The Zoonotic Potential of Poultry-Associated Nitrofurantoin-Resistant *Escherichia coli*

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Abstract

Escherichia coli is the most common cause of urinary tract infection (UTI) in humans. Nitrofurantoin is the most frequent UTI therapy in the United Kingdom. Here we identify poultry as a potential zoonotic reservoir of nitrofurantoin resistant (NFT-R) and pre-resistant (one mutation away from NFT-R) *E. coli*. We found NFT-R *E. coli* excreted by dogs fed a raw meat diet, as well as NFT-R and pre-resistant *E. coli* contaminating chicken meat sold for human consumption and chicken-based raw dog food. By whole genome sequence analysis, with reference to additional data from public repositories, we identified NFT-R or pre-resistant *E. coli* clones. These were ST919/8874, ST665, ST6805, ST69, ST752 and ST7529, which were dominated by isolates from poultry sources in Europe, though the ST752 clone is globally disseminated. Five of these clones included isolates from human clinical samples, of which several were confirmed urinary isolates. Our findings have important implications for public health and domestic hygiene, particularly for people being treated for UTI. They also increase concerns about the roles of raw-fed dogs as vectors for the entry of resistant *E. coli* into the home. It is likely that nitrofuran use in poultry production was the initial driver for the emergence of NFT-R on poultry farms. Therefore, whilst NFT-R *E. coli* sample-level positivity in poultry meat was far lower than resistance to more recently used fluoroquinolones, NFT-R and pre-resistant *E. coli* appear to have persisted among poultry flocks despite 30 years of zero legal use in Europe.

Introduction

Nitrofurantoin is the recommended first line treatment for uncomplicated urinary tract infection (UTI) in adults in the United Kingdom, most of which are caused by *Escherichia coli*.[1] Despite widespread use, nitrofurantoin resistance is rare.[2]

The ability of nitrofurantoin to kill relies on two oxygen-insensitive nitroreductases produced in the bacterium, NfsA and NfsB, which reduce nitrofurantoin to liberate highly reactive intermediates.[3] Nitrofurantoin resistance occurs predominately by loss of function mutations, deletions or insertional inactivation of both *nfsA* and *nfsB*, and in rare cases, by mutation of *ribE* or carriage of the plasmid-mediated *oqxAB* efflux pump genes.[2,4-6] We have recently identified the presence of nitrofurantoin "pre-resistant" urinary *E. coli*, where one gene (more commonly *nfsA*) is disrupted, but the other remains intact.[2]

The requirement for mutations in two genes – *nfsA* and *nfsB* – may form a barrier to nitrofurantoin resistance emerging among *E. coli* in an infection[7] and there is currently no evidence for nitrofurantoin-resistant (NFT-R) *E. coli* clones circulating in the human population.[2] If such clones emerge, this could spark a rise in NFT-R UTIs. The primary aim of this work, therefore, was to consider the possibility of animal reservoirs of NFT-R or pre-resistant *E. coli* with the potential to transfer into the human population.

Nitrofurans were widely used in food producing animals, particularly poultry, until the 1990s when the European Union banned this due to the risks associated with toxic chemical residues in meat. Similar bans are in place in the USA, Australia, the Philippines, Thailand and Brazil.[8] In some countries, nitrofurantoin is used to treat ornamental birds and fish, and as an off-label therapy for recurrent UTI in dogs, cats

and horses[9], however, in the United Kingdom, nitrofurantoin is not licensed for use in any animal.[10]

Surveys of NFT-R bacteria from animals are scarce, and in the few studies that have considered NFT-R *E. coli*, resistance was very rare or non-existent.[11-14], but emergence of NFT-R *E. coli* in dogs during treatment of UTI has been reported.[15]

We have established a 50 x 50 km study region in the South West of England in which we have sequenced resistant *E. coli* from human infections[2,16-18], and from the faeces of farmed[17,19], companion[20,21], and zoo animals.[22] In the work reported here, we investigated whether NFT-R *E. coli* are excreted by dogs in our study region or are found on meat being sold in stores in the area, and used whole genome sequence (WGS) data to test whether these NFT-R *E. coli* are shared across One Health compartments, including human infections. In so doing we have identified that there is a zoonotic reservoir of NFT-R and pre-resistant *E. coli* in poultry and in dogs fed raw meat.

Results

NFT-R E. coli excreted by dogs being fed raw (uncooked) meat.

