

Intermingled *Klebsiella pneumoniae* Populations Between Retail Meats and Human Urinary Tract Infections

Gregg S. Davis,¹ Kara Waits,² Lora Nordstrom,² Brett Weaver,² Maliha Aziz,^{1,2} Lori Gauld,³ Heidi Grande,² Rick Bigler,² Joseph Horwinski,² Stephen Porter,⁴ Marc Stegger,^{2,5} James R. Johnson,^{4,6} Cindy M. Liu,^{2,7} and Lance B. Price^{1,2}

¹George Washington University Milken Institute School of Public Health, Washington D.C.; ²Translational Genomics Research Institute, and ³Flagstaff Medical Center, Arizona; ⁴VA Healthcare System Minneapolis, Minnesota; ⁵Statens Serum Institut, Copenhagen, Denmark; ⁶Department of Medicine, University of Minnesota, Minneapolis; and ⁷Johns Hopkins School of Medicine, Baltimore, Maryland

(See the Editorial Commentary by Manges on pages 900–2.)

Background. *Klebsiella pneumoniae* is a common colonizer of the gastrointestinal tract of humans, companion animals, and livestock. To better understand potential contributions of foodborne *K. pneumoniae* to human clinical infections, we compared *K. pneumoniae* isolates from retail meat products and human clinical specimens to assess their similarity based on antibiotic resistance, genetic relatedness, and virulence.

Methods. *Klebsiella pneumoniae* was isolated from retail meats from Flagstaff grocery stores in 2012 and from urine and blood specimens from Flagstaff Medical Center in 2011–2012. Isolates underwent antibiotic susceptibility testing and whole-genome sequencing. Genetic relatedness of the isolates was assessed using multilocus sequence typing and phylogenetic analyses. Extraintestinal virulence of several closely related meat-source and urine isolates was assessed using a murine sepsis model.

Results. Meat-source isolates were significantly more likely to be multidrug resistant and resistant to tetracycline and gentamicin than clinical isolates. Four sequence types occurred among both meat-source and clinical isolates. Phylogenetic analyses confirmed close relationships among meat-source and clinical isolates. Isolates from both sources showed similar virulence in the mouse sepsis model.

Conclusions. Meat-source *K. pneumoniae* isolates were more likely than clinical isolates to be antibiotic resistant, which could reflect selective pressures from antibiotic use in food-animal production. The close genetic relatedness of meat-source and clinical isolates, coupled with similarities in virulence, suggest that the barriers to transmission between these 2 sources are low. Taken together, our results suggest that retail meat is a potential vehicle for transmitting virulent, antibiotic-resistant *K. pneumoniae* from food animals to humans.

Keywords. food; meat; poultry; antibiotic resistance; *Klebsiella*.

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Correspondence: Lance B. Price, PhD, George Washington University Milken Institute School of Public Health, 950 New Hampshire Ave NW, 713-Flr 7, Washington D.C. 20052 (lprice@email.gwu.edu).

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Classic foodborne bacterial pathogens, such as *Listeria monocytogenes*, *Salmonella enterica*, *Campylobacter* species, and diarrheagenic *Escherichia coli*, have driven most food-safety science and policy to date [1]. However, the bacterial species that contaminate meat products extend well beyond these “usual suspects” [2–5]. Today, it is increasingly recognized that colonizing opportunistic pathogens such as extraintestinal pathogenic *E. coli*, *Enterococcus* species, and *Clostridium difficile* may also be transmitted from food animals to humans

via retail meat [2, 3, 6–9]. Furthermore, antibiotic resistance has been increasing among Enterobacteriaceae that contaminate retail meats, particularly poultry products [10]. Thus, there is a continued need to characterize more fully the breadth and public health relevance of bacterial pathogens in our food supply.

