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Investigation of the Species Distribution of Tissue Invasive *Klebsiella pneumoniae* Complex in a
New York Hospital System Using Whole Genome Sequencing

Running Title: *Klebsiella pneumoniae* Complex Species

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ABSTRACT

BACKGROUND: Current research indicates that *Klebsiella pneumoniae* is a complex of closely related organisms (*K. pneumoniae sensu stricto*, *K. quasipneumoniae*, *K. variicola*, *K. quasivariicola*, and *K. africana*). There is a paucity of research into the distribution of *K. pneumoniae* complex members in clinical specimens and the pathogenic potential of complex members other than *K. pneumoniae sensu stricto*.

METHODS: To assess the rates and characteristics of different *K. pneumoniae* complex members in our 886 bed tertiary care facility in Rochester, New York, we performed whole genome sequencing using the Illumina MiSeq on all inpatient, sterile-source isolates identified by routine culture work-up as *K. pneumoniae* from October 2018 - October 2019 (n=35). We additionally sequenced all *K. pneumoniae* liver isolates available in our collection, spanning 2017-2019 (n=12). Analyses were focused on delineating complex members, detection of antibiotic resistance genes, and gene clusters associated with hypervirulent phenotypes.

RESULTS: In the yearlong collection of 35 *K. pneumoniae* complex isolates, we found that 92.4% were *K. pneumoniae sensu stricto* (n=32), 5.7% were *K. quasipneumoniae* (n=2), and 2.9% were *K. variicola* (n=1). In the liver isolate collection, one additional *K. variicola* was identified. Both *K. quasipneumoniae* isolates and one *K. variicola* isolate were from liver. One *K. quasipneumoniae* isolate was a phenotypic and genotypic extended spectrum beta-lactamase producer. All other *K. quasipneumoniae* and *K. variicola* isolates were resistant only to ampicillin. Five *K. pneumoniae sensu stricto* isolates were identified which carried genes associated with hypervirulence. No *K. quasipneumoniae* or *K. variicola* carried any hypervirulence associated genes.

CONCLUSIONS:

The rates of *K. pneumoniae sensu stricto*, *K. quasipneumoniae*, and *K. variicola* observed in this study are consistent with those observed internationally and in blood isolates. Interestingly, all of our *K. quasipneumoniae* isolates were isolated from liver abscesses. Genetic loci associated with hypervirulence appear to be restricted to *K. pneumoniae sensu stricto*.

KEY WORDS

Klebsiella pneumoniae; next generation sequencing; *Klebsiella variicola*; *Klebsiella quasipneumoniae*

BACKGROUND

Klebsiella pneumoniae is a major human pathogen that causes a wide variety of nosocomial and community acquired infections. It is the second most common cause of gram negative bacteremia and a leading cause of both urinary tract infections and ventilator-associated pneumonia (1-4). Infections caused by this organism can be particularly lethal; mortality rates of up to 50% have been reported (5-7).

K. pneumoniae was recently determined to be a complex of closely related species. In addition to *K. pneumoniae sensu stricto*, the *K. pneumoniae* complex is composed of *K. variicola*, *K. quasipneumoniae*, *K. quasivariicola*, and *K. africana* (8-11). Standard laboratory methods are generally unable to differentiate these species, though the most recent Bruker and Vitek2 matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) databases allow “*Klebsiella pneumoniae*” and “*Klebsiella variicola*” to be distinguished (12-14). Understanding of the epidemiology and clinical significance of individual members of the *K. pneumoniae* complex is still emerging. Systematic studies of *K. pneumoniae* complex

clinical isolates have revealed that 2.8 - 24.5% of clinical isolates initially identified as *K. pneumoniae* are actually *K. variicola* upon sequencing analysis and 3 - 12.8% are *K. quasipneumoniae*, with considerable variance across studies (15-22).

