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

CHINA CDC WEEKLY

Vol. 7 No. 12 Mar. 21, 2025

中国疾病预防控制中心周报

ISSN 2096-7071

China CDC Weekly

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Perspectives

Climate Change, Cryosphere Retreat, and Human Health

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ABSTRACT

The cryosphere, encompassing glaciers, ice sheets, permafrost, and snow, plays a critical role in regulating climate and sustaining human wellbeing. However, climate change is driving widespread cryosphere degradation, intensifying geophysical and climate-related hazards that pose escalating risks to the public health and safety. The resulting decline in both the quantity and quality of cryosphere services also has severe consequences, particularly for populations in polar regions, high-altitude mountains, and their downstream areas. Furthermore, teleconnected climate systems can even extend cryosphere change impacts beyond these regions. It has been seen that increasing cryosphere-related hazards, such as glacial lake outburst floods and extreme winter events, heighten public health risks. Disrupted meltwater supply and ecosystem shifts inflict water and food insecurity in arid and semiarid regions, exacerbating malnutrition and disease burdens. Additionally, thawing permafrost may release ancient pathogens and toxic substances, increasing the risks of infectious disease outbreaks and severe environmental contamination. Addressing these cascading risks requires urgent interdisciplinary research, public awareness, and investment in adaptive strategies to strengthen societal resilience amid a rapidly changing cryosphere and safeguard public well-being.

Climate change induces far-reaching cascading effects on ecosystems, economies, and human health. Among the most vulnerable components of the Earth system is the cryosphere, where water is frozen, encompassing glaciers, snow cover, permafrost, ice sheets, sea ice, lake ice, and other frozen formations. The cryosphere typically exhibits seasonal variations, accumulating and retreating in recurring patterns following freeze-thaw cycles over years. Climate-driven retreats in the cryosphere can generate both adverse and beneficial cascading effects on environments and societies (*1*), with profound implications for public health, as illustrated in Figure 1. However, our understanding of how cryosphere changes impact public health remains insufficient, hindering the development of effective risk mitigation policies. We aim to bridge this knowledge gap by raising awareness and fostering new perspectives to strengthen public health risk mitigation strategies in a rapidly changing cryosphere.

The cryosphere generates natural hazards that can severely impact human societies. For example, glacier lake outburst flooding (GLOF) (*2–3*), caused by deterioration and eventual breach of moraine- or ice-dammed glacial lakes, can lead to catastrophic damage to downstream communities. Global warming exacerbates not only geohazards but also climate-related hazards associated with cryosphere changes, threatening human lives. The increasing activity of cold fronts moving from high latitudes to mid-latitudes — resulting from a weakened polar vortex associated with Arctic warming (*4*) — can trigger severe winters and snowstorms in mid-latitude regions (*5–6*), leading to elevated mortality and morbidity risks.

The cryosphere also provides essential services that benefit societies, collectively known as cryosphere services (*1*). The cryosphere functions as a "reservoir of solid water" that serves as a critical source of freshwater; its freeze-thaw dynamics play vital roles in regulating climate and hydrological cycles; and its cold environments support essential ecosystems in high-latitude and high-altitude regions. Additionally, the cryosphere embodies significant spiritual, heritage, and recreational values. However, global warming is profoundly altering both the quantity and quality of cryosphere services, consequently impacting the wellbeing of human societies.

The intensification of cryosphere hazards and deterioration of cryosphere services resulting from climate change can produce substantial cascading effects on human health (*7*), particularly for populations closely connected with the cryosphere in polar and high mountain regions, as well as in semiarid

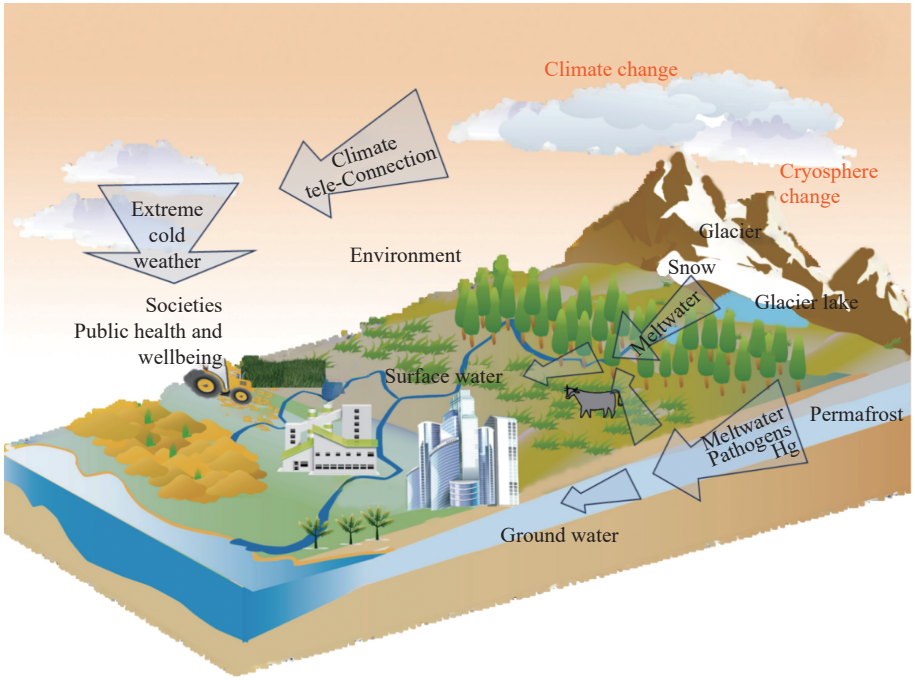

FIGURE 1. Cascading effects of cryosphere change due to global warming on environment and societies in terms of public health and wellbeing.

or arid downstream areas (*8*). The extreme weather and climate events in winter, influenced by teleconnected atmospheric circulation systems, extend cryosphere change impacts to regions even beyond these directly affected areas (*5*–*6*).

Cryosphere Change

Rapid climate change is driving unprecedented transformations in the cryosphere. Globally, glaciers have lost 273±16 gigatonnes (Gt) of mass annually from 2000 to 2023 (*9*–*10*). In High Mountain Asia, including Central and Northern Tien Shan as well as Eastern Pamir, glacier mass loss rates have progressively accelerated since the 1960s (*11*). While rising temperatures initially enhance glacier meltwater supply, this trend reverses once glacier mass diminishes beyond a critical threshold, ultimately leading to long-term freshwater availability decline.

Concurrently, snow cover has decreased in the Northern Hemisphere spring since 1950, particularly in North America, which exhibits a significant declining trend of 46 Gt per decade in March snow mass (*12*). In the European Alps, snow depth has declined at 8.4% per decade, accompanied by snow cover reduction at 5.6% per decade during 1971–2019, resulting in snow cover duration 36 days shorter than the six-century long-term mean (*13*). The warming climate not only reduces snow cover extent but also advances the timing of snowmelt (*14*). These changes subsequently diminish snowmelt water availability and alter annual snowmelt dynamics (*15*), affecting various ecological (*16*) and agricultural irrigation requirements (*17*).

Simultaneously, permafrost temperatures have increased globally by approximately 0.29 °C during the period from 2007–2016 (*18*). In the high-latitude Arctic, warming rates can reach up to 1°C per decade (*19*). Warming permafrost accelerates the release of soil organic carbon and nutrients, along with various toxic substances. Under high emission scenarios, projected mercury (Hg) release from thawing permafrost is expected to elevate Hg concentrations in Yukon River fish beyond the Environmental Protection Agency (EPA) guidelines by 2050 (*20*).

The Arctic continues to warm at more than twice the global rate (*21*). Compounded by declining sea ice and Arctic warming, the Northern Hemisphere has increasingly experienced extreme cold weather events (*5*,*22*), associated with a stratospheric polar vortex that has weakened over the past three decades, driving cold polar air masses toward mid-latitude regions (*6*). It is reasonable to conclude that the increasingly warmer Arctic and diminishing sea ice could further exacerbate stratospheric polar vortex variability, subsequently increasing the likelihood of extreme winter climate events in mid-latitude regions.

Impacts on Public Health

Growing impacts of cryosphere hazards under warming climate – increasing risks to public health: Evidence demonstrates that populations worldwide face unprecedented threats to their wellbeing, health, and survival from rapidly changing climate conditions and the subsequent increase in frequency and intensity of hazards, including floods, heatwaves, fires, and storms (*23*). Between 1961–1990 and 2014–2023, 61% of global land area experienced an increase in days with extreme precipitation, consequently elevating risks of flooding, infectious disease transmission, and water contamination (*24*). A comprehensive epidemiological analysis revealed that mortality risks increased and persisted for up to 60 days following flood events, with all-cause, cardiovascular, and respiratory mortality increasing by 2.1%, 2.6%, and 4.9%, respectively (*25*).

The changing cryosphere is generating increasingly severe hazards, such as GLOF and intensified snowmelt flooding during spring. In 2021, a catastrophic debris flow triggered by an avalanche involving approximately 27×10^6 m^3 of rock and ice resulted in devastating consequences across Himalayan regions of India (*26–28*). Extreme winter weather conditions, including snow storms induced by shifting stratospheric polar vortex patterns, affected 8.685 million people in January 2018 in China (*29*). During 2000–2019, cold spells contributed to 205,000 annual excess deaths globally, with Europe experiencing the highest mortality burden (*30*). More recently, in January 2025, historic snow storms swept across North America and Europe, triggering record-breaking low temperatures and hundreds of traffic accidents. These increasing cryospheric hazards substantially elevate risks of injury, morbidity, and mortality, comparable to the adverse impacts of other climate and hydroclimatic hazards previously described.

Revival of ancient pathogens and release of toxic substance from thawing permafrost – emerging risks to public health: Permafrost harbors diverse microbial communities (*31*). Thawing permafrost can expose preserved remains of infectious disease victims (both human and animal) (*32*). If viral pathogens maintain infectivity in permafrost, thawing could potentially trigger disease outbreaks (*33*) through three distinct pathways (*34*). The first involves reintroduction of human viral pathogens, such as influenza and smallpox, from thawing graves and mass burial sites. The second concerns reintroduction of viruses from thawed wildlife or domestic animal remains in permafrost, which could transmit directly to humans or infect animals before jumping to humans. The third pathway involves spillover of permafrost viruses with microbial hosts, including microeukaryotes, bacteria, and archaea, to humans or animals following permafrost thaw. Evidence suggests pathogenic microorganisms can emerge from thawing permafrost and cause disease. For example, in July-August 2016, a massive anthrax outbreak in reindeer (*Rangifer tarandus*) occurred on the Yamal Peninsula, Northwest Siberia in Russia, resulting in one human fatality, dozens of illnesses, and thousands of reindeer deaths (*35–36*). In 1991, Russian experts discovered a wooden vault containing frozen victims of a nineteenth-century smallpox epidemic in a small village near the North Pole (*37*). This discovery raised concerns about the possible resurgence of smallpox following permafrost thaw (*38*).