Klebsiella pneumoniae is a colonizing opportunistic pathogen of humans and animals, and a common contaminant of retail meat [4]. In animals, *K. pneumoniae* causes disease in cows, horses, and companion animals [11, 12]. In humans, *K. pneumoniae* frequently colonizes the gut and sporadically causes extraintestinal infections [13]. Although named for its capacity to cause pneumonia, *K. pneumoniae* also causes a wide range of other infections, including cystitis, pyelonephritis, osteomyelitis, meningitis, bacteremia, septicemia, liver abscess, and wound infections [13–15]. Additionally, increasing multidrug resistance among *K. pneumoniae* strains makes the clinical management of these infections more challenging [16–18]. The notoriety of *K. pneumoniae* as a multidrug-resistant pathogen can be attributed, in part, to successful lineages such as the carbapenem-resistant *K. pneumoniae* sequence type (ST) 258 [19]. However, broader analysis shows that the total *K. pneumoniae* population causing antibiotic-resistant infections in humans is genetically diverse [20, 21]. Thus, it is essential to characterize the origins and epidemiology of both epidemic and sporadic *K. pneumoniae* strains, of which meat may be an important reservoir.

To better understand the potential contribution of foodborne *K. pneumoniae* to human infections, we assessed the phenotypic and phylogenetic similarity of contemporaneously collected meat-source and clinical *K. pneumoniae* isolates. We compared the 2 populations using whole-genome sequence (WGS)-based phylogenetic analysis and in vivo virulence models.

MATERIALS AND METHODS

Sequence Data Accession Numbers

The sequences generated from the 82 isolates described in this study are available in the NCBI short read archive (SRA) under the accession number SRP060821.

Meat Sample Collection and Processing

Retail turkey, chicken, and pork products were purchased from all 9 major grocery store chains (1 store per chain) in Flagstaff, Arizona, from January to October 2012. No bodegas (convenience stores) were sampled. Retail meat samples were purchased as part of a larger study focusing on extraintestinal pathogenic *E. coli* (ExPEC). Beef was not included due to the low prevalence of ExPEC in these products [3, 22].

Samples were processed no later than 1 day past the sell-by date. From each package, 1 whole piece of meat, or 325 g \pm 10% of ground products, was transferred aseptically to a Stomacher bag (VWR, Radnor, Pennsylvania) containing 250 mL

MacConkey broth (Alpha Biosciences, Baltimore, Maryland). (For the first five collections 30 g \pm 10% of each ground sample was used during processing). After overnight enrichment at 44°C, a violet red bile agar plus 4-methylumbelliferyl- β -D-glucuronide (VRBA + MUG) (Teknova, Hollister, California) plate was inoculated with 10 μ L of broth and incubated at 37°C for 2 hours and then at 44°C for 22 hours. Four putative *K. pneumoniae* colonies from each VRBA + MUG plate were streaked onto CHROMagar (Hardy Diagnostics, Santa Maria, California) and incubated for 20–24 hours at 37°C. Finally, 1 putative *K. pneumoniae* colony, appearing blue in color on CHROMagar, was streaked for isolation on a second CHROMagar plate and incubated for 20–24 hours at 37°C. Isolates were confirmed as *K. pneumoniae* by DNA sequence analysis and stored at –80°C in Brucella broth with 20% glycerol.

Clinical *K. pneumoniae* Isolates

Klebsiella pneumoniae clinical isolates were collected from all routinely submitted clinical urine or blood specimens processed by the Flagstaff Medical Center from November 2011 to October 2012 in Flagstaff, Arizona, without regard for patient characteristics or clinical context. The Flagstaff Medical Center is the only hospital in Flagstaff, and its clinical laboratory serves healthcare centers throughout northern Arizona, except for certain outpatient clinics that utilize third-party diagnostic laboratories. The Northern Arizona Healthcare Institutional Review Board approved this study. A waiver of consent and Health Insurance Portability and Accountability Act authorization were obtained.

Phenotypic Susceptibility Testing

The antibiotic susceptibility profile of each isolate was determined by the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [23]. The antibiotics tested were as follows: amikacin, ampicillin, ampicillin-sulbactam, ceftazolin, cefoxitin, ceftriaxone, ceftazidime, cefotaxime, ciprofloxacin, gentamicin, imipenem, nalidixic acid, tetracycline, and trimethoprim-sulfamethoxazole. Isolates were classified as “susceptible,” “intermediate,” or “resistant” in accordance with 2011 breakpoint guidelines from the CLSI [23]. For statistical analyses, “intermediate” and “resistant” isolates were grouped together.

