

Global population genomics reveals the expansion of a chromosomally stabilized multidrug-resistant subclade of *Clostridium perfringens*

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Abstract

Clostridium perfringens is a key intestinal pathogen threatening human and animal health globally, yet its genomic diversity, antimicrobial resistance (AMR) evolution, and transmission remain incompletely understood. Here, we conducted a comprehensive genomic analysis of over 4,000 *C. perfringens* isolates collected worldwide over the past eight decades. We found that the AMR burden has significantly increased over time, and the distribution of AMR genes and their associated mobile genetic elements shows a clear host-related pattern. Phylogeographic analysis indicates that in China, Jilin and Hebei provinces may be the main transmission centers, promoting regional and inter-provincial transmission along livestock movement routes. Notably, we identified the emergence and expansion of a multi-antibiotics resistant clade 4.2 lineage, which gathered a diverse array of resistance determinants and mobile genetic elements in its chromosome through a serial of horizontal genetic transfers. This study establishes a global evolutionary framework and informs One Health surveillance and control of *C. perfringens*.

Introduction

Clostridium perfringens is a ubiquitous, spore-forming anaerobic bacterium that represents one of the most important zoonotic pathogens infecting both humans and animals [1, 2]. It is responsible for a broad spectrum of gastrointestinal and systemic diseases, including foodborne gastroenteritis, necrotizing enteritis, and enterotoxemia, resulting in the second and fourth most frequent infections in the US and Europe, respectively [3–5]. The pathogenicity of *C. perfringens* is primarily mediated by a panel of over 20 tissue-degrading toxins, which act synergistically to disrupt host barriers, facilitate bacterial dissemination, and amplify inflammatory damage [6–8]. Based on the composition of major toxins, the species is classified into seven toxinotypes (A–G) [9–12], each associated with distinct clinical manifestations and host ranges. Among them, type A and F strains are the most prevalent globally and account for the majority of human and animal infections.

Traditionally, *C. perfringens* has been regarded as a highly virulent yet low resistant pathogen. However, this perception is increasingly challenged by accumulated reports of multidrug-resistant (MDR) strains across diverse ecological settings [13–15]. The intensive use of antimicrobials in livestock production has created potent selective pressures, driving the emergence of MDR lineages that threaten to spill over into human populations [16–18]. The accumulation of resistance in such a highly virulent pathogen poses a dual threat, complicating treatment while simultaneously amplifying the risk of severe disease outcomes [19–21]. Despite these concerns, large-scale, longitudinal investigations into long-term evolutionary patterns of AMR and virulence in *C. perfringens* remain limited, particularly in rapidly developing regions with intensive livestock production systems [22–25]. Moreover, the genetic basis of resistance acquisition, stabilization, and dissemination, particularly the role of virulence-resistance co-adaptation in shaping emerging lineages, remains poorly defined.

To address these gaps, here we performed a comprehensive “One Health” genomic epidemiological analysis of over 4,000 *C. perfringens* isolates spanning nearly a century and comprehensive sources from humans, animals, and the environment. By combining phylogenomic reconstruction, resistome and virulome profiling, mobile element analysis, and transmission network modeling, we systematically characterized the evolutionary trajectories, host associations, and spatial diffusion patterns of this pathogen. Notably, we identified an emerging multidrug-resistant lineage, clade 4.2, exhibiting rapid expansion and extensive chromosomal integration of resistance modules. Together, our findings illustrate population dynamics of *C. perfringens*, offering genomic evidence to inform surveillance and intervention strategies within the One Health context.

Result

Comprehensive Genome Collection and Isolate Characterization

Between January 2023 and December 2024, systematic surveillance across clinical, veterinary, food, and environmental settings in China yielded 1,962 non-redundant *C. perfringens* isolates from 22 provinces (Fig. 1A,B). Following stringent quality control, we generated 1,776 high-quality genomes and integrated them with 2,295 publicly available *C. perfringens* genomes (May 2025), yielding a comprehensive collection of 4,071 genomes spanning 1930–2024 (Fig. 1C).

Geographically, isolates were predominantly derived from Asia (64.41%), followed by the Americas (19.38%) and Europe (13.93%), with limited representation from Africa and Oceania. Over half (51.61%) of isolates originated from animals, followed by humans (40.73%) and food and environmental sources (7.66%) (Fig. 1D). This broad host distribution highlights the pronounced ecological plasticity of *C. perfringens* and supports its classification as a quintessential One Health pathogen capable of sustained circulation across human, animal, and environmental reservoirs.

Temporal and Host-Associated Dynamics of Antimicrobial Resistance and Virulence

We observed a significant increase in AMR burden over time (Fig. 2A), particularly after the 1990s, which coincided temporally with the global expansion of industrial livestock production and intensified antimicrobial usage. Genes like *erm*(Q), *lnu*(P), and *tetA*(P) displayed the most prominent temporal enrichment (Fig. 2B), with sharp increase of detection after 1995 and continued elevation throughout the 2000s and 2010s. These genes confer resistance to macrolides, lincosamides, and tetracyclines, respectively, which are all antimicrobial classes widely deployed in both human medicine and veterinary practice, suggesting sustained antimicrobial exposure as a dominant selective pressure.

In contrast, virulence gene abundance remained relatively stable over time (Fig. 2C), with only moderate fluctuations across decades, indicating that resistance and virulence have responded to distinct selective regimes.

Geographical stratification revealed substantial regional heterogeneity, with higher resistance gene loads in Asian isolates relative to those from Europe and the Americas ($P < 0.001$) (Fig. 2D). While most resistance genes were shared across host categories, animal-derived isolates, particularly those from pigs, harbored significantly larger and more diverse resistomes than human or environmental isolates (Fig. 2E), consistent with differential antimicrobial exposure in livestock systems.

