ISSN 2096-7071 (Print)
ISSN 2096-3101 (Online)
CN 10-1629/R1

CHINA CDC WEEKLY

Vol. 7 No. 3 Jan. 17, 2025

CCDC weekly

中国疾病预防控制中心周报

ONE HEALTH ISSUE

ISSN 2096-7071
9 772096 707255 03>

Cover Image: adapted from Canva, https://www.canva.cn/.

Vital Surveillances

Molecular Epidemiology of Type F *Clostridium perfringens* Among Diarrheal Patients and Virulence-Resistance Dynamics — 11 Provinces, China, 2024

Zelin Yan[1,&]; Hanyu Wang[2,&]; Yanyan Zhu[1]; Xuejin Wang[1,3]; Yongning Wu[4]; Yang Wang[2,#]; Rong Zhang[1,#]

ABSTRACT

Introduction: Type F *Clostridium perfringens* (*C. perfringens*) represents a significant pathogen in human gastrointestinal diseases, primarily through its *cpe* gene encoding *C. perfringens* enterotoxin (CPE). This investigation examined the prevalence, antimicrobial resistance patterns, and genetic characteristics of Type F *C. perfringens* within the Chinese population.

Methods: The study analyzed 2,068 stool samples collected from 11 provincial hospitals in 2024. Antimicrobial susceptibility testing was conducted following Clinical & Laboratory Standards Institute (CLSI) guidelines, while whole-genome sequencing provided detailed genetic profiles. Evolutionary relationships and clonal transmission patterns were investigated through phylogenetic and genetic environment analyses.

Results: The prevalence of Type F *C. perfringens* was 2.38%, with isolates predominantly identified in human clinical samples and higher detection rates in gastroenterology departments. Notably, 47.1% of isolates demonstrated high resistance to metronidazole, while all exhibited intermediate resistance to erythromycin. Phylogenetic analysis revealed high similarity among isolates from patients within the same province (single-nucleotide polymorphism (SNPs)<100), and genetic environment analysis indicated potential horizontal gene transfer between animal and human strains.

Conclusions: This investigation predominantly identified Type F *C. perfringens* in human clinical cases, with sporadic detection in pets and food products. These findings highlight the emergence of Type F *C. perfringens* outbreaks among diarrheal patients, emphasizing the necessity for targeted interventions as virulence factors increase.

Clostridium perfringens (*C. perfringens*) is ubiquitously distributed across diverse environments, including soil, water, and animal gastrointestinal tracts (*1*). Based on the differential production of four major extracellular toxins (α, β, ε, and ι), toxin-producing strains are classified into five distinct toxinotypes (A through E) (*2*). Among these, Type F *C. perfringens* is particularly significant due to its *cpe* gene, which encodes enterotoxin CPE and is associated with non-foodborne gastrointestinal diseases (*3–4*). Type F *C. perfringens* has been implicated in large-scale diarrheal outbreaks, with strains harboring both *plc* and *cpe* genes identified in cases such as those reported in Beijing (*5*). Global epidemiological data indicate that Type F *C. perfringens* accounts for a substantial proportion of foodborne disease outbreaks in both developed and developing nations (*4*). In the United States alone, Type F food poisoning affects approximately 1 million individuals annually, resulting in economic losses exceeding $310 million (*6*). These infections can prove fatal even in otherwise healthy individuals (*7*).

The extensive deployment of antimicrobial agents has escalated antibiotic resistance among *C. perfringens* strains. Resistance mechanisms include β-lactamase production, multidrug efflux pumps, and plasmid-mediated gene transfer (*8*). Agricultural isolates demonstrate high resistance to multiple antibiotics, particularly tetracyclines and fluoroquinolones (*9*). In China, 13.8% of *C. perfringens* isolates exhibit resistance to six antibiotics, with 54.4% harboring multiple resistance genes (*10*). Similar multidrug resistance patterns have been documented globally, significantly impacting both animal and human health (*11–12*).

The investigation of Type F *C. perfringens* is therefore crucial, particularly in the context of diarrheal illness. Beyond its role in widespread foodborne outbreaks, this strain's capacity to cause severe gastrointestinal disorders represents a significant public health concern. This study aims to elucidate the molecular epidemiology and pathogenic mechanisms

of Type F *C. perfringens* in patients across 11 provincial-level administrative divisions (PLADs) in China, employing bioinformatics analysis to characterize resistance and virulence genes. This comprehensive approach is essential for addressing the challenges posed by *C. perfringens* and protecting both animal and human health.

METHODS

Sample Collection

From January 2 to May 28, 2024, we conducted a cross-sectional study to determine *C. perfringens* prevalence among inpatients at 11 provincial hospitals across China. The study included hospitals in Shandong (*n*=230), Guangxi (*n*=100), Henan (*n*=196), Gansu (*n*=190), Shaanxi (*n*=243), Fujian (*n*=238), Hunan (*n*=104), Guangdong (*n*=177), Jilin (*n*=200), Jiangxi (*n*=300), and Zhejiang (*n*=350) PLADs. Participating departments included Gastroenterology and Neurology. A total of 2,068 fecal or rectal swab samples were collected using ESwab^{TM} collection kits (Copan, Brescia, Italy). For *C. perfringens* isolation, we processed either a small fecal sample or 0.2 mL of transport medium with 50% ethanol, followed by centrifugation and plating on TSC agar for anaerobic incubation. Suspected colonies underwent further purification on blood agar and definitive identification using MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of *C. perfringens* isolates was performed using the Etest method following Clinical & Laboratory Standards Institute (CLSI) guidelines (M100-S29:2019). Nine antimicrobial agents were evaluated: metronidazole, penicillin, amoxicillin, tetracycline, ciprofloxacin, cefoxitin, linezolid, clindamycin, and erythromycin. For erythromycin and ciprofloxacin testing, we applied breakpoints equivalent to clindamycin and fluoroquinolones, respectively, due to the absence of specific CLSI guidelines for *C. perfringens*. *C. perfringens* ATCC 13124TM served as the quality control strain.

Whole-Genome Sequencing (WGS) and Analysis

Genomic DNA extraction was performed using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). DNA libraries were indexed using TruSeq DNA PCR-free Sample Preparation Kit (Illumina, Inc., San Diego, CA) and sequenced on the Illumina HiSeq X Ten System, generating 300-bp paired-end reads with minimum 150-fold coverage per isolate. Raw reads underwent trimming and assembly using SPAdes v3.11.1, followed by targeted analysis of AMR and virulence genes using ABRicate against relevant databases, employing thresholds of >90% identity and >75% coverage.

Phylogenetic Analysis

Type F *C. perfringens* isolates were retrieved from the National Center for Biotechnology Information (NCBI) database on August 27, 2024, using specific search criteria: "Toxin_genotypes: *cpe* & *plc*" and "species_taxid:1502." 91 Type F *C. perfringens* isolates, with their sources, countries of origin, and accession numbers are documented in Table 1 (*13*). Single-nucleotide polymorphisms (SNPs) were identified through sequence alignment using Snippy v4.6.0 (https://github.com/tseemann/snippy) (*14*), which generated a core genome alignment profile. Pairwise SNP distances were calculated using Snp-dists v0.6. We constructed a phylogenetic tree based on core-genome SNPs using Parsnp within the Harvest suite, with midpoint rooting and visualization enhanced through iTOL v6.25 (*15*).

Statistical Analysis

Clinical data were extracted from the hospital information system. We employed the Wilcoxon test to analyze differences in antimicrobial resistance and virulence genes, while Pearson chi-square and Fisher's exact tests were used to evaluate statistical significance (P<0.05) in gene frequencies and resistance phenotypes.

Data Availability

All supporting data for this study are included in this article and its Supplementary Information. The genome assemblies of *C. perfringens* have been deposited in NCBI under BioProject accession number PRJNA1154412. Additional data are available from the corresponding authors upon reasonable request.

RESULT

Epidemiological Information for Type F *C. perfringens* Isolates from China

Among 2,068 non-duplicated stool specimens

TABLE 1. Updated summary of the pathogenicity mechanisms of the currently identified/ characterized *Clostridium perfringens* toxins.

	Toxins	Gene	Toxin name	Alternative name	Mechanism of pathogenicity
1	Typing toxins	*plc/cpa*	Phospholipase	α-toxin	Disruption of cell membrane
2		*cpb*	β-toxin	–	Pore-formation
3		*etx*	ε-toxin	–	Pore-formation
4		*iap*	ι-toxin component Ia	–	Cytoskeleton disruption
5		*ibp*	ι-toxin component Ib	–	Cytoskeleton disruption
6		*cpe*	Enterotoxin (CPE)	–	Pore-formation and tight-junction disintegration
7	Non-typing toxins	*netB*	NetB	–	Pore-formation
8		*cpb2*	β2 toxin	–	Pore-formation
9		*lam*	λ-toxin	–	Potent protease
10		*pfo/pfoA*	Perfringolysin O	θ-toxin	Pore-formation
11		*cpd*	δ-toxin	–	Pore-formation
12		*ccp*	Clostripain	–	Digestion of collagen
13		*colA*	Microbial collagenase	κ-toxin	Digestion of collagen
14		*nanI*	Sialidase	–	Mucolysis
15		*nanJ*	Exo-α-sialidase	–	Mucolysis
16		*nanH*	Neuraminidase	–	Mucolysis
17		*nagH*	Hyaluronidase	μ-toxin	Digestion of connective tissue
18		*tpeL*	Glucosylating toxin	–	Induction of apoptosis
19		*becA*	Binary Enterotoxin Component A	–	Pore-formation
20		*becB*	Binary Enterotoxin Component B	–	Pore-formation
21		*netE*	NetE	–	Pore-formation
22		*netF*	NetF	–	Pore-formation
23		*netG*	NetG	–	Pore-formation

Note: "–" means no alternative toxins.

collected from patients across 11 provincial tertiary hospitals in 2024, 17 Type F *C. perfringens* isolates were identified, yielding a prevalence rate of 2.38% [95% (confidence interval) *CI*: 1.95%, 2.91%]. These isolates were distributed across 6 PLADs, with isolation rates varying from 0.9% in Shandong to 2.0% in Henan, Jilin, and Guangxi PLADs.

The demographic distribution of Type F *C. perfringens* cases closely mirrored the overall study population, with cases showing a mean age of 54.00±24.00 years and a gender distribution of 52.9% female versus 47.1% male (compared to the overall study population: age 37.51±11.98 years, 45.48% female versus 54.52% male). Notably, 64.7% of Type F *C. perfringens* isolates were recovered from patients presenting with diarrhea in gastroenterology departments.

Antimicrobial Susceptibility Profiles

Antimicrobial susceptibility testing revealed that most Type F *C. perfringens* isolates demonstrated susceptibility to linezolid, cefoxitin, and amoxicillin. However, 47.1% of isolates exhibited high resistance (>32 μg/mL) to metronidazole, and all strains showed intermediate resistance to erythromycin (Figure 1 and Supplementary Table S1, available at https://weekly.chinacdc.cn/).

Genomic Characteristics of 17 Type F *C. perfringens* Isolates

Phylogenetic analysis (Figure 2) revealed distinct clonal clusters of Type F *C. perfringens* isolates, each characterized by specific virulence and resistance determinants. Whole-genome sequencing identified

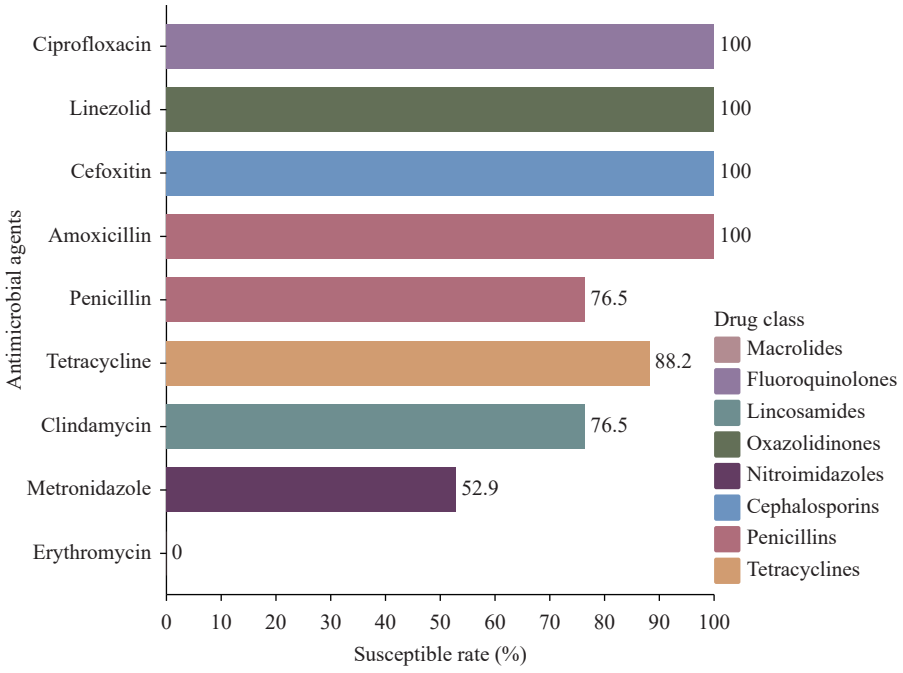


FIGURE 1. Distribution of antimicrobial resistance patterns among 17 F toxinotype *C. perfringens* isolates against 9 antimicrobial agents across 8 distinct categories.

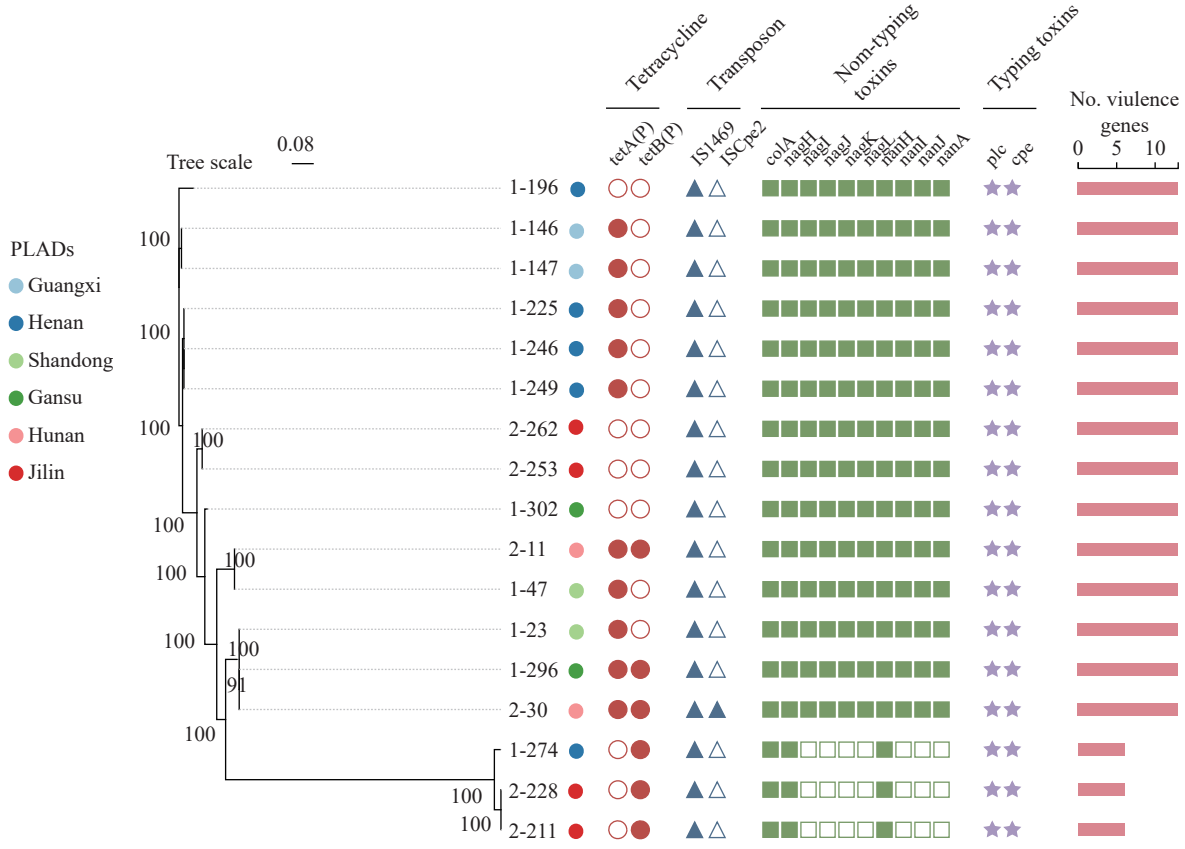


FIGURE 2. Phylogenetic relationships and corresponding antimicrobial resistance phenotypes, virulence characteristics, and genotypic profiles of 17 *C. perfringens* isolates from China.
Abbreviation: PLADs=provincial level administrative divisions.

tetracycline resistance genes [*tet*(A) and *tet*(B)], which corresponded with observed phenotypic resistance patterns. Among the analyzed isolates, we identified 12 distinct virulence factors: *colA, nagH, nagI, nagJ, nagK, nagL, nanH, nanI, nanJ, pfoA, plc*, and *cpe*. All isolates harbored the essential virulence determinants *cpe* (encoding enterotoxin CPE) and *plc* (encoding α-toxin). Notably, 18.0% (3/17) of isolates lacked *nagI, nagJ, nagK, nagL, nanI, nanJ*, and *pfoA* genes. The clustering patterns suggested the emergence of distinct clonal lineages, with an apparent inverse relationship between resistance gene carriage and virulence factor repertoire, indicating a potential fitness trade-off between resistance and virulence mechanisms.

Genetic Environment of the Type F *C. perfringens*

Analysis of the genetic environment (Figure 3) revealed high sequence homology between the IS*1469*-*cpe*-*hp*-IS*1151* gene cluster in our isolates (82.4%, 14/17) and sequences from a diarrheal canine isolate (strain D13122 plasmid pD13122_cpe, accession No. MG456815.1). This homology suggests potential horizontal gene transfer events between animal and human strains. The *cpe* gene, flanked by mobile genetic elements including transposons and insertion sequences (IS*1469*, IS*1151*), was found integrated into the chromosomal DNA of multiple isolates, indicating a mechanism for stable inheritance of virulence factors.

Phylogenetic Analysis of Type F *C. perfringens* Isolates in Global

Our 17 Type F *C. perfringens* isolates were analyzed in comparison with 91 Type F *C. perfringens* strains from the NCBI database, representing 13 countries and diverse sources (Figure 4). Comparative genomic analysis revealed that none of the NCBI database strains exhibited SNP distances less than 100 from our study isolates, suggesting distinct evolutionary trajectories. Notably, isolates 1-296 from Henan Province showed close genetic relatedness (SNPs<100) to isolates 2-30 and 1-23 from Hunan and Shandong, respectively. Within Henan Province, isolates 1-246, 1-225, and 1-249 demonstrated remarkable genetic similarity with less than 5 SNPs difference. Similarly, in Jilin Province, isolates 2-253 and 2-211 exhibited 100% sequence identity with isolates 2-262 and 2-228, respectively. Analysis of antimicrobial resistance genes revealed a relatively low prevalence of resistance determinants, with *tet(A)* and *tet(B)* being the most common at 46.7% and 15.2%, respectively.

CONCLUSION

This study characterized the epidemiological landscape of Type F *C. perfringens* across PLADs, revealing a 2.38% isolation rate. Although this prevalence appears relatively low, the exclusive detection of Type F *C. perfringens* in human samples, particularly from gastroenterology departments, emphasizes its clinical significance in human gastrointestinal health. The predominant isolation from gastroenterology departments aligns with established associations between Type F *C. perfringens* and diarrheal diseases, corroborating previous research linking these strains to gastrointestinal pathology through the *cpe* gene (*16*).

The enterotoxin CPE is a crucial virulence determinant in Type F strains (*17*), with historical studies reporting CPE detection rates of 40%–70% in gastroenteritis outbreaks (*18*). While research in Japan demonstrated a predominance of plasmid-mediated CPE with downstream IS*1151* sequences in food poisoning outbreaks (*19*), our analysis revealed a different pattern. The majority of our CPE-positive isolates (82.0%, 14/17) carried chromosomally-encoded CPE associated with IS*1469*, though some isolates harbored plasmid-borne *cpe*-IS*1151* loci, suggesting potential involvement in extraintestinal *C. perfringens* infections.

Antimicrobial susceptibility profiles revealed a concerning trend: while most Type F *C. perfringens* isolates maintained susceptibility to common antibiotics, 47.1% exhibited high resistance to metronidazole, a critical first-line treatment for anaerobic infections (*20*). The universal intermediate resistance to erythromycin among isolates suggests that antibiotic selective pressure in clinical settings may be driving the emergence of resistant strains, potentially compromising future therapeutic options.

Phylogenetic analysis revealed highly virulent strains (harboring both *cpe* and *plc* genes) with relatively few resistance elements, particularly tetracycline-associated transposons (IS*1469*, IS*cp2*). Core SNP analysis demonstrated evidence of clonal transmission within specific geographic regions, particularly among isolates from Henan, Hunan, Shandong, and Jilin provinces, suggesting localized spread patterns among diarrheal patients.

The identification of genetic similarities between human clinical isolates and those from a diarrheal dog

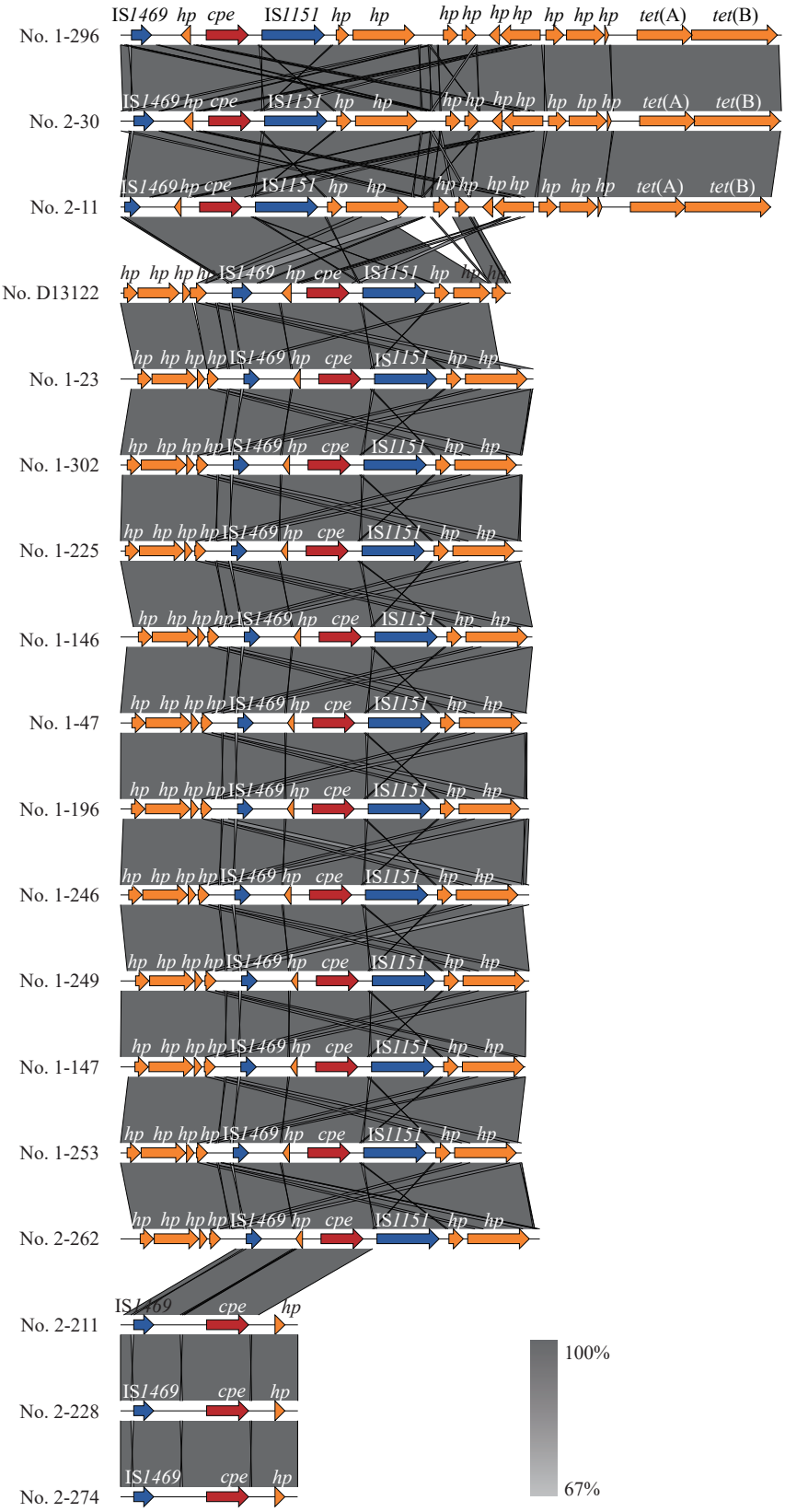


FIGURE 3. Genetic organization of the *cpe* locus in *Clostridium perfringens*.
Note: Arrows indicate gene orientation and function: red (toxin gene *cpe*), blue (mobile genetic elements), and orange (other protein-encoding genes).

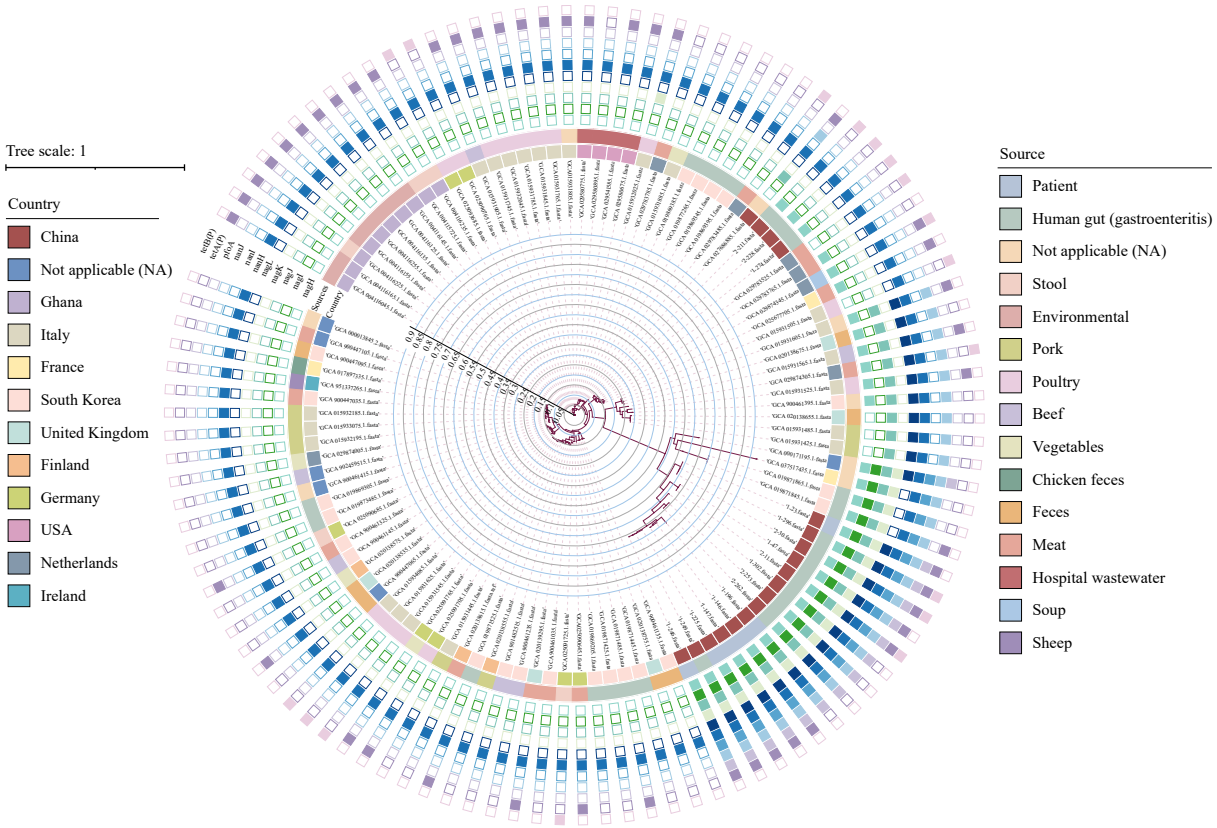


FIGURE 4. Global phylogenetic analysis of 108 F type *Clostridium perfringens* isolates based on core genome SNPs.

in an antibiotic-prevalent setting underscores the significance of horizontal gene transfer in virulence factor dissemination across species. Furthermore, the exclusive detection of Type F *C. perfringens* in human cases within our study suggests potential human-specific adaptation or a preferential ecological niche for human colonization. This host specificity, combined with the pathogen's virulence capabilities, emphasizes the importance of investigating its transmission dynamics, particularly regarding its persistence in human populations despite relatively low isolation rates.