Beyond the revival of ancient pathogens, thawing permafrost can release toxic substances (i.e., Hg) (*20*) and legacy hazardous industrial wastes (*39*) trapped in ice, contaminating soil and water supplies and impacting food webs. The Arctic contains a substantial reservoir of Hg, with approximately 597,000 Mg accumulated in the 0–3 m depth of permafrost soils (*40*). Exposure to Hg poses significant adverse health effects across life stages, including neurodevelopmental impairment in children and cardiovascular diseases in adults (*41*).

Increasing cryosphere hazards and relinquished cryosphere services – threats to people's wellbeing: Exposure to extreme weather and climate events significantly compounds mental health burdens, including depression, anxiety, and post-traumatic stress disorder (*42*). These psychological impacts manifest through direct pathways, such as infrastructure loss, displacement, and violence, as well as indirect mechanisms, including landscape degradation, protracted economic recovery, and perceived environmental instability (*43*).

The diminishing provisioning services of the cryosphere constrain meltwater supply, adversely affecting freshwater availability and quality for communities and economies. In high-altitude regions such as the Qinghai-Tibet Plateau (*44*), insufficient irrigation supply can compromise both food production and nutritional quality (*45–47*), precipitating food insecurity and subsequent malnutrition (*48*). Concurrently, diminished water availability can trigger regional livelihood crises and potential conflicts (*49*), fostering intergroup competition that generates anxiety and psychological

distress (*50*).

Furthermore, the decline in ice-dependent recreational practices, including tourism, winter sports, and pilgrimages, restricts access to nature-based stress relief mechanisms, particularly in high-latitude and high-altitude regions (*51–52*). Deteriorating cultural services can also undermine indigenous identity and traditions, catalyzing mental health challenges. For instance, in the Qullqipunqu Mountains of Peru, glacier retreat and restrictions on candle use during pilgrimages have profoundly disrupted religious practices and eroded glacier-related cultural values among native adherents (*53*).

More comprehensively, escalating cryosphere hazards impact overall human wellbeing, which encompasses multiple dimensions beyond physical health, including safety, place attachment, self-identity, and social belonging (*54*). This necessitates a paradigm shift in risk mitigation strategies toward a holistic approach centered on people, communities, and societies, rather than exclusively emphasizing food production and economic development that essentially support human wellbeing.

Strategies for Adaptation to Mitigate Public Health Risks to Cryosphere Change

Global efforts to reduce carbon emissions and mitigate climate change, such as the Paris Agreement, are currently underway. However, achieving the goal of limiting global warming to well below 2 °C above pre-industrial levels, with efforts to restrict the increase to 1.5 °C by the end of the 21st century, faces mounting challenges. Recent data indicate that global mean surface temperature exceeded 1.5 °C above pre-industrial levels for 12 consecutive months through June 2024, signaling an earlier breach of the Paris Agreement threshold (*55*). This development has profound implications for the cryosphere, which may undergo more rapid changes with subsequent intensification of public health risks.

Despite these concerning trends, public awareness and preparedness for these changes remain insufficient. Developing public health adaptation strategies to minimize risks associated with cryosphere change has become increasingly imperative. These strategies necessitate targeted, proactive measures that integrate broad climate resilience, healthcare infrastructure enhancement, and community preparedness, specifically addressing the escalating risks from cryosphere degradation. Implementation requires three critical steps: first, improving awareness among the public, governments, and health sectors about the health risks posed by cryosphere changes; second, developing climate adaptation action plans for public health that explicitly address cryosphere-related risks; and third, initiating targeted investments and implementation of public health adaptations, driven by either government policies or market opportunities. For example, to control and prevent the revival of ancient pathogens associated with thawing permafrost, the primary strategy should focus on mitigating GHG emissions, so ambitious climate action is imperative. Additionally, clinicians need to be aware that pathogenic microorganisms may emerge from thawing permafrost and cause disease. Thus, any suspected cases should be checked for their exposure to thawing permafrost. Furthermore, vaccines against ancient pathogens (e.g., smallpox) should be kept just in case of their possible comeback.

To facilitate progress in adaptation, it is essential to understand the potential hazards to public health posed by both geohazards and climate hazards emerging from rapid cryosphere changes in response to global warming. We must advance knowledge not only about the dynamics of cryosphere changes but also their immediate and long-term implications for public health across different regions and through various causal pathways. This necessitates interdisciplinary collaboration across diverse fields and international cooperation, especially in transboundary regions such as the Arctic and Tibetan Plateau.

An effective approach to adapting to cryosphere change and mitigating public health risks requires comprehensive efforts beyond environmental and public health sectors. In addition to strengthened global climate mitigation actions, the integration of proactive public health adaptation strategies centered on people, communities, and societies, fostered by improved water resource management, reinforced climate and environmental monitoring systems, and enhanced disaster risk management, will collectively yield substantial benefits for human health, and after all, for societal wellbeing.

Conflicts of interest: No conflicts of interest.

Ethical statement: This research involves no ethical concerns as it does not include human/animal subjects or sensitive data.

Funding: Supported by the National Key R&D Program of China (2022YFC3702705 to XS).

doi: 10.46234/ccdcw2025.062

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Submitted: February 28, 2025
Accepted: March 13, 2025
Issued: March 21, 2025

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Policy Notes

Policy Interpretation of the China National Climate Change Health Adaptation Action Plan (2024–2030)

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ABSTRACT

To better protect public health under climate change, in September 2024, the National Disease Control and Prevention Administration of the People's Republic of China, in collaboration with 12 other relevant departments, jointly released the China National Climate Change Health Adaptation Action Plan (2024–2030) (hereinafter referred to as the Action Plan), and innovatively proposed ten strategic prior actions. This study provides a systematic interpretation on the Action Plan, introducing its background, methodology and evidence used in production process, main concepts and content, the road map of implementation, as well as several possible challenges and solutions, which helps to give an overall understanding of the Action Plan.

Climate change has become one of the most complex public health issues of the 21st century (*1*). Since climate change trends are difficult to mitigate in the short term, improving the adaptation to climate change has become the key strategy in global efforts attempting to cope with climate change. In September 2024, the National Disease Control and Prevention Administration of the People's Republic of China, in collaboration with 12 other relevant departments, jointly released the China National Climate Change Health Adaptation Action Plan (2024–2030) (hereinafter referred to as the Action Plan) (*2*). This Action Plan outlines strategies for health adaptation to climate change in China and proposes ten key tasks, which is important for integrating health into climate policy and foster diverse, multi-level health adaptation policies and collaborative actions, as well as strengthening climate change adaptation and risk prevention capacities. The release of the Action Plan demonstrates China's role as a major country in the global climate change response process and reflects the national vision of prioritizing people's health in a strategic position. The World Health Organization (WHO) Representative Office in China highly commended the Action Plan (*3*). This article interprets the Action Plan's background, objectives, innovations, and challenges.

BACKGROUND

Climate Change and Its Health Impacts

As climate change characterized by global warming continues to intensify, extreme weather and climate events such as heatwaves, floods, cold waves, and typhoons have become more frequent and severe in recent years (*4*). According to the latest report of the Lancet Countdown on health and climate change, in 2023, the global average number of hours of high-temperature exposure per capita increased by 27.7%, and the proportion of land areas experiencing an increase in the number of days with extreme precipitation rose to 61% compared to the baseline of 1961 to 1990, both hitting new historical records (*5*). Extreme weather and climate events, such as global extreme heatwaves, typhoons, and floods, have resulted in 53% more direct economic losses compared to the historical average during 1992–2021 (*4*). In China, the average number of heatwave exposure days per person in 2023 reached over three times the historical average (1986–2005), which resulted in a 1.9 times surge in heatwave-related deaths. At the same time, drought and extreme rainfall led to a 15.1% and 2.4% higher excess risk of related infectious diarrhea from 2013–2022 (*6*). As the latest data in China Climate Bulletin (2024) indicated, the national average temperature reached 10.9 ℃ in 2024, breaking the historical record and leading to the hottest year. Human survival, life, and health are facing significant challenges.

Global and Domestic Policy Background

Enhancing health adaptation and risk prevention

capabilities in response to climate change is an urgent priority. The WHO has long called on countries to integrate health adaptation into their climate change policies and has identified "Respond to climate change, an escalating health threat in the 21st century" as one of its six strategic goals in its Fourteenth General Programme of Work, 2025–2028 (*7*). Countries should formulate and implement National Adaptation Plans (NAP) as well as Health National Adaptation Plans (HNAP), to achieve the goal of building climate-resilient health systems that can anticipate, absorb, and transform in a changing climate to protect population health (*8*). In China, national climate change adaptation actions are urgently needed to fulfill the mandate of comprehensive promotion of climate change health adaptation actions outlined in the National Climate Change Adaptation Strategy 2035 (*9*), and contribute to the "Healthy China" and "Beautiful China" initiatives.

METHODS

Who Was Involved in the Production of the Action Plan

The Action Plan was developed based on an in-depth investigation into the evidence linking climate change and health as well as adaptation, and extensive consultations and deliberations with experts and governors from the interdisciplinary fields, such as public health, ecology and environment, meteorology, water resources, agriculture, transportation, emergency management, finance, culture and tourism, and construction and urban-rural development. A clear and evidence-based framework for discussion as well as promote feedback mechanism were applied to balance the opinions of experts in different fields.

Evidence Considered in the Production of the Action Plan

To develop an evidence-based, needs-oriented, and capabilities-driven action plan, a comprehensive analysis of domestic and international evidence on climate change and health, as well as policy contexts of climate change adaptation, were conducted. Key evidence was adopted to ensure the scientific rigor, systematic consideration and rationality of policy formulation. These evidences include:

The core essence and new concept of global and national-level climate health policy and low-carbon strategy was taken into account, such as the guideline of WHO on how to build climate-resilient and low-carbon health system, the main concept of health adaptation delivered from the United Arab Emirates Declaration on Climate and Health of the 28th Conference of the Parties to the United Nations Framework Convention on Climate Change (COP28), the Building Resilience Against Climate Effect (BRACE) framework of the United States, and the experience of low-carbon construction from the 'Net Zero' National Health Service in United Kingdom England.

The latest domestic climate policy and actions, including the National Climate Change Adaptation Strategy 2035, etc.

Current trend and characteristics of climate change reported in series reports of the IPCC (*10*) as well as China Climate Bulletin in recent years; both national and regional climate change characteristics were taken into account, given the country's diverse climate and health challenges.

The public health impact of climate change (*5*,*6*) based on global evidence. Infectious diseases such as plague, dengue, malaria, Japanese encephalitis, zoonotic diseases, as well as chronic conditions influenced by climate, including cardiovascular diseases, respiratory diseases, mental health disorders, and allergies, were all considered.

Multi-sectoral actions and policies regarding climate change (*6*,*10*). Multiple sectors in China, in accordance with its respective responsibilities, have implemented a series of initiatives to address climate change mitigation and adaptation, with comprehensive coverage spanning policy regulatory frameworks, scientific and technological advancements, as well as engineering construction undertakings.

