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Norwegian patients and retail chicken meat share cephalosporin-resistant Escherichia coli and IncK/bla<sub>CMY-2</sub> resistance plasmids


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A B S T R A C T

Objectives: In 2012 and 2014 the Norwegian monitoring programme for antimicrobial resistance in the veterinary and food production sectors (NORM-VET) showed that 124 of a total of 406 samples (31%) of Norwegian retail chicken meat were contaminated with extended-spectrum cephalosporin-resistant Escherichia coli. The aim of this study was to compare selected cephalosporin-resistant E. coli from humans and poultry to determine their genetic relatedness based on whole genome sequencing (WGS).

Methods: Escherichia coli representing three prevalent cephalosporin-resistant multi-locus sequence types (STs) isolated from poultry (n = 17) were selected from the NORM-VET strain collections. All strains carried an IncK plasmid with a bla<sub>CMY-2</sub> gene. Clinical E. coli isolates (n = 284) with AmpC-mediated resistance were collected at Norwegian microbiology laboratories from 2010 to 2014. PCR screening showed that 29 of the clinical isolates harboured both IncK and bla<sub>CMY-2</sub>. All IncK/bla<sub>CMY-2</sub>-positive isolates were analysed with WGS-based bioinformatics tools.

Results: Analysis of single nucleotide polymorphisms (SNP) in 2.5 Mbp of shared genome sequences showed close relationship, with fewer than 15 SNP differences between five clinical isolates from urinary tract infections (UTIs) and the ST38 isolates from poultry. Furthermore, all of the 29 clinical isolates harboured IncK/bla<sub>CMY-2</sub> plasmid variants highly similar to the IncK/bla<sub>CMY-2</sub> plasmid present in the poultry isolates.

Conclusions: Our results provide support for the hypothesis that clonal transfer of cephalosporin-resistant E. coli from chicken meat to humans may occur, and may cause difficult-to-treat infections. Furthermore, these E. coli can be a source of AmpC-resistance plasmids for opportunistic pathogens in the human microbiota. E.S. Berg, Clin Microbiol Infect 2017;23:407.e9–407.e15 © 2017 European Society of Clinical Microbiology and Infectious Diseases. Published by Elsevier Ltd. All rights reserved.

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Introduction

Use of antimicrobials in human and veterinary medicine and in the livestock industry drives the emergence, selection and spread of bacterial resistance [1]. Food contaminated with antibiotic-resistant bacteria can be a source of resistance for human pathogens either by direct colonization of zoonotic bacteria, or by transfer of mobile genetic elements, e.g. plasmids, to other gut-colonizing bacteria [2].

The World Health Organization defines extended-spectrum cephalosporins as critically important antimicrobials for human medicine [3]. Many studies have shown that β-lactamase-producing Escherichia coli occurs in chicken meat [3,4]. Widespread use of cephalosporins in the broiler production pyramid and the international trade of breeding animals and hatching eggs enhance this into a global problem [5]. However, to what extent consumption of chicken meat contaminated by resistant bacteria affects human health has not been established [2,6].

With the exception of coccidiostats (not classified as an antibiotic in Europe), Norwegian broiler production has a very low usage of antimicrobials [7-10]. In spite of this, cephalosporin-resistant E. coli were detected in approximately one-third of the broilers and retail chicken fillets produced in Norway in the period from 2011 to 2014 [7-10]. A recent study showed that cephalosporin-resistant E. coli originating from Norwegian chicken fillets had low genetic diversity, mainly limited to a few multi-locus sequence types (STs), and typically carried the plasmid-mediated AmpC (pAmpC) blaCMY-2 gene on an IncK plasmid [11]. The poultry-associated E. coli had many of the same virulence factors as human extraintestinal pathogenic E. coli (ExPEC), which could facilitate human colonization and extraintestinal infections [11].