Rates of antimicrobial resistance and evaluations of clinical severity of *K. variicola* and *K. quasipneumoniae* infections in these studies have varied. Notably, *K. variicola* has been implicated in nosocomial outbreaks among neonates (23, 24). There is very little known about the clinical significance of *K. quasivariicola* and *K. africana*, aside from three case reports of *K. quasivariicola* infections (25-27). Although some strains of *K. pneumoniae*, known as hypervirulent *K. pneumoniae* and carrying additional siderophores and the hypermucoviscosity genes *rmpA* and *rmpA2*, are known for causing devastating invasive infections in healthy members of the community, it is unclear whether the same virulence potential exists for all members of the *K. pneumoniae* complex (28). It is generally believed that hypervirulent strains are *K. pneumoniae sensu stricto*, often of a single multi-locus sequence type (MLST), ST23. However, case reports of *K. variicola* and *K. quasipneumoniae* possessing genetic markers of hypervirulence do exist (16, 29-33).

There has not yet been any study on the prevalence of different members of the *K. pneumoniae* complex in the United States of America and previous studies have not focused on tissue invasive isolates. Furthermore, studies examining the frequency of virulence factors and antimicrobial resistance in species other than *K. pneumoniae sensu stricto* are few.

In this study, we sequenced all tissue invasive isolates (n=35) identified as “*Klebsiella pneumoniae*” by standard laboratory methods at our 886 bed tertiary care facility in Rochester, New York between October 2018 and October 2019 with the goal of determining the relative rates of isolation of *K. pneumoniae* complex members as well as their sequence types,

antimicrobial resistance, and the presence of hypervirulence associated genes. Upon finding that most of our non- *K. pneumoniae sensu stricto* isolates were liver isolates, we focused further on liver isolates and sequenced all qualifying *K. pneumoniae* available in our strain collection going back to 1/2017 (n=12).

METHODS

Aims, design and study site

In this study, we sought to determine the relative rates of isolation of *K. pneumoniae* complex members, as well as their sequence types, antimicrobial resistance, and the presence of hypervirulence associated genes, from tissue invasive isolates at the University of Rochester Medical Center (URMC) through the use of next generation genome sequencing. URMC is an 886 bed tertiary care facility located in Western New York with a large catchment area that includes the City of Rochester and surrounding rural counties. URMC Central Laboratories additionally provides reference microbiological services to a larger catchment area which includes several small community hospitals across much of Western New York.

Two sets of criteria were used for inclusion of isolates in this study: 1) all *K. pneumoniae* isolated from sterile site specimens collected from inpatients at Strong Memorial Hospital (SMH) between the dates of October 2018 and October 2019 (n=35) and 2) all additional *K. pneumoniae* isolated from liver specimens available at the URMC Central Laboratory from any facility in the URMC system going back to January 2017 (n=12). Only one isolate per patient was included in the study. For all isolates, patient data on age, sex, hospital site admitted to and inpatient/outpatient status were collected from the laboratory information system. The Institutional Review Board (IRB) at the University of Rochester approved this study.

Antimicrobial susceptibility testing

For isolates isolated prior to July 2019, antibiotic susceptibility testing was accomplished using the Biomerieux Vitek 2 Gram negative antimicrobial susceptibility testing (AST) card. For isolates isolated after July 2019, antibiotic susceptibility testing was accomplished using the BD Phoenix NMIC-300 panel.

DNA extractions and whole genome sequencing

Isolates were subcultured from frozen stocks onto BBL Trypticase Soy Agar with 5% Sheep Blood plates (BD, Franklin Lakes, NJ, USA). Overnight growth was lysed with lysozyme in Roche MagNA Pure Bacteria Lysis Buffer for 1 hour. Total nucleic acid was extracted from the lysate using the Roche MagNA Pure Compact Nucleic Acid Isolation Kit I and the MagNA Pure Compact instrument (Roche, Indianapolis, Indiana). Dual-indexed sequencing libraries were prepared from the lysates using Nextera XT DNA Library Preparation Kit (Illumina FC-131-1096), and sequenced on an Illumina MiSeq benchtop sequencer (Illumina, San Diego, CA) using Illumina MiSeq reagent kit V3, 600 cycles.

