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Transmission and infection of arboviruses, volume II

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Transmission and infection of arboviruses, volume II

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Mayaro Virus as the cause of Acute Febrile Illness in the Colombian Amazon Basin

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Introduction: Mayaro Fever (MF) is a tropical disease caused by the Mayaro virus (MAYV), with outbreaks documented in Latin America.

Methods: A hospital-based fever surveillance in Leticia, Colombian Amazon, collected sera from 1,460 patients aged 5-89 between December 2020 and April 2023.

Results: Dengue and malaria were the main diagnoses (19.4 and 5.8%, respectively), leaving 71.4% of cases unidentified after testing. Metagenomic sequencing and real-time RT-qPCR testing identified MAYV in two patients (25-year-old male and an 80-year-old female) exhibiting typical symptoms, of MF including rash, joint pain, and fever. Phylogenetics analysis of these two viruses revealed a close relationship to Peruvian strains within the MAYV D genotype.

Discussion: The study of AFI in Leticia, Colombia, identified dengue as prevalent, with malaria, COVID-19, Influenza, and Zika viruses also detected. Despite extensive testing, most cases remained unexplained until metagenomic sequencing revealed MAYV, previously unseen in Colombia but known in neighboring countries.

Conclusion: This study presents the first near full-length genomes of MAYV in Colombia, highlighting the need for further seroprevalence studies and enhanced surveillance to understand and control the spread of the virus in the region.

KEYWORDS

Mayaro Virus, Acute Febrile Illness, whole genome sequencing, Colombia, fever

Introduction

Mayaro Virus (MAYV) is an arthropod-borne, single-stranded RNA virus that belongs to the Semliki Forest antigenic sero-complex, a serological group within the alphavirus genus (family *Togaviridae*) (Acosta-Ampudia et al., 2018). MAYV was initially identified in the Mayaro county, Trinidad in 1954 and since then, several cases of Mayaro fever

(MF) have been reported in Latin America and the Caribbean (Caicedo et al., 2023). Four distinct MAYV genotypes have been recently identified in South America (Mavian et al., 2017). MAYV infections pose a significant health concern in Latin America, particularly in regions like South America, where the virus is endemic. Brazil, Peru, and Venezuela have documented the highest incidence and prevalence of Mayaro fever (Del Carpio-Orantes et al., 2022). Despite being relatively understudied compared to other mosquito-borne viruses like dengue and Zika, MAYV has been identified as a cause of Acute Febrile Illness (AFI) in the region. While cases vary due to underreporting and limited surveillance, sporadic outbreaks and localized transmission have been documented, particularly in areas with favorable ecological conditions for the virus and its mosquito vectors (Diagne et al., 2020). The impact of MAYV infections extends beyond the immediate health effects, often affecting vulnerable populations and burdening healthcare systems already strained by other infectious diseases. Understanding the epidemiology and impact of MAYV infections is crucial for effective public health responses and mitigating the spread of the virus in Latin America.

Acute Febrile Illness (AFI) represents a significant health challenge in tropical regions, where many infectious pathogens circulate, often leading to similar clinical presentations. Clinical manifestations may include arthralgia/arthritis, a maculopapular rash, and other symptoms such as headache, myalgia, retro-orbital pain, vomiting, and diarrhea (Acosta-Ampudia et al., 2018). Diagnosing MF can be difficult because its signs and symptoms can be easily confused with other co-occurring infections such as malaria and arboviral diseases such as dengue, chikungunya, and Zika (Arroyave et al., 2013). Consequently, confirmatory laboratory testing is required (Diagne et al., 2020). However, it is very limited in most of the tropical areas. Despite extensive laboratory screening, many AFI cases remain without a specific etiology identified. This diagnostic gap hampers individual patient management and impedes effective public health responses to emerging infectious threats (Barathan, 2024). Metagenomic next-generation sequencing (mNGS), a powerful molecular technique capable of detecting a broad range of pathogens directly from clinical samples, offers a promising solution to this diagnostic dilemma (Batool and Galloway-Peña, 2023). Metagenomic sequencing can unveil the presence of known and novel pathogens by analyzing the entire genetic content within a sample, providing crucial insights into disease causation and facilitating targeted interventions for diagnosed and undiagnosed AFI cases in tropical regions. Several studies on AFI in Colombia have observed a great proportion of febrile cases that remained with unknown diagnoses after routine testing or disease investigation. Therefore, this study aimed to employ mNGS as a diagnostic tool to identify the causative agents of AFI in different regions of Colombia where traditional laboratory methods often fail to provide etiological diagnoses. Through comprehensive genomic analysis of clinical samples, we aimed to elucidate the spectrum of pathogens contributing to AFI, including known and novel infectious agents. By characterizing the microbial diversity in these samples, our goal was to enhance our understanding of AFI epidemiology, inform clinical management strategies, and contribute to developing targeted interventions for improved public health outcomes in tropical settings such as Colombia.

TABLE 1 Diagnostics test results on serum samples from febrile patients in Leticia, Colombia.

Diagnostic test	Results (%)	Reference method
DENV RT-PCR	284/1460 (19.4)	Waggoner et al., 2016
DENV Duo RDT	263/1460 (18.0)	Bioline Dengue Duo (Dengue NS1 Ag + IgG/IgM), Abbott, Illinois, USA
	309/1460 (21.2)	
	973/1460 (66.6)	
Malaria PCR	85/1460 (5.8)	Kamau et al., 2013
Malaria RDT	83/1460 (5.7)	SD Bioline Malaria Ag P.f/Pan, Abbott, Illinois, USA
SARS-CoV-2 RT-PCR	43/1460 (2.9)	Corman et al., 2020
Influenza RT-PCR	4/1460 (0.3)	de-Paris et al., 2012
ZIKA RT-PCR	1/1460 (0.1)	Waggoner et al., 2016
MAYV RT-PCR	1/80 (1.25)	Waggoner et al., 2016
Negative to all laboratory tests	1043/1460 (71.4)	

RT-PCR, reverse transcription polymerase chain reaction; RDT, rapid diagnostic testing.

Materials and methods

Study setting

Repository samples used in this study were obtained from a cross-sectional hospital-based fever surveillance program (HFSP) conducted since December 2020 in four different regions of Colombia (Villavicencio, Apartado, Acacias, and Leticia; Figure 2A). This program is part of an ongoing Virus Discovery Research Plan of the Abbott Pandemic Defense Coalition (Averhoff et al., 2022) aimed at understanding infectious causes causing AFI of unknown origin. This study was reviewed and approved by the Ethics Committee of Corporacion Investigaciones Biologicas (CIB 10102022). Written informed consent was secured from adults (18 years or older). Informed assent was obtained from minors (<18 years), and written consent was obtained from their parents or guardians on their behalf. At the enrollment, participants agreed to use their data and left-over specimens for future studies. For the purpose of this study, acute-phase sera collected (from December 2020 to April 2023) from 1,460 febrile individuals (aged 5–89 years) from the municipality of Leticia (located in the Amazon region of Colombia) were used.

AFI case definition

For this study, AFI was defined as recent onset of fever (body temperature $\geq$ 38°C at the time of consultation or self-reported history of fever within the preceding seven days) without an obvious focus of infection. AFI was associated with non-specific symptoms such as headache, body rash, and muscle and joint pains (Tun et al., 2016).

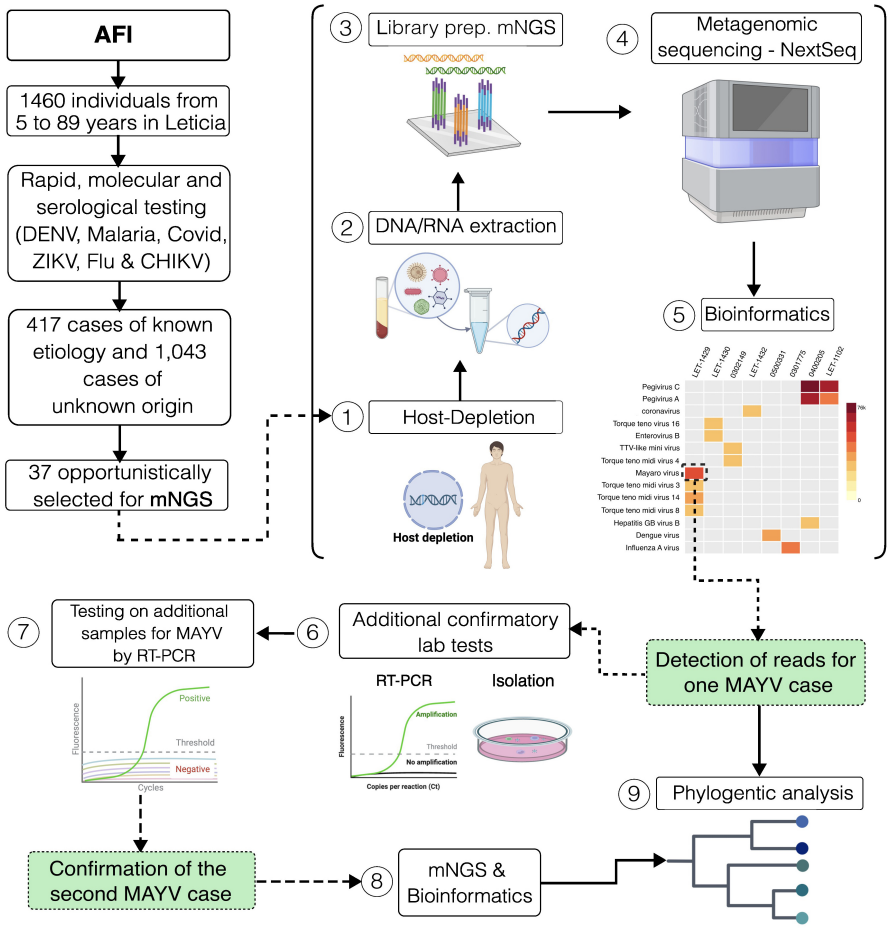

FIGURE 1
Graphical summary of the study design. Study setting and workflow for sample processing.

Laboratory testing

Samples were initially tested for dengue, malaria at the point-of-care using rapid diagnostic tests (SD Bioline Dengue Duo and Bioline Malaria Ag P.f/Pan, Abbott, IL, USA). Serum and whole blood samples were aliquoted and stored at −80°C until processing at the central lab (One Health Genomic Lab, Universidad Nacional de Colombia, Medellin). Samples were tested by molecular assays (reverse transcription RT-PCR and/or PCR) to detect Malaria, Dengue (DENV), Zika (ZIKV), Chikungunya (CHIKV), Influenza (IV), and Severe-Acute-Respiratory-Syndrome-related Coronavirus (SARS-CoV-2), following protocols described elsewhere (de-Paris et al., 2012; Kamau et al., 2013; Waggoner et al., 2016; Corman et al., 2020). All samples with unknown etiology were kept at −80°C for further testing.

Virus discovery and sequencing

After laboratory testing, a subset (n = 37) of the repository specimens with unknown etiology was non-randomly selected based on the reported clinical manifestations such as vomit, abdominal pain, skin rash, hemorrhages, and/or diarrhea. Selected samples were subjected to Metagenomic Next Generation Sequencing (mNGS) . mNGS was conducted using a NextSeq2000 (Illumina, California, USA) following a protocol described elsewhere (Berg et al., 2020). Contamination was identified when pathogens detected by mNGS did not align with the clinical diagnosis, had not been clinically confirmed, and were not regarded as the cause of the clinical disease. Sequencing data were analyzed using an open-source cloud-based metagenomics platform (Tun et al., 2016; Kalantar et al., 2020) using the following filters: category (viruses and viroids), subcategory (Viruses – Phage), threshold (NT rPM > = 10; NR rPM > = 5; NR L (alignment length in bp) > = 50), and pathogen tag (known_pathogens).

Bioinformatics and phylogeographic analysis

Sequence alignment was performed on the MAYV sequences from this study using Samtools v1.15 and BWA v0.7.17 (Li and Durbin, 2009; Li et al., 2009). Complete genome sequences of MAYV were downloaded from the public database GenBank (National Center for Biotechnology Information) and compared to the sequences from this work using MAFFT v7.520 (Katoh et al., 2019). A Bayesian phylogeographic analysis

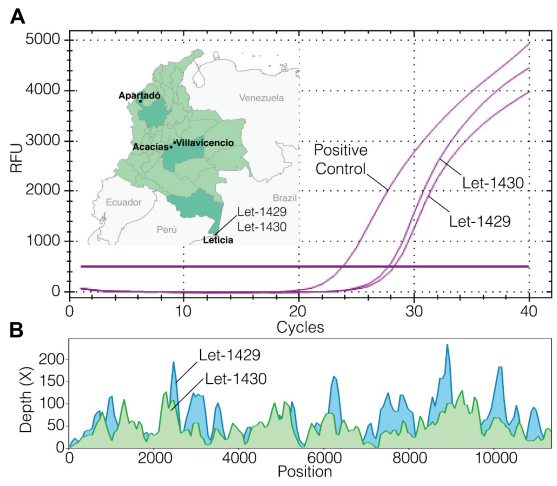

FIGURE 2
Fever surveillance, Mayaro virus (MAYV; Let-1429 and Let-1430) detection, viral isolation, and sequencing. **(A)** Map of Colombia showing the location where the Virus Discovery Research Program is conducted and origin of MAYV infections detected, and real-time RT-PCR test results on cell culture supernatants after 72 h of infection; **(B)** mNGS coverage plot for near full virus genomes.

was performed (Figure 2) on the MAYV sequences obtained as described previously (Ciuoderis et al., 2023).

Results

From December 2020 to April 2023, acute-phase sera were collected from 1,460 febrile individuals (aged 5–89 years, and 57.7% were female) from the municipality of Leticia, located in the Amazon region of Colombia. A total of 19.4% (284/1460) patients were confirmed as dengue after laboratory testing, while 5.8% (85/1460) were diagnosed as malaria, 2.9% (43/1460) as COVID-19, 0.3% (4/1460) as Influenza, and 0.1% (1/1460) as Zika, respectively (Table 1). However, 71.4% (1043 out of 1460) of these AFI cases remained unexplained (Figure 1). To address this, 37 samples were non-randomly selected for mNGS (Figure 1). Most confirmed dengue cases occurred in individuals aged 11–35 years, with peak incidences occurring during the rainy season (second and third trimester). Malaria cases were predominantly found in males aged above 35 years, with peak incidences occurring during July and September. COVID-19 cases were predominantly found in females aged 26–35 years, with incidences occurring throughout the period of study. Influenza cases were widely distributed among age groups and throughout the study period.

Results from mNGS revealed that a near full-length (94%) genome of MAYV was detected in one specimen (Figure 2B). This MAYV infection was referred to as the index case, occurring on April 2023 in a 25-year-old male (Let-1430) of Indigenous ethnicity. The subject reported fever, rash, joint pain, retro-orbital pain, shaking chills, abdominal pain, weakness, myalgia, headache, vomiting, and diarrhea. The subject did not report recent travel to areas outside of the vicinity. Additional serum specimens of unknown etiology (n = 80) from Leticia were tested for MAYV infection by real-time RT-qPCR, targeting the untranslated region of the nonstructural protein 1 gene of MAYV. The RT-qPCR method was conducted following a protocol described elsewhere (Waggoner et al., 2018). Serum samples were selected within a month before and after the index case was identified, and from these, another MAYV infection case was detected. This case occurred in an 80-year-old woman of mixed race (Let-1429) who reported the same symptoms as the index case except for the rash. Metagenomic NGS was also conducted in this specimen, identifying reads for MAYV with high identity (>95%) to the reference genome NC_003417. MAYV isolates were successfully obtained from both cases after inoculating infected serum samples onto sub-confluent Vero cells, following a protocol described elsewhere (Blohm et al., 2019). Virus isolation was confirmed by RT-qPCR (Figure 2A).

After mNGS, phylogenetic analysis revealed that MAYV from this study clustered together with genotype D sequences reported from Peru. In addition, phylogeographic analysis using available MAYV genomes revealed that MAYV strains in Colombia may have been introduced from Peru through two independent events (Figure 3) and, therefore, have not originated from a single outbreak. Two full-length genomes of MAYV analyzed in this work were deposited in the GenBank under accession numbers PP505831 and PP505832. Genomic sequencing data analysis for other samples revealed Pegivirus C (n = 1), *Prevotella melaninogenica* (n = 11), *Aeromona caviae* (n = 9), *Mycobacteroides chelonae* (n = 6), *Haemophilus influenzae* (n = 3), *Staphylococcus lugdunensis* (n = 2), *Mycobacterium avium* (n = 2), *Klebsiella aerogenes* (n = 1), *Bordetella bronchiseptica* (n = 1) as the most common atypical pathogens identified by mNGS.

Discussion

After investigating the landscape of AFI among a cohort of 1,460 individuals in the municipality of Leticia, situated within the Amazon region of Colombia, and primarily focusing on the identification and characterization of pathogens causing such illness, we found that a multifaceted picture emerged with the highest number of cases confirmed of dengue and smaller occurrences of malaria, COVID-19, Influenza and Zika. However, most (71.4%) of AFI cases remained unexplained. MNGS and other laboratory techniques were employed within this enigmatic pool, leading to the pivotal discovery and characterization of MAYV. To our knowledge, while MAYV is recognized as a source of AFI in South America, there have been no documented cases of MAYV infections in Colombia to date.

In agreement with our findings, genotype D of MAYV has been the most reported circulating genotype in South America (Mavian et al., 2017). Furthermore, there is strong evidence that MAYV is the cause of AFI outbreaks in neighboring countries, central and South America (Lorenz et al., 2019; Celone et al., 2023). A recent report from Peru showed that MAYV infections occurred in 17.3% of 496 febrile cases studied, of which 10.9% were MAYV mono-infections and 6.4% were co-infections with DENV (Aguilar-Luis et al., 2021). Similarly, another study in the same country confirmed MAYV infection by RT-PCR and sequencing in 11.1% (40/359) of the febrile patients tested (Aguilar-Luis et al., 2020). Confirmation of MAYV infections has recently been reported in mosquitoes and

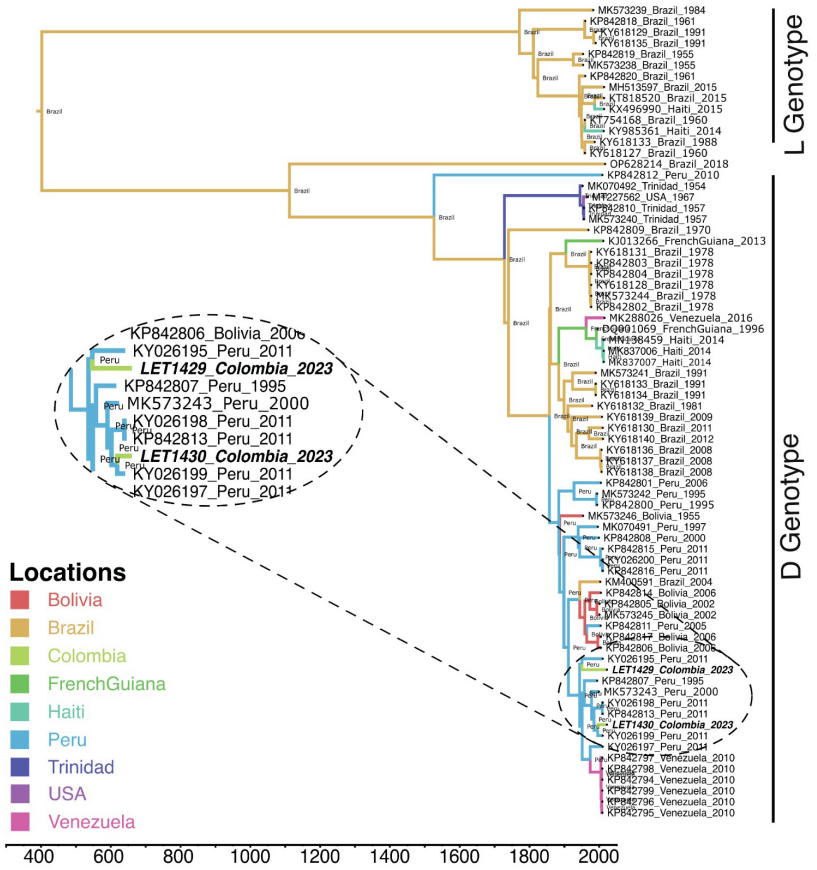

FIGURE 3
Phylogeographic and phylogenetic analysis of Mayaro virus from Colombia. A time-scaled phylogeographic maximum clade credibility tree was constructed using Bayesian Markov Chain Monte Carlo simulations with Beast v1.10.4. The model GTR + I + G was selected as the best-fit model based on Bayesian information criteria using ModelFinder from IQ-Tree program v2.1.2. The analysis included MAYV sequences from this study (bold italic) along with sequences available from GenBank. Branch colors indicate the phylogeographic origin. Bayesian posterior values (≥0.7) are shown in nodes.

humans in Brazil (Saatkamp et al., 2021; de Curcio et al., 2022). Natural MAYV infection has also been documented in Culicidae mosquito species in several South American countries, including Colombia (Caicedo et al., 2023), but additional evidence supporting their involvement in the MAYV transmission in Colombia is required.

There is some evidence of natural infection of MAYV in vector mosquitoes (*Psorophora albipes*) in the northeast region of Colombia (Caicedo et al., 2023). However, to our knowledge, MAYV infection in humans has not been previously reported in this country. In addition, detection of MAYV infection may also be challenging when using only molecular testing. The relatively short (approximately 3–10 days) viremia could limit MAYV detection (Pezzi et al., 2019). Therefore, further seroprevalence studies are highly recommended to comprehensively assess the burden of this disease in the Amazon River basin and Colombia. A recent review of population-based studies reported a seroprevalence of MAYV infection ranging between 6 and 67% for South American countries (Caicedo et al., 2023), and recently, a seroprevalence study in Mexico showed that MAYV infection occurred in 1% of adults who reported having suffered from an arboviral illness at some point in their lives (Del Carpio-Orantes et al., 2022). Consequently, our findings indicate that MAYV infections may be cryptically occurring in some regions of Colombia, and cases are being misdiagnosed, thus highlighting the need for active surveillance of MAYV in Colombia.

Atypical viral pathogens such as Pegivirus C and bacteria such as *Klebsiella pneumoniae* and non-tuberculous mycobacteria have been reported in febrile and patients with respiratory illness after metagenomic sequencing analysis (del Valle-Mendoza et al., 2017; Oguzie et al., 2023; Shen et al., 2023; Wei et al., 2023; Yang et al., 2024). However, further research is needed to assess its implications in disease pathogenesis, specifically in disease cases of AFI of unknown origin.

Our results contribute to the understanding of MAYV in the Americas and emphasize the importance of implementing metagenomic NGS to detect pathogens causing AFI of unknown origin, especially when other specific detection methods, such as PCR, are not available routinely used for diagnosis. There is also a need to develop commercially available diagnostic tests for MAYV to understand the disease burden better. Finally, we strongly suggest increasing educational campaigns about MAYV for the community, especially for healthcare personnel in high-risk areas of Colombia. Such targeted strategies are pivotal to ensure that communities are aware of this disease and that

healthcare professionals in the region are provided with the most recent knowledge to proficiently detect, manage, and address these MAYV infections.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: https://www.ncbi.nlm.nih.gov/genbank/, PP505831, https://www.ncbi.nlm.nih.gov/genbank/, PP505832.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Corporación de Investigaciones Biológicas. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

LP-R: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review and editing. KC: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review and editing. JU: Data curation, Methodology, Writing – review and editing. IM: Methodology, Writing – review and editing. VV: Methodology, Writing – review and editing. AA-A: Methodology, Writing – review and editing. MB: Formal analysis, Resources, Writing – review and editing. GC: Funding acquisition, Supervision, Writing – review and editing. JH-O: Writing – review and editing. JO: Funding acquisition, Supervision, Writing – review and editing.

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Conflict of interest

GC and MB are Abbott employees and shareholders. JO is a single owner of Vaccigen, LLC. JO and JH-O are employees and shareholders of VaxThera.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Drivers and epidemiological patterns of West Nile virus in Serbia

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Background: West Nile virus (WNV) is an emerging mosquito-borne pathogen in Serbia, where it has been detected as a cause of infection in humans since 2012. We analyzed and modelled WNV transmission patterns in the country between 2012 and 2023.

Methods: We applied a previously developed modelling approach to quantify epidemiological parameters of interest and to identify the most important environmental drivers of the force of infection (FOI) by means of statistical analysis in the human population in the country.

Results: During the study period, 1,387 human cases were recorded, with substantial heterogeneity across years. We found that spring temperature is of paramount importance for WNV transmission, as FOI magnitude and peak timing are positively associated with it. Furthermore, FOI is also estimated to be greater in regions with a larger fraction of older adult people, who are at higher risk to develop severe infections.

Conclusion: Our results highlight that temperature plays a key role in shaping WNV outbreak magnitude in Serbia, confirming the association between spring climatic conditions and WNV human transmission risk and thus pointing out the importance of this factor as a potential early warning predictor for timely application of preventive and control measures.

KEYWORDS

mosquito, vector-borne, mathematical model, West Nile virus, *Culex*

1 Introduction

West Nile virus (WNV) is a mosquito-borne virus, part of the genus Flavivirus which is rapidly becoming one of the most widespread emerging pathogens in Europe (1). It is maintained in an enzootic cycle between avian hosts and mosquito vectors, especially those belonging to the *Culex* genus (2). Mosquitoes acquire the infection after biting an infected bird and, after an incubation period, can then transmit the virus through subsequent blood meals. Mammals, including humans and equines, act as incidental dead end hosts in the natural transmission cycle, i.e., they cannot transmit the virus to mosquitoes (3). However, human-to-human transmission may occur through blood transfusions or organ transplantation (3).

Although most of the human infections are asymptomatic, about 25% present symptoms such as fever and headache, and less than 1% develop severe neurological complications which can have a fatal outcome (3).

WNV is characterised by high genetic diversity. Phylogenetic analysis has identified at least eight evolutionary lineages of which WNV lineages 1 (WNV-1) and 2 (WNV-2) are the most widespread and pathogenic, causing continuous outbreaks in humans and animals around the world (4–7). More specifically, WNV-2 accounts for 82% of all WNV sequences detected in Europe so far, being found in 15 European countries (6). Between 2012 and 2023, about 6,700 human infections were recorded in the European Union, with large inter-annual differences (8).

Different environmental factors may influence WNV transmission (9). For instance, temperature affects mosquito biology: warmer conditions increase the developmental rate of immature stages but also decrease survival (10–12). Higher temperatures also decrease the incubation period of the virus in the vector population (13). Land use plays a key role as well at shaping not only mosquito dynamics but also composition of both vector and host populations (9). Additionally, counterfactual simulations suggest that the establishment of the current areas of WNV circulation in Europe can be largely attributed to climate change (14), thus highlighting the importance of climatic conditions for WNV circulation.

In Serbia, WNV infection in humans was confirmed for the first time in 2012 (15), and *Culex pipiens* mosquitoes are considered to be the major vector for WNV transmission in the country (16, 17).

In this study, we analyzed and modelled WNV transmission patterns in Serbia between 2012 and 2023. We applied a previously developed modelling approach (18) aiming to quantify epidemiological parameters of interest and to identify the most important environmental drivers of the force of infection (FOI) in the human population in the country.

2 Methods

Serbia is a country 88,499 km^2 wide located in Central Europe with about 6.6 million inhabitants. WNV human case-based data were provided by the Serbian National Public Health Institute and include date of disease onset, importation status (i.e., whether the infection was acquired in Serbia or abroad), age group, gender and the probable place of infection at the NUTS (Nomenclature of territorial units for statistics) 3 level (19). We restricted our analysis to probable and confirmed autochthonous human cases with known place of infection.

As in Marini et al. (18), we denote by $h_{y,i}(w)$ the number of recorded WNV human cases with region *i* as place of infection with symptoms onset occurred during week *w* of year *y* ($w \in \{1, \ldots, 52\}$, $y \in \{2012, \ldots, 2023\}$), by $H_{y,i}$ the whole time series, i.e., $H_{y,i} = \bigcup_{w=1}^{52} h_{y,i}(w)$, and by $\Sigma_{y,i}$ the total number of cases with place of infection, identified as *i*, recorded during year *y*, i.e., $\Sigma_{y,i} = \sum_{w=1}^{52} h_{y,i}(w)$.

We modelled observed epidemiological curves using the FOI-model proposed in Marini et al. (18). We assumed $h_{y,i}(w)$ coming from a Poisson distribution with average $\sum_{t \in T_w} N_i \cdot \lambda_{y,i}(t)$, where $\lambda_{y,i}(t)$ denotes the WNV FOI (i.e., the rate at which susceptible humans acquire the infection) in region *i* and year *y* at day *t*, T_W represents the set of days in week *w* and N_i is the number of inhabitants of the region.

The number of inhabitants, also stratified by age group, for each considered NUTS3 region was retrieved from the Eurostat database (20).

As in Marini et al. (18), we assumed that the FOI for region *i* and year *y* could be modelled through the density function of a normal distribution, i.e.

$$\lambda_{y,i}(t) = c_{y,i} \cdot \frac{1}{\sigma_{y,i}\sqrt{2\pi}} e^{-\frac{1}{2}\left(\frac{t-\mu_{y,i}}{\sigma_{y,i}}\right)^2}$$

Where $\mu_{y,i}$ and $\sigma_{y,i}$ represent, respectively, the average and standard deviation of the distribution and $c_{y,i}$ is a magnitude rescaling factor.

