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Original Research

The Epidemiology of Invasive, Multiple-antibiotic–resistant *Klebsiella pneumoniae* Infection in a Breeding Colony of Immunocompromised NSG Mice

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Klebsiella pneumoniae **(*Kp*) is a gram-negative opportunistic pathogen that causes severe pneumonia, pyelonephritis, and sepsis in immunocompromised hosts. During a 4-mo interval, several NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}*/SzJ (NSG) breeders and pups in our facilities were diagnosed with *Kp* infections. An initial 6 adult and 1 juvenile NSG mice were submitted for necropsy and histologic examination because of acute onset of diarrhea and death. The evaluation revealed typhlocolitis in 2 of the mice and tritrichomoniasis in all 7. *Escherichia coli* positive for polyketide synthase (*pks*+) and *Kp* were isolated from the intestines. Given a history of sepsis due to *pks*$^{+}$ *E. coli* in NSG mice in our facilities and determination of its antimicrobial susceptibility, trimethoprim–sulfamethoxazole (TMP–SMX) was administered to the colony in the drinking water for 4 wk. After this intervention, an additional 21 mice became ill or died; 11 of these mice had suppurative pneumonia, meningoencephalitis, hepatitis, metritis, pyelonephritis, or sepsis. *Kp* was cultured from pulmonary abscesses or blood of 10 of the mice. Whole-genome sequencing (WGS) indicated that the *Kp* isolates contained genes associated with phenotypes found in pore-forming *Kp* isolates cultured from humans with ulcerative colitis and primary sclerosing cholangitis. None of the *Kp* isolates exhibited a hyperviscous phenotype, but 13 of 14 were resistant to TMP–SMX. Antimicrobial susceptibility testing indicated sensitivity of the *Kp* to enrofloxacin, which was administered in the drinking water. Antibiotic sensitivity profiles were confirmed by WGS of the *Kp* strains; key virulence and resistance genes to quaternary ammonia compounds were also identified. Enrofloxacin treatment resulted in a marked reduction in mortality, and the study using the NSG mice was completed successfully. Our findings implicate intestinal translocation of *Kp* as the cause of pneumonia and systemic infections in NSG mice and highlight the importance of identification of enteric microbial pathogens and targeted antibiotic selection when treating bacterial infections in immunocompromised mice.**

Abbreviations: *Kp, Klebsiella pneumoniae*; MKPV, mouse kidney parvovirus; NSG, NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}*/SzJ; pks, polyketide synthase; TMP–SMX, trimethoprim–sulfamethoxazole; WGS, whole-genome sequencing

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Severely immunocompromised NSG (NOD.*Cg-Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ) mice typically are used for oncology, stem cell biology, diabetes, and infectious disease research. The combination of mutations in these mice includes a unique MHC haplotype, a deletion in the C5 gene, a defect in the double-stranded repair mechanisms necessary for the gene rearrangement of antigen-specific receptors, and a knockout of the IL2 receptor γ chain.[21,37] These deficits result in mice with no mature B or T cells, dysregulation of antigen presenting-cells, no NK cells, no hemolytic complement, and impaired cytokine signaling.[41] These mice are routinely used for xenotransplantation, including implantation of human cell lines. Appropriate husbandry practices are critical because these mice are highly susceptible to infection with opportunistic agents such as *Escherichia coli* strains positive for polyketide synthase (*pks*$^{+}$), *Klebsiella oxytoca*, *Enterococcus* spp., and *Candida albicans,* which have been associated with meningitis or meningoencephalitis, myocarditis, pneumonia, pyelonephritis, cystitis, metritis, and septicemia in NSG and other immunocompromised mouse strains.[4,6,17,43]

Klebsiella pneumoniae (*Kp*) is a gram-negative encapsulated bacterium typically found in the environment and gastrointestinal tract of mammals.[22] In both human and veterinary medicine, *Kp* is considered an emergent and common opportunistic pathogen associated with an array of diseases.[33] This organism can acquire resistance to multiple antibiotics and is an important cause of nosocomial infections in humans that include urinary tract infections, pneumonia, septicemia, and soft-tissue

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infections and particularly occur in hospitalized immunocompromised patients.[30,33,34] Classic and hypervirulent strains of *Kp* have been implicated in cases of pneumonia, urogenital infections, and sepsis in laboratory, companion, and domestic animals.[15,35] In laboratory rodents, *Kp* commonly colonizes the gastrointestinal tract of healthy animals and rarely causes disease in immunocompetent hosts; however, *Klebsiella* spp. infections in immunocompromised mice have been associated with spontaneous disease, including sepsis, lymphadenitis, pneumonia, empyema, hepatic and renal abscesses, endocarditis and myocarditis, and thrombosis.[6,35]

This report documents an epidemiologic study of an outbreak of systemic *Kp* infection in a restricted-access colony of NSG mice that occurred despite the administration of trimethoprim sulfamethoxazole (TMP–SMX) in the drinking water. We hypothesized that a virulent phenotype was responsible for the invasive TMP–SMX-resistant *Kp* identified in this outbreak and that the systemic *Kp* originated from translocation of the organism from the intestine due to intestinal barrier compromise.[28,30,33,40,42]

Materials and Methods

Case presentation. An increase in diarrhea and mortality was noted primarily among NSG dams and their litters in 2 rooms (later combined into one). Some neonatal mice had undergone intracranial injection with human glial cells in a biologic hood located in a procedure room that was also used for procedures in immunocompetent mice. Over 3 wk, 7 mice (6 adults, 1 juvenile) were presented for clinical evaluation, with 4 animals having evidence of diarrhea and 2 of these showing acute neutrophilic enterotyphlocolitis (Figure 1A).

E. coli was isolated from the blood of the 2 mice with enterotyphlocolitis and the cecum of 4, and *Kp* was isolated from one blood sample and 4 cecal samples. The *E. coli* isolates were further characterized for the presence of genotoxic virulence factors. Three isolates contained the *pks* gene cluster encoding the genotoxin colibactin, previously shown to contribute to systemic disease in immunocompromised mice (*Il2rg*$^{-/-}$*Rag2*$^{-/-}$, *c-Kit*$^{W\text{-}sh/W\text{-}sh}$).[4] In addition, *E. coli* was identified from culture of rectal swabs from 5 mice in the room. In addition, a single sentinel CD1 mouse from the room had shown a positive nasal wash for *Kp* in the preceding year but had no gross lesions. Cecal colonization with *Tritrichomonas* spp. was noted commonly on fecal exams of immunocompetent CD1 sentinel mice and was also detected in all 7 NSG mice submitted for histopathological evaluation (Figure 1B). This protozoal infestation was considered to be an incidental finding, given that the large intestines of NSG mice were intermittently colonized with this protozoan without evidence of inflammation or clinical disease. Given these findings, *pks*$^+$ *E. coli* was presumed to be the causative agent of morbidity and mortality in these mice. Antimicrobial sensitivity testing revealed that all isolated *E. coli* strains were sensitive to enrofloxacin and trimethoprim sulfonamide (TMP-SMX); therefore, TMP–SMX was provided in the drinking water at 1.5 mg/mL (approximately 300 mg/kg) for 4 wk. During and after treatment, 21 additional mice were submitted and evaluated, as described below.