Among the 21 resistance genes identified in Chinese isolates, *tetA*(P), *tetB*(P), *optrA*, and *erm*(Q) were the most prevalent (Fig. 2G). Pig-derived isolates exhibited markedly elevated carriage rates of *lnu*(P), *ant*(6)-*lb*, and *optrA* compared with human-derived strains ($P < 0.001$), consistent with extensive exposure to lincosamides, aminoglycosides, and tetracyclines in intensive livestock production systems.

Virulence profiling identified 34 virulence-associated genes, including major toxins, enterotoxins, pore-forming toxins, and extracellular enzymes (Fig. 2F). Functional categorization demonstrated strong enrichment of factors involved in tissue degradation, such as collagenases and hyaluronidases. These enzymes facilitate extracellular matrix breakdown, enhance bacterial dissemination, and synergize with major toxins to exacerbate host damage. The long-term persistence of this virulence repertoire underscores its central role in ecological fitness and disease causation.

Mobile Genetic Elements and Mechanisms of Resistance Dissemination

To elucidate the genetic mechanisms underlying resistance propagation, we systematically examined the distribution of plasmids and insertion sequences across all isolates. Among the 4,071 genomes, 1,532 carried *Tcp* plasmids, 311 harbored *Pcp* plasmids, 626 possessed both types, whereas 1,602 lacked detectable plasmids.

Comparison of resistance burdens revealed that *Tcp*-positive isolates carried significantly more Antibiotics resistance genes (ARGs) than isolates harboring *Pcp* plasmids or mixed plasmid profiles (Fig. 3A). Notably, plasmid-free isolates also exhibited elevated resistance gene counts, which were attributed to extensive chromosomal integration of resistance determinants (see below).

Toxin gene abundance showed an inverse association with ARGs burden. Network analysis of ARGs–Virulence Factors (VFs) co-occurrence further supported this pattern, revealing pronounced mutual exclusivity between these gene classes (Fig. 3B), potentially attributed to evolutionary trade-offs between resistance acquisition and virulence maintenance.

Host-stratified network analyses revealed marked ecological differentiation. Pig-derived isolates exhibited the most complex ARG–IS interaction networks, involving multiple transposase families and

resistance determinants. In contrast, environmental isolates displayed simpler networks dominated by IS1469-associated tetracycline and macrolide resistance genes (Fig. 3C). These findings identify livestock-associated niches as major reservoirs for resistance diversification and innovation.

Insertion sequences belonging to the ISC_{pe} family occupied central positions in the co-occurrence network. IS1216E and ISC_{pe}6 displayed particularly strong associations with *optrA* and multiple auxiliary resistance genes, indicating their central role in resistance mobilization. These elements appear to function as molecular scaffolds facilitating recombination and transposition.

Long-read sequencing of 4 selected representative isolates demonstrated the high chromosomal integration of resistance genes. Particularly, *optrA* was embedded within composite transposons flanked by IS1216E elements, exhibiting high structural similarity to previously described plasmid-borne resistance modules (Fig. 3D). Comparative analysis indicated that these modules had undergone transposition from plasmids to chromosomes, generating stable chromosomal resistance islands. Note that the flanking insertion sequences remained intact, preserving potential for future mobilization.

Population Structure and Phylogenomic Organization

Maximum-likelihood phylogenetic reconstruction based on core single-nucleotide polymorphisms resolved all isolates into four major clades (Fig. 1D) associated with distinct geographic and ecological characteristics.

Clade 4 represented the dominant lineage, accounting for the majority of isolates, and was further subdivided into five well-supported subclades (4.1–4.5). Among these, subclade 4.5 was globally distributed, comprising 1,697 isolates from livestock, humans, and environmental sources, consistent with sustained circulation across interconnected ecological compartments. Other clades showed more restricted distributions and lower genetic diversity, suggesting localized adaptation or limited dissemination. Overall, the population structure reflects a balance between globally circulating generalist lineages and regionally confined specialist groups.

Notably, one subclade, subclade 4.2, displayed a markedly different evolutionary trajectory, characterized by recent emergence and rapid expansion, prompting a more detailed investigation.

Emergence and Expansion of Multidrug-Resistant Subclade 4.2

Spatiotemporal analysis revealed that subclade 4.2 first emerged in China in 2016 and subsequently expanded rapidly, accounting for increasing proportions of isolates in both China and Canada by 2023. Although its geographic distribution remains relatively constrained, its rapid growth indicates ongoing ecological establishment.

Consistent with the phylogenetic structure, cgMLST-based hierarchical clustering demonstrated strong concordance across multiple resolution levels. At the HC1840 threshold, all subclade 4.2 isolates were grouped into a single cluster that was fully consistent with the HC68 classification, supporting the genetic coherence and clonal nature of this lineage (Fig. S1). Moreover, the cgMLST minimum spanning tree revealed a star-like topology with a dominant central cluster, indicative of recent clonal expansion following emergence.

Subclade 4.2 isolates belonged uniformly to toxinotype A and were predominantly animal-derived (Fig. 5A). This subclade harbored a median of 150% more ARGs than other clades (median: 5 vs 2; $P < 10^{-6}$), particularly *erm*(Q), *lnu*(P), and *optrA* (146/611). Notably, subclade 4.2 isolates were rarely associated with plasmid, as revealed by long-read sequencings. Instead, more than 85% of resistance determinants were chromosomally encoded, contrasting sharply with non-4.2 isolates (Fig. S2).

In line with this, ARGs were not organized into large resistance islands but instead formed a limited number of discrete chromosomal clusters. Across the three complete genomes, 2–3 ARG-enriched regions were identified per strain, mainly comprising tetracycline resistance genes [*tetA*(P), *tetB*(P), *tet*(44)] and *ant*(6)-*lb*. These clusters were consistently flanked by insertion sequences, forming putative composite transposons, predominantly mediated by IS1216E and ISCpe family elements. In addition, several ARGs (e.g., *optrA*, *lnu*(P)) were present as single insertions across the chromosome, often associated with nearby IS elements. This pattern indicates a plasmid-independent resistance strategy, likely driven by stable chromosomal integration of resistance islands (Fig. S3).

