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ENTEROCOCCUS CECORUM **GENOMICS IN UK BROILERS**

Genomic characterisation of *Enterococcus cecorum* isolated from broiler chickens in the United Kingdom

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Abstract

Enterococcus cecorum is an important poultry pathogen associated with lameness and increased mortality, leading to major welfare and economic impacts. Treatment is often challenging because disease is frequently detected late and the organism can localise in bone and joints, limiting antimicrobial efficacy. Presence of antimicrobial resistance (AMR) genes may further complicate treatment. Despite increasing global genomic research, only one recent study has investigated the phylogeny of *E. cecorum* from conventional UK broiler farms, using limited samples and a restricted time frame.

In this study, 283 *E. cecorum* isolates were analysed, including 158 from the United Kingdom and 125 global non-UK isolates. UK isolates comprised 123 archived by the Animal and Plant Health Agency (APHA) between 2003 and 2022, predominantly from clinical outbreaks with increased welfare culling and mortality, and 35 isolates from a UK study including clinical and environmental samples. Genome sequencing was used to assess phylogeny, AMR determinants and virulence factors (VFs).

Single nucleotide polymorphism phylogenetic analysis identified eight major UK lineages with limited intra-lineage diversity, indicating that UK isolates were genetically distinct from non-UK populations. Using a 60-SNP threshold, APHA isolates formed 18 subclusters, consistent with long-term persistence and recurrent farm transmission, while multiple subclusters detected on some farms suggested repeated introductions. UK isolates carried fewer AMR genes than non-UK isolates, with *erm*(B), *lnu*(C), *tet*(M) and *tet*(L) most prevalent.

Screening of VF genes identified nine genes present in all isolates, with the remainder variably distributed. A subset of 60 UK isolates was examined for 13 previously described virulence-associated genes, with phylogenetic clustering indicating associations between gene presence or absence and clinical status or mortality classification. The capsular polysaccharide gene *cpsO* was assessed in this subset and most non-clinical or environmental isolates were *cpsO*-negative, although several isolates from high-mortality outbreaks also lacked this gene.

Overall, this study provides insight into the phylogeny, AMR profiles and virulence gene diversity of *E. cecorum* within the UK broiler sector, supporting targeted surveillance and investigation of pathogenic mechanisms.

Keywords: *Enterococcus cecorum*, genomics, broilers, antimicrobial resistance, virulence factors

Introduction

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Enterococcus cecorum, a Gram-positive facultative anaerobe, was first described in 1983 (Devriese et al., 1983) and can be found in poultry intestines. However, since the beginning of the millennium, it has also been recognised as a notable pathogen of the poultry industry (Souillard et al., 2022). In broiler chickens, *E. cecorum* can cause systemic bacterial infections, femoral head necrosis, septic arthritis and, in older birds, spinal osteomyelitis leading to paralysis. Once clinical disease is established in a flock, it can lead to welfare issues resulting in increased culling and mortality rates, poor production, and increased condemnation rates. Although very rare, human infections have been reported, indicating potential zoonotic risks, particularly for immunocompromised individuals (Delaunay et al., 2015). The epidemiology of *E. cecorum* in poultry is complex, involving horizontal and, possibly, vertical transmission, (Kense & Landman, 2011) potentially influenced by environmental or management-related factors (Jung et al., 2018). Clinically, *E. cecorum* systemic infections presents as depression and increased mortality due to sepsis; lesions include splenomegaly and pericarditis. In birds with subacute to chronic infections, septic arthritis, femoral head necrosis can result in joint swelling, reduced mobility or lameness, and spinal abscesses can cause paralysis (Jung et al., 2018).

A range of putative virulence factors associated with *E. cecorum* have been identified (Laurentie et al., 2023). These factors, which include mechanisms for adhesion, immune modulation, environmental adaptation, stress survival, and biofilm formation, are thought to be essential for the bacterium's capacity to evade host defenses, establish infections, and persist in adverse conditions. However, there is lack of comparison of virulence gene presence with real-life on-farm metadata of the scale of infection, which if confirmed, will be extremely important in treatment strategies and control measures required to mitigate *E. cecorum*'s impact on poultry health.

Research has shown that *E. cecorum* can carry antimicrobial resistance genes (ARGs), with multidrug resistance (MDR) commonly observed whether clinical signs were evident or not (Sharma et al., 2020), (Huang et al., 2024). This raises concerns since *E. cecorum* may serve as a reservoir for resistance genes, potentially facilitating the spread of MDR within poultry populations and complicating treatment protocols (Laurentie et al., 2023), (Wijetunge et al., 2012).
E. cecorum's genetic diversity has been studied globally using 16S rRNA gene analysis and whole-genome sequencing (WGS) (Laurentie et al., 2023); (Sharma et al., 2020); (Wijetunge et al., 2012); (Freitas et al., 2023); (Dolka, Heidemann Olsen, et al., 2015); (Dolka, Boyen, et al., 2015); (Borst et al., 2015); (Yulandi et al., 2020); (Zhong et al., 2017); (O'Dea et al., 2025). In the United Kingdom, clinical disease associated with *E. cecorum* infection was first reported in the early 2000s (Wood et al., 2002) although published data on UK isolates, to the best of our knowledge, remain limited to a 2025 study with sequenced samples collected in 2021-2022 (O'Dea et al., 2025). Our study aimed to

characterise its genetic diversity in depth using isolates collected between 2003 and 2022 on poultry farms in the UK, through the Animal and Plant Health Agency's (APHA) scanning surveillance program of diseased livestock that monitors emerging disease threats on UK farm (https://www.gov.uk/government/publications/avian-gb-disease-surveillance-and-emerging-threats-reports accessed on 23/07/2024) (accessed on 26/03/2026). WGS of *E. cecorum* isolates collected from UK broiler chickens by APHA and the O'Dea study (O'Dea et al., 2025) were compared with that from non-UK isolates (Laurentie et al., 2023) to investigate any influence of geography i.e. UK vs non-UK, on genetic diversity and the presence of ARGs. Additionally, we analysed the genetic diversity of *E. cecorum* isolates within and across farms, using WGS, to identify potential dominant lineages that may contribute to outbreaks in the UK. We also explored the presence of virulence factors in the full dataset, with a greater focus on a subset of isolates originating from poultry farms with high or low mortality, for APHA samples and reported "clinical" or "environmental/commensal" reported metadata for other UK isolates, to help pinpoint the correlation between virulence factors and scale of disease.