Mice. All original mice had been purchased from Jackson Laboratories (Bar Harbor, ME). The breeding colony had been maintained at an AAALAC-accredited facility for approximately 3 years, in polycarbonate microisolation caging with autoclaved hardwood bedding (Sani-Chips, PJ Murphy, Jewett, PA) and pelleted food (Prolab RMH 3000, Purina Mills, St. Louis, MO) and deionized water. Mice were housed in clean autoclaved cages with food and bedding twice weekly. The mice were transferred by grasping the base of the tail with rubber-tipped forceps that had been submerged in quaternary ammonia disinfectant solution (Quatricide PV, Pharmacal Research Laboratories, Waterbury, CT) prepared as a 1:64 dilution. The solution was refreshed monthly based on manufacturer's recommendations. The forceps were disinfected before use and between cages during cage changing. At the time of outbreak, cages containing bedding, feed pellets, and water bottle were autoclaved before housing mice; cages were changed on a bench top and maintained as static cages.

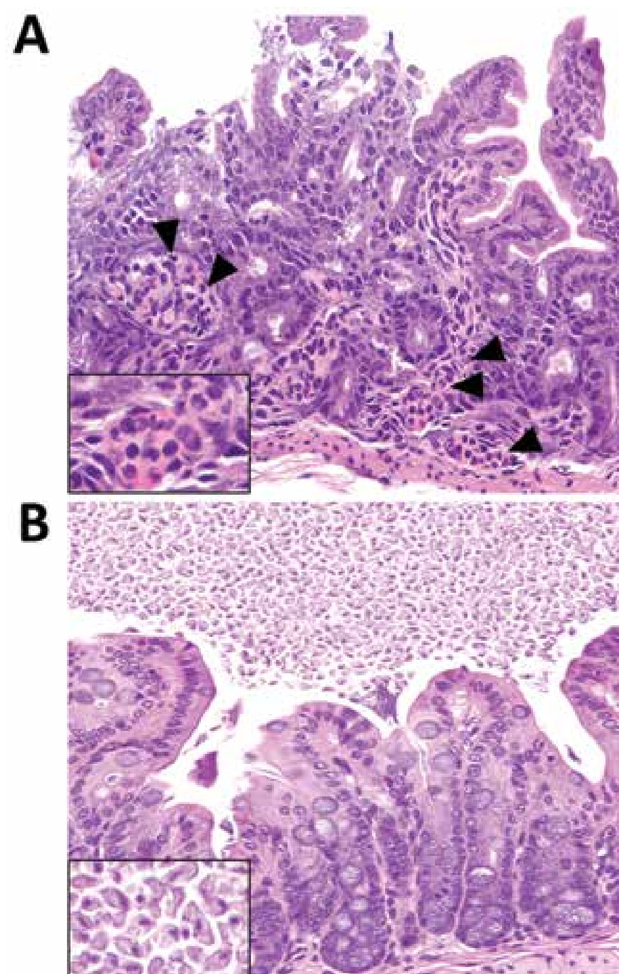

Figure 1. Representative histology of the gastrointestinal tract of NSG mice prior to antibiotic therapy. (A) Mild multifocal acute enteritis in an NSG mouse infected with *pks*$^+$ *E. coli*. The lamina propria of the small intestine is multifocally infiltrated by low numbers of neutrophils (arrowheads, inset). (B) Tritrichomoniasis in the large intestine of an NSG mouse. The cecum is colonized by numerous *Tritrichomonas* organisms (inset). Magnification, 40×.

All affected mice were on IACUC-approved protocols. Mice were either found dead or euthanized by using CO_2 at a 10% to 30% chamber replacement rate followed by intracardiac exsanguination. Health monitoring was performed via dirty-bedding sentinels (CD1 Elite, Charles River Laboratories, Wilmington, MA) that had been bred and maintained in a separate barrier facility. All sentinel mice during the previous and subsequent years had been negative on serology for mouse hepatitis virus, enzootic diarrhea of infant mice virus, minute virus of mice, Sendai virus, norovirus, pneumonia virus of mice, reovirus 3, Theiler encephalomyelitis virus, lymphocytic choriomeningitis virus, K virus, *Mycoplasma pulmonis*, and CAR bacillus and on

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ELISA for mouse parvovirus, *Ectromelia*, polyomavirus, and mouse adenoviruses. Rectal swabs were all negative for *Salmonella* and *Citrobacter rodentium*. Anal tape tests, skin scrapings, and sodium nitrate solution fecal flotation analyses were all negative for parasites. Direct duodenal smears were negative for protozoa, but cecal smears were intermittently positive for *Tritrichomonas* and *Entomoeba* spp., which are not excluded pathogens in mice maintained in this facility.

Postmortem examination. Full necropsies were performed at the Comparative Pathology Laboratory (Division of Comparative Medicine, Massachusetts Institute of Technology). Representative tissues from all organ systems were fixed in 10% neutral-buffered formalin, and paraffin-embedded sections (thickness, 5 μm) were stained with hematoxylin and eosin. To verify the presence of bacteria in tissue sections, Brown–Brenn modified Gram stains were performed in a subset of cases. To demonstrate the presence of neutrophils in lesions, formalin-fixed sections from a subset of cases were incubated for 1 h with rat antimouse Ly6G (dilution, 1:1000; clone 1A8, BioXCell, West Lebanon, NH). The immunohistochemical procedure was performed by using the antirat IgG horseradish peroxidase detection kit (BD Biosciences, San Jose, CA) and developed (ARK K3954, Dako, Agilent, Santa Clara, CA) according to manufacturer instructions.

Microbiology. In light of gross necropsy findings, culture samples were obtained from blood ($n = 10$), cecum ($n = 17$), lung ($n = 6$), liver ($n = 1$), and small intestine ($n = 1$). The samples were placed into *Brucella* broth containing 10% glycerol and homogenized. The homogenates were streaked onto chocolate agar and blood agar–MacConkey agar split plates (Becton Dickinson, Franklin Lakes, NJ), inoculated into trypticase soy broth (Becton Dickinson) for enrichment, and incubated for 18 to 24 h at 37 °C in 5% CO_2. Pure cultures were obtained by restreaking single morphologically different colonies onto another blood agar plate and speciated by using API 20E identification strips (bioMerieux, Marcy l'Etoile, France). To assess the elimination of *Kp* after antibiotic treatment, fecal samples saved in bacterial freeze media were plated on MacConkey, xylose–lysine–deoxycholate, and Hektoen enteric selective and differential agar or medium and on blood agar, a general-purpose enrichment medium. The bacterial plates were incubated for 18 to 24 h at 37 °C in 5% CO_2.

***K. pneumoniae* fluorescent in situ hybridization.** Fluorescent in situ hybridization was performed on formalin-fixed, paraffin-embedded tissues by using the probe 5′-CCT ACA CAC CAG CGT GCC-3′ (Integrated DNA Technologies, Coralville, IA), as previously reported.[4] Slides were deparaffinized in xylene and rehydrated in a descending ethanol series. After air drying, the slides were covered in hybridization buffer containing 10 ng/μL of probe at 74.5 °C and maintained overnight at 48 °C in a dark, humidified chamber. Slides were rinsed with water and incubated in 2 wash buffers for 15 min each at 48 °C. The slides were then rinsed, air dried, mounted in antifade reagent with DAPI (ProLong Gold, Thermo Fisher, Rockford, IL), and examined under fluorescence (Axioskop 2 plus, Zeiss, Oberkochen, Germany). Slides coated with *Kp* and *Enterococcus faecalis* colonies were used as positive and negative controls, respectively. Two negative controls were used for this experiment, one without a probe and one with a nontarget *E. coli*-specific probe.