Hence, $\mu_{y,i}$ indicates the Julian day of year *y* for which λ reaches its maximum in region *i*, $\sigma_{y,i}$ provides an estimate for the length (in days) of the epidemiological season and finally $c_{y,i}$ is a measure of the FOI magnitude in that year and geographical area. These three parameters were estimated by matching the generated epidemiological curve to the observed data through a maximum likelihood approach (considering only series with $\Sigma_{y,i} \geq 5$, i.e., NUTS3 regions and years with at least 5 cases). We denote with M, S and C the estimated distributions of $\mu_{y,i}$, $\sigma_{y,i}$ and $c_{y,i,\ respectively.}$ Additional modelling details can be found in Marini et al. (18).

We then quantified through linear models (see below) the relationships between the response variables S, M and C with a set of 6 covariates of potential interest defined as in Marini et al. (18):

1) $\eta(i)$: the total percentage of Corine Land Cover (CLC) labelled as urban or agricultural area. This measure can be interpreted as a proxy for the anthropogenic impact on the region *i*. Proportions of land cover classes for each spatial unit were derived from the 2018 CLC data inventory (21).

2–3) $T_{spring(y,i)}$ and $T_{summer(y,i)}$: respectively the average spring (April–May) and summer (June–July) Land Surface Temperature (LST) recorded in region *i* during year *y*. Monthly 5 km resolution Land Surface Temperature (LST) was derived from the MODIS (Moderate Resolution Imaging Spectroradiometer) MOD11c3 and VNP21A1D datasets (22, 23).

4–5) $P_{spring(y,i)}$ and $P_{summer(y,i)}$: respectively the cumulative spring (April–May) and summer (June–July) precipitation occurred in region i during year y. Monthly 5 km resolution cumulative precipitation data were derived from downscaled daily ECMWF (European Centre for Medium-Range Weather Forecasts) ERA5-Land datasets and downloaded from the Climate Data Store (24).

6) $\varepsilon(i)$: the fraction of people older than 65 years living in region *i* (20).

We first computed a full Linear Model which can be represented by the following equation:

$$Y \sim \eta + T_{spring} + T_{summer} + P_{spring} + P_{summer} + \varepsilon$$

Where Y can either be S, M or log(C) (we normalised the C distribution by log-transforming it).

We checked for potential collinearity among explanatory variables by computing Variance Inflation Factors (VIFs) (25). We then computed all possible submodels and selected as best the model with the lowest Akaike Information Criterion (AIC) score and whose coefficients were all statistically significant. Model assumptions were verified by checking residuals distributions and by plotting residuals versus fitted values and versus each covariate in the model (25).

All analysis was carried out in R v4.3.1 (26) using libraries "tidyverse" (27) and "MuMIn" (28). The R functions used to perform the model fit can be found at https://github.com/giomarini/epiCurve-repository.

3 Results

Between 2012 and 2023, a total of 1,387 autochthonous human cases were reported from 23 different NUTS3 regions (Figure 1A), all belonging to WNV-2 (29). Cases were mostly males (852, 61.4%). The lowest number of infections was observed in 2021 (20 cases), and the highest (415 cases) in 2018. For the year 2020 data collection was limited due the Covid-19 pandemic: 23 suspected WNV infection cases were notified, but they were not confirmed by laboratory analysis and they were not included in the analyses.

The cumulative epidemiological curve (total number of cases per week of symptoms onset across all years and regions, Figure 1C) clearly shows a peak around the 33rd week of the year (first half of August). Finally, Figure 1D reports recorded cases by age, thus highlighting the observed higher likelihood for older people to develop symptoms and thus being notified to the surveillance system.

We applied our FOI modelling approach to 51 epidemiological curves $H_{y,i}$ with on average 25.1 total cases (min = 5, max = 213, sd = 39.2). The majority of the considered epidemiological curves belonged to 2018 (10 curves), 2022 (9 curves) and 2013 (8 curves), when the three largest outbreaks occurred (Figure 1B).

We generated 100 stochastic realisations for each $H_{y,i}$ prediction and compared the frequencies of the observed and predicted values. More specifically, the number of human cases with day of symptoms onset t expected for region i and year y were drawn from a Poisson distribution Pois($\lambda_{y,i}(t)$). We found our model fits well observed cases as 97.4% of the simulated total number of weekly cases lie within the 95% Confidence Interval (CI) of model predictions. From Figure 2A, which shows observed and predicted frequencies for the total yearly number of WNV cases $\Sigma_{y,i}$, we can also note a very good agreement between the two quantities. Finally, there was a very good correlation (Pearson correlation coefficient = 0.94) between the predicted ($\overline{\Sigma_{y,i}}$) and observed ($\Sigma_{y,i}$) values (Figure 2B), with an average squared error $E\left(\Sigma_{y,i} - \overline{\Sigma_{y,i}}\right)^2 = 35.7$.

The estimated distributions of the three free FOI-model parameters (c, μ, σ) are characterised by a substantial temporal

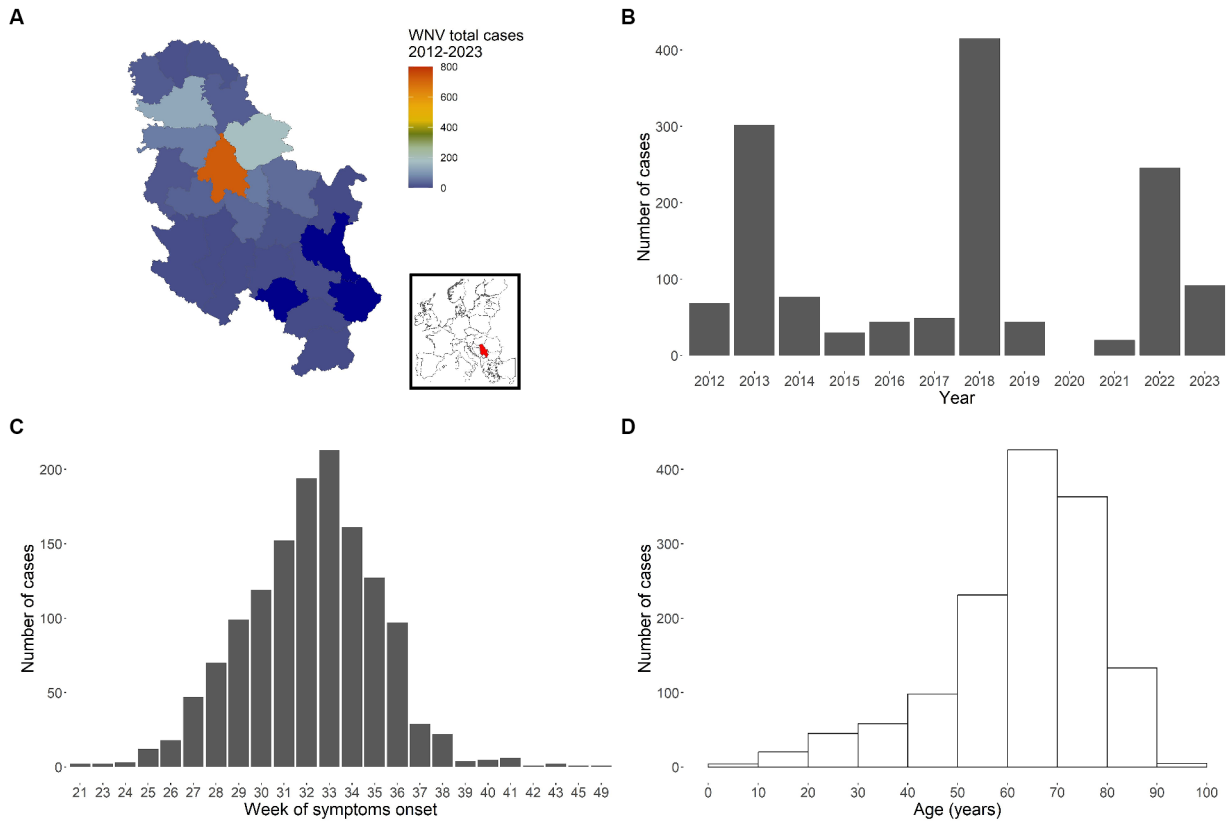

FIGURE 1
WNV cases recorded in Serbia between 2012 and 2023. Total number of cases by administrative area (NUTS3 level) (**A**, with inset map highlighting Serbia), by year **(B)**, by week of symptoms onset **(C)** and by age group **(D)**.

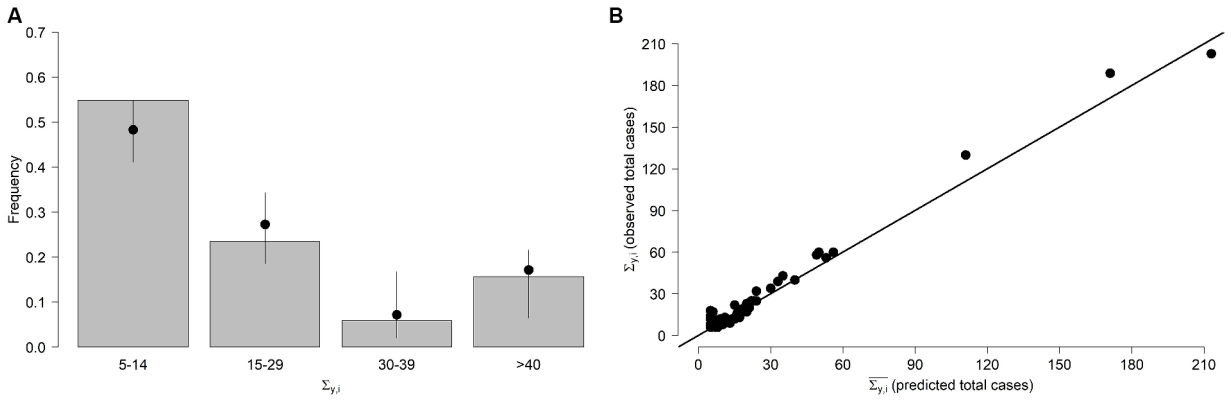

FIGURE 2
FOI model. **(A)** Frequencies of the stochastically predicted and observed $\Sigma_{y,i}$ (total number of WNV human cases recorded during year *y* in region *i*). Bars: observed $\Sigma_{y,i}$. Points and lines represent average and 95% quantiles of the frequencies of the stochastically predicted values, respectively. Values are shown aggregated by group. **(B)** Predicted ($\overline{\Sigma_{y,i}}$) and observed ($\Sigma_{y,i}$) total number of WNV human cases for each region and year.

heterogeneity, as shown in Figure 3. The WNV FOI peaked later in 2019 and 2021 and was higher in 2018 and 2022.

The average parameter μ (indicating when the FOI reaches its maximum) ranged between 196 and 260 (July 15–September 17) with a mean value of 224 (August 12) and a 95% Confidence Interval (CI) lying within 202–254 (July 21–September 11). The average for σ (length of the epidemiological season) is about 27 days (95%CI 13–48). Finally, the rescaling parameter *c* (FOI magnitude) is on average $5.89 \bullet 10^{-5}$ (95%CI $1.28 \bullet 10^{-5}$-$1.89 \bullet 10^{-4}$).

It is interesting to note that a shorter epidemiological season does not straightforwardly imply fewer human infections as σ values are mostly estimated to be below average for 2013 and 2018, when the FOI magnitude was greater.

As VIFs were all below 3 we did not discard any explanatory variable from the full statistical models (25). The best model for *C*, whose coefficients are reported in Table 1, included two covariates ($R^2 = 0.4$). As depicted in Figure 4A, we found that FOI magnitude is positively associated with spring temperature (T_{spring}) and is estimated to be greater in areas with higher proportions of older adult people (ε). The best model for *M* ($R^2 = 0.4$) included only the average spring temperature (see Table 1 and Figure 4B) indicating that warmer springs correspond to an earlier timing of the incidence peak. Finally, T_{spring} was found to be the only significant negative predictor also for *S*, meaning that epidemiological seasons are shorter with warmer springs ($R^2 = 0.09$), see Figure 4C.

4 Discussion

In this study we analysed and modelled WNV human transmission patterns in Serbia between 2012 and 2023. We applied a previously developed modelling framework (18) to investigate observed transmission patterns and identify the main environmental drivers of the FOI (the rate at which susceptible individuals acquire the infection) in the country. The deployed modelling framework, which aims at quantifying the WNV FOI using a limited number of parameters, was selected because it requires only data on recorded human infections. If more detailed data, such as entomological collections providing both mosquito abundance and WNV prevalence, are available, then other types of models might be developed to explicitly consider mosquito population dynamics and WNV transmission between vector and host populations (30, 31).

We found that spring temperatures are crucial at shaping WNV epidemiology, confirming previous findings demonstrating the importance of spring conditions in Europe for enhancing WNV circulation (18, 31–34). Warmer conditions are associated with an earlier peak of the FOI and a shorter epidemiological season but also with a larger FOI magnitude. Interestingly, estimated model coefficients for T_{spring} for the FOI magnitude (*c*, 0.134) are consistent with previous estimates at continental level [0.142, see (18)], suggesting spring temperature exerts a comparable effect at both spatial scales.

At European scale we found that infection peak tends to be earlier when summer temperature is higher (18), whereas for Serbia we found a similar association but with spring conditions. Indeed, summer temperatures did not seem to significantly affect WNV transmission in the country, probably because most of the amplification phase had occurred previously. As warmer conditions might amplify virus transmission (i) by increasing mosquitoes' biting rate (11) and (ii) the host-to-vector transmission probability (35, 36) and (iii) by shortening the mosquito viral incubation period (13), it is likely that favourable conditions during spring have a cascading effect later in the year, increasing human transmission risk. Consistently with our previous findings at European level (18), the FOI is estimated to be greater in areas with a higher number of older adult (age > 65 years) people. This association is unsurprising since age is one of the main risk factors for developing severe symptoms upon infection (37).

We found that model parameters are not significantly associated with precipitation-related variables, similarly to findings of our previous modelling efforts at continental level (18). Even though precipitation could indirectly influence the transmission dynamics of WNV by affecting mosquito breeding habitats and mosquito abundance, the direct effect of precipitation on WNV transmission may vary depending on local ecological and environmental conditions (9, 38).

Interestingly, land cover did not seem to significantly affect epidemiological parameters, whilst previously we found a negative

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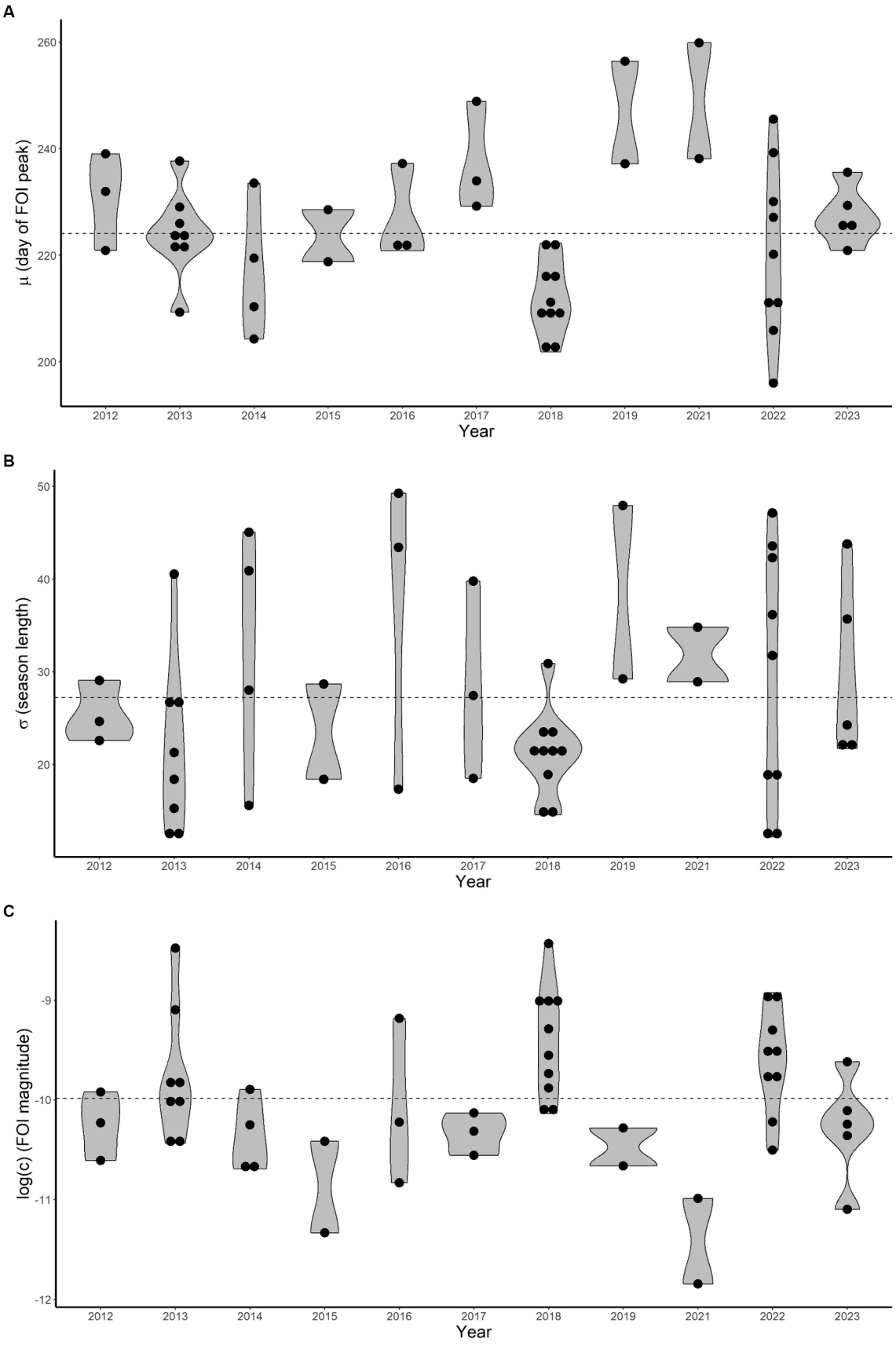

FIGURE 3
Estimated average (μ), standard deviation (σ) and magnitude (c, log-transformed) distributions (violin plots, **A**–**C** respectively) for each year. Dots represent estimated values for each epidemiological curve. Dashed horizontal lines are average values computed overall years.

TABLE 1 Estimates, standard errors, *t* values and *p*-values of the parameters of the best models for C, M and S.

Y	Parameter	Coefficient estimate	Standard error	*t* value	*p*-value
C	Intercept	−16.295	1.268	−12.855	<0.001
	ε	20.228	6.042	3.348	0.002
	T_{spring}	0.134	0.033	4.109	<0.001
M	Intercept	285.433	10.783	26.471	<0.001
	T_{spring}	−3.545	0.617	−5.746	<0.001
S	Intercept	49.740	10.404	4.781	<0.001
	T_{spring}	−1.302	0.595	−2.186	0.034

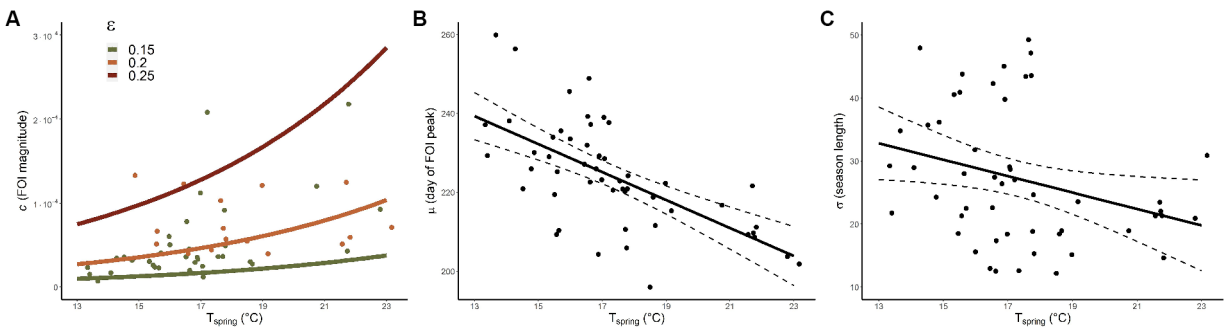

FIGURE 4
Model predictions. **(A)** Model predictions (lines) for *c* (FOI magnitude) conditional to T_{spring} (average spring temperature) and ε (fraction of inhabitants older than 65 years). Green (orange) dots represent FOI-model values with associated ε belonging to the 0.15–0.2 (0.2–0.25) interval. **(B,C)** Model predictions for μ (day of the year when the FOI reaches its maximum) and σ (length of the epidemiological season) respectively; continuous and dashed lines provide average and CIs respectively, dots represent FOI-model values.

association between η (total combined percentage of CLC labelled as urban or agricultural area) and the FOI magnitude *c* at European level (18). This result could depend on the narrower area under study and the lower variability of η across Serbia, with values ranging between 51 and 93% whilst at continental level we found it to vary between 22 and 97% (18). Our findings are consistent with recent phylodynamic models suggesting that WNV-2 is attracted to areas characterised by high crop and vegetation density, livestock cultivation, and urbanisation (6).

It is important to note that other factors could play an important role in shaping WNV circulation as well. For instance, a high avian immunity at the beginning of the epidemiological season, due to the previous year WNV circulation, might prevent pathogen transmission (39). Other climatic variables not explicitly considered in our model, such as drought or winter temperature might influence mosquito population dynamics and consequently WNV transmission as well (33, 40, 41).

We remark that the considered time series was incomplete as data collection in 2020 was limited because of the Covid-19 pandemic. This might also explain the low number of recorded cases in 2021, which however was consistent with other European countries (8).

As our modelling approach requires only human data, which are usually routinely collected by European health authorities, it might be easily applied to investigate the transmission of WNV or other vector-borne pathogens in other areas of interest. In fact, our proposed modelling framework is not specifically designed for WNV only, but it could be applied to any other vector-borne disease for which the FOI has a seasonal pattern and does not depend on the number of infectious humans such as tick-borne encephalitis or Usutu (18).

Our results point out the importance of weather anomalies at the beginning of the mosquito breeding season, which might amplify WNV circulation with a cascading effect later in the season. As previously highlighted in Farooq et al. (32), spring climate parameters should be given priority when developing climate-related WNV early warning systems.

Data availability statement

The data analyzed in this study is subject to the following licences/restrictions: the data that support the findings of this study are available from the Serbian National Public Health Institute but restrictions apply to the availability of these data, which were used under licence for the current study, and so are not publicly available. Requests to access these datasets should be directed to mitra_drakulovic@batut.org.rs.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/participants or patients/participants legal guardian/next of

kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

GM: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. MD: Data curation, Resources, Writing – review & editing. VJ: Data curation, Resources, Writing – review & editing. FD: Data curation, Methodology, Writing – review & editing. WW: Data curation, Resources, Writing – review & editing. VT: Writing – review & editing. MV: Data curation, Resources, Writing – review & editing. AR: Conceptualization, Writing – review & editing.

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Conflict of interest

WW was employed by Environmental Research Group Oxford Ltd, c/o Dept Biology.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The global incidence and trends of three common flavivirus infections (Dengue, yellow fever, and Zika) from 2011 to 2021

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Background: Flavivirus pose a continued threat to global health, yet their worldwide burden and trends remain poorly quantified. We aimed to evaluate the global, regional, and national incidence of three common flavivirus infections (Dengue, yellow fever, and Zika) from 2011 to 2021.

Methods: Data on the number and rate of incidence for the three common flavivirus infection in 204 countries and territories were retrieved from the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2021. The estimated annual percent change (EAPC) was calculated to quantify the temporal trend during 2011–2016, 2016–2019, and 2019–2021, respectively.

Results: In 2021, an estimated 59,220,428 individuals were infected globally, comprising 58,964,185 cases of dengue, 86,509 cases of yellow fever, and 169,734 cases of Zika virus infection. The age-standardized incidence rate (ASIR) of the three common flavivirus infections increased by an annual average of 5.08% (95% CI 4.12 to 6.05) globally from 2011 to 2016, whereas decreased by an annual average of −8.37% (95% CI −12.46 to −4.08) per year between 2016 to 2019. The ASIR remained stable during 2019–2021, with an average change of 0.69% (95% CI −0.96 to 2.37) per year globally for the three common flavivirus infections. Regionally, the burden of the three common flavivirus infections was primarily concentrated in those regions with middle income, such as South Asia, Southeast Asia, and Tropical Latin America. Additionally, at the country level, there was an inverted "U" relationship between the SDI level and the ASI. Notably, an increase in the average age of infected cases has been observed worldwide, particularly in higher-income regions.

Conclusion: Flavivirus infections are an expanding public health concern worldwide, with considerable regional and demographic variation in the incidence. Policymakers and healthcare providers must stay vigilant regarding the impact of COVID-19 and other environmental factors on the risk of flavivirus infection and be prepared for potential future outbreaks.

KEYWORDS

global burden of disease, flavivirus infections, dengue, yellow fever, Zika infection, age-standardized incidence rate, estimated annual percentage change importance

1 Introduction

Flaviviruses are single-stranded, positive-sense RNA viruses that are transmitted by insect vectors, and they belong to one of the four genera within the family Flaviviridae (Gould and Solomon, 2008). Over recent decades, notable flaviviruses such as Dengue virus (DENV), West Nile virus (WNV), Zika virus (ZIKV), and yellow fever virus (YFV) have been responsible for the emergence and re-emergence of numerous infectious diseases, posing enduring threats to global health (Pierson and Diamond, 2020). Flavivirus infections can be broadly categorized into two phenotypes: visceral and neurotropic. DENV and YFV typically cause systemic diseases involving hemorrhage, while WNV and ZIKV can result in severe neurological complications (Gould and Solomon, 2008; Pierson and Diamond, 2020). Additionally, ZIKV possesses a unique ability to infect the reproductive tract, facilitating sexual transmission and allowing the virus to reach the developing fetus. This can result in microcephaly, congenital malformations, and even fetal demise (Pierson and Diamond, 2020).

DENV is recognized as the fastest-spreading mosquito-borne virus, threatening roughly half of the global population with infection (Brady et al., 2012; Messina et al., 2019). Based on data from the lobal Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2019, there were an estimated 56.9 million cases of dengue and 36,055 deaths in 2019 alone, reflecting an 85.5% increase in global DENV incidence between 1990 and 2019, highlighting the escalating burden of DENV-related illnesses worldwide (Yang et al., 2021). Particularly endemic in South-East Asia and South Asia, dengue presents significant social, economic, and healthcare challenges in these regions (Shepard et al., 2016). Statistical models predict an expansion of DENV transmission geographically due to ongoing climate change and urbanization, with over 6 billion people projected to be at risk of DENV infection by 2080 (Messina et al., 2019). The exponential increase in DENV infections over the past few decades has made the search for a dengue vaccine a critical priority. Significant progress has been made in recent years in vaccine development; however, the long-term efficacy and safety of dengue vaccines in regions where the disease is endemic remain uncertain (Screaton et al., 2015; Thomas, 2023). YFV primarily afflicts tropical and subtropical regions of Africa, South, and Central America. In 2018, an estimated 109,000 severe infections and 51,000 deaths were attributed to yellow fever in these areas (Gaythorpe et al., 2021). Despite the availability of effective vaccines, YFV has sparked multiple pandemics and resurged as a major global health threat (Lindsey et al., 2022). YFV is mainly transmitted by the anthropophilic *Aedes* mosquitoes, which are prevalent in tropical and subtropical regions, but historically YFV is absent from the Asia-Pacific region. Increasing exchanges between Africa and Asia have led to imported YFV cases in non-endemic areas, posing a new viral threat to Asia (Wasserman et al., 2016). Asian-Pacific *Aedes* mosquitoes are competent vectors for YFV, with a higher potential to transmit the virus and pose a greater risk of transmission to human populations compared to *Aedes aegypti* from YFV-endemic regions in Africa (de Guilhem de Lataillade et al., 2020). The growing global interconnectedness facilitates YFV spread into low-risk or previously YFV-free regions, emphasizing the necessity of ongoing surveillance (Reno et al., 2020). Zika virus (ZIKV) has rapidly emerged since 2007, instigating epidemics across Micronesia, the South Pacific, and the Americas (Weaver et al., 2016; Musso et al., 2019). Designated as a Public Health Emergency of International Concern (PHEIC) by the World Health Organization (WHO) on February 1, 2016 (Gulland, 2016), the most recent outbreak in Brazil saw an estimated 440,000 to 1,300,000 cases of ZIKV infection reported (Bogoch et al., 2016). However, global mortality data for ZIKV is relatively limited compared to other viral infections, as most ZIKV infections are asymptomatic or result in mild symptoms such as fever, rash, joint pain, and conjunctivitis (Wikan and Smith, 2016). Notably, the outbreak in Brazil raised significant concerns due to the dramatic increase in cases of microcephaly (Wikan and Smith, 2016). The mortality rate was 52.6 deaths per 1,000 person-years among live-born children with congenital Zika syndrome, compared to 5.6 deaths per 1,000 person-years among those without the syndrome (Paixao Enny et al., 2022). ZIKV infections are predominantly concentrated in Latin American and Caribbean nations, with sporadic cases elsewhere (Guo et al., 2022). Studies predict a heightened risk of ZIKV transmission in forthcoming climate scenarios, particularly in regions like southern and Eastern Europe, northern America, and temperate areas of Asia such as northern China and southern Japan (Blagrove et al., 1930). The number of new people at risk of ZIKV infection is projected to exceed 1.3 billion by 2050 due to warming temperatures (Ryan et al., 2021). The rapid spread of flaviviruses, both locally and globally, is facilitated by various eco-epidemiological factors, including global warming, urban development, and increased intercontinental travel (Baker et al., 2022). Consequently, flaviviruses are recognized as potential candidates for future viral pandemics (Pierson and Diamond, 2020).