The antibiotics represented 7 different classes: aminoglycosides (amikacin and gentamicin), carbapenems (imipenem), cephalosporins (ceftazolin, cefoxitin, ceftriaxone, ceftazidime, and cefotaxime), folate pathway inhibitors (trimethoprim-sulfamethoxazole), penicillins and penicillin combinations (ampicillin and ampicillin-sulbactam), quinolones (nalidixic acid and ciprofloxacin), and tetracyclines (tetracycline). *Klebsiella pneumoniae* is intrinsically resistant to ampicillin; therefore, isolates were categorized as multidrug resistant if they were resistant to ≥ 2 additional antibiotic classes (ie, ≥ 3 classes total).

Extended-Spectrum β -Lactamase Confirmatory Test

Extended-spectrum β -lactamase (ESBL) phenotype was assessed by a standard disk diffusion method involving 30 μ g cefotaxime and 30 μ g ceftazidime disks, each with and without 10 μ g supplemental clavulanic acid (BD Diagnostic Systems, Sparks, Maryland). Isolates with a zone of inhibition ≥ 5 mm larger for either drug in combination with clavulanic acid vs the zone diameter for the drug alone were defined as ESBL producers [23].

Whole-Genome Sequence Analysis

For each isolate, 1–5 μ g DNA in 200 μ L was sheared to a size range of 200–1000 bp with the SonicMAN sonicator (Matrical BioScience, Spokane, Washington) and then purified using the Qiagen QIAquick polymerase chain reaction (PCR) Purification kit (Qiagen, Valencia, California). Enzymatic processing of the DNA was performed according to Illumina's recommendations using enzymes obtained from New England Biolabs (New England Biolabs, Ipswich, Massachusetts) and Illumina (Illumina, Inc, San Diego, California) oligonucleotides and adaptors.

After ligation of the adaptors, the DNA was separated electrophoretically on a 2% agarose gel for 2 hours, after which a gel slice containing 500- to 600-bp fragments of each DNA sample was isolated and then purified using the Qiagen QIAquick Gel Extraction kit (Qiagen). Individual libraries were quantified by quantitative PCR using a Kapa Library Quantification kit (Kapa Biosystems, Woburn, Massachusetts). Equimolar pools of ≤ 12 indexed *E. coli* libraries were prepared at a concentration of ≥ 1 nM using 10mM Tris-HCl (pH 8.0) with 0.05% Tween 20. The pooled paired-end libraries were sequenced on an Illumina Genome Analyzer Ix to a read length of at least 76 bp.

Multilocus Sequence Typing

In silico multilocus sequence typing (MLST) was done according to the scheme of Diancourt et al, which uses allelic variation at 7 housekeeping gene loci to assign STs [20]. Alleles were assigned to each isolate using the WGS data in the computer program short read sequence typing for bacterial pathogens [24]. Novel alleles were subjected to Sanger sequencing at ID Genomics (<http://idgenomics.com>) and were submitted to the Institut Pasteur's *K. pneumoniae* MLST database (<http://bigsdw.web.pasteur.fr/klebsiella/klebsiella.html>).

Single-Nucleotide Polymorphism Identification

Illumina WGS data sets were aligned against the *K. pneumoniae* strain MGH78578 chromosome (GenBank accession number CP000647) using the short-read alignment component of the Burrows-Wheeler aligner. Each alignment was analyzed for single-nucleotide polymorphisms (SNPs) using the genome analysis tool kit software [25]. Prior to calling SNPs, we employed a stringent downstream pipeline that filtered out low-

quality reads. In brief, we employed the following steps: verified that sequencing reads were of the expected length (100 bp), that the percentage of $\geq Q30$ bases was $>80\%$, and that the mean Phred quality score of each sample was approximately 30. SNP loci were excluded if they did not meet a minimum coverage of $10\times$ and if the variant was present in $<90\%$ of the base calls for that position. To be included in the final SNP matrix, the locus had to be present in all of the genomes. SNPs from duplicated or repetitive regions on the reference genome were discarded.

Whole-Genome Phylogenetic Analyses

The inferred phylogeny was estimated with FastTree2 software, version 2.1.8 [26], using a general time-reversible model of DNA sequence evolution. *Escherichia coli* NA114 (GenBank accession number CP002797) [27] was used as an outgroup to root the *K. pneumoniae* WGS tree. *Klebsiella pneumoniae* isolates in the clade closest to this bifurcation point were used to root the final tree.