To investigate if poultry-associated strains existed among human clinical E. coli isolates with pAmpC production, we screened isolates from microbiology laboratory and national strain collections. Isolates that were PCR positive for both IncK and blaCMY-2 were selected for whole genome sequencing (WGS). The aim of the study was to compare human- and poultry-associated cephalosporin-resistant E. coli by use of single nucleotide polymorphism (SNP) analyses to determine the genetic relatedness of selected AmpC-producing strains from the two reservoirs with the highest possible resolution.

Materials and methods

Bacterial isolates

The scheme for selection and analysis of the isolates in the present study is illustrated in Fig. 1.

Escherichia coli isolates from broilers. The poultry-associated E. coli strains were a subset of isolates originally collected from retail chicken meat (fillets) in 2012 (n = 4) and 2014 (n = 10) and from chicken faecal samples in 2011 (n = 3) as a part of the Norwegian monitoring programme for antimicrobial resistance in the veterinary and food production sectors (NORM/NORM-VET) [7-11]. The monitoring programme reported the following prevalence of pAmpC-positive E. coli isolates from broilers: 43% (108/252) from intestinal flora of healthy broilers in 2011, and from chicken fillet samples 32% (66/205) and 29% (58/201) in 2012 and 2014, respectively. Briefly, the 17 isolates selected for this study belonged to phylogroup D and carried similar conjugative IncK plasmids encoding blaCMY-2. The strains were typed to ST38 (n = 10), ST1158 (n = 5) and ST115 (n = 2). ST38 and ST1158 were the two most common STs among the...
cephalexin-resistant E. coli isolated from Norwegian chicken meat in 2012 and 2014 [11]. Furthermore, molecular characterization of the chicken isolates identified virulence factor genes (e.g. iroN, cma and iss) similar to those of ExPEC [11].

**Human clinical E. coli isolates.** The human clinical E. coli consisted of two collections of AmpC-producing ExPEC isolates. The first collection (A) included 158 isolates with a blaCMY-positive genotype tested as described by Brolund et al. [12]. These isolates were from Norwegian clinical microbiology laboratories sampled during 2010–2012 and originally submitted for verification of the presence of pAmpC at the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance. Submission criteria were resistance to cefotaxime (MIC >2 mg/L) and ceftazidime (MIC >4 mg/L) in combination with multi-drug resistance (MDR) (defined as resistance to at least two of the following groups of antibiotics: aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole or nitrofurantoin). The second collection (B) included 126 E. coli isolates displaying an AmpC phenotype identified at nine Norwegian clinical microbiology laboratories during 2013–2014. The phenotypic AmpC profile was determined according to EUCAST [13]. None of the isolates displayed classical serine extended-spectrum β-lactamase production. The original sample materials for the 284 human clinical E. coli isolates (of which 84% were from UTIs) are presented in Fig. 1.

**Ethical considerations**

This study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics (REC) [ref. 2014/419/REK sare-st].

**Molecular characterization**

**PCR screening.** All human clinical isolates were screened for the presence of IncK plasmids and for the blaCMY-2 gene. Twenty-nine E. coli isolates harboured both targets. The real-time PCR targeting the IncK replicon was based on previously published IncK primers [14]. The blaCMY-2 target was amplified in triplex-PCR format similar to a previously published real-time pAmpC PCR [12]. Further technical details for the PCR assays are presented in the Supplementary material (Appendix S1).

**Whole genome sequencing (WGS).** Human clinical (n = 29) and poultry (n = 17) isolates PCR-positive for blaCMY-2 and IncK were subjected to WGS. Paired-end genomic libraries with insert size 500 bp were made from approximately 100 ng purified DNA and sequenced on an Illumina HiSeq 2500 system (BGI Tech Solutions Co., Ltd., Hong Kong). Sequencing was performed twice for two poultry isolates (2011-01-2112 and 2012-01-1292) and one clinical isolate (E4-14) to produce WGS reproducibility controls.