We tested faecal samples from 600 healthy dogs living in our 50 x 50 km study area.[20] Two dogs living in the city of Bristol excreted NFT-R *E. coli* at detectable levels. Both NFT-R *E. coli* had loss of function mutations in *nfsA* and *nfsB*; one was ST6805 and carried horizontally-acquired genes conferring resistance to multiple antibiotics, the other was ST919 and did not carry any acquired resistance genes (**Table 1**).

We collected owner-completed survey data about the lives of the 600 dogs.[20,21] Since only two dogs excreted NFT-R *E. coli* it is not possible to be confident about any statistical assessment of associations between excretion and any lifestyle practice, as determined from the owner-completed surveys. Nonetheless, we noted (**Table S1**) that both dogs were fed raw meat at the time of faecal sampling, which was a practice observed for only 31/297 dogs living in the city of Bristol (Fisher Exact p=0.011).

NFT-R E. coli in uncooked chicken meat and chicken-based commercial raw dog food.

In the United Kingdom, raw meat fed to dogs is sourced as commercial raw dog food (RDF) or meat sold primarily for human consumption (once cooked).[23] Accordingly, to investigate whether meat potentially being fed to dogs in the city of Bristol was contaminated with NFT-R *E. coli* we tested 73 samples of meat from across the city: 15 packets each of beef, chicken and lamb, and 13 of pork purchased from large chain grocery stores, and 15 packets of chicken-based commercial RDF of various brands.[24]

Two out of 58 (3%) samples of meat from large chain grocery stores tested positive for NFT-R *E. coli*; both were chicken meat. WGS showed that the NFT-R *E. coli* had loss of function mutations in *nfsA* and *nfsB* and were ST665 (carrying multiple antibiotic resistance genes) and ST8874 (carrying none) (**Table 1**). One out of fifteen (7%) packets of chicken-based commercial RDF was positive for NFT-R *E. coli.* WGS showed this to be ST8874, the same ST found in one packet of chicken meat.

Despite their different origins, the two isolates carried the same mutations in *nfsA* and *nfsB* and both lacked mobile resistance genes (**Table 1**).

Poultry-associated NFT-R and NFT pre-resistant E. coli clones found across multiple countries and One Health compartments, including human clinical specimens

The NFT-R ST8874 (n=3) and ST919 (n=4) isolates found on meat or excreted by one dog in Bristol had the same mutations in *nfsA* and in *nfsB* and none of them carried acquired resistance genes (**Table 1**). We hypothesized that these STs are closely related and using multi-locus sequence typing found all except one of the alleles (*adk*38 in ST8874 versus *adk*861 in ST919) were identical across the two STs.

There was WGS data for only one ST8874 isolate on Enterobase (search date, 2nd July 2024) and 34 of ST919. The ST8874 isolate (Accession SAMN12241725) came from a chicken yoke sack infection in Czechia[25] and has the same *nfsA* and *nfsB* mutations found in the Bristol ST919/ST8874 isolates (**Table 1**). Of the 34 Enterobase ST919 isolates, 56% were from poultry or wildfowl sources and three had *nfsA* and *nfsB* mutations identical to the Bristol ST919/ST8874 isolates (**Table 1**). Two were of poultry origin, one from the United Kingdom (SAMN11444779) and one from Canada (SAMN32247434). The third (SAMEA7025515) is a published NFT-R *E. coli* from a human UTI from London, United Kingdom,[6] which is approximately 200 km from Bristol.

The Bristol ST8874 isolates and the Czech chicken isolate were very closely related to each other (<65 SNPs across a core genome alignment), and to the ST919 United Kingdom chicken isolate on Enterobase (<120 SNPs) which was only 57 SNPs

different from the NFT-R human urinary isolate from London. The remaining ST919 isolates were variable in their relatedness with the most diverse being from a Bristol dog (**Fig. 1**).

Among 94 ST665 sequences on Enterobase there was a source bias of 41% poultry and 10% human, including two isolates from the United Kingdom. One of these (SAMN21845675) had the same *nfsA* and *nfsB* mutations as the NFT-R ST665 isolate from uncooked chicken meat in Bristol, and the same seven mobile resistance genes (**Table 1**), with an additional *dfrA1*. The two isolates were 2394 SNPs, apart.

Of the seven other human ST665 isolates on Enterobase, multiple isolates from across Europe and into the Middle East had loss of function mutations in *nfsA* but wild-type *nfsB*, and so are considered nitrofurantoin pre-resistant.[2] In poultry ST665 isolates, *nfsA* loss but with a wild type *nfsB* was also seen in multiple countries, with, in some cases, identical *nfsA* mutations to those seen in human isolates (**Table S2**).

