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Implementation of WGS analysis of ESBL-producing *Escherichia coli* within EU AMR monitoring in livestock and meat

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Background: As WGS comes of age, changes in EU legislation implemented in 2021 allow its usage for systematic monitoring of ESBL-producing *Escherichia coli* from livestock and meat, replacing phenotypic testing. Presently, phenotypic testing correlates well with antimicrobial resistance predicted from WGS data. WGS has added value in the wealth of additional information that is present in the data.

Objectives: In this study we have detected the resistance phenotypes for a panel of antimicrobials while also analysing the molecular epidemiology of ESBL-producing *E. coli*.

Methods: Susceptibility testing was performed with broth microdilution of selectively isolated *E. coli*. Short-read WGS was performed in parallel and phenotypes predicted based on the sequence data, which was also used to determine the phylogeny of the isolates.

Results: The phenotypically determined resistance and the predicted resistance correlated 90%–100% for the different antimicrobial classes. Furthermore, clonal relationships were detected amongst ESBL-producing *E. coli* within livestock sectors and the meat produced by this sector.

Conclusions: Further implementation of WGS analysis of ESBL/AmpC-producing *E. coli* within the AMR monitoring programme of EU member states and global surveillance programmes will contribute to determining the attribution of livestock in the prevalence of ESBL/AmpC-encoding *E. coli* in humans.

Introduction

Transmission of antimicrobial resistance (AMR) from livestock or livestock-derived products is considered a risk for AMR in humans. As such, monitoring in livestock sectors has been mandatory as part of EU legislation since 2004 under directive 2003/99/EC (https://eur-lex.europa.eu/legal-content/NL/TXT/PDF/?uri=CELEX:02003L0099-20130701&from=EN). Monitoring is carried out by the national reference institutes from individual EU member states and the generated data are annually reported to the European Food Safety Authority (EFSA), which publishes the data collectively.[1] Several member states also report their own analysis of the data publicly (https://www.eurl-ar.eu/monitoring-reports.aspx). *Escherichia coli* is considered a suitable indicator for AMR in the Gram-negative microbiota due to its ubiquitous presence and tolerance for the uptake of resistance-encoding plasmids, while the zoonotic bacteria *Salmonella* spp., *Campylobacter jejuni* and *Campylobacter coli* are included as important causes of foodborne infections. Due to the increasing prevalence in Europe, selective isolation of ESBL/AmpC-producing *E. coli* has been mandatory since 2014 under Implementing Decision 2013/652/EU (http://data.europa.eu/eli/dec_impl/2013/652/oj). The use of WGS for the prediction of AMR has been studied extensively and several tools have been benchmarked demonstrating they have become progressively more accurate.[2–4] The EU monitoring programmes collect, by EU member states, vast amounts of phenotypic data via antimicrobial susceptibility testing, which are very suitable for detecting trends over time.[5] The use of WGS would allow the

molecular epidemiological analysis of plasmids, insertion sequences, virulence factors and phylogeny of isolates. As such, based on a scientific report by EFSA,[6] the legislation for AMR monitoring and reporting was updated, Commission Implementing Decision (EU) 2020/1729, (http://data.europa.eu/eli/dec_impl/2020/1729/oj), now permitting the reporting of WGS data instead of AST data of ESBL-, AmpC- or carbapenemase-producing *E. coli* isolates, which allows for the collection of a valuable new repository of molecular epidemiological interesting features besides AMR genes over time as each member states contributes.