Antimicrobial Resistance Phenotypes and Multidrug Resistance Profiles

Notably, *optrA*-positive strains were highly concentrated within subclade 4.2, accounting for 80% of the 154 *optrA*-carrying *C. perfringens* isolates. The majority originated from pigs (72%), with smaller contributions from poultry and humans (Fig. S4), highlighting a strong livestock association.

Phenotypically, *optrA*-positive isolates exhibited high-level resistance to multiple antimicrobial classes (Fig. S5). Resistance rates reached 98.7% for erythromycin, 95.3% for tetracycline, 96.7% for gentamicin, and 98.0% for linezolid, with elevated minimum inhibitory concentrations also observed for florfenicol and chloramphenicol. Most isolates were resistant to at least five antibiotic classes, fulfilling criteria for multidrug resistance (Table S1).

Genomic analysis further revealed frequent co-occurrence of *optrA* with *erm*(Q), *lnu*(P), *tetA*(P), *tetB*(P), and *ant*(6)-*lb*, forming a conserved resistance cassette (Fig. S4). This constellation indicates strong co-selection and suggests that oxazolidinone resistance is embedded within broader multidrug resistance platforms.

Temporally, *optrA* was absent before 2010 but increased sharply after 2016, paralleling intensified veterinary antimicrobial use. Together, these results establish *optrA* as a central driver of contemporary

MDR phenotypes.

Transmission Networks and Spatial Dissemination in China

Transmission networks were reconstructed among Chinese isolates using core-genome SNP distances. With a threshold of ≤ 10 SNPs (calibrated against background diversity, we identified 560 potential links, forming a host-resolved network (Fig. 4A).

Animal-to-animal transmission dominated (87%), whereas animal-to-human and human-to-human transmission accounted for 7% and 5%, respectively (Fig. 4B). Network topology analysis identified pigs as central hubs characterized by high connectivity and dense intra-host clustering (Fig. 4C).

Spatial analyses revealed strong provincial clustering, with secondary long-distance connections between northern and southern regions. High-density clusters were detected in Jilin, Hebei, and Guangxi (Fig. 4D). Multiple isolate pairs with zero SNP differences between Jilin and Hebei, two distantly located provinces, suggesting rapid long-distance transmissions likely associated livestock trade.

Overall, the network exhibited a “cluster-and-jump” structure characterized by persistent regional aggregation punctuated by directional long-distance transmission.

Phylogeography and Evolution of Subclade 4.2

Spatiotemporal phylogeographic reconstruction identified two major dissemination centers: a northern cluster (Beijing, Hebei, Jilin, Henan) and a southern cluster (Hunan, Guangxi, Jiangxi, Guangdong) (Fig. 5A B). Frequent bidirectional exchange was observed, with north-to-south movement predominating.

Moderate migration rates from Canada and Germany indicated sporadic international transmission. Some northern Chinese isolates showed close phylogenetic affinity to Canadian strains, suggesting occasional intercontinental exchange.

Evolutionary reconstruction supported a gradual, selection-driven emergence model (Fig. 5C). Initially susceptible lineages progressively accumulated resistance genes under sustained antimicrobial pressure. Subsequent IS-mediated recombination facilitated chromosomal integration of resistance modules, generating stable MDR clones. Homologous recombination among closely related strains further accelerated fixation.

Distinct Evolutionary Strategy of Type F Isolates

Type F isolates exhibited a contrasting evolutionary profile. Resistance gene carriage was generally low, with most isolates harboring only *tetA*(P) and *tetB*(P) and only a single isolate carrying *erm*(Q) (Fig. 6A). This pattern suggests that resistance acquisition is not the dominant adaptive strategy in this lineage.

Instead, Type F isolates were consistently enriched in insertion sequences, particularly ISC_{Cpe4}, IS1469, and IS1151. Comparative genomic analyses demonstrated that the enterotoxin gene *cpe* was embedded within a highly conserved module flanked by truncated IS elements (Fig. 6B). Gene arrangements were nearly identical across hosts and regions.

This structural conservation indicates that *cpe* is maintained as a stable, mobile virulence module capable of horizontal dissemination without extensive genomic remodeling. Collectively, these findings support a “low-resistance, high-virulence stability” strategy, in which pathogenic potential is preserved through insertion sequence–mediated modularity rather than resistance accumulation.

Discussion

This study provides a comprehensive genomic and ecological analysis of *C. perfringens* spanning nearly a century and multiple ecological sources, revealing how antimicrobial resistance, mobile genetic elements, and population structure have co-evolved under intensifying anthropogenic pressure. By integrating large-scale genomic surveillance with phenotypic and transmission analyses, we confirm that contemporary *C. perfringens* evolution is increasingly shaped by livestock-associated selection, resistance chromosome stabilization, and lineage-specific adaptive strategies. These findings deepen current understanding of how facultative pathogens maintain long-term ecological fitness under sustained antibiotic exposure.

A central observation of this study is the marked temporal increase in resistance gene burden since the 1990s, while virulence capacities have maintained remarkable stability. This decoupling indicates that resistance acquisition has become the dominant axis of recent adaptive evolution, imposed by the extensive use of antibiotics [26–28], while core virulence traits remain under strong purifying selection. Similar patterns have been observed in other opportunistic pathogens, including *Acinetobacter baumannii* and *Klebsiella pneumoniae*, where resistance expansion proceeds without parallel increases in virulence determinants [29, 30]. In *C. perfringens*, major pathogenic types (A and F), characterized by tissue-degrading enzymes and major toxins, persistence over decades [31–34], suggesting the role of pathogenicity in ecological adaptation, particularly in gut-associated and necrotic environments. The stability of this virulence core may constrain extensive genomic remodeling, favoring incremental resistance acquisition through mobile elements rather than wholesale genome reorganization.