The emergence of the COVID-19 pandemic reportedly triggered changes in the epidemiological patterns of various infectious diseases (Xiao et al., 2021; Hartner et al., 2024). Nonpharmaceutical interventions such as lockdowns, quarantine, universal masking, and physical distancing measures aimed at combating COVID-19 were estimated to have averted approximately 0.72 million dengue cases that would have otherwise occurred in 2020 across Latin America and Southeast Asia (Chen et al., 2022). Nevertheless, disruptions caused by COVID-19 have impeded public access to healthcare services, leading to a dual burden of COVID-19 and dengue (Harapan et al., 2021; Luo et al., 2024). Moreover, the efforts to combat SARS-CoV-2 came at the expense of flavivirus diagnosis and control practices, leading to the simultaneous circulation of SARS-CoV-2 and flavivirus in Brazil (da Silva et al., 2021). The disruption of immunization and drug administration campaigns during the COVID-19 era has left numerous children at risk of yellow fever and other neglected tropical diseases (Jafari et al., 2021). This complex interplay necessitates a thorough examination of potential shifts in the burden of flavivirus infections before and during the COVID-19 pandemic. Furthermore, robust assessments of flavivirus incidence and forecasts of future trends are indispensable for effective intervention planning to mitigate the risk of significant outbreaks.

2 Methods

2.1 Data source and data collection

The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) is a collaborative international effort led by the Institute for Health Metrics and Evaluation (IHME) and involving over 11,000

contributors (Ferrari et al., 2024). Its primary focus lies in estimating global population demographics, fertility rates, morbidity, and mortality. This cross-sectional study utilized annual estimations of region-, country-, and age-specific incidence numbers and crude rates of three prevalent flavivirus infections (Dengue, Zika, and yellow fever) obtained from the GBD 2021 through the Global Health Data Exchange (GHDx) query tool[1] (Ferrari et al., 2024). The study spanned individuals of all age groups across 204 countries and territories from 2011 to 2021, categorizing the population into twenty age brackets of five years each: <5, 5–9, 10–14, 15–19, 20–24, 25–29, 30–34, 35–39, 40–44, 45–49, 50–54, 55–59, 60–64, 65–69, 70–74, 75–79, 80–84, 85–89, 90–94, and >95 years of age. Furthermore, the 204 countries and territories are organized into 21 GBD regions based on epidemiological similarities and geographic proximity (Ferrari et al., 2024). All participants met the inclusion criteria set forth by the GBD Study. The study protocol received approval from the University of Washington's research ethics board and will be conducted in strict adherence to the university's policies and procedures, as well as compliance with relevant federal, state, and local laws (Ferrari et al., 2024).

2.2 Case definition

In the GBD 2021, all cases of dengue fever, including classical dengue [defined by the International Classification of Diseases version 10 (ICD-10) code under heading A90], and dengue hemorrhagic fever (ICD-10 code under heading A91) are accounted for. yellow fever cases are identified by any ICD-10 code under heading A95, while Zika virus (ZIKV) infections are defined by any ICD-10 code between U06 and U06.9 (Ferrari et al., 2024). A confirmed case of dengue is identified through one or more of the following methods, in accordance with the World Health Organization's criteria: isolation of the dengue virus in a cell culture; identification of the virus's genetic material via polymerase chain reaction (PCR); detection of the non-structural protein 1 (NS1) antigen using enzyme-linked immunosorbent assay (ELISA) or a rapid diagnostic test; and serological identification of immunoglobulin M (IgM) or immunoglobulin G (IgG) antibodies through ELISA, rapid tests, or hemagglutination inhibition assays. A diagnosis of yellow fever can be established based on the following criteria: (i) detection of yellow fever M (IgM) antibodies in a patient not vaccinated against yellow fever within 30 days prior to the onset of symptoms; or (ii) positive liver histopathology findings from a postmortem examination; or (iii) a clear epidemiological connection to a confirmed case or an outbreak, as per the World Health Organization's guidelines. Additionally, the diagnosis can be supported by either: (a) no yellow fever vaccination within 30 days before the illness began, coupled with one of the following: (i) identification of yellow fever-specific IgM antibodies; or (ii) a fourfold or greater increase in yellow fever IgM or IgG antibody levels between serum samples taken during the acute phase and the recovery phase, or both; or (iii) identification of yellow fever-specific neutralizing antibodies; or (b) no yellow fever vaccination within 14 days before the onset of symptoms, and one of the following: (i) detection of the yellow fever virus genome in blood or other organs through polymerase chain reaction (PCR); or (ii) detection of yellow fever antigen in blood, liver, or other organs via immunoassay; or (iii) isolation of the yellow fever virus. A patient is considered to have a confirmed case of recent Zika virus infection if they fulfill the criteria for a suspected case and also have laboratory evidence supporting this, which includes: (i) the presence of Zika virus RNA or antigen detected in any biological sample (such as serum, urine, saliva, tissue, or whole blood); OR (ii) a positive test for Zika virus-specific IgM antibodies along with a Zika virus neutralizing antibody titer (PRNT90) that is equal to or greater than 20 and is fourfold or greater in comparison to the titers against other flaviviruses, along with the exclusion of other flavivirus infections; OR (iii) in the case of postmortem specimens, identification of the Zika virus genome in fresh or paraffin-embedded tissue using molecular techniques, or identification through immunohistochemistry, according to the Pan American Health Organization's 2016 guidelines (Ferrari et al., 2024).

2.3 Global and national socioeconomic status

The socio-demographic index (SDI) is a composite indicator of background social and economic conditions that influence health outcomes in each location (Ferrari et al., 2024). This composite indicator encompasses three key indices: (1) total fertility rate for individuals under 25 years old; (2) mean education level among those aged 15 years and above; and (3) lag-distributed income *per capita*. The composite SDI is derived by standardizing these three indices for a specific location-year to yield the geometric mean. Based on the resulting SDI score, regions and countries are categorized into five distinct quintiles: low SDI (0–0.455), low-middle SDI (0.455–0.608), middle SDI (0.608–0.690), high-middle SDI (0.690–0.805), and high SDI (0.805–1). Additionally, the World Bank categorizes economies worldwide into four income tiers: low (<$1,045), lower-middle ($1,046 to $4,095), upper-middle ($4,096 to $12,695), and high-income (>$12,695) (World Bank Blogs, 2021). These classifications are determined by the gross national income *per capita* in current USD from 2020, utilizing the Atlas method exchange rates.

2.4 Statistical analysis

This study calculated the age-standardized incidence rate (ASIR) of flavivirus infections per 100,000 population employing the following formula:

$$\mathrm{ASIR} = \frac{\sum_{i=1}^{A} a_i w_i}{\sum_{i=1}^{A} w_i} \times 100{,}000$$

In this context, a_i signifies the age-specific incidence rate within the ith age subgroup, and w_i indicates the population count of individuals within the same age category sourced from the GBD Study Population Estimates 1950–2021 (Schumacher et al., 2024). To gauge the temporal trends in flavivirus infection burden, we computed the estimated annual percentage changes (EAPCs) in ASIR. This involved

1 http://ghdx.healthdata.org/gbd-results-tool

fitting a regression line to the natural logarithm of the rates, represented as $y = \alpha + \beta x + \varepsilon$, where $y = \ln(\text{ASR})$ and x = calendar year. The EAPC was derived as $100 \times (\exp(\beta) - 1)$, with corresponding 95% confidence interval (CI) obtained from the linear regression model (Gao et al., 2012). Additionally, we conducted Pearson correlation analysis to assess the association between ASIR and SDI quintile and visualized the results with locally weighted scatterplot smoothing (LOWESS) curves. All statistical analyses and mapping were performed using R software, version 4.1.0 (R Foundation for Statistical Computing), with significance set at $p < 0.05$.

3 Results

3.1 Global incidence of DENV, YFV, and ZIKV infections

In 2021, there were an estimated 59,220,428 flavivirus infections reported worldwide, with 27,480,266 incidents among males and 31,740,162 among females, marking a 3.5% increase from 2019 (Supplementary Table S1). According to the WHO, global yellow fever vaccination coverage, defined as the proportion of the target population receiving one dose of the vaccine in a given year, was 47% in 2021 (WHO, 2024). Notably, vaccination coverage in high-burden regions has declined from 2019 to 2021, with Africa seeing a drop from 47 to 45% and the Americas from 61 to 58%. In 2021, there were 290,766 cases and 4,602 deaths attributable to DENV and YFV infections, respectively, corresponding to 0.38 and 0.06 per 100,000 people. However, deaths related to ZIKV infection are rare and nearly negligible. The global ASIR of flavivirus infections stood at 715.69 per 100,000 population in 2021. Notably, the ASIR experienced an average annual increase of 5.08% (95% CI 4.12 to 6.05) from 2011 to 2016, followed by a decrease of −8.37% (95% CI −12.46 to −4.08) per year from 2016 to 2019. Furthermore, the ASIR remained stable during 2019–2021, with an average annual change of 0.69% (95% CI −0.96 to 2.37) globally (Supplementary Table S1).

The number of incident cases of DENV infection rose by 3.8%, from 56,799,358 in 2019 to 58,964,185 in 2021 (Table 1). Nevertheless, the number of both YFV and ZIKV infections declined between 2019 and 2021, with totals of 86,509 and 169,734 cases, respectively, in 2021 (Table 1). From 2011 to 2021, the temporal trends in the ASIR of DENV infection is consistent with those of the combined ASIR for the three prevalent flavivirus infections. However, the ASIR of YFV infection showed a declining trend over the same period. Additionally, a significant increase in the ASIR of ZIKV infection occurred during 2011–2016 [with an average annual change of 157.59% (95% CI 100.15 to 231.52)], while globally, the ASIR of ZIKV infection decreased by an annual average of −66.1% (95% CI −79.29 to −44.49) from 2016 to 2019, and −30.53% (95% CI −30.56 to −30.49) from 2019 to 2021 (Table 1).

Between 2011 and 2021, globally, the number of incident infection and ASIR were consistently higher among females compared to males (Figure 1A). In terms of specific flavivirus infections, the ASIR of DENV infection was slightly higher in females versus males among individuals under 95 years old (Supplementary Figure S1A). The ASIR of YFV infection was generally more frequent among males than females across all age groups, with the gap decreasing with increasing age (Supplementary Figure S1B). In the case of ZIKV infection, females under 70 years old had a higher ASIR than males of the same age, and this trend reversed after the age of 70 (Supplementary Figure S1C). The vast majority of incident cases worldwide were attributed to DENV infection (Figure 1C; Supplementary Figure S2). By age, the incidence of flavivirus infections peaked in the oldest age group among both sexes, even though the age group of 10–14 years had the highest number of cases (Figure 1B). Notably, the proportion of infected cases aged 60 years and above increased over the years globally, especially in higher-income regions (Figure 2 and Supplementary Table S2).

3.2 Incidence burden across four income-classified regions

In 2021, the incidence for flavivirus infections were highest in the region with lower-middle income (Supplementary Figure S3 and Supplementary Table S1). Between 2011 and 2016, the high-income region had the fastest increase in the ASIR [average annual change 10.65% (95% CI 2.61 to 19.32)], and a decrease in the ASIR was found only in low-income region [average change −1.79% per year (95% CI −3.28 to −0.28)]. Moreover, all income-classified regions showed a decreasing trend in the ASIR from 2016 to 2019 and then remained stable from 2019 to 2021 (Supplementary Table S1). The regions with the highest incidence of DENV, YFV, and ZIKV infection were the low-middle income region, the low-income region, and the upper-middle income region, respectively (Supplementary Figures S4, S5). Notably, in the 2016 global ZIKV epidemic, more than 80% of reported ZIKV cases originated from the upper-middle income region (Supplementary Figure S4C).

3.3 Incidence burden across 21 GBD regions

In 2021, South Asia documented the most incident cases of flavivirus infections, totaling 31,812,189, whereas Tropical Latin America had the highest ASIR for flavivirus infections, standing at 5464.54 per 100,000 population (Supplementary Table S1). Despite South Asia's high population and vulnerability to mosquito-borne infections, there were no recorded YFV or ZIKV infections in the region in 2021 (Table 1). Notably, no cases of DENV, YFV, or ZIKV infections were recorded in Central, Eastern, or Western Europe, nor in Central Asia, for either sex (Figure 1C). Between 2011 and 2016, the largest increases in ASIR of flavivirus infections occurred in the regions of Latin America (Southern, Andean, and Tropical) and High-income North America (Supplementary Table S1). From 2016 to 2019, the largest increases in ASIR of flavivirus infections were found in Oceania [2.6% (2.4 to 2.8)], East Asia [1.79% (1.6 to 1.97)], and Southern Sub-Saharan Africa [1.56% (1.32 to 1.81); Supplementary Table S1]. The most significant fluctuation in the ASIR for flavivirus infections occurred in High-income North America and Southern Sub-Saharan Africa during 2019 to 2021 (Supplementary Table S1). The Americas and the Caribbean witnessed the highest incidence of flavivirus infections during 2015–2017. Moreover, the regions of Australasia and North Africa and Middle East experienced a significant decrease in the incidence of flavivirus infections (Supplementary Figure S6).

TABLE 1 The number and age-standardized incidence rates (ASIR, per 100,000) of DENV, YFV, and ZIKV infections in 2021, as well as the temporal trends.

Characteristics	2021		2011–2016		2016–2019		2019–2021	
	Case number	ASIR per 100,000	Percent change (%)	EAPC (95% CI)	Percent change (%)	EAPC (95% CI)	Percent change (%)	EAPC (95% CI)
Dengue								
Overall	58,964,185	752.04	20.3	2.8 (1.53 to 4.08)	−10.2	−4.68 (−5.7 to −3.64)	3.8	0.92 (−0.86 to 2.73)
Sex								
Male	27,346,972	694.78	19.6	2.7 (1.48 to 3.95)	−9.7	−4.55 (−5.54 to −3.55)	3.6	0.83 (−0.88 to 2.56)
Female	31,617,213	810.65	20.8	2.87 (1.57 to 4.18)	−10.6	−4.79 (−5.85 to −3.72)	4	1 (−0.85 to 2.87)
World Bank classification								
High-income	685,622	56.35	13.3	1.97 (0.48 to 3.49)	−24	−9.51 (−11.05 to −7.94)	4	1.38 (0.88 to 1.89)
Upper-middle-income	17,444,892	724.12	29.1	4.98 (2.11 to 7.92)	−33.6	−13.75 (−15.7 to −11.76)	2.9	1.04 (−0.34 to 2.43)
Lower-middle-income	40,316,675	1185.11	14.9	1.18 (0.98 to 1.38)	6.3	0.54 (0.46 to 0.62)	4.2	0.77 (−1.19 to 2.76)
Low-income	496,614	70.35	18.1	0.89 (0.83 to 0.94)	9.5	0.65 (0.35 to 0.94)	1.5	−1.62 (−2.28 to −0.96)
GBD regions								
High-income Asia Pacific	485,712	294.01	30.7	5.8 (3.2 to 8.45)	−30.5	−11.59 (−13.3 to −9.84)	4.3	2.37 (1.05 to 3.71)
Central Asia	0	0	NA	NA	NA	NA	NA	NA
East Asia	61,440	4.27	7.8	0.63 (0.48 to 0.78)	6.8	1.45 (1.26 to 1.63)	4.1	1.47 (−2.93 to 6.06)
South Asia	31,812,189	1726.94	15.1	1.36 (1.16 to 1.56)	5.7	0.56 (0.51 to 0.61)	3.2	0.52 (−1.48 to 2.56)
Southeast Asia	6,728,444	971.89	31.3	4.71 (3.03 to 6.41)	−3.5	−2.35 (−3.29 to −1.41)	6.5	2.18 (0.3 to 4.09)
Australasia	18,448	58.99	−11.5	−4.05 (−4.27 to −3.84)	−8.3	−4.72 (−5.71 to −3.72)	6.7	1.41 (−1.65 to 4.56)
Caribbean	227,073	475.93	−34.2	−9.34 (−10.79 to −7.87)	−3.9	−2.05 (−2.61 to −1.49)	0.6	−0.38 (−2.29 to 1.57)
Central Europe	0	0	NA	NA	NA	NA	NA	NA
Eastern Europe	0	0	NA	NA	NA	NA	NA	NA
Western Europe	0	0	NA	NA	NA	NA	NA	NA
Andean Latin America	391,708	593.22	21.7	2.37 (1.29 to 3.46)	−3.3	−3.07 (−3.67 to −2.48)	9.6	2.97 (−1.01 to 7.1)
Central Latin America	2,886,641	1140.37	−10.3	−3.06 (−3.9 to −2.21)	−40	−16.66 (−18.87 to −14.39)	4.1	1.56 (1.03 to 2.1)
Southern Latin America	80,129	118.83	55.6	8.61 (5.51 to 11.8)	−5.5	−2.8 (−3.08 to −2.52)	0.2	−0.59 (−0.82 to −0.37)
Tropical Latin America	13,043,195	5774.82	39.2	6.41 (3.07 to 9.86)	−32.5	−13.42 (−15.26 to −11.54)	2.7	0.48 (−0.7 to 1.67)
North Africa and Middle East	53,391	8.5	−39.1	−12.01 (−14.5 to −9.44)	3.1	−0.54 (−0.58 to −0.49)	17.7	7.09 (−4.09 to 19.56)
High-income North America	1,376	0.36	10.3	1.07 (0.71 to 1.43)	2.9	0.13 (−0.26 to 0.53)	16.4	7.25 (−5.2 to 21.33)
Oceania	63,970	486.03	16.4	0.44 (0.05 to 0.84)	18.3	3.26 (2.76 to 3.76)	2.3	−1.16 (−7.49 to 5.61)
Central Sub-Saharan Africa	245,097	178.08	19.2	0.43 (0.34 to 0.52)	12.7	1.14 (0.67 to 1.61)	1.2	−2.02 (−3.03 to −1)

(Continued)

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TABLE 1 (Continued)

Characteristics	2021		2011–2016		2016–2019		2019–2021	
	Case number	ASIR per 100,000	Percent change (%)	EAPC (95% CI)	Percent change (%)	EAPC (95% CI)	Percent change (%)	EAPC (95% CI)
Eastern Sub-Saharan Africa	387,627	94.37	15.9	0.49 (0.23 to 0.75)	3.8	−1 (−1.11 to −0.9)	3.9	−0.17 (−0.75 to 0.41)
Southern Sub-Saharan Africa	1,087	1.32	−4	−2.04 (−2.83 to −1.23)	7.4	1.43 (1.2 to 1.66)	15.2	6.53 (0.86 to 12.51)
Western Sub-Saharan Africa	2,476,656	512.53	17.2	0.04 (−0.1 to 0.18)	12.9	1.09 (1.02 to 1.15)	10.1	2.07 (−3.07 to 7.47)
Yellow fever								
Overall	86,509	1.15	−30.7	−7.5 (−13.8 to −0.75)	−30.2	−11.13 (−14.84 to −7.26)	−5.5	−3.37 (−5.45 to −1.24)
Sex								
Male	60,460	1.57	−30.9	−7.6 (−13.82 to −0.92)	−30.1	−11.11 (−14.77 to −7.3)	−5.6	−3.43 (−5.59 to −1.22)
Female	26,048	0.70	−30.2	−7.3 (−13.76 to −0.37)	−30.4	−11.17 (−15 to −7.17)	−5.2	−3.25 (−5.15 to −1.32)
World Bank classification								
High-income	29	0	−23.6	−5.47 (−5.79 to −5.15)	−11.9	−4.37 (−5.05 to −3.68)	−4.6	−2.72 (−5.22 to −0.15)
Upper-middle-income	2,359	0.1	−21.1	−4.67 (−5.54 to −3.8)	−11.3	−0.41 (−60.29 to 149.78)	−5.1	−2.88 (−4.67 to −1.05)
Lower-middle-income	37,149	1.04	6.4	−2.11 (−9.44 to 5.81)	−43.9	−17.55 (−27.48 to −6.26)	−7.7	−4.79 (−6.5 to −3.06)
Low-income	46,943	6.42	−52.2	−13.75 (−19.9 to −7.13)	−13.8	−7.13 (−7.29 to −6.97)	−3.7	−4.25 (−6.56 to −1.89)
GBD regions								
High-income Asia Pacific	0	0	NA	NA	NA	NA	NA	NA
Central Asia	0	0	NA	NA	NA	NA	NA	NA
East Asia	0	0	NA	NA	NA	NA	NA	NA
South Asia	0	0	NA	NA	NA	NA	NA	NA
Southeast Asia	0	0	NA	NA	NA	NA	NA	NA
Australasia	0	0	NA	NA	NA	NA	NA	NA
Caribbean	38	0.08	−25.1	−6.09 (−6.37 to −5.81)	−12.6	−10.32 (−33.46 to 20.87)	−9.5	−5.09 (−9.47 to −0.51)
Central Europe	0	0	NA	NA	NA	NA	NA	NA
Eastern Europe	0	0	NA	NA	NA	NA	NA	NA
Western Europe	0	0	NA	NA	NA	NA	NA	NA
Andean Latin America	1,090	1.64	−19.3	−5.27 (−6.62 to −3.91)	−9.5	−6.08 (−10.7 to −1.22)	−3.3	−3.33 (−5.36 to −1.26)
Central Latin America	178	0.07	−19.1	−5.2 (−5.63 to −4.77)	−15	−6.45 (−10.37 to −2.36)	−9.6	−5.28 (−8.16 to −2.31)
Southern Latin America	709	1.07	−21.4	−5.2 (−5.93 to −4.46)	−10.8	−3.71 (−8.62 to 1.46)	−5.5	−3.43 (−5.15 to −1.68)

(Continued)

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TABLE 1 (Continued)

Characteristics	2021		2011–2016		2016–2019		2019–2021	
	Case number	ASIR per 100,000	Percent change (%)	EAPC (95% CI)	Percent change (%)	EAPC (95% CI)	Percent change (%)	EAPC (95% CI)
Tropical Latin America	187	0.09	−23.2	−5.52 (−6.21 to −4.83)	−16.1	−1.05 (−90.15 to 893.71)	−6.9	−3.93 (−4.68 to −3.16)
North Africa and Middle East	6,659	1.04	−31.3	−8.67 (−8.96 to −8.38)	−17.3	−7.65 (−7.97 to −7.32)	−5.8	−4.06 (−7.39 to −0.61)
High-income North America	0	0	NA	NA	NA	NA	NA	NA
Oceania	0	0	NA	NA	NA	NA	NA	NA
Central Sub-Saharan Africa	10,243	7.12	58.4	1.29 (−17.06 to 23.71)	−71	−33.12 (−53.41 to −3.98)	−6.2	−5.69 (−7.69 to −3.65)
Eastern Sub-Saharan Africa	24,416	5.41	−28.5	−8.65 (−9.74 to −7.54)	−14.1	−7.16 (−7.38 to −6.95)	−4.2	−4.5 (−7.17 to −1.75)
Southern Sub-Saharan Africa	0	0	NA	NA	NA	NA	NA	NA
Western Sub-Saharan Africa	42,988	8.13	−51.4	−14.16 (−20.67 to −7.11)	−13.1	−7.69 (−9.16 to −6.2)	−6	−5.85 (−7.36 to −4.33)
ZIKV infection								
Overall	169,734	2.13	24,856	157.59 (100.15 to 231.52)	−96.3	−66.1 (−79.29 to −44.49)	−50.8	−30.53 (−30.56 to −30.49)
Sex								
Male	72,833	1.83	25,702	158.77 (100.84 to 233.39)	−96.2	−66.04 (−79.03 to −45.02)	−50.8	−30.53 (−30.58 to −30.49)
Female	96,900	2.46	24,264	156.69 (99.6 to 230.11)	−96.3	−66.14 (−79.48 to −44.13)	−50.9	−30.53 (−30.56 to −30.5)
World Bank classification								
High-income	1,066	0.09	501,179	314.93 (134.45 to 634.34)	−99.7	−80.45 (−93.67 to −39.62)	−51	−30.15 (−30.22 to −30.07)
Upper-middle-income	153,753	6.24	48,249	195.54 (129.14 to 281.19)	−95.9	−65.43 (−79.38 to −42.04)	−50.8	−29.89 (−29.91 to −29.88)
Lower-middle-income	14,697	0.44	4,658	89.95 (59.69 to 125.94)	−97	−68.49 (−83.87 to −38.46)	−51	−31.01 (−31.02 to −30.99)
Low-income	0	0	NA	NA	NA	NA	NA	NA
GBD regions								
High-income Asia Pacific	0	0	NA	NA	NA	NA	NA	NA
Central Asia	0	0	NA	NA	NA	NA	NA	NA
East Asia	0	0	NA	NA	NA	NA	NA	NA
South Asia	0	0	NA	NA	NA	NA	NA	NA
Southeast Asia	0	0	NA	NA	NA	NA	NA	NA
Australasia	0	0	NA	NA	NA	NA	NA	NA
Caribbean	6,432	13.01	404,579	298.81 (124.68 to 607.9)	−99	−75.89 (−85.34 to −60.35)	−51.2	−30.69 (−30.7 to −30.68)
Central Europe	0	0	NA	NA	NA	NA	NA	NA

(Continued)

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TABLE 1 (Continued)

Characteristics	2021		2011–2016		2016–2019		2019–2021	
	Case number	ASIR per 100,000	Percent change (%)	EAPC (95% CI)	Percent change (%)	EAPC (95% CI)	Percent change (%)	EAPC (95% CI)
Eastern Europe	0	0	NA	NA	NA	NA	NA	NA
Western Europe	0	0	NA	NA	NA	NA	NA	NA
Andean Latin America	29,726	44.61	164,120	249.97 (133.4 to 424.78)	−81.5	−53.34 (−77.98 to −1.12)	−50	−30.55 (−30.56 to −30.53)
Central Latin America	51,041	19.99	18,660	141.68 (85.45 to 214.95)	−97.9	−69.15 (−86.19 to −31.09)	−51.1	−30.4 (−30.45 to −30.35)
Southern Latin America	107	0.16	1,481,650	384.54 (130.44 to 918.82)	−99.8	−86.32 (−90.34 to −80.65)	−51.4	−30.65 (−30.72 to −30.59)
Tropical Latin America	82,426	35.51	25100.4	167.35 (123.47 to 219.86)	−93.3	−59.22 (−83.96 to 3.69)	−50.9	−30.52 (−30.54 to −30.5)
North Africa and Middle East	0	0	NA	NA	NA	NA	NA	NA
High-income North America	1	0	1,232,261	373.1 (132.48–862.77)	NA	NA	NA	NA
Oceania	0	0	65,842	178.27 (59.38–385.83)	NA	NA	NA	NA
Central Sub-Saharan Africa	0	0	NA	NA	NA	NA	NA	NA
Eastern Sub-Saharan Africa	0	0	NA	NA	NA	NA	NA	NA
Southern Sub-Saharan Africa	0	0	NA	NA	NA	NA	NA	NA
Western Sub-Saharan Africa	0	0	NA	NA	NA	NA	NA	NA

NA: Not applicable.

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3.4 Incidence burden at the country level

In 2021, the ASIR for flavivirus infections was highest in Tonga (13388.9 per 100,000 population), followed by Seychelles (11565.2 per 100,000 population), Comoros (11075.1 per 100,000 population), and Marshall Islands (9646.5 per 100,000 population; Figure 3A). Meanwhile, 80 countries and territories reported no documented cases of DENV, YFV, or ZIKV infections. The global distribution of flavivirus infections has been predominantly shaped by the epidemic patterns of DENV infection (Figure 3B). Worldwide, YFV infection has been reported in only 47 countries and territories, with Burundi having the highest ASIR of 29.1 per 100,000 population (Figure 3C). In comparison, ZIKV infection affected 34 countries and territories, all within the American region (Figure 3D). The top three countries with the highest ASIR of ZIKV infection in 2021 were El Salvador (153.5 per 100,000), Belize (103 per 100,000), and Peru (79.1 per 100,000).

Additionally, the correlation between the ASIR of flavivirus infection and the SDI level exhibited an inverted "U" shape, peaking at SDI values around 0.6 and then declining as SDI values increased (Figure 4A). The patterns of the ASIR of DENV infection or ZIKV infection versus the SDI value were similar to those of the combined ASIR of the three flavivirus infections (Figures 4B,D). Furthermore, the ASIR of YFV infection decreased exponentially with increases in SDI level (Figure 4C).