Among four ST6805 isolates on Enterobase, one Danish poultry isolate (SAMEA112328101) shared the same *nfsA* mutation as a Bristol dog isolate (**Table 1**), but it had a wild type *nfsB*, hence is also considered pre-resistant. These two isolates are 2281 SNPs apart.

Based on the above findings, we suspected that there might be additional nitrofurantoin pre-resistant clones present on chicken meat being sold in Bristol. To test this, we sequenced 92 nitrofurantoin susceptible (NFT-S) *E. coli* from RDF and 76 from uncooked chicken meat, cultured from the same samples as the NFT-R isolates (above) and found 17 isolates from 12/15 samples of chicken meat and 10 isolates from 4/15 samples of chicken-based RDF, had obvious loss of function in

nfsA but wild-type *nfsB*, and so are defined as pre-resistant. These isolates, accounting for 16.1% of all NFT-S isolates from chicken meat or RDF sequenced, spanned 11 STs (**Table S3**). They included ST665 isolates from two chicken and one RDF samples with identical *nfsA* mutations, and pairwise SNP distances ranging from 84 to 880, reminiscent of the NFT-R clones described above. Furthermore, pre-resistant ST69 isolates, with identical *nfsA* mutations from six chicken meat and one RDF samples were identified with SNP distances of 10 to 41.

Evidence of a more longstanding nitrofuoranotin pre-resistant clone having *nfsA* loss of function was also seen for ST752, where isolates from different samples of chicken meat in Bristol were separated by in some cases as little as 88 SNPs (**Table S4**). Of ST752 isolates on Enterobase (n=503), the majority were from poultry (33.0%) or human (28.2%) sources. Of these, 91.9% of human isolates and 84.9% of poultry isolates carried the same *nfsA* mutation as also seen in the Bristol chicken meat isolates suggesting they are nitrofurantoin pre-resistant. We selected ST752 *nfsA* mutant Enterobase isolates from poultry and humans (n=10 for each) for comparison with the six isolates from chicken meat in Bristol. Two human isolates, one from the United Kingdom and the other from the Netherlands were 43 and 61 SNPs different, respectively, from one of the Bristol chicken meat isolates (**Table S4**). Furthermore, there were four incidences where ST752 isolates from humans were <100 SNPs from Enterobase poultry isolates, with the closest pair being 48 SNPs apart (**Table S4**).

Finally, for ST7529, a human clinical Enterobase isolate from the United Kingdom was found to be 103 SNPs different from the two Bristol RDF isolates, which were themselves identical. All carried the same *nfsA* loss of function mutation (**Table S3**).

Discussion

Because dogs in the United Kingdom are almost never treated with nitrofurantoin, we were surprised to find NFT-R *E. coli* in faeces excreted by two dogs out of a cohort of 600 based on testing one faecal sample per dog with a limit of detection of approximately 1000 viable bacteria per gramme of faeces. The NFT-R *E. coli* are clonally related to those found in poultry meat and poultry farm environment samples in the United Kingdom, Canada and Czechia. Given that we also found NFT-R *E. coli* in uncooked chicken meat, and chicken-based RDF purchased in the same city, the most likely explanation for these dogs excreting poultry-associated NFT-R *E. coli* is the fact – reported by their owners – that they were being raw fed at the time of sampling. Whilst we have shown compelling evidence that feeding raw meat to dogs is associated with them excreting fluoroquinolone resistant *E. coli*,[21] the evidence for NFT-R *E. coli* presented here is much weaker. This may be related to the relative degrees of contamination of meat with *E. coli* resistant to these different agents. For example, in parallel, we identified here 10% (3/30) of chicken meat or chicken-based RDF samples were positive for NFT-R *E. coli*, but 47% (14/30) of these same samples were positive for fluoroquinolone-resistant *E. coli*.[24]

A number of studies have reported sharing of resistant *E. coli* between dogs and their owners[26-29], so it is possible that dogs excreting NFT-R *E. coli* may pass these on to their owners. This is in addition to any associated risks of accidental ingestion when handling and preparing RDF or other uncooked poultry, which we have shown here can be contaminated with NFT-R or pre-resistant *E. coli*. Accordingly, it would seem prudent for those at risk of UTI, and particularly those who are taking

nitrofurantoin, to be cautious when handling uncooked meat or interacting with pets fed uncooked meat, and practice enhanced hygiene regimes. This is clearly sound advice for people taking other antibiotics, and particularly ciprofloxacin, but since the number of nitrofurantoin courses dispensed in the community in some countries is very high, and since resistance rates among urinary *E. coli* from humans are very low, the additional zoonotic burden exposed here is potentially important. Perhaps the most significant example of zoonotic antibiotic resistance transmission to be uncovered since the identification of porcine-associated *mcr*-positive *E. coli* with reduced colistin susceptibility in China.[30]

Whilst nitrofuran use in poultry production in many countries globally has been banned for decades due to toxicity concerns if found in meat,[11] it is likely that NFT-R and pre-resistant poultry-associated *E. coli* identified here evolved a long time ago. This is apparent due to the range of SNP distances between isolates from the various clones reported here.