This study's limitations include a relatively modest sample size that may not fully represent the diversity of China's population, potentially affecting the generalizability of the findings to different regions and demographic groups. Additionally, the potential selection bias introduced by hospital participation could skew the results, as the hospitals involved might differ from others in terms of patient characteristics, treatment protocols, and care quality.

In conclusion, our findings highlight the significant public health implications of virulence and antibiotic resistance patterns in Type F *C. perfringens*. The predominant detection of this pathogen in human cases emphasizes its clinical relevance and raises important questions about its transmission mechanisms and host adaptation. These observations underscore the necessity for targeted surveillance and preventive strategies to mitigate potential risks in both clinical and community settings.

Conflicts of interest: No conflicts of interest.

Ethics approval and consent to participate: Ethical approval was given by the Zhejiang University ethics committee (number 2024–0994). Informed patient consent was waived as samples were taken under a hospital surveillance framework for routine sampling. The research conformed to the principles of the Helsinki Declaration.

Funding: Supported by the National Key Research and Development Program of China (No. 2022YFD1800400) and the National Natural Science Foundation of China (No. 22193064).

doi: 10.46234/ccdcw2025.013

[#] Corresponding authors: Yang Wang, wangyang@cau.edu.cn; Rong Zhang, zhang-rong@zju.edu.cn.

[1] Department of Clinical Laboratory, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou City, Zhejiang Province, China; [2] Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Veterinary Medicine, China

Agricultural University, Beijing, China; [3] Key Laboratory of Medical Genetics of Zhejiang Province, Key Laboratory of Laboratory Medicine, Ministry of Education, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou City, Zhejiang Province, China; [4] Key Laboratory of Food Safety Risk Assessment, Ministry of Health and China National Center for Food Safety Risk Assessment, Beijing, China.
[&] Joint first authors.

Submitted: September 04, 2024
Accepted: November 13, 2024
Issued: January 17, 2025

REFERENCES

1. Kiu R, Hall LJ. An update on the human and animal enteric pathogen Clostridium perfringens. Emerg Microbes Infect 2018;7(1):141. https://doi.org/10.1038/s41426-018-0144-8.
2. Hassan KA, Elbourne LDH, Tetu SG, Melville SB, Rood JI, Paulsen IT. Genomic analyses of *Clostridium perfringens* isolates from five toxinotypes. Res Microbiol 2015;166(4):255 – 63. https://doi.org/10.1016/j.resmic.2014.10.003.
3. Grenda T, Jarosz A, Sapała M, Grenda A, Patyra E, Kwiatek K. *Clostridium perfringens*-opportunistic foodborne pathogen, its diversity and epidemiological significance. Pathogens 2023;12(6):768. https://doi.org/10.3390/pathogens12060768.
4. Kiu R, Caim S, Painset A, Pickard D, Swift C, Dougan G, et al. Phylogenomic analysis of gastroenteritis-associated *Clostridium perfringens* in England and Wales over a 7-year period indicates distribution of clonal toxigenic strains in multiple outbreaks and extensive involvement of enterotoxin-encoding (CPE) plasmids. Microb Genom 2019;5(10):e000297. https://doi.org/10.1099/mgen.0.000297.
5. Zhao FL, Gao X, Zhen BJ, Zhang P, Luo YX, Wang RP, et al. Laboratory detection and analysis of a suspected diarrhea outbreak caused by *Clostridium perfringens*. Chin J Food Hyg 2023;35(7):1109 – 13. https://doi.org/10.13590/j.cjfh.2023.07.021.
6. Scallan E, Griffin PM, Angulo FJ, Tauxe RV, Hoekstra RM. Foodborne illness acquired in the United States-unspecified agents. Emerg Infect Dis 2011;17(1):16 – 22. https://doi.org/10.3201/eid1701.P21101.
7. Bamford C, Milligan P, Kaliski S. Dangers of *Clostridium perfringens* food poisoning in psychiatric patients. S Afr J Psychiatr 2019;25:1339. https://doi.org/10.4102/sajpsychiatry.v25i0.1339.
8. Mak PHW, Rehman MA, Kiarie EG, Topp E, Diarra MS. Production systems and important antimicrobial resistant-pathogenic bacteria in poultry: a review. J Anim Sci Biotechnol 2022;13(1):148. https://doi.org/10.1186/s40104-022-00786-0.
9. Ngamwongsatit B, Tanomsridachchai W, Suthienkul O, Urairong S, Navasakuljinda W, Janvilisri T. Multidrug resistance in *Clostridium perfringens* isolated from diarrheal neonatal piglets in Thailand. Anaerobe 2016;38:88 – 93. https://doi.org/10.1016/j.anaerobe.2015.12.012.
10. Yan ZL, Fu B, Zhu YY, Zhang YY, Wu YC, Xiong PF, et al. High intestinal carriage of *Clostridium perfringens* in healthy individuals and ICU patients in Hangzhou, China. Microbiol Spectr 2024;12(7):e0338523. https://doi.org/10.1128/spectrum.03385-23.
11. Golden NJ, Crouch EA, Latimer H, Kadry AR, Kause J. Risk assessment for *Clostridium perfringens* in ready-to-eat and partially cooked meat and poultry products. J Food Prot 2009;72(7):1376 – 84. https://doi.org/10.4315/0362-028X-72.7.1376.
12. Zhang TF, Zhang WT, Ai DY, Zhang RR, Lu Q, Luo QP, et al. Prevalence and characterization of *Clostridium perfringens* in broiler chickens and retail chicken meat in central China. Anaerobe 2018;54:100 – 3. https://doi.org/10.1016/j.anaerobe.2018.08.007.
13. Matsuda A, Aung MS, Urushibara N, Kawaguchiya M, Sumi A, Nakamura M, et al. Prevalence and genetic diversity of toxin genes in clinical isolates of *Clostridium perfringens*: coexistence of alpha-toxin variant and binary enterotoxin genes (*bec/cpile*). Toxins (Basel) 2019;11(6):326. https://doi.org/10.3390/toxins11060326.
14. Kwong JC, Mercoulia K, Tomita T, Easton M, Li HY, Bulach DM, et al. Prospective whole-genome sequencing enhances national surveillance of *Listeria monocytogenes*. J Clin Microbiol 2016;54(2):333 – 42. https://doi.org/10.1128/JCM.02344-15.
15. Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res 2016;44(W1):W242 – 5. https://doi.org/10.1093/nar/gkw290.
16. Shrestha A, Mehdizadeh Gohari I, Li JH, Navarro M, Uzal FA, McClane BA. The biology and pathogenicity of *Clostridium perfringens* Type F: a common human enteropathogen with a new(ish) name. Microbiol Mol Biol Rev 2024;88(3):e0014023. https://doi.org/10.1128/mmbr.00140-23.
17. Sarker MR, Carman RJ, McClane BA. Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two *cpe*-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. Mol Microbiol 1999;33(5):946 – 58. https://doi.org/10.1046/j.1365-2958.1999.01534.x.
18. Kobayashi S, Wada A, Shibasaki S, Annaka M, Higuchi H, Adachi K, et al. Spread of a large plasmid carrying the *cpe* gene and the *tcp* locus amongst *Clostridium perfringens* isolates from nosocomial outbreaks and sporadic cases of gastroenteritis in a geriatric hospital. Epidemiol Infect 2009;137(1):108 – 13. https://doi.org/10.1017/S0950268808000794.
19. Tanaka D, Kimata K, Shimizu M, Isobe J, Watahiki M, Karasawa T, et al. Genotyping of *Clostridium perfringens* isolates collected from food poisoning outbreaks and healthy individuals in Japan based on the *cpe* locus. Jpn J Infect Dis 2007;60(1):68 – 9. https://doi.org/10.7883/yoken.JJID.2007.68.
20. Álvarez-Pérez S, Blanco JL, García ME. *Clostridium perfringens* type A isolates of animal origin with decreased susceptibility to metronidazole show extensive genetic diversity. Microb Drug Resist 2017;23(8):1053 – 8. https://doi.org/10.1089/mdr.2016.0277.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. Antimicrobial susceptibility profiles of 17 F toxinotyping *C. perfringens* strains

Drug class	Antimicrobial agents	MIC50 μg/mL	MIC90 μg/mL	Range μg/mL	R%	I%	S%
Macrolides	Erythromycin	2	2	1 to >2	0.0	100.0	0.0
Fluoroquinolones	Ciprofloxacin	0.25	0.5	0.064 to 1	0.0	0.0	100.0
Lincosamides	Clindamycin	0.5	2	≤0.064 to 2	0.0	23.5	76.5
Oxazolidinones	Linezolid	1	1	0.5 to 1	0.0	0.0	100.0
Nitroimidazoles	Metronidazole	1	>32	0.5 to >32	47.1	0.0	52.9
Cephalosporins	Cefoxitin	0.5	4	0.064 to 1	0.0	0.0	100.0
Penicillins	Penicillin	≤0.064	1	≤0.064 to 1	0.0	23.5	76.5
	Amoxicillin	≤0.064	0.064	≤0.064	0.0	0.0	100.0
Tetracyclines	Tetracycline	1	4	≤0.064 to 16	5.8	5.9	88.2

Note: MIC90 and MIC50 values were defined as the lowest concentration of the antibiotic at which 90% and 50% of the isolates were inhibited, respectively.
Abbreviation: S=susceptible; I=intermediate resistant; R=resistant.

Preplanned Studies

Prevalence and Risk Factors of Multidrug-Resistant *Enterococcal* Infection in Clinical Dogs and Cats — China, 2018–2021

Xukun Dang[1,2,&]; Junyao Jiang[3,&]; Siyu Chen[4,5]; Wei Huang[5]; Ying Jiao[5]; Siying Wang[5]; Zhiyu Zou[1,2]; Qi An[4]; Yu Song[4]; Lu Wang[1,2]; Run Fan[1,2]; Dejun Liu[1,2]; Stefan Schwarz[1,6,7]; Jianzhong Shen[1,2]; Zhaofei Xia[4,5]; Yang Wang[1,2]; Yanli Lyu[4,5,#]; Shizhen Ma[1,2,#]

Summary

What is already known about this topic?

Enterococcus spp., while naturally occurring as commensal bacteria in the gastrointestinal tract of animals and humans, have emerged as significant opportunistic pathogens in healthcare settings.

What is added by this report?

A comprehensive surveillance study revealed enterococci in 14.39% of clinical samples from dogs and cats across China during 2018–2021. Multidrug-resistant enterococcal infections showed significant correlation with urinary tract catheterization and extended hospitalization periods. Notably, pet-derived *Enterococcus faecalis* isolates demonstrated high genetic similarity with strains isolated from humans, farm animals, and environmental sources.

What are the implications for public health practice?

These findings underscore the critical need for enhanced surveillance of enterococcal infections and implementation of stringent aseptic protocols in veterinary clinical settings. Particular attention should be directed toward linezolid-resistant *Enterococcus faecalis* infections due to their demonstrated potential for transmission between pets and humans.

Enterococci, which naturally colonize the gastrointestinal tract of humans and animals, have emerged as significant healthcare-acquired pathogens (*1*). In veterinary medicine, *Enterococcus* spp. represents the third most prevalent pathogen in dogs and cats according to the China Antimicrobial Resistance Surveillance Network for Pets (CARPet) (*2*). These bacteria serve as reservoirs of antimicrobial resistance (AMR), presenting a public health concern through potential human transmission (*3*–*4*). The emergence of linezolid-resistant enterococci is particularly concerning as it limits therapeutic options. This study revealed enterococci in 14.39% of clinical samples from dogs and cats across China, with *Enterococcus faecium* (*E. faecium*) and *E. faecalis* identified as the predominant species. *E. faecium* exhibited higher resistance rates to daptomycin, rifampin, doxycycline, and amoxicillin-clavulanate compared to *E. faecalis*. Multidrug-resistant enterococcal infections were significantly associated with urinary tract catheterization history and extended hospitalization duration. Whole genome sequencing (WGS) analysis demonstrated genetic similarities between linezolid-resistant *E. faecium* isolates from this study and other pet isolates, while *E. faecalis* showed broader genetic relationships across various sources. To mitigate infection risks, particularly considering the potential for pet-to-human transmission of linezolid-resistant *E. faecalis*, enhanced aseptic practices and reduced hospitalization periods in veterinary clinics are recommended.

This study collected *Enterococcus* spp. from clinical samples of dogs and cats across 20 Chinese provinces and municipalities (2018–2021) through the CARPet surveillance system. Clinical samples and medical records were obtained from diseased animals at regional veterinary hospitals, with veterinarians collecting site-specific infected specimens. Antimicrobial susceptibility testing was conducted following CLSI guidelines (CLSI, VET01) using broth microdilution, with *E. faecalis* ATCC 29212 as the quality control strain. The antimicrobial panel included amoxicillin-clavulanate, doxycycline, azithromycin, rifampin, florfenicol, enrofloxacin, linezolid, vancomycin, and daptomycin. Results were interpreted according to CLSI VET01S and M100 breakpoints. Multidrug-resistant (MDR) isolates were defined as those resistant to three or more antimicrobial classes (*5*). Risk factors for MDR infections were evaluated using univariate analysis and logistic regression SPSS (version 22.0, International Business Machines Corporation, Armonk, USA). WGS of linezolid-resistant *Enterococcus* spp. isolates

was performed using the Illumina HiSeq X Ten platform. Draft genomes were assembled using SPAdes and analyzed for sequence types, antimicrobial resistance genes, and virulence genes using SRST2. Phylogenetic analysis compared pet-derived isolates with human, animal, and environmental isolates from China available in the NCBI database (2018–2021). Phylogenetic clusters were determined using fastBAPS software (https://github.com/gtonkinhill/fastbaps). The genome assemblies were deposited under BioProject accession no. PRJNA1039340.

From 2018 to 2021, surveillance across 20 Chinese provinces identified *Enterococcus* spp. in 460 (14.39%) of 3,197 clinical samples (2,247 canine and 950 feline). The highest detection rates were observed in ascites (24.00%), hepatobiliary system (22.00%), and urinary tract (18.68%) specimens. Feline samples exhibited a significantly higher detection rate (22.53%) compared to canine samples (10.95%) (Supplementary Figure S1, available at https://weekly.chinacdc.cn/). Among the 477 *Enterococcus* spp. isolates recovered, *E. faecium* (45.49%) and *E. faecalis* (43.40%) were predominant, with *E. gallinarum, E. avium* and others comprising the remainder (Figure 1). While most isolates originated from Beijing (n=413), others were distributed across Shanghai, Inner Mongolia, Liaoning, Fujian, Jiangsu, and 13 additional provinces (Supplementary Table S1, available at https://weekly.chinacdc.cn/).

Antimicrobial susceptibility testing revealed significantly higher resistance rates in *E. faecium* compared to *E. faecalis* for amoxicillin-clavulanate (86.75% *vs.* 1.14%), doxycycline (81.33% *vs.* 57.14%), rifampin (77.11% *vs.* 63.43%), and daptomycin (7.83% *vs.* 1.14%) (P<0.01) (Tables 1–2). Linezolid resistance was detected in 6.29% of *E. faecalis* and 5.42% of *E. faecium* isolates. All isolates were vancomycin-susceptible except for one intermediate *E. faecium* (Tables 1–2). Multidrug resistance was more prevalent among feline isolates (48.84%) than canine isolates (26.92%), with *E. faecium* exhibiting a substantially higher MDR rate (78.31%) compared to *E. faecalis* (4.57%).

Among 203 enterococcal isolates with complete case information, 76 were classified as MDR. Multivariate analysis initially considered four variables (P<0.05): duration of hospitalization, pet species, pet sex, and history of urinary tract catheterization. The final model, after backward selection, retained only two significant predictors: history of urinary tract catheterization (P=0.03) and duration of hospitalization (P=0.04). These findings indicate that pets with previous urinary tract catheterization or extended hospitalization periods had significantly higher risks of MDR *Enterococcus* infection (Supplementary Figure S2, available at https://weekly.chinacdc.cn/).

Analysis of 19 linezolid-resistant isolates, comprising 11 *E. faecalis* (LREfs) and 8 *E. faecium* (LREfm), revealed the presence of the *optrA* gene in all specimens. The LREfs represented eight distinct sequence types (STs). Phylogenetic analysis, incorporating 81 LREfs from the NCBI database, revealed distribution across six lineages with mixed-source isolates. Notably, seven LREfs showed close genetic relationships (19–94 SNPs) with isolates from humans, pets, farm animals, and plants, suggesting cross-species transmission potential (Figure 2A). The eight LREfm belonged to four known STs, with ST80 being predominant (n=4). Phylogenetic analysis of 56 isolates (including 48 from NCBI) identified three distinct lineages, with seven studied isolates clustering in lineage 3. Two isolates (20928 and 21196) from a cat and dog at the same hospital exhibited remarkable genetic similarity, differing by only five SNPs (Figure 2B), indicating clonal spread among pet isolates distinct from other sources.

DISCUSSION

This study provides a comprehensive analysis of enterococcal infections in Chinese veterinary clinics, encompassing diverse clinical samples from dogs and cats. Our findings, supported by CARPet surveillance data, revealed that *Enterococcus* spp. were present in 14.39% of clinical samples, establishing them as the third most prevalent bacterial pathogens in companion animals (*2*). This prevalence aligns with Korean data (19.3%) (*6*) and parallels reports from European countries, where *Enterococcus* spp. rank among the top five clinical pathogens in Spain (*7*), Portugal (*8*), and the Iberian Peninsula (*9*), comprising 5.6%–15.0% of isolates. Similarly, in Chinese human clinical settings, *Enterococcus* spp. account for 8.89% of isolates, ranking fourth (*10*). These parallel findings underscore the significance of *Enterococcus* as a pathogen in both veterinary and human medicine, emphasizing the critical need for enhanced surveillance protocols.

The emergence of antimicrobial resistance in *Enterococcus* spp., whether through genetic mutations or mobile genetic elements, presents significant therapeutic challenges. Our study revealed that over

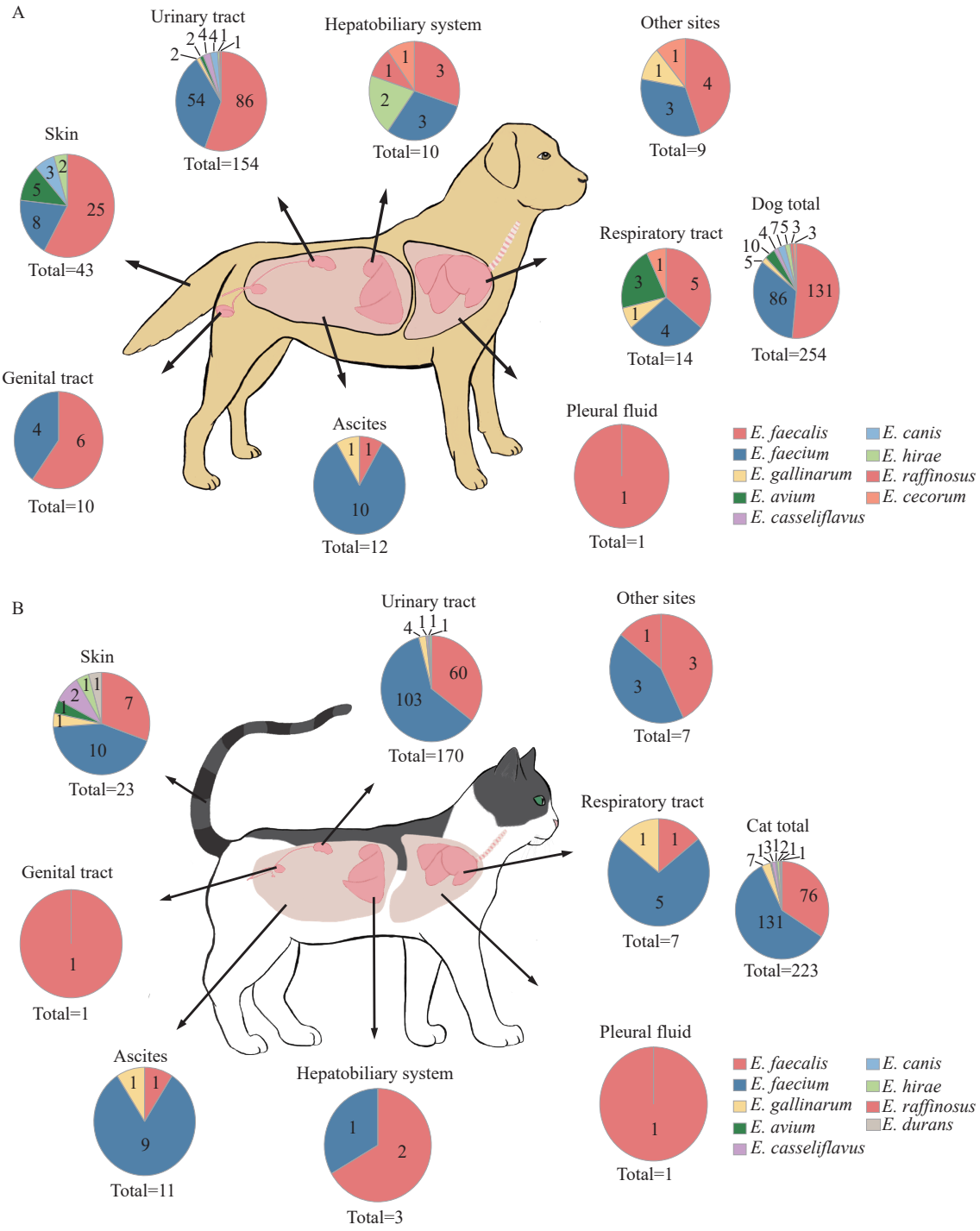


FIGURE 1. Distribution of *Enterococcus (E.)* spp. isolates in various samples. (A) From dogs; (B) From cats.

50% of isolates demonstrated resistance to doxycycline or exhibited elevated MICs for azithromycin and enrofloxacin, consistent with findings (43.2%–99.0%) from Japan (*11*), Australia (*12*), the UK (*13*), and Sweden (*14*). While enrofloxacin remains exclusive in veterinary medicine, both doxycycline and azithromycin are crucial antimicrobials in human and veterinary treatment of urinary and respiratory infections. Of particular concern, 86.75% of *E. faecium* isolates showed resistance to amoxicillin-clavulanate, a primary therapeutic option in both veterinary and human medicine. Encouragingly, resistance rates to last-resort antimicrobials — vancomycin, linezolid, and daptomycin — remained low (0–6.29%), preserving their efficacy against multidrug-resistant infections. These findings

TABLE 1. Minimal inhibitory concentrations and resistance rates of canine and feline *Enterococcus faecalis* isolates.

Antimicrobial agents	Number of *Enterococcal faecalis* corresponding to MIC (mg/L)											No. of resistant isolates (%)
	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	
Amoxicillin-clavulanate (2:1)			6	22	126	16	3	–	–	2		2 (1.14)
Doxycycline		12	8	4	2	1	4	44	76	24		100 (57.14)
Azithromycin			1	–	–	3	14	30	8	119		–
Florfenicol					6	42	93	20	–	–	14	–
Enrofloxacin		–	1	32	91	12	–	1	38			–
Rifampin			6	4	18	36	54	49	8			111 (63.43)
Vancomycin				2	121	49	3	–	–	–	–	0 (0)
Linezolid				4	40	111	9	11	–	–		11 (6.29)
Daptomycin				27	74	52	20	1	1	–		2 (1.14)

Note: The gray-shaded areas indicate untested antimicrobial concentrations. For isolates showing no growth at any concentration, the lowest MIC value was assigned. For isolates exhibiting growth at all tested concentrations, the next higher MIC value above the highest tested concentration was assigned (black numbers on gray background). MIC values for amoxicillin-clavulanic acid (2:1) are reported as amoxicillin MIC values. Color coding indicates susceptibility categories: susceptible (green), intermediate (yellow), and resistant (red).
Abbreviation: MIC=minimum inhibitory concentration.

TABLE 2. Minimal inhibitory concentrations and resistance rates of canine and feline *Enterococcus faecium* isolates.

Antimicrobial agents	Number of *Enterococcal faecium* corresponding to MIC (mg/L)											No. of resistant isolates (%)
	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	
Amoxicillin-clavulanate (2:1)			2	8	5	1	4	2	2	142		144 (86.75)
Doxycycline		12	16	1	1	2	–	–	39	95		134 (80.72)
Azithromycin			1	1	–	–	3	10	10	141		–
Florfenicol					1	14	94	38	2	4	13	–
Enrofloxacin		–	–	–	5	6	3	6	146			–
Rifampin			22	1	3	12	45	58	25			128 (77.11)
Vancomycin				24	117	13	11	1	–	–	–	0 (0)
Linezolid				–	19	118	21	3	5			8 (4.82)
Daptomycin				9	15	60	69	8	5			13 (1.81)

Note: The gray-shaded areas indicate untested antimicrobial concentrations. For isolates showing no growth at any concentration, the lowest MIC value was assigned. For isolates exhibiting growth at all tested concentrations, the next higher MIC value above the highest tested concentration was assigned (black numbers on gray background). MIC values for amoxicillin-clavulanic acid (2:1) are reported as amoxicillin MIC values. Color coding indicates susceptibility categories: susceptible (green), intermediate (yellow), and resistant (red).
Abbreviation: MIC=minimum inhibitory concentration.

emphasize the necessity for systematic antimicrobial susceptibility testing and resistance monitoring in veterinary practice.

Extended hospitalization significantly increases the risk of MDR enterococcal infections in companion animals, potentially through environmental transmission within hospital settings. Previous studies have demonstrated the potential for resistant *Enterococcus* spp. clone transmission within veterinary facilities through multiple vectors, including infected dogs, their owners, veterinary personnel, and hospital environmental surfaces (*6*). Similarly, pets requiring urinary tract catheterization exhibit elevated risks of MDR infections, potentially due to suboptimal aseptic technique during catheter placement and maintenance. The increased risk of healthcare-associated infections through invasive procedures is well-documented in veterinary medicine, emphasizing the critical importance of implementing rigorous cleaning and disinfection protocols to prevent MDR pathogen transmission (*15*).