Our analyses further reveal pronounced regional and host-associated heterogeneity in resistance accumulation, with Asian and pig-derived isolates exhibiting the highest resistance burdens. This pattern is consistent with global estimates indicating disproportionately high antimicrobial consumption in East Asian livestock systems [35, 36]. The enrichment of lincosamide-, tetracycline-, and aminoglycoside-resistance genes in pig-associated isolates reflects prolonged exposure to these compounds in intensive farming. Importantly, resistance profiles were largely shared across host categories, indicating frequent cross-sectoral exchange. This finding reinforces the concept that food-animal reservoirs function as

amplifiers of resistance evolution, with spillover into human and environmental compartments, a dynamic increasingly recognized across bacterial taxa [37–39].

A major contribution of this study is the demonstration that chromosomal stabilization serves as a primary strategy for the spread of drug resistance. Although plasmids have historically been considered primary vectors of ARGs transmission, our data show that many high-resistance isolates, particularly within subclade 4.2, lack detectable plasmids yet harbor substantial chromosomally integrated resistance islands, thereby establishing vertically stable multidrug-resistant genotypes. Similar stabilization processes have been described in *Staphylococcus aureus* and *Enterococcus* spp., where chromosomal integration enhances persistence under fluctuating selective regimes [40–42]. Our study reveals that *C. perfringens* has convergently evolved this strategy under high-pressure conditions, prioritizing genomic stability over horizontal transfer capacity.

The inverse relationship between resistance and virulence gene abundance suggests potential evolutionary trade-off. Although the underlying mechanisms remain unclear, constraints on resource allocation, regulatory disruption, or fitness costs associated with large resistance islands may limit the simultaneous optimization of these two traits. Experimental studies in other pathogens indicate that resistance acquisition can diminish virulence through metabolic burden or regulatory remodeling [43]. In *C. perfringens*, such trade-offs may promote ecological diversification, with highly resistant lineages dominating livestock reservoirs while more virulent, less resistant lineages persist in acute disease settings.

The emergence of subclade 4.2 marks a particularly significant evolutionary development. This lineage exhibits broad chromosomal resistance, high insertion sequence density, and rapid geographic expansion, hallmarks of successful MDR clones. Given the clinical importance of oxazolidinones as last-resort therapies, the dominant position of this subclade among *optrA*-positive isolates is especially concerning. *OptrA*-mediated linezolid resistance has been increasingly reported in Gram-positive pathogens [44, 45], yet the mechanism of its integration into a stable chromosomal platform remains poorly understood. Our data suggest that subclade 4.2 has resolved the mobility–stability trade-off by retaining IS-flanked resistance islands capable of occasional mobilization while ensuring vertical persistence.

The temporal association between the emergence of *optrA*-resistant strains and the surge in veterinary antimicrobial use further substantiates the causal role of agricultural selection. This pattern echoes observations with *Enterococcus faecalis* and *Enterococcus faecium*, where livestock-associated oxazolidinone resistance preceded widespread clinical detection [46]. These findings highlight the limitations of compartmentalized surveillance systems and underscore the need for integrated monitoring of critically important antimicrobials across human and animal sectors.

Transmission network reconstruction reveals that *C. perfringens* circulation in China is dominated by dense livestock-associated clusters connected by episodic long-distance dissemination. Pigs occupy a central hub position in transmission, reflecting their high population density, extensive trade networks,

and frequent exposure to antimicrobials. A similar “cluster-and-jump” pattern has been documented in zoonotic *Salmonella* and *Staphylococcus aureus* populations [38, 47]. In *C. perfringens*, this structure facilitates both sustained regional transmission and rapid expansion of successful clones. The detection of nearly identical isolates across provinces highlights the epidemiological importance of livestock movement in shaping bacterial population structures.

Phylogeographic analyses further indicate that subclade 4.2 has established dual dissemination centers with frequent bidirectional exchange, suggesting a metapopulation structure maintained by agricultural connectivity. Occasional international transmission events, particularly those Canadian isolates, indicate that MDR *C. perfringens* is not geographically confined. Although remained sporadic, such events raise concerns about global dissemination analogous to that observed for MDR *E. coli* ST131 and *K. pneumoniae* ST258 [48, 49].

In contrast to MDR-dominated lineages, Type F isolates exhibit a distinct “low-resistance, high-virulence stability” strategy. The conservation of *cpe*-containing modules flanked by truncated IS elements suggests long-term stabilization of enterotoxin-mediated pathogenicity. This emerging pathotype [50] generally lacks broad-spectrum resistance, suggesting acute virulence, rather than antimicrobial tolerance, remains their primary adaptive advantage. This differentiation highlights multiple evolutionary solutions within *C. perfringens*, shaped by niche-specific selection mechanisms.

Several limitations warrant consideration. First, despite extensive geographic coverage, sampling remains uneven across regions and historical periods, potentially introducing temporal bias. Second, resistance phenotype assessments primarily target Chinese isolates, limiting global genotype-phenotype comparisons. Third, the trade-off between resistance and virulence remains functionally unvalidated, necessitating experimental evolution and fitness assays to elucidate the causal mechanisms underlying genomic patterns. Finally, while chromosomal integration suggests stability of resistance islands, their long-term mobility remains uncertain.

Despite these limitations, our findings hold significant translational implications. The dominance of chromosomally stabilized MDR lineages implies that interventions targeting plasmid transmission alone will be insufficient. Instead, surveillance systems must incorporate high-resolution genomic monitoring capabilities to detect emerging chromosomal resistance mechanisms. Furthermore, the strong association between livestock production and the evolution of resistance underscores the urgency of implementing antimicrobial stewardship in agriculture, particularly for critically important drugs.

In conclusion, this study reveals that contemporary *C. perfringens* exhibits lineage-specific optimization of resistance, virulence, and genomic stability under sustained human activity pressure. The emergence of chromosomally stable multidrug-resistant clones, exemplified by the 4.2 sublineage, signifies a fundamental shift in the ecology of resistance with significant public health implications. By providing an open-access atlas of resistomes, virulomes, and transmission networks, this study supplies a foundational framework for predictive modeling and intervention design in interconnected one-health systems.