Antimicrobial sensitivity. Antimicrobial susceptibility testing of strains of *Kp* and *E. coli* was performed by using the disc diffusion method recommended by the Clinical and Laboratory Standards Institute.[10,11] Antimicrobials tested included ampicillin, ampicillin–clavulanic acid, ampicillin–sulbactam, cephalothin, enrofloxacin, gentamicin, and TMP–SMX. *E. coli* ATCC 29322 was included as the reference control. Minimum inhibitory concentrations for enrofloxacin and a quaternary ammonium compound (Quatricide PV) were determined by using the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute and others.[10,11] Minimum bacteriocidal concentration values for microbiocides were obtained by using a modification of a previously published protocol.[23]

String test. The hyperviscosity phenotype of *Kp* isolates was initially evaluated by using the string test.[11] Briefly, a sterile plastic inoculation loop was placed on top of and slowly removed from a colony grown on an agar plate. The string test was positive when a viscous string measuring 5 mm or longer was produced by the colony clinging to the loop.[30,33,40]

Whole-genome and molecular characterization. Representative mouse *Kp* isolates ($n = 15$), comprising 13 isolates previously described in brief[27] and 2 additional isolates, were submitted for whole-genome sequencing (WGS). Genomic DNA was purified from *Kp* isolates that had been grown overnight in LB broth. Barcoded libraries were created by using QIAseq FX DNA library kit (Qiagen, Germantown, MD), sequenced with MiSeq (Illumina, San Diego, CA), and trimmed by using BBDuk (Geneious, San Diego, CA); contigs were assembled and annotated by using SPAdes and RAST hosted by PATRIC.[27] For comparative genomic analysis, genomes of representative *Klebsiella* spp. were acquired from PATRIC.[12] In addition, the Similar Genome Finder Tool hosted by PATRIC was used to identify the 10 genomes most closely related to the mouse *Kp* genomes. Whole-genome phylogenetic analysis was performed by using OrthoFinder (version 2.3.1).[13] Average nucleotide identity values were calculated by using PYANI (version 0.2.10).[31] All *Klebsiella* spp. genomes were analyzed by using Kleborate to predict multilocus sequencing type, K and O antigen types, virulence genes, and antibiotic-resistance gene profiles.[25] In addition, mouse *Kp* genomes were analyzed against the Virulence Factor Database by using BLAST to identify other potential virulence factor genes as well as homologs to 97 gene sequences associated with the pore-forming *Kp* strains isolated from human patients with ulcerative colitis and primary sclerosing cholangitis.[26,28]

Plasmid DNA was extracted by using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacture's protocol. Antibiotic-resistance gene cassettes were detected from purified plasmid DNA through PCR analysis of *intl1* (forward primer, 5′-GAC TTG CGC TGC CCT ACC TCT CAC-3′; reverse primer, 5′-TTG GCG GCC TTG CTG TTC TAC-3′) and *sul1* (forward primer, 5′-AGG CGG ACT GCA GGC TGG TTA-3′; reverse primer: 5′-GGC CGG AAG GTG AAT GCT A-3′) by using the following thermocycling program: initial denaturation at 95 °C for 3 min, followed by 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 63.7 °C for 1 min, and elongation at 72 °C for 2 min, with a final incubation at 72 °C for 10 min.

***E. coli* PCR amplification.** *E. coli* samples were boiled and centrifuged at 12,000 × *g*. The resulting supernatant was used for PCR analysis to identify genotoxins. Colibactin is a genotoxic peptide encoded by *clb* genes that lie within the polyketide synthase (*pks*) pathogenicity island. These genes were identified by using 2 sets of primers: *clbA* (forward, 5′-CAG ATA CAC AGA TAC CAT TCA-3′; reverse, 5′-CTA GAT TAT CCG TGG CGA TTC-3′) and *clbQ* (forward, 5′-TTA TCC TGT TAG CTT TCG TTC-3′; reverse, 5′-CTT GTA TAG TTA CAC AAC TAT TTC-3′).[16]

***Tritrichomonas muris* PCR analysis.** The histologic diagnosis of *T. muris* was confirmed and speciated through PCR analysis of frozen samples of cecal tissue or contents. DNA was extracted

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by using QIAamp Fast DNA Stool Mini Kit (Qiagen); PCR amplification was performed in a 25-µL reaction volume by using the Expand High Fidelity PCR System according to manufacturer instructions (Sigma-Aldrich, St Louis, MO). Amplification of tritrichomonas DNA was performed by using the forward primer 5′-AGC GGA AAA GAA ACT AAC T-3′ and reverse primer 5′-CAT CGG TCT CAC AGC AT-3′, which targeted the 28S ribosomal RNA (expected amplicon, 260 bp). The cycling parameters consisted of an initial denaturing step of 3 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s, elongation at 72 °C for 30 s, and a final incubation at 72 °C for 4 min.

PCR amplification and quantitative PCR analyses for mouse kidney parvovirus (MKPV). Total DNA from frozen kidneys and formalin-fixed, paraffin-embedded kidneys of NSG mice was prepared as previously described.[19] PCR amplification was performed in a 25-µL reaction volume by using the Expand High Fidelity PCR System (Sigma-Aldrich) according to the manufacturer's instructions. MKPV-specific primers were designed to target NS1 region of the MKPV genome as described previously.[32] The double-stranded nucleotide sequences of selected PCR products were determined by using Sanger sequencing (Quintara Biosciences, Cambridge, MA).

Statistics. Fisher exact tests were used to evaluate the associations between *Kp* infection, age, sex, tritrichomonas status, systemic lesions, and MKPV infection. A P value of 0.05 or less was considered significant (JMP, version 14, SAS Institute, Cary, NC).

Data availability. Genomes of *Kp* have been deposited in GenBank under the following accession numbers: SULO00000000, SULP00000000, SULQ00000000, SULR00000000, SULS00000000, SULT00000000, SULU00000000, SULV00000000, SULW00000000, SULX00000000, SULY00000000, SULZ00000000, SUMA00000000, JAEAGK000000000, and JAEAGL000000000.

