Enterobacter cloacae complex ST-171 Isolates Expressing KPC-4 Carbapenemase Recovered from Canine Patients in Ohio, USA

Running title: *Enterobacter* ST171 with KPC-4 in US Dogs

Joshua B. Daniels,[1*] Liang Chen,[2] Susan V. Grooters,[3] Dixie F. Mollenkopf,[3] Dimitria A. Mathys,[3] Barry N. Kreiswirth,[2] Thomas E. Wittum[3]

Department of Veterinary Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado[1]

Public Health Research Institute Tuberculosis Center, New Jersey Medical School, Rutgers University, Newark, New Jersey[2]

Department of Veterinary Preventive Medicine, The Ohio State University College of Veterinary Medicine, Columbus, Ohio[3]

*Corresponding author

ABSTRACT

Carbapenem resistant *Enterobacteriaceae* (CRE) have emerged as a critical public health threat. Organisms expressing the *Klebsiella pneumoniae* carbapenemase (KPC) were first recognized in the US in the late 1990s and continue to be the predominant CRE genotype reported in clinical isolates. Strains harboring bla_{KPC} alleles have been observed in multiple species of *Enterobacteriaceae*, including the *Enterobacter cloacae* complex. A major *E. cloacae* clone, *Enterobacter xiangfangensis* ST171, has emerged as an important cause of hospital associated infections (HAI) and has been shown to carry different alleles of KPC in the context of Tn*4401*, residing on plasmids of multiple incompatibility groups. While CRE are commonly isolated from infected humans, their recovery from animals has been rare, particularly from companion animals. In the US, only six CRE have been reported from companion animals, and one from livestock, none of which were bla_{KPC}. This report describes two *E. xiangfangensis* sequence type ST171 isolates each with a large IncHI2 plasmid bearing bla_{KPC-4} recovered from dogs with infections at the Ohio State University Veterinary Medical Center. Our phylogenetic comparison of these canine isolates with available sequences from clinical human isolates of KPC-4 identified in ST171 suggest an epidemiologically significant clonal strain.

INTRODUCTION

Carbapenem class antimicrobial agents are critically important to human health and are considered drugs of last-resort. Carbapenem resistant *Enterobacteriaceae* (CRE) are a growing threat to global public health. In 2013, the CDC classified CRE as an “urgent” public health threat, and estimated that there were approximately 9300 CRE infections, and 610 related deaths in the US (1).

Carbapenem resistance among clinically-relevant *Enterobacteriaceae* is often conferred by plasmid-borne β-lactamase encoding genes that may be categorized in three of the four Ambler β-lactamase

classes. KPC and OXA carbapenemases respectively belong to classes A and D and function as serine proteases whose activity is inhibited by agents such as clavulanate and sulbactam. The NDM, VIM and IMP enzymes are class B metallo-β-lactamases which are inhibited by the presence of EDTA, but not clavulanate and sulbactam (2). In the US, the *K. pneumoniae* carbapenemase (KPC) emerged in the late 1990s and have become the predominant carbapenemase encoded by clinical CRE isolates primarily due to clonal dissemination of strains of *K. pneumoniae*, *Enterobacter spp.,* and *Escherichia coli* (3, 4). While healthcare associated infections (HAI) comprise the majority of cases involving CRE overall, community associated CRE infections (CAI) comprise up to 10.8% of infections in the US. Active surveillance performed by hospitals as part of infection control programs indicate that asymptomatic carriage may be up to 41%; however, the rate of carriage in healthy individuals remains unknown (5).

Antimicrobial-resistant bacteria and resistance genes are shared among human beings and animals through either direct or foodborne zoonotic transmission. As a result, veterinary use of antimicrobial drugs in both food-producing and companion animal species has the potential to impact antibiotic resistance of human pathogens (6). While carbapenem antimicrobial drugs are illegal to use in food-producing animals in the US, there is relevant selective pressure favoring CRE in production animal niches resulting from the use of the third-generation cephalosporin, ceftiofur, and other agents that may co-select for carbapenemase encoding genes due to co-localization with other resistance-encoding genes on plasmids (7, 8). To date, there have been two reports of multiple CRE genera expressing $bla_{\text{IMP-64}}$ located on an IncQ1 plasmid that were recovered from the same US swine farm (9, 10).

In US companion animals (e.g. dogs and cats), it is permissible for a veterinarian to prescribe carbapenem-class drugs off-label, as long as there is a valid veterinarian-client-patient relationship under the Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA; (11)). While an assessment of