In Vivo Virulence Model

Five pairs of genetically similar meat-source and clinical isolates were selected to determine their virulence in a mouse model of subcutaneous sepsis that measures both illness severity and lethality [28]. Each isolate was tested in 10 mice, 5 of which received a low-dose inoculum (1.5×10^8 – 3.3×10^8 colony-forming units [CFU]/mL) and 5 a high-dose inoculum (8.0×10^8 – 1.4×10^9 CFU/mL). Mice were injected with log-phase organisms and then observed for health status twice daily for the following 3 days. Maximal illness severity was scored daily on a 5-point scale, where 1 = no illness and 5 = death. The infection experience of each mouse during the observation period was summarized as the mean of the daily illness severity scores.

Statistical Analyses

For categorical data, statistical significance was assessed with 2-tailed Fisher exact tests. Extent of resistance was assessed by comparing groups of isolates according to the number of antibiotics, and antibiotic classes to which each isolate was resistant, using the Kruskal-Wallis test. Virulence was assessed by comparing mean mouse illness severity scores between groups of isolates, again using the Kruskal-Wallis test. Central tendencies were expressed as group medians (range). Statistical analyses were implemented in R version 3.0.1 [29]. The threshold for statistical significance was $\alpha = .05$.

RESULTS

Study Isolates

During the collection period, *K. pneumoniae* accounted for 174 of the 1728 (10%) human clinical isolates obtained from the Flagstaff Medical Center. Concurrently, *K. pneumoniae* was recovered

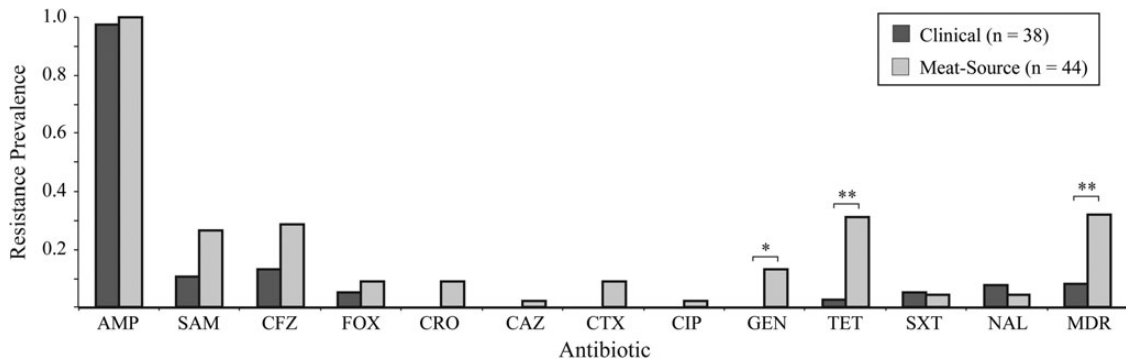


Figure 1. Antibiotic resistance prevalence of meat-source vs clinical *Klebsiella pneumoniae* isolates. The antibiotics tested were as follows: ampicillin (AMP), ampicillin-sulbactam (SAM), cefazolin (CFZ), ceftazidime (CAZ), cefotaxime (CTX), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), tetracycline (TET), and trimethoprim-sulfamethoxazole (SXT). Multidrug resistance (MDR) was defined as resistance to ≥ 3 classes of antibiotics. None of the isolates were resistant to amikacin or imipenem (not shown). * $P < .05$; ** $P < .01$.

from 241 of the 508 (47%) locally purchased retail meat products. Meat product contamination varied significantly in prevalence by meat type, along a gradient as follows: 58% (65/112) for pork, 47% (128/272) for chicken, and 38% (48/128) for turkey (for turkey vs pork, $P = .003$; for turkey vs chicken, $P = .02$).

A subset of 82 *K. pneumoniae* isolates was randomly chosen for WGS, including 38 clinical and 44 meat-source isolates, to give approximately equal numbers per meat type. Among the clinical isolates, 20 qualified as community-acquired, 4 as hospital-acquired, and 6 as healthcare-acquired, whereas 8 lacked data regarding acquisition source. The 44 meat-source isolates were recovered from chicken ($n = 16$), turkey ($n = 17$), and pork ($n = 11$) (Supplementary Table 1).