The presence of MDR bacteria in companion animals, including colistin-resistant and ESBL-producing *E. coli* (*16*) and methicillin-resistant staphylococci (*17–18*), represents a substantial transmission risk to pet owners. Companion animals can serve as reservoirs for antibiotic-resistant enterococci, facilitating their dissemination across human, animal, and environmental interfaces. Our genomic analyses revealed that LREfs demonstrate high

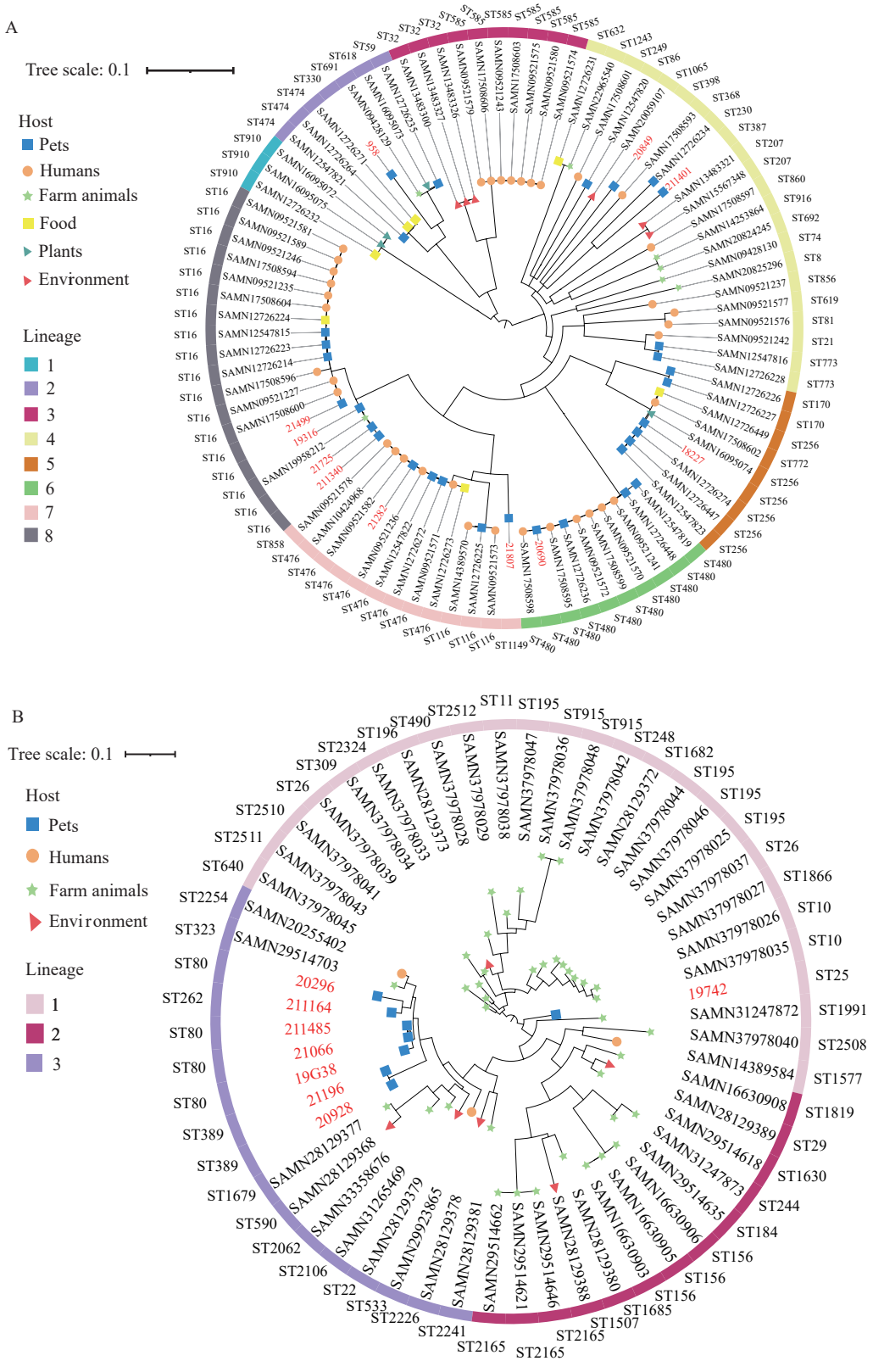


FIGURE 2. Phylogenetic tree of (A) linezolid-resistant *Enterococcus faecalis* and (B) linezolid-resistant *Enterococcus faecium* from pets in this study and humans, pets, farm animals, food, environments, and plants available from NCBI based on the core genome analysis.

genetic similarity across human, animal, and environmental isolates, whereas LREfm exhibits closer genetic relationships primarily among pet isolates. This pattern suggests that LREfs have achieved broader host adaptation and dissemination compared to LREfm, indicating potentially higher transmission risks between pets and their owners.

Study limitations include the restricted whole genome sequencing analysis of only linezolid-resistant isolates, which constrains our ability to comprehensively evaluate transmission patterns. Furthermore, the geographical distribution of samples was notably skewed, with 86.58% of pet-derived enterococci originating from Beijing, highlighting the need for broader surveillance across China to accurately assess national prevalence patterns.

This investigation establishes *E. faecium* and *E. faecalis* as the predominant enterococcal species in Chinese veterinary clinical settings. The study demonstrates that multidrug-resistant enterococcal infections correlate significantly with urinary catheterization procedures and extended hospitalization periods. To mitigate MDR transmission, we recommend implementing dedicated isolation facilities for infected animals, establishing rigorous cleaning and disinfection protocols, and ensuring thorough sterilization of medical instruments. Additionally, enhanced veterinary staff training in aseptic techniques and evidence-based antimicrobial selection, guided by pathogen identification and susceptibility testing, is crucial. Sustained surveillance efforts are essential to prevent the bidirectional transmission of MDR organisms between companion animals and humans.

Conflicts of interest: No conflicts of interest.

Funding: Supported by the National Key Research and Development Program of China (2022YFD1800400), National Natural Science Foundation of China (81991531, 32002339), 2115 Talent Development Program of China Agricultural University.

Acknowledgements: We are grateful to Yang Liu, Yinying Lou, Beibei Liang, Tianli Xie, and Yunke Chen at Beijing Zhongnongda Veterinary Hospital Co., Ltd for their invaluable assistance in sample collection.

doi: 10.46234/ccdcw2025.017

[#] Corresponding authors: Yanli Lyu, luyanli@cau.edu.cn; Shizhen Ma, shizhenma@cau.edu.cn.

[1] Key Laboratory of Animal Antimicrobial Resistance Surveillance, Ministry of Agriculture and Rural Affairs, College of Veterinary Medicine, China Agricultural University, Beijing, China; [2] National Key Laboratory of Veterinary Public Health Safety, College of Veterinary Medicine, China Agricultural University, Beijing, China; [3] Technology Innovation Center for Food Safety Surveillance and Detection (Hainan), Sanya Institute of China Agricultural University, Sanya City, Hainan Province, China; [4] Department of Clinical Veterinary Medicine, College of Veterinary Medicine, China Agricultural University, Beijing, China; [5] Beijing Zhongnongda Veterinary Hospital Co., Ltd., Beijing, China; [6] Institute of Microbiology and Epizootics, Centre for Infection Medicine, School of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany; [7] Veterinary Centre for Resistance Research, School of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany.

[&] Joint first authors.

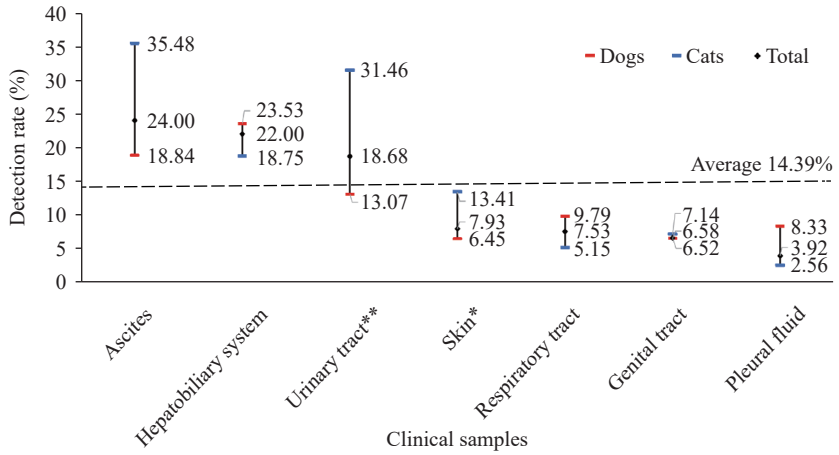
Submitted: September 23, 2024
Accepted: January 08, 2025
Issued: January 17, 2025

REFERENCES

1. Werner G, Coque TM, Franz CMAP, Grohmann E, Hegstad K, Jensen L, et al. Antibiotic resistant enterococci—tales of a drug resistance gene trafficker. Int J Med Microbiol 2013;303(6-7):360 – 79. https://doi.org/10.1016/j.ijmm.2013.03.001.
2. Ma SZ, Chen SY, Lyu YL, Huang W, Liu Y, Dang XK, et al. China antimicrobial resistance surveillance network for pets (CARPet), 2018 to 2021. One Health Adv 2023;1(1):7. https://doi.org/10.1186/s44280-023-00008-w.
3. De Graef EM, Decostere A, Devriese LA, Haesebrouck F. Antibiotic resistance among fecal indicator bacteria from healthy individually owned and kennel dogs. Microb Drug Resist 2004;10(1):65 – 9. https://doi.org/10.1089/107662904323047826.
4. Genath A, Hackmann C, Denkel L, Weber A, Maechler F, Kola A, et al. The genetic relationship between human and pet isolates: a core genome multilocus sequence analysis of multidrug-resistant bacteria. Antimicrob Resist Infect Control 2024;13(1):107. https://doi.org/10.1186/s13756-024-01457-7.
5. Feßler AT, Wang Y, Burbick CR, Diaz-Campos D, Fajt VR, Lawhon SD, et al. Antimicrobial susceptibility testing in veterinary medicine: performance, interpretation of results, best practices and pitfalls. One Health Adv 2023;1(1):26. https://doi.org/10.1186/s44280-023-00024-w.
6. Chung YS, Kwon KH, Shin S, Kim JH, Park YH, Yoon JW. Characterization of veterinary hospital-associated isolates of *Enterococcus* species in Korea. J Microbiol Biotechnol 2014;24(3):386 – 93. https://doi.org/10.4014/jmb.1310.10088.
7. Darwich L, Seminati C, Burballa A, Nieto A, Durán I, Tarradas N, et al. Antimicrobial susceptibility of bacterial isolates from urinary tract infections in companion animals in Spain. Vet Rec 2021;188(9):e60. https://doi.org/10.1002/vetr.60.
8. Marques C, Belas A, Franco A, Aboim C, Gama LT, Pomba C. Increase in antimicrobial resistance and emergence of major international high-risk clonal lineages in dogs and cats with urinary tract infection: 16 year retrospective study. J Antimicrob Chemother 2018;73(2):377 – 84. https://doi.org/10.1093/jac/dkx401.
9. Li YL, Fernández R, Durán I, Molina-López RA, Darwich L. Antimicrobial resistance in bacteria isolated from cats and dogs from the Iberian Peninsula. Front Microbiol 2021;11:621597. https://doi.org/10.3389/fmicb.2020.621597.
10. Hu FP, Guo Y, Zhu DM, Wang F, Jiang XF, Xu YX, et al. CHINET surveillance of antimicrobial resistance among the bacterial isolates in

2021. Chin J Infect Chemother 2022;22(5):521 – 30. https://doi.org/10.16718/j.1009-7708.2022.05.001.
11. Furuya Y, Matsuda M, Harada S, Kumakawa M, Shirakawa T, Uchiyama M, et al. Nationwide monitoring of antimicrobial-resistant *Escherichia coli* and *Enterococcus* spp. isolated from diseased and healthy dogs and cats in Japan. Front Vet Sci 2022;9:916461. https://doi.org/10.3389/fvets.2022.916461.
12. Scarborough R, Bailey K, Galgut B, Williamson A, Hardefeldt L, Gilkerson J, et al. Use of local antibiogram data and antimicrobial importance ratings to select optimal empirical therapies for urinary tract infections in dogs and cats. Antibiotics (Basel) 2020;9(12):924. https://doi.org/10.3390/antibiotics9120924.
13. Fonseca JD, Mavrides DE, Graham PA, McHugh TD. Results of urinary bacterial cultures and antibiotic susceptibility testing of dogs and cats in the UK. J Small Anim Pract 2021;62(12):1085 – 91. https://doi.org/10.1111/jsap.13406.
14. Windahl U, Holst BS, Nyman A, Grönlund U, Bengtsson B. Characterisation of bacterial growth and antimicrobial susceptibility patterns in canine urinary tract infections. BMC Vet Res 2014;10:217. https://doi.org/10.1186/s12917-014-0217-4.
15. Traverse M, Aceto H. Environmental cleaning and disinfection. Vet Clin North Am Small Anim Pract 2015;45(2):299 – 330. https://doi.org/10.1016/j.cvsm.2014.11.011.
16. Lei L, Wang YQ, He JJ, Cai C, Liu QZ, Yang DW, et al. Prevalence and risk analysis of mobile colistin resistance and extended-spectrum *β*-lactamase genes carriage in pet dogs and their owners: a population based cross-sectional study. Emerg Microbes Infect 2021;10(1):242 – 51. https://doi.org/10.1080/22221751.2021.1882884.
17. Gómez-Sanz E, Ceballos S, Ruiz-Ripa L, Zarazaga M, Torres C. Clonally diverse methicillin and multidrug resistant coagulase negative staphylococci are ubiquitous and pose transfer ability between pets and their owners. Front Microbiol 2019;10:485. https://doi.org/10.3389/fmicb.2019.00485.
18. Nienhoff U, Kadlec K, Chaberny IF, Verspohl J, Gerlach GF, Schwarz S, et al. Transmission of methicillin-resistant *Staphylococcus aureus* strains between humans and dogs: two case reports. J Antimicrob Chemother 2009;64(3):660 – 2. https://doi.org/10.1093/jac/dkp243.

SUPPLEMENTARY MATERIAL

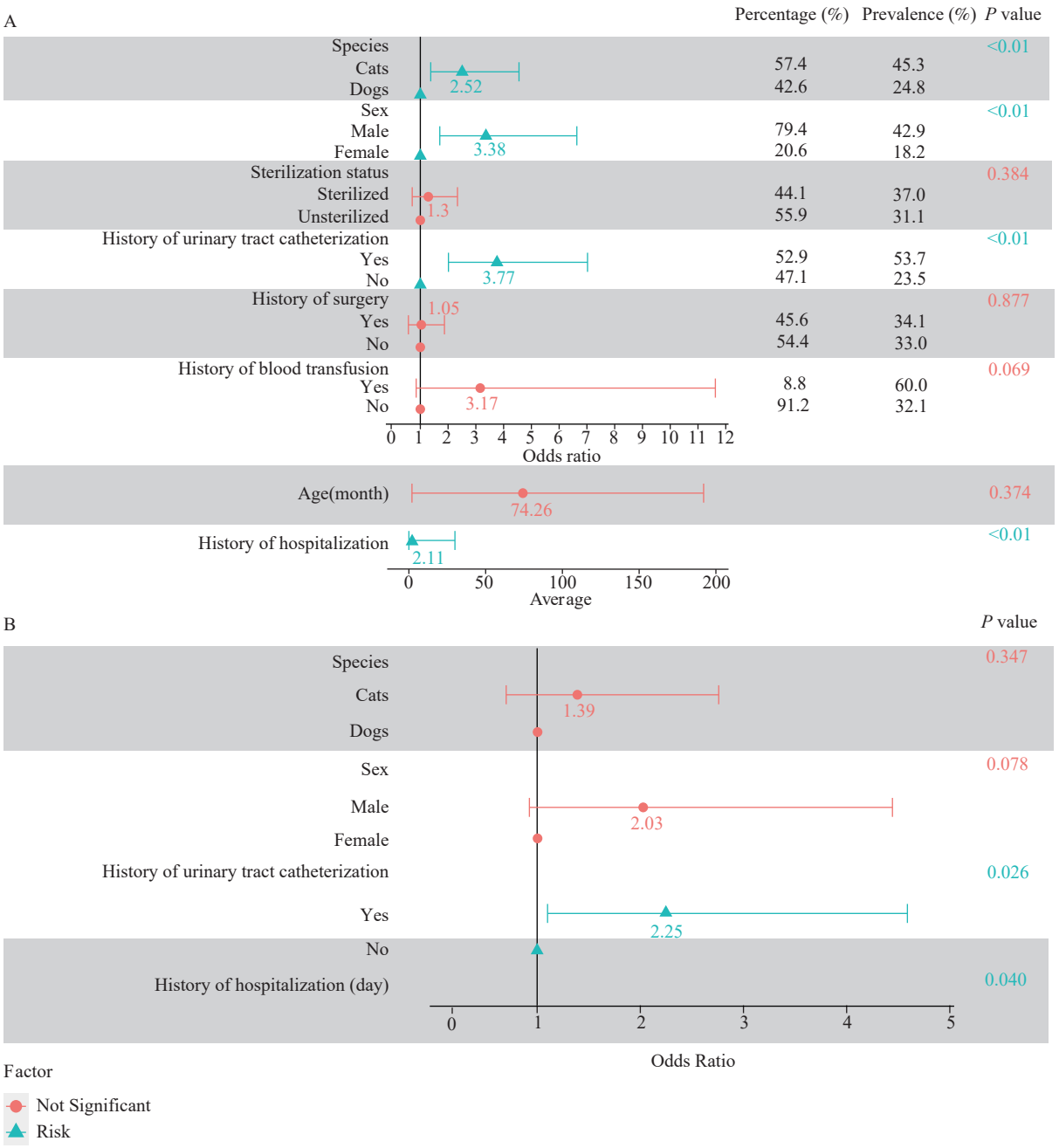


SUPPLEMENTARY FIGURE S1. Detection rates of *Enterococcus* spp. isolates from different clinical samples from dogs and cats in animal hospitals from 2018 to 2021 in China.

Note: The blue bar represents the detection rate of cat samples; the red bar represents the detection rate of dog samples; and the black dot represents the combined rate of total samples of dogs and cats. The dashed line indicates the average prevalence of *Enterococcus* isolates among dogs and cats. Statistical differences in detection rates between dogs and cats are indicated by asterisks.

* $P<0.01$.

** $P<0.001$.



SUPPLEMENTARY FIGURE S2. Forest plots of (A) univariate and (B) multivariate analyses of the risk factors associated with MDR *Enterococcus* spp. infections in dogs and cats.
Abbreviation: MDR=multidrug resistance.

SUPPLEMENTARY TABLE S1. Distribution of *Enterococcus* spp. isolates across 20 provincial-level administrative divisions in China.

Source	Beijing		Shanghai		Inner Mongolia		Liaoning		Fujian		Jiangsu		Shandong		Chongqing		Anhui		Henan	
	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat
Urinary tract	130	139	3	12	2	5	3	2	2	2	3	2	1	1		2	2		2	
Skin	39	21		1	1					1			1		1					
Ascites	13	11																		
Respiratory tract	14	7																		
Hepatobiliary system	9	3	1																	
Genital tract	9	1					1													
Pleural fluid	1	1																		
Other sites	8	7																		

Source	Hunan		Jiangxi		Hebei		Guangdong		Hubei		Shanxi		Guangxi		Guizhou		Hainan		Zhejiang	
	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat	Dog	Cat
Urinary tract	1	1			2		1	0	1	0	1	0	0	1	0	1	0	1	0	1
Skin			1																	
Other sites			1																	

Review

Prevalence and Risk Factors of Waterborne and Foodborne Protozoan Pathogens in Kenya: A One Health Perspective

Joseph Okoth Ogalo[1,2]; Chang Cai[3,#]; Ziming Han[1,2]; Yu Zhang[1,2]; Min Yang[1,2]

ABSTRACT

Intestinal infections affect approximately 450 million people globally, predominantly impacting children and immunocompromised individuals in low- and middle-income countries (LMICs) due to inadequate water, sanitation, and hygiene (WASH) conditions, poverty, malnutrition, and low literacy. In Kenya, the prevalence of intestinal infections is elevated by warm tropical climates and socioeconomic factors. This scoping review evaluates the national prevalence, risk factors, and contamination sources of intestinal protozoa in Kenya, using a One Health approach to synthesize existing data from various human, animal, and environmental studies. A comprehensive literature search identified 292 studies, of which 67 met the inclusion criteria, covering the period from 1966 to 2024. The review found that most studies utilized stool microscopy, a method with limited sensitivity, and largely focused on vulnerable human populations, with minimal investigation into environmental reservoirs. Key protozoa identified included *Entamoeba histolytica*, *Cryptosporidium*, and *Giardia*, with transmission driven by poor WASH conditions, environmental factors, and close human-animal interactions. The findings highlight significant gaps in environmental surveillance and suggest the need for a robust, integrated One Health approach to better understand and control protozoan infections in Kenya.

INTRODUCTION

Intestinal infections affect approximately 450 million people globally, predominantly children and immunocompromised individuals in low- and middle-income countries (*1*). These regions are particularly vulnerable due to poor water, sanitation, and hygiene (WASH) conditions, coupled with poverty, malnutrition, and low literacy levels (*2*). It is estimated that over 3.5 billion people worldwide host at least one species of intestinal pathogen during their lifetime, contributing to extended hospital stays, increased healthcare costs, and higher disability-adjusted life years (DALY) (*3*–*6*).

In Kenya, the prevalence of intestinal infections is high due to the warm tropical climate and socioeconomic conditions (*6*–*7*). These infections, caused by bacteria, viruses, or parasites such as protozoa and helminths, include common protozoa like *Giardia*, *Cryptosporidium*, *Blastocystis* spp., *Entamoeba*, and *Cyclospora* (*8*–*10*). Protozoa, transmitted primarily through the fecal-oral route, is spread by asymptomatic carriers and causes symptoms such as diarrhea, vomiting, and abdominal pain (*11*–*12*). Despite the increasing impact of these infections, data on morbidity and mortality in Kenya are limited (*13*). With 80% of the country classified as arid or semi-arid and many relying on surface water, urgent interventions are needed (*14*–*15*).

To address the rising burden of intestinal protozoan infections in Kenya, large-scale surveillance and comprehensive One Health studies are needed to evaluate the prevalence, synthesize data, and identify risk factors for targeted interventions and informed policymaking.

METHODS

The scoping review protocol was adapted from established methodologies (Figure 1). Scoping reviews are systematic reviews that map the breadth and scope of literature on a specific topic, in this case, protozoan pathogens in Kenya's source water. The primary research questions were: What is the national prevalence of protozoan pathogens in Kenya's source water intended for domestic consumption? What are the common genes/genotypes, contamination pathways, and available detection methods?

Literature searches were conducted between May 1 and May 21, 2024, using academic databases, including Science Direct, Google Scholar, African Journals Online (AJOL), and Springer Link. The

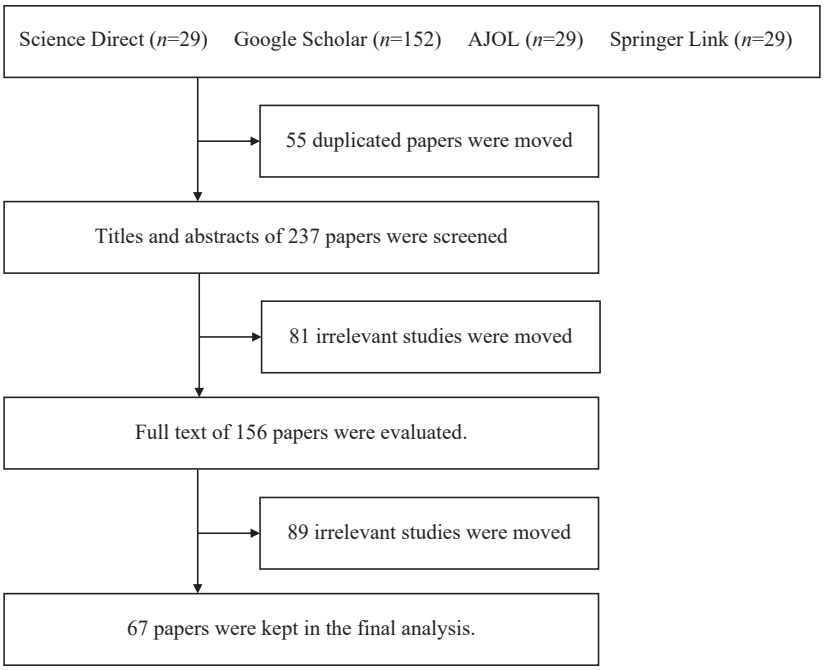


FIGURE 1. Flow diagram for the scoping review.
Abbreviation: AJOL=African Journals Online.

search strategy focused on keywords related to enteric protozoa, including *Cryptosporidium*, *Giardia*, Kenya, urban centers, informal settlements, population groups (including children and adults), food handlers, and vulnerable groups such as immunocompromised individuals. Specific search term combinations included: "Protozoa and Kenya", "*Cryptosporidium* and Kenya", "*Giardia* and Kenya", "Protozoa in informal settlements in Kenya", "Protozoan infections of children in Kenya", and "Protozoa in source water in Kenya".

The search, unrestricted by year but limited to English-language articles, excluded review articles. Boolean operators were used to refine the search. Titles and abstracts were screened, and full texts were evaluated according to the following inclusion and exclusion criteria. Papers meeting both the inclusion and exclusion criteria were retained for the final analysis.

The inclusion criteria were: 1) articles written in English; 2) original, full-text research articles; 3) studies based on One Health sample sources (human, animal, and environmental); and 4) analysis of enteric protozoa (*Cryptosporidium*, *Giardia*, *Entamoeba*, *Cyclospora*, *Isospora*, *Blastocystis*, and/or their oocysts or genetic markers) in One Health samples. Exclusion criteria were: 1) review papers that synthesized previously published data; 2) studies involving countries other than Kenya; 3) experimental studies focusing on protozoa detection efficiency using various methods; and 4) articles without extractable data.

RESULTS

A total of 292 studies were initially retrieved from 4 databases. After removing 55 duplicates, 237 studies were screened by title and abstract, excluding 81 irrelevant studies. The remaining 156 articles underwent full-text evaluation, resulting in the exclusion of 89 papers that did not meet the inclusion or exclusion criteria. Ultimately, 67 articles met the inclusion criteria and were included in the final analysis.

General Characteristics of Protozoan-Related Studies in Kenya

The studies included in this review were conducted between 1966 and 2024, with 54% (36 studies) published between 2010 and 2020 (Figure 2A). These studies predominantly focused on adults and children, with only 6% (4 studies) utilizing an "environmental surveillance" approach, sampling water and plants (*16*) (Figure 2B). Most were conducted in rural areas, followed by peri-urban and urban settings (Figure 2C). While 40% of the studies examined a single protozoan species, some investigated multiple species (Figure 2D).