Genomic analysis

Genome assembly and analysis was performed using the URM C Bacterial Genomic Analysis Pipeline version 3.0.3 or the URM C Bacterial Genomic Analysis Pipeline version 3.1.1, a previously published in-house bioinformatics pipeline written in Python, sqlite3, BASH, JavaScript, D3, JQuery, HTML, and Bootstrap which incorporates a modified CFSAN SNP Pipeline v.1.0.0 for reference-based single nucleotide polymorphism (SNP) calling and phylogenetic analysis and SPAdes v3.11.1 for genome assembly (34-36). Updates between version 3.0.3 and 3.1.1 addressed ease of use and did not impact pipeline functionality. Sequences were aligned against the *K. pneumoniae sensu stricto* strain MGH78758 (NCBI:

NC_009648.1) using bowtie2 v2.2.9 (37). Alignment resulted in an average coverage of 86X and an average depth of 55X (excluding regions below 12X). Annotation was accomplished using Prokka version 1.12 (38).

SNP calling and phylogenetic analysis

A maximum likelihood tree was produced using FastTree (39). Species assignment was based on the following reference genomes: *K. pneumoniae sensu stricto* MGH78758 (NCBI: NC_009648.1), *K. pneumoniae sensu stricto* HS11288 (NCBI: NC_016845.1), *K. quasipneumoniae* CAV2018 (NCBI: NZ_CP029432.1), *K. quasipneumoniae* spp. *similipneumoniae* 700603 (NCBI: NZ_CP014696.2), *K. quasivariicola* KPN1705 (NCBI: NZ_CP022823.1), *K. variicola* DSM15968 (NCBI: NZ_CP010523.2), and *K. variicola* X39 (NCBI: NZ_CP018307.1).

Prediction of virulence and antimicrobial resistance genes

Kleborate version 0.2.0 (<https://github.com/katholt/Kleborate>) was used with default parameters and --kaptive and --resistance enabled to determine sequencing type, determine K locus type, identify virulence factors, and identify antibiotic resistance genes (40-43). Isolates found to simultaneously contain the aerobactin operon and *rmpA* or *rmpA2* were classified as hypervirulent.(44)

RESULTS

Thirty-six isolates were identified as *Klebsiella pneumoniae* from sterile source specimens from adult inpatients at SMH during the October 2018 - October 2019 time period. Of these, 35 isolates were available for sequencing. An additional 12 isolates identified as *K. pneumoniae* isolated from liver were available in freezer collections dating back to 2017. These

included patients in other hospitals in the catchment area and outside of the October 2018 – October 2019 time period. Sequencing, genome assembly, and annotation were successful for all isolates. Characteristics of these isolates are available in Table 1.

Table 1. Summary of Patient and Isolate Characteristics

Characteristic	October 2018 – October 2019 Collection	Additional Liver Isolates (2017- 2019)
N	35	12
Female Sex	13 (37)	1 (8.3)
Mean patient age	65 years (range: 40 - 85 years)	59 years (range: 14 - 74)
Source	Sterile body fluid: 15 (42.9) Liver: 6 (17.1) Bone: 3 (8.6) Kidney: 3 (8.6) Other sterile tissue: 3 (8.6) Joint/joint fluid: 2 (5.7) CSF shunt: 1 (2.9)	Liver: 12 (100)
Polymicrobial specimen	20 (57)	9 (75)
Species	<i>K. pneumoniae sensu stricto</i> : 32 (92.4) <i>K. variicola</i> : 1 (2.9) <i>K. quasipneumoniae</i> : 2 (5.7)	<i>K. pneumoniae sensu stricto</i> : 11 (91.7) <i>K. variicola</i> : 1 (8.3) <i>K. quasipneumoniae</i> : 0 (0)