73 the frequency of carbapenem usage in companion animals has not been reported, pharmacokinetic
74 studies have been performed and companion animal dosage recommendations for imipenem and
75 meropenem are available in veterinary medical textbooks (12-14). Moreover, ESBL and AmpC
76 β-lactamase mediated resistance among *Enterobacteriaceae* isolated from dogs and cats is commonly
77 reported, indicating a potential clinical justification for carbapenem administration (15-20).
78
79 This potential for the veterinary application of carbapenem drugs suggests that the role of companion
80 animals as reservoirs for the zoonotic transmission of CRE should be considered, particularly for CAIs
81 (21). There has been only one report of CRE isolated from companion animals in the US, six *E. coli*
82 expressing $bla_{\text{NDM-1}}$ recovered from a variety of infections in cats and dogs between May 2008- May
83 2009 (22). Subsequent analysis by pulsed-field gel electrophoresis (PFGE) indicated that the isolates
84 represented diverse *E. coli* strains, which is consistent with the dissemination of diverse CRE expressing
85 bla_{NDM} in human patients.
86
87 CRE can be challenging to diagnose in clinical laboratories because they frequently exhibit clinical
88 susceptibility to carbapenem drugs upon routine antimicrobial susceptibility testing via broth
89 microdilution and Kirby-Bauer disc testing (23). Several strategies, both molecular and phenotypic, have
90 been used to increase the sensitivity of detecting carbapenemase production by clinical isolates (24, 25).
91 In this report, we describe two clinical isolates of *E. xiangfengensis* and their mobile genetic elements
92 recovered from canine patients in central Ohio, US. and belong to a disseminated clone that has been
93 responsible for major clusters of human CRE infections in the Northeastern and Upper Midwestern
94 United States (26).
95
96

97 **RESULTS**

98 **Antimicrobial Susceptibility Testing and Detection of KPC** Both isolates, identified by MALDI-TOF as *E.*
99 *cloacae*, had the same susceptibility pattern (agent, MIC (µg/ml)): Amikacin, ≤ 4; Amoxicillin/Clavulanic
100 acid, >8; Ampicllin, >8; Cefazolin, >32; Cefovecin, >32; Cefpodoxime, >8; Ceftazidime, >16;
101 Chloramphenicol, 8; Doxycycline >8; Enrofloxacin, >4; Gentamicin, 0.5; Imipenem, ≤ 1; Marbofloxacin,
102 >4; Piperacillin/Tazobactam, 16; Pradofloxacin, >2; Tetracycline, >16; and
103 Trimethoprim/Sulfamethoxazole, >4. Notably, they were phenotypically susceptible to imipenem. Both
104 isolates were observed to produce a carbapenemase by a CarbaNP test, and conventional PCR indicated
105 that both isolates harbored bla_{KPC}.
106

107 **Whole Genome Sequence and Annotation of Plasmid Containing KPC-4:** Subsequent Kmer analysis of
108 whole genome sequence identified the isolates as *E. xiangfangensis*, which is included in the *E. cloacae*
109 complex. Both isolates were identified as ST171 on MLST, and each contained two plasmids belonging to
110 IncF1B and IncHI2. The IncHI2 plasmids (Figure 1) were 351,806 (pOSUVMCKPC4-1) and 354,256 bps
111 (pOSUVMCKPC4-2), respectively, and were each identified as ST1 on pMLST. The plasmids both
112 contained bla_{KPC-4} in the context of Tn*4401b* (Figure 2), consistent with other reports of
113 *Enterobacteriaceae* expressing bla_{KPC-4}. Additional resistance genes in the two genomes were: *strAB*,
114 *aadA1*, $bla_{OXA-129}$, *sul1*, *tet(B)*, and *dfrA21*. Chromosomal analysis also indicated that they both carry
115 resistance genes bla_{ACT-16} and *fosA* in the two isolates. In comparison to pOSUVMCKPC4-1,
116 pOSUVMCKPC4-2 has two additional IS*3* at nt 62,536 to 63,760 and nt 180,063 to 181,287, respectively.
117 In addition, a 21kb region (nt 94,348-115,575 in pOSUVMCKPC4-2), containing the *strA, strB*, *aadA1*,
118 $bla_{OXA-129}$, *dfrA21, sul1* flanked by two IS*4321*, is inverted in comparison to pOSUVMCKPC4-1. This region
119 was identified as In524 (Figure 3), using the INTEGRALL database (27). The *intI1* is disrupted by the
120 Tn*5393* insertion sequence, which encodes genes *strA* and *strB* that confer resistance to aminoglycoside

121 antibiotics. Downstream of *strB* is a truncated integrase gene followed by three antimicrobial resistance
122 gene cassettes, *dfrA21*, $bla_{OXA-129}$, and *aadA1*. The 3' region following the integron harbors genes
123 encoding resistance to quaternary ammonium compounds, *qacEΔ1*, and sulfonamide antibiotics, *sul1*.
124 This region also contains a putative gene for N-Acyltransferase, Acyl-coA, and the insertion sequence
125 IS*6100*, which is a transposase shown to increase the expression of *strA* and *strB* genes. The IncF1B
126 plasmids in the two isolates were identical in size, 108,403 bp, and contained no identifiable resistance
127 genes.
128
129 Core SNP phylogenetic analysis placed these two *E. xiangfangensis* strains in the previously described
130 ST171 cluster II (Figure 4)(26). The two isolates formed a subclade in cluster II, and differ from each
131 other by 14 core snps. In cluster II, isolates have an average of 75 (10-137) core snp differences
132 compared with each other, while they showed ~530 core snps difference in comparison to isolates from
133 cluster I. Of interest, nearly all isolates from cluster II harbor the bla_{KPC-4} on the Tn*4401b* element.
134 Further analysis of the bla_{KPC-4}-harboring contigs from other clade II isolates revealed that similar to
135 OSUVMCKPC4-1 and OSUVMCKPC4-2, the majority (except for SMART-264 and BIDMC94) harbor bla_{KPC-4}
136 and IncH plasmid backbones.
137
138 **DISCUSSION**
139 This first reported isolation of KPC-producing CRE from US companion animals has important
140 implications for both veterinary medicine and public health, because animals may serve as reservoirs of
141 significant opportunistic human pathogens. Notably, these two cases represent an epidemiologically
142 significant clone of *Enterobacter* sp., ST171 that has been described in regional HAI clusters in the US
143 (28, 29), and which may now be expanding into the community. Analysis of contemporaneous human
144 clinical *E. cloacae* isolates from The Ohio State University Medical Center collected between 2011-2016