Material and methods

Origin of *E. cecorum* isolates

The study included 283 *E. cecorum* isolates of which 125 were non-UK/global isolates sourced from literature (Laurentie et al., 2023). Global isolates were collected between 1999 (n=1) and 2007-2017 (n=117), plus seven samples where the collection date was not reported. Their hosts included broiler (n=109), duck (n=1), goose (n=1), layer hen (n=1) and human (n=6), with seven hosts not reported. The countries of collection were France (n=103), Belgium (n=2), Germany (n=1), Poland (n=5) and the United States (n=6) and 8 were unknown countries of origin. The 35 isolates from O'Dea study (O'Dea et al., 2025) were obtained from UK clinical and commensal/environmental samples isolated from broilers sourced from farms, submitted by veterinarians, or collected at supermarkets and processing houses.

The remaining 123 isolates originated from the APHA bacteriology archive (Table S1). All isolates were derived from commercial broiler chickens. The majority of isolates were derived from birds between the ages of two and 39 days (median age 21 days), however the age was not available for 26 isolates. Five of the isolates were obtained from a previous study (Watson et al., 2024) and the remaining isolates (n=118) were collected through the APHA's scanning surveillance program between 2003 and 2022. The distribution of isolates across farms was uneven, as this is not a mandatory surveillance program, and participation requires agreement for sample collection and analysis. Farm details were available for 104 isolates that originated from 34 separate farms,

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whereas no farm details were available for 14 isolates as farm details were anonymised by the submitting veterinarians.

A subset of 25 APHA isolates from six farms with detailed metadata available were analysed in greater depth. These isolates were classified as 'high' or 'low' mortality based on mortality attributable to *E. cecorum* on a flock level (Table S2). Five farms presented with high mortality on a flock level and on one of these farms (FM38), isolates were collected from three subsequent flocks, all presenting with high leg culls, attributable to *E. cecorum*; all 21 isolates were classed as "high mortality". Four isolates were from one farm (FMB) without a significant *E. cecorum* problem, and used in a previous study (Watson et al., 2024), were included as "low mortality" isolates. This subset of 25 isolates were compared to the 35 O'Dea isolates (O'Dea et al., 2025) where we considered the clinical and environmental samples reported in the metadata by the authors without making the assumption of correspondence clinical = high mortality and environmental/commensal = low mortality.

All APHA isolates were stored at -80 °C in cryopreservation bead tubes (Protect, Technical Service Consultants Ltd, Lancashire, UK) and cultured on 5% Sheep Blood Agar at 37 °C in microaerophilic conditions when recovered for further analysis. Species confirmation of all samples was performed using Matrix-assisted laser desorption/ionization (MALDI-TOF, Bruker Ltd, Coventry, UK). DNA was isolated using KingFisher™ Flex Magnetic Particle Processor and MagMAX™ CORE Nucleic Acid Purification Kit.

Whole genome sequencing

WGS was conducted at the APHA Central Sequencing Unit using Illumina NextSeq 550 (Duggett et al., 2020) and *E.* cecorum confirmed by classification by KRAKEN2 (v. 2.1.3) (Wood et al., 2019). Raw sequences were quality assessed using FASTQC (v. 0.12.1) (Andrews, 2020) and summarised with MultiQC (v. 1.18) (Ewels et al., 2016). The metrics analysed were average quality scores (threshold > 23) and reads average length (> 147 bp) for raw sequences. Raw reads were assembled with Unicycler (v. 0.5.0) (Wick et al., 2017) and the assembly quality evaluated using Quast (v. 5.2.0) (Gurevich et al., 2013); GC percentage content and total contigs length were compared with the *E. cecorum* reference isolate NCTC12421, using thresholds 36.5 ± 0.5 and 2,421,598bp ± 20% respectively (Table S3). Two archived isolates (S66 and S77) were long-read sequenced using Nanopore technology following manufacturer instructions. For these isolates, hybrid assemblies using the short and long reads were assembled using Unicycler to produce more complete reference genomes.

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Raw sequences have been deposited in the SRA database and are available with the project number PRJNA1266129.

Detection of ARGs and virulence factors

APHA SeqFinder software (v.6.0.0) (Anjum et al., 2016); (Duggett et al., 2023) and Abricate (v. 1.0.1) (https://github.com/tseemann/abricate) (accessed on 26/03/2026), (AbuOun et al., 2021) was used to identify ARGs. Detected ARGs were then grouped by antimicrobial class and categorised according to the 2024 WHO List of Medically Important Antimicrobials for human medicine (https://cdn.who.int/media/docs/default-source/gcp/who-mia-list-2024-lv.pdf?sfvrsn=3320dd3d_2) (accessed on 26/03/2026) and according to their importance for veterinary medicine using the latest World Organisation for Animal Health (WOAH) List of Antimicrobial Agents of Veterinary Importance (https://www.woah.org/app/uploads/2024/05/91gs-tech-03-amr-working-group-report-en.pdf) (accessed on 26/03/2026).

For virulence factors, two custom-made virulence factor databases were created. The first custom-made database was obtained by merging virulence factors from Laurentie et al. in (Laurentie et al., 2023) with VFDB (Chen et al., 2005). Duplicate nucleotide sequences were eliminated using the software seqtk (v. 1.4-r122) (https://github.com/lh3/seqtk) (accessed on 26/03/2026). This custom-made database, which contained 32,752 virulence factor genes, was used with Abricate to investigate the presence of other virulence factors that may be present not only in *E. cecorum* (Laurentie's database), but from a wide range of bacteria.