Data are presented as frequency (percentage)

Phylogenetic clustering was used to determine the species of the *K. pneumoniae* complex isolates. Forty-three isolates were found to cluster with *K. pneumoniae sensu stricto* (84.1% - 90.2% identity to reference) (Additional File 1). Of the remaining isolates, two, URM-555 and URM-560, were found to be *K. quasipneumoniae* (87.5 – 88.6%, identity to reference) and two, URM-553 and URM-603, were found to be *K. variicola* (92.1 - 92.2% identity to reference). Both *K. quasipneumoniae* were from liver and represented 33% of the liver isolates from the October 2018 – October 2019 collection and 11% of all liver isolates tested. URM-603 was also a liver isolate, while URM-553 was from joint fluid. Looking strictly at the October 2018 – October 2019 collection, thirty-two isolates clustered with *K. pneumoniae sensu stricto*, one clustered with *K. variicola*, and two clustered with *K. quasipneumoniae*. These results correspond to a yearly isolation rate among tissue invasive isolates of 92.4% *K. pneumoniae sensu stricto* (n=32), 5.7% *K. quasipneumoniae* (n=2), and 2.9% *K. variicola* (n=1). Neither *K. quasivariicola* nor *K. africana* were identified among the strain sets.

General characteristics of the 2018 - 2019 sterile site collection

The population structure of isolates from the 2018 - 2019 sterile site collection was heterogeneous, separated by more than 200 SNPs across 26 sequence types (Additional File 2). Among isolates on which antimicrobial susceptibility testing was performed, none were resistant to carbapenems. Four strains exhibited phenotypic extended spectrum beta-lactamase (ESBL) activity, detected as ceftriaxone resistance, all of which carried *bla*_{CTX-M-15} (Additional File 3). Two of these isolates, URM-547 and URM-561, were ST307, an international *bla*_{CTX-M-15} associated clone. URM-555 was a *K. quasivariicola* isolate.

All isolates were assessed for the presence of genes associated with hypervirulence in *K. pneumoniae sensu stricto*: the hypermucoviscosity associated genes *rmpA* and *rmpA2* and the

siderophore gene clusters *ybt* (yersiniabactin), *clb* (colibactin), *iuc* (aerobactin), and *iro* (salmochelin). In the 2018 - 2019 collection, 20% of strains carried genes for yersiniabactin (n=7), 2.9% for colibactin (n=1), 5.7% for aerobactin (n=2), 5.7% for salmochelin (n=2), and 5.7% (n=2) for hypermucoviscosity. Two isolates, URM-475 and URM-549, met the genetic determinants for hypervirulence by carrying both *rmpA/rmpA2* and the aerobactin gene cluster (*iuc*), for an overall yearly isolation rate of 5.3% (n= 2 of 35) (Additional File 4). All isolates carrying a hypervirulence associated gene were *K. pneumoniae sensu stricto*.

Characteristics of *K. variicola* and *K. quasipneumoniae* isolates

URM-553 (*K. variicola*) was isolated in May of 2019 from a polymicrobial infection of joint fluid from a 45 year old female. It typed as ST729 with four variant loci according to the *K. pneumoniae* MLST scheme. Although a *K. variicola* specific MLST scheme has been developed, it remains standard practice to use *K. pneumoniae sensu stricto* MLST typing for all members of the *K. pneumoniae* complex (45). As expected for a *K. variicola* isolate, URM-553 carried a *bla_{LEN}* beta-lactamase, *bla_{LEN-24}*. (46) It carried no other antimicrobial resistance genes and was phenotypically resistant only to ampicillin (Additional File 3 and Additional File 4).