**Bioinformatic analysis of WGS data**

**Genome assembly and in silico genotyping.** The raw sequence data were initially trimmed and cleaned for adapters (BGI Tech Solutions). The paired-end reads were further cleaned for errors and de novo assembled into contigs (draft genomes) by the SPAdes-3.6.1 pipeline using default settings [15]. The contigs were submitted to the Centre for Genomic Epidemiology (CGE), Technical University of Denmark (DTU), for bioinformatics analysis (https://cge.dtu.dk/services/).

**Identification of SNPs and phylogenetic analysis.** The paired-end read sets, error-corrected by SPAdes, were uploaded to CSI Phylogeny 1.1 [16] on the DTU server by use of the default minimum settings (https://cge.dtu.dk/services/). The assembled scaffold of one of the poultry isolates (2012-01-1292) was used as the reference sequence by the mapping of the read sets. A selection of E. coli reference genomes downloaded from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genome/167) was included in the analysis to limit the SNP calling to the phylogenetic informative genomic sequences assumed to be representative for the E. coli core genome [17]. The rationale was that only the evolutionarily conserved sequences would remain as shared core genome sequences after the sequence alignment of highly different E. coli strains [18]. A final maximum-likelihood tree was constructed using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

**IncK plasmid assembly and plasmid SNP analysis.** Draft sequences of the IncK plasmid were made by mapping of the trimmed read sets against several IncK plasmids available from GenBank using BiNumerics, version 7.5 (Applied Maths, Sint-Martens-Latem, Belgium). The best match was obtained using the poultry-associated strain 53C unamended 3 IncK plasmid with blaCMY-2 assembled by de Been et al. as reference (Accession no. NZ_JXM01000007.1) [https://www.ncbi.nlm.nih.gov/nuccore/NZ_JXM01000007.1?report=GenBank] [19]. Finally, paired-end read sets from each isolate were uploaded to CSI phylogeny for SNP-based plasmid similarity analysis using the 53C IncK plasmid as reference.

**BEAST simulation.** To estimate a timeline for the diversification of the most closely related isolates of poultry and human origin, Bayesian Evolutionary Analysis Sampling Trees, BEAST version 1.7 software was used with input from CSI Phylogeny analysis of the 15 most closely related isolates [20]. A basic assumption for the time estimation was that any SNPs located in recombination sites of the E. coli genomes had been removed in the CSI Phylogeny analysis, i.e. by pruning the SNPs. Among several models of evolution, the chosen model with the best fit assumed an uncorrelated relaxed molecular clock, expansion growth in population size and a lognormal distribution of the mutation rate. All the BEAST Monte Carlo Markov Chain simulations were run for 150 million steps, and samples were saved every 10 000 steps. A single maximum clade credibility tree was produced using the TreeAnnotator tool in the BEAST pipeline, where 10% of the Monte Carlo Markov Chain steps were removed as burn-in. A final tree was constructed using FigTree.

**Results**

In total, 29 of the 284 clinical E. coli isolates were PCR positive for both blaCMY-2 and IncK. In collection A, consisting of 158 genotypic blaCMY-2-positive MDR clinical isolates, 16 carried IncK plasmids (14 isolates from UTIs, one isolate from bloodstream infection, and one isolate from an unknown body site). In collection B with 126 phenotypic AmpC-positive clinical isolates, 53 isolates were blaCMY-2-positive and 13 of these were also IncK positive (11 isolates from UTIs, one isolate from a perianal abscess and one from an unknown body site) (see Supplementary material, Table S1, Appendix S1).