RATIONALE AND EVIDENCE

The Overall Target of the Action Plan by 2030

This Action Plan aims to prevent climate change related health risks, strengthen health adaptation actions, and advance health promotion capabilities. This emphasizes moving the focus of disease prevention forward, prioritizing an adaptation interconnected mechanism driven by construction of the health early warning systems, fostering diverse and multi-level health adaptation policies and collaborative actions, and strengthening adaptation and risk prevention capacities.

Innovations and Strategic Prior Actions

The Action Plan is a groundbreaking policy representing a strategic leap towards a better incorporated health considerations throughout the entire course of climate policies. A distinctive innovation of this Action Plan is the emphasis of a balanced approach to mitigation and adaptation and the integration of health adaptation principles throughout the climate change policy system.

To align with the global framework of health adaptation, and comprehensively integrate the country' s health priorities and climate change challenges, the Action Plan outlines ten specific measures for national climate change adaptation by 2030, referred to as the "Ten Actions for Health", including:

1) Strengthening the inter-departmental collaboration mechanism on climate change and health;

2) Improving the policy and standards system for climate change and health;

3) Strengthening early warnings for climate-sensitive diseases;

4) Enhancing dynamic assessments of climate change-related health risks, vulnerabilities, and adaptation capacities;

5) Enhancing climate change related health risk prevention and comprehensive intervention capabilities;

6) Strengthening health guarantee capabilities for climate change;

7) Enhancing climate resilience of healthcare and public health systems;

8) Creating friendly environments for climate change health adaptation across the entire society;

9) Accelerating technological innovation for climate change health adaptation;

10) Advancing global initiatives on climate change health adaptation.

PRESENTATION

The Road Map of Action Plan Implementation

The Action Plan established goals for two phases. By 2025, a collaborative, multi-departmental mechanism addressing climate change and health will be improved, and a policy and standards framework will be constructed; the construction of climate-sensitive diseases monitoring systems will be strengthened, and climate change and health evaluation indicators will be developed; the first round of assessments on health risks, vulnerabilities, and adaptation capacities related to climate change will be completed. By 2030, a system of policies and standards for climate change and health will be primarily established; monitoring and early warning capacities will be consistently strengthened; assessment system for health risks, vulnerabilities, and adaption capacities will be well-developed; the climate resilience of key regions and sectors, as well as health systems, will be significantly enhanced; a friendly environment supporting climate change health adaptation across the entire society will be preliminarily formed.

To fulfill the goals in 2025 and 2030, a road map for a systematic implementation of the Action Plan is designed. It includes the following actions:

Construct a collaborative, multi-departmental working mechanism: The Action Plan emphasizes the construction of a collaborative, multi-departmental mechanism addressing climate change and health, establish a comprehensive and integrated cross-departmental system encompassing 'Planning–Monitoring–Warning–Assessment–Intervention'.

Build up health risk early warnings: The Action Plan encourages to build up health risk early warnings to strengthen public health intervention. This effort includes delivering health reminders to key populations and advancing diversified public health services.

Strengthen the health emergency response to extreme weather disasters: The Action Plan addresses strengthening health emergency response capabilities for infectious disease prevention and control, drinking water safety, environmental hygiene, and health emergency response. It also emphasizes enhancing the resilience and recovery capabilities of healthcare facilities for extreme weather and climate events.

Provide health protection guidance to the public: The Action Plan focuses on providing targeted health protection guidelines to enhance public awareness of risks and improve self-protection and self-rescue abilities, particularly to vulnerable populations such as pregnant women, children, the elderly, individuals with chronic diseases, and outdoor workers.

Integrate climate mitigation and adaptation: On one hand, the Action Plan emphasizes educating and guiding the public to adopt green and low-carbon lifestyles voluntarily. On the other hand, it also promotes the development of green and low-carbon healthcare facilities, by applying land-, energy-, water-, and material-efficient construction and technological integration (e.g., IoT, AI-driven platforms). The Action Plan finally aims to foster a friendly

environment for climate change health adaptation across the entire society.

Challenges and Support Measures

Interdepartmental collaboration: Considering the different responsibilities and work focuses of each department, there may be difficulties in effective coordination in data sharing and joint work. The Action Plan emphasizes enhancing organizational leadership to promote effective collaborations at both the local and national levels, such as to promote the establishment of interdepartmental information sharing platforms, joint work groups and mechanism.

Climate and health professionals: Climate change health adaptation is a highly interdisciplinary field that requires individual with knowledge in climate change, public health, environmental science, and other related fields. The shortage of such professionals may make it difficult to meet the needs of plan implementation. The Action Plan emphasizes the need to promote training in climate change and health skills and practical exercises to increase professional skillsets. Also, the Action Plan mandates the establishment of a National Expert Committee on Climate Change and Health to consolidate interdisciplinary expertise.

Dynamic adjustment of action implementation: Climate change and health issues are dynamic and uncertain, and the plan needs to be evaluated and adjusted dynamically according to the actual situation. Integrated indicators and metrics for the evaluation should be developed. The Action Plan requires public health departments to strengthen guidance on implementation and perform effectiveness evaluations as needed.

CONCLUSION

Amid intensifying health threats posed by climate change, the Action Plan underscores China's resolve to safeguard population health while advancing its dual carbon neutrality and resilience-building goals. As an active promoter and contributor to global climate action, China will vigorously promote the implementation of this Action Plan and provide support for global climate change health governance.

doi: 10.46234/ccdcw2025.063

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Submitted: March 11, 2025
Accepted: March 13, 2025
Issued: March 21, 2025

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Preplanned Studies

Human Infection with *Chlamydia pneumoniae* ST16 — Lishui City, Zhejiang Province, China, 2024

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Summary

What is already known about this topic?

Chlamydia pneumoniae (*C. pneumoniae*) is an important pathogen associated with respiratory infections. In China, *C. pneumoniae* pneumonia is not a notifiable infectious disease and is frequently overlooked in clinical detection protocols for community-acquired pneumonia. Consequently, the prevalence and genotypic distribution of chlamydial infections remain inadequately characterized.

What is added by this report?

We investigated four patients with *C. pneumoniae* pneumonia in Lishui City, Zhejiang Province, China, between April and May 2024. All patients exhibited decreased levels of retinol-binding protein and prealbumin, with two patients presenting with co-infections. Analysis of the 16S rRNA and *ompA* gene sequences demonstrated 98% to 100% homology with known *C. pneumoniae* strains. To further characterize these isolates, we sequenced seven housekeeping genes, which revealed that all four patients were infected with the ST16 sequence type.

What are the implications for public health practice?

Our findings underscore the necessity for enhanced surveillance and research on chlamydial infections, as well as the implementation of next-generation sequencing methodologies to improve pathogen identification, particularly in complex cases involving co-infections.

ABSTRACT

Introduction: This study aims to analyze the clinical characteristics and pathogenic features of *Chlamydia pneumoniae* pneumonia, providing a scientific basis for the diagnosis and treatment of *C. pneumoniae* infections.

Methods: Clinical data from four patients diagnosed with *C. pneumoniae* pneumonia in Lishui City, Zhejiang Province, between April and May 2024, were collected to analyze clinical manifestations and pathogenic findings. Multi-locus sequence typing (MLST) analysis of the pathogen was conducted using seven housekeeping genes.

Results: All patients exhibited decreased levels of retinol-binding protein and prealbumin, findings not previously reported in earlier studies. Additionally, co-infections were identified in two cases. Analysis of the 16S rRNA and *ompA* gene sequences indicated a homology of 98% to 100% with known *C. pneumoniae* strains. To further characterize these strains, sequencing of the seven housekeeping genes confirmed that all cases were infected with the ST16 genotype.

Conclusions: *C. pneumoniae* infections in Lishui City are predominantly caused by the ST16 genotype, highlighting the need for enhanced research into these infections. The decrease in retinol-binding protein and prealbumin levels may serve as auxiliary diagnostic biomarkers in clinical practice. Next-generation sequencing methods demonstrate significant potential for pathogen identification, particularly in diagnosing co-infections.

Humans are the only known reservoir of *Chlamydia pneumoniae* (*C. pneumoniae*), a gram-negative, obligate intracellular bacterium. This pathogen represents a common etiological agent of community-acquired pneumonia, with diagnosis and treatment frequently delayed due to its presentation with atypical clinical manifestations. Beyond respiratory infections, emerging evidence suggests associations between *C. pneumoniae* and various pathological conditions, including asthma, atherosclerosis, and Alzheimer disease (*1*).

In China, neither *C. pneumoniae* pneumonia nor *C. psittaci* pneumonia are classified as notifiable infectious diseases, and these pathogens are not routinely included in standard clinical diagnostic

panels for respiratory infections. Consequently, the epidemiological prevalence and genotypic distribution of chlamydial infections remain inadequately characterized. To date, only a single serovar of *C. pneumoniae* has been identified. Previously, our research team investigated the prevalence of *C. psittaci* infections in Lishui, revealing the presence of a novel *C. psittaci* strain responsible for local infections (*2*). In the current study, to elucidate the prevalence and genotypic characteristics of *C. pneumoniae* in Lishui, we conducted comprehensive clinical and laboratory investigations of four cases of severe community-acquired pneumonia.

In this study, an analysis was conducted for consecutive cases of *C. pneumoniae* infection in patients admitted to Lishui People's Hospital of Zhejiang Province between April 23 and May 14, 2024. Nested polymerase chain reaction targeting Chlamydia-specific genes of 16s rRNA and outer membrane protein A (*ompA*) were amplified. To further characterize the *C. pneumoniae* isolates through multi-locus sequence typing (MLST), we employed nested polymerase chain reaction methods to sequence seven housekeeping genes (*enoA*, *fumC*, *gatA*, *gidA*, *hemN*, *hflX*, and *oppA*) (*3*). Targeted next-generation sequencing (tNGS) was utilized for pathogen diagnosis in patients 2 and 3. Additionally, we attempted to obtain the complete genome sequence of *C. pneumoniae* using the NGS-based hybrid capture method.