Antibiotic Susceptibility

As expected for *Klebsiella*, resistance to ampicillin was nearly ubiquitous (1 clinical isolate was susceptible). In contrast, no resistance was detected to amikacin or imipenem. For the remaining antibiotics, resistance prevalence varied by antimicrobial agent and by isolate source (Figure 1). Resistance prevalence was greater among meat-source isolates for all antibiotics except trimethoprim-sulfamethoxazole and nalidixic acid, and significantly so for tetracycline ($P = .001$) and gentamicin ($P = .029$). Among the clinical isolates, antimicrobial resistance prevalence did not vary by acquisition source (ie, community, healthcare, or hospital). Among the meat-source isolates, those from turkey displayed the greatest resistance prevalence to all antibiotics tested except for ceftazidime, for which resistance was most prevalent among pork isolates. Resistance prevalence differences by meat type were significant for ampicillin-sulbactam ($P = .007$), cefazolin ($P = .004$), and tetracycline ($P < 0.001$) (Supplementary Figure 1). ESBL production was confirmed phenotypically in 2 isolates, both from poultry (1 each from chicken and turkey).

Approximately 22% of isolates overall were multidrug resistant, including 32% of meat-source isolates but only 8% of clinical isolates ($P = .014$). Compared with human isolates, meat-source isolates were resistant to significantly more individual antibiotics (median, 1 [range, 1–9] vs 1 [range, 0–5]; $P = .038$) and more antibiotic classes (median, 1 [range, 1–5] vs 1 [range, 0–4]; $P = .043$).

Multilocus Sequence Typing

Overall, 60 STs were identified (Supplementary Table 1), 21 (35%) of which were novel. Four STs (ST14, ST76, ST111, and ST188) included both meat-source and clinical isolates (Supplementary Figure 2). The ST188 isolates had heterogeneous susceptibility profiles, whereas the clinical and meat-source ST14, ST76, and ST111 isolates were susceptible to all antibiotics except ampicillin.

Phylogenetic Analyses

Phylogenetic analysis based on core SNPs identified 2 distinct clades, each containing both meat-source and clinical isolates (Figure 2). The smaller clade was well resolved and corresponds to *K. pneumoniae* phylogroup III (a.k.a., *Klebsiella variicola*) [30,31]. The larger clade, corresponding to *K. pneumoniae* phylogroup I [30], appeared to have undergone a rapid radiation with subsequent, but varying, degrees of diversification, as evidenced by the short internal branches and variable-length terminal branches. Although some subclusters were source specific, meat-source and clinical isolates were intermingled in both main clades.

Core-genome phylogenetic analysis showed that there were 5 closely related isolate pairs in which each pair contained 1 clinical and 1 meat-source isolate. Based on MLST, 4 of the isolate pairs shared identical STs (ST14, ST76, ST111, and ST188), whereas 1 pair displayed discordant STs (ST17 and ST1123) (Figure 2). ST1123 and ST17 were single-locus variants that