145 with an extended-spectrum ß-lactamase phenotype (*n*=8) revealed that two isolates belonged to ST171,
146 and both contained bla_{KPC-2} (data not shown), suggesting that these canine strains may have been
147 uniquely community-associated during that time period. However, our SNP analysis comparing the
148 canine isolates to other available ST171 whole genome sequence data in GenBank indicated that they
149 are closely related to historical human clinical isolates from the Northeastern US, the UK, and Colombia.
150 Together, these observations indicate the diversity regarding the dissemination of ST171 strains
151 harboring bla_{KPC}, including transcontinental movement and circulation in the community over multiple
152 years, with sporadic HAI activity.
153
154 The role of direct selection pressure by antimicrobial agents associated with the two infected dogs is
155 unclear. The first dog had a recent history of doxycycline administration for treatment of a chronic
156 pyoderma, which may have selected for the multidrug resistant *Enterobacter*, as the isolate was also
157 tetracycline resistant. However, co-selection for carbapenem resistance by tetracycline antibiotics has
158 not been reported. The second dog had no recent history of antimicrobial drug administration; however,
159 because the organism and the infection was the result of a dog bite from another dog, it is possible that
160 the biting dog had undergone antimicrobial selection pressure and inoculated the open wound with the
161 carbapenem-resistant *Enterobacter*. The dog with the bite wound had not previously been seen at this
162 facility and the organism was isolated from a specimen acquired on the day of presentation,
163 approximately two months after the first dog, thus it is reasonable to conclude that there was not a
164 common nosocomial source. Moreover, medical records indicated that the dogs resided in cities greater
165 than 240 km apart, suggesting that there is significant regional community dissemination of ST171 in
166 Ohio.
167

168 Laboratory detection of CRE is a challenge to all clinical diagnostic laboratories, but represents an even a
169 greater challenge to those in the veterinary setting because of the low prevalence in clinical isolates
170 from animals. *bla*KPC-4 can be particularly difficult to detect with conventional phenotypic (MIC)
171 methods because of its low hydrolytic activity (30), which may be compounded by the weak promoter
172 activity of TN*4401b,* as in these two isolates (31). The isolates described in this study were detected as
173 part of a surveillance program at a tertiary-referral veterinary hospital that is associated with a
174 university. Before the implementation of the surveillance program, only conventional AST would have
175 been performed, and these CRE would not have been detected. Veterinary diagnostic laboratories in the
176 US typically utilize AST performance standards with susceptibility breakpoints that are based on animal
177 pharmacokinetics-pharmacodynamics which are published in the CLSI VET01 document (32) and
178 supplemented with human breakpoints published in the same document when no animal-specific
179 breakpoints are available. While CLSI VET01 does not mention the need for enhanced detection
180 methods for carbapenemase activity, its human counterpart, the CLSI M100S27 document, discusses the
181 need for specific testing (33).
182
183 As fewer antimicrobial drugs retain their efficacy in the face of the proliferation of multidrug resistant
184 bacteria, there is a need to take a "One Health" approach that encompasses factors beyond
185 antimicrobial agent usage and infection control in human medicine. The identification of KPC-encoding
186 *E. cloacae* complex organisms in a companion animal species is more evidence that there are biological
187 reservoirs and potentially vectors for transmission to human beings in community circulation. Additional
188 surveillance work and studies of veterinary antimicrobial usage, as well as increasing the capabilities of
189 veterinary diagnostic laboratories will be critical to slowing the dissemination of CRE.
190
191

192 **MATERIALS AND METHODS**

193 **Bacterial Isolates** Both isolates, OSUVMCKPC4-1 and OSUVMCKPC4-2, were originally sourced from
194 clinical veterinary patients (dogs) that were treated at The Ohio State Veterinary Medical Center. The
195 first dog, seen in July 2016 for acute bacterial cystitis (UTI), was a 13-year old female,
196 ovariohysterectomized (spayed) Shetland Sheepdog that had a two-month history of chronic kidney
197 disease and a two-year history of pyoderma. The second dog, seen in September 2016, was a 6-year old
198 male, castrated, mixed-breed dog that presented for an infected bite wound that resulted from a fight
199 with another dog. The first dog was an established patient of the hospital for ongoing management of
200 her chronic health problems and had received doxycycline during the previous year; however, the
201 second dog had never been seen at the facility prior to presenting with the bite wound (which was
202 sampled for culture on the day of presentation), and had no recent history of antimicrobial
203 administration.

204

205 **Identification and Antimicrobial Susceptibility Testing** After routine aerobic cultivation from clinical
206 specimens, isolates were identified via MALDI-TOF (Biotyper, Bruker Daltonics, Billerica, MA) and
207 antimicrobial susceptibilities were determined via broth microdilution in accordance with CLSI VET01A4
208 (COMPGN1F plate, Trek Sensititre, Thermo-Fisher). Carbapenemase production was assessed using a
209 CarbaNP test (34). Isolates were screened for the presence of the transmissible carbapenemase gene,
210 bla_{KPC}, by conventional PCR using previously reported primers (35, 36).