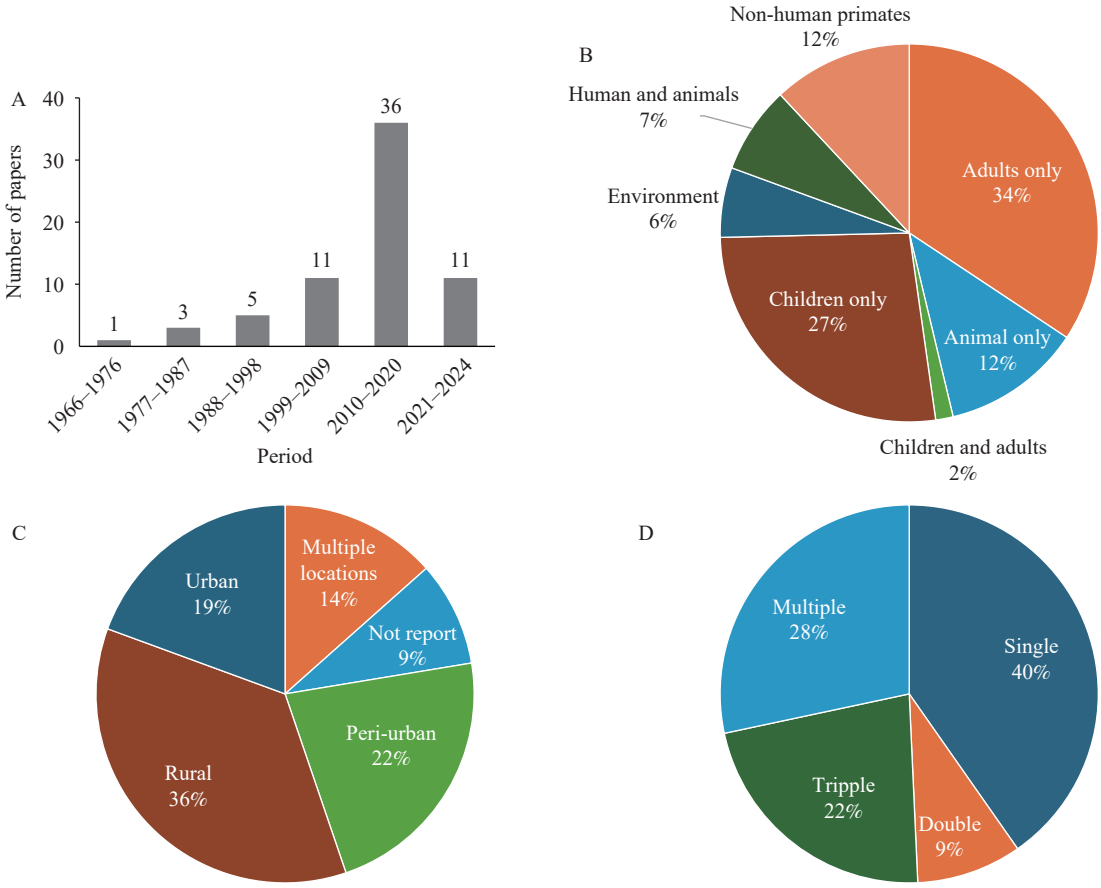


FIGURE 2. The distribution of a total of 67 studies under different classification criteria. (A) Number of publications in different periods of time. (B) Distribution of sampling targets. (C) Distribution of study locations. (D) Distribution of types of protozoa studied per article.

Study Design and Detection Methods

Of the 67 studies, 37% used a cross-sectional design. Two used a retrospective design, and 2 used a prospective design.

Microscopy was the most commonly used detection method (64%), primarily focusing on stool samples (Figure 3A and Figure 3C). Molecular studies, mainly polymerase chain reaction (PCR) (36%), were mostly conducted in the last decade. The sampling duration was frequently unspecified. However, 20% of the studies were conducted for less than 6 months, while 24% were completed within 6 to 12 months (Figure 3B). Over 80% of the studies collected stool samples, with a few collecting environmental samples, such as water or sewage (Figure 3C).

Prevalence of Protozoa and Associated Predisposing Factors in Kenya

The top three prevalent protozoa in Kenya were *Entamoeba histolytica*, *Giardia*, and *Cryptosporidium* (Figure 3D). Detection numbers for most protozoa, including these three, exhibited an increasing trend over time, peaking between 2010 and 2020 (Figure 4). This trend aligns with the temporal distribution of research activities (Figure 2A).

Entamoeba histolytica remains a significant public health concern, having been previously isolated from various sources, including food handlers with valid health certificates, schoolchildren, inpatients and outpatients, De Brazza monkeys, and pigs (*17–24*). However, many studies used microscopy, which does not differentiate between *E. histolytica* and *E. dispar*.

Cryptosporidium is the second most prevalent protozoan, identified through microscopy and PCR in children (*25–26*), baboons (*27*), rivers (*16*,*28–29*), as well as during outbreaks of new genotypes in a military camp (*30*). Human studies frequently involved routine hospital samples from individuals with watery stools (*31–33*). The presence of livestock and untreated/contaminated water sources was identified as risk factors, but some studies suggested anthroponotic transmission due to overcrowding and poor WASH

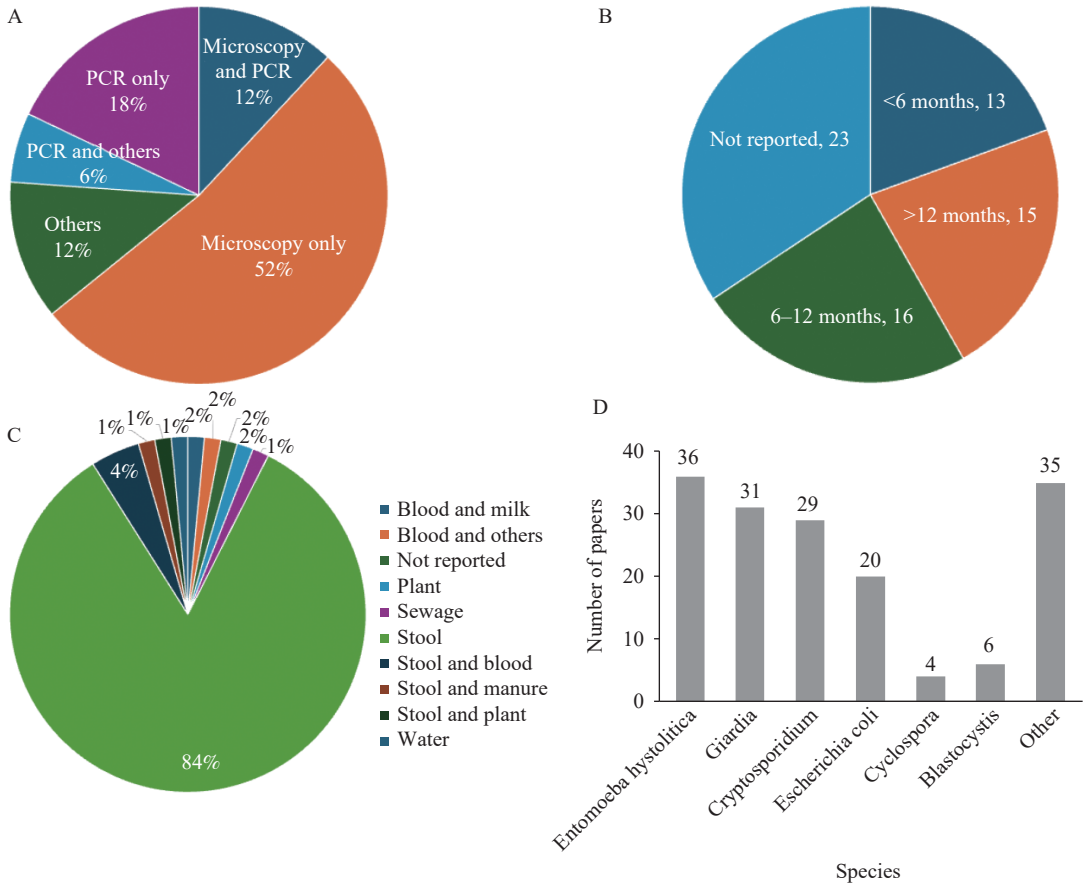


FIGURE 3. Main findings of a total of 67 studies included in this study. (A) Distribution of detection methods. (B) Distribution of sampling periods. (C) Distribution of types of samples. (D) Distribution of papers on different protozoan species detected.

conditions (*28*,*34*–*35*). *C. hominis* was the most prevalent species in human infections, whereas *C. parvum* was more common in environmental and animal samples (*36*). Other *Cryptosporidium* species detected in Kenya include *C. canis*, *C. felis*, *C. muris C. ryanae*, and *C. andersoni* (*35*,*37*).

Giardia lamblia is recognized as one of the most prevalent intestinal protozoan infections both in Kenya and globally (*38*). It has been detected in children (*22*,*39*) and food handlers (*18*,*40*). Studies have identified unhygienic conditions, improper sewage disposal, and low socioeconomic status as significant risk factors for *Giardia* infections, while factors such as sex, constipation, and loss of appetite were not associated with its prevalence (*41*–*42*). Notably, there is limited information on the prevalence of *Giardia* in domesticated animals and its national distribution in Kenya (*43*). To date, only one study has investigated the prevalence and risk factors of *Giardia* infections in dogs, the most commonly kept pets in Kenya (*44*).

Entamoeba coli (*E. coli*) has been found in food handlers (*17*), preschool and school-aged children (*6*,*39*,*45*), households in low-altitude rural areas (*41*), pregnant women (*46*), pigs (*47*), and non-human primates (*48*–*49*). However, no studies specifically address its environmental prevalence despite its impact on human immunodeficiency virus (HIV) infected patients (*1*,*22*,*50*) .

DISCUSSION

Overview of Current Intestinal Protozoa Studies in Kenya

Current research on intestinal protozoa in Kenya relies primarily on patient-based data, often collected through questionnaires. However, studies investigating environmental sources, such as watersheds, to identify infection pathways are lacking. This limitation may be due to resource constraints, resulting in the use of stool microscopy and flow cytometry for diagnostics. While conventional, these methods lack the sensitivity and specificity of molecular diagnostic tests, leading to inaccurate estimations of the true prevalence of

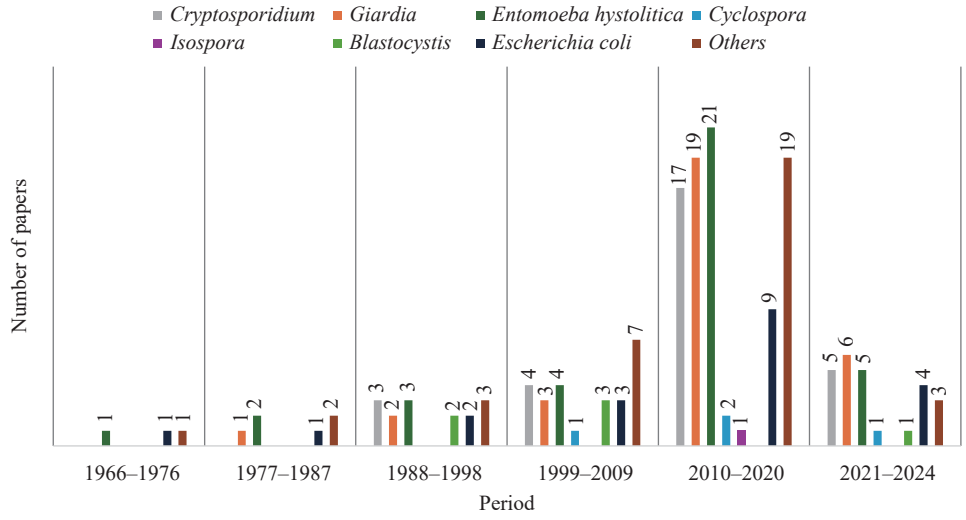


FIGURE 4. The temporal distribution of detection numbers for different protozoa.

intestinal protozoa in the environment.

Socioeconomic status remains a critical factor influencing the prevalence of intestinal protozoan infections in Kenya (*17*). Environmental conditions such as rainfall, temperature, and humidity further impact parasite prevalence, highlighting the role of socio-demographic and geographic factors (*51*). In peri-urban areas, poor WASH conditions and overcrowding contribute to anthroponotic transmission (*37*). In rural settings, the common presence of domestic animals such as cattle, sheep, and dogs in close proximity to human dwellings is associated with higher infection rates (*52*). Rapid rural-to-urban migration has led to the growth of informal settlements, characterized by overcrowding, poor services, and high poverty levels (*6*,*18*,*53*). Despite these factors, no studies have quantified environmental contamination, particularly during rainy seasons when transmission may increase.

This scoping review provides a comprehensive analysis of the national prevalence of protozoan infections in Kenya, highlighting that current research primarily targets vulnerable groups, such as children and patients with diarrhea. However, significant gaps remain in the surveillance of environmental reservoirs, including watersheds, drinking water sources, irrigation water, and soil. Water treatment in Kenya predominantly relies on chlorination, which is ineffective against many protozoa. Consequently, there is limited data on the prevalence of intestinal protozoa in environmental and animal reservoirs, including domesticated animals, pets, and wildlife. Enhanced surveillance of these reservoirs, aligned with the One Health approach, is crucial for identifying infection sources and improving public health interventions.

Limitations and Recommendations

A key limitation of this review is the insufficient epidemiological data on the transmission dynamics of protozoan infections in Kenya, highlighting the need for further studies to elucidate transmission risks and inform targeted public health interventions. Additionally, many existing studies focus on specific areas, often neglecting comprehensive environmental sampling and diverse animal reservoirs. The One Health approach, integrating human, animal, and environmental health, is essential for addressing zoonotic diseases. Although Kenya's Zoonotic Disease Unit (ZDU) has made progress, its capacity for overseeing zoonotic parasite surveillance remains limited and requires enhancement. Increasing human encroachment into wildlife habitats, tourism, the exotic pet trade, and bushmeat demand, create new infection pathways. There is an urgent need for research on zoonotic parasites in wildlife, particularly primates, as no studies in Kenya have concurrently collected specimens from human, animal, and environmental sources for comparative analysis.

To improve future surveillance of intestinal infections within the One Health framework, developing integrated health monitoring systems that incorporate data from humans, animals, and the environment is crucial. Collaborative research initiatives among public health agencies, veterinary services, and environmental organizations should be promoted to facilitate data sharing and resource

optimization. Engaging local communities, especially vulnerable populations, will significantly enhance data collection and awareness. A strategic focus on these areas is essential for mitigating the impact of intestinal infections and promoting overall health.

CONCLUSIONS

This review identifies significant gaps in the surveillance of intestinal protozoa, particularly within the environmental and animal health sectors. Socioeconomic and environmental factors, such as inadequate WASH conditions and human-animal interactions, influence intestinal protozoan infections in Kenya. The predominant protozoa — *Entamoeba histolytica*, *Cryptosporidium*, and *Giardia* — are often detected using less accurate stool microscopy. Persistent gaps in environmental and animal reservoir surveillance underscore the need for a comprehensive One Health strategy that includes broader sampling. Developing integrated health monitoring systems, fostering collaboration among relevant sectors, and engaging local communities, are crucial steps to strengthen surveillance efforts and mitigate the public health impact of intestinal infections.

Conflicts of interest: No conflicts of interest.

Funding: Supported by the National Key Research and Development Program of China (2022YFC3204703), and the China Postdoctoral Innovation Talents Support Program (BX20230400).

doi: 10.46234/ccdcw2025.014

[#] Corresponding author: Chang Cai, chang.cai@njau.edu.cn.

[1] State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China; [2] University of Chinese Academy of Sciences, Beijing, China; [3] College of Veterinary Medicine, Nanjing Agricultural University, Nanjing City, Jiangsu Province, China.

Submitted: September 06, 2024
Accepted: November 19, 2024
Issued: January 17, 2025

REFERENCES

1. Otieno BIA, Matey EJ, Bi XQ, Tokoro M, Mizuno T, Panikulam A, et al. Intestinal parasitic infections and risk factors for infection in Kenyan children with and without HIV infection. Parasitol Int 2023;94:102717. https://doi.org/10.1016/j.parint.2022.102717.
2. WHO. Ending the neglect to attain the Sustainable Development Goals: a global strategy on water, sanitation and hygiene to combat neglected tropical diseases, 2021-2030. Geneva: WHO; 2021. https://iris.who.int/bitstream/handle/10665/340240/9789240022782-eng.pdf.
3. Zibaei M, Bahadory S, Saadati H, Pourrostami K, Firoozeh F, Foroutan M. Intestinal parasites and diabetes: a systematic review and meta-analysis. New Microbes New Infect 2023;51:101065. https://doi.org/10.1016/j.nmni.2022.101065.
4. Saki J, Khademvatan S, Foroutan M, Gharibzadeh M. Prevalence of intestinal parasitic infections in haftkel county, southwest of Iran. Int J Infect 2017;4(4):e15593. https://doi.org/10.5812/iji.15593.
5. Coulibaly G, Ouattara M, Dongo K, Hürlimann E, Bassa FK, Koné N, et al. Epidemiology of intestinal parasite infections in three departments of south-central Côte d'Ivoire before the implementation of a cluster-randomised trial. Parasite Epidemiol Control 2018;3(2):63 – 76. https://doi.org/10.1016/j.parepi.2018.02.003.
6. Chege NM, Ondigo BN, Onyambu FG, Kattam AM, Lagat NJ, Irungu T, et al. The prevalence of intestinal parasites and associated risk factors in school-going children from informal settlements in Nakuru town, Kenya. Malawi Med J 2020;32(2):80 – 6. https://doi.org/10.4314/mmj.v32i2.5.
7. Nguhiu PN, Kariuki HC, Magambo JK, Kimani G, Mwatha JK, Muchiri E, et al. Intestinal polyparasitism in a rural Kenyan community. East Afr Med J 2010;86(6):272 – 8. https://doi.org/10.4314/eamj.v86i6.54138.
8. Lauwers GY, Mino-Kenudson M, Kradin RL. Infections of the gastrointestinal tract. In: Kradin RL, editors. Diagnostic pathology of infectious disease. 2nd ed. Amsterdam: Elsevier. 2018; p. 232-71. http://dx.doi.org/10.1016/B978-0-323-44585-6.00010-2.
9. Das R, Palit P, Haque A, Levine MM, Kotloff KL, Nasrin D, et al. Symptomatic and asymptomatic enteric protozoan parasitic infection and their association with subsequent growth parameters in under five children in South Asia and sub-Saharan Africa. PLoS Negl Trop Dis 2023;17(10):e0011687. https://doi.org/10.1371/journal.pntd.0011687.
10. Ahmed M. Intestinal parasitic infections in 2023. Gastroenterol Res 2023;16(3):127 – 40. https://doi.org/10.14740/gr1622.
11. Kyany'a C, Eyase F, Odundo E, Kipkirui E, Kipkemoi N, Kirera R, et al. First report of *Entamoeba moshkovskii* in human stool samples from symptomatic and asymptomatic participants in Kenya. Trop Dis Travel Med Vaccines 2019;5:23. https://doi.org/10.1186/s40794-019-0098-4.
12. Stephen Phillips R. Parasitism: life cycle and host defences against parasites. In: eLS. Chichester: John Wiley & Sons, Ltd. 2012. http://dx.doi.org/10.1002/9780470015902.a0003809.pub2.
13. Njambi E, Magu D, Masaku J, Okoyo C, Njenga SM. Prevalence of intestinal parasitic infections and associated water, sanitation, and hygiene risk factors among school children in Mwea Irrigation Scheme, Kirinyaga County, Kenya. J Trop Med 2020;2020:3974156. https://doi.org/10.1155/2020/3974156.
14. USAID. KENYA: a high priority country plan-Global Water Strategy | 2022-2027. 2022. https://www.globalwaters.org/sites/default/files/kenya_gws_high_priority_country_plan_2023_1.pdf. 2024-10-1.
15. Karanja L, Karumba M, Opondo M, Macharia E, Kiriro M, Kipruto S, et al. Influence of household sanitation on child stunting in Kenya. Nairobi: Kenya Institute for Public Policy, Research and Analysis, Kenya National Bureau of Statistics; 2023. https://repository.kippra.or.ke/xmlui/bitstream/handle/123456789/4172/SP3%20Influence%20of%20Household%20Sanitation.pdf?sequence=1.
16. Muchiri JM, Ascolillo L, Mugambi M, Mutwiri T, Ward HD, Naumova EN, et al. Seasonality of *Cryptosporidium* oocyst detection in surface waters of Meru, Kenya as determined by two isolation methods followed by PCR. J Water Health 2009;7(1):67 – 75. https://doi.org/10.2166/wh.2009.109.
17. Biwott GK, Wanjala PM, Ngeiywa MM. Prevalence of gastrointestinal parasitic infections among food handlers in Eldoret Municipality, Kenya. J Biol Agric Healthc 2014;4(27):160-71. https://core.ac.uk/download/pdf/234660479.pdf.
18. Kamau P, Aloo-Obudho P, Kabiru E, Ombacho K, Langat B, Mucheru O, et al. Prevalence of intestinal parasitic infections in certified food-handlers working in food establishments in the City of Nairobi, Kenya. J Biomed Res 2012;26(2):84 – 9. https://doi.org/10.1016/S1674-8301

(12)60016-5.
19. Kavili DM, Simbauni JA, Gicheru MM, Mungiria JN. Prevalence and risk factors of entamoeba histolytica amongst children attending primary schools in Kyuso Zone, Kyuso District, Kitui County, Kenya. J Appl Life Sci Int 2016;7(1):1 – 12. https://doi.org/10.9734/JALSI/2016/27681.
20. Kimosop RJ, Mulambalah CS, Ngeiywa MM. Prevalence of enteric parasitic diseases among patients referred at a teaching hospital in Kenya. J Health Res Rev 2018;5:78 – 85. https://doi.org/10.4103/jhrr.jhrr_7_18.
21. Mulinge E, Mbae C, Ngugi B, Irungu T, Matey E, Kariuki S. *Entamoeba* species infection in patients seeking treatment for diarrhea and abdominal discomfort in Mukuru informal settlement in Nairobi, Kenya. Food Waterborne Parasitol 2021;23:e00122. https://doi.org/10.1016/j.fawpar.2021.e00122.
22. Kipyegen CK, Shivairo RS, Odhiambo RO. Diarrhea and intestinal parasites among HIV infected patients in Baringo, Kenya. J Biol Agric Healthc 2013;3(14):21-5. https://core.ac.uk/download/234659152.pdf.
23. Karere GM, Munene E. Some gastro-intestinal tract parasites in wild De Brazza's monkeys (*Cercopithecus neglectus*) in Kenya. Vet Parasitol 2002;110(1-2):153 – 7. https://doi.org/10.1016/S0304-4017(02)00348-5.
24. Kanyari PWN, Kagira JM, Mhoma RJ. Prevalence and intensity of endoparasites in small ruminants kept by farmers in Kisumu Municipality, Kenya. Livest Res Rural Dev 2009; 21(11), article#202. https://www.researchgate.net/publication/286122782_Prevalence_and_intensity_of_endoparasites_in_small_ruminants_kept_by_farmers_in_Kisumu_Municipality_Kenya.
25. Nyamwange CI, Mkoji G, Mpoke S, Nyandieka HS. Cryptosporidiosis and its genotypes among children attending moi teaching and referral hospital in Eldoret, Kenya. East Afr Med J 2012;89(1):11-9. https://pubmed.ncbi.nlm.nih.gov/26845806/.
26. Deichsel EL, Hillesland HK, Gilchrist CA, Naulikha JM, McGrath CJ, Van Voorhis WC, et al. Prevalence and correlates of *cryptosporidium* infections in kenyan children with diarrhea and their primary caregivers. Open Forum Infect Dis 2020;7(12):ofaa533. https://doi.org/10.1093/ofid/ofaa533.
27. Li W, Kiulia NM, Mwenda JM, Nyachieo A, Taylor MB, Zhang XC, et al. Cyclospora papionis, *Cryptosporidium hominis*, and human-pathogenic *Enterocytozoon bieneusi* in Captive Baboons in Kenya. J Clin Microbiol 2011;49(12):4326 – 9. https://doi.org/10.1128/JCM.05051-11.
28. Walter EM, Charles M, Elick O, Manfred M, Domitila K. Prevalence of zoonotic *cryptosporidium spp*. isolates in Njoro Sub-county, Nakuru County, Kenya. Afr J Infect Dis 2021;15(2):3 – 9. https://doi.org/10.21010/ajid.v15i2.2.
29. Essendi WM, Muleke C, Miheso M, Otachi E. Genetic diversity of *cryptosporidium* species in Njoro Sub County, Nakuru, Kenya. J Parasit Dis 2022;46(1):262 – 71. https://doi.org/10.1007/s12639-021-01444-4.
30. Toriro R, Pallett S, Woolley S, Bennett C, Hale I, Heylings J, et al. Outbreak of diarrhea caused by a novel *cryptosporidium hominis* subtype during british military training in Kenya. Open Forum Infect Dis 2024;11(1):ofae001. https://doi.org/10.1093/ofid/ofae001.
31. Mutai DC, Owili PO, Muga MA. Trend of *cryptosporidium* infection among children below 24 months in an informal urban settlement, Kenya. Open J Med Microbiol 2020;10(3):153 – 61. https://doi.org/10.4236/ojmm.2020.103013.
32. Mbae CK, Nokes DJ, Mulinge E, Nyambura J, Waruru A, Kariuki S. Intestinal parasitic infections in children presenting with diarrhoea in outpatient and inpatient settings in an informal settlement of Nairobi, Kenya. BMC Infect Dis 2013;13:243. https://doi.org/10.1186/1471-2334-13-243.
33. Hossain MJ, Powell H, Sow SO, Omore R, Roose A, Jones JCM, et al. Clinical and epidemiologic features of *cryptosporidium*-associated diarrheal disease among young children living in Sub-Saharan Africa: the vaccine impact on diarrhea in Africa (VIDA) study. Clin Infect Dis 2023;76(Supplement_1):S97 – 105. https://doi.org/10.1093/cid/ciad044.
34. Njuguna C, Njeru I, Mgamb E, Langat D, Makokha A, Ongore D, et al. Enteric pathogens and factors associated with acute bloody diarrhoea, Kenya. BMC Infect Dis 2016;16(1):477. https://doi.org/10.1186/s12879-016-1814-6.
35. Kang'ethe EK, Mulinge EK, Skilton RA, Njahira M, Monda JG, Nyongesa C, et al. Cryptosporidium species detected in calves and cattle in Dagoretti, Nairobi, Kenya. Trop Anim Health Prod 2012;44 Suppl 1:25-31. http://dx.doi.org/10.1007/s11250-012-0202-5.
36. Mbae C, Mulinge E, Waruru A, Ngugi B, Wainaina J, Kariuki S. Genetic diversity of *cryptosporidium* in children in an urban informal settlement of Nairobi, Kenya. PLoS One 2015;10(12):e0142055. https://doi.org/10.1371/journal.pone.0142055.
37. Gatei W, Wamae CN, Mbae C, Waruru A, Mulinge E, Waithera T, et al. Cryptosporidiosis: prevalence, genotype analysis, and symptoms associated with infections in children in Kenya. Am J Trop Med Hyg 2006;75(1):78-82. https://pubmed.ncbi.nlm.nih.gov/16837712/.
38. Mbae C, Mulinge E, Guleid F, Wainaina J, Waruru A, Njiru ZK, et al. Molecular characterization of *Giardia duodenalis* in children in Kenya. BMC Infect Dis 2016;16:135. https://doi.org/10.1186/s12879-016-1436-z.
39. Chunge RN, Nagelkerke N, Karumba PN, Kaleli N, Wamwea M, Mutiso N, et al. Longitudinal study of young children in Kenya: intestinal parasitic infection with special reference to *Giardia lamblia*, its prevalence, incidence and duration, and its association with diarrhoea and with other parasites. Acta Trop 1991;50(1):39 – 49. https://doi.org/10.1016/0001-706X(91)90071-Q.
40. Nematian J, Nematian E, Gholamrezanezhad A, Asgari AA. Prevalence of intestinal parasitic infections and their relation with socio-economic factors and hygienic habits in Tehran primary school students. Acta Trop 2004;92(3):179 – 86. https://doi.org/10.1016/j.actatropica.2004.06.010.
41. Iseki M, Hayashi K, Gatika SM, Siongok TKA. The prevalence of intestinal protozoa in naivasha, kitui, machakos, taveta and nandi hills areas in Kenya. Jpn J Trop Med Hyg 1983;11(3-4):249 – 56. https://doi.org/10.2149/tmh1973.11.249.
42. Barasa E, Indieka B, Shaviya N, Osoro E, Maloba G, Mukhongo D, et al. Assemblages and subassemblages of *Giardia duodenalis* in Rural Western, Kenya: association with sources, signs, and symptoms. J Parasitol Res 2024;2024:1180217. https://doi.org/10.1155/2024/1180217.
43. Thiongó J, Mucheru O, Muite F, Langat B, Kamau P, Ireri L. Spatial distribution of *Giardia intestinalis* in children up to 5 years old attending out-patient clinic at provincial general hospital, Embu, Kenya. Res J Parasitol 2011;6(4):136 – 43. https://scialert.net/abstract/?doi=jp.2011.136.143. https://doi.org/10.3923/jp.2011.136.143.
44. Maingi HN, Mwangi WE, Chege JN, Kimeli P, Makau DN, Chepkwony MC, et al. Prevalence, risk factors and genetic identity of *Giardia* infection in dogs in Nairobi, Kenya. J Vet Med Anim Health 2023;15(3):21 – 34. https://doi.org/10.5897/JVMAH2023.1032.
45. Njenga D, Mbugua AK, Okoyo C, Njenga SM. Intestinal parasite infections and associated risk factors among pre-school aged children in kibera informal settlement, Nairobi, Kenya. East Afr Health Res J 2022;6(1):86 – 97. https://doi.org/10.24248/eahrj.v6i1.683.
46. Nyambura AW, Muita Gicheru M. Intestinal parasites infection among pregnant women in Nyeri County, Kenya. Int J Health Sci Res 2022;12(7):172 – 80. https://doi.org/10.52403/ijhsr.20220725.
47. Kagira JM, Githigia SM, Ng'ang'a JC, Kanyari PWN, Maingi N, Gachohi JM. Prevalence of gastrointestinal protozoa and association with risk factors in free-range pigs in Kenya. J Protozool Res 2010;20(1):1 – 9. https://doi.org/10.32268/jprotozoolres.20.1_1.
48. Mbora DNM, Munene E. Gastrointestinal parasites of critically endangered primates endemic to tana river, kenya: tana river red colobus (*Procolobus rufomitratus*) and crested mangabey (*Cercocebus galeritus*). J Parasitol 2006;92(5):928 – 32. https://doi.org/10.1645/GE-798R1.1.
49. Muriuki SMK, Murugu RK, Munene E, Karere GM, Chai DC. Some gastro-intestinal parasites of zoonotic (public health) importance