Methods

Selective cultures from caecal content or meat samples and phenotypic testing were set up as described by protocols from the EU Reference Laboratory for AMR (https://www.eurl-ar.eu/protocols.aspx). Broth microdilution was carried out using Sensititre panels EUVSEC2 and EUVSEC3. MIC values were determined with broth microdilution according to the CLSI standard. Results were interpreted using epidemiological cut-off values from EUCAST (https://eucast.org/mic_distributions_and_ecoffs/). WGS was performed on Illumina NextSeq and assemblies were performed with SPAdes-based Shovill v1.1.0 (https://github.com/tseemann/shovill). Genotypic prediction was performed using a local installation of ResFinder v4.1.0 and BLAST+, and local copies of the ResFinder and PointFinder databases, accessed on 24 April 2022.[14–16] Isolates for which no ESBL/AmpC gene could be detected were reanalysed using the non-assembled sequence reads for detection. *E. coli* phylotype was detected with ClermonTyper v0.7.0.[17] MLST was detected using the Achtman scheme with the MLST tool v2.16.4 (https://github.com/tseemann/mlst). Whole (draft) genome sequence comparisons were made using pairwise comparisons with in-house scripts based on MUMmer 2.[18] Isolates with a MUMi distance below 0.017 were subsequently used as a cluster for within core-genome comparison ROARY v3.12.0 after annotation by Prokka v1.12.[19,20] Core genomes over 3.9 Mb were considered complete enough to calculate pairwise SNP distance with snp-dists v0.8.2 (https://github.com/tseemann/snp-dists).

Results and discussion

Suspected ESBL/AmpC-producing *E. coli* were selectively isolated from caecal/faecal samples and meat. Caecal/faecal samples came from broiler chickens ($n=34$), slaughter pigs ($n=47$), veal calves ($n=103$) and dairy cattle ($n=42$), as well as from fresh meat collected at retail produced in the EU, from chicken ($n=47$), beef ($n=8$), veal ($n=14$), pork ($n=6$), other meat ($n=4$) or from imported meat collected at border control posts, which included beef ($n=1$) and chicken ($n=32$).

In total, 338 ESBL/AmpC-producing *E. coli* were isolated in 2021 from 2370 caecal or meat samples. The correlation between the phenotype as measured by broth microdilution and the WGS-predicted AMR phenotype was 90% or higher for all included antimicrobial classes; see Table 1. All isolates were phenotypically resistant to cefotaxime and ampicillin, which correlated 99.4% and 100% with genotypic predictions, respectively. Based on the detected ESBL or AmpC genes, various β-lactam phenotypes were identified, which correlated 94%–100%. Of the isolates, 5.6% were phenotypically resistant to ertapenem but not to other carbapenems. As no carbapenemases were detected and isolates were susceptible to other carbapenems, it is expected that these isolates would be susceptible upon re-testing, or that mutations in the outer membrane proteins in combination with ESBL or AmpC production may be responsible.[7] Co-resistance to non-β-lactam antibiotics was abundantly present with resistance to tetracyclines, sulphonamides and quinolones at the highest prevalence; see Table 1. Based on the genotypic predictions, 84.9% were classed as MDR, encoding resistance to three or more classes of antibiotics, compared with 1.3% to 31.3% of non-selectively isolated indicator *E. coli* from the same year (MARAN 2022; https://wur.eu/maran).

Table 1. Percentage of phenotypic and genotypic resistance and correlation between these, measured in 338 selectively cultured *E. coli* isolates from livestock and meat

	% R phenotype	% R genotype	% correlation
Amikacin	1.8	1.2	97.1
Ampicillin	100.0	100.0	100.0
Azithromycin	5.0	10.9	90.0
Cefepime	83.8	84.4	95.3
Cefotaxime	100.0	99.4	99.4
Cefotaxime/clavulanic acid	15.9	19.2	96.8
Cefoxitin	17.7	15.9	96.5
Ceftazidime	98.5	99.4	97.9
Ceftazidime/clavulanic acid	15.9	19.2	96.8
Chloramphenicol	27.1	27.7	99.4
Ciprofloxacin	48.7	49.9	98.8
Colistin	0.9	0.9	100.0
Ertapenem	5.6	0.0	94.4
Gentamicin	14.2	13.3	98.5
Imipenem	0.6	0.0	99.4
Meropenem	0.0	0.0	100.0
Nalidixic acid	29.2	26.0	95.0
Sulfamethoxazole	59.9	59.3	98.8
Temocillin	0.6	0.0	99.4
Tetracycline	59.3	59.6	99.7
Tigecycline	0.0	0.3	99.7
Trimethoprim	44.2	42.2	97.9