The second custom-made database was used to carry out a more in-depth analysis on a preliminary basis. It aimed to assess genes associated with isolates classified as high or low mortality (Laurentie et al., 2023); (Borst et al., 2015). This was used on the subset of 60 APHA/O'Dea isolates (O'Dea et al., 2025). The preliminary analysis combined the six mannitol metabolism genes , reported by Borst et al. (Borst et al., 2015), whose absence indicates pathogenicity, with seven virulence genes identified by Laurentie et al. (Laurentie et al., 2023), as associated with pathogenicity. The capsular polysaccharide gene *cpsO* associated with pathogenicity (Combar et al., 2024; Suyemoto et al., 2025) was also added.

Phylogenetic analysis

Assembled sequences were annotated using Prokka (v. 1.13) (Seemann, 2014). To identify conserved genes across all isolates, pangenome analysis was conducted on the 283 isolates using Roary (Page et al., 2015). Raw sequences were used to obtain the core genome SNPs identified by using Snippy

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(Seemann, 2015) taking the hybrid-assembly of isolate S77 as the reference. The phylogenetic tree was generated with RAxML (GTRGAMMA substitution model, 100 bootstraps, 100 maximum likelihood (ML) runs) (Stamatakis, 2014). Core phylogeny, along with metadata, was visualized using Microreact (Argimón et al., 2016). Analysing the ML tree we categorized, by looking at the terminal branches of each lineage on the phylogenetic tree, groups that we defined as lineages and sub-lineages.

After this first categorization, we identified clusters based on an intra-cluster pairwise SNP distance of less than 60 SNPs in the snp-dists matrix obtained with the software snp-dists (https://github.com/tseemann/snp-dists) (accessed on 26/03/2026). The 60-SNP threshold used to define clusters was chosen as a compromise between capturing biologically meaningful genetic variation and maintaining a manageable number of clusters for interpretation.

Results

E. cecorum genomic characterisation and phylogenetic analysis

This study comprised 283 *E. cecorum* isolates which included 123 isolates from UK with 118 from APHA's scanning surveillance program and the remaining five UK isolates were from Watson et al, 2024, a farm where no significant disease outbreak was reported. A further 35 UK isolates were included from O'Dea et al, 2025. The non-UK isolates comprised 125 isolates from five countries and a range of poultry hosts, and humans (Laurentie et al., 2023).

Pangenome analysis across the 283 *E. cecorum isolates* identified 9,843 genes, with a core genome comprising 1075 genes (49.58% of the reference genome, S77). There were 92,203 informative SNPs identified in the core genome which was used to generate the phylogenetic tree. We identified eight distinct lineages/clusters on the phylogenetic tree (A-H); six UK isolates from O'Dea et al were outside these clusters (Figure 1). Most UK isolates (n=142) formed the four main lineages (A, B, E, and H), as shown in Figure 1. The number of isolates, the minimum, maximum and average number of SNPs for each lineage is given in Table 1. Lineage A was the largest phylogenetic group for UK isolates comprising 80 APHA isolates primarily collected in 2022 and originating from 21 farms. The isolates were on average 116.15 SNPs apart from a maximum SNP distance of 1774 and minimum of 0, the isolates divided into four sublineages but the majority belonged to sublineage A4 and only five isolates belonged to sublineages A1 to A3. Lineage B included APHA isolates collected from 2003 to 2005; it also comprised four sublineages with an average SNP distance of 425.11, a maximum SNP distance of 1414, and a minimum of 0 for identical isolates. There were a comparable number of isolates in sublineages B1 (n=11) B2 (n=6) and B3 (n=8), and only 1 isolate in sublineage B4. The

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higher average SNP distances between isolates in lineage B indicated the isolates were even more diverse in this lineage than in lineage A. Only 14 UK isolates formed lineage E, which also harboured non-UK isolates (APHA-UK n=5, O'Dea-UK = 9; non-UK n=11). This lineage was divided in three sublineages (E1, E2, E3) showing the highest heterogeneity of all the lineages with an average SNP distance of 4905.3 and a maximum SNP distance of 8154 (minimum=0). Lineage H included 25 APHA isolates, as well as 5 O'Dea/UK and 8 non-UK isolates; they exhibited an average SNP distance of 157, with maximum SNP distance of 1769 and minimum distance = 0, with one cluster consisting of two identical isolates. This lineage is also divided into three sublineages (H1, H2, H3). The remaining UK isolates (n=16) merged within lineages including non-UK isolates (lineages C (n=1), D (n=1), F (n=3), G (n=2) and UKL (n=9); the latter nine isolates were all non-APHA isolates and were interspersed throughout the tree.

Non-UK isolates were generally clustered in distinct lineages (n=5) away from UK isolates. The average number of SNPs between UK and its nearest non-UK cluster was 1617, with a minimum of 0 (some UK and non-UK isolates were identical) and a maximum of 11994 SNPs. The two non-UK isolates originating from human samples formed a distinct group away from all other isolates included in the analysis, indicating an evolutionary distinct lineage (L103 and L104 in Figure 1).

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Genetic relatedness of *E. cecorum* on UK Farms (APHA isolates)

Based on the phylogenetic tree and SNP-distance matrix, hierarchically clustered with Pearson method (Table S4; https://github.com/tseemann/snp-dists) , we identified 18 dominant clusters for APHA isolates with pairwise SNP distances below 60, which we used as our cut-off for clones. Several clusters were observed in isolates from 2022 (C1 and C8 to C18; Table S5, column SNPs Groups and Figure 2); the year with the highest number of UK isolates. Despite the smaller number of isolates available in earlier years such as 2003, SNP-based clustering was still observed (Figure 2).

