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Characterization of *Klebsiella pneumoniae* complex isolates from pigs and humans in farms in Thailand: population genomic structure, antibiotic resistance and virulence genes

Thongpan Leangapichart[1], Kamonwan Lunha[2], Jatesada Jiwakanon[3], Sunpetch Angkititrakul[3], Josef D. Järhult[4], Ulf Magnusson[2] and Marianne Sunde[1]*

[1]*Section for Food Safety and Animal Health Research, Department of Animal Health, Welfare and Food Safety, Norwegian Veterinary Institute, Oslo, Norway;* [2]*Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden;* [3]*Research Group for Animal Health Technology, Khon Kaen University, Khon Kaen, Thailand;* [4]*Zoonosis Science Centre, Department of Medical Sciences, Uppsala University, Uppsala, Sweden*

*Corresponding author. E-mail: marianne.sunde@vetinst.no

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Objectives: To define characteristics of *Klebsiella pneumoniae* complex (hereafter KP) isolates from healthy pigs, farm workers and their household members in Thailand.

Methods: A total of 839 individual rectal swabs from pigs on 164 farms and 271 faecal samples of humans working on pig farms and persons living in the same household in Khon Kaen, Thailand were screened for gut colonization by KP. Genomic sequences were investigated for antibiotic resistance and virulence genes. Phylogenetic analyses were performed in addition to comparison with isolates from previous studies from Thailand.

Results: KP was detected in approximately 50% of pig and human samples. In total, 253 KP isolates were obtained: 39% from pigs, 34% from farmers and 26% from individuals living on the same farm but without animal contact. MLST revealed high genetic diversity with 196 different STs distributed over four phylogroups (Kp1 to Kp4). Low prevalence of ESBL-KP (7.5%) and colistin-resistant KP (3.2%) was observed among pigs and humans. Remarkably, four convergent MDR and hypervirulent strains were observed: one from pigs (ST290) and three from humans [ST35, ST3415 (strain 90CP1), ST17 (strain 90CM2)]. Sharing of KP clones among pigs and humans was identified for some STs including ST4788, ST661, ST3541 and ST29.

Conclusions: The study indicated a low prevalence of ESBL and *mcr* genes among KP isolated from pigs and healthy humans in Thailand and suggested the possibility of zoonotic transmission for a subset of circulating KP clones.

Introduction

Klebsiella pneumoniae (KP) is an important MDR (resistant to three or more antimicrobial classes) pathogen that has rapidly spread worldwide causing nosocomial and community-acquired infections.[1] High prevalence of third-generation cephalosporin (3GC), carbapenem and colistin resistance in KP has been reported, in addition, hypervirulent strains are circulating.[2,3] MDR and hypervirulent KP usually belong to separate clonal populations, but recent findings of strains exhibiting both antimicrobial resistance (AMR) and hypervirulence have been increasingly reported.[4] KP complex members can cause different infections in humans and animals.[5] Studies showing zoonotic transmission of Enterobacterales from companion animals, livestock and wildlife have been reported.[6] Transmission routes can occur via human-to-human contact, via close contact with animals and/or from other sources such as food and the environment.[7] However, to what degree members of the KP are exchanged between humans and animals and environmental compartments is largely unknown.

To date, there are few published reports on KP transmission between healthy animals and humans.[8] Here, we studied the genomic characteristics of KP based on antibiotic resistance and virulence and investigated possible transmission among pigs and humans in North-Eastern Thailand.

Materials and methods

Ethics

The study was conducted according to the Helsinki Declaration for the human subjects and the EU Directive 2010/63/EU for animal experiments; the protocol involving human participants and animals was approved by the Khon Kaen University Ethics Committee (Project ID: HE612268 and 0514.1.75/66, respectively). Pig samples were collected at the farms with the permission of the owner of the pig herd. All participants gave their informed consent.

Sample collection

Pig and human faecal isolates were obtained through a cross-sectional study carried out between September and December 2018 in Khon Kaen, Thailand.[9] Samples were cultured for KP isolation using Simmons citrate agar (SCA) containing 1% inositol (SCAI)[10] and antibiotic-enriched media including MacConkey agar (Oxoid, Hampshire, England) containing cefotaxime 1 μg/mL (Sigma-Aldrich, Saint Louis, USA), CHROMagar™ mSuperCARBA™, and CHROMagar™ COL-APSE (CHROMagar, Paris, France). Species were identified by MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany) and refined using whole-genome sequences.