Analysis of the variation in *E. coli* lineages was performed based on the WGS data. Detected phylotypes were spread over all sources of samples and included most of the described phylotypes: A ($n=130$), B1 ($n=103$), B2 ($n=2$), C ($n=37$), D ($n=23$), E ($n=8$), F ($n=7$), G ($n=25$) or cryptic ($n=3$). A total of 113 different MLST types were detected, although the four most prevalent accounted for nearly a third of the isolates (105/338). These dominant types were ST10 ($n=34$), ST58 ($n=27$), ST88 ($n=26$) and ST117 ($n=18$), which were all found in both caecal/faecal or meat samples from broilers, pigs, dairy cattle or veal calves. ST58 was more prevalent in caecal/faecal samples ($n=26$) and ST117 was mostly detected in meat ($n=13$), ST10 and ST88 were detected regularly in both sources. ST10, ST58, ST88 and ST117 have all previously been described as prevalent STs in livestock studies.[8,9] Both isolates of phylotype B2 belonged to the MDR clonal lineage MLST 131 and were isolated from samples of chicken meat; see Figure S1 (available as Supplementary data at *JAC* Online).[10] A circular cladogram based on the MUMmer analysis is shown in Figure 1, indicating

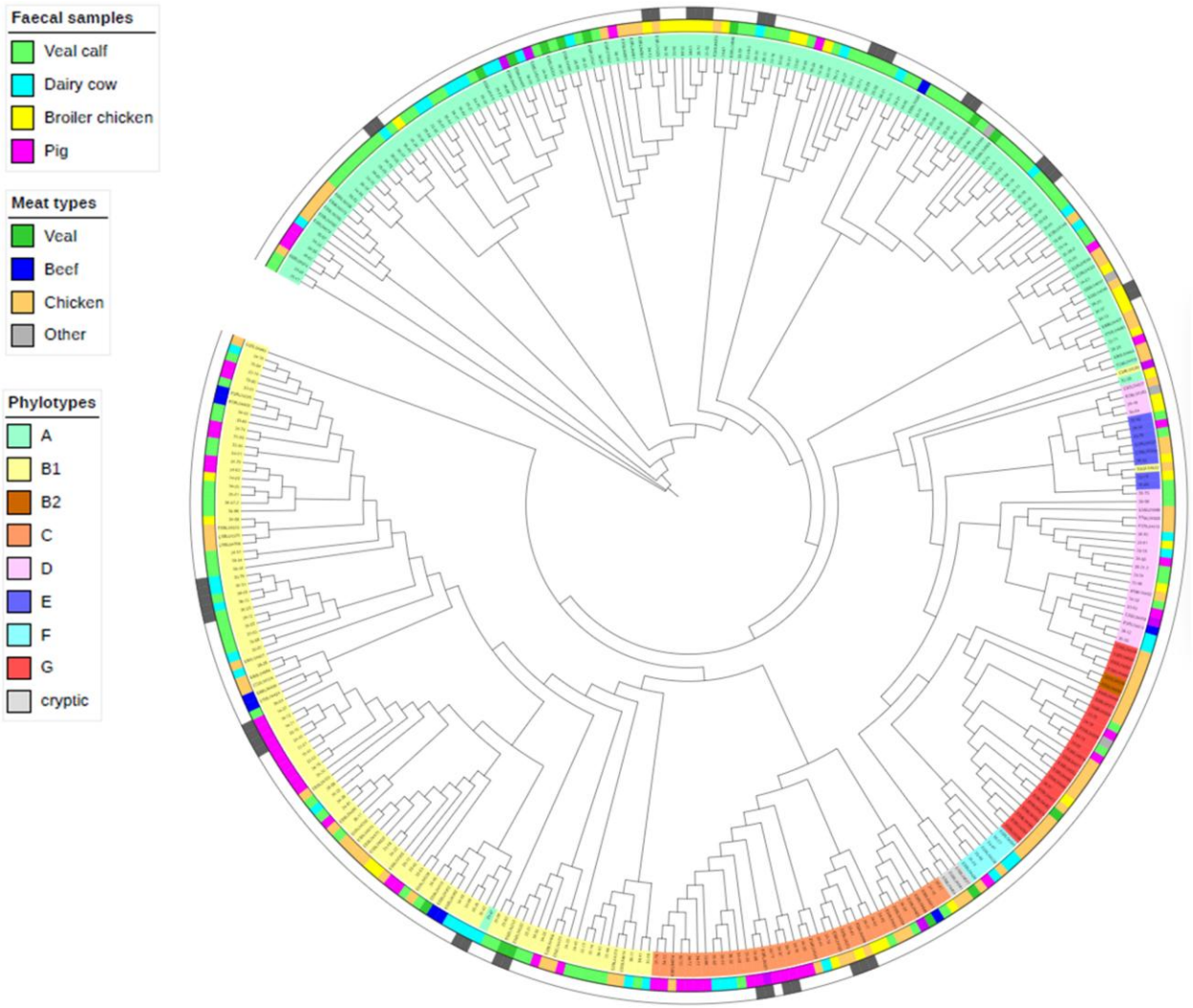

Figure 1. Circular cladogram of the complete genome comparisons of 338 ESBL/AmpC-producing *E. coli* genomes. Metadata is plotted in circles, from inside to outside: (1) Isolate ID, coloured per *E. coli* phylotype; (2) sample type, meat or faecal; (3) grey bars connect possible clones of ≤ 40 SNPs.

a diverse set of *E. coli* in all animal hosts, although some clusters of possible clonal lineage are present. A total of 15 clusters were detected for which core genomes per cluster were determined. For 39 isolates in these clusters, the total core genome contained 40 SNPs or less. Of these, two clusters were detected between veal faecal samples, one cluster between meat samples of veal, four clusters between veal and dairy cow faecal samples, and two clusters between faecal samples of dairy cattle. Furthermore, two clusters were detected between pig faecal samples and two clusters between pig faecal and pork meat samples. Finally, one cluster was detected consisting of broiler