The study cohort comprised three females and one male, with a median age of 22 years (range: 14–36 years). Medical records indicated that three patients presented with high fever. All four patients were hospitalized with cough, with three exhibiting productive sputum. Only one patient reported chills and myalgia. Computed tomography (CT) chest scans revealed unilateral pulmonary inflammation in all patients. Hematological analysis demonstrated markedly decreased retinol-binding protein and prealbumin levels across all four cases. Three patients exhibited elevated plateletcrit values. Additional abnormal laboratory parameters were observed in two patients, including neutrophilia, elevated high-sensitivity C-reactive protein, hyperfibrinogenemia, increased d-dimer, prolonged activated partial thromboplastin time, and elevated total bile acids, concurrent with lymphopenia and hypoalbuminemia. Serological testing for IgM antibodies against *Legionella pneumophila* and *Mycoplasma pneumoniae* yielded negative results in three patients. Throat swab specimens from two patients were analyzed for common respiratory pathogens, including influenza virus, parainfluenza virus, metapneumovirus, bocavirus, respiratory syncytial virus, coronavirus, rhinovirus, *M. pneumoniae*, and adenovirus. Patient 4 tested positive for adenovirus, while no other pathogens were detected in any patient. All patients recovered following treatment with azithromycin or doxycycline and were subsequently discharged.

tNGS of bronchoalveolar lavage fluid samples from Patients 2 and 3 identified *C. pneumoniae* in Patient 2 (10,681 reads), while Patient 3 demonstrated a polymicrobial infection with *C. pneumoniae* (87 reads), *Haemophilus influenzae* (35,231 reads), and human adenovirus 5 (157 reads) (Table 1). Sanger sequencing confirmed *C. pneumoniae* infection, with 99%–100% homology to known *C. pneumoniae* 16S rRNA sequences and 98%–100% homology to the *ompA* gene. The sequences were deposited in GenBank (accession numbers PQ510372-PQ510375 for 16S rRNA, PQ522244-PQ522247 for *ompA*). Complete *C. pneumoniae* genomic sequences were attempted to be obtained using NGS-based hybrid capture methodology. Only Patient 4 yielded a sufficient genomic draft (GenBank BioProject PRJNA1177653), with phylogenetic analysis demonstrating close clustering with the prototype strain AR39 (Figure 1). MLST analysis revealed that all four patients were infected with sequence type 16 (ST16) (*enoA*:5, *fumC*:4, *gatA*:4, *gidA*:6, *hemN*:3, *hflX*:5, and *oppA*:7).

DISCUSSION

In this study, we identified *C. pneumoniae* infection in four patients with severe pneumonia from Lishui City, Zhejiang Province, China. Analysis of the 16S rRNA and *ompA* gene sequences demonstrated 98% to 100% homology with known strains of *C. pneumoniae*. To further characterize the molecular epidemiology of these isolates, we sequenced seven housekeeping genes for MLST. Results revealed that all four patients were infected with the ST16 genotype. Based on currently available sequencing data for *C. pneumoniae*, MLST classification encompasses four distinct sequence types: ST16, ST17, ST18, and ST215. Among these, ST215 is exclusively derived from Australian koalas and *Perameles bougainville*, while the remaining three sequence types (ST16, ST17, and ST18) are associated

TABLE 1. Demographic and basic clinical features of the four patients.

Characteristics	Case #1	Case #2	Case #3	Case #4
Age (years)/sex	28/Female	36/Female	16/Male	14/Female
Signs/symptoms	37.1 ℃ Productive cough	37.5 ℃ Chills, Myalgia Productive cough	36.5 ℃ Productive cough	38.5 ℃ Fever, Cough Moist rales
IgM detection*	(−)	(−)	/	(−)
Nucleic acid detection of common respiratory pathogens†	/	(−)	/	Adenovirus (+)
tNGS	/	*C. pneumonia* (10,681 reads)	*C. pneumonia* (87 reads) *Haemophilus influenzae* (35,231 reads) Human Adenovirus 5 (157 reads)	/
Blood test	↑ (APTT, D-dimer, FIB, PCT, TBA) ↓ (PA, RBP)	↑ (D-dimer, FIB, hs-CRP, NEUT, PCT) ↓ (ALB, LYM, PA, RBP)	↑ (PCT, TBA) ↓ (ALB, PA, RBP)	↑ (ADA, APTT, AST, β-2-MG, CK, HBDH, hs-CRP, LDH, NEUT) ↓ (LYM, PA, RBP)

Note: "/" means the test was not performed; (−) means a negative result; ↑ and ↓ means increased and decreased test values, respectively.
Abbreviation: *C. pneumoniae*=*Chlamydia pneumoniae*; tNGS=Targeted next-generation sequencing; ADA=Adenosine Deaminase; ALB=Albumin; APTT=Activated partial thromboplastin time; AST=Aspartate aminotransferase; β-2-MG=Beta-2-microglobulin; CK=Creatine Kinase; FIB=Fibrinogen; HBDH=Alpha hydroxybutyrate dehydrogenase; hs-CRP=Hypersensitive C-reactive protein; LDH=Lactate dehydrogenase; LYM=Lymphocyte; NEUT=Neutrophil; PA=Prealbumin; PCT=Plateletcrit; RBP=Retinol-binding protein; TBA=Total bile acid.
* Simultaneous testing for IgM antibodies against *Legionella pneumophila* and *Mycoplasma pneumoniae*.
† Common respiratory pathogens include influenza virus, parainfluenza virus, metapneumovirus, bocavirus, respiratory syncytial virus, coronavirus, rhinovirus, *Mycoplasma pneumoniae*, and adenovirus.

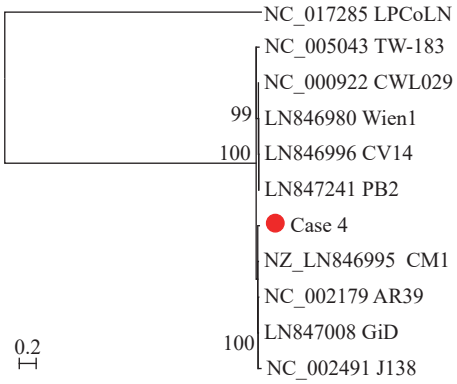

FIGURE 1. Phylogenetic analysis of *C. pneumoniae*. The phylogenetic data were obtained through the alignment of concatenated core genes with the recombination region removed.

with human infections (*4*–*5*).

All four patients with *C. pneumoniae* pneumonia initially presented with cough and fever of unknown origin, with productive cough observed in three cases. CT scans revealed unilateral pneumonia in all patients. Laboratory analyses demonstrated consistent abnormalities across all cases, notably decreased levels of retinol-binding protein and prealbumin. This finding represents a novel clinical observation not previously documented in the literature on *C. pneumoniae* infection (*6*–*8*). Both retinol-binding protein and prealbumin are hepatically synthesized proteins involved in vitamin A transport, and alterations in their concentrations serve as indirect indicators of systemic inflammatory responses (*9*–*10*). Therefore, monitoring these biomarkers in patients with *C. pneumoniae* pneumonia may provide valuable clinical insights, enabling physicians to better assess disease severity and potentially offering actionable biomarkers for clinical management.

C. pneumoniae frequently occurs as a co-infecting pathogen in pneumonia cases. In our cohort, two patients exhibited co-infections: one with a dual pathogen profile (adenovirus) and another with a triple pathogen profile (adenovirus and *H. influenzae*). Such co-infections typically present greater therapeutic challenges, underscoring the critical importance of accurate pathogen identification for targeted intervention. In this context, tNGS represents an increasingly valuable diagnostic modality for severe clinical infections, particularly for identifying complex co-infections. However, standard hospital pathogen detection protocols rarely include *C. pneumoniae*, highlighting how tNGS implementation could substantially enhance detection rates for this pathogen.

In conclusion, the epidemiology and predominant sequence types of *C. pneumoniae* in China remain inadequately characterized. While our findings

contribute meaningful data to the existing literature, the limited sample size necessitates further comprehensive investigations into *C. pneumoniae* and other chlamydial infections.

Conflicts of interest: No conflicts of interest.

Ethical statement: The study was approved by the Ethics Committee of the National Institute of Communicable Disease Prevention and Control, China CDC (Approval no. ICDC-202115).

Funding: This work was supported by grants from the Tengfei initiative by the National Key Laboratory of Intelligent Tracking and Forecasting for Infectious Diseases (grant number 2024NITFID504), grants from the National Key Research and Development Program of China (grant number 2021YFC2301202), and the National Natural Science Foundation of China (grant number 82102390).

doi: 10.46234/ccdcw2025.064

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Submitted: November 06, 2024
Accepted: February 05, 2025
Issued: March 21, 2025

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Methods and Applications

A Quadruplex Digital PCR Assay for the Simultaneous Detection of Four Intestinal Bacterial Pathogens and Its Application in Wastewater Samples

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ABSTRACT

Introduction: A quadruplex digital polymerase chain reaction (dPCR) method was developed for the simultaneous detection of *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, and *V. parahaemolyticus* in wastewater to enhance pathogen identification velocity and efficiency. This study established detection limits for these bacterial pathogens and validated the method using environmental wastewater samples.

Methods: Specific primers and probes were designed targeting the *invA* gene of *Salmonella*, *ipaH* gene of *Shigella*, *tlh* gene of *V. parahaemolyticus*, and cholera toxin gene *ctxA* of *V. cholerae*. The quadruplex dPCR assay underwent rigorous evaluation for analytical sensitivity and specificity. Detection limits were determined using spiked wastewater samples, and the method's effectiveness was assessed through preliminary testing of 60 environmental wastewater samples.

Results: The quadruplex dPCR assay was optimized at an annealing temperature of 58°C. In spiked wastewater samples, the detection limits were 390 CFU/100 mL for *Salmonella*, 11 CFU/100 mL for *Shigella*, 660 CFU/100 mL for *V. cholerae*, and 640 CFU/100 mL for *V. parahaemolyticus*. Analysis of 60 municipal wastewater samples revealed pathogen concentrations ranging from 100.9–14,560 copies/100 mL for *Shigella*, 86.5–7,329 copies/100 mL for *Salmonella*, and 84.5–865.7 copies/100 mL for *V. parahaemolyticus*.

Conclusions: The developed quadruplex dPCR assay demonstrates robust capability for comprehensive surveillance of intestinal bacterial pathogens in wastewater, offering reliable detection even at low concentrations.

Wastewater-based epidemiology (WBE) has emerged as a pivotal and cost-effective public health tool, offering a swift and efficient means to evaluate the prevalence and dissemination of pathogens within a community through wastewater analysis (*1*). This method supports a comprehensive approach called One Sample, Multiple Analyses (OSMA) (*2*), allowing simultaneous monitoring of various health indicators, including antibiotic resistance genes, drug-resistant bacteria, pathogens, and chemical contaminants. This enhances traditional surveillance techniques. WBE's non-intrusive nature augments clinical surveillance by detecting pathogens in wastewater, which helps provide early warnings of disease outbreaks and track infection trends (*3*–*4*).

The utility of WBE was especially evident during the COVID-19 pandemic, where it effectively traced SARS-CoV-2 transmission, demonstrating its value in infectious disease surveillance (*4*–*5*). The monitoring of enteric pathogens including *Salmonella*, *Shigella*, and *Vibrio species* in wastewater has provided crucial epidemiological insights into foodborne and waterborne diseases. These pathogens, responsible for conditions like gastroenteritis, dysentery, and cholera, are consistently detected in wastewater systems (*6*–*8*). Surveillance of these pathogens is vital for understanding disease patterns, assessing intervention effectiveness, identifying contamination sources, quantifying disease burden, and guiding targeted surveillance efforts.