commonly observed in old world non-human primates in Kenya. Acta Trop 1998;71(1):73 – 82. https://doi.org/10.1016/S0001-706X(98)00040-0.
50. Wanyiri JW, Kanyi H, Maina S, Wang DE, Steen A, Ngugi P, et al. Cryptosporidiosis in HIV/AIDS patients in Kenya: clinical features, epidemiology, molecular characterization and antibody responses. Am J Trop Med Hyg 2014;91(2):319 – 28. https://doi.org/10.4269/ajtmh.13-0254.
51. Eberhard ML, Njenga MN, DaSilva AJ, Owino D, Nace EK, Won KY, et al. A survey for *Cyclospora* spp. in kenyan primates, with some notes on its biology. J Parasitol 2001;87(6):1394 – 7. https://doi.org/10.1645/0022-3395(2001)087[1394:ASFCSI]2.0.CO;2.
52. Peter GS, Gitau GK, Mulei CM, Vanleeuwen J, Richards S, Wichtel J, et al. Prevalence of Cryptosporidia, Eimeria, Giardia, and Strongyloides in pre-weaned calves on smallholder dairy farms in Mukurwe-ini district, Kenya. Vet World 2015;8(9):1118 – 25. https://doi.org/10.14202/vetworld.2015.1118-1125.
53. Sakketa TG. Urbanisation and rural development in sub-Saharan Africa: a review of pathways and impacts. Res Globalization 2023;6:100133. https://doi.org/10.1016/j.resglo.2023.100133.

Review

A Database on Antibiotics and Antibiotic Resistance in Wastewater and Solid Waste from Pharmaceutical Industry Based on a Systematic Review

Jingwei Wu[1,2,3]; Ziming Han[1]; Xiaomin Ma[4]; Ming Su[1,3]; Amir Hossein Hamidian[5]; Yu Zhang[1,2,3,#]; Min Yang[1,2,3]

ABSTRACT

Residual antimicrobial agents in wastewater and solid waste from antimicrobial manufacturing facilities can potentially contaminate environments. The World Health Organization has established technical guidelines for managing antimicrobial resistance (AMR) in pharmaceutical wastewater and solid waste. However, the scarcity of publicly available data on antimicrobial manufacturing processes impedes the development of effective mitigation strategies. To address this knowledge gap, we developed a comprehensive database documenting antibiotics and antibiotic resistance genes (ARGs) in actual wastewater and solid waste samples, primarily fermentation residues. Through systematic review methodology, we compiled data from extensive searches of English-language article databases, including Web of Science and PubMed. The database contains data from 270 distinct samples collected across 45 fermentation residue treatment systems and 46 wastewater treatment systems, derived from 70 published English-language articles spanning 2008 to 2024. In operational pharmaceutical facilities, antibiotic concentrations ranged from 82 to 1,663 mg/L in raw wastewater and from 1,000 to 10,182 mg/kg dry matter (DM) in antibiotic fermentation residues. Various treatment technologies demonstrated significant reductions in both antibiotic concentrations and ARG levels within wastes. This database provides the first global perspective on antibiotic and ARG contamination from antibiotic production processes, supporting AMR management initiatives. It establishes a dynamic, continuously updated platform accessible to researchers and industry stakeholders via the link: https://dash.drwater.net/antiboard/.

Antimicrobial resistance (AMR), driven by high concentrations of active pharmaceutical ingredients (API), poses a significant threat to disease treatment effectiveness (*1*–*2*). Pharmaceutical manufacturing processes release substantial quantities of antibiotics into the environment through wastewater and solid waste, with fermentation residue representing a major solid waste component during antibiotic production. The complex matrix of contaminants and microbiota in these waste streams presents significant management challenges. The World Health Organization (WHO) and the United Nations Environment Programme (UNEP) jointly released the *Guidance on wastewater and solid waste management for manufacturing of antibiotics* (*3*) in September 2024, highlighting antibiotic production's crucial role in global AMR surveillance and control.

Antibiotic manufacturing facilities require dedicated collection and treatment infrastructure for both wastewater and solid waste streams, providing opportunities to control the environmental release of antibiotics and antibiotic resistance genes (ARGs) (*4*–*6*). However, comprehensive data and scientific understanding of AMR pollution characteristics within the pharmaceutical industry remain limited. To address this knowledge gap, we developed a systematic review-based database documenting antibiotics and ARGs in pharmaceutical wastewater and solid waste streams. Fermentation residues constitute the primary solid waste generated during antibiotic production processes. The database development incorporated diverse scientific literature, including English-language review articles and research papers on treatment technologies and applications for pharmaceutical wastewater and solid waste (*5*,*7*), as well as relevant grey literature from local, national, and regional sources (*8*–*10*). China has emerged as a leader in this field, demonstrating substantial research achievements and extensive international collaboration. This database serves as an essential resource for understanding the current status of antibiotics and ARGs in pharmaceutical manufacturing while supporting enhanced management and control strategies.

LITERATURE SEARCH STRATEGY

Following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (*11*), we conducted a comprehensive literature search using the Science Citation Index (SCI) and Social Sciences Citation Index (SSCI) databases in Web of Science (WOS) and PubMed. The search covered articles published from January 2000 to December 2023. In WOS, we employed the following search strategy: "TS = {[(pharmaceutic* OR production OR fermentation) NEAR (waste* OR residue OR mycelia)] NEAR antibiotic*}", with a comparable approach used in PubMed. We supplemented these database searches with relevant grey literature (Figure 1). Using CiteSpace (*12*), we analyzed and visualized collaboration networks among countries, institutions, and researchers based on the 1,000 most relevant papers.

RESULTS

Analysis of publication trends revealed 2,108 unique publications following duplicate removal, demonstrating a marked increase during the 2000–2023 period (Figure 2A). Prior to 2015, research on antibiotic production wastes received limited attention, with minimal publications. From 2016 onward, publication rates exhibited a significant upward trajectory. Publication volumes in 2022 and 2023 exceeded 320 annually, representing more than a fourfold increase compared to 2015's 79 publications. This trend indicates the growing recognition of pharmaceutical waste management as a critical research focus.

China emerged as a pioneer in addressing pharmaceutical wastewater and residue management, with Chinese researchers contributing 371 publications, substantially surpassing other nations. China's pivotal role in international collaboration is evidenced by its high betweenness centrality (0.54), calculated from collaboration frequency. India ranked second with 87 publications (centrality 0.18), followed by the United States with 63 publications (centrality 0.35). Notably, while Iran produced 56 publications, its low centrality (0.13) indicates limited international collaboration. Conversely, Spain demonstrated strong international engagement with high centrality (0.32) despite fewer publications (Table 1).

Within China's research landscape, the Chinese

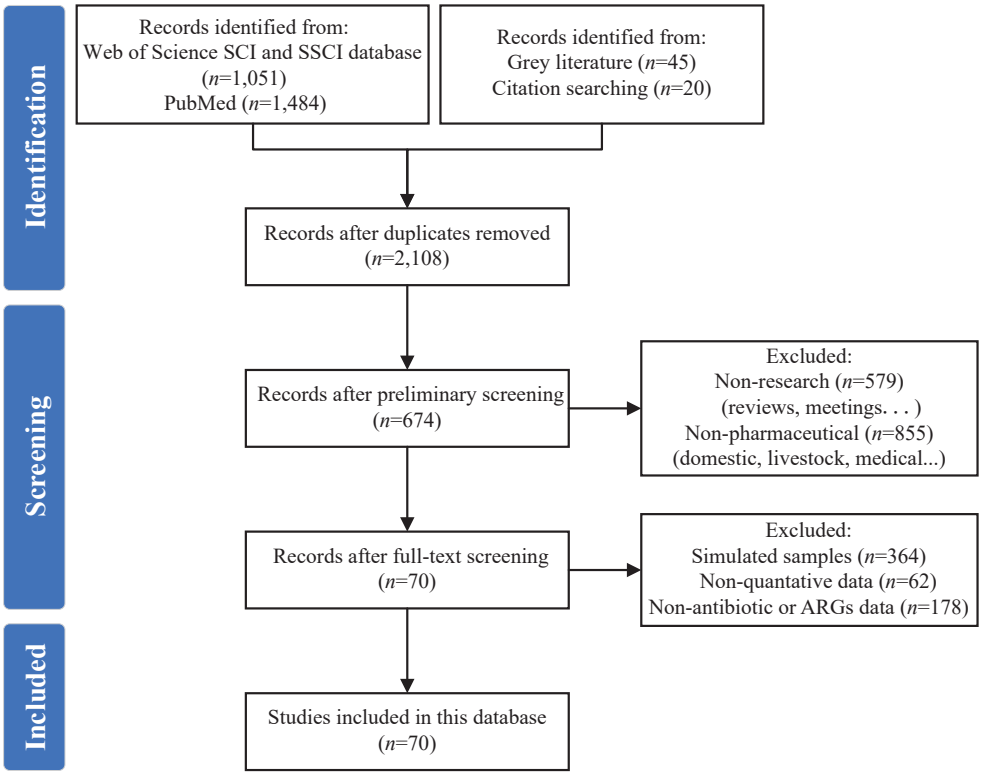


FIGURE 1. Flow diagram of the studies selection procedure.
Abbreviation: ARGs=antibiotic resistance genes.

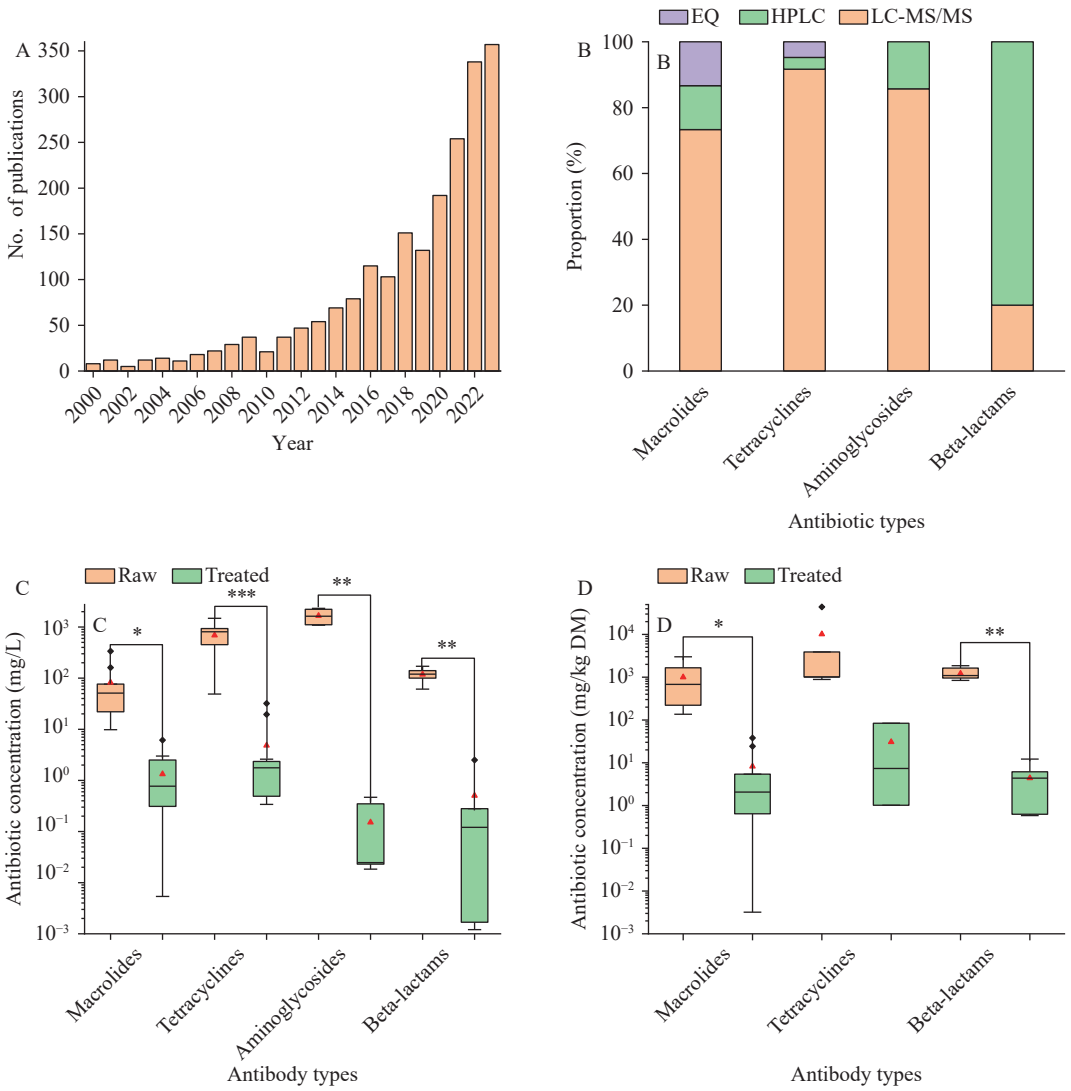


FIGURE 2. Analysis of publication trends and antibiotic measurements. (A) Annual publication numbers; (B) Distribution of antibiotic detection methods; (C) Antibiotic concentrations in pharmaceutical wastewater; (D) Antibiotic concentrations in fermentation residues.
Note: Red triangles (C, D) indicate mean values; black squares represent outliers. Statistical significance of pre- versus post-treatment concentration differences was determined using Paired-Sample t-Tests, with asterisks denoting significance levels.
Abbreviation: EQ=equivalent quantity; HPLC=high-performance liquid chromatography; LC-MS/MS=Liquid Chromatography-Mass Spectrometry/Mass Spectrometry.

Academy of Sciences (CAS) and Tsinghua University (THU) distinguished themselves through substantial publication output and high centrality metrics. The University of Chinese Academy of Sciences (UCAS) and the Research Center for Eco-Environmental Sciences (RCEES), both CAS affiliates, achieved significant research outcomes. The research group led by Min Yang and Yu Zhang has gained particular prominence, earning substantial citations for their sustained investigations into pharmaceutical wastewater and fermentation residue. In India, the Indian Institute of Technology System (IIT System) and in Turkey, Istanbul Technical University, emerged as leading institutions. While China and India dominate bulk antibiotic production, the challenges of antibiotic pollution and AMR risks have attracted significant attention from international organizations including UNEP and WHO, as well as industry groups like the Antimicrobial Resistance Industry Alliance, resulting in comprehensive guidelines addressing pharmaceutical supply chain pollution (*8*,*13*). The IIT system in India has conducted extensive research on

local pharmaceutical wastewater treatment systems, emphasizing the crucial role of regulatory standards in AMR control (*14*).

Data Records

Following PRISMA guidelines, 70 papers reporting actual samples from 2008-2024 were incorporated into the database (Figure 1). The database comprises 16 comprehensive columns documenting: antibiotic and sample types, geographical location, studied systems, treatment methodologies, experimental scale, detection methods, antibiotic concentrations, ARG testing methods and results, publication year, source ID, and reference source (Figure 3). Each entry's source ID links directly to detailed publication information, facilitating efficient data retrieval.

The database encompasses 270 samples across five major categories: wastewater, sludge, and receiving

TABLE 1. International collaboration analysis across countries, institutions, and researchers.

Country/Institution/Author	Publications	Centrality	Year of first publication
Country			
China	371	0.54	2007
India	87	0.18	2002
Spain	33	0.32	2006
France	30	0.08	2010
Brazil	29	0.07	2009
America	63	0.35	2002
Iran	56	0.13	2014
England	23	0.22	2001
Institution			
Chinese Academy of Sciences	78	0.02	2008
Research Center for Eco-Environmental Sciences (RCEES)	41	0.08	2008
Tsinghua University	39	0.05	2013
University of Chinese Academy of Sciences	31	0.03	2017
Istanbul Technical University	16	0.08	2004
Indian Institute of Technology System (IIT System)	10	0.01	2016
Author			
Yang, Min	24	0.13	2008
Zhang, Yu	20	0.08	2008

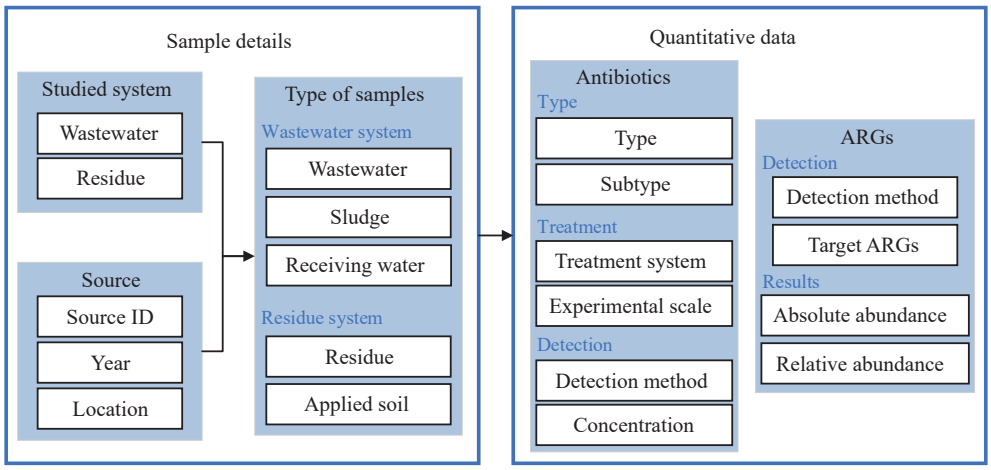


FIGURE 3. Framework of the original and quantitative samples data.
Abbreviation: ARGs=antibiotic resistance genes.

water from wastewater treatment systems; and fermentation residue and applied soil from fermentation residue research systems. It contains 256 antibiotic records and 100 ARG records. The antibiotic records are distributed as follows: 96 for residues, 1 for applied soil, 126 for wastewater, 26 for sludge, and 7 for receiving water. ARG documentation includes 39 records for residues, 3 for applied soil, 31 for wastewater, 27 for sludge, with no records for receiving water. The most extensive antibiotic concentration datasets were collected in 2023 (*n*=50), 2015 (*n*=30), and 2013 (*n*=21). Similarly, ARG data peaked in 2023 (*n*=28), followed by 2019 (*n*=17) and 2013 (*n*=16). Sampling locations primarily span 14 provincial-level administrative divisions (PLADs) in China, with a concentration in the northern region due to the prevalence of antibiotic production facilities. Additional reports originated from Poland and Croatia.

The geographical distribution of pharmaceutical facilities shows a significant dispersion, with notable clustering in northern China. Manufacturing facilities in Hebei Province and Shandong Province demonstrate diverse production capabilities, manufacturing antibiotics across five major categories. Macrolide and aminoglycoside production facilities show the broadest geographical distribution, operating across six PLADs or municipalities. Tetracycline and β-lactam production facilities are distributed across five PLADs or municipalities each.

Detection Methods and Concentration of Antibiotics and ARGs

The primary quantitative methods for antibiotic detection comprise liquid chromatography-tandem mass spectrometry (LC-MS/MS) and high-performance liquid chromatography (HPLC) (Figure 2B). The selection of detection methodology is determined by the specific antibiotic type and molecular structure. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) is preferentially employed for macrolides, tetracyclines, and aminoglycosides due to its superior sensitivity. Conversely, HPLC enables precise separation and quantitative analysis of β-lactam antibiotics in complex matrices. Additionally, Zhang et al. (*15*) introduced an innovative potency assay method to evaluate residual antibacterial effects, expressed as antibiotic equivalent quantity (EQ). Compared to conventional chemical methods, the EQ approach comprehensively accounts for the antibacterial activities of both parent compounds and their transformation products.

Antibiotic concentrations in production wastewaters were substantially elevated, with mean values ranging from 82 to 1,663 mg/L, markedly higher than the μg/L levels typically observed in municipal and domestic wastewaters (*16*–*18*). Tetracycline and aminoglycoside antibiotics exhibited particularly high concentrations (Figure 2C). Post-treatment analysis revealed significant reduction in residual antibiotics, with mean effluent concentrations below 5.0 mg/L across various antibiotic classes. However, these levels remained substantially higher than the 12.5 μg/L typically found in domestic wastewater treatment plant effluent (*19*).

Among fermentation residues, macrolides, tetracyclines, and beta-lactam antibiotics have been most extensively investigated. Raw sample concentrations averaged between 1,000 to 10,182 mg/kg dry matter (DM), substantially higher than the 4.0 mg/kg observed in municipal sewage sludge (*20*). Hydrothermal treatment and other remediation approaches achieved removal rates exceeding 90% (Figure 2D). Given the potential ecological risks and antimicrobial resistance concerns, fermentation residue has been classified as hazardous waste requiring efficient remediation treatment.

Researchers primarily employed quantitative PCR (qPCR) and metagenomic sequencing for ARG detection. Both production wastewater and fermentation residues exhibited significant ARG enrichment, predominantly showing resistance to the antibiotics being manufactured (Table 2). While biological treatment systems effectively removed antibiotics and certain ARGs from wastewater (*5*,*21*), treatment system sludge showed ARG enrichment, with abundance levels surpassing those found in fermentation residue from the same facility (*22*). ARG abundance in fermentation residues consistently reached approximately 5 log copies/mg.

Notably, treatment experiments remain predominantly confined to laboratory scale. Among research groups, the RCEES, CAS team has conducted the most extensive field studies investigating antibiotic and ARG occurrence and removal, with the explicit goal of scaling findings to pilot and full-scale applications (*4*,*23*–*26*).

Treatment Method and System

Biological treatment processes, particularly the

TABLE 2. Detection of antibiotic resistance genes in pharmaceutical-related matrices.

Antibiotic	Sample	Methods	Target ARGs	Abundance	Ref.
SPM	Sludge	qPCR	4 *erm*, *ere*A, *mph*B, *mef*A, *msr*D	1.4×10^{7} copies/mg; 4.3×10^{-1} copies/16S rRNA gene	(*22*)
	Residue	qPCR	4 *erm*, *msr*D	1.6×10^{5} copies/mg; 1.6×10^{-3} copies/16S rRNA gene	
	Residue	qPCR	*ere*A, 2 *erm*, *mef*A	1.64×10^{7} copies/mL, decrease 83.6%	(*38*)
	Compost	qPCR	4 *erm*, *mef*A, *mph*A	Lowest in mature phase	(*39*)
ERY	Effluent	qPCR	5 *erm*	8 log copies/mL	(*40*)
	Residue	qPCR	2 *erm*, *ere*A, mpfB, *mef*A	1.45–5.68 log copies/mL, decreased by 96%–99% after treatment.	(*41*)
	Residue	qPCR	2 *erm*, *ere*B	7.42×10^{-9}–6.82×10^{-4} copies/16S rRNA gene, decreased 99% after treatment.	(*42*)
	Effluent	HT-PCR	3 *erm*, *maA/mel*, *floR*, *sul*2, *tet*M, *mef*A	1.8–5 log copies/mL	(*43*)
	Influent	qPCR	4 *erm*, 1 *ere*, 2 *mph*, 1 *mef*	4.3×10^{8} copies/mL	(*5*)
	Effluent	qPCR	4 *erm*, 1 *ere*, 2 *mph*, 1 *mef*	2.1×10^{7} copies/mL	
OTC	Influent	qPCR	9 *tet*	7.0×10^{9} copies/mL; 3.2×10^{0} copies/16S rRNA gene	(*21*)
	Effluent	qPCR	9 *tet*	1.8×10^{8} copies/mL; 1.4×10^{0} copies/16S rRNA gene	
	Residue	qPCR	9 *tet*	1.4×10^{5} copies/mg; 2.4×10^{-2} copies/16S rRNA gene	
	Sludge	qPCR	5 *tet*	9-13 log copies/g dry matter	(*44*)
	Residue	qPCR	3 *tet*	6.5×10^{5} copies/mg in raw residue, decreased to 1.28×10^{4} copies/mg after treatment.	(*45*)
	Compost	Metagenomics	Multi-drugs	After composing, multiple ARGs increased by 57.38 %.	(*46*)
	Sludge	Metagenomics	*sul*1, *aph(6)-I*, *tet*A	Total: 2.41-6.55 copies/16S rRNA gene Tetracycline:0.82±0.21 copies/16S rRNA gene	(*47*)
TC	Production wastewater	qPCR	2 *sul*, 4 *tet*, 2 *bla*, *erm*B, *qnr*D	Average (4.80 ± 12.84) × 10^{5} copies/mL each.	(*48*)
	Effluent	qPCR	2 sul, 3 *tet*, 2 *bla*, *qnr*D	0.4-5.1×10^{7} copies/mL each.	
PG	Residue	qPCR	bla_{TEM}	4.17±0.19 log copies/mg	(*49*)
	Compost	qPCR	bla_{TEM}	8.98±0.27 log copies/mg	

Abbreviation: SPM=spiramycin; ERY=erythromycin; OTC=oxytetracycline; TC=tetracycline; PG=penicillin; qPCR=quantitative polymerase chain reaction; ARGs=antibiotic resistance genes; Ref.=reference.

combination of anaerobic digestion and activated sludge treatment, represent the predominant approach for managing antibiotic-containing pharmaceutical wastewater (*5*,*21*,*27*). However, high concentrations of antibiotic residues in wastewater have been demonstrated to promote ARG development and lead to treatment system failure (*4*,*28*).