WGS-based in silico genotyping of the 29 IncK/blaCMY-2-positive isolates revealed a diversity of other plasmid replications (e.g. IncFIB, IncFII and IncI1), acquired antibiotic resistance genes (e.g. blaTEM-1B, sul1/2 and strA/B), STs (e.g. ST38, ST69 and ST131), serotypes (e.g. O7:H18, O17/O77:H18 and O25:H4) and virulence factors (e.g. iroN, cma, iss, eilA and iut) (see Supplementary material, Appendix S1, Table S2 and Fig. S2). A subset of the human isolates (n = 7), all from UTIs, showed genotypic features similar to the most prevalent poultry-associated E. coli strains (ST38, phylogroup D, serotype O7:H18, carriage of IncK, IncFIB-, IncFII- and pO111 plasmids, and iroN, cma, iss and eilA virulence factor genes) [11] (see Supplementary material, Appendix S1, Table S2 and Fig. S2).
The CSI Phylogeny pipeline identified 56,610 valid SNP positions in the shared core genome of 2.51 Mbp of the 29 clinical isolates, the 17 poultry strains and the 23 NCBI reference strains. The phylogenetic analysis revealed that the majority of the 29 clinical isolates were genetically diverse and not closely related to the poultry isolates (Fig. 2). However, from the subset of the seven ST38 UTI isolates, five clustered together with the ten ST38 poultry isolates (Fig. 3). The number of SNP differences between these UTI and poultry isolates was only 1–13 (Fig. 2 and see Supplementary material, Appendix S1 and Fig. S2). The remaining ST38 clinical isolates, E2-21 and E4-13 had 34 and 529 SNP differences, respectively, compared with the ST38 reference poultry isolate (Fig. 2 and see Supplementary material, Appendix S1 and Fig. S2). In contrast, pairwise comparison of any of the ST38 poultry isolates to the non-

Fig. 2. Genome single nucleotide polymorphism (SNP) differences in human clinical Escherichia coli isolates compared with one AmpC-producing poultry E. coli (ST38) strain collected from Norwegian retail chicken fillets. Multiple sequence alignment resulted in 56,610 valid SNPs in the core genome of 2.51 Mbp positions shared by all strains. The data labels show the span in SNP differences within the group of isolates.

Fig. 3. Dendrogram after whole genome comparison with single nucleotide polymorphism (SNP) calling and phylogenetic analysis of Escherichia coli isolates from human clinical infections, poultry samples and a selection of GenBank E. coli reference genomes retrieved from GenBank. The colours of the boxes reflect the origin of the isolates as indicated. The multilocus sequence types (STs) of the included poultry isolates and of isolates of clinical relevance are indicated on the figure. STs of all investigated isolates can be found in supplementary material. The length of the scale bar represents the average number of substitutions per site at the variable sites.
ST38 human clinical isolates as well as to the NCBI reference E. coli genomes, resulted in 4000–10 600 SNP differences. The poultry strains clustered in three genetically diverse groups according to their STs and showed few SNP differences within each group (ST38 group up to eight SNP differences, ST1158 group up to five SNP differences, and ST115 group one SNP difference) (Figs. 2 and 3). For the three isolates that were sequenced twice as WGS reproducibility controls, no SNP differences were observed between each pair (see Supplementary material, Appendix S1 and Fig. S2).

Mapping of the paired-end read sets to the 53C unnamed 3 IncK plasmid sequence re-constructed by de Been et al. [19] showed that the 29 clinical isolates and the poultry strains had IncK plasmid variants highly similar to the 53C reference plasmid (85.9 kb) albeit with a size range of 80–85 kb. The closely related human clinical and chicken isolates as well as the rest of the clinical and poultry isolates shared an IncK plasmid backbone with a 79-kb core sequence. The CSI phylogeny analysis clustered the IncK variants from most of the isolates into one large group whereas the five ST1158 poultry isolates formed a separate group (see Supplementary material, Appendix S1 and Fig. S3).

The genome sequences of the closely related clinical ST38 UTI isolates (n = 5) and the poultry strains (n = 10) were subjected to a new CSI Phylogeny analysis to produce data input to the BEAST simulation. The SNP alignments were used as input to the BEAST analyses that estimated the point in time for the diversification of the ancestor of the 15 closely related isolates to year 2005 with a mutation rate of 0.0098 mutations/genome/year (Fig. 4). The final tree illustrates that the five clinical UTI E. coli isolates belonged to three different clusters (Fig. 4). Apparently, the genetic diversity within this group of UTI isolates was within the same order of magnitude as the genetic diversity between the UTI and poultry isolates.