It still remains unclear how prevalent NFT-R or NFT pre-resistant *E. coli* are in meat sold for human consumption around the world, and whether other poultry-associated clones exist, but the findings of this study should stimulate interest in answering that question through widespread surveillance in a number of One Health compartments.

Methods

Canine faecal sample collection and processing

Ethical approval was obtained from the Faculty of Health Research Ethics Committee, University of Bristol (ref: 89282) alongside Ethical Approval of an Investigation Involving Animals (ref: UB/19/057). Details of canine faecal sample

collection are published[20,21], but briefly, 303 rural dogs and 297 urban dogs were recruited at local dog-walking sites between September 2019 and September 2020. All owners gave informed consent to participate. Faecal samples were collected and a standardized questionnaire was provided to collect demographic data and variables chosen as being potentially associated with carriage of ABR *E. coli* in dogs. Samples were refrigerated and processed within 48 h. Faecal samples were weighed (0.1–0.5 g) and PBS was added at 10 mL/g before vortexing and mixing with an equal volume of 50% sterile glycerol. Samples were stored at -75°C.

Meat sample collection and processing

Details of meat sample collection and processing are published[24], but briefly, we sampled beef, chicken, lamb and pork meat collected from large chain grocery stores in the city of Bristol between October and November 2022, totalling 15 samples per type of meat, except for pork where due to availability only 13 were collected. Samples were either processed on the day or kept at 4°C and processed within the use by date. For raw chicken sold as RDF, one of each brand sold across five RDF stores in Bristol was purchased. A total of 15 packets were collected in September 2023, covering 11 brands of RDF.

Under sterile conditions, all meat and RDF samples were weighed to 200g and placed into a stomacher bag with 200 mL of EC broth (Oxoid LTD). Bags were sealed and incubated for 5 hours at 37°C, 180 rpm. The liquid culture was aliquot into Eppendorf tubes with 25% (v/v) glycerol and stored at -75°C.

Microbiology

Twenty microlitres of each sample was spread onto Tryptone Bile X-Glucuronide agar plates (Sigma) containing Nitrofurantoin (64 mg/L) based on EUCAST resistance breakpoints for human infections[31] and incubated overnight at 37°C. Putative NFT-R *E. coli* were re-streaked to confirm resistance. Isolates were stored on Cryobeads at -75°C. Resistance was confirmed by MIC using broth dilution according to EUCAST methodology.

WGS and phylogenetic analysis

WGS was performed by MicrobesNG to achieve a minimum 30-fold coverage. Preparation of isolates was carried out following MicrobesNG strain submission procedures as described in detail elsewhere.[32] Genomes were sequenced on an Illumina NovaSeq 6000 (Illumina, San Diego, USA) using a 250 bp paired end protocol. Sequencing data was analysed using ResFinder 4.4.2[33], Hound[34] and MLST 2.0.[35]

Sequence alignment and phylogenetic analysis was carried out using the computational facilities of the Advanced Computing Research Centre, University of Bristol (http://www.bristol.ac.uk/acrc/) and the Bioconda environment management system.[36] Alignment of sequences and reference sequence (**Table S5**) were performed using Snippy and Snippy-Core software (https://github.com/tseemann/snippy) and SNP distances were determined using SNP-dists software (https://github.com/tseemann/snp-dists). Maximum likelihood trees were produced using RAxML and the GTRGAMMA model of rate of heterogeneity.[37] Phylogenetic trees were illustrated using Microreact.[38]

Data Availability

WGS data are deposited with ENA under accessions PRJEB74489 (Isolates from meat) and PRJEB74488 (isolates from canine faeces).

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Transparency declaration

M.B.A. is married to the owner of a veterinary practice that sells various mass-manufactured dog foods amounting to a value less than 5% of total turnover. Otherwise, the authors declare no competing interests.

Author Contributions

Conceived the Study: J.E.S., M.B.A.

Collection of Data: J.E.S, B.A, K.R.

Cleaning and Analysis of Data: J.E.S.

Initial Drafting of Manuscript: J.E.S., M.B.A.