211

212 **Whole Genome Sequencing.** Both isolates were first sequenced using Illumina MiSeq and subsequently
213 by the PacBio RS II system at the University of Maryland Institute for Genome Sciences (OSUVMCKPC4-
214 1) and at the University of Delaware Sequencing and Genotyping Center (OSUVMCKPC4-2) using two
215 SMRT cells per isolate, with genomes assembled using Canu v1.4 (37). Isolate DNA was prepared for

216 PacBio sequencing using a commercial DNA extraction kit (Qiagen Blood & Cell Culture DNA Maxi Kit
217 (Qiagen, Germantown, MD). Initially, sequences were submitted to the Center for Genomic
218 Epidemiology website (https://cge.cbs.dtu.dk) for Multi Locus Sequence Typing (MLST), plasmid
219 identification and resistance gene detection using PlasmidFinder and ResFinder (38-40). Additional
220 antimicrobial resistance databases were used to identify genotypes (41, 42). Identification of insertion
221 sequences that encode transposases were identified by IS Finder, as well as annotation of the
222 corresponding IRL, IRR (43). The curated integron database, INTEGRALL (27), identified the class 1
223 integron. Annotation of plasmid regions of interest were first examined for functional genes with NCBI's
224 Conserved Domain Database search (44). Regions that contained antimicrobial resistance genes were
225 then used as query sequences to search the NCBI database with BLAST for similar regions (45).
226 Sequences are available in GenBank through NCBI (accession numbers CP024908 and CP029246) and
227 were additionally annotated using the NCBI automated annotation pipeline.
228
229 An additional 16 KPC-producing ST171 genomes from the GenBank Whole Genome Shotgun (WGS)
230 database were downloaded and compared OSUVMCKPC4-1 and OSUVMCKPC4-2. A core single-
231 nucleotide polymorphism (SNP) (defined as SNPs shared across all genomes) analysis was conducted
232 using kSNP3.0 (46), and a core SNP maximum-likelihood tree was produced by RAxML 8.2.4 (47) using
233 the GTRGAMMA model and 100 bootstrap replicates. Furthermore, BLASTn comparisons between each
234 isolate's de novo assembly and the reference pOSUVMCKPC4-2 plasmid were conducted using the
235 method described previously (26), and the phylogenetic tree was annotated using iTOL (48).
236
237 **FUNDING**
238 This work was supported in part by the following grants from the USDA NIFA (2014-67005-21709 to T.W
239 and J.D.) and the National Institutes of Health (R01AI090155 and R21AI135250 to B.K, and R21AI117338

to L.C). The contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA NIFA or the National Institutes of Health.

……..

REFERENCES

1. Centers for Disease Control and Prevention, Antibiotic Resistance Threats in the United States, 2013. https://www.cdc.gov/drugresistance/threat-report-2013/index.html. Accessed April 17, 2018.
2. Diene SM, Rolain JM. 2014. Carbapenemase genes and genetic platforms in Gram-negative bacilli: Enterobacteriaceae, Pseudomonas and Acinetobacter species. Clin Microbiol Infect 20:831-8.
3. Guh AY, Bulens SN, Mu Y, Jacob JT, Reno J, Scott J, Wilson LE, Vaeth E, Lynfield R, Shaw KM, Vagnone PM, Bamberg WM, Janelle SJ, Dumyati G, Concannon C, Beldavs Z, Cunningham M, Cassidy PM, Phipps EC, Kenslow N, Travis T, Lonsway D, Rasheed JK, Limbago BM, Kallen AJ. 2015. Epidemiology of Carbapenem-Resistant Enterobacteriaceae in 7 US Communities, 2012-2013. JAMA 314:1479-87.
4. Gupta N, Limbago BM, Patel JB, Kallen AJ. 2011. Carbapenem-resistant Enterobacteriaceae: epidemiology and prevention. Clin Infect Dis 53:60-7.
5. Kelly AM, Mathema B, Larson EL. 2017. Carbapenem-resistant Enterobacteriaceae in the community: a scoping review. Int J Antimicrob Agents 50:127-134.
6. Singer RS, Finch R, Wegener HC, Bywater R, Walters J, Lipsitch M. 2003. Antibiotic resistance--the interplay between antibiotic use in animals and human beings. Lancet Infect Dis 3:47-51.
7. Rosengren LB, Waldner CL, Reid-Smith RJ. 2009. Associations between antimicrobial resistance phenotypes, antimicrobial resistance genes, and virulence genes of fecal Escherichia coli isolates from healthy grow-finish pigs. Appl Environ Microbiol 75:1373-80.
8. Wittum TE. 2012. The challenge of regulating agricultural ceftiofur use to slow the emergence of resistance to extended-spectrum cephalosporins. Appl Environ Microbiol 78:7819-21.
9. Mollenkopf DF, Stull JW, Mathys DA, Bowman AS, Feicht SM, Grooters SV, Daniels JB, Wittum TE. 2017. Carbapenemase-Producing Enterobacteriaceae Recovered from the Environment of a Swine Farrow-to-Finish Operation in the United States. Antimicrob Agents Chemother 61.
10. Mollenkopf DF, Mathys DA, Feicht SM, Stull JW, Bowman AS, Daniels JB, Wittum TE. 2018. Maintenance of Carbapenemase-Producing Enterobacteriaceae in a Farrow-to-Finish Swine Production System. Foodborne Pathog Dis doi:10.1089/fpd.2017.2355.
11. United States Food and Drug Administration. Feb. 2, 2018 1994. Animal Medicinal Drug Use Clarificaiton Act of 1994 (AMDUCA).