4 Discussion

To the best of our knowledge, this is the first study to evaluate the incidence and trends of the three common flavivirus infections—DENV, YFV, and ZIKV—at global, regional, and national levels. To help inform the optimal implementation of public health interventions, robust estimates of flavivirus incidence and trends of future dynamics are essential (Gaythorpe et al., 2021). In this study, we provided the most up-to-date estimates of flavivirus infection incidence across the globe from 2011 to 2021. In 2021, nearly 60 million flavivirus infections were estimated to have occurred in 124 countries and territories, marking a 1.12-fold increase from 2011. Globally, the incidence of flavivirus infections peaked in 2016, which may be attributed to the 2015–2016 El Niño climate phenomenon (Anyamba et al., 2019). A significant overlap between the El Niño phenomenon, regional climate anomalies, and hyperendemic for DENV in South America and Southeast Asia have been demonstrated by previous studies (Anyamba et al., 2019; Ferreira et al., 2022; Liyanage et al., 2022). Additionally, the unique climatic conditions caused by the El Niño event were optimal for the transmission of ZIKV in the regions of America (Paz and Semenza, 2016; Caminade et al., 2017; Anyamba et al., 2019). The geographical distribution of flaviviruses has the potential to expand further, as their primary vectors are predicted to spread into temperate regions (Kraemer et al., 2019). The trajectory of ZIKV's spread in the Western Hemisphere illustrates the introduction of a previously obscure vector-borne disease into new ecological systems and populations, leading to swift dissemination with significant implications for human health (Lazear and Diamond, 2016). Therefore, timely surveillance to detect changes in pathogen distribution is essential for providing early warnings to public health officials to implement interventions, as evidenced by the global COVID-19 pandemic (Dong et al., 2020).

Notably, Pierson and Diamond (2020) indicated that flaviviruses are now globally distributed, infecting up to 400 million people annually, a figure significantly higher than the GBD estimation. This discrepancy could be attributed to several factors, including variations in data sources and modeling methodologies. Pierson and Diamond (2020) investigation included the presence of flavivirus infections derived from both peer-reviewed literature and HealthMap alerts. The GBD used cases of flavivirus infections reported by countries to the WHO and other global monitoring entities (Ferrari et al., 2024). This likely led to an underestimation of flavivirus infections due to under-reporting caused by limited health system capacity or misdiagnosis, even in many hyperendemic countries (Kakkar, 2012; Petersen et al., 2016; Shearer et al., 2018). Most flaviviruses are known to cause subclinical infections that are typically undetectable by existing clinical-based disease surveillance programs. For example, it was estimated that only 96 million of the 390 million global dengue cases in 2010 manifest apparent sign or symptom (Bhatt et al., 2013). Additionally, approximately 20% of individuals infected with ZIKV develop a clinically apparent febrile illness (Lazear and Diamond, 2016). More than 85% of YFV infection cases were either asymptomatic or presented with mild illness (Ndeffo-Mbah and Pandey, 2020). Consequently, we need to be cautious about the limitations of clinical-based surveillance programs when interpreting our estimations of the global burden of flavivirus infections (Chandra et al., 2021).

Given the lack of highly effective vaccines for mosquito-borne infections other than yellow fever and Japanese encephalitis, public health interventions have primarily focused on reducing human exposure through vector control (Ferguson, 2018). Over the past century, the use of insecticide-treated nets, long-lasting insecticidal nets, and indoor residual spraying has become the primary and recommended means of mosquito vector control (Wilson et al., 2020). Despite the use of these strategies and the accelerated development of long-lasting insecticidal nets and indoor residual spraying with different compounds, the global burden of mosquito-borne diseases on public health and economies continues to rise (Franklinos et al., 2019). Although the ASIR of flavivirus infections fluctuated worldwide from 2011 to 2021, a significant decrease in the incidence of flavivirus infections was observed in some non-endemic settings during this period, such as Australasia and North Africa and Middle East. Data from the Australia National Notifiable Disease Surveillance System showed that the release of Wolbachia-infected mosquitoes notably decreased local dengue transmission from 2011 to 2019 (Garske et al., 2024). Moreover, there was a substantial decrease in imported dengue notifications amid the COVID-19 pandemic due to travel restrictions. Similarly, several North African countries (Algeria, Libya, Morocco, and Tunisia) have not documented any outbreaks of DENV during 2011–2021 (Petersen et al., 2022). However, the burden of flavivirus infections, as measured by seroprevalence in many countries within the Middle East and North Africa, does not accurately reflect the incidence of flavivirus infections in this area (Humphrey et al., 2016). Despite our results showed an estimated declining trend in the ASIR of flavivirus infections in the Middle East and North Africa, further regional investigations are needed to characterize the epidemiological patterns of flavivirus in this region.

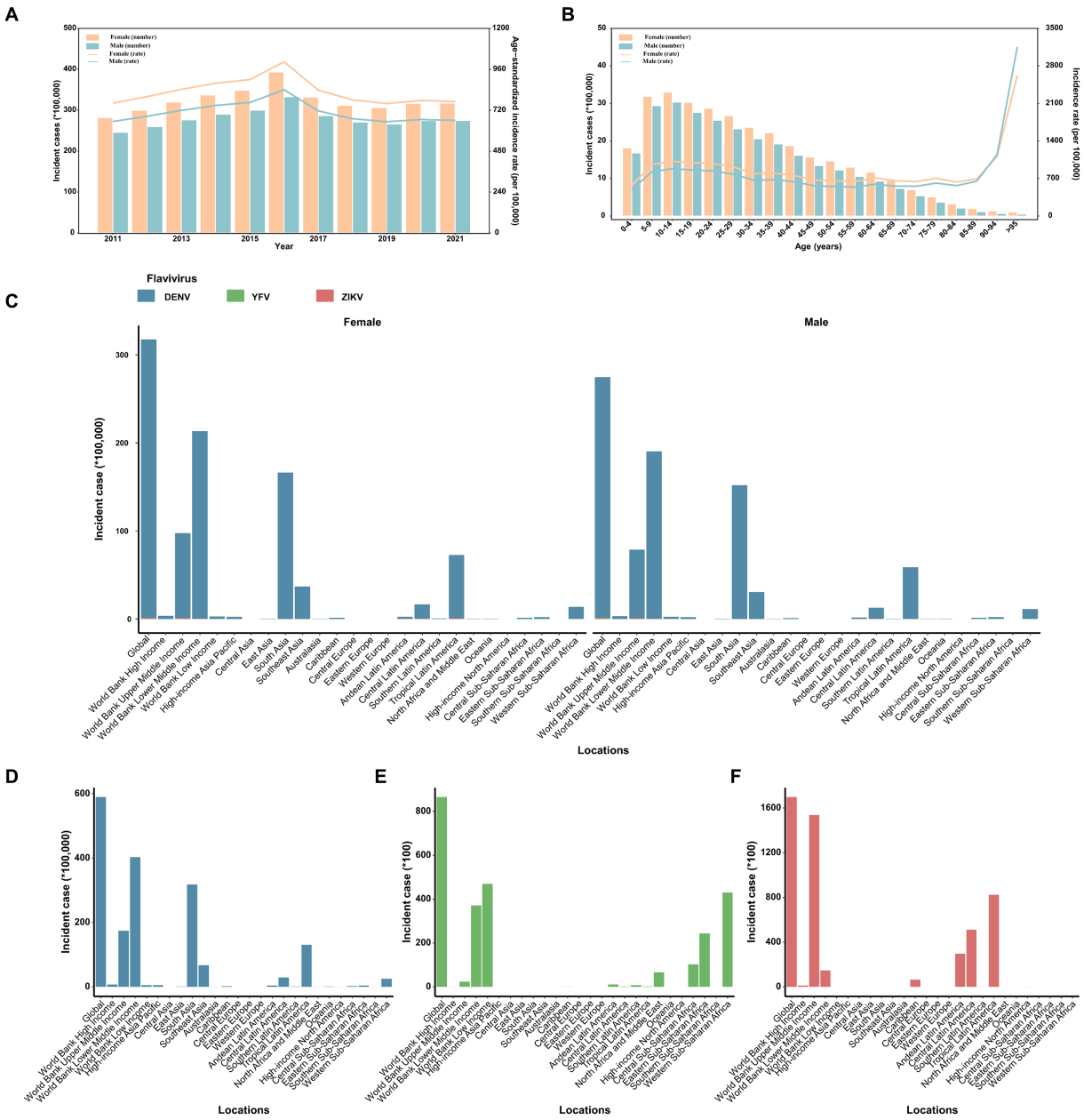

FIGURE 1
The distribution and trend of the number and age-standardized rate of incidence for three prevalent flavivirus (DENV, YFV, and ZIKV) infections, by sex. **(A)** The number and age-standardized rate of incidence from 2011 to 2021. **(B)** The number and rate of incidence in 2021 across age groups. **(C)** The distribution of incident cases across the globe, in different World Bank income classification and 21 GBD regions, 2021. **(D)** The distribution of incident cases of dengue across the globe, in different World Bank income classification and 21 GBD regions, 2021. **(E)** The distribution of incident cases of yellow fever across the globe, in different World Bank income classification and 21 GBD regions, 2021. **(F)** The distribution of incident cases of ZIKV infection across the globe, in different World Bank income classification and 21 GBD regions, 2021. DENV, Dengue virus; YFV, yellow fever virus; ZIKV, Zika virus.

In contrast, we have found that the regions of Asia (except for High-income Asia Pacific) observed a moderate increasing trend in the ASIR of flavivirus infections during the same period. Some studies of these regions have also documented increasing trends in dengue incidence. Results from the National dengue surveillance data for Cambodia revealed that the dengue incidence increased between 2002 and 2020 (Yek et al., 2023). Substantial increases in the number of dengue case have also been found in China for the period 2005–2020 (Yue et al., 2022). The dengue incidence in Southeast Asia is expected to continue rising in the short to medium term; however, this prediction does not consider the effects of COVID-19 restrictions on dengue risk (Colón-González et al., 2023). Chen and colleagues reported a reduced annual dengue incidence across most countries in Latin America and Southeast Asia following the implementation of COVID-19 interventions (Chen et al., 2022). Since it's unsustainable to continue limiting community mobility in the post-COVID-19 era, vector control interventions remain the best choice for managing flavivirus

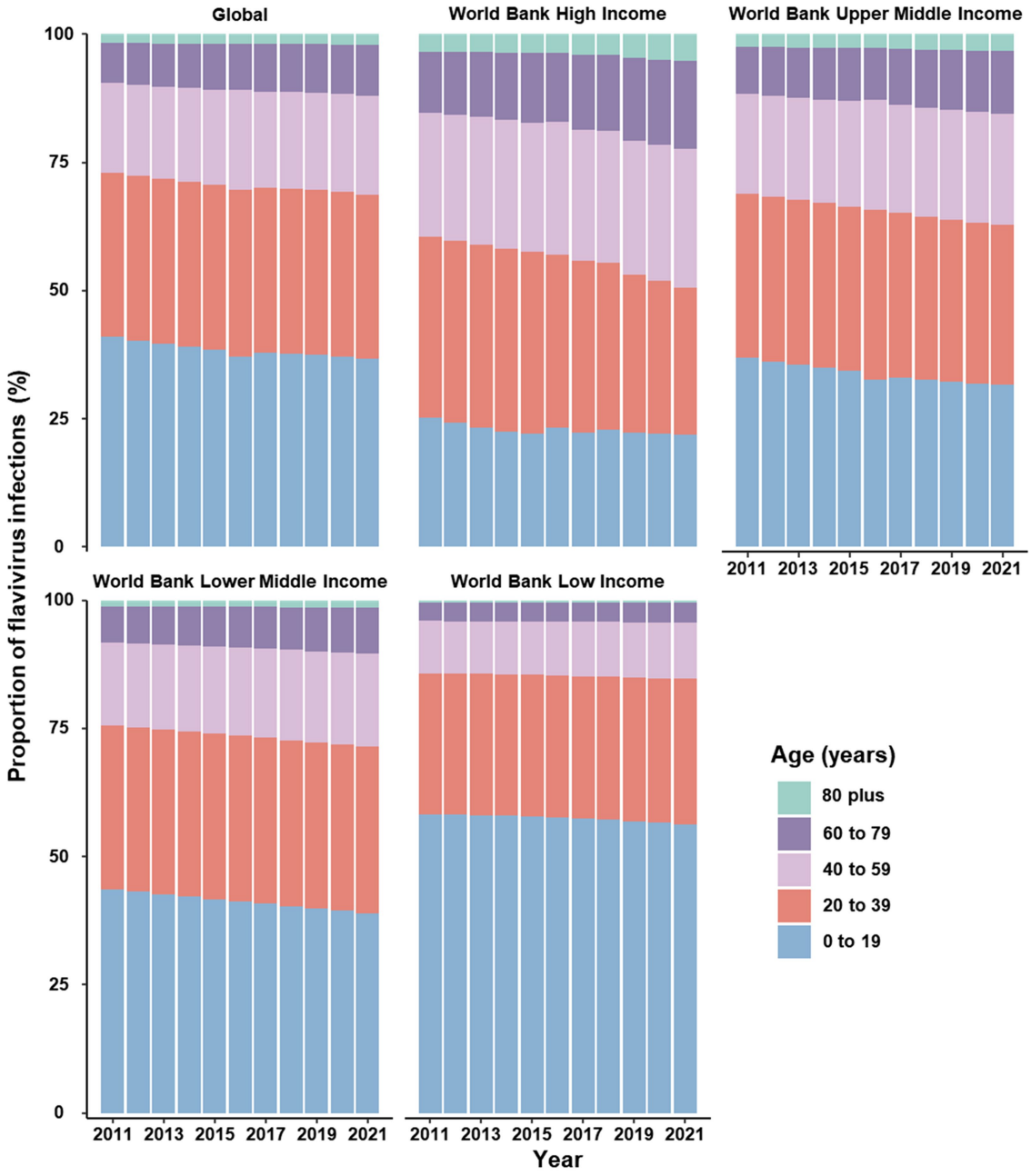

FIGURE 2
Proportion of cases infected with flavivirus stratified by age groups, worldwide and across four income levels, 2011–2021.

infections (Sasmono and Santoso, 2022). Notably, greenhouse warming would increase the frequency of disastrous climatic change such as extreme El Niño events, which is highly congenial for the breeding of mosquitoes (Colón-González et al., 2018; Rao et al., 2019). Therefore, sophisticated early warning systems that integrate comprehensive climate indices and provide extended prediction windows enhance global preparedness, enabling more efficient control and prevention of flavivirus epidemics (Chen et al., 2024).

It is encouraging that the global incidence of yellow fever continued to decline between 2011 and 2021, which may be attributed to the inclusion of vaccination against yellow fever in routine infant immunization programs among countries at high risk of yellow fever (Garske et al., 2014). Nevertheless, the global COVID-19 pandemic and other public health priorities have eroded healthcare delivery and access, leading to decreased coverage of yellow fever vaccines (Lindsey et al., 2022). Modeling analysis indicated that achieving and maintaining a 90% population immunity is recommended for the global elimination of yellow fever epidemics (Ndeffo-Mbah and Pandey, 2020). However, vaccination coverage in 2016 was estimated to be substantially below the recommended threshold (Shearer et al., 2018). Meanwhile, the need for a new vaccine against flaviviruses is growing as climate change has increased the number of people exposed to flaviviruses, leading

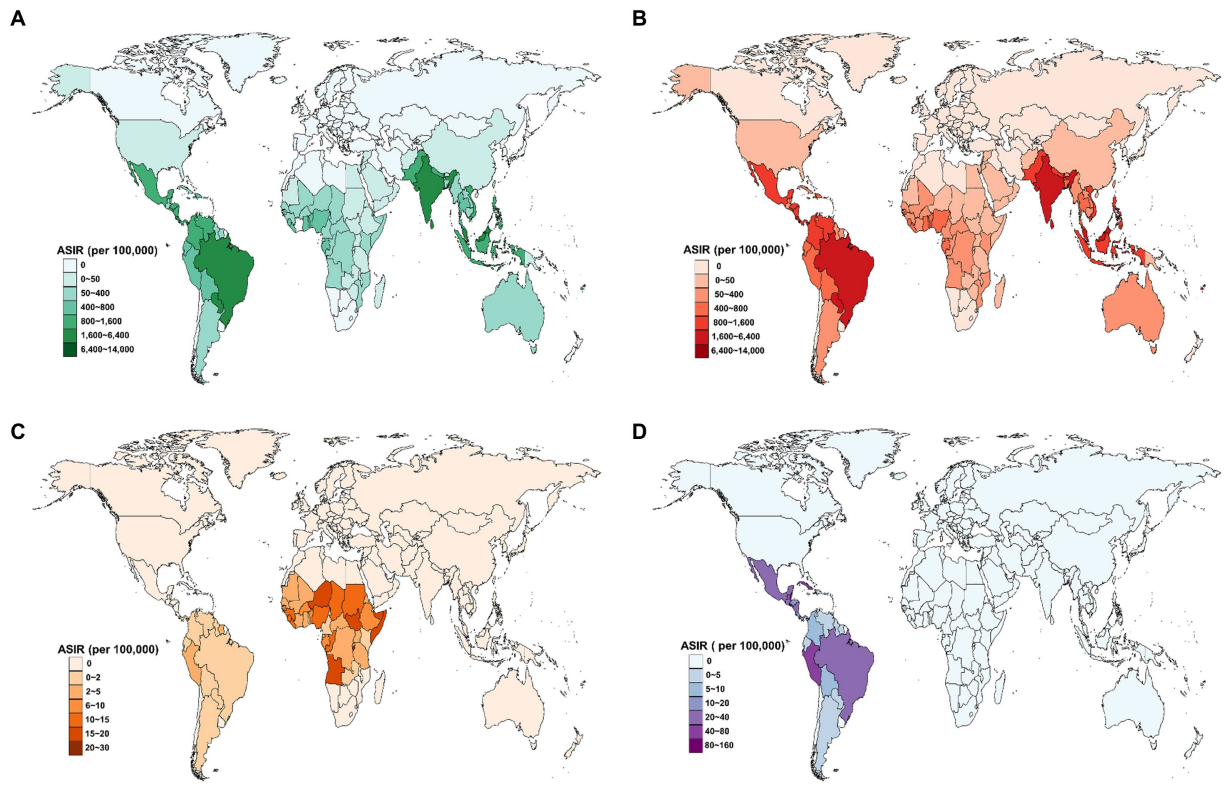

FIGURE 3
Estimated age-standardized incidence rate (ASIR) for three prevalent flavivirus infections in 2021, by country. **(A)** The combined ASIR of DENV, YFV, and ZIKV infections. **(B)** The ASIR of DENV infection. **(C)** The ASIR of YFV infection. **(D)** The ASIR of ZIKV infection. DENV, Dengue virus; YFV, yellow fever virus; ZIKV, Zika virus.

to a limited supply of vaccines produced with existing technology (Lindsey et al., 2022). Children and young adults under 40 years old remain the most affected by flavivirus infections. However, the proportion of cases in older age groups has increased over the years. This changing pattern can be attributed to population aging, as higher proportions of cases in older age groups are observed in higher-income regions, which correspond to lower fertility rates and higher life expectancies in these areas (Schumacher et al., 2024). Our finding is coincident with the impact of demographic transition on the age distribution of dengue in several endemic countries in Southeast Asia (Cummings et al., 2009). Hence, age- and region-appropriate health-care resource planning and allocation should be prioritized.

5 Limitations

Our study has some limitations. Firstly, the estimates for flavivirus infections in the GBD Study relies on complex statistical modeling and extrapolation techniques. The definition of cases or measurement approaches may differ geographically and temporally. Therefore, the accuracy and robustness of estimates can fluctuate between regions and health scenarios (Ferrari et al., 2024). Additionally, it's likely that these burdens are underestimated as mild cases of flavivirus infections frequently pass unnoticed owing to nonspecific symptoms and the limited capacity for surveillance or laboratory diagnostics in numerous vulnerable regions (de Araújo Lobo et al., 2016; Musso et al., 2019; Lindsey et al., 2022). Nonetheless, the GBD 2021 compute an adjustment factor aimed at rectifying underreporting. These adjustment factors were estimated using MR-BRT (meta-regression—Bayesian, regularized, trimmed), which factored in variables such as SDI level and reported incidence rate (Ferrari et al., 2024). Moreover, the flavivirus genus includes many members such as West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV) (Pierson and Diamond, 2020). However, due to limited data, the incidence of these other flaviviruses has not been evaluated in this study. Despite these limitations, our study provides useful information for public health professionals and policymakers to prevent the potential threats posed by the substantial global flavivirus burden.

6 Conclusion

In summary, the global burden of flavivirus infections is substantial, with considerable regional and demographic variations in incidence. Our research provides updated evidence of the changing global threat of flavivirus infections and will help decision-makers, healthcare providers, and at-risk communities

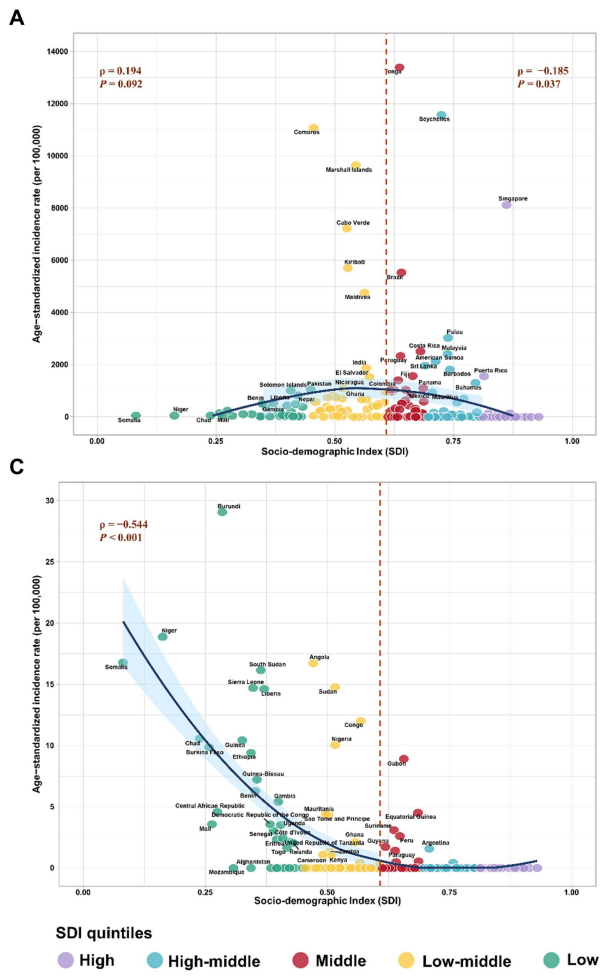

FIGURE 4
The correlation between age-standardized incidence rate (ASIR) of three prevalent flavivirus infections and socio-demographic index (SDI). **(A)** The combined ASIR of DENV, YFV, and ZIKV infections versus SDI. **(B)** The ASIR of DENV infection versus SDI. **(C)** The ASIR of YFV infection versus SDI. **(D)** The ASIR of ZIKV infection versus SDI. The different colors stand for different SDI quintiles. The ρ indices and p-values were derived from Pearson correlation analysis. DENV, Dengue virus; YFV, yellow fever virus; ZIKV, Zika virus.

worldwide to better prepare for and respond to future flavivirus pandemics.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The GBD study's protocol has been approved by the research Ethics Board at the University of Washington (UW). The GBD studies must be conducted in full compliance with UW policies and procedures, as well as applicable federal, state, and local laws. Therefore, all ethical standards are justified by properly citing the respective sources (http://ghdx.healthdata.org/gbd-results-tool).

Author contributions

YL: Conceptualization, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. XD: Data curation, Formal analysis, Funding acquisition, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Development and evaluation of two rapid lateral flow assays for on-site detection of African swine fever virus

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Introduction: African swine fever (ASF) is a lethal and highly contagious transboundary animal disease with the potential for rapid international spread. In the absence of a widely available and definitively proven vaccine, rapid and early detection is critical for ASF control. The quick and user-friendly lateral flow assay (LFA) can easily be performed by following simple instructions and is ideal for on-site use. This study describes the development and validation of two LFAs for the rapid detection of ASF virus (ASFV) in pig serum.

Methods: The highly immunogenic antigens (p30 and p72) of ASFV Georgia 2007/1 (genotype II) were expressed in plants (*Nicotiana benthamiana*) and were used to immunize BALB/c mice to generate specific monoclonal antibodies (mAbs) against the p30 and p72 proteins. mAbs with the strongest binding ability to each protein were used to develop p30_LFA and p72_LFA for detecting the respective ASFV antigens. The assays were first evaluated using a spike-in test by adding the purified p30 or p72 protein to a serum sample from a healthy donor pig. Further validation of the tests was carried out using serum samples derived from experimentally infected domestic pigs, field domestic pigs, and feral pigs, and the results were compared with those of ASFV real-time PCR.

Results: p30_LFA and p72_LFA showed no cross-reaction with common swine viruses and delivered visual results in 15 min. When testing with serially diluted proteins in swine serum samples, analytical sensitivity reached 10 ng/test for p30_LFA and 20 ng/test for p72_LFA. Using real-time PCR as a reference, both assays demonstrated high sensitivity (84.21% for p30_LFA and 100% for p72_LFA) with experimentally ASFV-infected pig sera. Specificity was 100% for both LFAs using a panel of PBS-inoculated domestic pig sera. Excellent specificity was also shown for field domestic pig sera (100% for p30_LFA and 93% for p72_LFA) and feral pig sera (100% for both LFAs).

Conclusion: The results obtained in this study suggest that p30_LFA and p72_LFA hold promise as rapid, sensitive, user-friendly, and field-deployable tools for ASF control, particularly in settings with limited laboratory resources.

KEYWORDS

African swine fever, lateral flow assay, rapid, sensitive, development

1 Introduction

ASF is a devastating and highly contagious transboundary animal disease with the potential for rapid international spread (Dixon et al., 2020). The causative agent, African swine fever virus (ASFV), is a large, double-stranded DNA virus belonging to the *Asfarviridae* family. This complex virus genome is approximately 170–194 kilobase pairs (kbp) and contains over 150 open reading frames (ORFs), depending on the strain (Karger et al., 2019; Wang et al., 2021; Li et al., 2022). While warthogs and specific soft ticks act as natural reservoirs for ASFV, harboring the virus with no signs of illness, domestic pigs face a different fate. Infection in domestic pigs triggers a severe and often fatal disease with high mortality rates (Karger et al., 2019; Netherton et al., 2019; Wang Y. et al., 2021; Li et al., 2022). Based on the p72 major capsid p72 protein gene (*B646L*), 24 ASFV genotypes (I–XXIV) have been identified (Quembo et al., 2018). Genotype II ASFV that emerged in the Caucasus region in 2007 is responsible for the contemporary pandemic. The situation worsened dramatically when ASF reached China in 2018, swiftly spreading across Asia (Le et al., 2019; Berends et al., 2021; Mighell and Ward, 2021). Notably, the disease re-emerged in the Caribbean in 2021, impacting the Dominican Republic and Haiti after approximately four decades of absence (Jean-Pierre et al., 2022; Ramirez-Medina et al., 2022). Currently, ASF remains widespread in sub-Saharan Africa, parts of West Africa, and Sardinia and continues to spread in Europe, Asia, the Pacific, and the Caribbean regions (Shi et al., 2021; Wang et al., 2023).

The lack of effective tools makes prevention and control extremely challenging. Attempts to immunize animals using vaccine formulations prepared by conventional means and comprising infected cell extracts, supernatants of infected pig peripheral blood leukocytes, purified and inactivated virions, infected glutaraldehyde-fixed macrophages, or detergent-treated infected alveolar macrophages failed to induce protective immunity (Escribano et al., 2013; Rock, 2021; Pikalo et al., 2022). Similarly, subunit vaccines targeting specific viral proteins, even in combination, have not provided complete protection (Gaudreault and Richt, 2019; Rock, 2021). Live-attenuated vaccine (LAVs) candidates, though promising, face hurdles related to stable production, safety concerns, and the lack of methods to differentiate between vaccinated and infected animals (DIVAs) (Bosch-Camós et al., 2020; Rock, 2021; Wang T. et al., 2021; Brake, 2022; Truong et al., 2023). There is currently no ASF vaccine commercially available outside of Vietnam. Consequently, control strategies currently rely heavily on strict sanitation measures and the culling of infected and potentially exposed animals (Gallardo et al., 2015; Wang et al., 2023). Therefore, rapid and accurate diagnosis of ASFV infection is urgently required for the prevention, control, and eradication of the disease in affected countries. The OIE-recommended tests for ASFV detection include virus isolation, fluorescent antibody testing, real-time PCR, and conventional PCR (World Organisation for Animal Health (OIE), 2023). However, these methods are time-consuming and require well-equipped laboratories and trained personnel, which can lead to delays in disease diagnosis in remote or underserved areas. Newer molecular tests, such as portable PCR and LAMP assays, offer promise for field use but still require some technical expertise for nucleic acid extraction (Fan et al., 2020; Fu et al., 2021; Yang et al., 2022; Bohorquez et al., 2023).

LFAs offer a compelling solution for detecting pathogens in the field, especially in resource-limited settings. These tests are rapid, inexpensive, and user-friendly, requiring minimal training and equipment. They are ideal for point-of-care testing (POCT), providing results quickly and conveniently. Several lateral flow assays (LFAs) have been developed for detecting various viral and bacterial antigens (Magambo et al., 2014; Carrio et al., 2015; Schramm et al., 2015; Ang et al., 2016; Onyilagha et al., 2022). In this report, we expressed the highly immunogenic antigens (p30 and p72) of ASFV Georgia 2007/1 (genotype II) in the well-established *Nicotiana benthamiana* plant expression system (Alcaraz et al., 1990; Revilla et al., 2018; Park et al., 2019; Kim et al., 2023; Shin et al., 2023). Plant-based systems offer several advantages for recombinant protein production compared to bacterial or mammalian cell platforms. These include safety, low cost, compatibility with green technologies, minimal contamination risks, and widespread societal acceptance (Lee and Ko, 2017; Islam et al., 2019; Kim et al., 2023). Importantly, the potential for large-scale production, optimized growth conditions, low cultivation costs, and the ability to produce complex proteins further highlight the benefits of plant expression systems for recombinant protein manufacturing (Burnett and Burnett, 2020). By utilizing mAbs targeting plant-expressed ASFV p30 and p72 proteins, we successfully developed two novel LFAs for ASFV detection. These LFAs demonstrate high sensitivity and excellent specificity and are suitable for rapid, user-friendly, and field-deployable ASF surveillance.