Antimicrobial susceptibility testing (AST)

KP isolates were susceptibility tested by disc diffusion and by the use of the EUVSEC Plate (Trek diagnostics, Thermo Scientific, USA). The results were interpreted using EUCAST clinical breakpoints[11] with the exception of tetracycline and sulfamethoxazole that were interpreted according to CLSI guidelines (M100-ED30:2020).

WGS and analysis

WGS was performed using Illumina HiSeq 4000 (Illumina, San Diego, USA) platform or Illumina NovaSeq 6000 platform with 2 × 150 bp reads. Sequence assembly was performed with SPAdes version 3.13.0. An average coverage depth of 150-fold was achieved from both Hiseq and Novaseq platforms. Detailed microbiological methods and genomics analysis are presented in the Supplementary Materials and methods (Supplementary Materials and methods are available as Supplementary data at *JAC* Online).

Statistical analysis

Statistical analysis was performed with Microsoft Excel. Fisher's exact test was used with a P value <0.05 considered significant.

Availability of data and materials

Reads (fastq files) from the study have been submitted to the European Nucleotide Archive (www.ebi.ac.uk/ena) under study accession number PRJEB38540.

Results and discussion

This study investigated the natural ecology and genetic characteristics of KP in pigs and humans. KP was detected in approximately 50% of pig and human samples (Figure S1). A total of 253 KP were isolated in this study: 39% (n = 99/253) originated from pigs, 34% (n = 87/253) from contacts, and 26.4% (n = 67/253) from non-contacts (Table S1 and Figure S1). Most of them were isolated from SCAI agar (85%, n = 215/253), enabling growth of all KP variants and generating an unbiased strain collection. Since we used four different media on all samples, multiple isolates (identical and different clones) were isolated from the same sample as follows: 19 samples contained two different KP isolates (seven pooled pig samples/five contact samples/seven non-contact samples). Three human samples (one contact/two non-contacts) harboured three different KP isolates. In total, our findings showed that, from 15 humans, two or three clones were simultaneously present in the faecal content, demonstrating polyclonal KP carriage. Future studies on KP colonization should take this into account as investigation of one randomly chosen KP may only represent a minority of an individual's intestinal KP population.

The frequency of tetracycline and trimethoprim/sulfamethoxazole resistance and MDR in pig isolates was significantly higher than in human isolates (P < 0.05) (Figure S1). Unsurprisingly, high rate of tetracycline resistance was observed in pigs that may likely relate to an extensive use of tetracycline derivatives in pigs.[12] When isolates from SCAI agar were analysed separately, most of them (79%, n = 170/215) were susceptible to all antibiotics tested (ampicillin excluded) (Table S1).

Phylogenetic analysis revealed considerable genetic diversity with 196 STs clustering into four distinct phylogroups including Kp1 (n = 173, 68.3%), Kp2 (n = 34, 13.5%), Kp3 (n = 13, 5.1%) and Kp4 (n = 33, 13%) (Figure 1). Of 85 novel STs, ST4746 to ST4829 were submitted to the BIGSdb database (https://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html). Eighteen STs occurred in both animal and human hosts including the most dominating STs (≥4 isolates): the novel ST4788 (n = 9), followed by epidemic and international clones, ST661 (n = 6), ST17 (n = 5), ST35 (n = 5), ST3541 (n = 4), ST29 (n = 4) and ST1213 (n = 4).[13–15] Intermixing of pig and human isolates was observed to some degree. High genetic diversity was also observed when our isolates were analysed with 241 genomes from previous Thailand studies.[13,14] Some isolates displayed close relationship, including isolates from pigs, humans (clinical and carrier) and environmental reservoirs (Figure S2).