Digital polymerase chain reaction (dPCR), a third-generation PCR technology, provides exceptional accuracy and sensitivity for absolute quantification. This study establishes a quadruplex dPCR method for the simultaneous detection of *Salmonella*, *Shigella*, *V. parahaemolyticus*, and *V. cholerae* in wastewater samples. By monitoring bacterial levels in wastewater with dPCR, this approach serves as an early warning system, enabling timely identification of outbreak risks and prompt implementation of preventive measures.

METHODS

Wastewater Samples

A total of 60 wastewater samples were collected from piped wastewater networks serving large residential communities, subway stations, educational institutions, and hospitals across 4 districts of Beijing Municipality (Chaoyang, Haidian, Daxing, and Fengtai), China. The collected samples were categorized as follows: 27 from residential communities, 18 from healthcare institutions, and 15 from other locations. All samples were collected in November 2024.

Artificially Spiked Wastewater

Fresh bacterial colonies of the four positive control strains were washed three times with phosphate-buffered saline (PBS, pH 7.4) and subjected to serial twofold dilutions in PBS. For each dilution, 1 mL was spiked into 100 mL of pre-screened wastewater that had tested negative for all four target pathogens.

Bacterial Strains

The positive control strains utilized for method development were *V. cholerae* strain N16961, *Salmonella enterica* serovar Typhimurium strain LT2 (*9*), *S. flexneri* strain SH1, and *V. parahaemolyticus* strain ICDC-VP1329. The specificity of the dPCR assay was validated using a comprehensive panel of 90 bacterial strains, comprising 51 target strains and 39 non-target strains. The panel included 16 *Salmonella* strains (5 *S. Enteritidis*, 5 *S. typhi*, and 6 *S. Typhimurium*); 9 *Shigella* strains (2 *S. flexneri* and 7 *S. sonnei*); 11 *V. parahaemolyticus*; 13 *V. cholerae* (7 O1 serogroup and 6 O139 serogroup, with 12 *ctxA*-positive strains); 2 Enteroinvasive *Escherichia coli* (EIEC); 20 other pathogenic *E. coli* (excluding EIEC); and various other species including 1 *V. alginolyticus*, 3 *V. fluvialis*, 2 *V. mimicus*, 2 *V. vulnificus*, 1 *Aeromonas hydrophila*, 3 *Plesiomonas shigelloides*, 1 *Yersinia enterocolitica*, 2 *Clostridium perfringens*, 1 *Edwardsiella tarda*, 1 *Listeria monocytogenes*, 1 *Citrobacter sp.*, and 1 *Clostridium difficile*. All strains were maintained in laboratory collection.

Primers and Probes

Target gene sequences were selected based on comprehensive sequence alignments from the NCBI NR database (https://www.ncbi.nlm.nih.gov/nucleotide/). All primers and probes were designed using Beacon Designer V8.20 and synthesized by Sangon Biotech (Shanghai, China).

The primer and probe sequences (5' to 3') for pathogen detection are as follows: for the *invA* gene of *Salmonella*, the forward primer is CCGCCAAACCT AAAACCAG, the reverse primer is GGCTCTTCG GCACAAGTA, and the probe is FAM-CGCCAA TCAGTCCYAACGACGACCCTT-BHQ1; for the *ipaH* gene of *Shigella*, the forward primer is GCAGAG AAACTTCAGCTCTC, the reverse primer is CAGT GCGGAGGTCATTTG, and the probe is HEX-TCACTCCCGACACGCCATAGAAACGC-BHQ1; for the *tlh* gene of *V. parahaemolyticus*, the forward primer is CGAACGAGAACGCAGACATTA, the reverse primer is GCAACCACTTTGTTGATTTG ATCT, and the probe is ROX-TTCTTCGCCGCTG ACAATCGCTTCTCA-BHQ2; and for the *ctxA* gene of *Vibrio cholerae*, the forward primer is AGGGGCTACAGAGATAGATATTACA, the reverse primer is GCGGTGCATGATGAATCCA, and the probe is Cy5-ACCTGCCAATCCATAACCATCTG CTGC-BHQ3.

Quadruplex dPCR Assay

The quadruplex dPCR analysis was performed using the multiplex QIAcuity Digital PCR system (QIAGEN, Hilden, Germany). Reactions were conducted using the QIAcuity Probe PCR Kit (QIAGEN) according to manufacturer specifications. Each 40 µL reaction mixture contained 10 µL of 4× dPCR™ Supermix for Probes (QIAGEN), 10 µL of template DNA, 800 nM of each primer, 400 nM of each probe, and 6.25 units/mL EcoRI-HF® (New England Biolabs, United Kingdom). The reaction mixture was distributed into a 24-well 26k QIAcuity Nanoplate (QIAGEN), sealed, and analyzed using the QIAcuity dPCR instrument (QIAGEN). Each sample was analyzed in triplicate, with a no-template control (NTC) serving as the negative control. The thermal cycling protocol consisted of initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 58 °C for 30 s. Positive results were determined by the presence of positive droplets in the dPCR assay.

Analytical Specificity

The specificity of primers and probes underwent comprehensive evaluation using both the BLASTn algorithm (within the non-redundant nucleotide database) and Primer-BLAST (within the non-redundant database restricted to Enterobacterales)

(https://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequent validation was performed against genomic DNA extracted from 90 bacterial strains, comprising 51 target strains and 39 non-target strains as detailed above.

Limit of Detection (LOD)

The analytical sensitivity of the assay was determined using artificially spiked wastewater samples. Bacterial concentrations in each dilution were quantified by colony-forming unit (CFU) counting on LB agar plates. The spiked wastewater samples underwent centrifugation at 10,000 g for 30 minutes, and DNA was extracted from the resulting pellet using a DNeasy® PowerSoil Kit (QIAGEN, USA) according to the manufacturer's protocol. The extracted DNA was resuspended in 100 μL of nuclease-free water, with 10 μL used as template for dPCR detection. The entire process, from spiked sample preparation through dPCR detection, was performed in triplicate. The LOD was defined as the lowest CFU concentration in wastewater that yielded positive results across all replicates(*10*). Process repeatability was assessed using the coefficient of variation (CV) of the measured copy number per reaction.

Statistical Analysis

The dPCR data were analyzed using the QIAcuity Software Suite (version 2.5.0.1; Qiagen, Germany). A database was established using Microsoft Excel 2010. Statistical analysis and visualization were conducted using R software (version 4.4.2, R Core Team, Vienna, Austria). Differences in target gene concentrations in nucleic acid extracted from 100 mL wastewater samples across different sampling sites were assessed using the Wilcoxon test, implemented with the "wilcox_test" function from the "rstatix" package. Whisker plots were created using the "ggplot2" package.

RESULTS

Establishment and Optimization of the Quadruplex dPCR Assay for Four Bacterial Pathogens

Following primer and probe design, the amplification efficiency of each target gene using serial dilutions of positive control strain was initially evaluated DNA via real-time PCR. The analysis demonstrated optimal amplification efficiency (90%–110%) for each target gene in both singleplex (single target gene with corresponding primers and probe) and quadruplex (all four target genes with corresponding primers and probes) systems. After confirming amplification efficiency, the quadruplex dPCR reaction conditions were optimized. Through systematic evaluation of annealing/extension temperatures between 58°C and 62°C, 58°C provided optimal discrimination between positive and negative partitions. Based on these results, 58°C was the optimal annealing and extension temperature for the quadruplex dPCR assay (Figure 1).

Analytical Specificity and Sensitivity

In silico specificity analysis confirmed high target specificity for all primers and probes across the four target genes. However, two important considerations emerged: the *ipaH* gene is present in both *Shigella* and EIEC, and while the *ctxA* gene is predominantly found in *V. cholerae*, it can occasionally occur in other species such as *V. mimicus*. Subsequent experimental validation using 90 bacterial strains (51 target and 39 non-target) demonstrated robust analytical specificity of the quadruplex dPCR assay. The assay showed complete concordance with expected results for target organisms, with no observed cross-reactivity or false-positive results across the tested strain panel.

Using artificially spiked wastewater samples, the following LOD values were established for the quadruplex dPCR assay: 390 CFU/100 mL for *Salmonella*, 11 CFU/100 mL for *Shigella*, 660 CFU/100 mL for *V. cholerae*, and 640 CFU/100 mL for *V. parahaemolyticus* (Table 1).

Pathogen Detection and Quantification in Municipal Wastewater Samples Using Quadruplex dPCR Assay

The quadruplex dPCR assay was employed to analyze four bacterial pathogens across 60 municipal wastewater samples collected from the pipe network. Analysis revealed differential detection rates among the target pathogens: *Shigella* exhibited the highest prevalence at 78.3% (47/60), followed by *Salmonella* at 46.7% (28/60), and *V. parahaemolyticus* at 33.3% (20/60) (Table 2). No *V. cholerae* was detected in any of the samples. Quantitative analysis demonstrated pathogen-specific concentration ranges: *Shigella* showed the highest concentrations at 100.9–14,560 copies/100 mL, followed by *Salmonella* at 86.5–7,329