279 https://www.fda.gov/AnimalVeterinary/GuidanceComplianceEnforcement/ActsRulesRe
280 gulations/ucm085377.htm. Accessed April 18, 2018.
281 12. Byun SY, Jeong JW, Choi JH, Lee KP, Youn HY, Maeng HJ, Song KH, Koo TS, Seo KW. 2016.
282 Pharmacokinetic study of meropenem in healthy beagle dogs receiving intermittent
283 hemodialysis. J Vet Pharmacol Ther 39:560-565.
284 13. Plumb DC. 2015. Plumb's veterinary drug handbook, Eighth edition. ed. PharmaVet Inc.
285 Distributed by John Wiley & Sons, Stockholm, Wisconsin
286 Ames, Iowa.
287 14. Bidgood T, Papich MG. 2002. Plasma pharmacokinetics and tissue fluid concentrations
288 of meropenem after intravenous and subcutaneous administration in dogs. Am J Vet Res
289 63:1622-8.
290 15. Zogg AL, Zurfluh K, Schmitt S, Nuesch-Inderbinen M, Stephan R. 2018. Antimicrobial
291 resistance, multilocus sequence types and virulence profiles of ESBL producing and non-
292 ESBL producing uropathogenic Escherichia coli isolated from cats and dogs in
293 Switzerland. Vet Microbiol 216:79-84.
294 16. Adams RJ, Kim SS, Mollenkopf DF, Mathys DA, Schuenemann GM, Daniels JB, Wittum TE.
295 2018. Antimicrobial-resistant Enterobacteriaceae recovered from companion animal
296 and livestock environments. Zoonoses Public Health doi:10.1111/zph.12462.
297 17. Aslantas O, Yilmaz ES. 2017. Prevalence and molecular characterization of extended-
298 spectrum beta-lactamase (ESBL) and plasmidic AmpC beta-lactamase (pAmpC)
299 producing Escherichia coli in dogs. J Vet Med Sci 79:1024-1030.
300 18. Liu X, Thungrat K, Boothe DM. 2016. Occurrence of OXA-48 Carbapenemase and Other
301 beta-Lactamase Genes in ESBL-Producing Multidrug Resistant Escherichia coli from Dogs
302 and Cats in the United States, 2009-2013. Front Microbiol 7:1057.
303 19. Falgenhauer L, Schmiedel J, Ghosh H, Fritzenwanker M, Yao Y, Bauerfeind R, Imirzalioglu
304 C, Chakraborty T. 2014. Resistance plasmids in ESBL-encoding Escherichia coli isolates
305 from humans, dogs and cats. Berl Munch Tierarztl Wochenschr 127:458-63.
306 20. Damborg P, Gaustad IB, Olsen JE, Guardabassi L. 2011. Selection of CMY-2 producing
307 Escherichia coli in the faecal flora of dogs treated with cephalexin. Vet Microbiol
308 151:404-8.
309 21. Abraham S, Wong HS, Turnidge J, Johnson JR, Trott DJ. 2014. Carbapenemase-producing
310 bacteria in companion animals: a public health concern on the horizon. J Antimicrob
311 Chemother 69:1155-7.
312 22. Shaheen BW, Nayak R, Boothe DM. 2013. Emergence of a New Delhi metallo-beta-
313 lactamase (NDM-1)-encoding gene in clinical Escherichia coli isolates recovered from
314 companion animals in the United States. Antimicrob Agents Chemother 57:2902-3.
315 23. Hrabak J, Chudackova E, Papagiannitsis CC. 2014. Detection of carbapenemases in
316 Enterobacteriaceae: a challenge for diagnostic microbiological laboratories. Clin
317 Microbiol Infect 20:839-53.
318 24. Rood IGH, Li Q. 2017. Review: Molecular detection of extended spectrum-beta-
319 lactamase- and carbapenemase-producing Enterobacteriaceae in a clinical setting. Diagn
320 Microbiol Infect Dis 89:245-250.
321 25. Aguirre-Quinonero A, Martinez-Martinez L. 2017. Non-molecular detection of
322 carbapenemases in Enterobacteriaceae clinical isolates. J Infect Chemother 23:1-11.