Methods

Ethical approval

All the experimental procedures in this study comply with the relevant ethical regulations. The collection of bacterial strains was approved by the ethics committee of the Second Affiliated Hospital of Zhejiang University School of Medicine before the start of the study. The ethical certification was approved by the Human Research Ethics Committee of the hospital. All participants gave verbal informed consent before sample collection.

Bacterial isolation, identification and preservation

From January 2023 to December 2024, we collected samples from humans, animals and the environment in 22 provinces of China, and conducted a retrospective genomic study on the *C. perfringens* strains isolated from these samples. The collected animal feces, human feces and environmental samples were stored at -80°C . The samples were inoculated into anaerobic meat liver broth within 24 hours after collection, and cultured under anaerobic conditions for 8 hours. Then, the culture liquid was centrifuged and spread on a triple sugar iron (TSC) agar plate, and cultured under anaerobic conditions for 18–20 hours [51, 52].

Single typical colonies were selected from the TSC plates and transferred to blood agar plates containing 5% sheep blood for further cultivation. The initial screening strains were identified by their growth characteristics on the blood plates, and then identified using 16S rDNA sequencing and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) [53]. The strains confirmed to be *C. perfringens* were further detected for the α toxin and enterotoxin genes (Table S2) by PCR. The purified strains were stored in brain heart infusion (BHI) medium containing 30% glycerol and preserved at -80°C for long-term storage.

Antimicrobial susceptibility test

The minimum inhibitory concentrations (MICs) of eight antibacterial antibiotics (penicillin, tetracycline, linezolid, erythromycin, clindamycin, gentamicin, florfenicol, and vancomycin) against the isolates of *C. perfringens* were determined using the broth microdilution method. The concentration gradient ranges of each antibiotic were as follows: penicillin, tetracycline, and linezolid were 0.125–256 mg/L; erythromycin and clindamycin were 0.06–128 mg/L; gentamicin was 1–2048 mg/L; florfenicol was 0.25–512 mg/L; and vancomycin was 0.015–32 mg/L [54]. The fresh culture isolates were standardized to approximately 1×10^6 CFU/mL and mixed with twofold serial dilutions of antibiotics before being inoculated into 96-well microplates and cultured under anaerobic conditions at 37°C for 24 hours. The MIC was defined as the lowest antibiotic concentration that completely inhibited the visible growth of bacteria. The ATCC 13124 strain of *C. perfringens* was used as the quality control strain. The results were interpreted according to the CLSI guidelines (M100-Ed34: 2024).

DNA extraction, sequencing and de novo genome assembly

Extract genomic DNA using the PureLink Genomic DNA Mini Kit (Invitrogen). Construct the library using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina) and sequence it on the Illumina HiSeq X Ten platform, obtaining 300 bp paired-end reads with an average sequencing depth of no less than 150×. The raw sequencing data are quality-controlled and then assembled from scratch using SPAdes v3.11.1 [55]. The genomic quality is evaluated by CheckM v1.0.12 [56], and genomes with a completeness of $\geq 85\%$ and contamination $< 5\%$ are included in the subsequent analysis.

Collection and organization of public genomic data

As of October 20, 2025, genomic data of *C. perfringens* was collected from the NCBI database. Only those genomes with clear collection time, isolation source and geographical location information were retained for subsequent analysis. Eventually, 2295 public genomes were obtained and merged with the sequencing data from this study for comprehensive analysis.

Identification of VFs, ARGs and plasmids

All the strains were typed for A–G type toxins using TOXlper v1.1 [57]. The virulence-related genes and colonization factors were searched through ABRicate v1.0.1 [58] (parameters: --minid 90, --mincov 80). The ARGs were identified based on the ResFinder v4.0 database [59].

To identify the conjugative plasmids pCW3 and pCP13 families, a custom database was constructed based on the *Tcp* and *Pcp* loci (reference sequences: NC_010937.1 and AP003515.1). Strains carrying more than 3 *Tcp* or *Pcp* encoding genes were determined to contain the corresponding plasmids [60, 61]. The heat map was drawn using Sangerbox 3.0 [62].

Phylogenetic and population structure analysis

The genome annotation was completed using Prokka v1.14.6 [63], and the pan-genome analysis was constructed by Roary v3.13.0 [64] to build the core gene matrix. Core genome SNPs were extracted using EToKi [65], and the maximum likelihood phylogenetic tree was constructed based on IQ-TREE2 (model parameters: -m MF) [66]. Phylogenetic clustering was accomplished using rhierBAPS [67], and the tree structure was visualized and annotated using iTOL [68].

cgMLST and transmission analysis

Construct a cgMLST dataset for all strains, conduct core genome analysis using rapidCGA_v2, and achieve multi-level clustering through HierCC [69]. Genomes with insufficient cgMLST locus coverage were excluded from HierCC clustering. Evaluate the biological rationality of different clustering levels using HCCeval. Visualization was accomplished using Grapetree [70]. The SNP distance is calculated by SNP-dists v0.7.

Temporal scale and geographical transmission inference

The TreeTime v0.11.3 [71] software was used to reconstruct the time scale of the phylogenetic tree. Firstly, the linear correlation between the root tip distance and the sampling time was evaluated to detect the molecular clock signal. Given that the majority of the strains were collected in 2023, the overall molecular clock signal was relatively weak. Further, the migration module of TreeTime was used, treating the countries as discrete traits, to conduct maximum likelihood inference of the ancestral geographical state, thereby assessing the migration events between different countries and the potential transmission paths of emerging antibiotics-resistant branches.

Declarations

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Contributions

J.W., Z.Z., H.W. and Z.Y. conceived and designed the study. Z.Z., Y.Z., R.Z., L.X., Y.T., X.T., Y.Y. and H.Z. designed the methodology. H.Z., L.Z. and K.W. conducted bioinformatics analyses and interpreted data. X.T. and R.Z. collected the data. Z.Y., M.F., Y.Z., X.W. and performed the genetic interaction screens. All authors provided comments in editing the manuscript.