We noted that several clusters were detected across multiple farms within the same year (Figure 2). For instance, *E. cecorum* belonging to cluster C14 was identified on 16 farms and most were isolated in 2021-2022. Similarly, *E. cecorum* belonging to cluster C11 was present on six farms during 2022. There were fewer isolates present in the remaining clusters and Table S5 provides the farm-level distribution of all clusters. Notably, nine farms yielded isolates present in multiple clusters, while for 25 farms, isolates belonged to one cluster only (Figure 2). Of these farms, Farms 23, 29 and 38 had the largest numbers of isolates per farm in our collection.

From Farm 23, 29 and 38, multiple isolates from different management groups (e.g. from broilers in separate houses, or from separate all-in, all-out production cycles) were analysed to understand

intra-farm genomic heterogeneity. Twelve isolates from 2022 from Farm 38 belonged to lineage A, specifically cluster 13, indicating a clonal population was persistently present in 2022. In contrast, the 11 isolates from Farm 23, also all from 2022, represented greater diversity, spanning two distinct lineages/clusters. The majority were from lineage A, cluster 14, while four isolates belonged to lineage H, cluster 11. Seven isolates were obtained from Farm 29, also from 2022, which were distributed across lineages A (n=2) and H (n=5) and five different clusters, indicating significant heterogeneity in the *E. cecorum* recovered from Farm 29.

Antimicrobial resistance genes (ARGs)

A total of 37 distinct ARGs, associated with AMR resistant phenotypes to nine antimicrobial classes, were detected. ARGS and antibiotic classes are reported in Table 2, together with their importance according to WHO List of Medically Important Antimicrobials guidelines (https://cdn.who.int/media/docs/default-source/gcp/who-mia-list-2024-lv.pdf?sfvrsn=3320dd3d_2) (accessed on 26/03/2026) and to WOAH List of Antimicrobial Agents of Veterinary Importance (June 2024) (https://www.woah.org/app/uploads/2021/06/amended-91gs-tech-03-amr-working-group-report-en.pdf (accessed on 26/03/2026). Overall, UK isolates in this study were found to carry fewer ARGs compared to non-UK isolates, with ~49% UK isolates not harbouring any ARGs (Table 2).

The most prevalent ARGs across both datasets were *tet(*M), erm(B), *tet*(L), and *lnu*(C). The macrolide resistance gene *erm*(B) was present in 22% of UK and 68% of non-UK isolates, while *lnu*(C), conferring lincosamide resistance, was detected in 11% of UK and 24% of non-UK isolates. For tetracycline resistance, *tet*(M) was identified in 33% of UK and 67% of non-UK isolates, whereas *tet*(L) was found in 22% and 51%, respectively.

Analysis of APHA isolate assemblies showed that co-occurrence of *tet*(M) and *tet*(L) was observed in 22% of APHA-UK isolates and 50% of non-UK isolates (Table S5). When present on the same contig (n = 74 isolates), the two genes were located 515 bp apart and flanked by transposon-associated sequences, suggesting potential mobility.

All other ARGs were detected in ≤6% of isolates in both UK and non-UK datasets (Table 2). Genes associated with vancomycin resistance were identified in a single non-UK isolate, corresponding to the presence of the *vanA* operon.

When examining the distribution of ARGs across the main UK lineages (Table S5), lineage A showed minimal resistance, with 76% of isolates lacking ARGs. For those carrying ARGs, isolates harboured between one and nine ARGs. In lineage B, all isolates harboured ARGs, with at least one to five ARGs being present. For lineage E, 96% of isolates harboured ARGs, with one isolate harbouring eight

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ARGs. While in Lineage H, 50% of isolates were fully sensitive with the remaining 50% harbouring between one to eight ARGs.

Putative virulence determinants and Virulence Factors Analysis

We considered the distribution of the most discriminatory putative virulence determinants from Laurentie et al, (2023) and Borst et al, (2015) in all 283 *E. cecorum isolates,* in our data set (Table S6), it indicated lineage specific variability of virulence genes, with lineage A being the only lineage where all isolates except UKL8, consistently harboured six out seven genes identified by Laurentie (Laurentie et al., 2023). It also showed that lineage C, a non-UK *E. cecorum*, was the only lineage harbouring all 12 out of 13 putative virulence determinants included in the list. The distribution of pathogenicity markers (Laurentie et al. 2015) and (Borst et. al. 2015) across our dataset is shown in Figure 3.

Therefore to better define virulence factors, we extended the pathogenicity analysis against our *E. cecorum* UK and non-UK isolates by considering the Virulence Factors' Database (VFDB) and Laurentie's complete gene database (Laurentie et al., 2023) plus *cpsO*. The analysis identified 138 distinct VF genes with >70% identity across the 283 isolates, each classified according to their putative functional role in microbial pathogenesis. There were nine VFs that were present in all 283 isolates which included genes involved in metabolism, regulation, adherence, immune modulation/capsule, stress survival as well as two with unknown functions (Table S7).

To further study VFs likely to be associated with clinical disease outbreaks, we compared VFs present in 60 isolates for which the mortality metadata (25 APHA) or clinical/non-clinical classification (35 UKL isolates) were available. APHA samples included one representative outbreak isolate from three farms with high-mortality flocks (FM18, FM32, and FM36); 12 isolates from farm FM38 collected from high-mortality flocks over three separate production cycles; five isolates from high-mortality flocks at farm FMD; and four from low-mortality flocks at farm FMB where three were from caeca and one was from a bird with arthritis. In total we analysed 4 low-mortality isolates and 21 high-mortality isolates for APHA samples and 17 environmental/commensal and 18 clinical samples for the other UK samples. The distribution of Laurentie and Borst putative virulence factors across these 60 isolates is reported in Figure 4/Table S8. The *cpsO* gene was consistently absent in low-mortality (APHA) and all but two environmental/commensal (other UK) isolates. Most high-mortality/clinical isolates were cpsO positive, however, four isolates (S114, S60, S62, and S69), which were isolated from high-mortality outbreaks, also lacked this gene.