To address this challenge, pretreatment of production wastewater to remove antibiotics prior to biological treatment has emerged as the optimal strategy for controlling ARG development (*8*,*29*–*30*). Enhanced hydrolysis has established itself as the leading pretreatment method in full-scale pharmaceutical wastewater treatment systems, demonstrating removal efficiencies exceeding 99% for oxytetracycline (*4*,*31*–*32*). For fermentation residues, hydrothermal treatment has shown exceptional effectiveness in eliminating both antibiotics and ARGs, achieving approximately 90% removal efficiency (*33*–*34*).

TECHNICAL VALIDATION

To ensure data accuracy and validity across diverse reporting formats, we implemented a comprehensive validation protocol. For ARG data, we maintained distinct recording formats: specific target genes for qPCR analyses and comprehensive gene profiles for metagenomic studies. Sample-specific units were standardized according to matrix type (mass-based for solids and volume-based for liquids). We employed a dual-verification system where one researcher entered the records while a second researcher independently validated the dataset to prevent errors and eliminate duplicate entries.

USAGE NOTES

This comprehensive database encompasses 270 records documenting antibiotics and ARGs in

pharmaceutical wastewater and solid waste, developed through systematic literature review. The database is accessible through an online platform https://dash.drwater.net/antiboard/, providing researchers with validated scientific data for analysis and reference. The Environmental Microbiology Technology Research Group of RCEES, CAS maintains continuous data collection and performs periodic updates to the website. Future development of the database aims to enhance pharmaceutical industry management through collaborative support from pharmaceutical industry associations.

This study provides a comprehensive overview of antibiotic production industry waste research and establishes a database tracking antibiotics and ARGs in actual industrial samples. While numerous treatment technologies demonstrate promising laboratory-scale removal efficiencies for both antibiotics and ARGs, validation through full-scale implementation remains crucial. Given that India and China dominate global antimicrobial manufacturing, enhancing pollution control measures in these nations offers the greatest potential for reducing worldwide AMR risks from pharmaceutical production. The implementation of enhanced hydrolysis pre-treatment to reduce antibiotic concentrations in manufacturing wastewater has proven particularly effective in controlling AMR development during biological treatment. This approach has been successfully scaled to full-scale wastewater treatment facilities in China, positioning Chinese AMR prevention technologies at the forefront of global pharmaceutical industry practices. As the field continues to evolve, this database serves as a vital resource for researchers and industry stakeholders addressing these challenges.

In 2008, while China's Ministry of Environmental Protection established discharge limits for key pollutants in pharmaceutical wastewater, the absence of specific standards for residual antibiotics highlighted the need for further research on resistance thresholds (*35*). The Ministry of Ecology and Environment's 2023 technical guidelines for wastewater treatment represent progress, though many emerging technologies remain in experimental phases (*10*). Notably, antibiotic fermentation residues have maintained their classification as hazardous waste since their initial inclusion in the Directory of National Hazardous Wastes in 2008.

The current database iteration, while ensuring accuracy through manual literature curation, faces challenges in keeping pace with the rapidly expanding volume of publications on pharmaceutical industry antibiotic pollution and resistance. Integration of artificial intelligence (AI), particularly Large Language Models (LLMs) and Retrieval-Augmented Generation (RAG) systems, offers potential solutions for enhancing data retrieval, automating updates, and transforming the database into a dynamic, self-updating knowledge system (*36*–*37*). Furthermore, future expansions will incorporate data from Chinese theses, dissertations, and grey literature, including relevant local, national, and regional documents, extending beyond the current English-language research paper focus.

Conflicts of interest: No conflicts of interest.

Funding: Supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB0750400), the Basic Science Center Project of the Natural Science Foundation of China (52388101), and the China Postdoctoral Innovation Talents Support Program (BX20230400).

doi: 10.46234/ccdcw2025.015

[#] Corresponding author: Yu Zhang, zhangyu@rcees.ac.cn.

[1] State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China; [2] Sino-Danish College, University of Chinese Academy of Sciences, Beijing, China; [3] University of Chinese Academy of Sciences, Beijing, China; [4] Agricultural Information Institute, Chinese Academy of Agricultural Sciences, Beijing, China; [5] Department of Environmental Science and Engineering, Faculty of Natural Resources, University College of Agriculture & Natural Resources, University of Tehran, Karaj, Iran.

Submitted: September 23, 2024
Accepted: December 09, 2024
Issued: January 17, 2025

REFERENCES

1. Han ZM, Feng HD, Wang C, Wang XG, Yang M, Zhang Y, et al. Mitigating antibiotic resistance emissions in the pharmaceutical industry: global governance and available techniques. China CDC Wkly 2023;5(46):1038 – 44. https://doi.org/10.46234/ccdcw2023.195.
2. Hui XS, Fang WJ, Wang G, Liu HL, Dai XH. Waste recycling of antibiotic mycelial residue: the feasible harmless treatment and source control of antibiotic resistance. J Cleaner Prod 2023;401:136786. https://doi.org/10.1016/j.jclepro.2023.136786.
3. World Health Organization. Guidance on wastewater and solid waste management for manufacturing of antibiotics. Geneva: World Health Organization; 2024. Licence: CC BY-NC-SA 3.0 IGO. https://www.who.int/publications/i/item/9789240097254.[2024-9-13].
4. Yi QZ, Zhang Y, Gao YX, Tian Z, Yang M. Anaerobic treatment of antibiotic production wastewater pretreated with enhanced hydrolysis: simultaneous reduction of COD and ARGs. Water Res 2017;110:211 – 7. https://doi.org/10.1016/j.watres.2016.12.020.

5. Tang L, Feng HD, Luan X, Han ZM, Yang M, Zhang Y. Occurrence, distribution, and behaviors of erythromycin A, production byproducts, transformation products, and resistance genes in a full-scale erythromycin A production wastewater treatment system. Water Res 2023;245:120640. https://doi.org/10.1016/j.watres.2023.120640.
6. He YP, Tian Z, Luan X, Han ZM, Zhang Y, Yang M. Recovery of biological wastewater treatment system inhibited by oxytetracycline: rebound of functional bacterial population and the impact of adsorbed oxytetracycline on antibiotic resistance. Chem Eng J 2021;418:129364. https://doi.org/10.1016/j.cej.2021.129364.
7. Feng MM, Liu YW, Yang L, Li ZJ. Antibiotics and antibiotic resistance gene dynamics in the composting of antibiotic fermentation waste - a review. Bioresour Technol 2023;390:129861. https://doi.org/10.1016/j.biortech.2023.129861.
8. World Health Organization. Technical brief on water, sanitation, hygiene (WASH) and wastewater management to prevent infections and reduce the spread of antimicrobial resistance (AMR). Geneva: World Health Organization. 2020. https://www.who.int/publications/i/item/9789240006416. [2024-9-21]
9. Zhang Y, Walsh TR, Wang Y, Shen JZ, Yang M. Minimizing risks of antimicrobial resistance development in the environment from a public one health perspective. China CDC Wkly 2022;4(49):1105 – 9. https://doi.org/10.46234/ccdcw2022.224.
10. Ministry of Ecology and Environment of the People's Republic of China. Guideline on available techniques of pollution prevention and control for pharmaceutical industry——Active pharmaceutical ingredients (fermentation, chemical synthesis, extraction) and preparation categories. https://www.mee.gov.cn/ywgz/fgbz/bz/bzwb/kxxjszn/202309/t20230926_1041928.shtml. [2024-11-25]. (In Chinese).
11. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. PLoS Med 2021;18(3):e1003583. https://doi.org/10.1371/journal.pmed.1003583.
12. Chen CM. CiteSpace Ii: detecting and visualizing emerging trends and transient patterns in scientific literature. J Am Soc Inf Sci 2006;57(3):359 – 77. https://doi.org/10.1002/asi.20317.
13. AMR Industry Alliance. Minimizing risk of developing antibiotic resistance and aquatic ecotoxicity in the environment resulting from the manufacturing of human antibiotics. Geneva: AMR Industry Alliance. 2022. https://www.amrindustryalliance.org/wp-content/uploads/2022/06/AMRIA_Antibiotic-Manufacturing-Standard_EMBARGOED-UNTIL-JUN-14-8-am-EDT.pdf. [2024-12-5].
14. Kotwani A, Kapur A, Chauhan M, Gandra S. Treatment and disposal practices of pharmaceutical effluent containing potential antibiotic residues in two states in India and perceptions of various stakeholders on contribution of pharmaceutical effluent to antimicrobial resistance: a qualitative study. J Pharm Policy Pract 2023;16(1):59. https://doi.org/10.1186/s40545-023-00562-z.
15. Zhang H, Zhang Y, Yang M, Liu MM. Evaluation of residual antibacterial potency in antibiotic production wastewater using a real-time quantitative method. Environ Sci: Processes Impacts 2015;17(11):1923 – 9. https://doi.org/10.1039/C5EM00228A.
16. Kamal A, Kanafin YN, Satayeva A, Kim J, Poulopoulos SG, Arkhangelsky E. Removal of carbamazepine, sulfamethoxazole and aspirin at municipal wastewater treatment plant of Astana, Kazakhstan: paths to increase the efficiency of the treatment process. J Chem Technol Biotechnol 2024;99(11):2248 – 58. https://doi.org/10.1002/jctb.7713.
17. Zhang YY, Hu YF, Li XW, Gao LJ, Wang SY, Jia SY, et al. Prevalence of antibiotics, antibiotic resistance genes, and their associations in municipal wastewater treatment plants along the Yangtze River basin, China. Environ Pollut 2024;348:123800. https://doi.org/10.1016/j.envpol.2024.123800.
18. Lin Q, Yu CS, Chen KY, Yasir H, Luo AC, Liang ZW, et al. Occurrence of micropollutants in rural domestic wastewater in Zhejiang Province, China and corresponding wastewater-based epidemiology analysis. Sci Total Environ 2024;931:172686. https://doi.org/10.1016/j.scitotenv.2024.172686.
19. Hu JR, Lyu Y, Chen H, Li S, Sun WL. Suspect and nontarget screening reveal the underestimated risks of antibiotic transformation products in wastewater treatment plant effluents. Environ Sci Technol 2023;57(45):17439 – 51. https://doi.org/10.1021/acs.est.3c05008.
20. Meng F, Sun SJ, Geng JL, Ma LX, Jiang JP, Li B, et al. Occurrence, distribution, and risk assessment of quinolone antibiotics in municipal sewage sludges throughout China. J Hazard Mater 2023;453:131322. https://doi.org/10.1016/j.jhazmat.2023.131322.
21. Liu MM, Zhang Y, Yang M, Tian Z, Ren LR, Zhang SJ. Abundance and distribution of tetracycline resistance genes and mobile elements in an oxytetracycline production wastewater treatment system. Environ Sci Technol 2012;46(14):7551 – 7. https://doi.org/10.1021/es301145m.
22. Liu MM, Ding R, Zhang Y, Gao YX, Tian Z, Zhang T, et al. Abundance and distribution of Macrolide-Lincosamide-Streptogramin resistance genes in an anaerobic-aerobic system treating spiramycin production wastewater. Water Res 2014;63:33 – 41. https://doi.org/10.1016/j.watres.2014.05.045.
23. Li D, Yang M, Hu JY, Ren LR, Zhang Y, Li KZA. Determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river. Environ Toxicol Chem 2008;27(1):80 – 6. https://doi.org/10.1897/07-080.1.
24. Deng YQ, Zhang Y, Gao YX, Li D, Liu RY, Liu MM, et al. Microbial Community Compositional Analysis for Series Reactors Treating High Level Antibiotic Wastewater. Environ Sci Technol 2012;46(2):795 – 801. https://doi.org/10.1021/es2025998.
25. Zhang Y, Xie JP, Liu MM, Tian Z, He ZL, van Nostrand JD, et al. Microbial community functional structure in response to antibiotics in pharmaceutical wastewater treatment systems. Water Res 2013;47(16):6298 – 308. https://doi.org/10.1016/j.watres.2013.08.003.
26. Tian Y, Tian Z, He YP, Sun GX, Zhang Y, Yang M. Removal of denatured protein particles enhanced UASB treatment of oxytetracycline production wastewater. Sci Total Environ 2022;816:151549. https://doi.org/10.1016/j.scitotenv.2021.151549.
27. Bengtsson-Palme J, Milakovic M, Švecová H, Ganjto M, Jonsson V, Grabic R, et al. Industrial wastewater treatment plant enriches antibiotic resistance genes and alters the structure of microbial communities. Water Res 2019;162:437 – 45. https://doi.org/10.1016/j.watres.2019.06.073.
28. Amin MM, Zilles JL, Greiner J, Charbonneau S, Raskin L, Morgenroth E. Influence of the antibiotic erythromycin on anaerobic treatment of a pharmaceutical wastewater. Environ Sci Technol 2006;40(12):3971 – 7. https://doi.org/10.1021/es060428j.
29. Grela A, Kuc J, Klimek A, Matusik J, Pamuła J, Franus W, et al. Erythromycin scavenging from aqueous solutions by zeolitic materials derived from fly ash. Molecules 2023;28(2):798. https://doi.org/10.3390/molecules28020798.
30. Liu MM, Zhang Y, Zhang H, Zhang HF, Li KX, Tian Z, et al. Ozonation as an effective pretreatment for reducing antibiotic resistance selection potency in oxytetracycline production wastewater. Desalination Water Treat 2017;74:155 – 62. https://doi.org/10.5004/dwt.2017.20731.
31. Tang M, Gu Y, Wei DB, Tian Z, Tian Y, Yang M, et al. Enhanced hydrolysis of fermentative antibiotics in production wastewater: hydrolysis potential prediction and engineering application. Chem Eng J 2020;391:123626. https://doi.org/10.1016/j.cej.2019.123626.
32. Feng HD, Hu YQ, Tang L, Tian Y, Tian Z, Wei DB, et al. New hydrolysis products of oxytetracycline and their contribution to hard COD in biological effluents of antibiotic production wastewater. Chem Eng J 2023;471:144409. https://doi.org/10.1016/j.cej.2023.144409.
33. Han ZM, Feng HD, Luan X, Shen YP, Ren LR, Deng LJ, et al. Three-year consecutive field application of erythromycin fermentation residue following hydrothermal treatment: cumulative effect on soil antibiotic resistance genes. Engineering 2022;15:78 – 88. https://doi.org/10.1016/j.eng.2022.05.011.
34. Han ZM, Luan X, Feng HD, Deng YQ, Yang M, Zhang Y. Metagenomic insights into microorganisms and antibiotic resistance genes of waste antibiotic fermentation residues along production,

storage and treatment processes. J Environ Sci 2024;136:45 – 55. https://doi.org/10.1016/j.jes.2022.10.035.
35. Ministry of Ecology and Environment of the People's Republic of China. Discharge standard of water pollutants for pharmaceutical industry Fermentation products category. https://www.mee.gov.cn/ywgz/fgbz/bz/bzwb/shjbh/swrwpfbz/200807/t20080701_124699.shtml. [2024-11-25]. (In Chinese).
36. Ofori-Boateng R, Aceves-Martins M, Wiratunga N, Moreno-Garcia CF. Towards the automation of systematic reviews using natural language processing, machine learning, and deep learning: a comprehensive review. Artif Intell Rev 2024;57(8):200. https://doi.org/10.1007/s10462-024-10844-w.
37. Foppiano L, Lambard G, Amagasa T, Ishii M. Mining experimental data from materials science literature with large language models: an evaluation study. Sci Technol Adv Mater: Methods 2024;4(1):2356506. https://doi.org/10.1080/27660400.2024.2356506.
38. Awad M, Tian Z, Gao YX, Yang M, Zhang Y. Pretreatment of spiramycin fermentation residue using hyperthermophilic digestion: quick startup and performance. Water Sci Technol 2018;78(9):1823 – 32. https://doi.org/10.2166/wst.2018.256.
39. Wang G, Liu HL, Gong PC, Wang J, Dai XH, Wang P. Insight into the evolution of antibiotic resistance genes and microbial community during spiramycin fermentation residue composting process after thermally activated peroxydisulfate pretreatment. J Hazard Mater 2022;424:127287. https://doi.org/10.1016/j.jhazmat.2021.127287.
40. Hua T, Li SN, Li FX, Ondon BS, Liu YWJ, Wang HN. Degradation performance and microbial community analysis of microbial electrolysis cells for erythromycin wastewater treatment. Biochem Eng J 2019;146:1 – 9. https://doi.org/10.1016/j.bej.2019.02.008.
41. Chu LB, Chen D, Wang JL, Yang ZL, Shen YP. Degradation of antibiotics and antibiotic resistance genes in erythromycin fermentation residues using radiation coupled with peroxymonosulfate oxidation. Waste Manage 2019;96:190 – 7. https://doi.org/10.1016/j.wasman.2019.07.031.
42. Zhang YX, Liu HL, Xin YJ, Shen YP, Wang J, Cai C, et al. Erythromycin degradation and ERY-resistant gene inactivation in erythromycin mycelial dreg by heat-activated persulfate oxidation. Chem Eng J 2019;358:1446 – 53. https://doi.org/10.1016/j.cej.2018.10.157.
43. Chu LB, Wang JL, Chen CH, He SJ, Wojnárovits L, Takács E. Advanced treatment of antibiotic wastewater by ionizing radiation combined with peroxymonosulfate/H_2O_2 oxidation. J Cleaner Prod 2021;321:128921. https://doi.org/10.1016/j.jclepro.2021.128921.
44. Pei J, Yao H, Wang H, Ren J, Yu XH. Comparison of ozone and thermal hydrolysis combined with anaerobic digestion for municipal and pharmaceutical waste sludge with tetracycline resistance genes. Water Res 2016;99:122 – 8. https://doi.org/10.1016/j.watres.2016.04.058.
45. Awad M, Tian Z, Zhang Y, Yang M, Yin WJ, Dong LP. Hydrothermal pretreatment of oxytetracycline fermentation residue: removal of oxytetracycline and increasing the potential for anaerobic digestion. Environ Eng Res 2020;26(4):200258. https://doi.org/10.4491/eer.2020.258.
46. Chen SN, Zhong WZ, Ning ZF, Niu JR, Feng J, Qin X, et al. Effect of homemade compound microbial inoculum on the reduction of terramycin and antibiotic resistance genes in terramycin mycelial dreg aerobic composting and its mechanism. Bioresour Technol 2023;368:128302. https://doi.org/10.1016/j.biortech.2022.128302.
47. Tian Y, Tian Z, Feng HD, Luan X, Han ZM, Zhang Y, et al. Unveiling the threshold values of organic and oxytetracycline loadings for nitrification recovery of a full-scale pharmaceutical wastewater treatment system. Chem Eng J 2023;463:142487. https://doi.org/10.1016/j.cej.2023.142487.
48. Hou J, Chen ZY, Gao J, Xie YL, Li LY, Qin SY, et al. Simultaneous removal of antibiotics and antibiotic resistance genes from pharmaceutical wastewater using the combinations of up-flow anaerobic sludge bed, anoxic-oxic tank, and advanced oxidation technologies. Water Res 2019;159:511 – 20. https://doi.org/10.1016/j.watres.2019.05.034.
49. Yang L, Zhang SH, Chen ZQ, Wen QX, Wang Y. Maturity and security assessment of pilot-scale aerobic co-composting of penicillin fermentation dregs (PFDs) with sewage sludge. Bioresour Technol 2016;204:185 – 91. https://doi.org/10.1016/j.biortech.2016.01.004.

Methods and Applications

Development of a High-Throughput qPCR Assay for Detecting Waterborne Protozoa and Helminths Across Different Environmental Media in China

Fuming Duan[1,2]; Ziming Han[1]; Tiantian Tian[3]; Huican Zhang[1,4]; Min Yang[1,2]; Yu Zhang[1,2,4,#]

ABSTRACT

Introduction: The establishment of a high-throughput quantification approach for waterborne pathogenic protozoa and helminths is crucial for rapid screening and health risk assessment.

Methods: We developed a high-throughput quantitative polymerase chain reaction (HT-qPCR) assay targeting 19 waterborne protozoa and 3 waterborne helminths and validated its sensitivity, specificity, and repeatability. The assay was then applied to test various environmental media samples.

Results: The HT-qPCR assay's limit of detection (LOD) was 5×10^2 copies/μL DNA, and its specificity was confirmed using *Giardia* and *Cryptosporidium* standards. Repeatability, assessed through intra- and inter-group experiments, yielded a coefficient of variation (*CV*) of 1.0%–4.6% and 1.2%–6.4% at concentrations of 1×10^5 and 1×10^4 copies/μL, respectively. The R^2 values of the 22 standard curves ranged from 0.983 to 0.998, with amplification efficiencies between 80% and 107%. In drinking water sources, sludge from municipal wastewater treatment plants (MWTPs), and livestock manure samples, 17 of 22 targets were detected, with *Acanthamoeba* genus (50.0%), *Acanthamoeba castellanii* (11.8%), and *Enterocytozoon bieneusi* (11.8%) showing high prevalence. *Cryptosporidium* spp., *Enterocytozoon bieneusi*, and *Cyclospora cayetanensis* were simultaneously found in all three sample types.

Discussion: This study presents a useful tool for the rapid detection of waterborne protozoa and helminths in complex environmental microbiomes, providing scientific data for monitoring cross-media transmission and controlling microbial risk from a One Health perspective.

Water is a common transmission vector for numerous pathogens. Many waterborne protozoa and helminths are zoonotic, posing significant risks to both humans and animals, particularly through the ingestion of fecally contaminated water. Even at relatively low environmental concentrations, these pathogens can present substantial public health risks, with vulnerable populations such as the elderly, children, and individuals with acquired immunodeficiency syndrome being particularly susceptible (*1*). A notable example is the 1993 cryptosporidiosis outbreak in Milwaukee, United States, which resulted in approximately 400,000 infections (*2*). From a One Health perspective, cross-media monitoring and understanding transmission mechanisms are crucial for controlling waterborne protozoa and helminths in the environment-animal-human continuum.

Among waterborne protozoa and helminths, *Cryptosporidium* and *Giardia* are included in the National Standard for Drinking Water Quality of China (GB 5749-2022), the European Union's drinking water quality standards, and the World Health Organization's (WHO) Guidelines for drinking-water quality. They are also classified as Class B pathogenic microorganisms by the National Institutes of Health, USA (*3*–*6*). No other waterborne protozoa are included in these standards and guidelines. In addition to *Cryptosporidium* and *Giardia*, other pathogens such as *Echinococcus ameba*, *Microsporidium*, *Enterocytozoon bieneusi*, *Toxoplasma gondii*, and *Schistosoma* have also been linked to waterborne diseases in both humans and animals (*1*,*7*). Therefore, there is an urgent need for an efficient detection method that can cover a broad spectrum of these waterborne protozoa and helminths. Traditional detection methods for pathogenic protozoa and helminths mainly include microscopy, quantitative polymerase chain reaction (qPCR), nested PCR, and loop-mediated isothermal amplification (LAMP) (*8*). Recent advancements in high-throughput technologies

have enabled the detection of microorganisms within complex microbiomes. High-throughput qPCR (HT-qPCR) has been widely employed for monitoring of bacterial pathogens and viruses in environmental samples (*9*–*11*). However, a high-throughput approach specifically targeting waterborne protozoa and helminths is still lacking, impeding the rapid screening of these pathogens in the environment. In this study, we developed and validated an HT-qPCR method for the detection of protozoa and helminths and applied it to screen for waterborne pathogens in environmental samples and related pollution sources from a One-Health perspective.

METHODS

Sampling Based on a One Health Perspective

A One Health perspective emphasizes the close links between human, animal, and environmental health. This study selected environmental samples, including: 1) drinking water source samples from the Yangtze River in August 2023 (n=23) and Yellow River in October 2023 and January 2024 (n=23) in China (n=46); 2) sludge samples from municipal wastewater treatment plants (MWTPs) in 2015 (n=12); and 3) livestock manure in 2020 and 2021 from pigs (n=6), chickens (n=2), sheep (n=1), and cattle (n=1). These samples are closely related to human, animal, and environmental health, respectively, and were used to investigate the potential risks of pathogenic microorganisms. Studying and managing these samples will help us better understand and respond to various health risks and achieve common health goals for humans, animals, and the environment. For drinking water source samples, 10 L of water was concentrated using the calcium carbonate flocculation method, and the concentrate was transferred for DNA extraction. Sludge and livestock manure samples were used directly for DNA extraction. Environmental DNA was extracted from 0.5 g of sample concentrate using the FastDNA SPIN Kit for Soil and eluted in 75 μL of DES. Extracted DNA was stored at −20 °C until use.

High-throughput qPCR Setup

Twenty-two pathogenic taxa, including 19 protozoa and 3 helminths, were selected as detection targets (Table 1). These taxa were chosen based on their waterborne characteristics and potential environmental dissemination risk as outlined in the WHO Water Quality Guidelines (4th edition) (*3*). Additionally, 18S rRNA and 16S rRNA genes served as quality controls. DNA was diluted to approximately 20 ng/μL, and 400 ng/μL bovine serum albumin (BSA) was added to each qPCR system to improve amplification efficiency and mitigate potential inhibition. The amplification efficiency of all reactions was between 80% and 120%.

The PCR primers and probes were designed using a Thermo Fisher proprietary process, with T_m values between 58 °C and 62 °C (*12*). Primer lengths were between 9 bp and 40 bp, with GC content ranging from 30% to 80%. Primer and probe information, including assay IDs, is shown in Supplementary Table S1 and stored in the Odyssey data by Thermo Fisher. The HT-qPCR assay for detecting waterborne protozoa and helminths was conducted on OpenArray chips using an Applied Biosystems QuantStudio 12K Flex instrument (Thermo Fisher, USA). The OpenArray Module enables simultaneous operation of up to four OpenArray chips, allowing testing of up to 192 samples over a 3-hour period, significantly increasing reaction throughput.

Validation and Application of the HT-qPCR Assay

For the sensitivity test, the standard plasmid (1×10^7 copies/μL) was diluted to a gradient of 1×10^6, 1×10^5, 1×10^4, 1×10^3, 5×10^2, 4×10^2, 3×10^2, 2×10^2, and 1×10^2 copies/μL (3 replicates of each sample), and HT-qPCR assays were performed. The minimum concentration of gene copies from the standard series was considered the qPCR limit of detection (LOD). The unit of LOD was gene copies per microliter of DNA (*13*). For the specificity test, quality control standards of *Giardia lamblia* and *Cryptosporidium parvum* (Waterborne™, USA) were used for the qPCR assay. For the repeatability test, plasmid standards of 1×10^5 and 1×10^4 copies/μL were used for intra- and inter-group experiments, and the average Cycle threshold (Ct) value and coefficient of variation (*CV*) were calculated.