2 Materials and methods

2.1 Animals, viruses, and cells

Specific pathogen-free female Balb/c female mice (6 weeks old) were purchased from Orient Bio, Sungnam, Korea. All animal experiments were authorized by the Institutional Animal Care and Use Committee of MEDEXX (IACUC# AEC-20160713-0001). All animal experiments were performed under strict adherence to the IACUC protocol.

Murine myeloma cell line Sp2/0Ag14 was purchased from the American Type Culture Collection (ATCC-CRL-1581, Rockville, MD, United States) and was maintained in RPMI-1640 (Gibco, New York, NY, United States) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, United States) at 37 °C with 5% CO_2.

A virulent VNUA-ASFV-05 L1 strain (genotype II) was isolated from the spleen of a domestic pig with typical acute ASF during an ASF outbreak in Northern Vietnam in 2020 (Truong et al., 2021). It is maintained in BSL-3 laboratories of Kansas State University. This virus was used to make the standards for the Quantitative ASFV Real-Time PCR.

2.2 Porcine serum samples

This study utilized serum samples from domestic pigs and feral pigs in Dr. Jishu Shi's laboratory at Kansas State University. These samples include the following:

i ASFV negative pig sera: serum samples from pigs inoculated with phosphate-buffered saline (PBS, pH 7.4, Thermo Scientific, Bridgewater, NJ, United States).

ii iASFV-infected pig sera: serum samples from pigs infected with the ASFV VNUA-ASFV-05 L1 at different time points: day 0, day 7, and the day of euthanasia.
iii Other common swine virus-infected pig sera: serum samples from pigs infected with classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), and bovine viral diarrhea virus (BVDV).
iv Feral pig sera: serum samples from feral pigs caught in Kansas (collaboration with USDA APHIS Wildlife Services, Kansas Wildlife Services, United States).

Additionally, serum samples from field pigs with no known exposure to ASFV were collected and tested with the presented LFAs in four farms within South Korea.

2.3 Protein expression and generation of monoclonal antibodies

DNA sequences encoding p30 and p72 protein of ASFV Georgia 2007/1 (GenBank accession number FR682468.2) were codon optimized for *N. benthamiana*. For the purification, six histidine tags or porcine Fc domain were fused at the C-terminus of p30 or p72, respectively. Each fusion gene was additionally fused with the ER signal sequence of binding protein (BiP) and then inserted into the pCAMBIA1300 vector harboring CaMV 35 s promoter and NoS terminator. Expression of each protein in *N. benthamiana* was performed according to a previously described protocol (Sohn et al., 2023). To confirm the purity of the isolated proteins, samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% gel. Gels were then stained with Coomassie Brilliant Blue R-250 (BioSolutions, Suwon, South Korea) according to the manufacturer's instructions.

For the generation of mAbs against p30 and p72 proteins, 100 µL of protein p30 or p72 (2.5 µg/mL) mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich Inc., St. Louis, MO, United States) was used as immunogens to inject (intraperitoneal injection, IP) each of five female Balb/c mice. Two booster injections, each with the same protein dose and equal volume of Freund's incomplete adjuvant (Sigma-Aldrich Inc., St. Louis, MO, United States), were administered at 2-week intervals. One week after the second booster, blood samples were collected for antibody titer testing. The mouse with the highest antibody titer received a final injection of 2.5 µg protein without adjuvant. Three days later, these mice were euthanized, and their spleen cells were fused with SP2/0Ag14 cells using 50% polyethylene glycol (Sigma-Aldrich Inc., St. Louis, MO, United States) at a 5:1 ratio. Following fusion, cells were resuspended in a HAT-selective medium (Sigma-Aldrich Inc., St. Louis, MO, United States) at a concentration of 10^5 cells/ml. Then, 100 µL of this cell suspension was added to each well of a 96-well plate and incubated at 37°C with 5% CO_2. After 10 days, culture supernatants were screened for the presence of antibodies against p30 or p72 using an indirect ELISA. Positive wells were subjected to multiple rounds of single-cell cloning through limiting dilution until monoclonals were achieved.

2.4 Indirect ELISA

ELISA plates were coated overnight at 4°C with 100 µL/well of protein (1 µg/mL) in PBS (pH 7.4, Thermo Scientific, Bridgewater, NJ, United States). To perform the ELISA assay for screening the hybridomas, the plates were washed three times with PBS containing 0.05% Tween 20, blocked with 300 µL of 5% skimmed milk in PBS, and incubated at 37°C for 1 h. After washing, the plates were incubated with 100 µL/well of primary Ab (culture supernatants) for 1 h. After washing, the plates were incubated with secondary Ab (0.8 mg/mL at 1:10,000 dilution Goat anti-Mouse IgG HRP, 100 µL/well) at 37°C for 1 h. Enzyme assay was performed using TMB (Invitrogen, Carlsbad, CA, United States). The reaction was allowed to occur for 10 min and then stopped with 100 µL/well of 1 M sulfuric acid. The absorbance at 450 nm was measured using a microtiter plate reader.

2.5 Nucleic acid extraction and quantitative ASFV real-time PCR

Viral nucleic acids were extracted using an automated King Fisher™ Duo Prime DNA/RNA extraction system (Thermo Fisher Scientific, Waltham, MA, United States) with MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA, United States), according to the manufacturer's protocols. Serially diluted Genotype II ASFVs were added to the extraction plate as standards for quantification. ASFV DNA was then detected using Path-ID Multiplex One-Step RT-PCR Kit (Applied Biosystems, Grand Island, NY, United States) on StepOnePlus™ Real-Time PCR System (Applied Biosystems, Grand Island, NY, United States) using previously described primers and probes (Shi et al., 2016). The reaction condition involved a 95°C incubation for 5 min, followed by 45 cycles of denaturation at 95°C for 15 s and a combined annealing and extension step at 60°C for 45 s. Upon completion, standard curves, Ct values, and virus quantities in each sample were recorded. All experiments were performed in duplicate.

2.6 Development and assembly of the lateral flow device

Lateral flow device development and assembly incorporated minor modifications from previously described methods (Sastre et al., 2016). Briefly, a dispenser applied specific capture solutions: goat anti-rabbit IgG (1 mg/mL) for the control line and either anti-p30 mAb 89G6 (2 mg/mL) or anti-p72 mAb 5G11 (2 mg/mL) for the test line, at a rate of 1 µL/cm. The membranes were then dried for over 4 h at a low humidity (below 20%).

Colloidal gold conjugation began by heating 90 mL of distilled water to 100°C. After reaching boiling, 10 mL of 1% (w/v) gold chloride trihydrate and 1 mL of 1% sodium citrate tribasic dihydrate were added and stirred until completely dissolved. The solution turned a final red color and was then stirred for an additional 10 min at room temperature. Following dilution, the solution's absorbance was measured using a spectrophotometer. Antibody conjugation involved adjusting the pH of 50 mL of colloidal gold solution to 7.2 with 0.1 M potassium carbonate. Either anti-p30 mAb 91G3 (7 µg/mL in PBS) or anti-p72 mAb 7D11 (7 µg/mL in PBS) was slowly added dropwise and

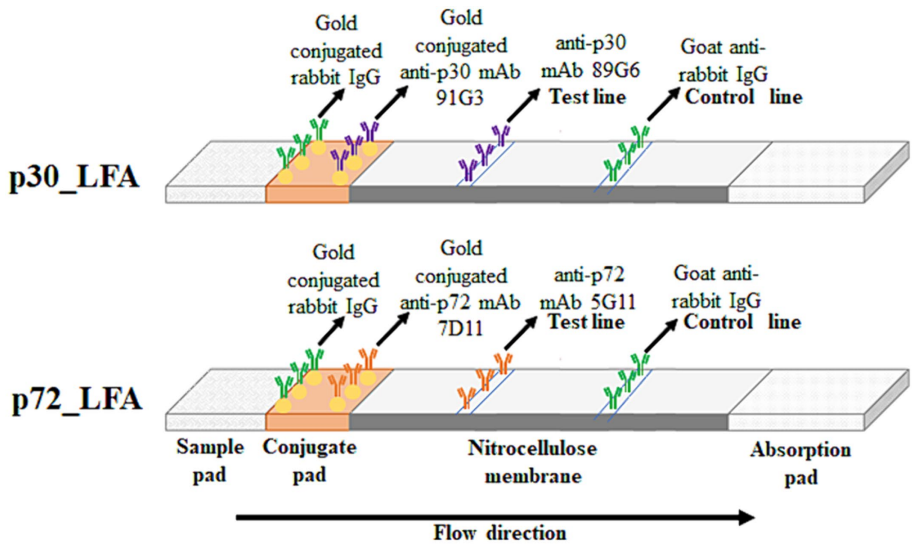

FIGURE 1
Schematic diagram of p30_LFA and p72_LFA. The sample (pig serum or plasma) migrates through the conjugate pad and the nitrocellulose membrane by capillarity. In the presence of ASFV, the p30/p72 protein is captured first by the gold-conjugated mAb91G3/mAb7D11, forming an antibody–antigen immune complex. This immune complex then reacts with the immobilized mAb89G6/mAb5G11 on the membrane, making the test line visible along with the control line (gold-conjugated rabbit IgG captured by goat anti-rabbit IgG). In the case of a negative test, only a control line appears.

stirred for 30 min. Subsequently, 5 mL of 10% (w/v) bovine serum albumin (BSA) was incorporated, and the mixture was stirred for another 30 min. The conjugate was centrifuged (10,000 rpm, 30 min) to separate the liquid (supernatant) from the solid (pellet). The pellet was then resuspended in 1% (w/v) BSA in PBS and stored in a refrigerator.

The conjugation pad was prepared with a solution containing 3% sucrose, 0.1% sodium azide, 1% BSA, and the appropriate antibody-gold conjugate (with an optical density (OD) of 2 at 540 nm) in PBS. Gold-conjugated rabbit IgG (OD 0.7 at 540 nm) was included for testing the control line. This solution was absorbed onto a glass fiber conjugate pad and dried for over 4 h at low humidity (below 20%). Finally, the membrane with capture antibodies, the conjugate pad, and a sample pad were assembled in a plastic housing to create the final LFA kit (Figure 1).

2.7 Test procedure

The test is designed for use with porcine plasma or serum samples without dilution. Samples should be brought to room temperature (15–30°C) prior to test. A measure of 120 µL of the samples is applied to the sample pad. The sample will flow along the result window by capillarity. The results are interpreted 15 min after adding the sample. In the presence of ASFV, the test line (red/pink) is visible along with the red/pink control line. In the case of a negative test, only a red/pink control line appears. The control line must appear always. Otherwise, the test has to be considered invalid and needs to be repeated with a new LFA cassette.

2.8 Statistical analysis

For this study, quantitative ASFV real-time PCR was regarded as the standard (reference) for pathogen detection. Sensitivity and specificity analyses were carried out by the web-based MedCalc statistical software.[1]

3 Results

3.1 Development of the lateral flow assay

The ASFV proteins p30 and p72 were successfully expressed and purified from *N. benthamiana* as expected protein size (Figure 2). To establish the LFA test, we selected mAbs with the strongest binding ability to each plant-derived protein. A specific mAb for p72 (mAb 5G11) was used as the capture reagent on the test line for the p72_LFA. Similarly, a high-affinity mAb for p30 (mAb 89G6) was used on the test line in the p30_LFA. For increased sensitivity, we designed the LFA to avoid diluting the sample. Pig serum or plasma can be directly applied to the designated well on the device. After optimization, we established a user-friendly testing procedure:

i Add 120 µL of the sample directly to the sample well (or use the provided dropper to add four drops).
ii Wait for 15 min.
iii Interpret the results in the designated window.

The appearance of a pink test line and a pink control line indicates a positive result, while only the pink control line indicates a negative result (Figure 3).

1 https://www.medcalc.org/calc/diagnostic_test.php, accessed on 21 June 2024.

3.2 Analytical specificity and sensitivity of p30_LFA and p72_LFA

To confirm that our p30_LFA and p72_LFA tests only detect ASFV and do not react to other viruses, we tested them with various serum sample categories. These categories include serum samples from pigs injected with PBS ($n = 30$), serum samples from pigs infected with CSFV ($n = 12$), serum samples from pigs infected with PRRSV ($n = 12$), serum samples from pigs infected with PRV ($n = 10$), and serum samples from pigs infected with BVDV ($n = 2$). The results are encouraging (Table 1). Both p30_LFA and p72_LFA tests are negative for all samples (100% specificity). This indicates that the tests are highly specific for ASFV and do not react with other common swine viruses.

In order to evaluate the analytical sensitivity, spike-in tests were carried out. We added known amounts of purified ASFV p30 and p72 proteins to serum samples from a healthy donor pig. We then progressively diluted these samples by two-fold serial dilutions. These diluted samples were then analyzed using both p30_LFA and p72_LFA. The p30_LFA test can detect ASFV p30 protein as low as 10 nanograms per test (ng/test) (Figure 4A). The p72_LFA test can detect ASFV p72 protein down to a concentration of 20 ng/test (Figure 4B).

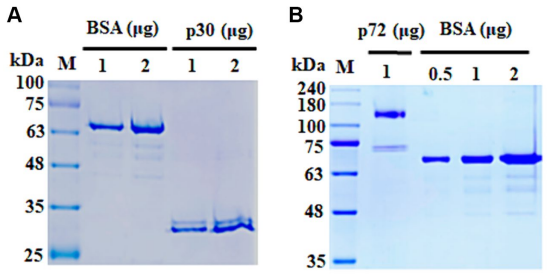

FIGURE 2
SDS-PAGE analysis of purified ASFV proteins. **(A)** Purified p30 protein. **(B)** Purified p72 protein. Bovine serum albumin (BSA) was used as a loading control. kDa, kilo Dalton.

3.3 Validate p30_LFA and p72_LFA with experimental samples

To further evaluate p30_LFA and p72_LFA tests, we used serum samples collected at 0-day post-infection (DPI), 7 DPI, and the day of euthanasia from 10 pigs infected with ASFV VNUA-ASFV-05 L1 (genotype II). This virus was isolated from a domestic pig during an ASF outbreak in Northern Vietnam in 2020 and caused typical clinical

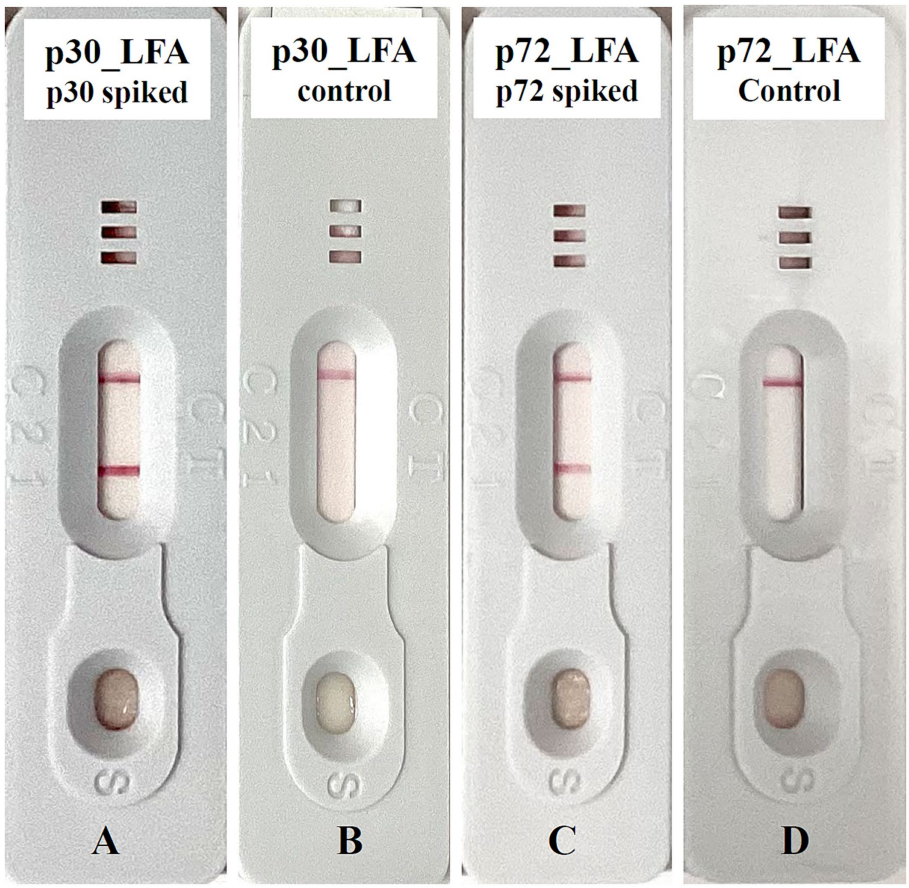

FIGURE 3
Exemplary lateral flow device for ASFV detection. **(A)** p30_LFA positive: test line and control line are detected. **(B)** p30_LFA is negative: only the control line is detected. **(C)** p72_LFA is positive: test line and control line are detected. **(D)** p72_LFA is negative: only the control line is detected.

TABLE 1 Analytical specificity test of p30_LFA and p72_LFA with various swine serum categories.

Pig #	DPI 0				DPI 7				Day of euthanasia				
	RT-PCR		p30 LFA	p72 LFA	RT-PCR		p30 LFA	p72 LFA		RT-PCR		p30 LFA	p72 LFA
	Ct value	Quantity (HAD_{50})			Ct value	Quantity (HAD_{50})				Ct value	Quantity (HAD_{50})		
1	UD	UD	–	–	40.67	2	–	+	DPI 16	35.78	44	–	+
2	UD	UD	–	–	20.04	4,395,958	+	+	DPI 8	23.70	321,269	+	+
3	UD	UD	–	–	23.99	259,958	+	+	DPI 9	25.89	66,709	+	+
4	UD	UD	–	–	20.81	2,549,016	+	+	DPI 8	23.76	306,789	+	+
5	UD	UD	–	–	22.25	906,527	+	+	DPI 11	26.98	30,539	+	+
6	UD	UD	–	–	36.98	113	–	+	DPI 8	20.34	2,497,416	+	+
7	UD	UD	–	–	UD	UD	–	–	DPI 7	22.21	805,733	+	+
8	UD	UD	–	–	UD	UD	–	–	DPI 13	20.98	1,695,203	+	+
9	UD	UD	–	–	UD	UD	–	–	DPI 14	24.62	126,019	+	+
10	UD	UD	–	–	UD	UD	–	–	DPI 14	19.25	4,830,258	+	+

DPI, days post-infection; HAD_{50}, 50% hemadsorption doses; UD, underdetermined; "–," negative; "+," positive.

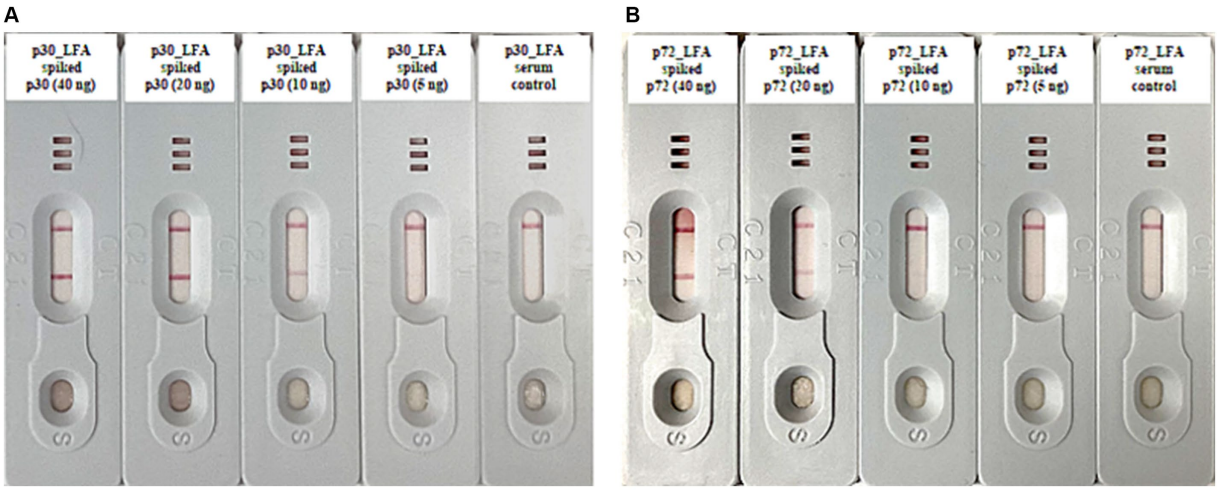

FIGURE 4
Analytical sensitivity tests of p30_LFA and p72_LFA. **(A)** Two-fold serial dilutions of purified ASFV p30 protein (40, 20, 10, 5, and 0 ng) in serum samples (from healthy donor pig) tested by p30_LFA. **(B)** Two-fold serial dilutions of purified ASFV p72 protein (40, 20, 10, 5, and 0 ng) in serum samples (from healthy donor pig) tested by p72_LFA.

signs of acute ASF (Truong et al., 2021). Six pigs tested positive for ASFV using quantitative ASFV real-time PCR at 7 DPI and all pigs tested positive on the day they were euthanized (between 8 and 16 DPI). The amount of ASFV in their serum samples (viral load) varied, ranging from a high level (Ct values of 19.25, ASFV quantity 4,830,258 HAD_{50}) to a very low level (Ct values of 40.67, ASFV quantity 2 HAD_{50}) (Table 2).

The results showed the following:

i For p72_LFA: All serum samples that were tested positive by real-time PCR were also tested positive on the p72_LFA test. This indicates that the p72_LFA test has 100% sensitivity (95% confidence interval: 79.41 to 100%) for detecting ASFV in these samples.

ii For p30_LFA: Three samples (pig #1 at 7 DPI and 16 DPI, pig#6 at 7 DPI) with very low viral loads (Ct values of 40.67, 35.78, and 36.98, respectively) tested negative for p30_LFA. This indicates that the p30_LFA test has 84.21% sensitivity (95% confidence interval: 60.42 to 96.62%) for detecting ASFV in these samples.

Importantly, both the p30_LFA and p72_LFA tests did not show any false positives, meaning they have 100% specificity for detecting ASFV in these samples.

3.4 Validate p30_LFA and p72_LFA with field samples

To validate the performance of p30_LFA and p72_LFA with field samples, we collected serum samples from domestic pigs ($n = 100$) across four farms in different geographical regions of South Korea (Table 3). These farms had no history of exposure to ASFV, and all samples tested negative for ASFV using an ASFV real-time PCR assay.

The p30_LFA test results are ideal, showing negative results for all 100 serum samples, demonstrating 100% specificity. This means the p30_LFA accurately identified pigs without ASFV. However, the p72_LFA test produced unexpectedly positive results. Despite these farms being ASFV-free, seven samples reacted with the p72_LFA test. This translates to a specificity of 93% for the p72-LFA test.

In addition, we tested serum samples from feral pigs ($n = 6$) with p30_LFA and p72_LFA. Both p30_LFA and p72_LFA showed negative results for these samples, demonstrating 100% specificity.

4 Discussion

While highly sensitive and specific molecular tests such as real-time PCR exist for ASFV detection (Fan et al., 2020; Fu et al., 2021; Yang et al., 2022; Bohorquez et al., 2023; World Organisation for Animal Health (OIE), 2023), these assays can be expensive, require specialized training, and are limited to laboratory use. LFA offers a promising alternative. It is cost-effective, portable, requires no additional equipment, can easily be performed outside the laboratory, and provides results within minutes. These features make LFA a valuable POCT. A large number of such assays have been applied as efficient tests for the on-site analysis of biomarkers, such as proteins, small molecules, and nucleic acids, from a variety of different biological samples, including serum, blood, urine, saliva, and many other types (Magambo et al., 2014; Carrio et al., 2015; Schramm et al., 2015; Omidfar et al., 2023). A key limitation of LFAs is their generally lower sensitivity compared to molecular assays such as real-time PCR, which can lead to false-negative results (Onyilagha et al., 2022). However, this limitation might be less critical for ASFV detection. Pigs infected with virulent ASFV strains, such as the contemporary pandemic genotype II ASFV strain, develop high levels of the virus in their blood within a few days after infection. These virulent strains have a short incubation (2–3 days), followed by early clinical signs such as fever within 3–5 days. The fever in ASFV-infected animals coincides with viremia (the number of viruses in the blood), which quickly peaks (within 1–2 days of fever) up to 10^9 HAD_{50}/ml (Dixon et al., 2020; Onyilagha et al., 2022). The high viral load during early infection makes LFA a viable option for detecting ASFV antigens in the early stage of ASFV infection.

This study focused on developing rapid and highly sensitive LFA for detecting the infection of ASFV in pigs. We developed p30_LFA, which is designed to detect the early viral structural protein p30 (encoded by the *CP204L* gene), expressed as early as 2–4 h post-infection and throughout the infection cycle (Revilla et al., 2018; Omidfar et al., 2023). In the meantime, we developed p72_LFA, which is designed to detect the p72 capsid protein (encoded by the *B646L* gene), the main structural protein of ASFV, accounting for approximately 33% of the total viral mass (Alcaraz et al., 1990; García-Escudero et al., 1998). Our tests achieved high analytical sensitivity in the spike-in test. p30_LFA detects as low as 10 ng of p30 protein per test (Figure 4A). p72_LFA detects down to 20 ng of p72 protein per test (Figure 4B). Both p30_LFA and p72_LFA showed no cross-reactions with other tested viruses (CSFV, PRRSV, PRV, and BVDV) (Table 1).

Compared to real-time PCR using samples from pigs experimentally infected with ASFV, our p30_LFA and p72_LFA assays demonstrated promising sensitivity and specificity. The p30_LFA detected ASFV in 84.21% of positive samples, while the p72_LFA

TABLE 2 Comparing results of p30_LFA and p72_LFA tests with the quantitative ASFV real-time PCR using serum samples from ASFV VNUA-ASFV-05 L1 (genotype II) experimentally infected pigs.

Category	Details	Test number	Positive (p30_LFA/p72_LFA)	Negative (p30_LFA/p72_LFA)	Specificity (p30_LFA/p72_LFA)
1	Serum from PBS injected pig	30	0/0	30/30	100%/100%
2	Serum from CSFV-infected pig	12	0/0	12/12	100%/100%
3	Serum from PRRSV-infected pig	12	0/0	12/12	100%/100%
4	Serum from PRV-infected pig	10	0/0	10/10	100%/100%
5	Serum from BVDV-infected pig	2	0/0	2/2	100%/100%

TABLE 3 Validation of the p30_LFA and p72_LFA with field samples collected in South Korea.

Farm locations	Number of samples	ASFV RT-PCR	p30_LFA			p72_LFA		
			Positive	Negative	Specificity	Positive	Negative	Specificity
Farm 1 (Gyeonggi-do)	18	Negative	0	18	100%	1	17	94.4%
Farm 2 (Gyeonggi-do)	40	Negative	0	40	100%	5	35	87.5%
Farm 3 (Gyeongsangbuk-do)	20	Negative	0	20	100%	0	20	100%
Farm 4 (Jeju Island)	22	Negative	0	22	100%	1	21	95.5%
Total	100	Negative	0	100	100%	7	93	93%

achieved 100% sensitivity. Both assays exhibited 100% specificity, meaning no false positives were observed (Table 2). This is a significant improvement over previously reported ASFV LFAs, which only detected 68% of real-time PCR-positive samples and required sample dilution before testing (Sastre et al., 2016). Our assays achieve high sensitivity (low false negatives) due to two key factors. First, we utilize high-affinity mAbs in p30_LFA and p72_LFA. Second, these assays are designed for direct use with porcine plasma or serum samples, eliminating the need for dilution steps. Since serum samples were used, p72_LFA is expected to be more sensitive than p30_LFA. The p30_LFA targets the inner core shell protein of ASFV, while the p72_LFA targets the outer capsid protein of ASFV, granting it direct access to assay capture antibodies (Alcaraz et al., 1990; Revilla et al., 2018). To further improve p30_LFA sensitivity, we are incorporating an additional sample lysis step to liberate inner antigens. We will report these results in the near future.

Field testing using serum samples from South Korean farms revealed high specificities of 100 and 93% for p30_LFA and p72_LFA, respectively (Table 3). Seven unexpected false positives were observed with p72_LFA out of 100 field samples. These discrepancies might be due to farm-specific factors, such as underlying disease conditions or vaccination protocols, warranting further investigation. Further assessment of the specificity and sensitivity of p72_LFA under field conditions, as well as the exploration of strategies to enhance its diagnostic accuracy, are planned for future studies. Encouragingly, both LFAs exhibited 100% specificity with feral pig serum, suggesting they could be a reliable tool for identifying ASFV infection in wild pigs. These findings highlight the potential of p30_LFA and p72_LFA as field-deployable tools for identifying ASFV-infected farms and monitoring ASFV in wild pigs, particularly in areas with limited access to molecular assays or centralized laboratories.

We are presently conducting additional field validation studies using samples collected from both domestic and wild pigs to validate these promising findings. The validations will encompass the quality of the samples and environmental variables. Field samples primarily consist of carcasses from animals discovered either deceased or harvested, a crucial aspect to consider during LFA sample analysis. Factors including cold temperatures have the potential to decelerate reactions, potentially leading to skewed results. Windy conditions could introduce dust or debris that might disrupt the testing process. We will investigate the specific influence of these variables on the p30-LFA and p72-LFA assays and report the findings soon.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of MEDEXX. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

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Conflict of interest

JK, HK, and E-JS were employed by the BioApplications Inc. H-JP and M-JL were employed by the MEDEXX Co., Ltd. S-HH and C-WS were employed by the Celltrix Co., Ltd.