faecal samples and one cluster between broiler faecal samples and chicken meat. No clusters were detected amongst samples of caecal content or meat across the different livestock species and no clusters were detected concerning imported meat. Further metadata of the isolates are displayed in Figure S1, including detected ESBL/AmpC genes, MLST types and genotypic AMR profiles.

The ESBL monitoring is carried out annually on a relatively small proportion of animals, indicating that clonal transmission of ESBL/AmpC-producing *E. coli* throughout the production chain occurs frequently. The current cut-off for putative clonal transmission used here was 40 SNPs in the core genome, as previously described.[11] As this was a pairwise comparison between isolates, and some isolates fell just outside this cut-off, it is to be expected that the percentage of isolates that are part of a cluster will increase with the addition of further data in future years. Currently, there are few examples of direct evidence for clonal transmission of ESBL/AmpC-producing *E. coli* from livestock onto raw meat at consumer level in the Europe.[12,13] Further implementation of WGS analysis of ESBL/AmpC-producing *E. coli* within the AMR monitoring programme of EU member states and global surveillance programmes, and FAIR access of this data, will contribute to determining the attribution of livestock in the prevalence of ESBL/AmpC-encoding *E. coli* in humans. The apparent clonal transmission of ESBL/AmpC-producing

E. coli in Dutch livestock indicates that these bacteria circulate amongst farms, rather than receiving multiple independent introductions from other sources, suggesting that measures against between-farm transmission are necessary to further reduce prevalence.

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Transparency declarations

The authors declare that there are no competing interests.

Data availability

Sequence data and AST data that support the findings of this study have been deposited in GenBank as BioProject PRJNA885502 (https://www.ncbi.xyz/bioproject/PRJNA885502).

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online.

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