Table 1. Culture results of NSG mice after TMP–SMX treatment

Site cultured (number of samples)	Culture results[a]
Lungs (n = 6)	
With pneumonia (n = 5)	*K. pneumoniae* (n = 5) *E. coli* (n = 1)
Without pneumonia (n = 1)	*K. pneumoniae* (n = 1)
Blood (n = 10)	
With other systemic lesions[b] (2)	*K. pneumoniae* (n = 1) No growth (n = 1)
No other systemic lesions (7)	*K. pneumoniae* (n = 5) *E. coli* (n = 1) No growth (n = 1)
Cecum (n = 18)	*K. pneumoniae* (n = 16) *Enterococcus faecalis* (n = 10) *E. coli* (n = 4) *Enterococcus cloacae* (n = 3) *Aeromonas hydrophilia* (n = 1) *Enterococcus amnigenus* (n = 1) *Enterococcus faecium* (n = 1) *Streptococcus uberis* (n = 1) *Streptococcus xylosus* (n = 1)

[a]In cases that grew Kp (n = 16), 12 yielded pure cultures of Kp from lungs, blood, or other organs

[b]Other systemic lesions include meningitis, epicarditis, metritis, splenitis, hepatitis, peritonitis, pyelonephritis, and cystitis

Results

During and after TMP–SMX treatment, 21 NSG mice (13 females, 8 males; 14 adults, 7 pups) were submitted for diagnostic evaluation. *Kp* was isolated from cecal contents, blood, or extraintestinal lesions in 17 of the 21 mice examined. *Kp* was isolated from the cecum of adult mice (P = 0.0254) and from extraintestinal tissues, including lungs, blood, and liver, in 57% (12 of 21) of the mice (Table 1). *Kp* was exclusively isolated from the cecum in 4 additional mice. Cecal cultures from 12 mice recovered *Kp* and other bacterial organisms, including *Enterococcus faecalis* (n = 10), *E. coli* (n = 4), *Enterococcus cloacae* (n = 3), and one each of *Enterococcus amnigenus*, *Enterococcus faecium*, *Streptococcus uberis*, *S. xylosus*, and *Aeromonas hydrophilia* (Table 1). Two of the *E. coli* isolates from the ceca of 2 different mice were *pks*$^{+}$.

Histologic findings. Histologically, the lungs of 5 NSG mice contained multifocal to coalescing abscesses that completely effaced the architecture of the bronchi, bronchioles, and alveolar septa (Figure 2A through C). These abscesses contained large numbers of degenerate neutrophils intermixed with karyorrhectic debris, necrotic cells, mineralization, fibrin, edema, and coccobacilli forming microcolonies. At the periphery, these abscesses were surrounded by fibrous connective tissue, large to moderate numbers of foamy macrophages, and fewer neutrophils. *Kp* was recovered from bacterial cultures of the pulmonary abscesses and cecum (Table 1). Fluorescent in situ hybridization specific for *Kp* highlighted the presence of rod-shaped bacteria associated with leukocytes along the edges of pulmonary abscesses (not shown). The meninges, lateral and fourth ventricles, and Virchow–Robin spaces of 2 additional mice were filled with large numbers of degenerate neutrophils admixed with microcolonies of rod-shaped bacteria (Figure 2D through F), fewer macrophages, congestion, and polymerized fibrin. The adjacent cerebral cortex was infiltrated by multifocal clusters of neutrophils intermixed with areas of malacia, gliosis, and edema (not shown). Two other mice had areas of multifocal random neutrophilic hepatitis, one of which also had concurrent suppurative neutrophilic pyelonephritis with papillary necrosis. The uterine walls of 2 breeders were multifocally infiltrated by low to moderate numbers of degenerate neutrophils admixed with fibrinous exudate and few hemosiderin-laden macrophages. These breeders exhibited suppurative inflammation in the peritoneum and/or mediastinum. One mouse had suppurative pyelonephritis and chronic tubulointerstitial nephropathy.

Whole-genomic and molecular characterization. WGS was performed on 15 representative *Kp* isolates to further study their pathogenic potential and antibiotic resistance profiles. Whole-genome phylogenetic and average nucleotide analyses determined that the mouse *Kp* isolates were most similar to each other (range, 99.96% to 100.00%). Using the Similar Genome Finder Tool hosted by PATRIC, the 10 genomes most closely related to the mouse *Kp* isolates were human strains cultured from human blood, rectal swabs, sputum, throat, or urine. Overall, the mouse *Kp* isolates were 98.93% to 99.30% similar to these genomes and to representative *Kp* strains from humans and rats (Table S1). In general, average nucleotide identity values for mouse *Kp* strains were more similar to nonmouse strains not encoding *rpmA* (99.21% ± 0.00%) than to genomes harboring this hypervirulence gene (99.13% ± 0.00%). Unlike the representative *Kp* genomes from humans and rats, all 15 mouse *Kp* genomes were ST1166 and had the predicted K capsular and O antigens of K45:O2.v2 (Figure 3).

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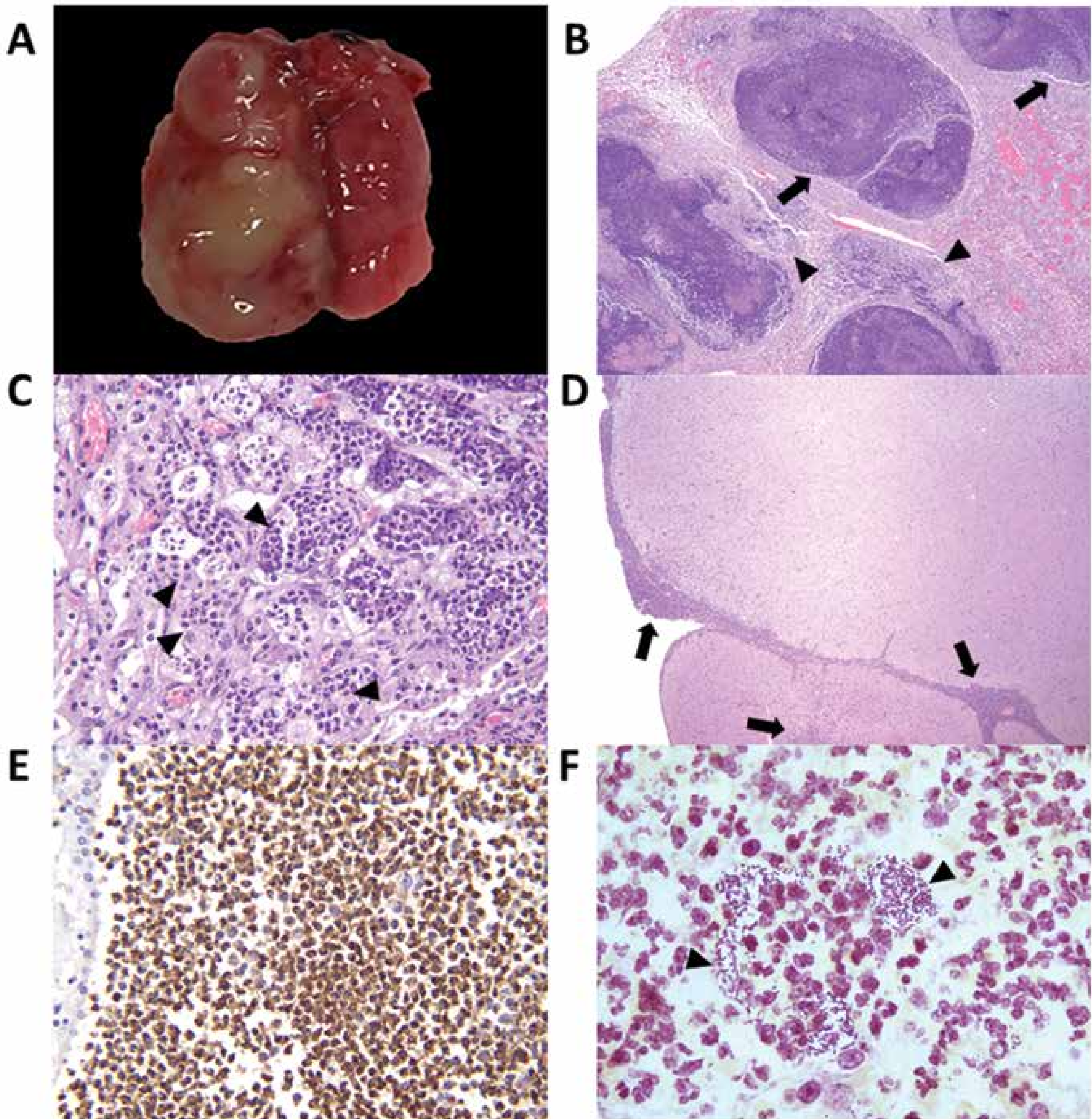