Figure 4, which presents the phylogenetic comparison of the 60 isolates, reveals a clear clustering

pattern associated with flock mortality or isolate clinical/environmental classification. Among APHA isolates, those classified as originating from high-mortality cases group together into distinct clades, while those associated with low mortality form separate clusters. In contrast, for the other UK isolates, classification is based on “clinical” or “environmental” origin rather than mortality; nevertheless, these categories broadly follow the same phylogenetic separation. This pattern indicates a strong concordance between phylogenetic structure and pathogenic phenotype, suggesting that genomic background captures biologically meaningful differences between isolates. A small number of exceptions are observed among the non-APHA UK isolates; however, the overall pattern demonstrates a clear delineation between high-mortality/clinical and low-mortality/environmental groups, supporting the validity of the phylogenetic signal.

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Discussion

This study aimed to elucidate the genomic diversity, antimicrobial resistance, and virulence potential of *E. cecorum* in broiler chickens, to better understand its epidemiology and provide a foundation for future control strategies. The phylogenetic analysis of *E. cecorum* isolates revealed a distinct separation of lineages of most UK isolates from global ones. This analysis identified eight lineages with smaller SNP variation within each lineage, when compared to the variation between lineages, with the two human isolates being most distal. These findings highlight a complex genetic landscape characterised by pronounced divergence, suggesting distinct evolutionary trajectories and possibly differing adaptive strategies within the studied populations. However, it's important to note that due to the sample size and most UK isolates being from outbreaks on farms, it might mean that even lineages with only a few isolates could be more significant, as they could reflect specific sampling patterns rather than true population size. For this reason, we refined this initial analysis using a threshold of 60 SNPs to define clones. While thresholds such as 2–7 SNPs for clones and 25–309 SNPs for different strains have been used for *E. faecium* (Raven et al., 2018), similar data for *E. cecorum* are, to the best of our knowledge, unavailable. Therefore, the SNP differences between isolates in our data was used as a guideline to inform the choice of 60 SNPs for *E. cecorum.* By defining the SNP threshold, it allowed more precise delineation of clusters, which facilitated better tracking of transmission on UK farms collected through APHA surveillance.

Across all APHA isolates, 18 clusters were identified using the threshold of 60 SNPs for clones. The presence of these clusters across different farms and months or years in UK suggests that some *E. cecorum* genotypes persist through various production cycles, potentially surviving standard sanitation efforts and re-infecting new flocks. This concurs with previous findings where *E. cecorum* has been detected by PCR from disinfected farm buildings (Tessin et al., 2024; Watson et al., 2024).

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The prevalence of specific clusters in multiple UK farms in 2021-2022, highlights *E. cecorum's* ability to spread, underscoring the need for improved understanding of persistence and transmission so appropriate control measures can be devised. For example, cluster C14 was detected on 16 farms and C11 on 6 farms during 2021–2022, pointing to the possible circulation of these dominant clones in clinically affected poultry farms.

Also, a more detailed analysis of isolates from three UK farms revealed there were different patterns in the distribution of *E. cecorum* clusters depending on the farm. On Farm 38, all 12 isolates belonged to a single cluster, although isolates were collected at different times, suggesting the persistence of a clonal population. Research has shown that pathogenic *E. cecorum* strains can survive for extended periods under various conditions commonly found in poultry houses (Gregg et al., 2025); (Watson et al., 2024); (Grund et al., 2021). In contrast, Farms 23 and 29 exhibited greater genetic diversity, with multiple clusters identified on the same farm. This variation raises important questions about the transmission routes of *E. cecorum* clones. Although investigating transmission pathways was beyond the scope of this study, the presence of distinct clones temporally in different management groups on the same farm suggests there may be several different sources of introduction to the flocks. Vertical transmission via the hatchery has so far not been unequivocally demonstrated for *E. cecorum* (Kense & Landman, 2011),although several recent studies suggest this may be possible. Suyemoto et al. (Suyemoto et al., 2025) cultured *E. cecorum* from hatchery samples, with identical PFGE profiles to isolates cultured from broilers within the same production complex. Watson *et. al* (Watson et al., 2024) detected *E. cecorum* from day-old chick papers by PCR. As most of our study was carried out during an avian influenza (AI) outbreak, this impeded farm visits other than the most essential ones, so, it was difficult to obtain *E. cecorum* isolates from other farms in which this bacterium did not present as a primary problem. Future studies with larger datasets will further examine E. cecorum farm transmission and persistence dynamics, particularly whether certain *E. cecorum* clones are circulating within and between clinically affected flocks and farms.

When comparing ARGs between UK and non-UK isolates, we found that UK isolates generally harbored fewer ARGs. This difference may, however, be due to the bias in the isolates collection. The most common ARGs detected included *erm(B)*, *lnu(C)*, *tet(M)*, and *tet(L)*, conferring resistance to macrolides and tetracyclines, respectively. Notably, the co-localisation of *tet(L)* and *tet(M)* on the same contigs in several isolates suggests a physical linkage within the bacterial genome that may facilitate the co-inheritance and spread of resistance traits. Differences in ARG profiles between countries may reflect distinct evolutionary pressures arising from variations in both the quantity and type of antibiotics used; for example, macrolide use in the UK poultry meat sector is extremely low.

The UK Veterinary Medicines Directorate and British Poultry Council reported a 99% reduction in macrolide use and a 78% overall reduction in antibiotic use in broiler chickens between 2014 and 2024. Tetracyclines remained the second-most commonly used class, despite a 94% reduction over the same period (see https://assets.publishing.service.gov.uk/media/69808ca601dffa64655800da/UK-VARSS_2024_Supplementary_Material_1.pdf, and https://britishpoultry.org.uk/wp-content/uploads/2024/08/BPC-Antibiotic-Report-2024-Singles.pdf).