The other *K. variicola* isolate, URM-603 was isolated in March of 2018 from a polymicrobial liver abscess of a 67 year old male. It typed as ST347 with a single variant loci. Like URM-553, URM-603 carried *bla_{LEN-24}*, had no other antimicrobial resistance genes and was phenotypically resistant only to ampicillin (Additional File 3 and Additional File 4).

The *K. quasipneumoniae* isolates, URM-555 and URM-560, were isolated in June 2019 from a polymicrobial liver abscess and October 2018 from a monomicrobial liver abscess, respectively. URM-555 was isolated from a 64 year old male and typed as ST414. Phenotypically, it was resistant to ampicillin, ciprofloxacin, ceftriaxone, cefazolin, moxifloxacin,

and trimethoprim-sulfamethoxazole, and intermediate to ampicillin/sulbactam and tobramycin (Additional File 3). URM-555 carried an assortment of antibiotic resistance genes encoding resistance to fluoroquinolones (*qnrS1*), rifampin (*arr-3*), sulfonamides (*sulI* and *sulII*), and trimethoprim (*dfrA27*), and the extended spectrum beta-lactamase *bla_{CTX-M-15}*. As expected for a *K. quasipneumoniae* isolate, URM-555 also carried a *bla_{OKP}* beta-lactamase, *bla_{OKP-B-8}*.(46) URM-560 was isolated from a 70 year old female and typed as ST1584. It only the carried the *sulI* gene encoding resistance to sulfonamides and was resistant only to ampicillin (Additional File 3 and Additional File 4). As expected, URM-560 carried a *bla_{OKP}* beta-lactamase, *bla_{OKP-B-10}*.

DISCUSSION

In our collection of sterile site specimens from between October 2018 and October 2019, we observed an isolation rate of 92.4% for *K. pneumoniae sensu stricto* (n=32), 5.7% for *K. quasipneumoniae* (n=2), and 2.9% for *K. variicola* (n=1). There was no evidence of clonal spread within our collection, with all isolates differing by more than 200 SNPs (Additional File 4) (47). Our rates of *K. quasipneumoniae* are comparable to the 3 - 12.8% isolation rates previously reported from studies of blood, CSF, and respiratory specimens (15-22). Likewise, our observed rates of *K. variicola* are low, but within the range (2.8 - 24.5%) that has been previously reported (15-22). Notably, unlike previous collections, which assessed the frequency of *K. quasipneumoniae* and *K. variicola* primarily from blood, our collection exclusively examined tissue isolates. The consistency of our rates of *K. quasipneumoniae* and *K. variicola* with previous studies, therefore, suggests that *K. quasipneumoniae* and *K. variicola* do not have any deficits in tissue invasion relative to *K. pneumoniae sensu stricto*.

Our initial sample set suggested that *K. quasipneumoniae* was enriched among our *K. pneumoniae* complex liver isolates (33%). These cases were likely the result of hematogenous spread in bacteremic individuals. Both of the patients from whom our initial *K. quasipneumoniae* isolates were cultured had corresponding positive blood cultures identified by conventional work up as *K. pneumoniae*. As our conventional culture work up cannot differentiate *K. pneumoniae sensu stricto* from *K. quasipneumoniae*, it is likely that these positive blood cultures were also *K. quasipneumoniae*. However, this raised the possibility that tissue invasive *K. quasipneumoniae* may have a tropism for the liver.

An expanded analysis of *K. pneumoniae* complex liver isolates (2017 - 2019) in our laboratory failed to reveal additional *K. quasipneumoniae* isolates. Furthermore, a literature review revealed only one previous report of *K. quasipneumoniae* isolated from liver, with most previous reports of clinical *K. quasipneumoniae* isolates being isolated from bloodstream infections, with a lesser proportion isolated from urine, respiratory secretions, wound infections, and cerebral spinal fluid (CSF) (32). This may be explained by the relatively recent focus on and tools for identifying this complex member.