Figure 2. Suppurative pneumonia and meningoencephalitis in NSG mice infected with *K. pneumoniae*. (A) Representative gross image of an affected lung from an NSG mouse shows multifocal to coalescing abscesses in the left pulmonary lobe. (B) Bronchioles (arrows) and alveoli (arrowheads) are effaced by multiple abscesses and areas of necrosis. Magnification, 4×. (C) Alveoli are infiltrated by multiple dense clusters of degenerate neutrophils (arrowheads) admixed with few foamy macrophages, karyorrhectic debris, fibrin, and congestion. Magnification, 40×. (D) An affected brain from an NSG mouse. The meninges, cortex, and Virchow–Robins spaces are infiltrated by multifocal to coalescing regions of suppurative inflammation (arrows). Hematoxylin and eosin stain; magnification, 4×. (E) Immunohistochemistry for Ly6G in the cerebrum demonstrates the presence of neutrophils in areas of meningitis (brown staining of neutrophils). Magnification, 40× (F) Gram staining of the meninges shows dense clusters of extracellular, gram-negative, rod-shaped bacteria (arrowheads) associated with neutrophilic inflammation. Magnification, 100×.

None of the *Kp* mouse genomes harbored *rmpA*, *rmpA2*, or *magA* genes responsible for the hyperviscomucoid phenotype. In agreement with this finding, none of the 23 mouse *Kp* isolates evaluated had the hyperviscomucoid phenotype, as indicated by a negative string test. All 15 mouse *Kp* genomes contained multiple genes related to capsular production (*wzi*, *wza*, *wzb*, *wzc*, *gnd*, *wcaA*, *wcaJ*, and *galF*) but lacked other capsular genes traditionally found in this gene cluster (*cpsB*, *cpsG*). In addition, these 15 genomes contained the virulence factors for lipopolysaccharide synthesis, including the genes *uge* and *wabG*. Gene clusters responsible for type 1 fimbriae (*fimA–fimI*, *fimK*), type 3 fimbriae (*mrkA–mrkD*, *mrkF–mrkI*), and enterobactin siderophore biosynthesis were present in all 15 genomes, but none of these genomes encoded genes for

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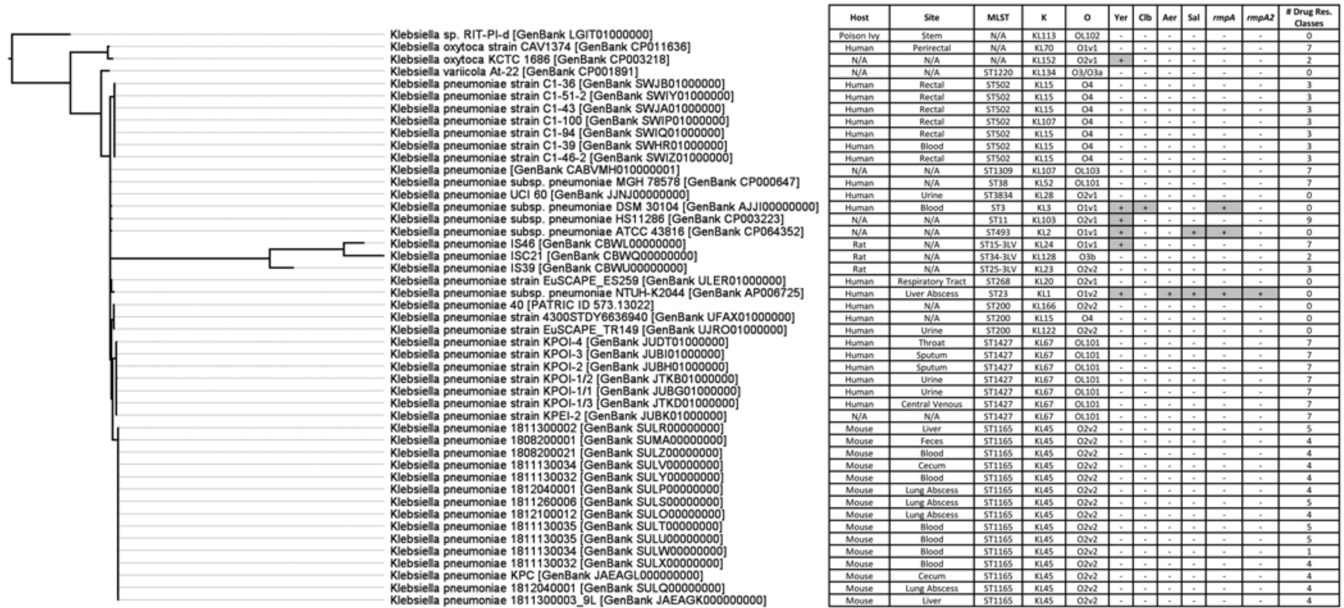