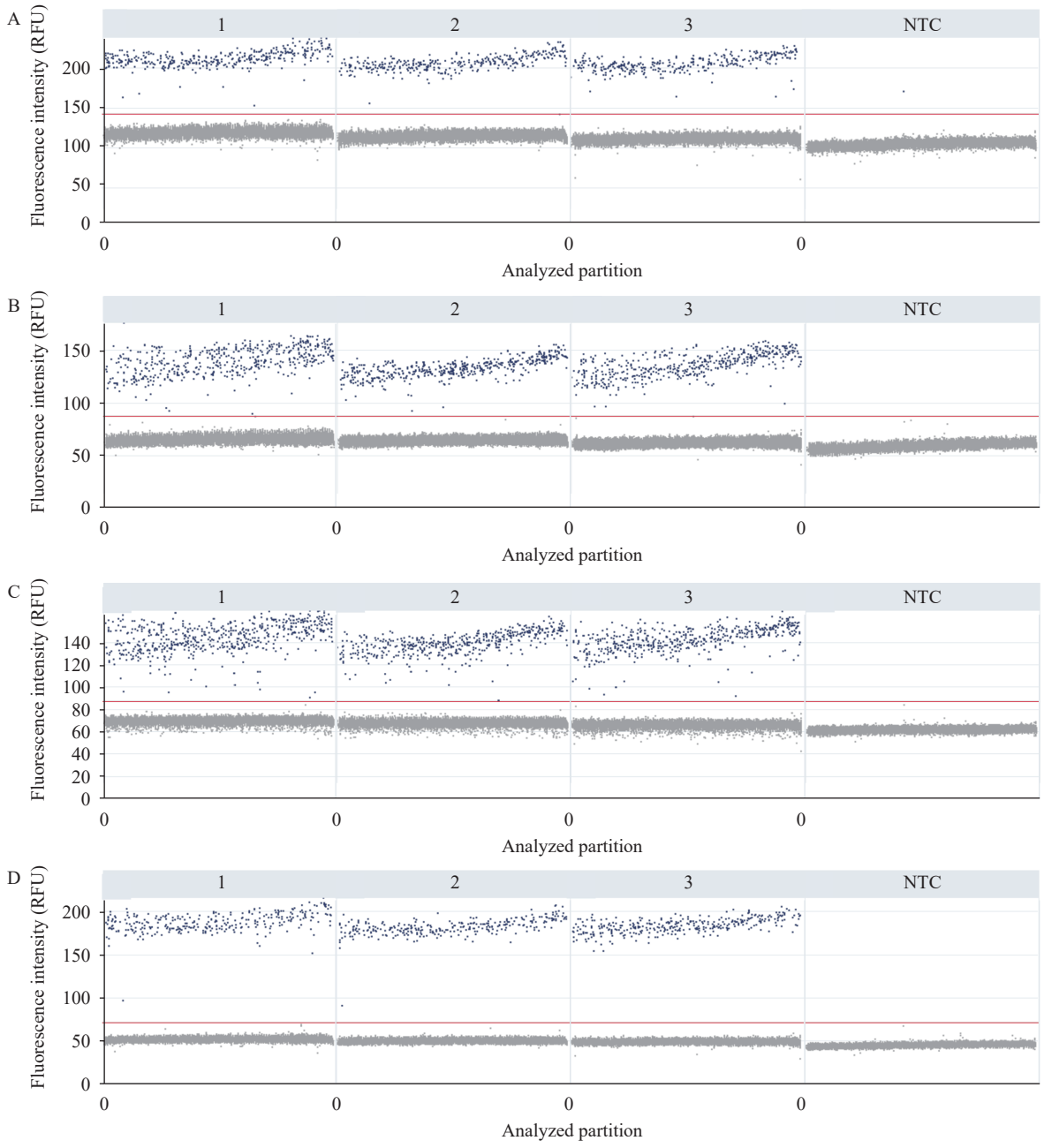

FIGURE 1. The fluorescence intensity of the target gene by digital PCR. (A) the *ipaH* gene of *Shigella* SH1; (B) the *invA* gene of *Salmonella* LT2; (C) the cholera toxin gene *ctxA* of *Vibrio cholerae* N16961; (D) the *tlh* gene of *Vibrio parahaemolyticus* ICDC-VP1329. 1, 2, and 3: Three replicates.
Note: The red horizontal lines denote the fluorescence threshold. Partitions represented by blue dots above this threshold line are interpreted as positive, while those represented by grey dots positioned below the threshold line are classified as negative.
Abbreviation: NTC=no-template control; PCR=polymerase chain reaction; RFU=relative fluorescence unit.

copies/100 mL, and *V. parahaemolyticus* at 84.5–865.7 copies/100 mL (Figure 2).

DISCUSSION

Current approaches for pathogen detection in wastewater encompass traditional culture-based methods, molecular techniques such as PCR, and next-generation sequencing (*11*). However, these methodologies exhibit significant limitations in sensitivity, specificity, and their capacity to detect low pathogen concentrations in complex wastewater matrices. dPCR has emerged as a transformative

TABLE 1. Results of dPCR for the detection of artificially spiked wastewater samples.

Bacterial strains	Expected CFU/mL of wastewater sample	Mean±SD (cp/μL DNA)	CV (%)	LOD (CFU/100 mL wastewater sample)
Shigella flexneri SH1	0.22	3.13±0.31	9.78	11
	0.11	1.31±0.36	27.49	
	0.05	1.16*±0.33	28.65	
Salmonella enterica serovar Typhimurium strain LT2	7.80	9.65±1.01	30.5	390
	3.90	3.33±1.02	30.5	
	1.95	0.95*±0.33	35.22	
Vibrio cholerae N16961	33.00	37.48±15.81	42.17	660
	6.60	2.23±1.39	62.30	
	3.30	0.15*±0.13	87.12	
Vibrio parahaemolyticus ICDC-VP1329	12.80	11.45±1.11	9.7	640
	6.40	4.56±0.40	8.83	
	3.20	1.38*±0.54	38.79	

Notes: Mean, average copy values (*n*=3) in a final reaction volume of 40 μL. CV between replicates (*n*=3), CV=SD/Mean ×100%. Expected CFU/mL of wastewater sample: the bacterial concentration calculated by colony counting results.
Abbreviation: CV=coefficient of variation; SD=standard deviation; LOD=limit of detection; CFU=colony-forming unit.
* In three replicates, a negative well appeared.

TABLE 2. Comparison of the quadruplex dPCR for detecting 60 wastewater samples of different types (community *n*=27, hospital *n*=15, other *n*=18) collected from Beijing Municipality, China.

Type of Place	*Shigella* spp.		*Salmonella* spp.		*Vibrio parahemolyticus*		*Vibrio cholerae*	
	POS (%)	POS/SUM	POS (%)	POS/SUM	POS (%)	POS/SUM	POS (%)	POS/SUM
Community	88.9	24/27	63.0	17/27	40.7	11/27	0.0	0/27
Hospital	53.3	8/15	13.3	2/15	13.3	2/15	0.0	0/15
Other	83.3	15/18	50.0	9/18	38.9	7/18	0.0	0/18
Total	78.3	47/60	46.7	28/60	33.3	20/60	0.0	0/60

Notes: Positive well: a well is considered positive when the number of positive droplets is greater than or equal to 3. Positive sample: a sample is determined to be positive when two or more of its three replicate wells are positive.
Abbreviation: POS=number of positive samples. SUM=the sum of positive and negative samples.

technology for wastewater surveillance, offering superior sensitivity and quantitative precision for pathogen detection (*12*). Its capacity for absolute quantification without calibration curves renders dPCR particularly advantageous for wastewater analysis, especially given its robust performance in the presence of PCR inhibitors commonly encountered in complex environmental matrices (*12*–*13*).

Previous studies have demonstrated the efficacy of multiplex dPCR for bacterial detection. A duplex dPCR assay developed for *Shigella* and *Salmonella* detection in fecal samples achieved detection limits of 12.3 copies/r and 23.7 copies/r, with corresponding CFU/mL values of 550 and 10,000(*14*), respectively. In this study, the detection limits were 13.1 copies/r and 33.3 copies/r. Comparatively, this method demonstrates enhanced sensitivity, with detection limits of 11 and 390 CFU/100 mL for these bacteria in wastewater matrices. These performance differences may be attributed to variations in sample type and processing procedures. While another triplex dPCR assay reported a detection limit of 0.23 copies/μL for *Salmonella* nucleic acid in food samples, our method achieved 3.33 copies/μL in wastewater matrices. This sensitivity variation likely reflects differences in both methodological approaches and matrix-specific bacterial recovery rates. The disparity underscores the critical importance of optimizing wastewater sample pretreatment protocols for real-world applications. In this analysis of environmental samples, the elevated detection rates for *Shigella* and *Salmonella*, with concentrations predominantly ranging from 394.55 to 1300 and 131.9 to 527.3 CFU/100 mL, respectively, indicate potential public health concerns that warrant enhanced surveillance measures. This sensitivity variation is likely attributable to differences in

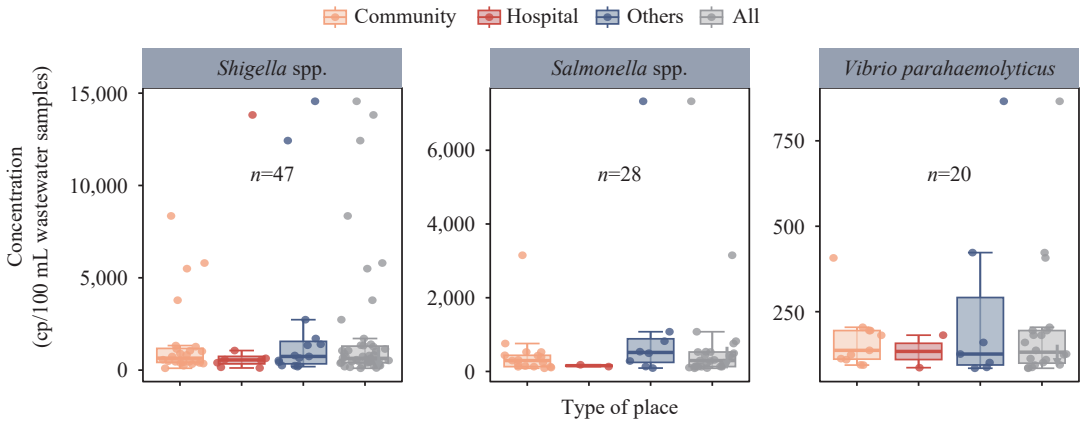

FIGURE 2. Whisker plots of the concentration of the target genes in the nucleic acid extracted from 100 mL wastewater samples from different sampling sites measured by quadruplex dPCR. Upper and lower fences display the 5th and 95th percentiles. The boxes display the interquartile range and median.
Abbreviation: dPCR=digital polymerase chain reaction.

methodological approaches, as well as potential variations in DNA extraction and PCR amplification efficiency, which may be influenced by matrix differences. These discrepancies highlight the critical need for standardized wastewater sample pretreatment methods in surveillance systems, particularly when comparing data across different sentinel sites. Since the *ipaH* gene is present in both *Shigella* and EIEC, and the *ctxA* gene can occasionally be found in other species, for positive detection of *Shigella* and *Vibrio cholerae*, it is recommended to isolate and culture the strains before performing biochemical identification. Alternatively, detection can focus on specific genes of EIEC and vibroid-like bacteria.

This study presents the first application of multiplex dPCR for simultaneous monitoring of these four pathogens in wastewater environments. The developed quadruplex dPCR method enhances detection throughput and facilitates large-scale wastewater monitoring programs. The method's capacity to detect pathogens at low concentrations enables earlier identification of potential disease outbreaks.

Despite these advantages, several challenges and limitations remain in implementing multiplex dPCR for wastewater monitoring. First, the technology has not yet achieved full process automation and still relies on operator technical proficiency, potentially affecting result accuracy. Second, samples with extremely low concentrations often exhibit high CV values across repeated measurements, with discrepancies between detected values and actual copy numbers — a phenomenon which was also encountered in this experiment.

Therefore, to enhance result reliability and accuracy in practical applications, it is recommended to perform at least three replicate measurements and calculate the average. Furthermore, while dPCR assays remain relatively costly and the complexity of analysis may pose barriers for laboratories with limited resources, potentially hindering widespread application in public health monitoring, exploring collaborations with external laboratories to share equipment or providing simplified data analysis software could help lower entry barriers. Due to the nascent stage of wastewater monitoring in our country and the absence of standardized protocols for multiplex dPCR in wastewater surveillance, result variability across different laboratories has hindered cross-study comparability. Therefore, establishing national standards promptly is essential to unify workflows and practices among laboratories.

Conflicts of interest: No conflicts of interest.

Acknowledgements: The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences and the Chinese Urban Wastewater Surveillance System for their valuable support.

Ethical statements: This study did not involve human or animal subjects, thus, no ethical statement was required.