Discussion

The aim of this study was to use WGS-based SNP analysis to achieve the best discriminatory power by the determination of the genetic relatedness of a selected group of AmpC-producing human clinical ExPEC isolates compared with cephalosporin-resistant E. coli found in retail chicken fillets. To focus the investigation on isolates that might have received their AmpC-resistance from poultry-associated E. coli, the presence of IncK and blaCMY-2 was used as a selection criterion for the human clinical isolates [11]. The PCR screening showed that 29 of the 284 AmpC-producing human clinical isolates were IncK/blaCMY-2-positive. This indicates that the majority (n = 182) of our blaCMY-2-positive clinical ExPECs have acquired the pAmpC from other sources than the cephalosporin-resistant E. coli found in chicken fillets and were therefore unrelated to the chicken meat E. coli. Sequence analysis after WGS confirmed that all of the 29 clinical isolates selected in the screening carried a plasmid backbone with high similarity to the IncK/blaCMY-2 plasmid in the poultry-associated E. coli.

The WGS-based in silico low-resolution E. coli genotyping of the 29 clinical strains showed that most of these ExPEC isolates were distantly related to the poultry E. coli. However, seven of them, all from UTIs, belonged to ST38. The dominating cephalosporin-resistant E. coli isolated from the Norwegian retail chicken fillets also belonged to this ST [11]. Several European studies have shown that ST38 is associated with human UTIs, and in addition, may occur as a contaminant of chicken meat [21–25]. The phylogenetic SNP-based analysis provided higher resolution than multilocus sequence typing and showed that only five of the seven clinical ST38 ExPEC isolates were nearly identical to the dominating ST38 E. coli isolates collected from poultry (n = 10). The high-resolution comparisons of informative SNPs within the multiple sequence

Fig. 4. Bayesian Evolutionary Analysis Sampling Trees (BEAST) analysis of the closely related human and chicken Escherichia coli isolates. Yellow boxes; poultry isolates, pink boxes; urinary tract isolates.
alignment-defined common ‘core’ genome (2.5 Mbp) of the reference strains, the poultry and human isolates revealed very few SNP differences (<15) between these 15 ST38 isolates. Hereby, the SNP-based phylogenetic result strongly suggests a link between these isolates. Extrapolation and comparison of our findings to similar studies using E. coli core genomes derived from proteome analyses indicate that our ten poultry and five UTI isolates were as closely related as would be expected for clones in a foodborne E. coli outbreak [6,19]. With the assumption that the isolates were clonal, the BEAST analyses determined the time when these 15 human and poultry E. coli isolates diverged from a common ancestor to year 2005. The BEAST output showed that the five clinical E. coli isolates were positioned among the poultry isolates in three different clusters. Furthermore, the diversity within these human ExPEC isolates was in the same low order of magnitude as the diversity between the human and poultry E. coli isolates.