Host	Site	MLST	K	O	Yer	Clb	Aer	Sal	*rmpA*	*rmpA2*	# Drug Res. Classes
Poison Ivy	Stem	N/A	KL113	OL102	-	-	-	-	-	-	0
Human	Perirectal	N/A	KL70	O1v1	-	-	-	-	-	-	7
N/A	N/A	N/A	KL152	O2v1	+	-	-	-	-	-	2
N/A	N/A	ST1220	KL134	O3/O3a	-	-	-	-	-	-	0
Human	Rectal	ST502	KL15	O4	-	-	-	-	-	-	3
Human	Rectal	ST502	KL15	O4	-	-	-	-	-	-	3
Human	Rectal	ST502	KL15	O4	-	-	-	-	-	-	3
Human	Rectal	ST502	KL107	O4	-	-	-	-	-	-	3
Human	Rectal	ST502	KL15	O4	-	-	-	-	-	-	3
Human	Blood	ST502	KL15	O4	-	-	-	-	-	-	3
Human	Rectal	ST502	KL15	O4	-	-	-	-	-	-	3
N/A	N/A	ST1309	KL107	OL103	-	-	-	-	-	-	7
Human	N/A	ST38	KL52	OL101	-	-	-	-	-	-	7
Human	Urine	ST3834	KL28	O2v1	-	-	-	-	-	-	0
Human	Blood	ST3	KL3	O1v1	+	+	-	-	+	-	0
N/A	N/A	ST11	KL103	O2v1	+	-	-	-	-	-	9
N/A	N/A	ST493	KL2	O1v1	+	-	-	+	+	-	0
Rat	N/A	ST15-3LV	KL24	O1v1	+	-	-	-	-	-	7
Rat	N/A	ST34-3LV	KL128	O3b	-	-	-	-	-	-	2
Rat	N/A	ST25-3LV	KL23	O2v2	-	-	-	-	-	-	3
Human	Respiratory Tract	ST268	KL20	O2v1	-	-	-	-	-	-	0
Human	Liver Abscess	ST23	KL1	O1v2	+	-	+	+	+	+	0
Human	N/A	ST200	KL166	O2v2	-	-	-	-	-	-	0
Human	N/A	ST200	KL15	O4	-	-	-	-	-	-	0
Human	Urine	ST200	KL122	O2v2	-	-	-	-	-	-	0
Human	Throat	ST1427	KL67	OL101	-	-	-	-	-	-	7
Human	Sputum	ST1427	KL67	OL101	-	-	-	-	-	-	7
Human	Sputum	ST1427	KL67	OL101	-	-	-	-	-	-	7
Human	Urine	ST1427	KL67	OL101	-	-	-	-	-	-	7
Human	Urine	ST1427	KL67	OL101	-	-	-	-	-	-	7
Human	Central Venous	ST1427	KL67	OL101	-	-	-	-	-	-	7
N/A	N/A	ST1427	KL67	OL101	-	-	-	-	-	-	7
Mouse	Liver	ST1165	KL45	O2v2	-	-	-	-	-	-	5
Mouse	Feces	ST1165	KL45	O2v2	-	-	-	-	-	-	4
Mouse	Blood	ST1165	KL45	O2v2	-	-	-	-	-	-	4
Mouse	Cecum	ST1165	KL45	O2v2	-	-	-	-	-	-	4
Mouse	Blood	ST1165	KL45	O2v2	-	-	-	-	-	-	4
Mouse	Lung Abscess	ST1165	KL45	O2v2	-	-	-	-	-	-	4
Mouse	Lung Abscess	ST1165	KL45	O2v2	-	-	-	-	-	-	5
Mouse	Lung Abscess	ST1165	KL45	O2v2	-	-	-	-	-	-	4
Mouse	Blood	ST1165	KL45	O2v2	-	-	-	-	-	-	5
Mouse	Blood	ST1165	KL45	O2v2	-	-	-	-	-	-	5
Mouse	Blood	ST1165	KL45	O2v2	-	-	-	-	-	-	1
Mouse	Blood	ST1165	KL45	O2v2	-	-	-	-	-	-	4
Mouse	Cecum	ST1165	KL45	O2v2	-	-	-	-	-	-	4
Mouse	Lung Abscess	ST1165	KL45	O2v2	-	-	-	-	-	-	4
Mouse	Liver	ST1165	KL45	O2v2	-	-	-	-	-	-	4

Figure 3. Whole-genome phylogenetic tree of mouse *Kp* isolates compared with representative *Klebsiella* spp. genomes. The *Kp* mouse genomes were most similar to classic *Kp* strains with multidrug antibiotic resistance profiles that had been isolated from humans. # Drug Res. Classes, number of antibiotic resistant gene classes; Aer, aerobactin; Clb, colibactin; K, K capsule antigen type; MLST, multilocus sequence type; O, O antigen type; rpmA, regulator of mucoid phenotype A; rpmA2, regulator of mucoid phenotype A2; Sal, salmochelin; Yer, yersiniabactin.

the synthesis of aerobactin, salmochelin, yersiniabactin, or colibactin. In addition, the 15 mouse *Kp* genomes contained the *khe* gene, which encodes for hemolysin, but hemolytic activity was not observed phenotypically. The *Kp* mouse genomes also encoded 28 homologous gene sequences with a putative type VI secretion system, reactive oxygen species degradation, and carbohydrate update or metabolism functions, and have previously been associated with the pore-forming cytotoxicity caused by *Kp* strains isolated form human patients with primary sclerosing cholangitis.[28]

Fourteen of the 15 mouse *Kp* isolates encoded identical antibiotic-resistance gene cassettes containing *aadA2*, *cmlA1*, *sul1*, *dfrA15*, and *qacE*Δ*1* for aminoglycoside, phenicol, sulfonamide, trimethoprim, and quaternary ammonium compound resistance, respectively (Figure 4A). BLAST analysis against the nr/nt database revealed that this cassette was most homologous to class 1 integron plasmid sequences from *Aeromonas caviae*, *Kp*, *E. coli*, *Enterobacter cloacae*, *Salmonella enterica*, and *Enterobacter hormaechei* (coverage, 57.0% to 98.0%; percentage identity, 97.7% to 100.0%; Table S2). PCR assays using purified plasmid DNA and targeting the *intI* and *sul1* genes of this antibiotic-resistance gene cassette confirmed its presence in the 14 mouse *Kp* isolates. In total, plasmid DNA from 20 of 23 mouse *Kp* isolates were positive for both antibiotic resistance gene cassettes (Figure 4B and C). Furthermore, all isolates also encoded the SHV61 extended spectrum β-lactamase gene. None of the genomes contained fluoroquinolone resistance genes, but *qacE*Δ*1* and *cepA*, cation efflux pumps associated with chlorhexidine resistance, were present in the genomes of all 15 mouse *Kp* isolates and were located on the chromosome.

Antimicrobial sensitivity profile of *Kp*. Antimicrobial sensitivity testing by disc diffusion demonstrated that 13 of 14 *Kp* isolates were resistant to TMP–SMX, but all were susceptible to enrofloxacin and gentamicin, as were the original *E. coli* strains (Table 2). All isolates ($n = 14$) were resistant to ampicillin; 13 of 14 were sensitive to ampicillin–clavulanic acid, ampicillin–sulbactam, and cephalothin. For confirmation, minimal inhibitory concentration determinations by broth dilution were performed for enrofloxacin; all 14 *Kp* isolates were susceptible at 0.25 µg/mL or less. Enrofloxacin was provided in the water at 0.25 mg/mL continuously thereafter. The treatment eliminated deaths associated with *Kp* infection in this breeding colony of NSG mice. Furthermore, to assess treatment efficacy and elimination of *Kp*, fecal samples saved in bacterial freeze media from several cages were cultured in general-purpose, selective, and differential media. Whereas bacterial growth occurred on general-purpose blood agar plates, no growth was observed in selective and differential media for gram-negative bacteria. These findings suggest that *Kp* was successfully eliminated from the colony after treatment with enrofloxacin. With regard to the quaternary ammonium product, the minimum inhibitory concentration for the *Kp* isolates ranged from 0.7 to 5.4 µg/mL and the minimum bacteriocidal concentration ranged from 0.7 to 21.4 µg/mL.

Coinfection with *T. muris* and MKPV. The ceca of NSG mice (19 of 22) frequently contained a heavy burden of protozoal organisms that exhibited morphologic features consistent with *T. muris*. These mice did not have typhlocolitis but did show goblet cell hyperplasia and epithelial cell degeneration and tufting, with numerous exfoliated epithelial cells in the intestinal lumina. *Tritrichomonas* DNA was detected in 4 of 6 cecal samples submitted for evaluation. Sequence analysis of 28s rRNA amplicons revealed 100% identity with *T. muris* (accession no., Z18255). *Kp* was significantly ($P = 0.0254$) detected in ceca cocolonized with *T. muris* versus those without *T. muris*.

Because MKPV has been detected in NSG breeders housed in the same room where the outbreaks occured,[19] we prospectively collected kidneys from NSG mice, including the 7 early diarrhea cases and the 21 subsequent cases. MKPV was detected in the kidneys of 15 of the 28 NSG mice submitted for evaluation; of these 15 MKPV cases, 2 were from the initial 7 mice with diarrhea, and 13 were from the subsequent cohort. Five mice were either bacteremic or had lesions consistent with *Kp* infection; however, MKPV status was not significantly associated with *Kp* infection or the presence of systemic inflammatory lesions.