323 26. Chavda KD, Chen L, Fouts DE, Sutton G, Brinkac L, Jenkins SG, Bonomo RA, Adams MD,
324 Kreiswirth BN. 2016. Comprehensive Genome Analysis of Carbapenemase-Producing
325 Enterobacter spp.: New Insights into Phylogeny, Population Structure, and Resistance
326 Mechanisms. MBio 7.
327 27. Moura A, Soares M, Pereira C, Leitao N, Henriques I, Correia A. 2009. INTEGRALL: a
328 database and search engine for integrons, integrases and gene cassettes. Bioinformatics
329 25:1096-8.
330 28. Hargreaves ML, Shaw KM, Dobbins G, Snippes Vagnone PM, Harper JE, Boxrud D,
331 Lynfield R, Aziz M, Price LB, Silverstein KA, Danzeisen JL, Youmans B, Case K, Sreevatsan
332 S, Johnson TJ. 2015. Clonal Dissemination of Enterobacter cloacae Harboring blaKPC-3 in
333 the Upper Midwestern United States. Antimicrob Agents Chemother 59:7723-34.
334 29. Gomez-Simmonds A, Hu Y, Sullivan SB, Wang Z, Whittier S, Uhlemann AC. 2016.
335 Evidence from a New York City hospital of rising incidence of genetically diverse
336 carbapenem-resistant Enterobacter cloacae and dominance of ST171, 2007-14. J
337 Antimicrob Chemother 71:2351-3.
338 30. Wolter DJ, Kurpiel PM, Woodford N, Palepou MF, Goering RV, Hanson ND. 2009.
339 Phenotypic and enzymatic comparative analysis of the novel KPC variant KPC-5 and its
340 evolutionary variants, KPC-2 and KPC-4. Antimicrob Agents Chemother 53:557-62.
341 31. Cheruvanky A, Stoesser N, Sheppard AE, Crook DW, Hoffman PS, Weddle E, Carroll J,
342 Sifri CD, Chai W, Barry K, Ramakrishnan G, Mathers AJ. 2017. Enhanced Klebsiella
343 pneumoniae Carbapenemase Expression from a Novel Tn4401 Deletion. Antimicrob
344 Agents Chemother 61.
345 32. Clincal and Laboratory Standards Institute. 2015. Performance Standards for
346 Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals.
347 3rd ed. CLSI Supplement VET01S. Clinical and Laboratory Standards Institute, Wayne,
348 PA.
349 33. Clinical and Laboratory Standards Institute. 2017. Performance Standards for
350 Antimicrobial Susceptibility Testing. 27th ed. CLSI Supplement M100. Clinical Laboratory
351 Standards Institute, Wayne, PA.
352 34. Poirel L, Nordmann P. 2015. Rapidec Carba NP Test for Rapid Detection of
353 Carbapenemase Producers. J Clin Microbiol 53:3003-8.
354 35. Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD,
355 Alberti S, Bush K, Tenover FC. 2001. Novel carbapenem-hydrolyzing beta-lactamase,
356 KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae. Antimicrob Agents
357 Chemother 45:1151-61.
358 36. Lob SH, Kazmierczak KM, Badal RE, Hackel MA, Bouchillon SK, Biedenbach DJ, Sahm DF.
359 2015. Trends in susceptibility of Escherichia coli from intra-abdominal infections to
360 ertapenem and comparators in the United States according to data from the SMART
361 program, 2009 to 2013. Antimicrob Agents Chemother 59:3606-10.
362 37. Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. 2017. Canu: scalable
363 and accurate long-read assembly via adaptive k-mer weighting and repeat separation.
364 Genome Res 27:722-736.

365 38. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM,
366 Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob
367 Chemother 67:2640-4.
368 39. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L, Sicheritz-
369 Ponten T, Ussery DW, Aarestrup FM, Lund O. 2012. Multilocus sequence typing of total-
370 genome-sequenced bacteria. J Clin Microbiol 50:1355-61.
371 40. Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, Moller
372 Aarestrup F, Hasman H. 2014. In silico detection and typing of plasmids using
373 PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother
374 58:3895-903.
375 41. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, Rolain JM.
376 2014. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in
377 bacterial genomes. Antimicrob Agents Chemother 58:212-20.
378 42. McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, Bhullar K, Canova MJ,
379 De Pascale G, Ejim L, Kalan L, King AM, Koteva K, Morar M, Mulvey MR, O'Brien JS,
380 Pawlowski AC, Piddock LJ, Spanogiannopoulos P, Sutherland AD, Tang I, Taylor PL,
381 Thaker M, Wang W, Yan M, Yu T, Wright GD. 2013. The comprehensive antibiotic
382 resistance database. Antimicrob Agents Chemother 57:3348-57.
383 43. Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA
384 sequences. J Comput Biol 7:203-14.
385 44. Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, Chitsaz F, Derbyshire MK, Geer
386 RC, Gonzales NR, Gwadz M, Hurwitz DI, Lu F, Marchler GH, Song JS, Thanki N, Wang Z,
387 Yamashita RA, Zhang D, Zheng C, Geer LY, Bryant SH. 2017. CDD/SPARCLE: functional
388 classification of proteins via subfamily domain architectures. Nucleic Acids Res 45:D200-
389 D203.
390 45. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search
391 tool. J Mol Biol 215:403-10.
392 46. Gardner SN, Slezak T, Hall BG. 2015. kSNP3.0: SNP detection and phylogenetic analysis
393 of genomes without genome alignment or reference genome. Bioinformatics 31:2877-8.
394 47. Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis
395 of large phylogenies. Bioinformatics 30:1312-3.
396 48. Letunic I, Bork P. 2016. Interactive tree of life (iTOL) v3: an online tool for the display
397 and annotation of phylogenetic and other trees. Nucleic Acids Res 44:W242-5.
398