Ethics declarations

Competing interests

The authors declare no competing interests.

Additional information

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Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures

the number of *C. perfringens* isolates per Chinese province, ordered by abundance. (C) Global distribution of *C. perfringens* isolates, with country-specific stacked bar charts and pie charts illustrating the proportional breakdown of isolates by clade and year of collection. (D) Maximum-likelihood phylogenetic tree of 4,071 *C. perfringens* strains based on single-nucleotide polymorphisms (SNPs). Concentric rings from inside to outside denote: clade (numbered 1–4.5), plasmid (*Pcp*, *Tcp*), geographic origin (Africa, America, Asia, Europe, Oceania), host (animal, environment, food, human), pathotype (A–G), year of isolation (1930–2024), number of ARGs, and number of VFs.

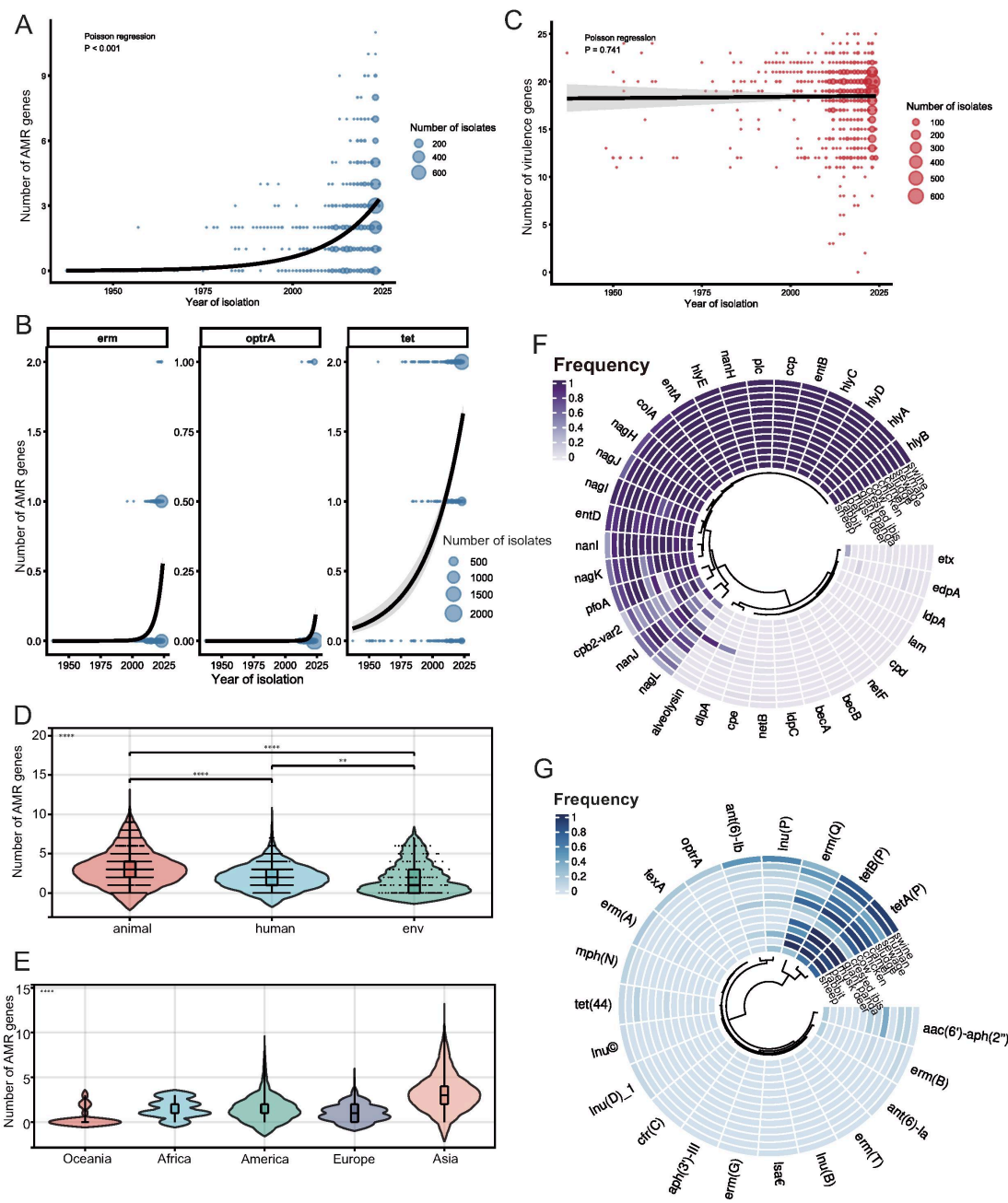


Figure 2

Temporal and spatial dynamics of antimicrobial resistance and virulence genes in *C. perfringens*

(A) Temporal trend in the total number of antimicrobial resistance (AMR) genes per *C. perfringens* isolate, analysed by Poisson regression ($P = 0.001$). The size of each data point is proportional to the number of isolates (50, 100, 200, 300, 600). (B) Temporal trend in the total number of virulence genes per isolate, analysed by Poisson regression ($P = 0.741$). Data points are colored and sized according to the number of isolates, as in a. (C) Temporal trends in the abundance of three key AMR genes (*erm*, *optrA*, and *tet*) per isolate, with point size and color indicating the number of isolates (50, 100, 200, 2000). (D) Distribution of the total number of AMR genes per isolate stratified by isolation source (animal, human, and environment). Data are presented as violin plots with embedded boxplots; the horizontal line denotes the median, and box limits indicate the interquartile range. Statistical significance was determined by a non-parametric test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (E) Distribution of the total number of AMR genes per isolate across continents (Oceania, Africa, America, Europe, and Asia), presented as violin plots with boxplots (median and interquartile range). (F) Circular heatmap depicting the prevalence of virulence genes across the entire *C. perfringens* isolate collection; color intensity (blue gradient) indicates the frequency of each gene. (G) Circular heatmap showing the prevalence of AMR genes across isolates, with color intensity (blue gradient) representing gene frequency.