Our analysis of previously identified putative virulence determinants yielded mixed results in the 283 *E. cecorum* isolates included in this study. Genes previously linked to pathogenicity (Laurentie et al., 2023); (Borst et al., 2015); (Combar et al., 2024) were variably present across all *E. cecorum* isolates including those from human samples with few exceptions represented by five O'Dea isolates, which could possibly be due these being sequenced only by Oxford Nanopore Technology rather than Illumina so lacking depth/coverage in certain areas of the genome. Lineage A was the only lineage that consistently exhibited gene presence and absence patterns aligned with findings from prior studies , specifically demonstrating presence of all six genes identified as relevant to pathogenic strains and lacking two mannitol PTS system genes (Borst et al., 2015), (Laurentie et al., 2023).

The presence of *cpsO* in most high-mortality (APHA) and clinical (UKL) isolates, and its absence from all low mortality/non-clinical isolates (APHA) and in all but two isolates of environmental/commensal isolates (O'Dea) supports other studies which have used *cpsO* PCR to distinguish pathogenic from commensal *E. cecorum* (Combar et al., 2024);(Suyemoto et al., 2025). However, in our study four isolates derived from independent high mortality outbreaks were *cpsO* negative. This indicates an imperfect specificity of *cpsO* as a marker for distinguishing pathogenic from commensal isolates, in agreement with previous findings by Combar et al. (Combar et al., 2024). Also, three of these *cpsO*-negative, high-mortality isolates were from Farm F38, where all 12 isolates otherwise had a high level of core genetic similarity, suggesting other VFs are likely to have been involved in these outbreaks.

Some inconsistencies were also observed between the reported clinical/environmental status of certain isolates (UKL12, UKL15, and UKL25) and their phylogenetic clustering. These isolates grouped with low-mortality/environmental/commensal strains, despite being described as clinical in O'Dea et al. (Table S1); (O'Dea et al., 2025). The *cpsO* gene in UKL12 and UKL15 was absent while present for UKL25 . These discrepancies may also reflect limitations in the reported metadata and inconsistency in clinical/commensal classification of these isolates. Isolate UKL15 (reported as strain 40 in their dataset) was classified as “clinical” yet was isolated from the caeca of a healthy 364-day-old bird. Isolate UKL12 (their strain 22) was reported as clinical and associated with bacteraemia, although

the source of the isolate is not specified. UKL25 (their strain 6) was classified as clinical, but no bird age, isolate source or clinical history metadata were reported. Taken together, these observations highlight that discrepancies between metadata-based classifications and phylogenetic clustering can arise. WGS provides an independent, data-driven framework that can help identify such inconsistencies, emphasising the importance of integrating genomic data with appropriate isolate selection, including well-curated and standardised metadata. Future work incorporating harmonised data collection approaches may further improve the reliability of associations between genotype, phenotype, and epidemiological context.

Our study identified 138 distinct VF genes which were variably present across all 283 isolates. As nine VFs were conserved in all isolates, this suggests they are essential for *E. cecorum* survival. The remaining VF genes exhibited a more sporadic occurrence, potentially reflecting strain-specific adaptations, niche specialization, or differential selective pressures associated with virulence. However, it is important to note that as neither the UK nor the non-UK sets of isolates were selected randomly, and given the relatively small sample size, the results may not be fully representative of the entire *E. cecorum* population and require further validation with larger sets of isolates. In addition, it is important to note that mortality on flock levels is influenced also by other factors such as early disease detection, stress, management and disease control measures, which further complicates the picture. In this context, bacterial virulence determinants may represent only one component of a complex disease process (Higuita et al., 2025).

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In conclusion, this study provides an insight into phylogeny, antimicrobial resistance, and genetic VFs of *E. cecorum* in the UK broiler sector. As certain *E. cecorum* clones appear to have been disseminated from farm to farm and occur repeatedly over several production cycles on the same farm, these findings underscore the urgent need for targeted research into *E. cecorum* environmental persistence and transmission pathways so control measures can be developed to effectively manage this emerging pathogen. It is likely that robust biosecurity protocols, effective sanitation practices, and continuous monitoring will be essential in preventing the spread of *E. cecorum* and mitigating its impact on poultry health and production.

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Figure captions

Figure 1: Phylogenetic tree of *Enterococcus cecorum* isolates included in this study. From the innermost to the outermost rings, the metadata represent the source (APHA-UK, O'Dea-UK, non-UK), lineage and sublineage designation. Lineages A–H were identified among UK isolates Few O'Dea samples (n=6) that were not included in A-H lineages were marked as UKL. Bootstrap values > 70 are reported

Figure 2: Scatter plot of clonal cluster and farms. The SNP cluster groups (60 SNPs difference maximum) are given on the X-axis and the different farms are on the Y-axis. Samples are color coded by years (reported in the legend).

Figure 3: Distribution of pathogenicity markers (Laurentie *et*

al. 2015) and (Borst *et. al.* 2015) across different isolates from literature (UK and non-UK) and APHA. Lineages and sublineages found for all UK isolates are also reported. For the virulence factors, green indicates absence of a specific gene, yellow its presence. Bootstrap values >70 are reported.

Figure.4: Phylogenetic tree of 60 isolates for which High/Low pathogenicity (APHA) or clinical/environmental (other UK isolates) data were available. Red and blue squares are for reported high and low mortality orange and light green for clinical / environmental samples. For each isolate, the presence (yellow) or absence (light green) is reported for each putative virulence factor. Bootstap values > 70 are reported

Table 1: Lineages and sublineages found for UK isolates. The minimum, maximum average and median SNP values for each lineage are reported together with the AMR profiles.