Funding: Supported by the National Key Research and Development Program (2022YFC3201704-01) from the Ministry of Science and Technology of the People's Republic of China and the Key Project of the Capital's Funds for Health Improvement and Research, China (2022-1G-4231).

doi: 10.46234/ccdcw2025.065

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Submitted: December 23, 2024
Accepted: March 02, 2025
Issued: March 21, 2025

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Outbreak Reports

The First Imported Case of Lassa Fever — China, 2024

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Summary

What is already known about this topic?

Lassa fever, an acute viral hemorrhagic illness caused by Lassa virus, is primarily transmitted through contact with rodents, particularly *Mastomys* species. The disease is endemic to West Africa and is not classified as a notifiable infectious disease in China. Prior to August 2024, no cases of Lassa fever had been reported in China.

What is added by this report?

The clinical manifestations of Lassa fever (LF) are nonspecific and may closely resemble those of other febrile illnesses, posing challenges for detection at points of entry and during medical evaluations. In this severe case, the patient experienced coma and developed persistent hearing loss even after discharge, emphasizing the critical importance of early diagnosis and treatment.

What are the implications for public health practice?

For cases with unclear diagnoses, healthcare providers should prioritize obtaining a detailed 30-day travel history to assess the potential risk of imported infectious diseases. Public health strategies in Africa must prioritize: 1) community-tailored health education campaigns, 2) enhanced syndromic surveillance coupled with pathogen-specific research, and 3) sustainable stockpiling of rapid diagnostic tests (RDTs) for endemic infections alongside accelerated vaccine development pipelines.

ABSTRACT

Introduction: On August 3, 2024, a suspected case of Lassa fever was identified in Sichuan Province, likely imported from an endemic region. Local CDCs promptly initiated investigation and implemented preventive measures upon receiving this report.

Methods: The response included epidemiological investigations, contact tracing and management, hospital infection control measures, environmental disinfection, laboratory testing, biological sample management, and risk communication strategies.

Results: The patient was confirmed as China's first imported case of Lassa fever on August 6. The investigation identified 12 close contacts and 71 general contacts. By August 24, all contacts had completed medical observation without developing any symptoms consistent with Lassa fever. The patient recovered with residual hearing loss and was discharged on September 24 following expert verification.

Conclusions: The increasing frequency of international travel and ongoing globalization have elevated the risk of novel infectious disease importation, including Lassa fever. Simultaneously, the widespread adoption of metagenomic sequencing for diagnosing unexplained illnesses in healthcare settings has enhanced pathogen detection sensitivity, enabling more precise identification of emerging infectious agents.

Lassa fever (LF) is an acute viral hemorrhagic illness caused by Lassa virus (LASV), which was first identified in 1969 in the Nigerian town of Lassa (*1*). The disease is primarily transmitted through contact with rodents, particularly *Mastomys natalensis*, which serves as the primary natural reservoir, along with black house mice and small chevrotains (*2*). Human infection occurs through direct contact with infected animals and their excreta, as well as exposure to blood, bodily fluids, or excretions from infected patients. Healthcare workers may become infected when treating LF patients without proper protective equipment, including during the handling of deceased patients. Additionally, laboratory-acquired infections can occur through improper biosafety practices. There is no documented evidence of airborne person-to-person transmission (*3*–*6*).

This report describes the first imported case of LF in China, from Guinea, and details the public health

response measures implemented to prevent secondary transmission.

INVESTIGATION AND RESULTS

Case Report

A 49-year-old female, employed as a cook in Guinea, Africa since March 2024, presented with lumbar pain, abdominal pain, frequent urination with urgency, nausea, vomiting, dry mouth, and general malaise upon returning to China on July 24. She subsequently sought medical evaluation at four different hospitals. The patient's initial presenting symptoms included altered consciousness and unexplained fever. Following treatment, her vital signs stabilized, consciousness cleared, though she developed severe hearing impairment (near-complete deafness). While her reading and writing abilities remained intact, she exhibited scattered cutaneous hemorrhages and bruising at venipuncture sites. Laboratory findings revealed acute renal insufficiency and elevated transaminases, though cardiopulmonary function remained adequate. On August 5, the patient was transferred to a negative pressure isolation room at a designated infectious disease hospital.

During her stay in Guinea, the patient was stationed in Mamou, a region bordering Sierra Leone, where she worked as a camp cook at a factory. The patient reported unremarkable living conditions with no apparent rodent activity and no similar cases among local residents. On July 17, 2024, she developed high fever and anorexia while working in the Mamou Region. Local hospital evaluation yielded positive results for both Plasmodium via microscopic blood smear examination and typhoid fever via Widal test. Treatment with artemisinin and levofloxacin initially provided symptomatic relief.

The patient departed Guinea on July 23, 2024, and arrived in China on July 24 afternoon. She traveled by private vehicle to Mianyang City, Sichuan Province, arriving at the bus terminal that evening. After taking a taxi to the first hospital, she underwent medical evaluation and overnight observation. On July 25, she was discharged and returned to her apartment via private transport. She remained in isolation at home until July 30, when her daughter-in-law found her collapsed and incontinent. The patient was subsequently transported to the second hospital in Jiangyou City. On August 1 and 2, she was transferred via ambulance to the third hospital in Mianyan City and the fourth hospital in Chengdu City, respectively. Cerebrospinal fluid and blood samples were collected on August 3 and 4. On August 5, she was transferred to the ICU negative-pressure isolation ward at the fifth hospital (infectious disease hospital) for treatment. By September 24, the patient had recovered except for persistent hearing loss and was discharged following expert verification. The complete epidemiological timeline is illustrated in Figure 1.

Laboratory Testing

Initial blood samples tested negative for Plasmodium at both Jiangyou CDC and Sichuan CDC on August 1 and 2, 2024, respectively. On August 2, cerebrospinal fluid collected at the third hospital was analyzed using targeted next-generation sequencing (tNGS) at a commercial medical laboratory, which yielded positive detection of LASV. Subsequent fluorescence quantitative Polymerase Chain Reaction (PCR) testing

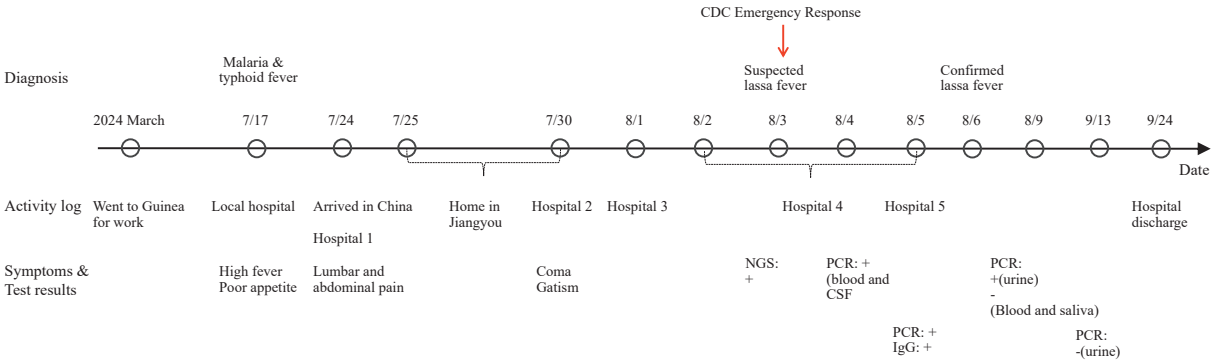

FIGURE 1. Epidemiological timeline of the first imported Lassa fever case in China, 2024.
Note: "−" means LASV negative; "+" means LASV positive.
Abbreviation: PCR=Polymerase Chain Reaction; NGS=Next-Generation Sequencing; CSF=Cerebrospinal fluid; LASV=Lassa virus.

by Sichuan CDC confirmed positive results in both cerebrospinal fluid and blood samples. China CDC conducted comprehensive testing on 5 non-inactivated samples (2 cerebrospinal fluid and 3 blood), with all samples testing positive by fluorescence quantitative PCR and all 3 blood samples showing positive IgG antibodies. Fluorescence quantitative PCR analysis for *Salmonella typhi*, *Salmonella paratyphi* and *Salmonella* yielded negative results. On August 6, following expert consultation and review of epidemiological characteristics, clinical presentation, and laboratory findings confirming both LASV nucleic acid and IgG antibodies, the case was definitively diagnosed as severe LF. Post-treatment monitoring showed negative PCR results for blood and saliva samples, though urine samples remained positive for approximately one month before converting to negative prior to hospital discharge. Whole genome sequencing performed independently by China CDC and Sichuan provincial CDC on cerebrospinal fluid and urine samples revealed LASV genotype IV, consistent with known geographical and epidemiological distribution patterns. Detailed results and specific sampling time are shown in Table 1.

Contact Tracing and Infection Control Measures

Close contacts were defined as: 1) individuals who lived or worked with the patient; 2) personnel involved in medical diagnosis, treatment (invasive or non-invasive), caregiving, laboratory testing, and sample handling who may have had direct contact with the patient's blood, bloody secretions, excreta, or contaminated materials without taking standard protective measures. General contacts were defined as individuals who had contact with the patient outside these categories and did not take standard protective measures. The investigation identified 12 close contacts and 71 general contacts. All contacts underwent temperature monitoring and symptom screening twice a day for 21 days following their last exposure. Three general contacts developed fever symptoms, while all other contacts remained asymptomatic. Blood, saliva, and urine samples were collected from all 12 close contacts both at initial management and at the end of the medical observation period. Blood samples were also collected from the three general contacts with fever. All samples tested negative for LASV by PCR analysis. By August 24, all contacts had completed their medical observation period without developing any suspected clinical symptoms of LF.

Additional public health measures implemented by CDCs included: 1) enhanced infection control protocols at all medical facilities where the patient received treatment; 2) comprehensive environmental disinfection of potentially contaminated areas; 3) stringent management of clinical specimens; and 4) targeted health education about LF for the patient, family members, medical staff, and other contacts.

International Alert

In accordance with the International Health Regulations (IHR), China's national focal point officially reported the case to the World Health Organization (WHO). Following WHO's request, supplementary epidemiological investigation details regarding the patient's residence in Guinea were provided. However, since the airline had already departed when the case was identified, further investigation of potential exposure among flight passengers and crew members could not be conducted.

DISCUSSION

Research indicates that LF exhibits significant regional variation in case fatality rates across West Africa, ranging from 16.5% to 50.0%. An estimated 300,000 new infections and 5,000 deaths occur annually throughout Western Africa, with recent Nigerian outbreaks reporting fatality rates of up to 25.4% (*7*). Cases exported from Africa have been documented in various countries and regions, with some instances resulting in secondary transmission (*3*–*4*). The disease demonstrates particularly high mortality among symptomatic cases and poses severe risks to pregnant women and fetuses. The WHO has designated LF as a priority disease for global research and development. Given the strengthening ties between China and Africa, the risk of LF importation into China is likely to increase, necessitating proactive preparedness measures.