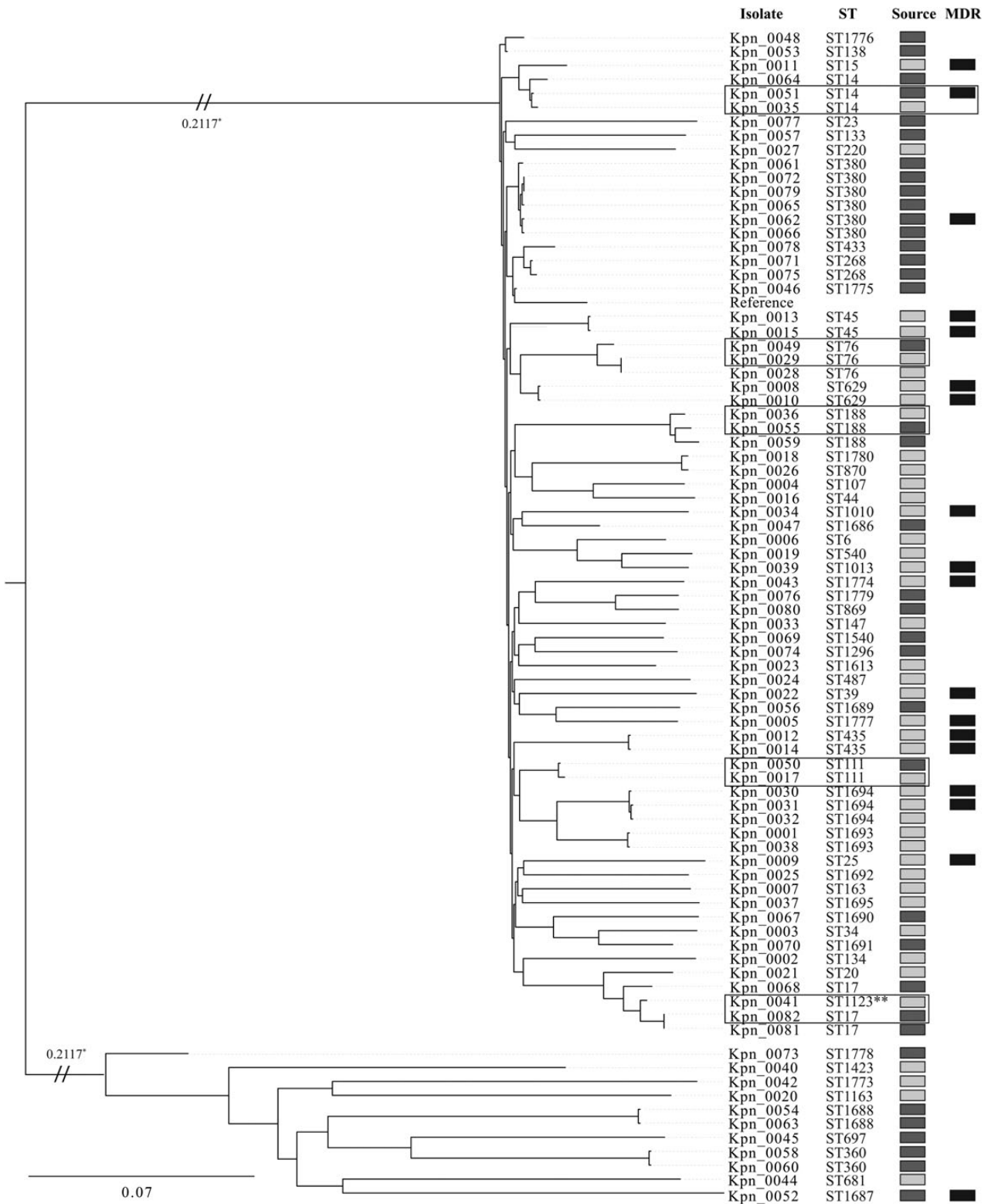


Figure 2. Rooted phylogeny of 83 *Klebsiella pneumoniae* isolates. The inferred phylogeny was based on 62 437 core single-nucleotide polymorphisms and rooted with *Escherichia coli* NA114. Isolates are coded by source (ie, clinical [dark gray] or meat source [light gray]), multilocus sequence type (ST), and multidrug resistant (MDR [black boxes]). The large upper clade corresponds to *K. pneumoniae* phylogroup I and the smaller well-resolved clade corresponds to *K. pneumoniae* phylogroup III (a.k.a., *Klebsiella variicola*) [30, 31]. The scale bar reflects genetic distance, reported as the expected number of nucleotide changes per site. The 5 most closely related meat–human isolate pairs, indicated with boxes, were tested in a murine sepsis model. *Branches leading to the 2 main clades were manually shortened; the length of each branch is indicated on each branch. **ST1123 and ST17 differ by a single nucleotide.