A limitation of our study is the lack of epidemiological data of patient exposure to chicken meat to strengthen the hypothesis of resistance transmission from poultry to human. Alternatively, data of exposure to other potential sources of antimicrobial resistance could weaken the hypothesis. In addition, the BEAST analysis that was based on only 15 isolates collected over a short time period could not show the evolution of the strains in the two compartments over time. Another limitation of our study is the narrow focus on comparison of SNPs in E. coli from the two reservoirs without a view to identify the genes harbouring the SNPs to see a potential biological significance or to study the evolution of ST38 ExPEC. Consumption of chicken meat contaminated with cephalosporin-resistant E. coli can be a driver for increasing occurrence of pAmpC-positive pathogens causing human infections. Previous findings have demonstrated the same plasmid-mediated resistance genes (e.g. blaCTX-M, blaKPC, blaTEM and blaCMY-2), the same mobile genetic element, and highly similar genotypes of E. coli in humans and retail chicken meat. These studies have indicated a common link between these reservoirs [21,25–30]. The WGS-based genome comparisons enabled us to investigate if transmission of resistance may occur by whole bacterial clonal transfer between poultry and humans. Furthermore, the high-resolution sequence comparison also enabled us to investigate if the resistance transmissions may have occurred by horizontal dissemination of plasmids between bacteria. The results of this study provide support for the hypothesis that clonal transmission of AmpC-producing E. coli between poultry and humans may occur. Our results based on the SNP comparisons and the BEAST analysis showed that certain ST38 strains from the two reservoirs are very closely related and that they may have a common ancestor. Since the strains are highly similar, we cannot firmly decide in which direction the transmission between the two reservoirs occurred. However, contaminated poultry meat is a recognized source for infections with other zoonotic bacteria such as Campylobacter. This strengthens the hypothesis that a recent clonal transfer of resistant E. coli might have occurred from a poultry reservoir to human hosts. With the low number of SNP differences (<6 SNPs per Mbp core genome) and close genetic relatedness between the ST38 strains from human and poultry, our results differ from the data from the Netherlands [19]. De Been et al. identified 1263 SNP differences per Mbp core genome between their most closely related human and poultry isolates collected in 2006–2011 [19]. The dissimilar results of the two studies may be explained by different sampling strategies and selection of isolates.

We found similar IncK plasmid variants with blaCMY-2 both in the clonally related human clinical isolates (n = 5) and the poultry strains (n = 10), and in the rest of the clinical isolates (n = 24) and the poultry strains (n = 7). This suggests that horizontal transfer of resistance plasmids seems to occur with higher frequency than clonal transmission of resistant bacteria. Our results are in line with the results from other studies, and supports that plasmids are significant vectors for antimicrobial resistance dissemination [19,31]. Building an IncK/blaCMY-2 plasmid scaffold by positioning the WGS read sets against the 53C unnamed 3 IncK plasmid (86.0 kb), resulted in a common plasmid backbone sequence of 79 kb for all of our 29 ExPEC isolates. This plasmid backbone might be a part of many of the variants of blaCMY-2-containing IncK plasmids detected in the E. coli population of broilers in parts of Europe during the last few years [11,19,21–25,32]. Notably, in addition to blaCMY-2, the poultry-associated IncK plasmid backbone has two plasmid addiction systems that ensure stable maintenance of the plasmid within each of the daughter cells after cell division [11,33]. Plasmid addiction systems increase the potential for persistence regardless of any antimicrobial selection pressure. In addition, they may increase the potential for resistance plasmid transmission to opportunistic pathogens or to other bacteria in the human and poultry gut microbiota or in environmental reservoirs [33].

Even if only a small fraction of pAmpC-producing ExPEC in human infections is attributable to contaminated food, this is a significant public health concern [6]. Our approach based on targeted selection of resistant isolates for WGS-based high-resolution comparisons supports that transmission of antimicrobial resistance may occur both by clonal transfer of E. coli from poultry to humans and more frequently by the transfer of plasmids to pathogenic E. coli adapted to humans.

In conclusion, 20% of the 29 selected IncK/blaCMY-2-positive ExPEC isolates showed very close relatedness to cephalosporin-resistant E. coli found in retail chicken fillets. The remaining 80% of the clinical isolates exhibited a pAmpC vector highly similar to the IncK plasmid carried by the cephalosporin-resistant E. coli associated with poultry. This study supports the hypothesis that transfer of antimicrobial resistance may occur between poultry and humans both by clonal transmission and, more frequently, by plasmid dissemination.

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Transparency declaration

The authors have no conflict of interests.

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Authors contribution

ESB, ALW, MST, ØS, URD and MSu designed and conceived the study. ESB, ALW, SSM, JSS, ØS, NG, CSS, IHL, SBJ, ST and MSu performed initial strain selection and analysis. ESB, JA, SSM, JSS, MS,
Appendix A. Supplementary data

Additional Supporting Information may be found in the online version of this article at http://dx.doi.org/10.1016/j.cmi.2016.12.035.

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