Corrected and Approved Manuscript: All authors

Table 1 NFT-R *E. coli* isolates excreted by dogs, and from raw poultry in Bristol, United Kingdom.

Isolates were from raw chicken meat sold for human consumption (RCM), chicken raw dog food (RDF), and canine faeces (DOG), sequence type (ST) and missense and nonsense mutations, deletions and insertions affecting NfsA and NfsB sequences are noted. Green represents those variants predicted to be functionally wild- type.[2] Red represents those conferring loss of function.

			Effect of non-synonymous mutations relative to *E. coli*		
Isolate	ST	Phylo-group	NfsA	NfsB	Other resistance genes
RCM1	ST8874	B2	I117T, K141E, G187D, 212Stop	46Stop	None
RCM2	ST665	A	138FS	M75I, V93A, 101Stop	*aadA1*, *aph(3")-Ib*, *aph(6)-Id*, *sul1*, *sul2*, bla_{TEM-1B}, *tet*(A)
RDF15	ST8874	B2	I117T, K141E, G187D, 212Stop	46Stop	None
DOG1030	ST919	B2	I117T, K141E, G187D, 212Stop	46Stop	None
DOG1110	ST6805	A	Insertional inactivation after codon 225	M75I, 79Stop	*aadA5*, *dfrA17*, *sul2*, *tet*(A)

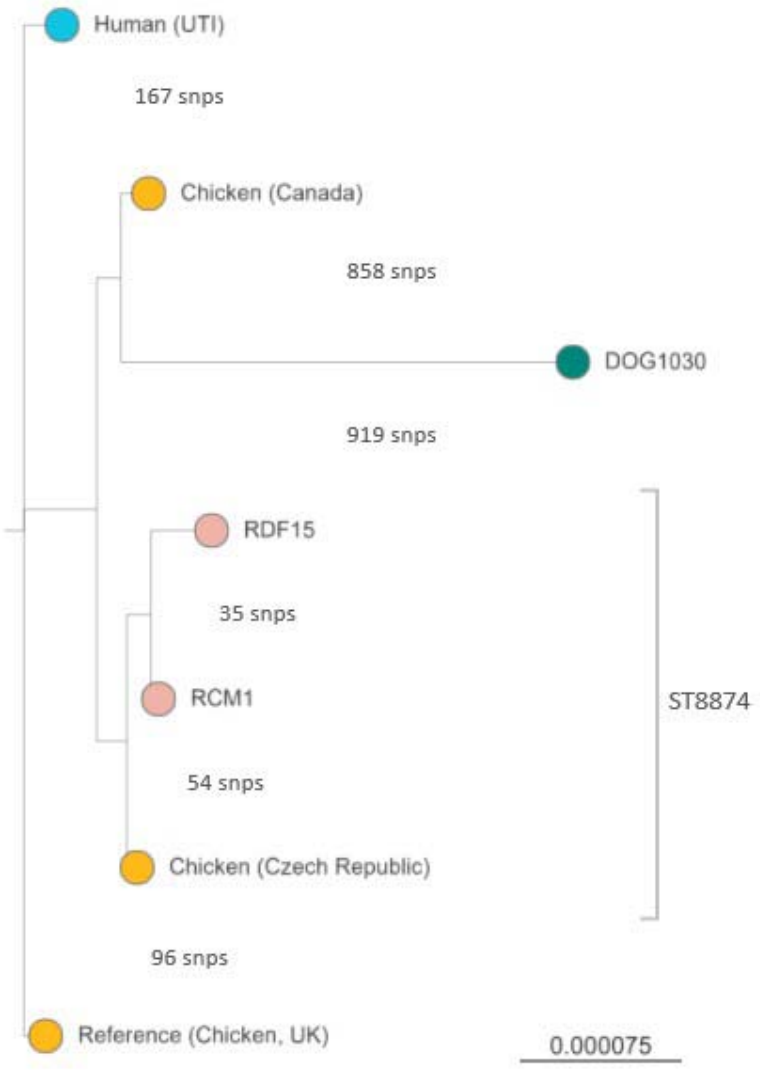

Figure 1 Maximum Likelihood phylogenetic tree based on a core genome alignment illustrating the evolutionary relationship between NFT-R ST919 and ST8874 isolates. Core genomes from a human UTI isolate from Enterobase (blue), dog faecal isolate (green), chicken isolates from Enterobase (yellow) and raw chicken meat (pink) were aligned using Snippy and Phylogenetic trees were constructed based on RaXML analysis. Reported SNP distances between pairs of isolates were determined using SNPdist. RCM, Raw Chicken Meat.

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