The overall rates of antimicrobial resistance and hypervirulence markers among our collection of sterile site specimens from between October 2018 and October 2019 was unremarkable. 5.3% of isolates had the genetic markers of hypervirulence, which is consistent with the rates observed in previous studies of North American hospitals (48-50), all of which were *K. pneumoniae sensu stricto*. No carbapenemase producers were isolated and 12% were ESBL producers, all of which carried *bla*_{CTX-M-15}. These rates are comparable to the rates observed in other studies in the United States.(51, 52) The limited literature on this topic has suggested that *K. quasipneumoniae* and *K. variicola* may have lower rates of antimicrobial

resistance compared to *K. pneumoniae sensu stricto*. (15, 20, 21, 53) In our study, one of our *K. quasipneumoniae* was an ESBL producer. However, the total number of *K. quasipneumoniae* and *K. variicola* isolates is too low to draw conclusions about the rates of ESBL production in these species.

There are several limitations to this study. The first limitation is the small size of this study. The low number of *K. quasipneumoniae* and *K. variicola* isolates precluded the ability to draw conclusions about the prevalence of virulence factors and antimicrobial resistance among these species. Furthermore, all of the isolates in the October 2018 and October 2019 collection were from a single institution. Since a substantial amount of inter-study variability in rates of *K. quasipneumoniae* and *K. variicola* exists, it is possible that these findings will not be replicated at other institutions. Finally, the isolates in this study were only collected from adults and were predominantly collected from male patients. As a results, findings of this study may not be representative of all patient populations.

CONCLUSIONS

The rates of *K. pneumoniae sensu stricto*, *K. quasipneumoniae*, and *K. variicola* observed in this study are consistent with those observed internationally and in blood isolates. The total number of *K. quasipneumoniae* and *K. variicola* isolates were too low to draw conclusions about utilization of virulence factors, rates of antimicrobial resistance, or tissue preference. Genes associated with hypervirulence were found only in *K. pneumoniae sensu stricto* at rates consistent with previous studies in the United States. Unusually, all of our *K. quasipneumoniae* isolates were isolated from liver abscesses.

281

282 **LIST OF ABBREVIATIONS**

283 URM - University of Rochester Medical Center

284 MALDI-TOF - matrix assisted laser desorption ionization-time of flight mass spectrometry

285 MLST - single multi-locus sequence type

286 SMH - Strong Memorial Hospital

287 AST - antimicrobial susceptibility testing

288 SNP - single nucleotide polymorphism

289 ESBL - extended spectrum beta-lactamase

290 CSF - cerebral spinal fluid

291

292 **DECLARATIONS**

293 **Ethical approval and consent to participate**

294 Testing of the *Klebsiella* isolates and limited collection of patient data were approved by the
295 Institutional Review Board of the University of Rochester Medical Center (URMC). All methods
296 were performed in accordance with the relevant guidelines and regulations. As only de-identified
297 patient data was collected for this study, the requirement to collect informed consent was waived
298 by the Institutional Review Board of the URM.

299 **Consent for publication**

300 Not applicable.

301 **Availability of data and materials**

302 Sequence data that support the findings of this study have been deposited in National Center for
303 Biotechnology Information database with the primary accession code [PRJNA673499](https://www.ncbi.nlm.nih.gov/submit/PRJNA673499).

304 **Competing interests**

305 The authors declare that they have no competing interests.

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Authors' contributions

J.B. participated in project conceptualization, methodology design, data analysis and curation, table preparation, and writing and editing the manuscript. ST participated in methodology and software design, validation of methods, data analysis and curation, and writing and editing the manuscript. J.W. participated in methodology design, validation of methods, data analysis, and editing of the manuscript. D.H. participated in resource provision, supervision, project administration, and funding acquisition. N.P. participated in project conceptualization, methodology design, resource provision, supervision, project administration, funding acquisition, table preparation, and writing and editing the manuscript. All authors reviewed the manuscript.

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