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Complex transmission epidemiology of neglected Australian arboviruses: diverse non-human vertebrate hosts and competent arthropod invertebrate vectors

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More than 75 arboviruses are indigenous to Australia, of which at least 13 are known to cause disease in humans. Alphaviruses are the most common arboviruses, notably including Ross River and Barmah Forest viruses, which contribute a significant public health and economic burden in Australia. Both can cause febrile illness with arthritic symptoms. Each circulates nationally across diverse climates and environments, and has multi-host, multi-vector dynamics. Several medically important flaviviruses also circulate in Australia. Infection with Murray Valley encephalitis or Kunjin viruses is less common but is associated with brain inflammation. Key research priorities for Australian arboviruses aim to understand clinical manifestations, develop timely diagnostics, and identify transmission cycles that permit the maintenance of arboviruses. While these can now be answered for a handful of notifiable alpha- and flaviviruses there are others for which non-human vertebrate hosts and competent arthropod invertebrate vectors are still to be identified and/or whose role in transmission is not well understood. One or more of these 'neglected' arboviruses may be the causative agent of a proportion of the many thousands of fever-related illnesses reported annually in Australia that at present remain undiagnosed. Here, what is known about enzootic cycling of viruses between arthropod vectors and mammalian and avian reservoir hosts is summarised. How and to what extent these interactions influence the epidemiology of arbovirus transmission and infection is discussed.

KEYWORDS

arbovirus, neglected, transmission, arthropod, vector, reservoir host, enzootic, Australia

1 Introduction

Arthropod-borne viruses, commonly known as arboviruses, are a polyphyletic group of RNA viruses that circulate between different arthropod vectors (insects, usually mosquitoes, but also midges, sand flies and black flies; and arachnid ticks) and human and non-human vertebrate hosts (Franz et al., 2015). While these are predominantly mammals

and birds the possible role of reptiles and amphibians in arbovirus transmission cycles has been discussed (Bosco-Lauth et al., 2018). These viruses are typically transmitted through the bite of an infectious arthropod vector, which acquires the virus by feeding on a viraemic vertebrate host (Lequime et al., 2016).

Arboviruses are significant health threats in tropical and subtropical regions with 3.9 billion people at risk, leading to an estimated disease burden of 300,000 to 5 million disability-adjusted life years lost annually (Labeaud et al., 2011). Arboviral infection can cause disease in vertebrates but does not trigger significant pathology in arthropods (Franz et al., 2015). While some human arboviral infections are asymptomatic or present with a mild influenza-like illness, arboviral pathogens causing serious illness ranging from rash and arthritis to encephalitis and haemorrhagic fever are an increasing threat to global health security (Wilder-Smith et al., 2017). The most striking illustration of this is the worldwide advance of dengue over the last 70 years (Gyawali et al., 2016a), while Japanese encephalitis virus (JEV) causes the majority of viral encephalitis cases in Asia (World Health Organization, 2019). Moreover, recent epidemics of chikungunya (CHIKV) and West Nile virus infection, plus the transcontinental spread of Zika virus (ZIKV), exemplify the growing risk posed by previously obscure pathogens and hence justify the concern over such emerging arboviruses (Gyawali et al., 2016b).

The interactions between invertebrate arthropod vectors and vertebrate hosts play a crucial role in the transmission and maintenance of arboviruses in nature (Kuno and Chang, 2005). Arthropod vectors serve as both the primary natural reservoirs and the means of transmission for arboviruses, while vertebrate hosts can act as amplifiers of viral replication (Kuno and Chang, 2005). The transmission cycle between these hosts allows for the persistence and spread of arboviruses in the environment. Arboviruses generally establish lifelong infection in vectors but exhibit transient infection of variable magnitude and duration in vertebrate hosts (Althouse and Hanley, 2015). Host factors such as tissue barriers, immune responses, genetic diversity, and replication dynamics all contribute to the complex dynamics of arbovirus transmission. Important ecological factors include population abundance, vector-host contact rate, and host migratory and other behaviours.

Only by fully understanding interactions between vectors and hosts can arbovirus transmission be controlled and/or prevented. Nowhere is this of more relevance than Australia, where more than 75 arboviruses that are indigenous to the country have been identified (Centers for Disease Control and Prevention, 2024). The alphaviruses Ross River (RRV) and Barmah Forest (BFV), and the flavivirus Murray Valley encephalitis (MVEV), are established as causative agents of debilitating diseases (e.g., Fraser, 1986), each of which may be detected by both antibody-based recognition and nucleic-acid amplification. However, for most of the remaining arboviruses (e.g., Alfuy [ALFV], Edge-Hill [EHV], Kokobera (KOKV], Sindbis [SINV], and Stratford [STRV]), that are or may be associated with pathology in humans, including some undifferentiated febrile illnesses routine tests are not available to diagnose infection (Gyawali et al., 2017a; Gyawali et al., 2019a). Prominent among public health challenges in parts of Australia north of the Tropic of Capricorn, as well as occasionally in more southerly latitudes, are so-called 'neglected' Australian arboviruses. Some of these viruses cause acute undifferentiated febrile illness, for which over half of all cases that occur annually in Australia are not diagnosed (Susilawati and McBride, 2014). Instigating a rigorous identification program would reduce the possibility of significant outbreaks of these indigenous arboviruses at a time when population growth accelerates across regional Australia, thereby bringing humans into closer proximity of native wildlife and vectors (Gyawali et al., 2017a).

Until very recently, JEV was limited to Far North Australia (Torres Strait Islands and Tiwi Island). During the hot, wet summers of 2021-22 and 2022-23, however, there was a dramatic geographical expansion of JEV across central and southern Australia (Pendrey and Martin, 2023). Of 45 clinical cases (35 laboratory-confirmed and 10 suggestive epidemiologically and/or symptomatically), there were seven deaths (Yakob et al., 2023). Given that local transmission occurred over two consecutive mosquito seasons, it is likely that JEV is now established endemically on the Australian mainland, placing as much as 750,000 people, 3% of the national population, at risk of JEV (Yakob et al., 2023). While sporadic cases of the closely related MVEV are largely confined to Northern Australia, major outbreaks were reported across southern and eastern regions of the country in 1951 (45 cases), 1974 (58 cases) and 2011 (17 cases) (Selvey et al., 2014a). In early 2023, six cases of MVEV infection were notified in the south-eastern state of Victoria, three of which were fatal (Braddick et al., 2023).

The concurrent (re-)emergence and co-circulation of JEV with MVEV (McGuinness et al., 2023) highlights key knowledge gaps in vector ecology, transmission dynamics and intervention efficacy. Integral to tailoring a control and prevention strategy to suit all Australian arboviruses is a better understanding of the interactions between their arthropod vectors and vertebrate hosts, which underpins their transmission epidemiology. While the number of arthropods from which these viruses have been recovered is considerable, less is known about the non-human vertebrate hosts that may be involved in their environment cycling or in the biting preferences of different mosquito species for these different reservoir hosts (Gyawali et al., 2019b; Gyawali et al., 2020). Information on the epidemiology and ecology of most of the neglected arboviruses is sketchy but they are known, or at least assumed, to be largely maintained in zoonotic cycles rather than exclusively by human-to-human transmission.

2 Transmission cycles of arboviruses

The transmission cycle of an arbovirus is determined by virus-vector host interactions. Arboviruses are transmitted between hosts by their arthropod vectors. The transmission cycle starts when an arthropod feeds on viraemic blood. Arboviruses can establish infection in the midgut epithelial cells of the arthropod vector and subsequently replicate in various tissues, including the salivary glands, enabling transmission to occur subsequently when the vector takes a blood meal (Lequime et al., 2016). Many arboviruses (e.g., MVEV, KUNV and JEV) have complex transmission cycles that include multiple host and vector species in maintenance and spillover (Kuno and Chang, 2005). Some hosts develop sufficiently high viraemias to infect susceptible vectors that feed on them while others do not. Failure to develop a viraemia sufficient to infect

a vector does not mean that the host will not develop clinical symptoms. Cycles of transmission may involve only arthropods and humans (e.g., epidemic cycle of dengue virus, DENV, and RRV) or only non-human vertebrates and vectors (e.g., Akabane virus) or there may be transmission of viruses between human and non-human hosts (zoonoses, e.g., BFV, MVE, RRV). Most Australian arboviruses are zoonotic and maintain enzootic cycles involving birds and mammals as reservoir hosts (Go et al., 2014; Russell and Kay, 2004). In this cycle, the virus is continuously maintained in nature and may or may not cause disease in the enzootic host. Infections and epidemics in human populations can arise from direct spill-over of these enzootic and epizootic (exploiting domestic animals, e.g., JEV) cycles when amplification achieves a viraemia high enough for transmission (Weaver and Barrett, 2004).

3 Arboviruses and their vertebrate hosts

3.1 Mammals

Vertebrates can be considered as a possible reservoir for an arbovirus when a minimum of three commonly used criteria are met: viraemia; virus isolation; and relatively high antibody titres. While the presence of serum antibody against arboviruses in a host does not, *per se*, prove that an animal has been viraemic or involved in virus transmission as a reservoir, serological surveys do provide information about which animals might be involved in the transmission cycles of arboviruses (Table 1).

3.1.1 Marsupials

Macropods (notably, kangaroos and wallabies) are reservoirs and likely focal hosts of RRV. Laboratory infections and transmission of RRV from different kangaroos species to mosquitoes (Doherty et al., 1964; Doherty et al., 1966; Kay and Aaskov, 1989; Kay et al., 1986; Lindsay et al., 2005; Potter et al., 2014), isolation of RRV from agile wallabies (*Macropus agilis*) at Mitchell River Mission in Cape York Peninsula, Far North Queensland (Doherty et al., 1971), and viraemias of maximum titre of 4.6–5.6 suckling mouse intracerebral inoculation (SMIC) LD_{50}/mL for between 4 and 6 days after introduction of RRV by the bite of infected mosquitoes (Kay et al., 1986) to Eastern grey kangaroos (*Macropus giganteus*) and agile wallabies have strongly suggested that marsupials are suitable hosts for RRV. While experimental studies of infection of macropods with all contemporary arboviruses have not been undertaken, one study reported titres of MVEV in eastern grey kangaroos as high as 10^3 SMIC LD_{50}/mL up to 6 days following the bite of an infected mosquito (Kay et al., 1986). Eastern grey kangaroos are terrestrial and widespread throughout eastern Australia, from Cape York to Victoria (Kay et al., 1985a). In Western Australia, the equivalent macropod is the closely related Western grey kangaroo (*Macropus fuliginosus*), which recent preliminary research (based on antibody detection) suggests is a common host for RRV and BFV (Gyawali et al., 2020). Common brushtail possums (*Trichosurus vulpecula*), a semi-arboreal marsupial, abound in the forest and urban areas throughout the eastern, northern, and south-western regions of Australia (How and Hillcox, 2000), and have been found to produce high titre viraemias following the bite of RRV-infected mosquitoes (Boyd et al., 2001), suggesting they may play a role in the urban transmission of RRV. Brushtail possums also generate mild viraemia for JEV (Daniels et al., 2000).

3.1.2 Ungulates

The epidemic spread of RRV infection across the Western Pacific region in 1979–80 (Aaskov et al., 1981; Rosen et al., 1981; Tesh et al., 1981), and presence of historical antibody of RRV in animal sera collected from the south Pacific (Togami et al., 2020) have demonstrated endemic transmission of RRV in the absence of marsupial reservoirs. Serological studies have detected anti-RRV antibodies in domestic ungulates (large mammals with hooves), including cows and horses (Table 1). Comprehensive experimental studies of infection of cattle and horses with all arboviruses have not been undertaken. However, when cattle were infected with MVEV using orally infected *Culex annulirostris*, no viraemia was detected (Kay et al., 1985b). Similarly, when horses were infected with RRV by intravenous injection or the bite of infected *Cx. annulirostris*, virus could not be recovered in cell culture when sera from infected horses were cultured. Yet, the viraemia was sufficient to infect laboratory mosquitoes when *Ae. vigilax* and *Cx. annulirostris* were fed on the infected horses (Kay et al., 1987; Ryan et al., 1997). The presence of anti-RRV antibodies in horses has led some arbovirologists to propose that they may act as amplifying hosts for RRV and to the further suggestion that viraemic horses could transport RRV from peri-urban to urban or city environments (Doherty et al., 1966; Gard et al., 1977; Cloonan et al., 1982; McManus and Marshall, 1986; Pascoe et al., 1978). Apart from RRV, antibodies to BFV, MVEV, and Sindbis virus (SINV) have also been detected in horses (Table 1). In addition, isolation of West Nile virus Kunjin strain (KUNV) following experimental infection (Badman et al., 1984) and natural infection (Frost et al., 2012) indicate the possible role of horses in the transmission cycle of each of these viruses. KUNV was responsible for a large outbreak of neurological disease in horses in 2011 (Frost et al., 2012; Roche et al., 2013). While neutralising antibodies against neglected Australian arboviruses have been detected in cattle (Table 1), Kay et al. (1985b) were unable to detect viraemia in cows infected by MVEV using *Cx. annulirostris*. Intensive pig farming and a large feral pig population may have aided to recent Australian JEV outbreaks (Williams et al., 2022). The latter serve as amplifying hosts of JEV and MVEV, and their geographic range and large number (~ 3.2 million) provide a latent risk of spillover to domestic piggeries and humans (Mackenzie and Smith, 2024). It is unknown how population dynamics and distribution of amplifying hosts influence arbovirus transmission.

3.1.3 Cats and dogs

No viraemia sufficient to infect mosquitoes developed in small domestic animals such as dogs and cats following experimental infection with RRV or BFV through the bite of infected *Ae. vigilax* (Boyd and Kay, 2002). A relatively low antibody prevalence (~ 10%) in serological survey of these animals suggested them less likely to be significant reservoirs of RRV, BFV and JEV.

TABLE 1 Association of Australian arboviruses in domestic and native animals.

Vertebrate	Location	RRV	BFV	SINV	ALFV	EHV	KOKV	KUNV	MVEV	STRV	Reference
Cow	New South Wales	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Cloonan et al., 1982
	Eastern Queensland	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	N/T	Doherty et al., 1964
	Brisbane	+	N/T	+	N/T	N/T	N/T	N/T	N/T	N/T	Doherty et al., 1966
	Mitchell River Mission	+	N/T	+	+	+	+	+	+	N/T	Doherty et al., 1971
	Northern Australia	+	N/T	N/T	N/T	N/T	N/T	N/T	+	N/T	Doherty et al., 1973
	Queensland	+	N/T	+	N/T	+	+	+	+	+	Sanderson, 1969
	New South Wales	+	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Vale et al., 1991
Horse	Eastern Australia	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	N/T	Anderson et al., 1952
	Victoria	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Azuolas, 1997
	South coast New South Wales	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Cloonan et al., 1982
	Gympie, Brisbane outer suburb	+	N/T	+	N/T	N/T	N/T	N/T	+	N/T	Doherty et al., 1966
	Mitchell River Mission	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Doherty et al., 1971
	Brisbane	+	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Kay et al., 2007
	New South Wales	+	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Vale et al., 1991
Dog	North and south Queensland	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	N/T	Doherty et al., 1964
	Redcliffe, Brisbane outer suburb	+	N/T	+	N/T	N/T	N/T	N/T	N/T	N/T	Doherty et al., 1966
	Mitchell River Mission	+	N/T	+	+	+	+	+	+	N/T	Doherty et al., 1971
	Brisbane	+	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Kay et al., 2007
Cat	Brisbane	+	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Kay et al., 2007
Wallaby	North and south Queensland	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	N/T	Doherty et al., 1964
	Gympie, Brisbane outer suburb	+	N/T	+	N/T	N/T	N/T	N/T	N/T	N/T	Doherty et al., 1966
	Mitchell River Mission	+	N/T	+	+	+	+	+	+	N/T	Doherty et al., 1971
	New South Wales	+	–	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Vale et al., 1991
Kangaroo	North and south Queensland	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	N/T	Doherty et al., 1964
	Mitchell River Mission	+	N/T	+	+	+	+	+	+	N/T	Doherty et al., 1971
	Western Queensland	+	N/T	+	N/T	N/T	N/T	N/T	N/T	N/T	Doherty et al., 1966
	New South Wales	+	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Vale et al., 1991
Fruit bat	Innisfail, north Queensland	–	N/T	–	N/T	N/T	N/T	N/T	N/T	N/T	Doherty et al., 1966
	Mitchell River Mission	+	N/T	–	+	–	+	+	+	N/T	Doherty et al., 1971
	Brisbane	+	–	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Kay et al., 2007

(Continued)

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TABLE 1 (Continued)

Vertebrate	Location	RRV	BFV	SINV	ALFV	EHV	KOKV	KUNV	MVEV	STRV	Reference
Rat	North and south Queensland	N/T	N/T	N/T	N/T	N/T	N/T	N/T	–	N/T	Doherty et al., 1964
	Northeast Queensland	+	N/T	+	N/T	N/T	N/T	N/T	N/T	N/T	Doherty et al., 1966
Wild pig	Mitchell River Mission	+	N/T	–	–	–	+	+	+	N/T	Doherty et al., 1971
Common brushtail possum	Eastern Australia	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	N/T	Anderson et al., 1952
	All Australian states & territories	+	–	+	N/T	N/T	N/T	–	+	N/T	Azuolas, 1997
	Mitchell River Mission	+	N/T	–	N/T	N/T	N/T	N/T	+	N/T	Doherty et al., 1971
	Brisbane	+	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Kay et al., 2007
Bandicoot	Northeast Queensland	+	N/T	+	N/T	N/T	N/T	N/T	+	N/T	Doherty et al., 1966

Virus name abbreviations: RRV, Ross River; BFV, Barmah Forest; SINV, Sindbis; ALFV, Alfuy; EHV, Edge Hill; KOKV, Kokobera; KUNV, West Nile virus Kunjin strain; MVEV, Murray Valley encephalitis; STRV, Stratford. N/T, not tested; +, antibodies detected; –, antibodies not detected. Mitchel River Mission in Far North Queensland is now named Kowanyama.

3.1.4 Bats

Bats have been associated with several zoonotic pathogens including viruses causing Ebola (filo virus), Lassa fever (Lassa virus) and COVID-19 (SARS-CoV-2). They are also suggested for having association with arboviruses. Fruit bats, also called flying foxes are found to have low viraemia to an experimental infection with RRV and JEV, but still were capable of infecting susceptible mosquitoes (Ryan et al., 1997; van den Hurk et al., 2009).

3.2 Birds

As early as 70 years ago, Anderson postulated that birds might be the primary reservoir of MVEV. The detection of anti-MVEV antibodies in many *Ciconiiformes* (storks) and *Pelecaniformes* (ibises, herons and pelicans) (Anderson, 1953; Anderson, 1954; Anderson et al., 1958; Doherty, 1964; Gard et al., 1976; Liehne et al., 1976; Boyle et al., 1983), and the high prevalence of anti-MVEV antibodies in birds (from 44% in adults to 96% in juveniles) after the MVEV epidemics of 1974–1975 (Marshall et al., 1982a) supported Anderson's hypothesis. Galahs, sulphur-crested cockatoos, corellas, and black ducks produced MVEV viraemias with titres of 10^2 to 10^6 SMIC LD_{50}/mL for 1–9 days following the bite of infected *Cx. annulirostris* mosquitoes (Kay et al., 1985b). With this viraemia in birds, approximately 10% of recipient *Cx. annulirostris* acquired virus infection.

Many avian species, particularly members of the family Ardeidae (herons, egrets, and allies), whose distribution overlaps with *Culex* mosquitoes, exhibit high prevalence of MVEV and JEV (Soman et al., 1977). Ardeids are considered the main vertebrate hosts of MVEV (Selvey et al., 2014b). As these avian species migrate to and across Australia (Guay et al., 2012), they share habitats with numerous other resident host species, such as cattle egrets (*Bubulcus ibis*) and feral pigs. The current role of migratory birds and feral pigs in the maintenance and transmission of arboviruses is a key health research priority for Australia. Where new wetlands, viraemic birds, and high mosquito densities converged near piggeries, the probability of "spillover" and rapid amplification in domestic pigs increased, causing the 2022 JEV outbreak in southern Australia. However, the mechanism of interaction between feral pigs, wildlife, and domestic animals is not clearly known.

The detection of antibodies to MVEV and KUNV in chickens (Doherty et al., 1968), was followed by isolation of MVEV from a sentinel chicken at Echuca, a town on the banks of the Murray River in northern Victoria, during the MVEV epidemic of 1974 (Campbell and Hore, 1975). Subsequently, the health department of several Australian state governments have employed flocks of sentinel chickens to monitor transmission of MVEV and KUNV as an early warning surveillance system to identify the threat of outbreaks (Doherty et al., 1976; Mackenzie et al., 1992). Besides MVEV, other arboviruses including Alfuy virus (ALFV), KUNV, RRV and SINV were also isolated from wild birds collected at Mitchell River Mission between 1963 and 1967 (Whitehead et al., 1968; Doherty, 1972; Doherty, 1977; Doherty et al., 1971). Ongoing research also suggests that birds may contribute to transmission dynamics of RRV and BFV, although their role in maintaining these viruses is still unclear (Vieira et al., 2023).

Furthermore, the increase in rainfall in southern Australia and the migration of water birds due to flowing inland rivers

could lead to heightened activity of MVEV in certain areas (Selvey et al., 2014a). These findings underscore the significance of understanding the association of Australian arboviruses with water birds in the context of public health challenges and disease transmission dynamics.

4 Arboviruses and their arthropod vectors

4.1 Mosquitoes

Australia harbours a diverse mosquito fauna of more than 300 species (Webb et al., 2016). However, arboviruses have been recovered from only around 30 of these, mainly from species of *Aedes*, *Anopheles* and *Culex* mosquitoes (Webb et al., 2016). This includes several species and subgenera of *Aedes* that were reclassified by some authorities as belonging to the *Ochlerotatus* genus (Reinert, 2000). Isolation of a virus from a mosquito does not imply that it is competent to transmit the virus or that the mosquito plays a significant role in the transmission of that virus (Kain et al., 2022). A mosquito is called a competent vector when an arbovirus is isolated from it in its wild-caught stage; the mosquito can transmit the arbovirus to a host; and the mosquito itself is infected when feeding upon a viraemic host.

Some mosquito species such as *Anopheles annulipes*, *Cx. annulirostris* and *Cx. australicus* are cosmopolitan throughout Australia (Russell, 1998). KOKV, KUNV, MVEV and SINV were each first isolated from *Cx. annulirostris* collected at Mitchell River Mission in 1960 (Doherty R. L. et al., 1963). BFV was isolated from *Cx. annulirostris* in northern Victoria in 1974 (Marshall et al., 1982b). *Cx. annulirostris* is a freshwater mosquito species that is most active from spring to late autumn (Russell, 1995). Females are opportunistic feeders that readily take a blood meal from a wide variety of vertebrates, including humans, mammals, and birds, depending on host availability (Kay et al., 2007; Gyawali et al., 2019b), proliferate under optimal conditions, and are capable of dispersing more than 4 km per day (O'Donnell et al., 1992). *Cx. annulirostris* is also the primary vector of JEV in Australia. The virus was isolated from this vector during a JE outbreak in 1995 on Badu Island (Ritchie et al., 1997a), and later also on other islands of the Torres Strait (Hanna et al., 1999). The competence of the vector to virus was further demonstrated when JEV infecting a laboratory colony of *Cx. annulirostris* was transmitted to mice (van den Hurk et al., 2003) and to flying foxes (van den Hurk et al., 2009) via vector bite.

Limited understanding of the spatiotemporal importance of individual *Culex* species in transmitting endemic MVEV and emerging JEV is attributed to the lack of longitudinal vector and arbovirus surveillance. Other endemic mosquito species that may play a role in JEV maintenance in Australia include two recently established vectors with limited distributions – *Culex gelidus* has been implicated in previous Australian JEV outbreaks, whereas *Culex tritaeniorhynchus* is responsible for most of the JEV transmission in Asia (van den Hurk et al., 2022).

Culex quinquefasciatus, *Cx. sitiens*, *Ae. camptorhynchus*, *Ae. notoscriptus*, and *Ae. vigilax* are other common Australian mosquitoes (Russell, 1995). *Culex sitiens* is usually found around pools, puddles, ponds, wells, ditches, and rock pools, and often frequents tidal marshes and mangrove swamps. Females are primarily ornithophagic (i.e., feed on birds) but do feed on humans as well (Webb et al., 2016). *Culex quinquefasciatus* is active only during the warmer months, is generally ornithophagic and feeds on humans during the middle of the night (Webb et al., 2016). *Ae. vigilax* is a coastal saltmarsh mosquito that breeds in the brackish waters of mangrove swamps and salt marshes. Females are highly active at sunset, very aggressive biters and feed on humans and domestic animals (Belkin, 1962). The first isolate of RRV was taken from *Ae. vigilax* (Doherty R. et al., 1963). *Ae. notoscriptus*, a peri-domestic mosquito, is a competent vector of RRV and for these reasons it has been suggested that this species be considered more seriously in the context of urban transmission (Watson and Kay, 1998).

Aedes aegypti, a major global vector of DENV, may have been introduced into Australia in the early or mid-19th century (Mackenzie et al., 1996) and is now widespread throughout urban tropical north Queensland. Although this mosquito was widely distributed across south-east Queensland until the 1950s, it is now limited to an area bounded by Wondai in the south, Goomeri in the south-east and Charleville in the south-west (Queensland Health, 2015; Gyawali et al., 2016c).

Aedes albopictus, the Asian tiger mosquito, is also very able to transmit DENV and is distributed throughout the Torres Strait Islands to the north of Queensland (Ritchie et al., 2006). JEV was isolated from this vector in Malaysia and in Taiwan (Vythilingam et al., 1995; Weng et al., 1999; Su et al., 2014). Laboratory experiments in Australia demonstrated infection to Australian *Ae. albopictus* by feeding an infectious blood meal with virus titre $10^{3.5}$ TCID50/mL (Nicholson et al., 2014). The ability of infected *Ae. albopictus* transmitting JEV to hosts (i.e. laboratory weanling mice) has been demonstrated in Taiwan (Weng et al., 1997). Overall, findings of vector competence within and outside Australia strongly suggest *Ae. albopictus* as a potential vector for JEV. However, it is not apparent that this mosquito has played a role in any outbreaks in Australia to date.

The Australian arboviruses associated with different mosquito species, whether in terms of competence or evidence of experimental virus transmission, are presented in Table 2.

4.2 Ticks

Very little is known about tick-borne arboviruses in Australia (Dehhaghi et al., 2019). Approximately 70 species of ticks are found in Australia, 16 of which are known to feed on humans (Australian Government Department of Health and Aged Care, 2023). Upolu virus, a bunyavirus, was isolated from the widely distributed soft-bodied tick, *Ornithodoros capensis* on the Great Barrier Reef, in 1966 (Doherty et al., 1969). Nugget (Orbivirus) and Taggert (Nairovirus) are Kemerovo and Sakhalin group viruses and have been isolated from hard-bodied ticks (*Ixodes uriae*) from Macquarie Island (Doherty et al., 1975), in the Southern Ocean south-east of Tasmania. Saumarez Reef virus is a flavivirus that was isolated from *Or. capensis* and *Ix. eudyptidis* in the Australian region (St George et al., 1977). The transmission cycles and the importance of these viruses in human infection are unresolved.

TABLE 2 Arbovirus vectors (mosquitoes) and their distribution in Australia.