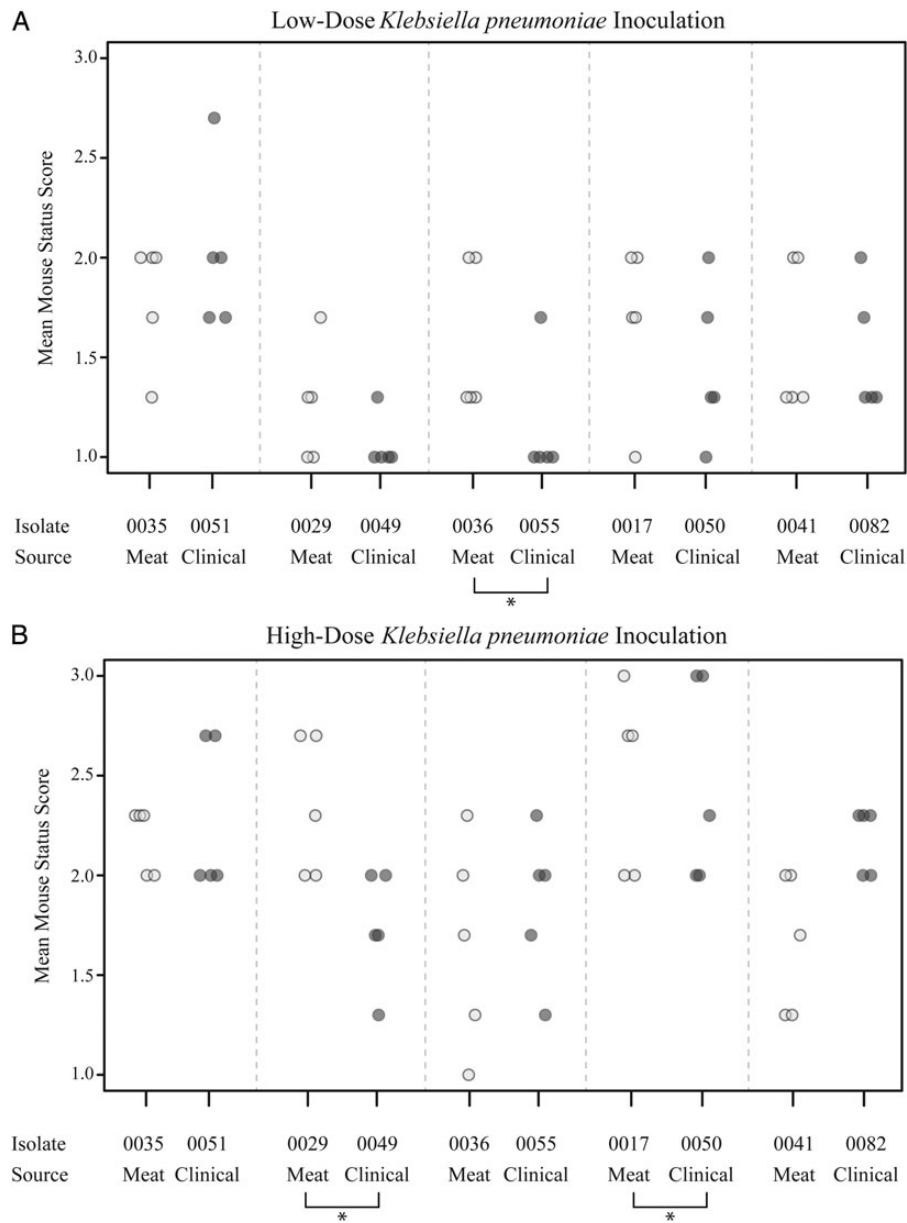


Figure 3. Virulence of 5 pairs of closely related meat-source and clinical isolates in a murine subcutaneous sepsis model. Isolates were tested at a low dose (A) and a high dose (B). Illness severity (mean mouse status) was scored on a 5-point scale where 1 = no illness and 5 = death. Each circle represents the mean status score for a given mouse (n = 5) over a 3-day observation period. None of the isolates were lethal. * $P < .05$.

differed by only 1 SNP in *gapA*; both isolates were susceptible to all antibiotics except ampicillin. Thus, of the 38 human clinical study isolates, 5 (13%) were closely related to meat-source isolates based on core-genome SNP analysis.

In Vivo Virulence Model

These 5 *K. pneumoniae* isolate pairs, each containing a clinical isolate and a genomically matched meat-source isolate (Figure 2), were assessed for virulence using a standard murine

subcutaneous sepsis model. In this model, *E. coli* pyelonephritis strain CFT073 routinely kills 80%–100% of challenged mice, whereas laboratory strain MG1655 (K-12) causes no discernible mouse illness. For the 10 *K. pneumoniae* isolates, with 100 total mice tested (10 per isolate; 5 each at a high- vs a low-inoculum dose), the overall median per-isolate mouse illness severity score was 2 (range, 1–3), with no lethality. Disease severity increased in a dose-dependent manner for 7 of 10 isolates (3 meat-source, 4 clinical) (Supplementary Table 2).