The standard plasmid (1×10^7 copies/μL) was diluted to six gradient concentrations of 1×10^6, 1×10^5, 1×10^4, 1×10^3, 5×10^2, and 1×10^2 copies/μL, with three replicates per concentration. RNase-free ddH_2O served as a negative control. The kinetic amplification curve and PCR amplification cycle threshold (Ct value) were obtained, and a standard curve was constructed by plotting the Ct value (vertical axis) against the $\log_{10}$ (plasmid copy number) (horizontal axis). Method

TABLE 1. Basic information of waterborne protozoa and helminths.

Pathogen	Detection target	Health significance	Persistence in water supplies	Resistance to chlorine	Relative infectivity	Important animal source
Acanthamoeba	*Acanthamoeba*	–	–	–	–	–
	Acanthamoeba culbertsoni	High	May multiply	Low	High	No
	Acanthamoeba castellanii	High	May multiply	Low	High	No
	Acanthamoeba polyphaga	High	May multiply	Low	High	No
Balantioides	*Balantidium coli*	High	Moderate	High	High	No
Blastocystis	*Blastocystis hominis*	High	May multiply	High	Moderate	Yes
Cryptosporidium	*Cryptosporidium* spp.	–	–	–	–	–
	Cryptosporidium parvum	High	Long	High	High	Yes
	Cryptosporidium hominis	High	Long	High	High	Yes
	Cryptosporidium meleagridis	High	Long	High	High	Yes
Cyclospora	*Cyclospora cayetanensis*	High	Long	High	High	Yes
Enterocytozoon	*Enterocytozoon bieneusi*	High	Long	High	High	Yes
Entamoeba	*Entamoeba histolytica*	High	Moderate	High	High	No
Encephalitozoon	*Encephalitozoon*	High	Moderate	High	Moderate	Yes
Giardia spp.	*Giardia* spp.	–	–	–	–	–
	Giardia lamblia	High	Moderate	High	High	Yes
Naegleria	*Naegleria fowleri*	High	May multiply	Low	Moderate	No
Isospora	*Isospora belli*	Moderate	Moderate	High	High	Yes
Toxoplasma gondii	*Toxoplasma gondii*	High	Long	High	High	Yes
Echinococcus	*Echinococcus*	High	Long	Low	Moderate	Yes
Dracunculus	*Dracunculus medinensis*	Low	Moderate	Moderate	High	No
Schistosoma	*Schistosoma* spp.	High	Short	Moderate	High	Yes

Note: "–"=not available.

validity was verified based on the correlation coefficients, slopes, and amplification efficiencies of the standard curves for each detection target. Following development, the HT-qPCR assay was used to detect waterborne protozoa and helminths in various environmental samples.

RESULTS

Establishment and Validation of HT-qPCR Assay

Table 2 outlines the sensitivity of the HT-qPCR assay, evaluated using a gradient of positive plasmid concentrations. The results showed that all 24 detection targets were reliably detected at positive plasmid concentrations exceeding 5×10^2 copies/μL DNA. At concentrations of 4×10^2 and 3×10^2 copies/μL DNA, 15 and 12 targets were stably detected, respectively. When the concentration dropped below 2×10^2 copies/μL DNA, none of the 24 targets were consistently detected. Thus, the limit of detection for the HT-qPCR assay was established at 5×10^2 copies/μL DNA.

The specificity of the method was verified using standard samples of *Cryptosporidium parvum* and *Giardia lamblia*, alongside a negative control of RNase-free water. For *Cryptosporidium parvum*, only the qPCR assays for *Cryptosporidium* spp. and *Cryptosporidium parvum* produced positive results with Ct values less than 35 in the amplification curves, while no amplification was observed in the other assays. Similarly, for *Giardia lamblia*, only the qPCR assays targeting *Giardia* spp. and *Giardia lamblia* showed positive results with Ct values below 35. No positive results were observed for any of the 24 detection targets in the negative control. To assess the repeatability of the method, intra- and inter-group experiments were performed using positive plasmid concentrations of 10^5 and 10^4 copies/μL DNA. When the plasmid concentration was 10^5 copies/μL DNA, the *CV* for intra- and inter-group experiments ranged

TABLE 2. Sensitivity test results of the HT−qPCR assay.

Positive plasmid copy number (copies/μL)	Ct value within group			Note
	Average value	Standard deviation	Coefficient of variation (%)	
1×10^6	17.4–19.3	0.08–0.4	0.4–2.2	Stable detection of 24 targets
1×10^5	20.8–23.0	0.2–0.9	1.0–4.1	Stable detection of 24 targets
1×10^4	25.0–27.3	0.4–1.2	1.7–4.6	Stable detection of 24 targets
1×10^3	27.9–30.7	0.5–2.6	1.5–8.6	Stable detection of 24 targets
5×10^2	28.8–31.4	0.03–1.0	0.1–3.3	Stable detection of 24 targets
4×10^2	ND–33.5	0.6–9.3	1.9–50.7	Stable detection of 15 out of 24 targets
3×10^2	ND–32.3	0.4–8.6	1.2–37.6	Stable detection of 12 out of 24 targets
2×10^2	ND–32.5	0.2–9.6	0.5–46.4	All 24 target detections are unstable
1×10^2	ND–32.9	0.2–7.2	0.9–48.5	All 24 target detections are unstable

Abbreviation: HT-qPCR=high-throughput quantitative polymerase chain reaction; Ct=Cycle threshold.

from 1.0% to 4.1% and 1.2% to 5.5%, respectively. At 10^4 copies/μL DNA, the *CV* range for intra- and inter-group experiments was 1.7%–4.6% and 3.0%–6.4%, respectively. A *CV* of less than 10% confirmed the method's acceptable repeatability.

A total of 24 standard curves for 19 protozoa, 3 helminths, and 18S rRNA and 16S rRNA genes were generated (Supplementary Table S1, available at https://weekly.chinacdc.cn/). The correlation coefficients (R^2) ranged from 0.983 to 0.998, with amplification efficiencies between 80% and 107%, indicating reliable stability across all detected targets under the experimental conditions.

Application to Diverse Environmental Samples

Table 3 presents the diagnostic performance of the HT-qPCR assay for detecting protozoa and helminths in environmental samples from drinking water sources (n=46), sludge from MWTPs (n=12), and livestock manure (n=10). In total, 17 of 22 targets were detected across all samples, with the three most prevalent being the *Acanthamoeba* genus (50.0%), *Acanthamoeba castellanii* (11.8%), and *Enterocytozoon bieneusi* (11.8%). The distribution of protozoa and helminths varied among the three media types. Thirteen were detected in drinking water sources, 13 in MWTP sludge, and 11 in livestock manure.

In drinking water sources, the frequently detected targets were *Acanthamoeba* genus, *Naegleria fowleri*, and *Acanthamoeba castellanii*, with concentrations ranging from n.d.–5.0×10^5, n.d.–1.5×10^5, and n.d.–4.7×10^5 gene copies/L. In MWTP sludge, the dominant targets were *Acanthamoeba* genus, *Isospora belli*, and *Acanthamoeba polyphaga*, with concentrations of n.d.–7.2×10^5, n.d.–1.2×10^5, and n.d.–1.1×10^5 gene copies/g, respectively. In livestock manure, *Balantidium coli*, *Cyclospora cayetanensis*, and *Enterocytozoon bieneusi* were detected most frequently, with concentrations of n.d.–9.9×10^5, n.d.–4.7×10^4, and n.d.–1.0×10^4 gene copies/g, respectively (Supplementary Table S2, available at https://weekly.chinacdc.cn/). Notably, *Cryptosporidium* was detected more frequently than *Giardia*.

Importantly, *Cryptosporidium* spp., *Enterocytozoon bieneusi*, *Cyclospora cayetanensis*, *Balantidium coli*, and *Isospora belli* were simultaneously detected in drinking water sources, MWTP sludge, and livestock manure.

DISCUSSION

This study developed and validated an HT-qPCR assay capable of detecting 22 waterborne protozoa and helminths. This assay addresses the limitations of low throughput in existing diagnostic approaches while improving accuracy and providing a more cost-effective solution for assessing the risks of protozoa and helminths from a One Health perspective.

Compared to current detection methods, this approach significantly enhances detection efficiency. Using four OpenArray chips in a single run on the Applied Biosystems QuantStudio 12K Flex instrument, 22 targets across up to 192 samples can be analyzed within 3 hours. This rapid data generation is advantageous for large-scale monitoring and initial screening, facilitating subsequent risk assessments.

Infectious diseases caused by pathogenic bacteria, viruses, and parasites (e.g., protozoa and helminths) are the most prevalent health risks linked to drinking water. The primary threat to public health from

TABLE 3. Protozoa and helminth DR in environmental samples by HT-qPCR.

Type	No.	Target	Drinking water sources (DR%)	MWTP sludge (DR%)	Livestock manure (DR%)
Protozoa	1	*Acanthamoeba genus*	54.3	58.0	20.0
	2	*Acanthamoeba culbertsoni*	4.3	8.3	10.0
	3	*Acanthamoeba castellanii*	10.9	8.3	20.0
	4	*Acanthamoeba polyphaga*	2.2	25.0	–
	5	*Balantidium coli*	2.2	8.3	40.0
	6	*Blastocystis hominis*	–	–	10.0
	7	*Cryptosporidium* spp.	8.7	16.7	10.0
	8	*Cryptosporidium meleagridis*	–	–	–
	9	*Cryptosporidium hominis*	–	–	–
	10	*Cryptosporidium parvum*	–	8.3	–
	11	*Cyclospora cayetanensis*	4.3	8.3	20.0
	12	*Isospora belli*	2.2	33.3	10.0
	13	*Encephalitozoon*	2.2	–	–
	14	*Entamoeba histolytica*	–	8.3	–
	15	*Enterocytozoon bieneusi*	8.7	16.7	20.0
	16	*Giardia* spp.	–	–	–
	17	*Giardia lamblia*		16.7	–
	18	*Naegleria fowleri*	13.1	–	–
	19	*Toxoplasma gondii*	–	–	10.0
Helminths	20	*Echinococcus*	–	–	–
	21	*Dracunculus medinensis*	2.2	8.3	10.0
	22	*Schistosoma* spp.	–	–	–

Note: "–"=not detectable.
Abbreviation: MWTP=municipal wastewater treatment plant; DR=detection rates; HT-qPCR=high-throughput quantitative polymerase chain reaction.

waterborne microbes stems from drinking water contaminated with human and animal excreta (*3*). In this study, several parasites, including *Cryptosporidium* spp., *Enterocytozoon bieneusi*, and *Cyclospora cayetanensis*, were simultaneously detected in drinking water sources, MWTP sludge, and livestock manure. Animal breeding near the drinking water sources may be a potential source of contamination (*14–15*). For instance, waterborne transmission of *Enterocytozoon bieneusi* was reported during the 2013 "Pig Carcass Disposal Incident" in Shanghai, China, where dead pigs in the Huangpu River contributed to *Enterocytozoon bieneusi* contamination with animal and human genotypes, posing a threat to drinking water safety (*16*). The widespread existence of zoonotic *E. bieneusi* genotypes (D, EbpC, Type IV) in dogs and cats indicates that they are potential sources of environmental contamination and human infections (*17*). Thus, beyond *Cryptosporidium* and *Giardia*, which are already included in the National Standard for Drinking Water Quality of China (GB 5749-2022), monitoring the cross-media transmission of a broader range of protozoa and helminths is crucial for future public health management from a One Health perspective. Considering that RNA may reflect the viable pathogens in the samples, in the future, we will simultaneously quantify DNA and RNA from protozoa and helminths to improve the identification of viable pathogens (*11*).

This study provides a valuable tool for rapid screening and source tracking of waterborne protozoa and helminths. It is primarily used for high-throughput screening to determine the prevalence of a wide range of protozoa and helminths, characterize prevalence, and provide baseline data for identifying reference pathogens. It should be noted that the current HT-qPCR method does not allow for genotype identification of the detected pathogens. While suitable for routine monitoring, further research is needed to explore the potential for animal-environment-human transmission of waterborne pathogens.

Conflicts of interest: No conflicts of interest.

Funding: Supported by the National Key Research and Development Program of China (2022YFC3204703), and the China Postdoctoral Innovation Talents Support Program (BX20230400).

doi: 10.46234/ccdcw2025.016

[#] Corresponding author: Yu Zhang: zhangyu@rcees.ac.cn.

[1] State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China; [2] University of Chinese Academy of Sciences, Beijing, China; [3] College of Life Sciences, Henan Agricultural University, Zhengzhou City, Henan Province, China; [4] Henan Institutes of Advanced Technology, Zhengzhou University, Zhengzhou City, Henan Province, China.

Submitted: September 06, 2024
Accepted: November 12, 2024
Issued: January 17, 2025

REFERENCES

1. Ma JY, Li MY, Qi ZZ, Fu M, Sun TF, Elsheikha HM, et al. Waterborne protozoan outbreaks: an update on the global, regional, and national prevalence from 2017 to 2020 and sources of contamination. Sci Total Environ 2022;806:150562. https://doi.org/10.1016/j.scitotenv.2021.150562.
2. Plutzer J, Karanis P. Neglected waterborne parasitic protozoa and their detection in water. Water Res 2016;101:318 – 32. https://doi.org/10.1016/j.watres.2016.05.085.
3. WHO. Guidelines for drinking-water quality, 4th edition, incorporating the 1st addendum. Geneva: World Health Organization; 2017. https://www.who.int/publications/i/item/9789241549950.
4. National Institutes of Health, USA. NIAID Biodefense Research Agenda for Category B and C Priority Pathogens. https://www.hsdl.org/c/view?docid=441288. [2024-8-15].
5. National Health Commission of the People's Republic of China. Standards for Drinking Water Quality (GB 5749-2022). Mandatory national standards. https://www.ndcpa.gov.cn/jbkzzx/c100201/common/content/content_1665979083259711488.html. [2024-8-15].
6. European Union. Directive (EU) 2020/2184 of the European parliament and of the council of 16 December 2020 on the quality of water intended for human consumption. https://eur-lex.europa.eu/eli/dir/2020/2184/oj. [2024-8-15].
7. Stentiford GD, Feist SW, Stone DM, Bateman KS, Dunn AM. Microsporidia: diverse, dynamic, and emergent pathogens in aquatic systems. Trends Parasitol 2013;29(11):567 – 78. https://doi.org/10.1016/j.pt.2013.08.005.
8. Efstratiou A, Ongerth J, Karanis P. Evolution of monitoring for *Giardia* and *Cryptosporidium* in water. Water Res 2017;123:96 – 112. https://doi.org/10.1016/j.watres.2017.06.042.
9. Tuo JH, Shen Y, Jia SY, Liu SN, Zhang QF, Wang DP, et al. HPB-chip: an accurate high-throughput qPCR-based tool for rapidly profiling waterborne human pathogenic bacteria in the environment. Water Res 2024;260:121927. https://doi.org/10.1016/j.watres.2024.121927.
10. Xie ST, Ding LJ, Huang FY, Zhao Y, An XL, Su JQ, et al. VFG-Chip: a high-throughput qPCR microarray for profiling virulence factor genes from the environment. Environ Int 2023;172:107761. https://doi.org/10.1016/j.envint.2023.107761.
11. Brooks YM, Spirito CM, Bae JS, Hong AN, Mosier EM, Sausele DJ, et al. Fecal indicator bacteria, fecal source tracking markers, and pathogens detected in two Hudson river tributaries. Water Res 2020;171:115342. https://doi.org/10.1016/j.watres.2019.115342.
12. Lappan R, Jirapanjawat T, Williamson DA, Lange S, Chown SL, Greening C. Simultaneous detection of multiple pathogens with the TaqMan Array Card. MethodsX 2022;9:101707. https://doi.org/10.1016/j.mex.2022.101707.
13. Ahmed W, Zhang Q, Ishii S, Hamilton K, Haas C. Microfluidic quantification of multiple enteric and opportunistic bacterial pathogens in roof-harvested rainwater tank samples. Environ Monit Assess 2018;190(2):105. https://doi.org/10.1007/s10661-018-6482-0.
14. Galván AL, Magnet A, Izquierdo F, Fenoy S, Rueda C, Fernández Vadillo C, et al. Molecular characterization of human-pathogenic microsporidia and *Cyclospora cayetanensis* isolated from various water sources in Spain: a year-long longitudinal study. Appl Environ Microbiol 2013;79(2):449 – 59. https://doi.org/10.1128/AEM.02737-12.
15. Ben Ayed L, Yang WL, Widmer G, Cama V, Ortega Y, Xiao LH. Survey and genetic characterization of wastewater in Tunisia for *Cryptosporidium* spp. , Giardia duodenalis, Enterocytozoon bieneusi, Cyclospora cayetanensis and Eimeria spp. J Water Health 2012;10(3):431 – 44. https://doi.org/10.2166/wh.2012.204.
16. Hu Y, Feng YY, Huang CC, Xiao LH. Occurrence, source, and human infection potential of *Cryptosporidium* and *Enterocytozoon bieneusi* in drinking source water in Shanghai, China, during a pig carcass disposal incident. Environ Sci Technol 2014;48(24):14219 – 27. https://doi.org/10.1021/es504464t.
17. Wang HY, Lin XH, Sun YX, Qi NS, Lv MN, Xiao WW, et al. Occurrence, risk factors and genotypes of *Enterocytozoon bieneusi* in dogs and cats in Guangzhou, southern China: high genotype diversity and zoonotic concern. BMC Vet Res 2020;16(1):201. https://doi.org/10.1186/s12917-020-02421-4.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. Standard curves, correlation coefficients, amplification efficiencies for detection target of HT−qPCR.

Type	No.	Target	Standard curve	Correlation coefficient	Amplification efficiency (%)	Assay ID*
Protozoa	1	*Acanthamoeba*	y=−3.80x+41.12	R^2=0.997	83	AP7DU7W
	2	*Acanthamoeba culbertsoni*	y=−3.69x+41.58	R^2=0.993	87	APEPZZ9
	3	*Acanthamoeba castellanii*	y=−3.91x+41.68	R^2=0.996	80	APDJ7GC
	4	*Acanthamoeba polyphaga*	y=−3.81x+41.84	R^2=0.996	83	APAAJCZ
	5	*Balantidium coli*	y=−3.70x+40.84	R^2=0.995	86	AP2XA9F
	6	*Blastocystis hominis*	y=−3.68x+40.52	R^2=0.995	87	APGZFRJ
	7	*Cryptosporidium hominis*	y=−3.80x+41.12	R^2=0.998	83	APAAJDD
	8	*Cryptosporidium meleagridis*	y=−3.93x+41.66	R^2=0.998	80	AP9HTZD
	9	*Cryptosporidium* spp.	y=−3.72x+40.53	R^2=0.987	86	Pr07922253_s1
	10	*Cryptosporidium parvum*	y=−3.16x+37.60	R^2=0.985	107	APGZGDP
	11	*Cyclospora cayetanensis*	y=−3.47x+38.39	R^2=0.997	94	Pr07922173_s1
	12	*Encephalitozoon*	y=−3.67x+40.76	R^2=0.987	87	APDJ7HX
	13	*Entamoeba histolytica*	y=−3.69x+40.00	R^2=0.998	87	AP2XCMY
	14	*Enterocytozoon bieneusi*	y=−3.88x+41.16	R^2=0.999	81	AP7DWCR
	15	*Giardia* spp.	y=−3.74x+40.42	R^2=0.996	85	AP473C7
	16	*Giardia lamblia*	y=−3.21x+37.45	R^2=0.983	109	APZTK62/AP33ACW/ AP7DXGR
	17	*Isospora belli*	y=−3.64x+39.34	R^2=0.996	88	APNKRWX
	18	*Naegleria fowleri*	y=−3.85x+41.33	R^2=0.997	82	AP2XERK
	19	*Toxoplasma gondii*	y=−3.63x+39.23	R^2=0.998	88	APDJY3F
Helminths	20	*Dracunculus medinensis*	y=−3.94x+40.89	R^2=0.997	80	APPRVCD
	21	*Echinococcus*	y=−3.79x+40.48	R^2=0.994	84	APGZMAC
	22	*Schistosoma* spp.	y=−3.74x+38.16	R^2=0.995	85	APNKXJZ/APU69VP / APWC3FM/APXGWZJ
Quality control	23	16S rRNA gene	y=−3.20x+36.56	R^2=0.995	105	Ba04930791_s1
	24	18S rRNA gene	y=−3.71x+38.47	R^2=0.997	86	Pa99999901_s1

* The PCR primers and probes were designed by a ThermoFisher proprietary process. Primer and probe information are stored in the Odyssey data by ThermoFisher.

SUPPLEMENTARY TABLE S2. Concentration (mean value and range) of detection targets in different media.

Type	No.	Target	Drinking water sources		MWTP Sludge		Livestock manure	
			Mean value (gc/L)	Range (gc/L)	Mean value (gc/g)	Range (gc/g)	Mean value (gc/g)	Range (gc/g)
Protozoa	1	*Acanthamoeba genus*	7.5×10^{4}	nd−5.0×10^{5}	2.3×10^{5}	nd−7.2×10^{5}	6.7×10^{3}	nd−5.3×10^{4}
	2	*Acanthamoeba culbertsoni*	3.4×10^{3}	nd−8.8×10^{4}	4.8×10^{3}	nd−5.7×10^{4}	6.0×10^{2}	nd−6.0×10^{3}
	3	*Acanthamoeba castellanii*	2.4×10^{4}	nd−4.7×10^{5}	5.2×10^{3}	nd−6.2×10^{4}	2.2×10^{3}	nd−1.7×10^{4}
	4	*Acanthamoeba polyphaga*	2.6×10^{3}	nd−1.2×10^{5}	1.5×10^{4}	nd−1.1×10^{5}	nd	nd
	5	*Balantidium coli*	2.0×10^{3}	nd−9.0×10^{4}	7.4×10^{3}	nd−8.9×10^{4}	1.0×10^{5}	nd−9.9×10^{5}
	6	*Blastocystis hominis*	nd	nd	nd	nd	2.5×10^{3}	nd−2.5×10^{4}
	7	*Cryptosporidium* spp.	3.5×10^{3}	nd−4.5×10^{4}	1.9×10^{4}	nd−1.8×10^{5}	4.1×10^{2}	nd−4.1×10^{3}
	8	*Cryptosporidium meleagridis*	nd	nd	nd	nd	nd	nd
	9	*Cryptosporidium hominis*	nd	nd	nd	nd	nd	nd
	10	*Cryptosporidium parvum*	nd	nd	6.9×10^{3}	nd−8.3×10^{4}	nd	nd
	11	*Cyclospora cayetanensis*	4.3	nd−5.7×10^{4}	1.2×10^{5}	nd−1.0×10^{4}	4.9×10^{3}	nd−4.7×10^{4}
	12	*Isospora belli*	1.3×10^{3}	nd−6.0×10^{4}	2.3×10^{4}	nd−1.2×10^{5}	6.7×10^{3}	nd−6.7×10^{4}
	13	*Encephalitozoon*	9.6×10^{2}	nd−4.4×10^{4}	nd	nd	nd	nd
	14	*Entamoeba histolytica*	nd	nd	4.5×10^{3}	nd−5.4×10^{4}	nd	nd
	15	*Enterocytozoon bieneusi*	3.5×10^{3}	nd−6.5×10^{4}	9.0×10^{3}	nd−4.9×10^{4}	1.4×10^{3}	nd−1.0×10^{4}
	16	*Giardia* spp.	nd	nd	nd	nd	nd	nd
	17	*Giardia lamblia*	nd	nd	1.8×10^{4}	nd−1.8×10^{5}	nd	nd
	18	*Naegleria fowleri*	8.9×10^{3}	nd−1.5×10^{5}	nd	nd	nd	nd
	19	*Toxoplasma gondii*	nd	nd	nd	nd	6.7×10^{3}	nd−6.7×10^{4}
Helminths	20	*Echinococcus*	nd	nd	nd	nd	nd	nd
	21	*Dracunculus medinensis*	9.0×10^{2}	nd−4.1×10^{4}	4.6×10^{3}	nd−5.6×10^{4}	3.0×10^{3}	nd−3.0×10^{4}
	22	*Schistosoma* spp.	nd	nd	nd	nd	nd	nd

Abbreviation: MWTP=municipal wastewater treatment plant; nd=not detectable; gc=gene copies.

Outbreak Reports

Infection Tracing and Virus Genomic Analysis of Two Cases of Human Infection with Avian Influenza A(H5N6) — Fujian Province, China, April–May 2024

Yanhua Zhang[1]; Jingjing Wu[1]; Qi Lin[1]; Jianming Ou[1]; Xiaoqi Qi[1]; Youxian Zheng[2]; Fengping Li[2]; Yuwei Weng[1,#]

Summary

What is known about this topic?

Global human cases of zoonotic influenza A(H5N6) have increased significantly in recent years, primarily due to widespread circulation of clade 2.3.4.4b virus since 2020. Concurrent with this trend, sporadic human infections with clade 2.3.4.4h H5N6 avian influenza virus continue to occur. The high mortality rate associated with H5N6 virus infections has emerged as a critical public health concern.

What is added by this report?

Through comprehensive field epidemiological investigations and laboratory analyses, we identified the infection sources for these cases and conclusively ruled out human-to-human transmission. Genetic analyses revealed that while the virus maintains its avian host tropism, it has acquired mutations that may enhance human receptor binding affinity, viral replication capacity, pathogenicity, and neuraminidase inhibitor resistance.

What are the implications for public health practice?

The ongoing viral mutations increase the potential for H5 subtype avian influenza viruses to overcome species barriers and cause human epidemics. Enhanced surveillance strategies incorporating advanced technologies, such as metagenomic sequencing, are essential for early risk detection and management. Special attention should be directed toward cancer patients and immunocompromised individuals, who demonstrate increased susceptibility to avian influenza virus infections and require targeted prevention and control measures.

In recent years, avian influenza viruses, particularly those of the H5 subtype, have emerged as significant public health concerns. While these viruses primarily circulate in wild birds and domestic poultry with sporadic human infections, they possess pandemic potential. Although sustained human-to-human transmission of H5 subtypes has not been documented, the reporting of two human H5N6 avian influenza cases in Quanzhou City within a one-month period warranted immediate investigation as a potential infection cluster. This study employed integrated field epidemiological investigations and genetic analyses to trace the infection sources of the 2.3.4.4h clade H5 subtype avian influenza virus, aiming to identify potential risks and establish evidence-based prevention and control strategies.

INVESTIGATION AND RESULTS

Tracing the Source of Infection

From April to May 2024, two cases of human H5N6 avian influenza infection were reported in Quanzhou City, Fujian Province, China. Following case identification, local health authorities and CDC teams immediately initiated comprehensive epidemiological investigations, laboratory analyses, and control measures.

Case A, a previously healthy 52-year-old female from Quangang District, developed symptoms following exposure to heavy cold rain on April 13, 2024. Laboratory sampling on April 21 yielded H5N6-positive results on April 23, and the patient succumbed to the infection on April 30. The patient worked at a salt field within 2 kilometers of her residence. Family members reported that she had remained within Quangang District with no travel history in the 10 days preceding symptom onset. She maintained a small poultry flock in a shed 50 meters from her home, where several poultry had recently fallen ill. Eight days before symptom onset, she had slaughtered and consumed these diseased poultry. Following symptom onset, she sought medical attention sequentially at a village clinic, township health center, and hospital.