Resistome analysis of 253 isolates showed that *aad*A2, *aph*(3′)-*Ia*, *dfr*A1, *dfr*A12, *tet*(A), *tet*(C), *sul*1, sul3, *qnr*S1 and *fos*A5 were more common in pigs compared with humans (Table S2). Whereas *aac*(6′)-*Ib-cr* was found to be significantly more frequent in human isolates (P = 0.048). The prevalence of *fos*A7 was higher in non-contacts (11%) compared with pigs and contacts (3% each). No carbapenemase genes were detected. Moreover, our findings confirmed low prevalence of ESBL and colistin-resistant KP. Twenty isolates (7% of pig isolates and 7.8% of human isolates) carried at least one ESBL gene including $bla_{\text{CTX-M-15}}$ (n = 6), $bla_{\text{CTX-M-55}}$ (n = 4), $bla_{\text{CTX-M-3}}$ (n = 3), $bla_{\text{CTX-M-14}}$ (n = 2), $bla_{\text{CTX-M-40}}$ (n = 1), $bla_{\text{CTX-M-63}}$ (n = 1), $bla_{\text{SHV-27}}$ (n = 4), $bla_{\text{SHV-12}}$ (n = 1) and $bla_{\text{SHV-13}}$ (n = 1) (Table S1). A previous Thailand study showed that 5% of healthy pigs carried 3GC-resistant *Klebsiella* spp., which was comparable to our results (7.6%).[16] However, prevalence of 3GC-resistant KP in humans (7.8%) was relatively low when compared with the same study where 26.7% of farm workers carried 3GC-resistant KP.[16] This might be due to the different study areas, population density and the access to antibiotics. Several variants of *mcr* genes (*mcr*-1.1, -2.3, -3.19, -3.40, -3.5 and -8; Table S1) were detected in eight isolates showing colistin MICs from 8 to >16 mg/L. One contact carried two *mcr*-positive isolates: strain 90CP1 (ST3415) harbouring *mcr-1*; and strain 90CM2 (ST17) harbouring $bla_{\text{CTX-M-63}}$, *mcr-2.3* and *mcr-3.40*. Co-harbouring of $bla_{\text{CTX-M}}$ and *mcr* genes was found in two other isolates: strain 57PM1 (ST4770, pig) containing $bla_{\text{CTX-M-55}}$ and *mcr-3.5*; and strain 106CM1 (ST35, contact)

Figure 1. Phylogeny of core gene SNPs (372059 SNPs) from 253 *K. pneumoniae* isolates and distribution of their antibiotic resistance genes.

with $bla_{CTX\text{-}M\text{-}55}$ and *mcr-3.19*. This evidence is worrisome, since the spread of *mcr* or ESBL genes could be facilitated and transmitted to other pathogens and could spread to other hosts or environments.[17]

Additionally, diversity of capsular serotype (K-loci) and LPS serotype (O-loci) was investigated (Table S1). The most common K-loci were KL58 ($n = 15$; including 9 ST4788), KL107 ($n = 11$; numerous STs including 2 ST661), KL30 ($n = 10$; numerous STs), KL103 ($n = 8$;