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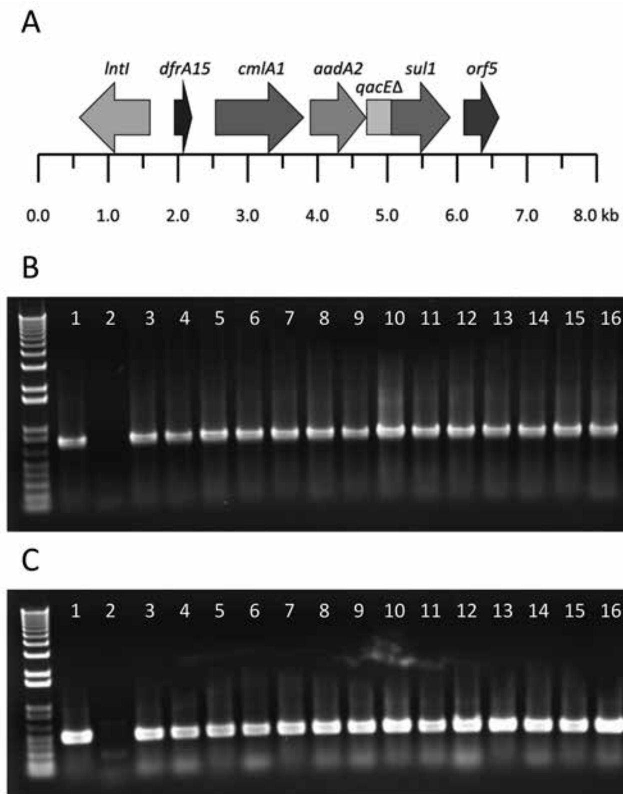

Figure 4. (A) Gene structure of plasmid-encoded class 1 integron antibiotic resistance gene cassettes present in mouse *Kp* genomes. (B) PCR analysis for *intI* sequence from purified plasmid DNA of representative mouse *Kp* genomes. (C) PCR for *sul* sequence from purified plasmid DNA of representative mouse *Kp* genomes. Lanes indicate 16 different representative isolates.

Of the 15 MKPV cases, 2 had chronic tubulointerstitial lesions consistent with MKPV-induced inclusion body nephropathy, whereas the remaining 13 kidneys did not. The mice with inclusion body nephropathy had MKPV levels that were quantified as over 10,000 times higher than in MKPV-positive mice without histologic lesions. The majority of the latter MKPV cases had fewer than 10 copies of MKPV per µg of mouse DNA. MKPV in situ hybridization signals (RNAscope) were detected in the nucleus and cytoplasm of affected tubular epithelial cells in the 2 mice with chronic inclusion body nephropathy (data not shown). MKPV infection was not associated with sex, age, or tritrichomonas status. A subset of these mice (n = 10) was included in a separate report of MKPV infection from our group.[19]

Discussion

In the current epidemiologic study, we determined that the disease outbreak in our NSG mice was a classic presentation of opportunistic *Kp* infection, which typically results in pulmonary abscesses, bacteremia, or systemic suppurative inflammation in infected immunocompromised mice.[5,35] Contrary to what we initially suspected, the *Kp* strains isolated were neither phenotypically hyperviscous nor genetically characterized as hypervirulent, but they still contributed to significant morbidity and mortality. *Kp* strains vary greatly in their pathogenicity, which stems from a variety of virulence factors. Although *Kp* hypervirulent strains are increasing in medical importance, even classic *Kp* strains contain multiple virulence factors and can have antibiotic resistance.[24,33,36] For example, classic *Kp* strains produce a thick capsule, lipopolysaccharide, types 1 and 3 fimbriae, and siderophores,[30] all of which were present in these isolates. Virulence factors specifically associated with hypervirulent strains, such as the genes *rmpA* and *magA* that mediate capsule production,[30] were not present in our isolates. Two isolates derived from liver and cecum of the same bacteremic mouse were originally identified as positive on the string test. When WGS did not support this finding due to the lack of *rmpA* and *magA*, these strains were retested and found to be string test negative. The viscosity of colonies can be affected by growing conditions, resulting in borderline viscosity that can result in a false positive.[40] In contrast, truly hyperviscous

Table 2. Antimicrobial disc diffusion data (millimeters) from *K. pneumoniae* isolates

Sample ID	Ampicillin	Amoxicillin–Clavulanic acid	Ampicillin–Sulbactam	Cephalothin	Enrofloxacin	Gentamicin	Trimethoprim–Sulfamethoxazole
1808210021 Bood	**0***	25	20	20	27	18	**0***
1808210001 Feces	**0***	**0***	**0***	**0***	29	19	**0***
1811130032 Blood	**0***	25	19	20	26	19	**0***
1811130032 Blood	**0***	20	18	20	27	21	**0***
1811130034 Blood	**0***	21	19	19	25	18	**0***
1811130034 Cecum	**0***	20	21	20	26	20	**0***
1811130035 Blood	**0***	24	17	20	26	21	20
1811130035 Blood	**0***	25	18	19	27	18	**0***
1811260006 Lung abscess	**0***	23	19	19	26	21	**0***
1811300002 Liver	**0***	22	20	19	27	21	**0***
1812040001 Lung abscess	**0***	23	19	20	26	19	**0***
1812100012 Lung abscess	**0***	22	19	20	27	21	**0***
1811300003 Cecum	**0***	21	19	19	27	20	**0***
1811300003 Liver	**0***	25	21	19	26	19	**0***
Resistant	≤ 13	≤ 13	≤ 11	≤ 14	≤ 16	≤ 12	≤ 10
Intermediate	14–16	14–17	12–14	15–17	17–20	13–14	11–15
Sensitive	≥ 17	≥ 18	≥ 15	≥ 18	≥ 21	≥ 15	≥ 16

E. coli ATCC 25922, Reference control
*Bolded values indicate antibiotic resistance

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colonies often produce strings that extend multiple centimeters, well beyond the 5-mm threshold we used. If the string test is not definitive, confirmatory tests such as the sedimentation test, or PCR analysis of *rmpA* or *magA* can be used. In one study, we used WGS to confirm our results of negative string tests.[40]

These apparently classic *Kp* strains harbored antibiotic resistance genes to a wide range of antibiotic classes, as confirmed via antimicrobial sensitivity testing and WGS. In the initial 7 cases, culture results had mixed growth of commensals and potentially pathogenic bacteria, such as *pks*+ *E. coli* and *Kp*. These polymicrobial results were difficult to interpret and required ancillary phenotypic and molecular assays to better characterize these organisms. Our PCR testing and clinical history lead us to believe *pks*+ *E. coli* was the major contributor to morbidity, and the choice to use of TMP–SMX was based on antimicrobial testing of these isolates. All *Kp* cases of pulmonary abscesses, bacteremia, or sepsis developed after the administration of TMP–SMX. In addition, our laboratory records indicated that *Kp* was rarely isolated from the nares of sentinel mice or lesions from sick mice. We suspect that *Kp* circulating in asymptomatic breeders was transmitted via the fecal–oral route to pups and other breeders in the colony. *Kp* strains isolated from humans and animals are well known to acquire resistance to various antibiotics, including TMP–SMX.[24,34] Treatment with TMP–SMX likely enriched the large intestines of NSG mice with antibiotic-resistant *Kp* that was then shed in the feces and spread to naïve mice. In addition, continuous antibiotic treatment in mice can create a dysbiosis accompanied by a superspreader phenotype of intestinal pathogens, in this case *Kp*.[42] Although we did not quantify *Kp* in the feces, the high percentage of *Kp* in cecal contents implies that *Kp* was high in feces. Our WGS analysis of *Kp* isolates and their antimicrobial susceptibility patterns confirmed that most *Kp* isolates were resistant to TMP–SMX, perhaps explaining the sudden increase in *Kp*-associated deaths in this colony of NSG mice. The fact that *Kp* was commonly found as a pure culture in blood and pulmonary abscesses further supports the source of the disease outbreak as invasive *Kp* and not one of the other commensal organisms originally isolated from the fecal samples of mice with diarrhea.