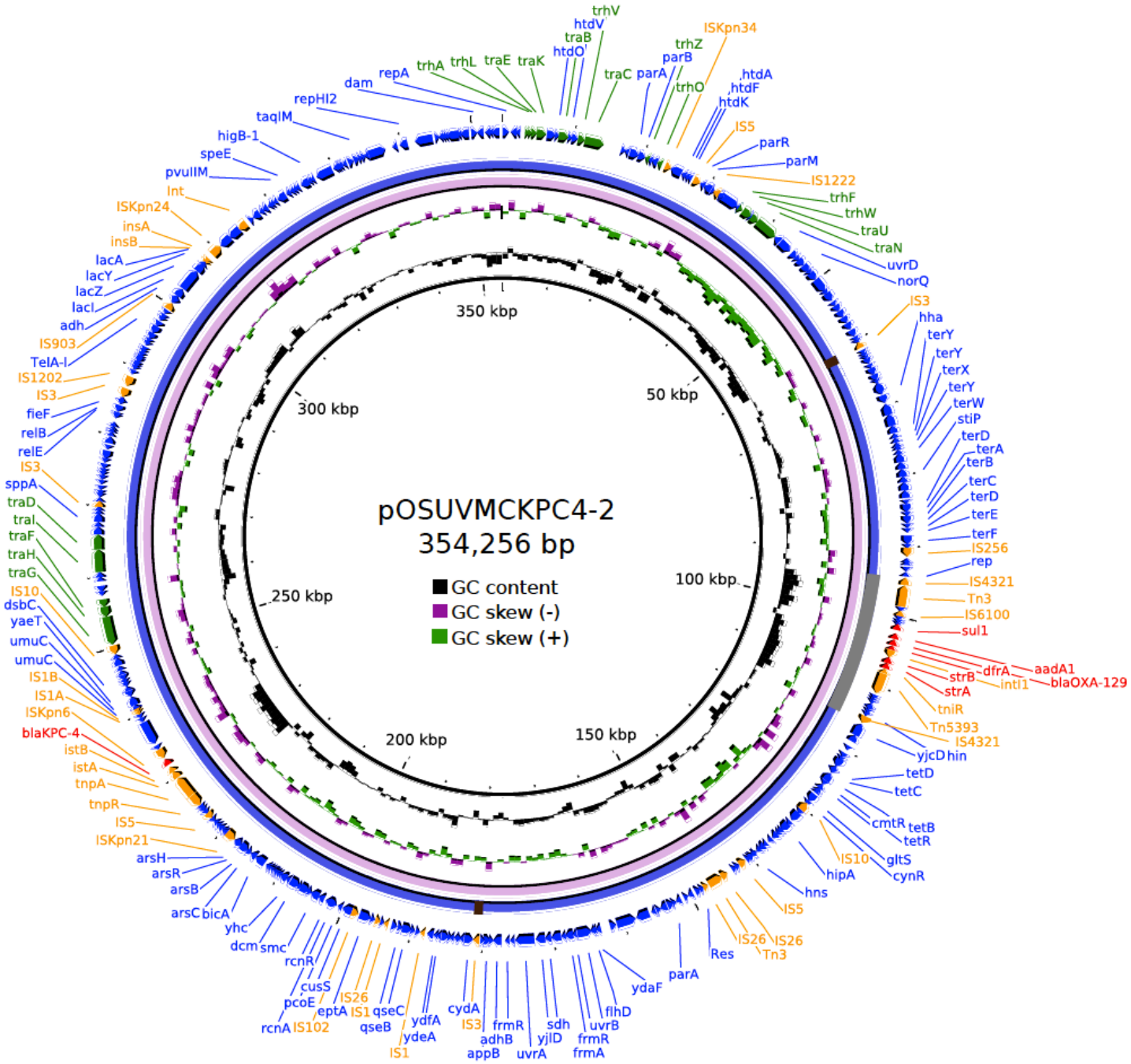
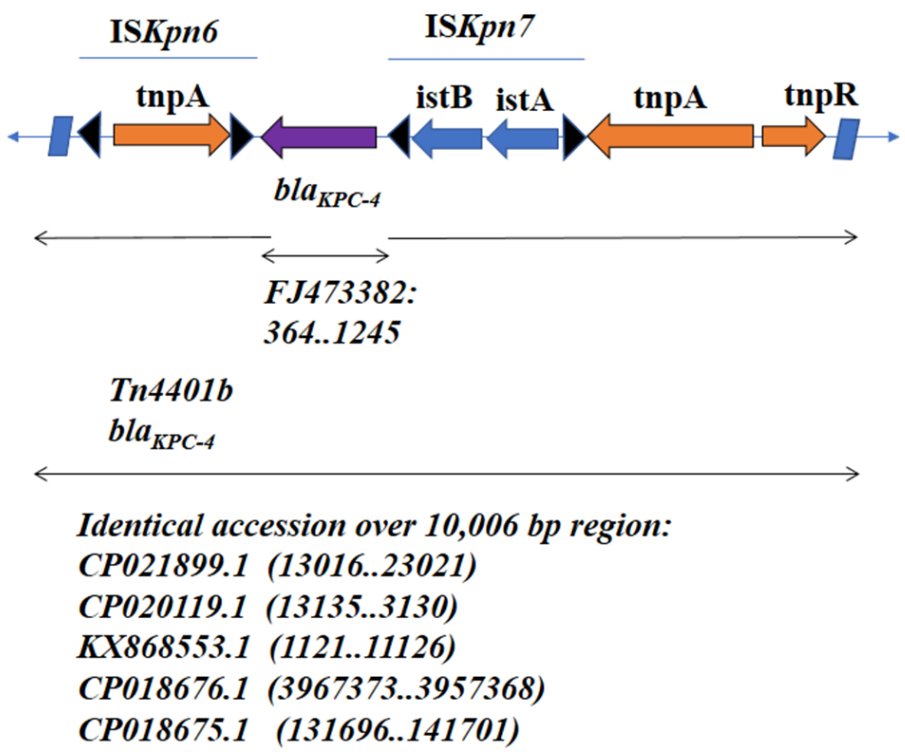