Lineage	Number of Isolates	Sublineages	Avg_SNPs	Med_SNPs	Min	Max	Years
A	80	4	116.15	14	0	1774	2021-2022
B	26	4	425.11	140	0	1414	2003-2010
C	18	2	44.14	54	7	94	2007-2011
D	5	1	2328.1	2966	0	3387	2004-2012
E	25	3	4905.03	4859.5	0	8154	1999-2022
F	3	1	2877	2958	2276	3397	2021-2022
G	4	2	835.83	1253	0	1254	2003
H	30	3	157	40	0	1769	2022

Table 2: Individual ARGs in UK and non-UK samples. The last column reports the importance according to WHO (Liaw, 2002) classification in CIA (Critically important antimicrobial), HIA (High important antimicrobial), HPCIA (Highest priority critically important antimicrobial). WOAH classification: VCIA: Veterinary Critically Important Antimicrobial Agents, VHIA: Veterinary Highly Important Antimicrobial Agents, VIA: Veterinary Important Antimicrobial Agents. N.A. means the antimicrobial class has not been classified.

Antibiotic resistance genes	Class	UK (n=158)	%	non-UK (n=125)	%	WHO/WOAH class
none		77	48.73	0	0	
erm(B)	Macrolide-Lincosamide-Streptogramin B (MLSB)	27	22.13	85	68	HIA-VCIA
tet-M	Tetracycline	52	32.91	84	67.2	HIA-VCIA
tet-L	Tetracycline	35	22.15	64	51.2	HIA-VCIA
lnu(C)	Lincosamide	18	11.39	30	24	HIA-VHIA
ant4-Ia_b	Aminoglycoside	2	1.27	5	4	CIA-VCIA
aadD	Aminoglycoside	2	1.27	5	4	CIA-VCIA

tet-44	Tetracycline	2	1.27	6	4.8	HIA-VCIA
lnuB	Lincosamide	7	4.43	2	1.6	HIA-VHIA
lsaE	Lincosamide-Streptogramin A (LSA)	7	4.43	2	1.6	HIA-VHIA
tet-U	Tetracycline	0	0	1	0.8	HIA-VCIA
ant6-Ia_c	Aminoglycoside	8	5.06	4	3.2	CIA-VCIA
mefG	Macrolide	1	0.63	0	0	HIA-VCIA
aad6_a	Aminoglycoside	1	0.63	5	4	CIA-VCIA
msrD	Macrolide-Streptogramin B (MSB)	1	0.63	6	4.8	HIA-VCIA
ant6-Ia_a	Aminoglycoside	10	6.3	4	3.2	CIA-VCIA
sat4A	Aminoglycoside	2	1.27	0	0	CIA-VCIA
lnuD	Lincosamide	0	0	1	0.8	CIA-VHIA
vanA-A	Glycopeptide	0	0	1	0.8	HPCIA-N.A.
vanH-A	Glycopeptide	0	0	1	0.8	HPCIA-N.A.
vanR-A	Glycopeptide	0	0	1	0.8	HPCIA-N.A.
vanS-Pt2	Glycopeptide	0	0	1	0.8	HPCIA-N.A.
vanX-A	Glycopeptide	0	0	1	0.8	HPCIA-N.A.
vanY-A	Glycopeptide	0	0	1	0.8	HPCIA-N.A.
vanZ-A	Glycopeptide	0	0	1	0.8	HPCIA-N.A.
aph3-IIIa	Aminoglycoside	2	1.27	1	0.8	CIA-VCIA
ant9-Ia_a	Aminoglycoside	0	0	2	1.6	CIA-VCIA
ermA	Macrolide-Lincosamide-Streptogramin B (MLSB)	0	0	2	1.6	HIA-VCIA
tet-O	Tetracycline	4	2.5	6	4.8	HIA-VCIA
ermT	Macrolide-Lincosamide-Streptogramin B (MLSB)	0	0	1	0.8	HIA-VCIA
ermG	Macrolide-Lincosamide-Streptogramin B (MLSB)	0	0	7	5.6	HIA-VCIA
mefA	Macrolide	0	0	8	6.4	HIA-VCIA

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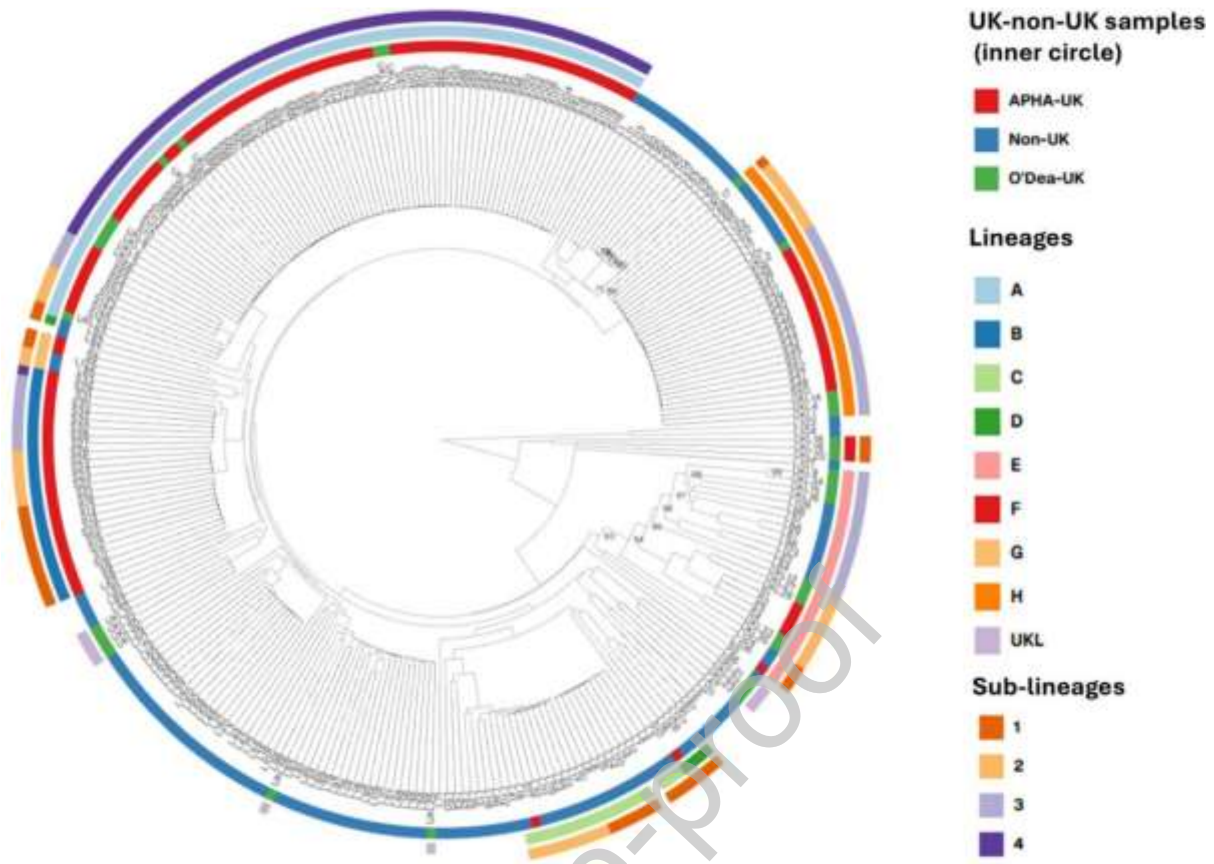