This imported case of LF was effectively contained, and no local transmission occurred. This conclusion is supported by the following factors. First, LF is endemic to West Africa, including Guinea, with no previous cases detected in China. The patient's work history in Guinea from March to July 2024, coupled with symptom onset prior to entering China, indicates infection occurred during her stay in Guinea. Second,

TABLE 1. Laboratory test results of biological samples from the patient.

Samples	Plasmodium by microscopic examination	*Salmonella typhi*, *Salmonella paratyphi* and *Salmonella* by fluorescence quantitative PCR	LASV by fluorescence quantitative PCR				Blood by LASV IgG antibody	LASV Genetic sequence
	Blood	Blood	CSF	Blood	Urine	Saliva		
Aug,1	Negative*	–	–	(+) 31.45§	–	–	(+)§	
Aug,2	Negative*	–	(+) 34.92*, 30.79§	–	–	–	–	(+)†
Aug,3	–	–	(+) 32.89§	(+) 36.66*, 35.28§	–	–	(+)§	(+)§ Clade Ⅳ
Aug,4	–	–	–	(+) 37.18§	–	–	(+)§	–
Aug,8	–	Negative¶	–	Negative*	–	–	–	–
Aug,9	–	–	–	Negative*	(+) 33.36*	Negative*	–	–
Aug,12	–	–	–	Negative*	(+) 31.62*	Negative*	–	–
Aug,16	–	–	–	–	(+) 31.52*	–	–	–
Aug,31	–	–	–	–	(+) 33.22*	–	–	–
Sept,10	–	–	–	–	(+) 36.57*	–	–	–
Sept,13	–	–	–	–	Negative*	–	–	–
Sept,17	–	–	–	–	Negative*	–	–	–

Note: "–" means no sample collected; "+" means positive.
Abbreviation: LASV=Lassa virus; CSF=Cerebrospinal fluid.
* Sichuan Provincial CDC.
† Third-party testing company.
§ China CDC.
¶ Chengdu CDC.

the primary reservoir host, *Mastomys* species, has not been documented in China. Human-to-human transmission typically occurs through contact with infected bodily fluids, with secondary cases predominantly occurring among caregivers, healthcare workers, and those handling deceased patients. Among the case's contacts, only three close contacts developed fever due to other illnesses, with no link to LF. All 83 contacts completed 21-day observation periods, demonstrating neither clinical signs of LF nor positive results on reverse transcription-polymerase chain reaction (RT-PCR) testing. Third, immediate implementation of comprehensive control measures — including patient isolation, contact tracing, medical observation, and environmental disinfection — effectively contained any potential spread. The patient was isolated and medically monitored until testing negative in blood, saliva, and urine.

Looking ahead, China faces increased risk of emerging infectious disease importation, including LF, particularly in major port cities due to international travel and commerce. This necessitates several strategic improvements: enhanced entry quarantine and disease screening capabilities in areas with significant international traffic; improved health declaration awareness among travelers; strengthened clinical vigilance for emerging infections in healthcare settings, including thorough travel history documentation; and expanded laboratory capacity for LF testing at all levels of disease control organizations. When feasible, implementation of advanced technologies such as multi-pathogen detection and metagenomic sequencing can facilitate rapid identification of unknown pathogens. Enhanced diagnostic capabilities could significantly improve clinical management and prevention of LF in at-risk populations (*8*). While no approved vaccines or therapeutics currently exist for LF, several promising candidates are undergoing clinical trials (*9*). Accelerated vaccine development and commercialization efforts remain crucial. For individuals traveling to or working in Africa, comprehensive education about LF prevention is essential, emphasizing rodent avoidance, food protection, and strict hygiene practices. Organizations should provide targeted health education regarding locally prevalent infectious diseases to Chinese residents abroad.

Conflicts of interest: No conflicts of interest.

Ethical statement: Received ethical approval from the Ethics Committee of the Chengdu Center for Disease Control and Prevention, China (approval number: 2025018).

Funding: Supported by Beijing Natural Science Foundation (L222027) and Major and Application Projects of Chengdu Science and Technology Key R&D Support Plan (2021-YF09-00061-SN).

doi: 10.46234/ccdcw2025.066

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Submitted: November 21, 2024
Accepted: February 20, 2025
Issued: March 21, 2025

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Notes from the Field

Identification and Evolution of the First Isolated *Brucella abortus* Strain from a Human Brucellosis Case — Tianjin Municipality, China, August 2024

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Brucellosis remains a significant global zoonosis with profound negative implications for human health and economic systems worldwide (*1*). The disease is caused by Gram-negative, facultative intracellular bacteria belonging to the genus *Brucella* (*2*). Currently, 13 recognized *Brucella* species exist, with *B. melitensis*, *B. abortus*, and *B. suis* representing the predominant human pathogens (*3*). Human infection with *Brucella* spp. typically occurs through consumption of contaminated animal products or direct contact with infected animals (*4*).

In August 2024, a 71-year-old male farmer from Baodi District, Tianjin Municipality, China presented with persistent fever, excessive sweating, muscle and joint pain, and fatigue. The patient reported regularly entering a sheep pen without protective equipment approximately every 7 days, though he had no history of travel to known brucellosis-endemic regions. A *Brucella* strain, designated "BD002," was isolated from the patient, confirming the diagnosis of brucellosis. Epidemiological investigation suggested that the sheep in the patient's care were the likely source of infection.

For isolation of the *Brucella* strain, 500 μL of blood sample was inoculated onto a blood agar plate and incubated at 37 °C with 5% carbon dioxide for 72 hours, yielding off-white single colonies. The bacterial suspension was subsequently prepared and inactivated for DNA extraction and polymerase chain reaction (PCR) analysis. The brucella cell surface protein 31 polymerase chain reaction (BCSP31-PCR) test for genus identification revealed the characteristic 224-bp DNA band, while the abortus, melitensis, ovis, and suis polymerase chain reaction (AMOS-PCR) species-identification test yielded no detectable DNA bands (*5*). Notably, AMOS-PCR can identify *B. abortus* biovars 1, 2, and 4, *B. melitensis*, and *B. ovis*, but does not encompass all biovars of *Brucella* spp.

To further characterize strain BD002, we conducted both multi-locus sequence typing (MLST) and next-generation sequencing (NGS) through GENEWIZ Co. and BGI-Shenzhen, China, respectively. For MLST, 9 *Brucella* housekeeping genes were amplified and subjected to Sanger sequencing, which identified BD002 as sequence type 2 according to the PubMLST database. NGS analysis generated 23 contigs using SPAdes v3.7, with a total genome length of 3,252,744 base pairs (*6*). The assembled genomic sequences have been deposited in the National Genomics Data Center (Accession No. GWHFJIX00000000.1) (*7*). We conducted phylogenetic analysis using the BD002 genome alongside 386 *B. abortus* genomic sequences from the NCBI Genome database, employing Snippy v4.6 and RAxML-ng v1.2.2 with strain 544 (Accession No. GCA_000369945.1) as the reference sequence for core-genome single-nucleotide polymorphism analysis (*8*). The resulting maximum likelihood phylogenetic tree was visualized using R v4.3.1 and *ggtree* v3.10.1 (*9*). Phylogenetic analysis revealed 4 distinct clades, with BD002 clustering within Clade B (Figure 1A). Notably, BD002 formed a sub-clade with strains from other Chinese provincial-level administrative divisions, including Inner Mongolia, Jilin, Heilongjiang, Beijing, Hebei, and Shanxi, as well as strains from Russia, Georgia, Mongolia, and other countries (Figure 1B).

Human brucellosis in Tianjin affects approximately 200 individuals annually, with *B. melitensis* identified as the predominant causative agent. This study documents the first isolation of *B. abortus* from a human case in Tianjin, indicating the circulation of multiple *Brucella* species within the region.

Compared with traditional AMOS-PCR methodology, MLST and NGS techniques provide superior accuracy in *Brucella* spp. identification and offer substantially higher resolution for phylogenetic analysis. These advanced molecular approaches should be fully integrated into routine etiological surveillance of human brucellosis.

Additionally, particular attention should be directed

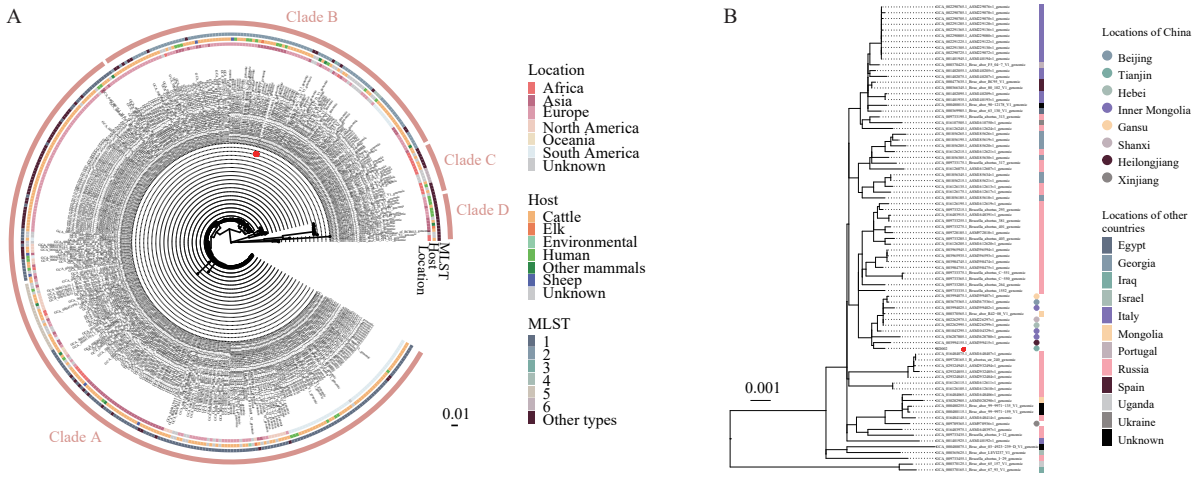

FIGURE 1. The ML phylogenetic tree of *B. abortus* genomes based on cg-SNP. (A) The ML phylogenetic tree was constructed using the genomic sequence of strain BD002 integrated with 386 additional *B. abortus* genomic sequences and associated metadata obtained from the NCBI genome database. (B) A detailed view of the subclade within clade B containing strain BD002 is presented, with countries of strain origin indicated by differently colored circles. The BD002 strain is specifically highlighted with a red circle.
Note: For (A), the analysis revealed four distinct worldwide clades of *B. abortus* strains, highlighted in orange. The continental origin, host species, and sequence types of the strains are represented by differently colored squares. Abbreviation: ML=maximum likelihood; cg-SNP=core-genome single-nucleotide polymorphism.

toward livestock handlers who neglect to appropriate personal protective equipment and fail to implement adequate disinfection and hygiene practices following occupational exposure (*10*).

doi: 10.46234/ccdcw2025.067

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Submitted: December 30, 2024
Accepted: March 13, 2025
Issued: March 21, 2025

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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. 12 Mar. 21, 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