Mosquito species	Distribution	Associated arbovirus(es)	Reference(s)
Anopheles amictus	NSW, QLD, WA	BFV, EHV, RRV, SINV	Doherty et al., 1979; Russell, 1995; van den Hurk et al., 2002
Anopheles annulipes	All states/territories	BFV, MVEV, RRV, SINV, TRUV	Doherty, 1972; Russell, 1995; van den Hurk et al., 2002
Anopheles bancroftii	NT, QLD, WA	MVEV, SINV	Russell, 1995
Anopheles hilli	WA, NT, QLD	RRV, SINV	Russell, 1995; Knope et al., 2016
Anopheles meraukensis	QLD, WA	EHV, SINV	Doherty R. L. et al., 1963; van den Hurk et al., 2002
Aedes aegypti	QLD	DENV	Knox et al., 2003
Ochlerotatus alternans	NSW, NT, QLD, SA, VIC, WA	RRV, SINV	Russell et al., 1991; Russell, 1995
Ochlerotatus bancroftianus	NSW, NT, QLD, SA, VIC, WA	BFV, EHV, GGV, RRV	Russell, 1995
Ochlerotatus camptorhynchus	NSW, SA, TAS, VIC, WA	BFV, KOKV, RRV, SINV	Marshall et al., 1982b; Ritchie et al., 1997b; Knope et al., 2016
Ochlerotatus clelandi	SA, TAS, VIC, WA	RRV	Russell, 1995
Ochlerotatus flavifrons	NSW, SA, TAS, VIC	RRV	Russell, 1995
Ochlerotatus funereus	NSW, NT, QLD	BFV, RRV	Lee et al., 1980; Russell, 1998; Ryan et al., 2000
Ochlerotatus eidsvoldensis	QLD, WA	BFV, GGV, MVEV, SINV	Russell, 1995
Ochlerotatus procax	NSW, QLD, VIC	BFV, RRV	Kay and Standfast, 1987; Ryan and Kay, 1999
Ochlerotatus lineatopennis	QLD	RRV	van den Hurk et al., 2002
Aedes multiplex	NSW, southern QLD, VIC	RRV	Ryan et al., 2000
Ochlerotatus normanensis	NSW, NT, QLD, WA	BFV, EHV, GGV, MVEV, RRV, SINV	Doherty et al., 1979; Broom et al., 1989; van den Hurk et al., 2002
Ochlerotatus pseudonormanensis	WA	BFV, MVEV, SINV	Sammels et al., 1999
Ochlerotatus theobaldi	NSW, QLD, SA, VIC, WA	GGV, RRV, SINV	Doherty, 1972
Ochlerotatus tremulus	WA	KUNV, MVEV, RRV, SINV	Liehne et al., 1981; Kay and Standfast, 1987
Ochlerotatus sagax	NSW, QLD, SA, VIC, WA	MVE, RRV	Kay et al., 1989; Russell, 1998
Ochlerotatus vigilax	All states/territories	BFV, EHV, GGV, KOKV, RRV, SINV, STRV	Doherty R. et al., 1963; Kay et al., 1975; Boyd and Kay, 1999; Ryan et al., 2000
Ochlerotatus notoscriptus	NSW, NT, QLD, WA	BFV, RRV	Ritchie et al., 1997b; Watson and Kay, 1998; Watson and Kay, 1999; Knope et al., 2016
Coquillettidia linealis	NSW, QLD, SA, VIC	BFV, EHV, GGV, RRV, TRUV	Russell, 1995; Jeffery et al., 2002
Culex annulirostris	All states/territories	ALFV, BCV, BFV, EHV, EUBV, GGV, JEV, KOKV, KOOV, KOWV, KUNV, MVEV, RRV, SINV, TRUV, WONV	Doherty et al., 1979; Kay et al., 1979; Kay et al., 1984; Kay et al., 1989; Ritchie et al., 1997b; Boyd and Kay, 2000; Ryan et al., 2000; van den Hurk et al., 2002; Colmant et al., 2016; Inglis et al., 2016
Culex australicus	All states/territories	KUNV, MVEV, RRV, SINV	Marshall et al., 1982b; Ryan et al., 2000
Culex fatigans	QLD	KUNV, SINV	Doherty et al., 1979
Culex quinquefasciatus	All states/territories	BFV, KUNV, MVEV, RRV, SINV	Doherty et al., 1979; Kay and Standfast, 1987
Culex palpalis	WA	MVE, RRV	Russell, 1995
Culex sitiens	WA, QLD, NSW	BFV, KUNV, MVE, RRV, SINV	Fanning et al., 1992; Boyd and Kay, 2000; van den Hurk et al., 2002
Mansonia uniformis	NSW, NT, QLD, VIC, WA	BFV, MVEV, RRV	Russell, 1998; Ryan et al., 2000

NSW, New South Wales; NT, Northern Territory; QLD, Queensland; SA, South Australia; TAS, Tasmania; VIC, Victoria; WA, Western Australia. *Virus name abbreviations*: ALFV, Alfuy; BCV, Bunyip Creek; BFV, Barmah Forest; DENV, Dengue; EHV, Edge Hill; EUBV, Eubenangee; GGV, Gan Gan; JEV, Japanese encephalitis; KOKV, Kokobera; KOOV, Koongal; KOWV, Kowanyama; KUNV, West Nile virus Kunjin strain; MVEV, Murray Valley encephalitis; RRV, Ross River; SINV, Sindbis; STRV, Stratford; TRUV, Trubanaman; WONV, Wongal. *Note:* Dark grey shading: vector is competent to transmit the virus. Light grey shading: vector is poorly competent to transmit the virus. No colour: vector competence data are not available for the virus.

4.3 Midges

Most of the viruses of the *Orbivirus* serological group (Bluetongue, Corriparta, Eubenangee, Palyam, Wallal and Warrego viruses) were isolated from biting midges such as *Culicoides brevitarsis* and *C.* marksi (Doherty et al., 1977; Standfast et al., 1984). There are reports of the alphavirus, BFV, replicating in, and being isolated from, *C. brevitarsis* and *C.* marksi (Standfast et al., 1984). However, it is not known if this vector is competent to transmit BFV. Another virus, Thimiri, from the Simbu serogroup, was isolated from *C. histrio* collected from northern Australia (Standfast and Dyce, 1982). The previous isolations of Thimiri virus were from birds in India and Egypt (Carey et al., 1971) but the vertebrate host in Australia is uncertain, and the role of these viruses in human infection is yet to be determined.

4.4 Sand flies and black flies

To date, no arbovirus that is indigenous to Australia has been identified to have a transmission cycle involving either a sand fly or a black fly. However, examples do exist of the *Phlebotomus* and *Simulium* genera providing competent vectors of arboviruses elsewhere in the world (Blair and Olson, 2015) – for sand fly-borne phleboviruses and black fly-borne rhabdoviruses, respectively (Kuno and Chang, 2005). Hence, these transmission routes should be considered alongside others for those many neglected Australian arboviruses for which our knowledge of their transmission epidemiology is incomplete.

5 Emerging public health threat

There is growing awareness among the Australian healthcare community that indigenous arboviral diseases have a serious impact on national public health (Taylor-Robinson, 2021). More widely, they pose a global epidemic risk (Gyawali et al., 2016d), as exemplified by RRV outbreaks across several Pacific islands (Aaskov et al., 1981; Rosen et al., 1981; Tesh et al., 1981). The projected escalation of human activity in the tropical north of Australia, including economic development and urbanisation, will bring humans into close contact with native reservoir wildlife and vector mosquitoes for Australian indigenous arboviruses (Gyawali and Taylor-Robinson, 2017). The expanded agriculture sector predicted for these locations will change the ecology of these mammals, birds, and insects (Gyawali et al., 2017b). Furthermore, unforeseen climatic and environmental variations (Inglis, 2009), such as increased incidence of cyclones, heavy rainfall, and resultant intensified flooding associated with outbreaks of RRV (Tall et al., 2014) and MVEV (Selvey et al., 2014b), have occurred of late with disconcerting regularity (Knutson et al., 2010), potentially effectuating an ecological change for Australian arboviruses. The projected future climatic suitability of Northern Australia for competent vector mosquito species needs to be evaluated. Moreover, it is worth noting that already this century close relatives of many of these neglected arboviruses have caused regional epidemics and global pandemics (Gyawali et al., 2016b; Mayer et al., 2017).

In recent years, the Australian Government has made significant efforts to develop the regional Australia, focusing northern tropical region of Australia in principle (Australian Government, 2015). As defined by the Northern Australia Infrastructure Facility Act 2016, this aims to harness water resources and improve trade, business, and transport infrastructure in order to stimulate employment and population growth in those historically underinvested area (Australian Government Department of Infrastructure, Transport, Regional Development, Communications and the Arts, 2024). One challenge of the federal government's commitment to facilitating growth across regional Australia is the potential emergence of unique and poorly understood healthcare threats. With increased human activity in this remote and medically underserved region, there is a major risk of encountering neglected arboviruses that have not been extensively studied (Gyawali and Taylor-Robinson, 2017; Gyawali et al., 2017a).

The economic and social development of the currently sparsely populated tropical north of Australia is set to bring infection-naïve humans into close contact with native reservoir hosts and vector mosquitoes. This convergence of factors may precipitate an increased prevalence of infection with neglected indigenous arboviruses. Moreover, the escalating rate and effects of climate change that are increasingly observed in the tropical north of the country will likely drive a population boom of arbovirus-transmitting mosquitoes. As a commensurate response, continuing assiduous attention to vector monitoring and control is required, harnessing artificial intelligence to rapidly process large volumes of data, thereby improving data analysis, prediction (Sinclair et al., 2019), and decision-making (Taylor-Robinson, 2023). In this overall context, improved epidemiological surveillance and diagnostic screening, including establishing novel, rapid pan-viral tests to facilitate early diagnosis and appropriate treatment of febrile primary care patients, should be considered a public health priority.

6 Future directions

This brief article summarises our current understanding of the complex transmission dynamics between virus, vector and host for neglected Australian arboviruses. It is apparent that there are large knowledge gaps that need closed as a future research priority. Yet, already from the available information, including a deep dive into the sources cited here and detailed elsewhere, public health stakeholders across the nation should be exhorted to consider the complex transmission dynamics between virus, vector, and host for neglected Australian arboviruses.

Key questions to address include:

(1) What are the specific arboviruses indigenous to Australia that are associated with neglected diseases, and what is their prevalence among human populations?
(2) How do arboviruses interact with their arthropod vectors and vertebrate hosts in transmission cycles, and what factors influence the efficiency of transmission between these entities?

(3) Are there alternative modes of transmission for arboviruses, such as vertically, that may impact their circulation and persistence in nature?
(4) How do environmental factors, including climate change, urbanization, and land use changes, influence the transmission dynamics of arboviruses and their vectors in Australia?
(5) What is the role of different vertebrate reservoir hosts in the maintenance and amplification of arboviruses, and how does this impact the risk of spillover to humans?
(6) How do within-host dynamics, such as viraemia levels and host immune responses, affect the transmission success of arboviruses and their ability to establish infection in new hosts?
(7) What are the implications of co-circulation of multiple arboviruses in a given region on transmission dynamics, vector competence, and disease outcomes?
(8) How can advanced technologies, such as artificial intelligence and genomic analyses, be leveraged to enhance surveillance, prediction, and response measures for arbovirus disease outbreaks in Australia?

By shedding light on these interrelated, multifactorial issues researchers can gain a comprehensive understanding of which vector(s) and virus(es) represents a potential threat to Australian public health, and of which geographical location(s), region(s) or state(s) should be targeted for routine vector and virus surveillance and control. Only through unravelling the intricate interactions between viruses, vectors, and hosts in the transmission dynamics of neglected Australian arboviruses will effective strategies for disease control and public health interventions be achieved.

While arbovirus species that are indigenous to Australia provide the focus of this review it should be noted that the same principles broadly apply to invasive species, such as DENV and JEV, and potentially CHIKV and ZIKV, that are mentioned briefly in context herein. As all of these are important human pathogens, given their widening global distribution in recent times there is a growing need for outbreak preparedness. Similar research to that described above is required to determine the capacity for reservoir infections in Australia's unique native animals and birds as well as the vector competence and ecology of the country's mosquito species.

7 Conclusion

Extremely little is known about the distribution, epidemiology and transmission ecology of neglected arboviruses that are native to Australia. There is also scant information on the immunopathology and true disease burden, including undiagnosed acute undifferentiated febrile illnesses, for which they are a likely cause. This is despite their predicted emergence as human pathogens in the rapidly developing Northern Australia, thus posing a significant public health threat to that vast region (Gyawali and Taylor-Robinson, 2017), and potentially more so globally (Gyawali et al., 2016d). Consideration of these focal points coupled with improved diagnostic protocols, including the preparation of first-line screening tests for a panel of arboviruses, would help to counter this emerging and hitherto neglected threat to the health of traditionally underserved communities. Moreover, understanding better the vector competence of mosquitoes is crucial for predicting and managing the spread of arboviruses that pose a risk to humans or livestock (Kain et al., 2022). Combatting the vectors that are the most competent, identifying the widest ranging reservoir hosts, assessing the risk of transmission in different locations, and developing targeted control strategies will all directly inform Australian public health efforts as well as contribute to global arbovirus surveillance and control initiatives.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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A bibliometric analysis of Oropouche virus

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Objectives: Oropouche virus (OROV) causes systemic infections including the nervous and blood systems, posing a significant and growing public health challenge. However, a comprehensive review of the bibliometric analysis of OROV is still lacking. Therefore, the objective of this study was to provide insight into the research dynamics and current hotspots of OROV.

Methods: This study used bibliometric analysis to explore the current status of research related to OROV. 148 publications from 1961 to 2024 were retrieved from the Scopus database. Countries, authors, institutions, journals, references, and keywords were visualized using VOSviewer, CiteSpace, R studio, and Bibliometrix. Microsoft Excel was used for statistical analysis.

Results: Brazil is the country with the highest number of publications, total cited frequency, and the most extensive international collaboration. The most popular journal in this field is the American Journal of Tropical Medicine and Hygiene. Instituto Evandro Chagas is the institution with the highest number of publications, and Eurico Arruda is involved in the highest number of publications. Keyword co-occurrence analysis showed that Oropouche bunyavirus, virology, bunyavirus, priority journal, and nucleotide sequence are the main research hotspots in this field.

Conclusion: Our study provides a comprehensive overview of the research trends and key areas of focus in OROV. The field is currently experiencing rapid growth, as evidenced by the rising number of annual publications, which not only highlights increased research activity but also lays a solid foundation for further in-depth investigations. This trend offers valuable insights for developing effective strategies for outbreak prevention and control in public health. Presently, researchers are concentrating on the detailed study of Bunyavirus infections, employing both virological and genetic approaches to elucidate their complex pathogenic mechanisms.

KEYWORDS

OROV, bibliometrics, VOSviewer, CiteSpace, Oropouche fever

1 Introduction

Oropouche virus (OROV) belongs to the family *Peribunyaviridae*, genus *Orthobunyavirus*, and is a single-stranded, negative-sense RNA virus enveloped in a spherical lipid membrane (Files et al., 2022; Kuhn et al., 2023). Initially isolated in 1955 from mosquitoes in Trinidad and Tobago, it was named after a patient in Vega de Oropouche, Trinidad (Anderson et al., 1961). OROV is the causative agent of Oropouche fever (OROF). Several outbreaks attributable to OROV have been documented in Latin

America, including Brazil, Peru, and Ecuador (Anderson et al., 1961; Watts et al., 1997; Romero-Alvarez and Escobar, 2018; Gutierrez et al., 2020; Bonifay et al., 2023). Currently, OROV is the second most prevalent arbovirus after dengue virus in the Brazilian legal Amazon, indicating its wide geographical distribution and significant epidemiological impact (Tilston-Lunel et al., 2015a). By late 2022, the initial cases of Oropouche fever were identified in Roraima, a northern state in Brazil. The outbreak then quickly spread to the eastern region (Moutinho, 2024). As of August 27, 2024, Brazil has reported a total of 7,848 cases (Lorenz and Chiaravalloti-Neto, 2024; Moutinho, 2024). On May 27, 2024, Cuba's Ministry of Public Health confirmed the first case of Oropouche fever in Santiago province (Castilletti et al., 2024). Additionally, on July 25, 2024, Brazil's Ministry of Health reported two fatalities due to Oropouche fever in Bahia. On August 2, 2024, Pernambuco recorded its first case of vertical transmission of the Oropouche virus, which resulted in fetal death (Lenharo, 2024; Martins-Filho et al., 2024a). OROV remains a concern as an endemic virus in tropical and subtropical regions (Romero-Alvarez and Escobar, 2018).

OROV similarities with Orthobunyaviruses suggest that OROV particles have an envelope ~90 nm in diameter, featuring surface distribution of glycoproteins Gn (N-terminal glycoprotein) and Gc (C-terminal glycoprotein), and containing three ribonucleoprotein (RNP) complexes, with each RNA fragment binding to multiple copies of nucleocapsid (N) and large (L) proteins (viral RNA-dependent RNA polymerase) (Obijeski et al., 1976). Analysis of N gene sequences has classified OROV into four genotypes (I, II, III, and IV), with an average nucleotide difference of ~5% between genotypes (Vasconcelos et al., 2011; Travassos da Rosa et al., 2017).

OROV, transmitted to humans via the bite of infected mosquitoes, has emerged as a key vector facilitating transmission between populations inhabiting forested areas and nearby water bodies (Romero-Alvarez and Escobar, 2018; Feitoza et al., 2023). The spillover of the virus from animal hosts to humans has raised profound public health concerns (Ellwanger and Chies, 2021). In the transmission cycle of OROV, Parakou midges serve as vectors for various arboviruses, with their reliance on human and wild mammalian blood constituting a global public health challenge (Sakkas et al., 2018). While human-to-human transmission of OROF has not been documented, nonhuman primates like howler monkeys, capuchin monkeys, and velvet monkeys, as well as mammals like sloths, are considered potential hosts for OROV in the sylvatic cycle (Pinheiro et al., 1981b, 1982a; Tilston-Lunel et al., 2015b; Travassos da Rosa et al., 2017; Sakkas et al., 2018). Two primary transmission cycles sustain the virus in nature: the urban cycle and the sylvatic cycle (Anderson et al., 1961; Pinheiro et al., 1981a; Romero-Alvarez and Escobar, 2017; Sakkas et al., 2018). Anthropogenic activities and increased populations of *C. paraensis* in urban areas promote human-vector interactions, potentially increasing the likelihood of vector-borne diseases (Pinheiro et al., 1981b).

After being bitten by an OROV-infected midge or mosquito, an incubation period of 3 to 8 days typically occurs (Pinheiro et al., 1981b, 1982b; Mercer et al., 2003; Sakkas et al., 2018). Patients infected with OROV usually exhibit symptoms of acute febrile illness, including fever, headache, muscle pain, joint pain, chills, photophobia, rash, and dizziness, but severe cases are rare (Pinheiro et al., 1981a; Travassos da Rosa et al., 2017; Romero-Alvarez and Escobar, 2018; Vernal et al., 2019). Some patients may also experience hemorrhagic symptoms, including spontaneous hemorrhage, or neurological complications such as encephalitis and meningitis associated with OROV infection (Vasconcelos et al., 1989; Mourão et al., 2009; Vasconcelos et al., 2009; Alvarez-Falconi and Ríos Ruiz, 2010; Sakkas et al., 2018; Chiang et al., 2021; Sciancalepore et al., 2022). Despite the absence of specific treatments or vaccines for OROV, managing symptoms and providing supportive care remain the best strategies (Sakkas et al., 2018). However, with increased interest in OROV vaccine development, several teams are progressing with the development of vaccine candidates using different approaches, such as inactivated viruses, live attenuated viruses, and recombinant protein vaccines (Files et al., 2022). Notably, about 60% of patients may experience a recurrence of these symptoms within 1 to 2 weeks of recovery (Sakkas et al., 2018).

Bibliometrics, as a computational and statistical methodology, focuses on in-depth quantitative and qualitative analyses of literature within specific research areas, using literature databases to assess the contributions and collaborations of authors, institutions, countries, and journals. This approach provides insight into the current state of a subject area, revealing evolutionary tracks and potential trends in scientific research. In the context of OROV research, we have leveraged bibliometric tools such as VOSviewer, CiteSpace, and Bibliometric R software to systematically highlight research advances in the field (Synnestvedt et al., 2005; van Eck and Waltman, 2010). By employing a robust research methodology and advanced visualization tools, this study aims to provide insights into the current state of the OROV field and comprehensively reveal its current research landscape and potential future directions.

2 Methods

2.1 Data collection

In this study, the Scopus database served as the primary data source for bibliometric analysis. Scopus stands as a comprehensive, multidisciplinary abstract and citation database, globally acknowledged as a high-impact repository of scholarly resources, offering extensive coverage of scientific literature. The bibliometric analysis was conducted on April 26th, 2024. Utilizing Scopus, the study was searched using the Boolean expression ("oropouche virus" OR OROV), resulting in a total of 197 records. As illustrated in Figure 1, the initial results were refined as follows: (1) Filtered for documents in "English" (excluding 15 records). (2) Limited the document type to "articles" and "reviews" (excluding 20 records). (3) Excluded studies not related to the Oropouche virus (excluding 14 records). In the end, 148

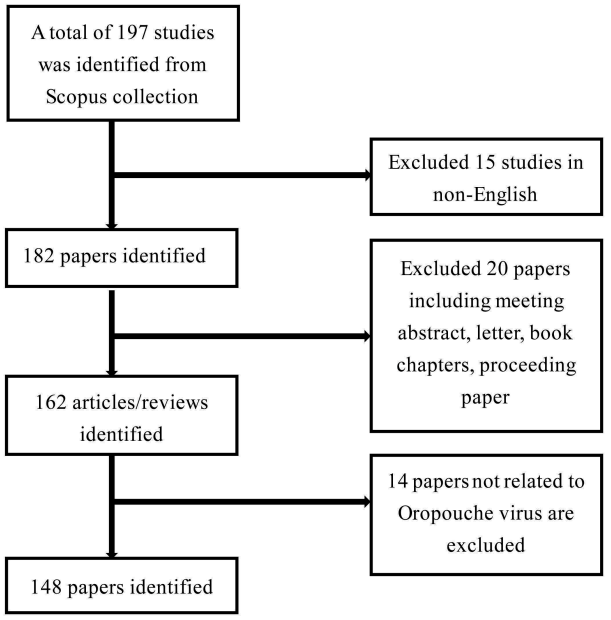

FIGURE 1
Flowchart of the screening process of publication on OROV.

studies were exported to a Comma-Separated Values (CSV) file in Microsoft Excel.

2.2 Data analysis and visualization

In this study, a variety of tools and software in scientometrics were employed for quantitative examination and analysis. VOSviewer (version 1.6.20), CiteSpace (version 6.3.R1), R Studio, Bibliometrix, and Microsoft Excel were utilized to analyze the study data from various angles. Microsoft Excel was used to measure and quantitatively analyze articles, countries, authors, and journals.

VOSviewer, a free tool, was utilized for managing and visualizing knowledge structures, creating bibliometric networks, and generating maps based on data such as country partnerships, author collaborations, and journal relationships. This aided in identifying research hotspots within the field by extracting keywords from bibliometric co-occurrence analysis.

CiteSpace, developed by Professor Chen at Drexel University, is a Java-based information visualization program designed for bibliometric analysis. This program enables researchers to visually identify leading-edge trends by presenting data in the form of a knowledge graph. Visual mappings of authors for each period were created using CiteSpace, where node types and sizes represent the weight of each parameter, and connecting lines indicate the strength of relationships between parameters. Timezone view analysis was also conducted to pinpoint research hotspots and trends.

Bibliometric analysis was conducted using R Studio version 2023.12.1 and the Bibliometrix tool version. This online analyzing platform facilitated the display of country cooperation on maps, identification of affiliate contributions, determination of the most cited literature and keywords, and generation of keyword clouds and treemaps.

3 Results

3.1 The trend of publication outputs

Based on the inclusion criteria of publication type and language, a total of 148 papers were included in this study for further bibliometric analysis. The temporal trend and the number of papers related to OROV are illustrated in Figure 2. From 1961 to 1996, there were fewer articles published, with a maximum of only one article per year. The relatively high number of publications in 1981 ($n = 5$) was attributed to the publication of a review covering the clinical, epidemiological, and ecological aspects of OROV, as well as the report of a series of epidemiological investigations into the 1975 OROV outbreak in Santarem. From 1997 to 2009, the number of annual publications remained relatively stable, ranging from 0 to 4 publications per year. Subsequently, from 2010 to 2022, there was a continuous increasing trend in the number of annual publications, except for 2014 and 2019, reaching a peak of 17 publications in both 2021 and 2022. However, from 2023 to 2024, there is a decreasing trend in the number of published articles.

3.2 Distribution of countries

A total of 28 countries have conducted relevant studies. Table 1 illustrates the top 10 countries that are globally productive. In terms of publication numbers, Brazil leads significantly with 90, followed by the United States with 58. As depicted in Figure 3A, Brazil's literature output in the field of OROV research reached 624 articles, this number significantly surpasses that of the United States ($n = 255$) and Peru ($n = 94$). Upon closer examination, the country cooperation network map reveals strong connections between countries like Brazil, the United States, Peru, the United Kingdom, Germany, and Spain, demonstrating extensive collaborative research efforts in this area (Figure 3B). The analysis indicates that Brazil leads in Single-Country Publications (SCPs), whereas the United States excels in Multi-Country Publications (MCPs), illustrating Brazil's dominance in OROV research and the United States' involvement in collaborative research with other nations (Figure 3C).

In this study, a co-authorship network analysis was conducted using VOSviewer software to map collaboration between countries. The minimum document count parameter for a country was set to 1, encompassing all 28 countries. Figure 3D displays the intensity of collaboration in OROV research among countries, with Brazil exhibiting an extensive network of international collaborations, particularly with the United States. Furthermore, Figure 3D highlights the significant presence of Brazil and the United States in the current research landscape. These visualizations offer valuable insights into collaborative networks and the substantial contributions of countries in OROV research.

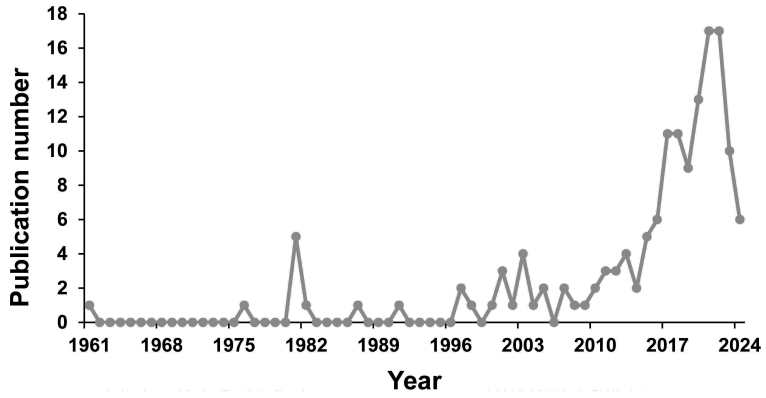

FIGURE 2
Annual number of publications on OROV from 1961 to 2024.

TABLE 1 Top 10 productive countries in the field of Oropouche virus.

Rank	Country	Documents	Citations	Average citation per paper
1	Brazil	90	2,103	23
2	United States	58	1,768	30
3	Peru	18	554	31
4	United Kingdom	13	492	38
5	France	10	500	50
6	Germany	7	249	36
7	Ecuador	6	272	45
8	Spain	6	88	15
9	Colombia	5	27	5
10	Paraguay	4	259	65

3.3 Distribution of authors and co-cited authors

Over 900 authors have contributed to OROV research. Table 2 provides details on the 10 most productive authors in the field. Notably, Eurico Arruda leads the list with 11 articles, followed by Gustavo Olszanski Acrani and Felipe Gomes Naveca, each with 8 articles, showcasing their significant impact. The networks formed by these authors and their collaborators not only highlight prominent scholars in OROV research but also offer valuable insights into collaborative relationships, crucial for future advancements. Figure 4A illustrates the collaborative network among authors, with each author contributing at least 3 documents, resulting in 9 clusters and 51 nodes. Figure 4B zooms in on the collaborations among current authors, with Eurico Arruda exhibiting the most collaboration, denoted by the green node. Together with their peers, these prolific authors have enriched the breadth and depth of OROV research, emphasizing the collaborative nature of scientific exploration in the field. Furthermore, in Figure 5, we conducted citation analysis using CiteSpace (version 6.3.R1) software, focusing on authors cited within 2-year time slices. The analysis revealed Pinheiro FP as the most cited author ($n = 68$), followed by Vasconcelos HB ($n = 42$), Elliott RM ($n = 41$), and Saeed MF ($n = 38$).

3.4 Distribution of the affiliations

In Figure 6, we present the 20 highly productive institutions that have garnered significant recognition in OROV research. Instituto Evandro Chagas leads the list with the highest number of articles received and published ($n = 69$), closely followed by the University of São Paulo ($n = 51$) and Universidad Peruana de Ciencias Aplicadas ($n = 44$). Remarkably, half of the top 20 institutions are based in Brazil, followed by the United States and France. The outstanding performance of these institutions, as evidenced by various metrics, underscores their substantial influence and significance in the field.

3.5 Journals and co-cited journals

In the realm of OROV research, Table 3 outlines the top 10 journals making significant contributions, along with the top 10

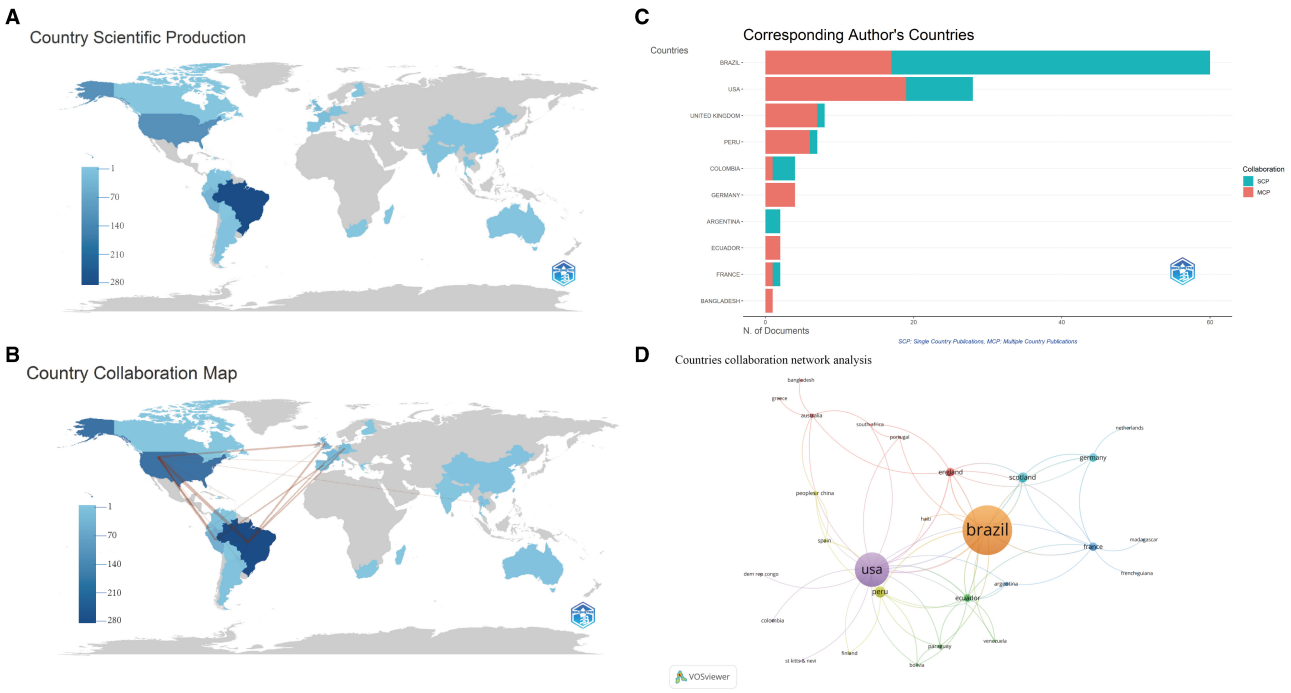

FIGURE 3
Visualization and analysis of the international collaboration networks in OROV research. The geographical distribution **(A)** and collaboration visualization **(B)** of countries and areas on the research of OROV. The choropleth map shows the geographical distribution of the cooperating countries. The change in color on the map (from light blue to dark blue) reflects the number of publications in each country. The number of any two countries connected by a red line is a direct reflection of the intensity of the cooperation between them. **(C)** The analysis of country co-authorships in the study, SCP, indicates that the authors of this paper are all from the same country, and MCP indicates that the authors of this paper are from different countries, where there is international collaboration. SCP, single country publications; MCP, multiple Country Publications. **(D)** Countries collaboration network analysis. Each node represents a country. The size of the node represents the number of publications, and the thickness of the connection between the nodes represents the intensity of cooperation.