Case B, a 40-year-old male from Luojiang District with nasopharyngeal cancer and pulmonary metastases,

developed symptoms on May 8, 2024. He tested positive on May 14 and died that evening. The patient, who primarily convalesced at home due to his cancer condition, resided with his parents who maintained a poultry shed west of their residence. While he had no direct contact with poultry or live poultry markets, his village featured widespread household poultry keeping, with reports of poultry illness and mortality approximately one month prior. His only potential exposure occurred during occasional walks along village paths adjacent to these poultry sheds.

The residences of Cases A and B were separated by approximately 27 kilometers. Although both patients received treatment at the same hospital, no epidemiological links were identified during their hospital stays, and no other direct or indirect connections were established. The spatio-temporal relationships between the two cases are illustrated in Figure 1.

During the outbreak response, surveillance identified 21 close contacts of Case A and 6 close contacts of Case B. All contacts underwent 10 days of medical observation with negative nucleic acid test results before release, confirming the absence of secondary infections.

Environmental sampling was conducted to identify potential infection sources for both cases. Following a risk-based sampling strategy and site disinfection protocols, we initially collected samples from the patients' outdoor poultry sheds and indoor environments, followed by sampling of neighboring sheds and nearby live poultry markets. For Case A, all 84 collected samples tested negative. For Case B, after initial negative results from 15 environmental samples, we expanded sampling to include the patient's poultry shed (10 samples), neighboring poultry sheds (110 samples), and local live poultry markets (92 samples). While the patient's shed remained negative, positive results were detected in neighboring sheds (2.73%, 3/110) and live poultry markets (5.43%, 5/92). Detailed positive sample information is presented in Supplementary Table S1 (available at https://weekly.chinacdc.cn/).

Nucleic acid-positive samples underwent next-generation sequencing (NGS) using the MiniSeq^{TM} DX-CN platform (Illumina, San Diego, USA). Raw data processing and assembly were performed using CLC Genomics Workbench 23.0 (Qiagen, Hilden, Germany). High-quality genomic sequences were obtained from two case samples and six of eight environmental samples, with A/Environment/Fujian/02-Env04/2024(H5N6) and A/Environment/Fujian/02-Env06/2024(H5N6) yielding insufficient quality. The virus strains were designated as A/Fujian/01/2024(H5N6) (FJ01) from Case A and A/Fujian/02/2024(H5N6) (FJ02) from Case B. Pairwise nucleotide sequence identity analysis was conducted using MegAlign software (version 7.1.0, DNASTAR, Inc., USA), with results presented in Table 1.

Sequence analysis revealed partial genetic divergence between Cases A and B, with 100% identity observed only in the matrix protein (MP) and nonstructural protein (NS) segments. Notably, Case B's viral sequences showed complete identity with those from neighboring poultry shed Z but differed from samples collected from other nearby sheds and markets. Significantly, shed Z was positioned on an elevated terrain approximately 50 meters from the patient's residence and 8 meters from his regular walking path.

Genomic Analysis of Viruses

Comparative genomic analysis was performed on the eight segments from the FJ01 and FJ02 strains using the Basic Local Alignment Search Tool (BLAST) against both the National Center for Biotechnology Information (NCBI, USA) and Global Initiative on Sharing All Influenza Data (GISAID) databases. The analysis revealed that the PB2, PB1, PA, and HA segments of both strains showed highest sequence homology with A/Guangdong/lgf/2021(H5N6), A/duck/Hunan/S40199/2021(H5N6), A/Neogale vison/China/FD/NV/SD/L4/2021(H5N6), and A/Rattus norvegicus/China/FS21/2021, respectively. The remaining segments (NP, NA, MP, and NS) demonstrated highest similarity to A/Guangdong/1/2021(H5N6). Detailed BLAST results are presented in Supplementary Table S2 (available at https://weekly.chinacdc.cn/).

Sequence alignment was conducted on the hemagglutinin (HA) gene segments of the FJ01 and FJ02 strains using MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/). The phylogenetic analysis was performed using MEGA software (version 7.0.26, Pennsylvania State University, USA), employing the Maximum Likelihood method with the Tamura-Nei model and 1,000 bootstrap replicates to ensure statistical robustness. Reference sequences were obtained from the GISAID database. Both HA genes were definitively classified within clade 2.3.4.4h, as illustrated in Supplementary Figure S1 (available at https://weekly.chinacdc.cn/).

Comprehensive molecular characterization of the

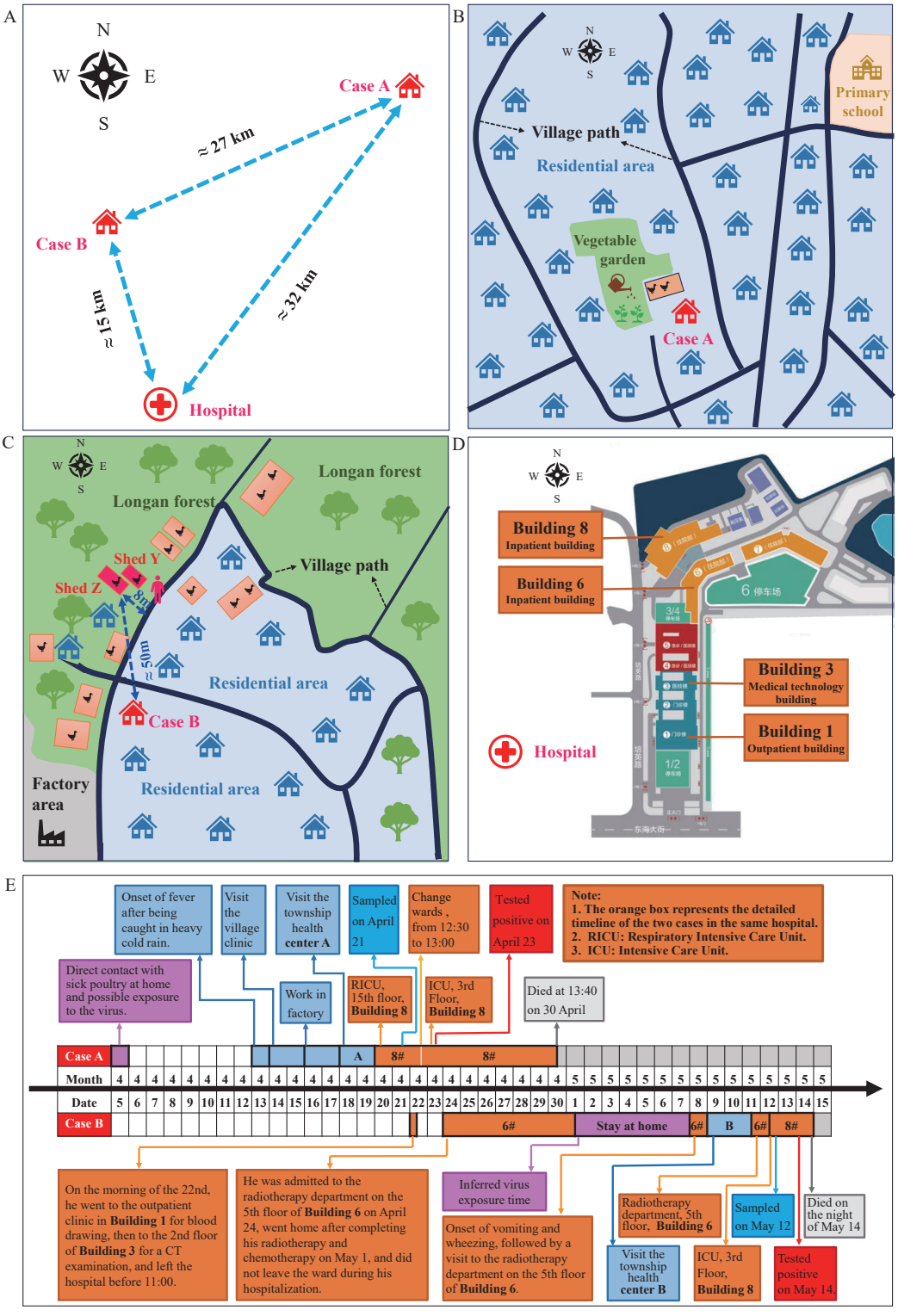


FIGURE 1. The spatio-temporal relationship analysis of the two cases. (A) Spatial distribution of cases and hospital locations; (B) Case A's residence (red house) and associated poultry shed (light orange box); (C) Case B's residence (red house) and sampled surrounding poultry sheds, with virus-positive sheds indicated in red; (D) Hospital layout diagram showing areas where both Case A and Case B received treatment; (E) Post-onset activity timeline for both cases, with the orange box highlighting their concurrent hospital stays.
Note: For (C) The patient's family poultry shed was situated west of the residence.

TABLE 1. Nucleotide sequence identity matrix comparing case samples and environmental isolates.

Virus strains	The percent identity between FJ02 and other strains							
	PB2	PB1	PA	HA	NP	NA	MP	NS
FJ01	99.92	99.91	99.91	99.76	99.93	99.71	100.00	100.00
A/Environment/Fujian/02-Env01/2024(H5N6)	99.98	100.00	99.95	100.00	99.93	99.93	100.00	99.88
A/Environment/Fujian/02-Env02/2024(H5N6)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
A/Environment/Fujian/02-Env03/2024(H5N6)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
A/Environment/Fujian/02-Env04/2024(H5N6)	94.26	86.60	85.85	–	89.44	86.04	96.04	89.64
A/Environment/Fujian/02-Env05/2024(H5N6)	99.89	99.87	89.68	99.82	100.00	99.85	99.96	99.88
A/Environment/Fujian/02-Env06/2024(H5N6)	96.12	94.99	99.91	99.17	94.77	98.01	97.55	95.29
A/Environment/Fujian/02-Env07/2024(H5N6)	99.38	98.75	98.68	99.29	98.92	97.86	99.49	98.30
A/Environment/Fujian/02-Env08/2024(H5N6)	99.41	98.80	98.73	99.29	99.06	98.01	99.49	98.30

Note: "–" means that the sequence quality is too low (less than 50% coverage) to be analyzed.

FJ01 and FJ02 strains was conducted through sequence analysis. The HA and NA protein coding followed H3N2 numbering conventions. Complete results are presented in Table 2.

PUBLIC HEALTH RESPONSE

Local health authorities and CDC teams implemented comprehensive public health measures in response to the outbreak, including: 1) enhanced 2-week surveillance of influenza-like illnesses (ILI) in outpatient settings and severe acute respiratory infections (SARI) in hospitalized patients, with pre-screening and triage protocols to prevent nosocomial transmission; 2) emergency 2-week citywide environmental monitoring of poultry-related environments; 3) immediate disinfection of outbreak sites with subsequent effectiveness evaluation; 4) coordinated prevention efforts with the Agriculture Department for at-risk poultry culling and free-range poultry immunization enhancement, and collaboration with the Market Regulation Authority to implement "three ones" measures in live poultry markets (daily cleaning, weekly disinfection, and monthly closure); and 5) strategic risk communication, health education, and public information dissemination.

The implemented control measures proved effective, with no new infections reported and all close contacts remaining uninfected as of May 29, leading to the formal declaration of the outbreak's conclusion.

DISCUSSION

Comprehensive analysis of epidemiological and laboratory data demonstrated that cases A and B were independent events with no epidemiological links. Medical observation of all close contacts revealed no signs of infection, providing strong evidence against human-to-human transmission in both cases.

Environmental investigation plays a crucial role in tracing avian influenza virus infections. Although both cases were associated with poultry illness or mortality prior to symptom onset, initial environmental sampling from the patients' poultry sheds yielded negative results. This unexpected outcome can be attributed to two factors: the rapid disposal of diseased poultry before patient illness onset, which eliminated environmental viral evidence, and the implementation of thorough disinfection measures by families or health authorities prior to sampling. Nevertheless, viral genetic sequencing analysis of samples from surrounding poultry sheds near Case B provided definitive evidence for outbreak source identification.

The transmission routes differed between the two cases. Case A's infection likely resulted from direct contact with diseased poultry, while Case B, who had no direct poultry exposure, suggested an alternative transmission pathway. Genetic analysis revealed 100% nucleotide sequence identity between Case B's virus and samples from poultry shed Z, while distinct genetic differences were observed in viruses from shed Y and local live poultry markets. This evidence strongly indicates shed Z as Case B's infection source. Although the viruses from both cases showed high sequence similarity, they were not identical, ruling out direct transmission between cases. The absence of infection among close contacts further confirms the lack of human-to-human transmission.

The location of poultry shed Z, approximately 8 meters from Case B's regular walking route, suggests

TABLE 2. Key molecular markers associated with influenza virus pathogenicity.

Genes	Molecular markers	FJ01	FJ02	Phenotypic effect
HA	Cleavage site*	RERRRKR↓GLF		Shows multiple basic amino acids and high pathogenicity to chickens.
	S127P (*1*)	S	S	Increases virus binding to α2–6 sialic acids (α2–6 SA).
	S137A (*1*)	**A**	**A**	**Increases virus binding to α2–6 SA.**
	G143R*	G	G	Increases virus binding to α2–6 SA.
	I155T (*2*)	**T**	**T**	**Increases virus binding to α2–6 SA.**
	N158D*	N	N	Increases virus binding to α2–6 SA, and increases airborne transmissibility.
	T160A*	**A**	**A**	**Increases virus binding to both α2,3-SA and α2,6-SA, and increase airborne transmissibility.**
	V214I*	V	V	Increases virus binding to α2–6 SA.
	N224K*	N	N	Increases virus binding to α2–6 SA, and increases airborne transmissibility.
	Q226L*	Q	Q	Increases virus binding to α2–6 SA.
	S227N*	R	S	Increases virus binding to α2–6 SA.
	G228S*	G	G	Increases virus binding to α2–6 SA.
NA	**58-68 Delete** (*3*)	**Delete**	**Delete**	**Associated with increased viral virulence in mammals.**
	E119V (*4*)	E	E	Shows different degrees of neuraminidase inhibitor resistance.
	D198N (*5*)	**N**	**N**	**Shows neuraminidase inhibitor resistance.**
	H274Y**	H	H	Shows neuraminidase inhibitor resistance.
PB2	**K389R** (*6*)	**R**	**R**	**Enhances growth capacity in human and mammalian cells.**
	Q591K**	Q	Q	Increases pathogenicity in mice.
	E627K**	E	E	Associated with mammalian adaptation, increases virus replication in mammalian cells, increases virulence in mice, increases transmissibility in ferrets, determinant of cold sensitivity.
	D701N**	D	D	Associated with mammalian adaptation, increases virus replication in mammalian cells, improves binding of PB2 to importin protein in mammalian cells.
PB1	**D3V***	**V**	**V**	**Enhances viral polymerase activity.**
	H99Y*	H	H	Increases viral polymerase activity, and associated with mammalian adaptation.
	M317I***	M	M	Increased pathogenicity in mice.
	L473V***	**V**	**V**	**Maintains efficient viral replication.**
	D622G*	**G**	**G**	**Enhances viral polymerase activity and virulence in mice.**
PA	I38T/L (*7*)	I	I	Reduces the susceptibility of polymerase acidic (PA) endonuclease inhibitor.
	T97I***	T	T	Associated with mammalian adaptation.
	K142Q***	K	K	Enhances replication and pathogenesis in mice when combined with PB2 627K, associated with mammalian adaptation.
	K615R***	**R**	**R**	**Increases viral virulence in mammals when combined with NP N319K.**
NS1	**P42S***	**S**	**S**	**Increases viral virulence in mice by preventing the dsRNA-mediated activation of the NF-κB and IRF − 3 pathways.**
	P212S**	P	P	Promotes viral replication in mice.
	D92E**	D	D	Increased virulence and/or cytokine resistance.
NP	N319K***	N	N	Increases viral virulence in mammals when combined with PA K615R.
M2	L26F**	L	L	Shows antiviral amantadine resistance.
	V27A**	V	V	Shows antiviral amantadine resistance.
	A30T**	A	A	Shows antiviral amantadine resistance.
	S31N**	S	S	Shows antiviral amantadine resistance.
	G34E**	G	G	Shows antiviral amantadine resistance.

Note: HA and NA mutation sites follow H3N2 numbering conventions. Bold text indicates that the mutations have occurred in the viruses reported in this study.
Abbreviation: HA=Hemagglutinin; NA=neuraminidase.
* Data derived from reference (*8*);
** Data derived from reference (*9*);
*** Data derived from reference (*10*).

potential aerosol transmission over this distance. Notably, both cases exhibited compromised immune status: Case A experienced heavy rain exposure before illness onset, while Case B had nasopharyngeal cancer with ongoing chemotherapy and radiotherapy. The absence of symptoms among family members despite potential viral exposure suggests that immunocompromised status may represent a significant risk factor for H5N6 avian influenza virus infection in humans.

While genomic analysis revealed no significant recombination compared to recent H5N6 viruses, several concerning features were identified, including a highly pathogenic HA protein cleavage site and mutations associated with increased virulence, human infection potential, enhanced viral replication, and possible neuraminidase inhibitor resistance. The ongoing H5N1 outbreak in mammals in the United States heightens concerns about potential cross-species transmission and human epidemic risk. Although sustained human-to-human transmission of H5 subtype viruses remains undocumented, these findings underscore the critical importance of enhanced surveillance and robust pandemic preparedness measures.

Conflicts of interest: No conflicts of interest.

Acknowledgments: The staff from the Quanzhou Municipal Center for Disease Control and Prevention, the Second Affiliated Hospital of Fujian Medical University, Quangang District Center for Disease Control and Prevention, and Luojiang District Center for Disease Control and Prevention.

Funding: Supported by the Fujian Natural Science Foundation (2021J01351) and the Major Scientific Research Program for Young and Middle-aged Health Professionals of Fujian Province, China (Grant No.2021ZQNZD006).

doi: 10.46234/ccdcw2024.274

[#] Corresponding author: Yuwei Weng, wengyuwei@fjcdc.com.cn.

[1] Fujian Provincial Center for Disease Control and Prevention, Fujian Provincial Key Laboratory of Zoonosis Research, Fuzhou City, Fujian Province, China; [2] Quanzhou Municipal Center for Disease Control and Prevention, Quanzhou City, Fujian Province, China.

Submitted: September 24, 2024
Accepted: November 25, 2024
Issued: January 17, 2025

REFERENCES

1. Cao L, Liu YH, Li KB, Lu JY, Lu EJ, Chen YY, et al. Epidemiological and genetic characteristics of H5 subtype avian influenza virus in Guangzhou, 2014-2019. Chin J Epidemiol 2020;41(7):1115 – 20. https://doi.org/10.3760/cma.j.cn112338-20190730-00565.
2. He DL, Wang FF, Zhao LM, Jiang XN, Zhang S, Wei F, et al. Epidemiological investigation of infectious diseases in geese on mainland China during 2018-2021. Transbound Emerg Dis 2022;69 (6):3419 – 32. https://doi.org/10.1111/tbed.14699.
3. He ZL, Wang X, Lin Y, Feng SY, Huang XY, Zhao LX, et al. Genetic characteristics of waterfowl-origin H5N6 highly pathogenic avian influenza viruses and their pathogenesis in ducks and chickens. Front Microbiol 2023;14:1211355. https://doi.org/10.3389/fmicb.2023.1211355.
4. Bialy D, Shelton H. Functional neuraminidase inhibitor resistance motifs in avian influenza A(H5Nx) viruses. Antiviral Res 2020;182: 104886. https://doi.org/10.1016/j.antiviral.2020.104886.
5. Hossain MG, Akter S, Dhole P, Saha S, Kazi T, Majbauddin A, et al. Analysis of the genetic diversity associated with the drug resistance and pathogenicity of influenza a virus isolated in Bangladesh from 2002 to 2019. Front Microbiol 2021;12:735305. https://doi.org/10.3389/fmicb.2021.735305.
6. Bi FY, Jiang LL, Huang LH, Wei JG, Pan XW, Ju Y, et al. Genetic characterization of two human cases infected with the avian influenza a (H5N6) viruses - Guangxi Zhuang autonomous region, China, 2021. China CDC Wkly 2021;3(44):923 – 8. https://doi.org/10.46234/ccdcw2021.199.
7. Hickerson BT, Petrovskaya SN, Dickensheets H, Donnelly RP, Ince WL, Ilyushina NA. Impact of Baloxavir resistance-associated substitutions on influenza virus growth and drug susceptibility. J Virol 2023;97(7):e00154 – 23. https://doi.org/10.1128/jvi.00154-23.
8. Liang YY. Pathogenicity and virulence of influenza. Virulence 2023;14 (1):2223057. https://doi.org/10.1080/21505594.2023.2223057.
9. Xiao CK, Xu JN, Lan Y, Huang ZP, Zhou LJ, Guo YX, et al. Five independent cases of human infection with avian influenza H5N6 - Sichuan Province, China, 2021. China CDC Wkly 2021;3(36):751 – 6. https://doi.org/10.46234/ccdcw2021.187.
10. Griffin EF, Tompkins SM. Fitness determinants of influenza a viruses. Viruses 2023;15(9):1959. https://doi.org/10.3390/v15091959.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. Information on nucleic acid-positive samples and corresponding virus strain nomenclature.

Virus strains	GISAID Accession No.	Sample source	Sample type
A/Fujian/01/2024(H5N6)	EPI_ISL_19132453	Case A	Bronchoalveolar lavage fluid
A/Fujian/02/2024(H5N6)	EPI_ISL_19145644	Case B	Oropharyngeal
A/Environment/Fujian/02-Env01/2024(H5N6)	/	Shed Y	Poultry drinking water
A/Environment/Fujian/02-Env02/2024(H5N6)	/	Shed Z	Poultry drinking water
A/Environment/Fujian/02-Env03/2024(H5N6)	/	Shed Z	Poultry drinking water
A/Environment/Fujian/02-Env04/2024(H5N6)	/	LPM	Poultry drinking water
A/Environment/Fujian/02-Env05/2024(H5N6)	/	LPM	Poultry drinking water
A/Environment/Fujian/02-Env06/2024(H5N6)	/	LPM	Poultry drinking water
A/Environment/Fujian/02-Env07/2024(H5N6)	/	LPM	Poultry drinking water
A/Environment/Fujian/02-Env08/2024(H5N6)	/	LPM	Cutting board swab

Note: Shed Y was located approximately 50 meters from Case B's residence, with Shed Z situated adjacent to Shed Y. LPM refers to live poultry markets in Case B's vicinity.

SUPPLEMENTARY TABLE S2. Similarity analysis of viral genome segments using BLAST.

Virus	Segment and length (bp)	Strain with the highest similarity (accession ID, percent identity)	
		NCBI database	GISAID database
FJ01	PB2:2250	A/Guangdong/lgf/2021(H5N6) (OL519558.1, 98.68%)	A/Guangdong/lgf/2021 (H5N6) (EPI2255274, 98.68%)
FJ01	PB1:2239	A/duck/Guangxi/293D21/2017(H1N2) (MH667662.1, 96.44%)	A/duck/Hunan/S40199/2021(H5N6) (H5N6) (EPI1997200, 98.42%)
FJ01	PA: 2131	A/Guangdong/1/2021(H5N6) (OK284448.1, 99.02%)	A/Neogale vison/ China/FD/NV/SD/L4/2021 (H5N6) (EPI3351954, 99.07%)
FJ01	HA: 1725	A/Rattus norvegicus/China/FS21/2021 (H5N6) (OQ291577.1, 97.24%)	A/Rattus norvegicus/China/FS21/2021 (H5N6) (EPI2394816, 97.24%)
FJ01	NP: 1551	A/Guangdong/1/2021(H5N6) (OK284449.1, 99.04%)	A/Guangdong/1/2021 (H5N6) (EPI2200687, 99.04%)
FJ01	NA: 1398	A/Guangdong/1/2021(H5N6) (OK284452.1, 98.04%)	A/Guangdong/1/2021 (H5N6) (EPI2200690, 98.04%)
FJ01	MP: 1021	A/Guangdong/1/2021(H5N6) (OK284450.1, 99.42%)	A/Bar-headed Goose/Tibet/XZQ17-1/2021 (H5N8) (EPI1949225, 99.42%)
FJ01	NS: 872	A/Guangdong/1/2021(H5N6) (OK284453.1,97.98%)	A/Guangdong/1/2021 (H5N6) (EPI2200691, 97.98%)
FJ02	PB2: 2247	A/Guangdong/lgf/2021(H5N6) (OL519558.1, 98.55%)	A/Guangdong/lgf/2021 (H5N6) (EPI2255274, 98.55%)
FJ02	PB1: 2239	A/duck/Guangxi/293D21/2017(H1N2) (MH667662.1, 96.44%)	A/duck/Hunan/S40199/2021(H5N6) (EPI1997200, 98.42%)
FJ02	PA: 2133	A/Guangdong/1/2021(H5N6) (OK284448.1, 99.12%)	A/Neogale vison/China/FD/NV/SD/L4/2021 (H5N6) (EPI3351954, 99.16%)
FJ02	HA: 1725	A/Rattus norvegicus/China/FS21/2021(H5N6) (OQ291577.1, 97.24%)	A/Rattus norvegicus/China/FS21/2021 (H5N6) (EPI2394816, 97.24%)
FJ02	NP: 1552	A/Guangdong/1/2021(H5N6) (OK284449.1, 99.11%)	A/Guangdong/1/2021 (H5N6) (EPI2200687, 99.11%)
FJ02	NA: 1400	A/Guangdong/1/2021(H5N6) (OK284452.1, 98.18%)	A/Guangdong/1/2021 (H5N6) (EPI2200690, 98.18%)
FJ02	MP: 1021	A/Guangdong/1/2021(H5N6) (OK284450.1, 99.42%)	A/Bar-headed Goose/Tibet/XZQ17-1/2021 (H5N8) (EPI1949225, 99.42%)
FJ02	NS: 872	A/Guangdong/1/2021(H5N6 (OK284453.1, 97.98%)	A/Guangdong/1/2021 (H5N6) (EPI2200691, 97.98%)

Abbreviation: BLAST=basic local alignment search tool; NCBI=national center for biotechnology information (USA); GISAID=global initiative on sharing all influenza data.

SUPPLEMENTARY FIGURE S1. Phylogenetic analysis of H5 virus HA genes using the maximum likelihood method.
Note: The phylogenetic tree encompasses major H5 clades (indicated at terminal nodes) and is rooted in the reference strain A/Goose/Guangdong/1/1996 (H5N1). The tree was constructed using 1,000 bootstrap replicates, with values shown above branches. Viral strains from this study are denoted by red spots (case A), red triangles (case B), and black triangles (environmental isolates).
Abbreviation: HA=hemagglutinin.

Youth Editorial Board

Indexed by Science Citation Index Expanded (SCIE), Social Sciences Citation Index (SSCI), PubMed Central (PMC), Scopus, Chinese Scientific and Technical Papers and Citations, and Chinese Science Citation Database (CSCD)

The inauguration of *China CDC Weekly* is in part supported by Project for Enhancing International Impact of China STM Journals Category D (PIIJ2-D-04-(2018)) of China Association for Science and Technology (CAST).

CCDC weekly

Vol. 7 No. 3 Jan. 17, 2025

Responsible Authority
National Disease Control and Prevention Administration

Sponsor
Chinese Center for Disease Control and Prevention

Editing and Publishing
China CDC Weekly Editorial Office
No.155 Changbai Road, Changping District, Beijing, China
Tel: 86-10-63150501, 63150701
Email: weekly@chinacdc.cn

CSSN
ISSN 2096-7071 (Print)
ISSN 2096-3101 (Online)
CN 10-1629/R1