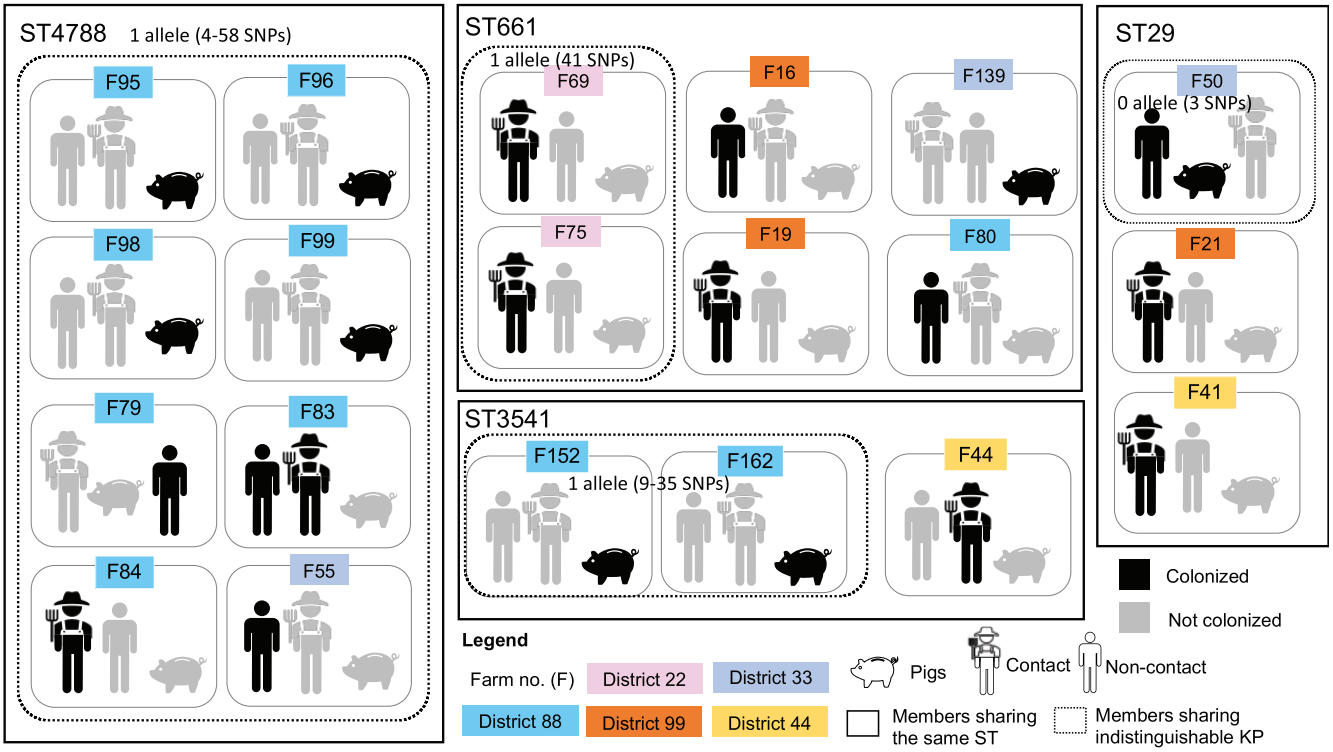

Figure 2. Faecal colonization and sharing of *K. pneumoniae* among pigs, farmers (contact) and household members (non-contact). Allelic and SNPs differences of identical clone were indicated. KP, *K. pneumoniae*.

including 4 ST1213) and KL123 ($n = 8$; including 4 ST3541). K-type-associated virulence factors were found only in human isolates; KL1 only in 1.2% ($n = 3$; ST23, ST3537, ST4760) and KL2 in 0.3% ($n = 1$; ST65). Genetic diversity of STs and K-loci made statistical comparison between pigs and humans impossible. However, a higher prevalence of O3/O3a in pig (21.2%) and O1v2 in human isolates (11.7%) was observed, which might imply host-associated specific serotypes[1] (Figure S3). In addition, the presence of virulence genes including main features identified in hypervirulent KP (*iuc, iro* and *rmpA*)[18] were rare, found in 28 isolates (0.4%–7% of all isolates, Figure S4). Among virulent KP, there were 12 isolates carrying *iuc*; 18 isolates with a single *ybt*+ (six of them had *ybt*+ and ICEKp). Interestingly, all nine ST4788 isolates, the most dominant ST (found in both pig and humans), were associated with KL58 and contained *ybt*+. Remarkably, four convergent MDR and hypervirulent strains[18] were observed: one from pigs (ST290) and three from humans [ST35, ST3415 (strain 90CP1), ST17 (strain 90CM2)] (Figure S4).

Possible transmission between reservoirs was investigated (Figure 2). Core-genome MLST (cgSTs) and core-genome SNPs (SNPs) analysis were calculated and described for each main ST (Figure S5). Isolates within ST4788 ($n = 9$) were identical to each other (one allelic difference), indicating a possible epidemiological relationship. No human–pig pair within the same farm was found. Furthermore, the evidence of inter-farm pig–pig or human–human sharing within the same district area was found in this study; three pig isolates within ST3541 (152PP1, 162PK, 162PP1) differed by one allele (9–35 SNPs apart), and human isolates within ST661 (69CK and 75CK) differed by one allele (41 SNPs apart). However, animal–human transmission was only identified in a human–pig pair on one farm, no. 50 (ST29, 3 SNPs apart), indicating limited zoonotic transmission. We did not observe related links between hosts for ST1213, ST17 and ST35 (Figure S5). However, a limitation of our study is that we were not able to fully explore the possibility of shared strains between pigs and humans as we only selected one colony per plate. Route and mode of transmissions are still unknown. Efficient monitoring systems are required to monitor prevalence of AMR bacteria and antimicrobial usage, which is essential for limiting future development of emergence of antibiotic resistance. There is a need for future studies based on larger scale such as comparing clones between countries with extensive antibiotic use and countries with restricted antibiotic usage. A global scale will be required to broadly assess the population of MDR KP and their possible virulence determinants.

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Transparency declarations

None to declare.

Supplementary data

Supplementary Materials and methods, Tables S1 and S2 and Figures S1 to S5 are available as Supplementary data at *JAC* Online.

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