FIG 1 IncHI2 plasmids isolated from *E. xiangfengensis* ST171 sourced from dogs (pOSUVMCKPC4-2 (violet) and pOSUVMCKPC4-1 (blue)). Outer ring denotes location and orientation of genes. Subplasmidic mobile element associated genes are indicated in orange and red and plasmid transfer genes are indicated in green.



404

405 **FIG 2** Schematic structure of Tn4401b containing *bla*$_{KPC-4}$ present on large IncH12 plasmids in

406 *Enterobacter xiangfangensis* ST171 recovered from two canine patients of The Ohio State University

407 Veterinary Medical Center in 2016. Genes and their corresponding transcription orientation are

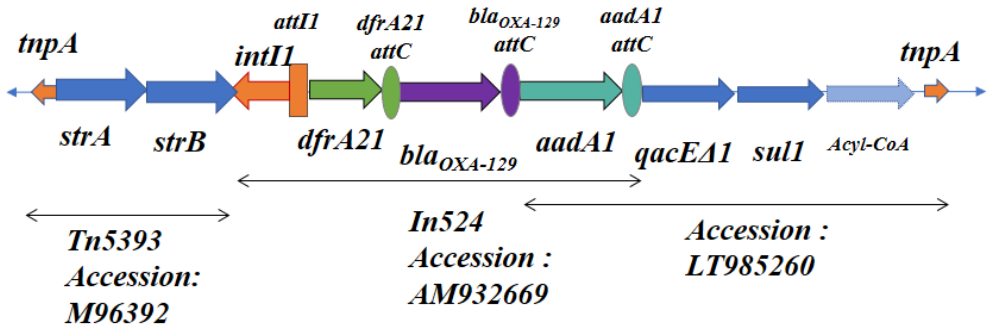
408 indicated by colored horizontal arrows. Corresponding insertion sequences (IS) that encode transposons

409 are indicated above the figure with a narrow blue line. Black triangles indicate inverted repeat right and

410 left sequences associated with the IS*Kpn6* and IS*Kpn7*. Flanking parallelograms represent the two

411 inverted repeat sequences associated with Tn4401 structures.

412



413

414 **FIG 3** Schematic structure of the In*524* class 1 integron and the surrounding region present on large

415 IncH12 plasmids in *Enterobacter xiangfangensis* ST171 recovered from two canine patients of The Ohio

416 State University Veterinary Medical Center in 2016. Genes and their corresponding transcription

417 orientations are indicated by colored horizontal arrows. Homologous alignments to references are

418 indicated with narrow, double sided black arrows with region description and corresponding accession

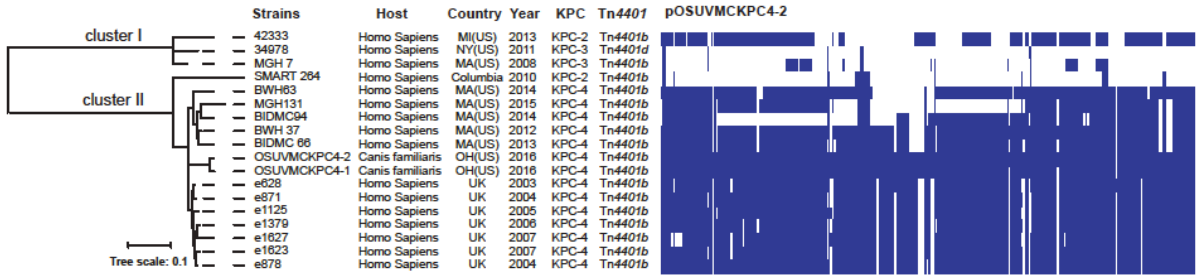
419 number below. Recombination sites, attC, of gene cassettes are illustrated as ovals with color

420 corresponding to the related gene. Integron gene cassettes are incorporated into the rectangular attI1

421 site corresponding to the class 1 integron.

422

423



424

425

426 **FIG 4** Heatmap of plasmids among KPC-4 bearing *Enterobacter* ST171 with additional KPC-2 and KPC-3

427 containing ST171 isolates included as references. (Left) Core SNP phylogenetic tree generated by RAxML.

428 (Center) Metadata, including host, isolation location, year, *Tn4401* isoform, and KPC allele. (Right)

429 Plasmid composition is illustrated by showing the BLASTn matches to each *Enterobacter* genome across

430 all of the genes on the pOSUVMCKPC4-2 plasmid.

431
432