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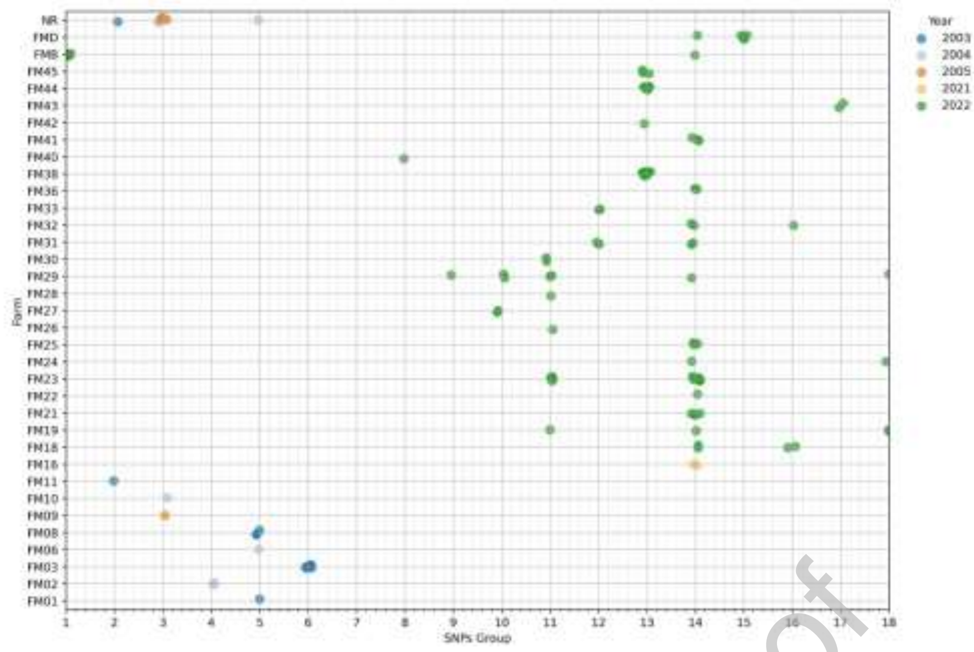

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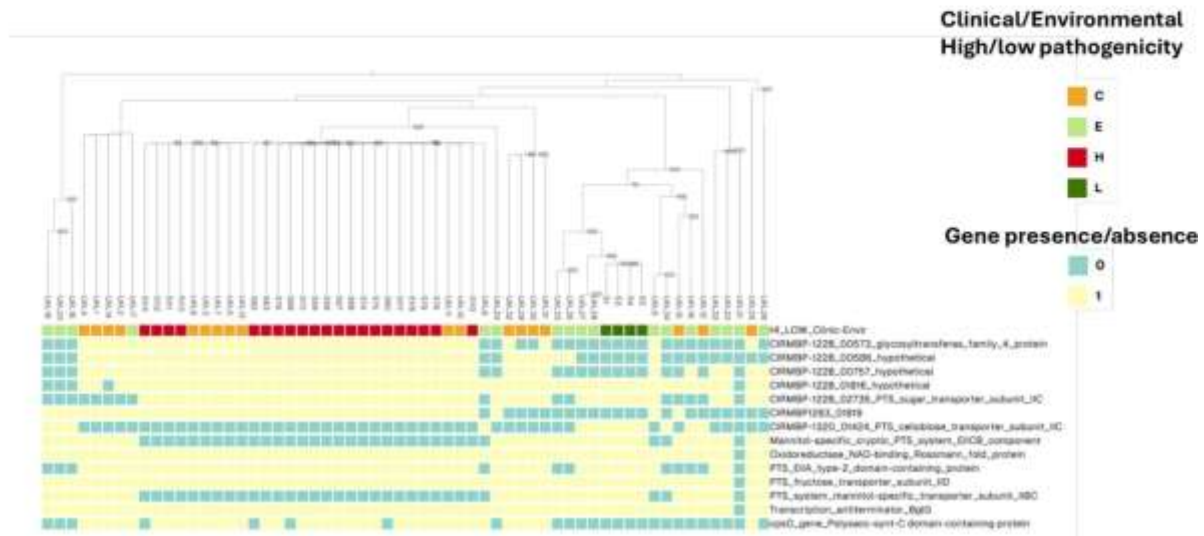

conflict_of_interest

The authors declare no conflict of interest.

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