	PDR	−	−	+
8	63/ Female	Obstetrics and Gynecology	Endometrium carcinoma, intraabdominal abcess	Blood + Abscess	PDR	−	−	+
9	63/ Female	Emergency medicine	Rectum cancer	Blood	n.s.	−	−	+
10	66/ Female	ICU-General surgery	Pancreatitis, intraabdominal abcess	Blood + Abscess	PDR	−	−	+
11	69/ Female	Internal Medicine	Orbital abcess	Blood	PDR	−	−	+
12	77/ Female	ICU-Internal medicine	Diabetes mellitus, gastrointestinal hemorrhage	Blood	PDR	−	−	+

n.s.: not significant; ICU: intersive care unit; XDR: extensive drug resistance, PDR: pan-antimicrobial drug resistance.

products in 8 isolates, comprising 88.9% (8/9) of the genotypic assays and 66.7% (8/12) of the total hvKP detections.

DISCUSSION

Serious health threats are posed by the increasing incidence and potential antimicrobial resistance of hvKP strains [24]. A reliable biomarker or laboratory assay, that can be used for detection, differentiation and screening for hvKP, is therefore urgently needed. However, despite evaluation of several approaches based on phenotypic properties or amplification of particular virulence associated genes, no specific target could so far fulfill main requirements of a surrogate biomarker for hvKP detection [24]. In this study, we have evaluated frequently employed assays based on phenotype or genotype, for the identification of hvKP in a clinical microbiology laboratory setting.

The string test, an easy and practical method that can be robustly performed during routine diagnostic bacteriology applications, was initially suggested for hvKP identification [5]. This approach was used to define hvKp in some of the initial studies. However, bacteria with the hypermucoviscous phenotype, as observed via the string test, have not been universally associated with hvKP and the test itself has been shown to be suboptimal, especially for screening in regions with lower prevalence [11]. In this study, we have observed a detection rate of 2.7% (3/111) for hypermucoviscous *K. pneumoniae* in our cohort. This prevalence is considerably lower compared to the findings in reports from single centers China, where detection rates of 33%–45.7% were documented [8, 25]. The performance characteristics of the string test was previously evaluated in detail and observed to be overall inferior to the genotypic biomarkers [11]. Moreover, a discrepancy between hypermucoviscosity phenotype and related virulence genes was observed [26]. Recently, an infection model in *Galleria mellonella* larvae was used to assess hvKp virulence where the strains could be clustered in high and moderate virulence groups, unrelated to the hypermucoviscosity phenotype [26]. Therefore, the string test cannot be considered a proper method for screening or virulence assessment in hvKP, despite its simplicity and ease of performance [27]. This is also indirectly observed in our study, where no association of string test reactivity and genotypic target detection was noted (Table 1).

Several loci on the *Klebsiella* genome, directly or indirectly associated with virulence or hypermucoviscous colonies on agar plates have been suggested as biomarkers to characterize hvKP strains [11]. In addition to the commonly-used regions such as *iroBCD*, *iucABCD* and *rmpA/A2*, several other genes have been identified as candidate virulence factors of *K. pneumoniae*, some of which were also associated with virulence in bacteria species other than *Klebsiella* [28]. We have selected some of these genes for PCR amplification, namely *rmpA*, *iucA* and *peg344* in this study, as they detect different targets and have been previously used in endemic regions. Despite lack of specific detection via *rmpA* and *peg344* PCRs, we could amplify and successfully characterize partial *iucA* sequences in 7.2% of all isolates (8/111), providing direct evidence for hvKP in the study cohort. The gene *iucA* is located in the operon encoding for the aerobactin, the abundant siderophore produced by hvKP strains, also a critical mediator for enhancing ex vivo and in vivo virulence [29, 30]. The amplification of *iucA* has been previously shown to have high accuracy for hvKp identification and could effectively differentiate hvKP from cKP in a murine sepsis model [11]. Therefore, it is widely considered as a stable genetic biomarker for hvKP strains. It has also been reported that a combination of *iucA* and *peg344* amplification increases diagnostic accuracy [11], an observation that could not be reproduced in this study.

We have further performed *magA* amplification in our *K. pneumoniae* cohort, using previously-published primers and identified one positive strain, comprising 0.9% of all isolates tested. The sequencing of the amplicon provided a section of the *wzc* gene and revealed the capsule type as K5, via pairwise sequence comparisons and BLAST analysis. It is known that 79 distinct capsular types exist in *Klebsiella* spp. and recent efforts provided a complete sequence repertoire of coding regions for each [31]. Based on this data, it is documented that wzy-based capsular genotyping could reliably differentiate major capsule types except for those lacking or identical wzy sequences [31]. The initial hvKP clones were mostly hypermucoviscous and K1 and K2 type capsules, prevalent in Asia and Europe/America, respectively, were linked to hypervirulence [27]. Moreover, capsule types K5, K20, K54 and K57 have also been frequently-detected in human infections caused by *K. pneumoniae*. It is known that hvKP strains does not always confer to K1/K2 and other capsule types can be associated with the hypervirulent phenotype [9, 27]. Serological screening have previously revealed the circulation of various *K. pneumoniae* capsular types in Turkey [32, 33] but PCR-based investigations for virulence factors associated with hvKP and capsule typing have failed to identify positive strains [34, 35].

The *rmpA* and *magA* amplifications we carried out in this study also produced non-specific products, sometimes within the expected amplicon range. Sequencing of all detectable products revealed that the corresponding primers could bind and amplify various regions of the bacteria genome. This indicates a need for revisiting previously-published primers with the current genome data and updating as necessary. Given the advent of whole genome sequencing for pathogenic bacteria and the subsequent availability of sequences [36], specificity and accuracy of a nucleic acid based assay should be improved, especially for the task of developing a universal assay for hvKP detection with FDA clearance.

The epidemiological impact of our findings require further elucidation. Originating from Turkey, an Eurasian country with no prior documentation of hvKP circulation, our data indicate an overall low prevalence. However, the observed antibiotic resistance patterns in individuals with evidence for hvKP infection is alarming, with possible PDR in 88.9% of the bacteria with detectable iucA sequences. So far, hvKP strains have mostly retained antimicrobial susceptibility to multiple drug classes [24]. However, recent reports describe hvKP clones obtaining resistance plasmids or acquisition of hvKP virulence plasmid by resistant cKP strains with serious outcomes [17, 37]. Therefore, active measures are required for hvKP screening, control and management. The main limitation of this effort is the single center origin of the evaluated strains. Given the findings of this pilot study, hvKP circulation in community and other health-care settings should be closely monitored, with the implemantation of appropriate infection control precautions. For this purpose, an internationally acclaimed sensitive, accurate and robust diagnostic assay is urgently needed.

In conclusion, we have identified hvKP strains, at a tertiary care institution from a previously unexplored region, in low prevalence via genotypic and phenotypic testing. Significant antibiotic resistance patterns were recognized in probable hvKP strains, and circulation of *K. pneumoniae* capsular type K5.

Conflict of interest: The authors have no conflicts of interest to declare.

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