AMR gene counts across plasmid types. Point size is proportional to the number of strains (50, 100, 150, 200, 250). (B) Correlation network between insertion sequences (ISs) and AMR/virulence genes. Edge color indicates Pearson's correlation sign (blue: negative; red: positive), edge thickness denotes absolute correlation strength (r_{abs}), and point size reflects significance (red: $P < 0.01$; grey: $P \geq 0.01$). The heatmap background visualizes pairwise Pearson's r values. (C) Co-occurrence networks of AMR genes and IS elements, stratified by isolation source (all, sheep, human, environment, chicken, cow, swine). Node size corresponds to degree, edge width indicates correlation strength, and colour intensity represents co-occurrence frequency. The average weighted degree is annotated for each network. (D) Comparative genomic analysis of representative *C. perfringens* plasmids harbouring AMR genes. Left: circular genome map of plasmid 9-A24, annotated with GC content, GC skew, coding sequences (CDSs), tRNA, rRNA, and CRISPR regions. Right: synteny plot showing genomic conservation and rearrangement between related plasmids.

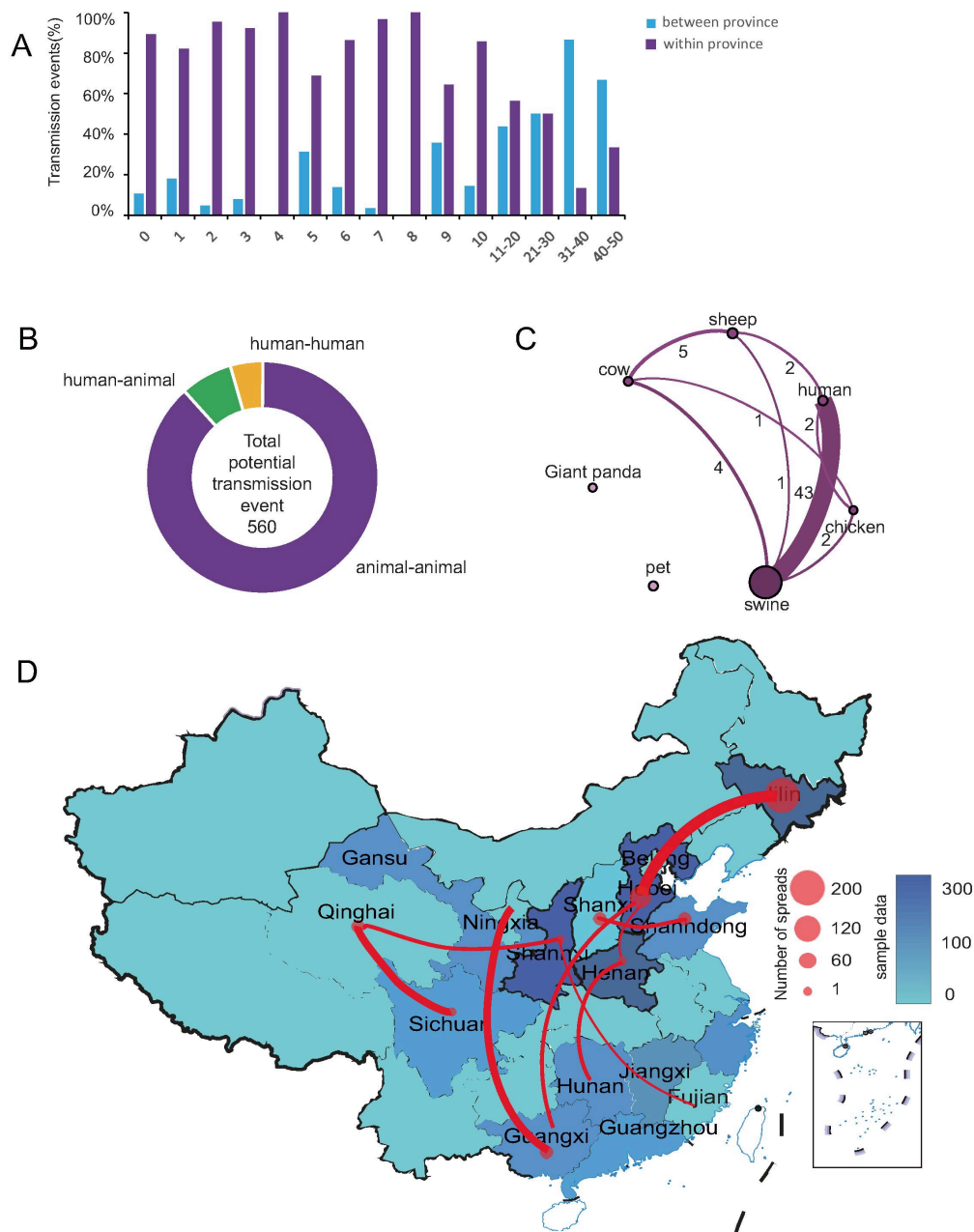


Figure 4

Population structure and transmission dynamics of *C. perfringens* in China

(A) Proportion of intra-provincial (purple bars) and inter-provincial (blue bars) transmission events across SNP distance thresholds (0–50 SNPs). (B) Donut chart illustrating the relative contribution of different transmission types (animal–animal, human–animal, human–human) to the total 560 potential

Figure 5

Global population structure and evolution of multidrug-resistant *C. perfringens*