Previously, 97 orthologous genes were identified that correlated with the pore-forming cytotoxicity caused by *Kp* strains isolated from human patients with primary sclerosing cholangitis.[28] A BLASTP analysis revealed that 28 of these orthologous sequences were also present in our 15 mouse *Kp* strains. These genes included putative functions associated with the type IV secretion system, reactive oxygen species degradation, and carbohydrate update or metabolism. Although the genes and mechanisms responsible for pore-forming cytotoxicity are unknown, the presence of these 28 genes indicates that the mouse *Kp* strains are genetically similar to extraintestinal *Kp* strains associated with intestinal barrier disruption and liver pathology. Further in vitro experiments are required to ascertain whether the presence of these 28 orthologous genes in the rodent *Kp* strains can exhibit pore-forming cytotoxicity that allows translocation of the bacteria and ensuing sepsis.[28]

In addition to traditional antibiotics, these *Kp* genomes were also resistant to quaternary ammonium compounds. The responsible genes, such as *qac* family, encode for efflux pumps that eliminate both drug residues and quaternary ammonium compounds from the bacterium.[7] Decreased susceptibility to these commonly used antiseptic and disinfectant agents makes elimination from the environment difficult and heightens the risk reinfection.[39]

Quaternary ammonium disinfectants are commonly used in vivaria as surface-acting detergents with antibacterial properties. The product used in our facilities, Quatricide (Pharmacal Waterbury, CT), combines 2 of these compounds, didacyldimethyl ammonia chloride and n-alkyl dimethyl benzyl ammonia chloride. The use of this detergent as a disinfectant of forceps used to transfer mice into clean cages raised concern due to our recognition that the *Kp* isolate recovered in this outbreak encoded an efflux-based system, the *qac*-mediated resistance genes *qacE*Δ*1* and *cepA*. The *qacE* gene and its attenuated variant *qacE*Δ*1* is commonly found in gram-negative bacteria, including the Enterobacteriaceae, to which *Kp* belongs.[39] Select studies conducted in the United Kingdom noted the presence of *qacE*Δ*1* and *cepA* genes and a reduced susceptibility to biocides.[1,2] In contrast, other reports indicated the presence of *qacE*Δ*1* and *cepA* genes had no significant effect on *Kp* biocide resistance.[3,29]

In this outbreak, we speculated that the presence of the *qacE*Δ*1* and *cepA* genes in the *Kp* isolates increased resistance to the quaternary ammonium product we used. The practical relevance of this possibility may be discounted given that the recommended concentration of the disinfectant is usually higher than what the targeted bacteria can tolerate.[38] To address this possibility, we determined the minimal inhibitory concentration and minimum biocidal concentration of the disinfectant.[11] The values obtained were well within the sensitivity ranges as compared with the concentration of the working solution (686 µg/mL). Nevertheless, the *qacE*Δ*1* and *cepA* genes, together with other mechanisms such as various multidrug efflux genes or modifications in the cell wall of *Kp*, may have contributed to the development of *Kp* resistance to the disinfectant and facilitated the spread of *Kp* during the current outbreak.[8] We recently changed our sanitation program; we have replaced the disinfectant used with Peroxigard (Virox Technologies, Ontario, Canada).

We viewed the association of cecal *Tritrichomonas* spp. with *Kp* cocolonization as incidental, given that typhlitis was not observed in these cases. *Tritrichomonas* spp. in the intestines of laboratory mice are considered commensal organisms.[5] Recent studies have shown that experimental infections of laboratory mice with *Tritrichomonas* spp. alters the epithelial and immune cell homeostasis in the intestinal mucosa, making them more susceptible to inflammation in the large intestine.[9,14,20] Such effects may have contributed to facilitating translocation of *Kp* from the intestines into the bloodstream in cases of enteric *Kp* infection and sepsis.

MKPV is a recently identified murine pathogen responsible for inclusion body nephropathy in immunocompromised mice, including NSG.[32] We recently detected MKPV from both archived and fresh kidney samples from immunocompromised mice with or without inclusion body nephropathy.[19] Although inclusion body nephropathy was rare in the current cohort, we found low levels of MKPV in 54% of mice analyzed. Whether these mice would have progressed to IBN had they reached advanced age is unknown. In the current cases, MKPV status was not associated with *Kp* infection; however, we have previously observed MKPV-positive immunocompromised mice with inclusion body nephropathy and concurrent urosepsis and meningitis from *pks*+ *E. coli*.[19] Similar to *Kp*, *pks*+ *E. coli* is a common gastrointestinal inhabitant of mice that can sometimes cause systemic disease.[18,19,41] The similarities between these 2 outbreaks of systemic infections prompts additional questions; future studies are needed to determine whether MKPV infections in immunocompromised mice enhance their susceptibility to systemic bacterial infections and other comorbidities.

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Once *Kp* was identified as the causative agent of this outbreak, we made appropriate changes to treatment and husbandry protocols. Mice were transitioned from autoclaved static cages to IVC and cages were changed in a biosafety cabinet. Autoclaved food, water, and bedding continued to be used. Enrofloxacin was provided in the water indefinitely. Although we originally had considered a shorter course of treatment, the investigators' prolonged experimental timeline and continued use of the colony required this long-term therapeutic approach. Feces collected from the colony after 2 y of enrofloxacin treatment did not grow *Kp*, suggesting that the organism had not developed resistance against this drug class. In addition, the colony continued to do well clinically, with no subsequent cases of systemic bacterial infection reported.

In summary, this study revealed that classic (nonhyperviscous) *Kp* strains have the potential to induce systemic disease in severely immunocompromised mice. The alteration of the intestinal microflora after TMP–SMX therapy may have enhanced the susceptibility of these NSG breeders and pups to *Kp* infections. Although we were unable to determine when and how *Kp* was introduced into this colony of NSG mice housed under SPF conditions, retrospective analysis of culture results from sentinel mice revealed that *Kp* was detected rarely in sentinel CD1 mice from the room in question. Treatment of the colony with oral antibiotics clearly contributed to increased colonization density of a *Kp* superspreader phenotype, promoting rapid spread of *Kp* in the colony. Our findings suggest that translocation of enteric *Kp* may have resulted in the systemic inflammatory lesions we observed. In view of the enhanced susceptibility of NSG mice to opportunistic pathogens, these mice should be housed under optimal and rigorous management practices. Importantly, the change of antibiotic treatment from TMP–SMX to enrofloxacin successfully controlled this outbreak of *Kp*.

Supplementary Materials

Table S1. Average nucleotide identity values
Table S2. Integron BLAST results

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