At the lower challenge dose, 1 of the closely related isolate pairs showed a significant within-pair difference in virulence that favored the meat-source isolate ($P = .04$). At the higher challenge dose, 2 of 5 isolate pairs exhibited a significant within-pair virulence difference, in 1 instance favoring the meat-source isolate ($P = .02$), in the other the clinical isolate ($P = .02$). Thus, in a murine sepsis model that was able to detect significant virulence differences between individual *K. pneumoniae* isolates and different dose levels, meat-source and human clinical isolates displayed relatively similar levels of virulence, without overall source group-specific differences (Figure 3).

DISCUSSION

In this study, we assessed 82 *K. pneumoniae* isolates from retail meat products and human clinical specimens for phenotypic and genotypic relatedness. Whereas meat isolates were more extensively antibiotic resistant, the 2 populations were genetically intermingled and, for closely related isolates, exhibited similar virulence in a murine sepsis model.

Antibiotic resistance has been increasing within *K. pneumoniae* [18], with the rapid dissemination of *K. pneumoniae* carbapenemases being of particular concern [16, 32]. The human clinical isolates studied here exhibited varied resistance profiles (Figure 1), but were generally less extensively resistant than those reported in other studies of US inpatient and outpatient populations [18, 33, 34]. Encouragingly, none of the clinical isolates in the current study were resistant to third-generation cephalosporins or imipenem. These findings may reflect regional differences in antibiotic use and dissemination of antibiotic-resistant *K. pneumoniae* strains.

Antibiotic resistance, including multidrug resistance, was more prevalent and extensive among meat-source *K. pneumoniae* isolates than human clinical isolates. Third-generation cephalosporin resistance—including ESBL production (2 isolates)—was confined to the meat-source isolates, and resistance to gentamicin and tetracycline was significantly more prevalent among meat-source isolates. All of the present meat-source isolates were collected in 2012, during which year >5.9 million kg of tetracyclines and 273 536 kg of aminoglycosides were sold or distributed for use in food-animal production in the United States [35]. Thus, the distinct resistance profiles of the 2 *K. pneumoniae* populations may reflect antibiotic selective pressures at their source—that is, food-animal production vs hospital and general community use. Unfortunately, the US Food and Drug Administration does not collect detailed data on antibiotic use in food-animal production, making it difficult to draw direct connections in this or other studies between antibiotic use and resistance [35].

In contrast to these phenotypic differences, genetic analyses demonstrated overlap between the meat-source and clinical *K. pneumoniae* populations. Specifically, MLST identified 4 STs and a

single-locus variant pair (ST1123 and ST17) that contained both meat-source and clinical isolates. High-resolution phylogenetic analysis confirmed that meat-source and clinical isolates do not represent distinct populations, but are intermingled across the phylogeny. Furthermore, closely related meat-source and clinical isolates displayed similar, albeit relatively low-level, virulence in a mouse subcutaneous sepsis model. Low-level virulence in mouse models has also been reported for the epidemic ST258 strain, suggesting that the increased morbidity and mortality associated with this clone in humans stems more from the clone's expanded antibiotic resistance repertoire than from any special virulence [36]. Taken together, the close phylogenetic relationships and similar in vivo virulence of meat- and clinical-source *K. pneumoniae* isolates suggests there may be a low barrier to transmission between these 2 environments.

This study identified a diverse population of antibiotic-resistant *K. pneumoniae* from retail meat sources. Although these isolates did not include some of the most notorious epidemic *K. pneumoniae* clones (eg, ST258), we did identify a turkey-source epidemic clone, ST15, that is common in both humans and companion animals [12]. It is critical to recognize that although epidemic lineages can be associated with severe disease and poor clinical outcomes, they account for only a minor portion of all *K. pneumoniae* infections, most of which are due to sporadic clones. Understanding the epidemiology of these sporadic clones, and their reservoirs and transmission pathways, will help elucidate the key drivers of resistance and may identify opportunities for intervention, both of which are critical to reducing the overall *K. pneumoniae* disease burden. Here we show that retail meat may be an important source of antibiotic-resistant *K. pneumoniae* of possible human health significance, which increases the range of potential public health risks associated with antibiotic use in food-animal production.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (<http://cid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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