(A) Maximum-likelihood phylogenetic tree of *C. perfringens* clade 4.2, with concentric annotation tracks showing year of isolation, pathotype, host, continent, plasmid type, and carriage of 11 antimicrobial resistance (AMR) genes (from top to bottom: *ant(6)-Ib*, *aph(2'')-Ih*, *erm(B)*, *erm(Q)*, *lnu(D)*, *lnu(P)*, *mph(B)*, *optrA*, *tet(44)*, *tetA(P)*, *tetB(P)*). Branches are coloured by geographic origin to highlight the emergence and spread of a multidrug-resistant (MDR) clade. (B) Geographical transmission map of MDR *C. perfringens* strains in China and Canada. Provinces/states are shaded by isolate count (light grey: 0–100; dark grey: 100–300). Purple and red ovals denote northern and southern dissemination clusters in China, respectively; blue arrows indicate cross-border transmission events between China and Canada. (C) Model for the evolution and chromosomal integration of *C. perfringens* clade 4.2. Left: Antibiotic selection pressure drives the emergence of MDR strains from susceptible clade 4 isolates, via IS-mediated recombination of the *optrA* AMR gene cassette. Right: Subsequent homologous recombination facilitates the integration of AMR determinants into the chromosome, leading to the stable OPCP (*optrA*-positive clade 4.2) lineage, which is transmitted between China and Canada.

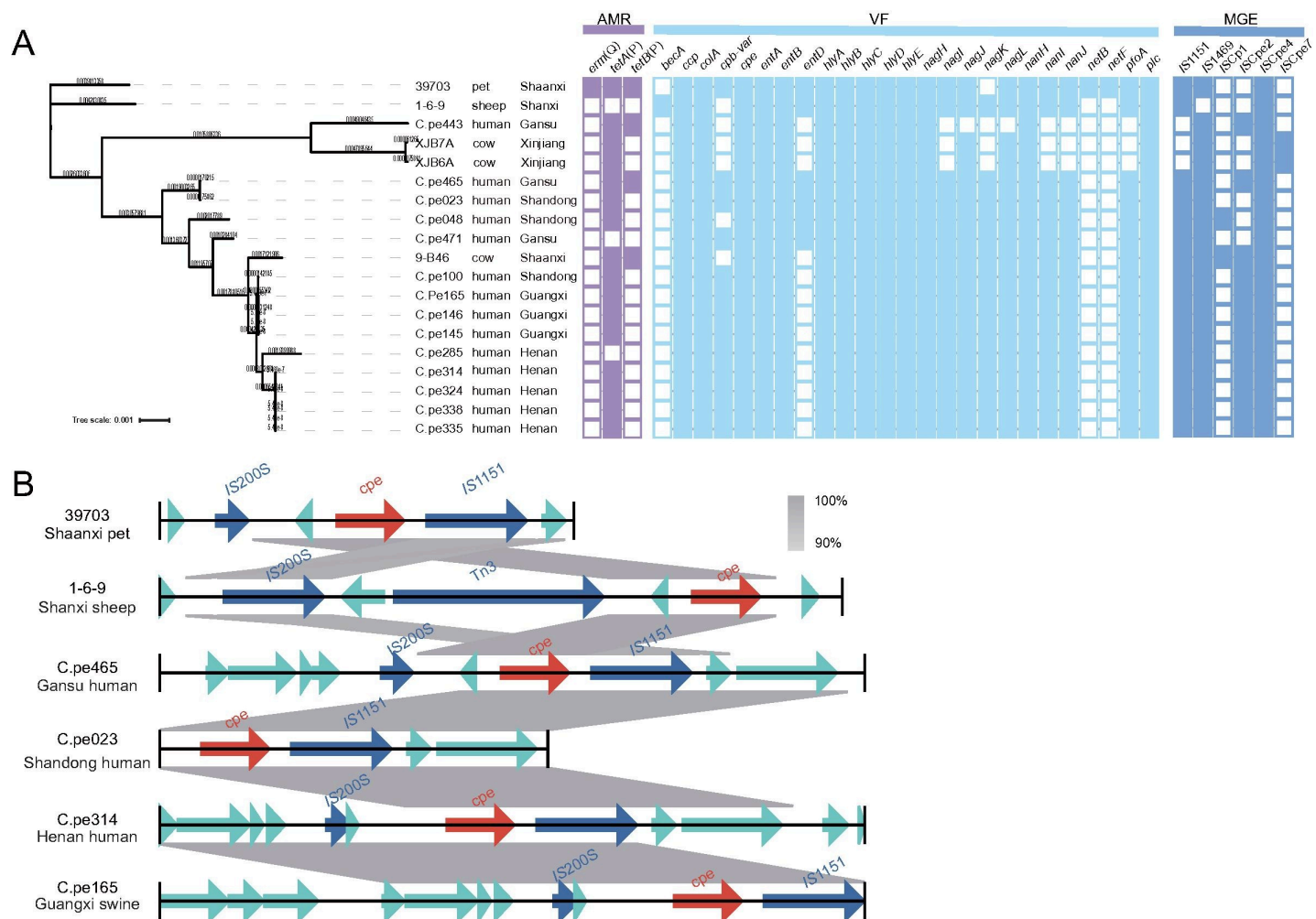


Figure 6

Phylogenetic analysis and structural comparison of the *cpe*-containing genomic island in type F *C. perfringens*

(A) Maximum-likelihood phylogenetic tree of type F *C. perfringens* isolates, annotated with host, location, and carriage of key gene classes: antimicrobial resistance (AMR) genes (*erm(Q)*, *tetA(P)*, *tetB(P)*), virulence factors (VFs, including *cpe* and other toxin genes), and mobile genetic elements (MGEs, including insertion sequences IS1151, IS1469, ISCp1, ISCp2, ISCp4, ISCp7). (B) Structural alignment of the *cpe*-containing genomic island across representative type F *C. perfringens* isolates from diverse hosts and regions (Shaanxi pet/sheep, Gansu human, Shandong human, Henan human, Guangxi swine). Genes are coloured by function: *cpe* (red), insertion sequences (blue: IS1151, IS200S), transposon Tn3 (grey), and other accessory genes (teal). Grey shading indicates regions of $\geq 90\%$ nucleotide identity, highlighting conserved synteny and IS-mediated rearrangements in the *cpe* island.

Supplementary Files

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