TABLE 2 Top 10 productive authors in the field of Oropouche virus.

Rank	Author	Documents	Citations	Average citation per paper
1	Arruda, Eurico	11	206	19
2	Acrani, Gustavo Olszanski	8	268	34
3	Naveca, Felipe Gomes	8	250	31
4	Watts, Douglas M.	7	211	30
5	Aguilar-Luis, Miguel Angel	6	79	13
6	Del Valle-Mendoza, Juana	6	79	13
7	Silva-Caso, Wilmer	6	79	13
8	Elliott, Richard M.	6	193	32
9	Chiang, Jannifer Oliveira	6	178	30
10	Pinheiro, F.P.	5	194	39

co-cited journals. The American Journal of Tropical Medicine and Hygiene and Viruses leads the pack with 12 publications each, underscoring their pivotal role in advancing the field. Following closely is the Journal of Virology, with seven articles. Figure 7A presents a visual analytic graph depicting the journals, where node size corresponds to the number of published articles, and line thickness indicates the strength of collaborations between journals.

Regarding co-cited journals (Table 3), the American Journal of Tropical Medicine and Hygiene tops the list with a substantial co-citation frequency of 435, followed by the Journal of Virology with 218 co-citations, and the Journal of General Virology with 164. Figure 7B illustrates a visual network of 41 journals with more than 16 co-citation frequencies, with node size directly reflecting the frequency of co-cited articles. These findings serve as valuable guidance for researchers in selecting appropriate journals for publication, while also shedding light on the prominence and impact of journals in OROV research.

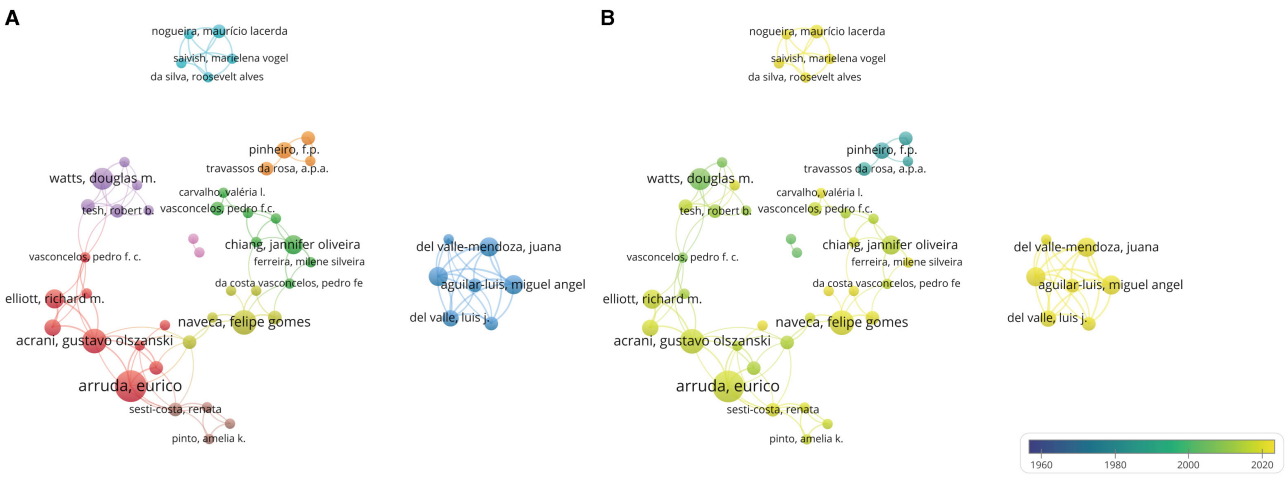

FIGURE 4
Visualization of author analysis. **(A)** Author collaboration network analysis. Minimum number of documents per author ≥3. The size of the node represents the number of author publications, and the thickness of the connection between the nodes represents the intensity of cooperation. **(B)** Cooperation time chart of author collaboration. Early occurrences of author collaborations are indicated by nodes labeled in purple or blue, and current author collaborations are indicated by nodes labeled in yellow.

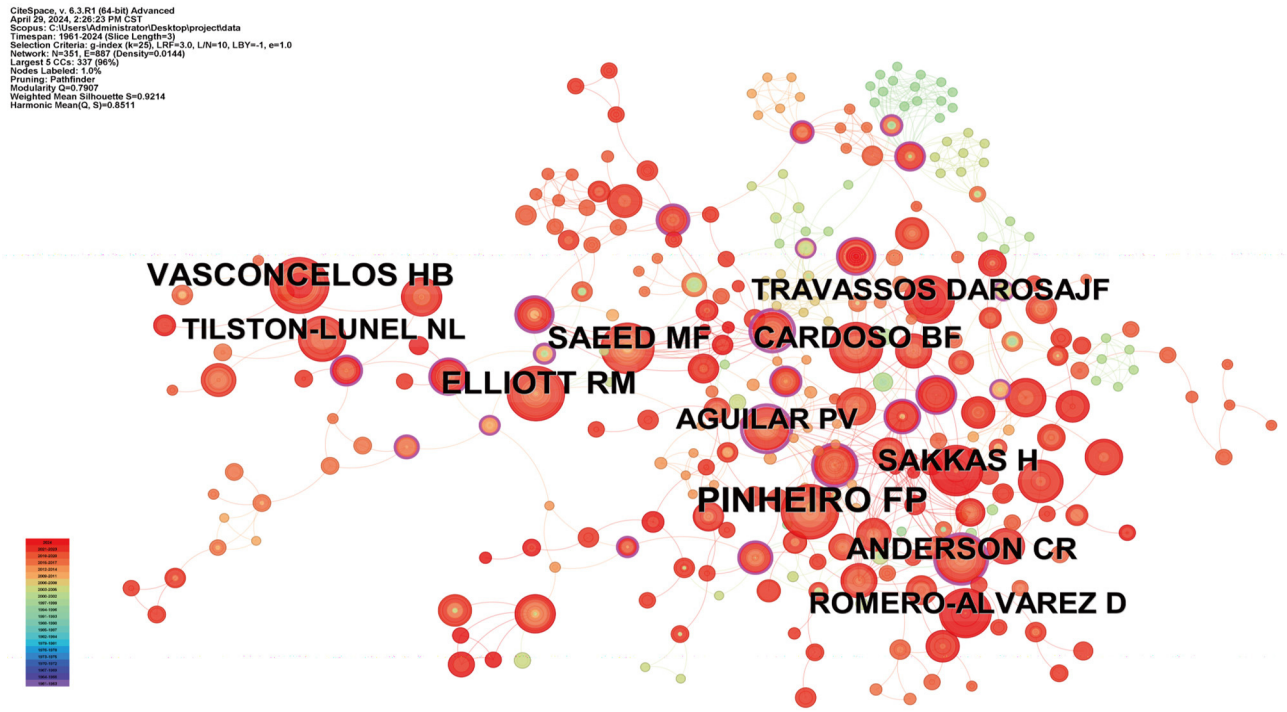

FIGURE 5
Co-citation of cited authors. The size of the circle indicates the number of cited papers, and the color of the link indicates the level of collaboration between the cited papers.

3.6 Reference analysis with strongest citation bursts

Figure 8 illustrates significant papers alongside their respective active time spans, with distinct lines denoting different periods, and prominent red lines marking intense citation bursts. Among these, the review article "*Oropouche Fever: A Review*" by Sakkas H. et al., published in 2018, garnered substantial attention and citations between 2021 and 2024. This literature underscores OROF as the second most common arboviral febrile illness in Brazil, following dengue fever. Transmission primarily occurs through cycles involving both urban and forest environments, with the

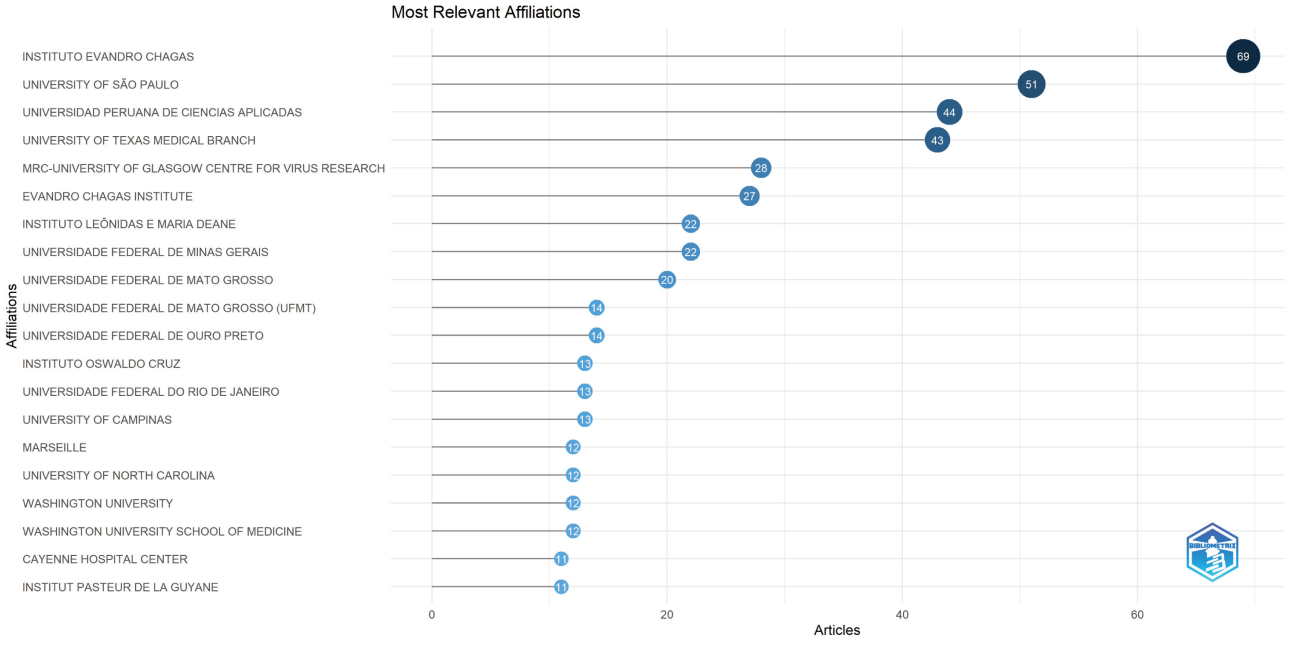

FIGURE 6
Top 20 most relevant affiliations on the research of OROV.

TABLE 3 Top 10 productive journals and co-cited journals in the field of Oropouche virus.

Rank	Journals	Documents	Citations	Average citation per paper	Co-cited journals	Co-citations
1	American Journal of Tropical Medicine and Hygiene	12	479	40	American Journal of Tropical Medicine and Hygiene	435
2	Viruses	12	206	17	Journal of Virology	218
3	Journal of Virology	7	226	32	Journal of General Virology	164
4	Memorias do Instituto Oswaldo Cruz	6	151	25	PLoS Neglected Tropical Diseases	128
5	Virus Research	6	126	21	Viruses	126
6	PLoS Neglected Tropical Diseases	6	231	39	PLoS One	123
7	PLoS ONE	6	118	20	Virology	109
8	Emerging Infectious Diseases	4	168	42	Emerging Infectious Diseases	86
9	Journal of General Virology	4	112	28	Nature	72
10	Vector-Borne and Zoonotic Diseases	4	133	33	Science	65

mosquito serving as a crucial vector in urban transmission. Despite extensive research, specific antiviral therapy for OROV remains elusive. Moreover, there's a looming risk of its further spread across the Americas, particularly under favorable climatic conditions, with potential expansion to other continents. The second notable burst of citations pertains to "*Oropouche fever, an emergent disease from the Americas*," published by Romero-Alvarez and Escobar (2018), which also gained prominence during the 2021–2024 period. This literature highlights the challenge of distinguishing OROF symptoms from those of other arboviral diseases prevalent in the Americas. Furthermore, it emphasizes the potential exacerbation of OROV emergence due to habitat loss in South America shortly. Lastly, the third noteworthy literature is "*Molecular Epidemiology of OROV, Brazil*," which gained prominence from 2015 to 2016.

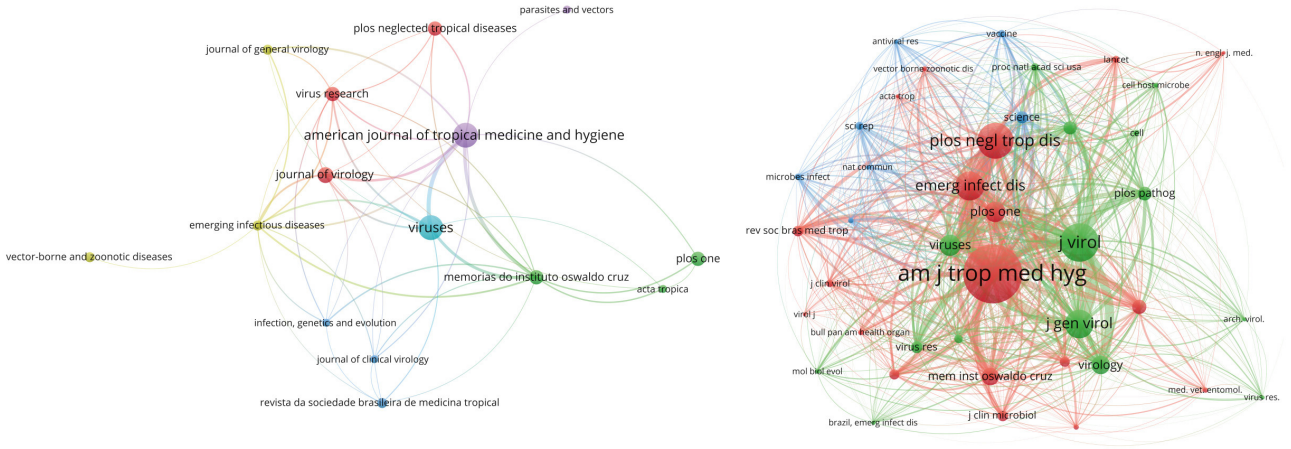

FIGURE 7
Analysis of journals involved in OROV. **(A)** The visualization of journals. Minimum number of documents per journal ≥3. As the largest node in the graph, the Journal of American Journal of Tropical Medicine and Hygiene has the most publications. **(B)** The visualization of co-cited journals. Minimum number of citations per journal ≥16. The size of the node is proportional to the frequency of co-citation, American Journal of Tropical Medicine and Hygiene has the most co-citations.

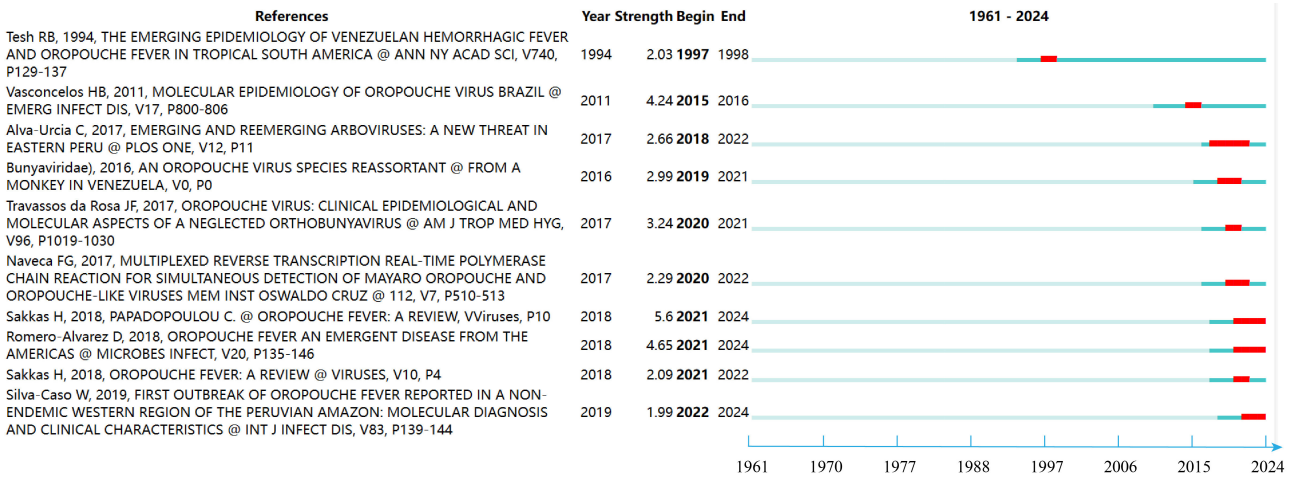

FIGURE 8
The top 10 references with the strongest citation bursts on OROV. The line represents the period and the red line represents the burst of strong citations.

3.7 Analysis of keywords

The role of keywords is pivotal in accurately summarizing the core content and theme of research. As depicted in Figure 9A, the keyword cloud map visually represents the frequency of keywords, intuitively unveiling the study's focus. The labels within the cloud map indicate the extent of researchers' attention to specific topics, with the frequency of keyword usage reflected by the number of labels; higher frequencies correspond to more labels. This figure highlights the central themes of orthobunyavirus, article, oropouche virus, human, and nonhuman.

Figure 9B illustrates the evolution of keywords, showcasing the top 25 keywords with the highest number of citation bursts. Each line represents a distinct period, with red lines denoting strong citation bursts. Notably, keyword citation bursts span from as early as 1976 to as late as 2024. The first cited keyword was "oropouche bunyavirus". The second most cited keyword, "arthropod" ceased in 1982.

Figure 9C depicts the research trend of OROV through a time zone view. The movement of nodes from left to right signifies changes in researchers' focus on specific topics over time, with the size of each node reflecting researchers' levels of interest in the respective topics. From 1976 to 1977, themes like human,

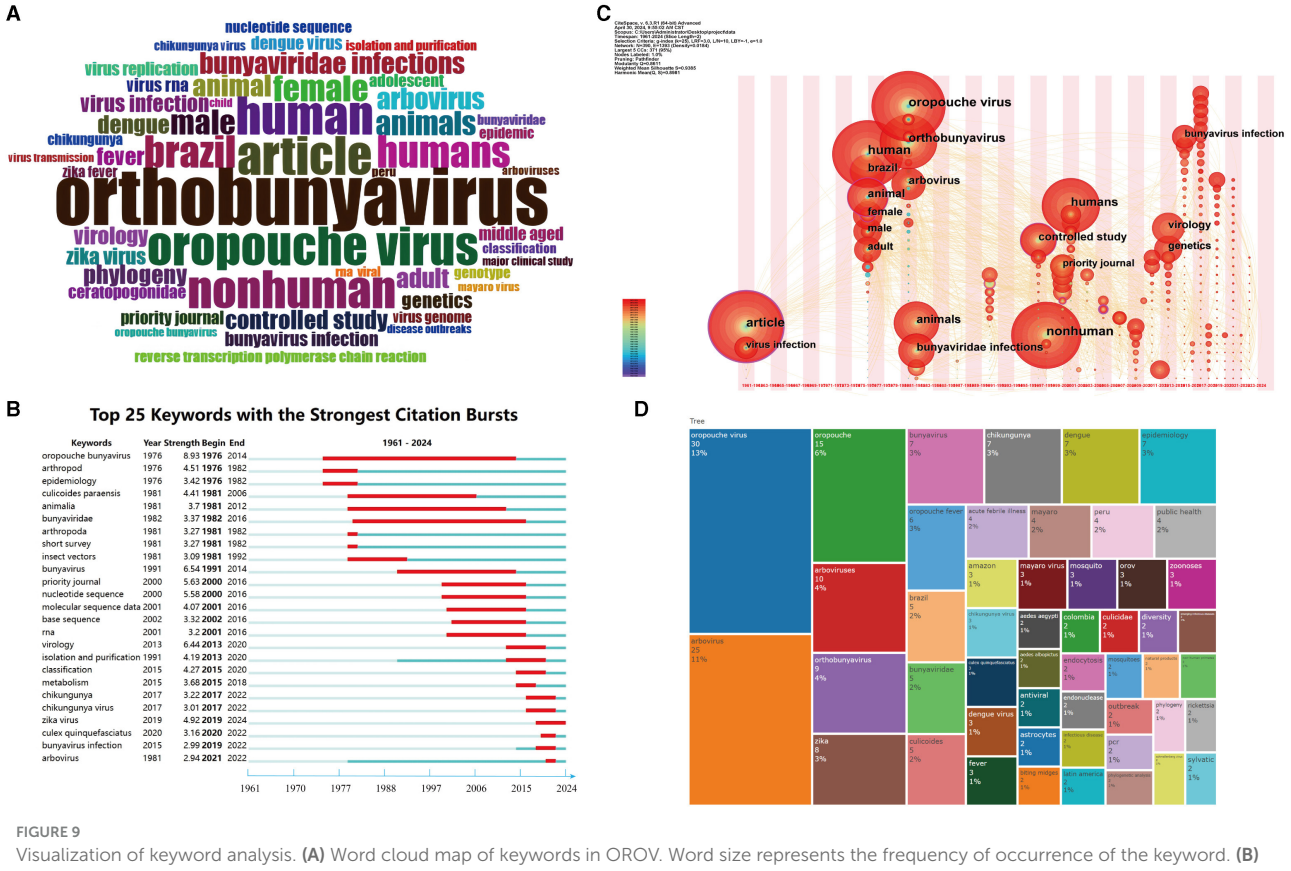

FIGURE 9
Visualization of keyword analysis. **(A)** Word cloud map of keywords in OROV. Word size represents the frequency of occurrence of the keyword. **(B)** The top 25 keywords with the strongest citation bursts on OROV. The line represents the period and the red line represents the burst of strong citations. **(C)** The time zone visualization map of co-occurring keywords in OROV. The timeline keywords indicate the initial appearance of each keyword, with node size reflecting its frequency. The connecting lines show common connections, and time is grouped in two-year intervals. **(D)** The clustering tree map of OROV. The size of the squares represents the frequency of occurrence of the keyword.

Brazilian, animal, and female dominated. In 1981-1982, research attention shifted to OROV, orthobunyavirus, arbovirus, animals, and bunyaviridae infections, among other topics. During 1997–2001, themes such as nonhuman, humans, controlled study, and priority journals garnered significant attention. The period of 2013–2015 saw increased emphasis on topics like virology, genetics, and bunyavirus infection.

Lastly, Figure 9D focuses on the frequency of keywords used by authors, with "oropouche virus" (n = 30, 13%), "arbovirus" (n = 25, 11%), "oropouche" (n = 15, 6%), "arboviruses" (n = 10, 4%), and "orthobunyavirus" (n = 9, 4%) among the most frequently used words.

4 Discussion

In academic research, bibliometric analysis has become an indispensable tool for providing a broad overview of the field and accurately capturing the trends and hotspots within it. Especially in the study of emerging and re-emerging viral diseases, bibliometric analyses of relevant publications are particularly important as they can provide insights to the global scientific and health communities on outbreak prevention and control strategies. This study is based on the widely recognized Scopus database with superior data quality and accuracy as a source of literature data, which covers a wide range of publications in the biomedical field and provides enriched features for bibliometric analysis (Burnham, 2006). To analyze in deep the research dynamics related to OROV, quantitative analysis software such as VOSviewer, CiteSpace, R studio, and Bibliometrix were used in this study. With these tools, 148 documents published in 72 academic journals from 1961 to 2024 were analyzed with 949 authors from 589 institutions in 28 countries/regions. The results of the study show that the amount of research literature on OROV shows a continuous increase over time, especially peaking in 2021 and 2022. The dynamics of this trend highlight the dynamic growth of this research area and may reflect continued advances in technology and research methodology. In addition, collaborative networks in research demonstrate the interdisciplinary quality of the field, while specific journals, prolific authors, and influential research institutions contribute significantly to the development of the academic field.

After an in-depth statistical analysis of the number of papers published by countries/regions in the field of OROV research, we found that although the total amount of relevant literature is not huge, certain key countries/regions have a significant influence

in the field and their collaborative relationships are visible. Of these, Brazil and the United States serve as the main research forces, with Brazil taking the lead in terms of the number of publications. It is worth mentioning that more than half of the top 20 affiliates most relevant to research on the virus are located in Brazil, followed by the United States and France. It is noteworthy that the intensity of OROV infections is particularly prominent in Brazil. This phenomenon may reflect the fact that international academic development in the field of virus research is somewhat limited by geographic location. To promote the in-depth development of OROV research, we call on academic institutions to strengthen cross-regional and cross-country cooperation and exchanges to jointly address this global public health challenge. Brazil's significant contribution to this field undoubtedly deserves our further attention and study. At the same time, we have observed close cooperation in research on the virus between Brazil and countries such as the United States, Peru, the United Kingdom, Germany, and Spain.

At the level of research institutions, Instituto Evandro Chagas is particularly strong, topping the list, followed by the University of São Paulo and Universidad Peruana de Ciencias Aplicadas. This data not only highlights Brazil's significant strengths in the field of OROV research but also maps the collaboration between Brazil and other countries in the field of scientific research, especially with the United States. Despite the existence of cooperative relationships, the frequency, scope, and depth of inter-agency cooperation could be improved. For example, cooperation between Brazilian and United States institutions is still insufficient, which to some extent constrains development in this area. Because of the significant advantages of Instituto Evandro Chagas in terms of the number of publications and its scientific strength, we strongly recommend that research organizations in various countries strengthen their cooperation and communication with each other to promote the further development of the research field of OROV. By taking advantage of each other's strengths, we hope to achieve greater breakthroughs in this field, and at the same time break down academic barriers and build a more solid foundation for in-depth research on OROV.

From the point of view of authors' contribution, Eurico Arruda, Gustavo Olszanski Acrani, and Felipe Gomes Naveca are among the top three authors in terms of the number of articles published and the significant impact of their research in the field, as well as the total citation frequency, which is a good proof of their importance in the study of OROV. The latest findings from Eurico Arruda's team show that human nerve cells can support the production of infectious viral particles through OROV infection. In addition, OROV infection triggered the release of the pro-inflammatory cytokine TNF-α (tumor necrosis factor-α) and led to a decrease in cell viability within 48 hours of infection, suggesting that OROV is capable of inducing an inflammatory response and tissue damage. The research data not only reveal the neuropathogenesis of OROV, but also help to improve the understanding of the possible acute and chronic consequences of OROV infection in the human brain (Almeida et al., 2021). On the other hand, Pinheiro FP has the highest number of co-citations. His latest review provides a comprehensive review of the epidemiology, pathogenesis, and molecular biological characterization of OROV. It covers key information such as the first isolation of the virus, outbreaks over the past six decades, clinical expression of infection, diagnostic techniques, genomic and genetic characterization, evolutionary history, and viral transmission pathways, providing valuable insights into the current state of OROV research (Anderson et al., 1961).

The research on OROV has been published in two journals, "American Journal of Tropical Medicine and Hygiene" (IF = 3.3) and "Viruses" (IF = 4.7), which are undoubtedly the most productive academic platforms in this field. From the perspective of co-cited journals, "American Journal of Tropical Medicine and Hygiene" is still the most influential journal in the field. These journals provide strong support for OROV research with their high-quality content and international outlook. It is worthwhile for researchers in related fields to use them as the first-choice journals for submission and reference.

"*Oropouche Fever: A Review*" demonstrated the highest intensity of citation bursts, topping the list with an intensity of 5.6. The review was written in 2018 by Sakkas H et al. The authors are from the Department of Microbiology, Faculty of Health Sciences, School of Medicine, University of Ioannina, and the Department of Physiology, Anatomy and Microbiology, La Trobe University. This literature provides a comprehensive review of current research advances in OROV fever and the knowledge gaps that exist (Sakkas et al., 2018). This was closely followed by "*Oropouche fever, an emergent disease from the Americas*" by Romero-Alvarez and Escobar (2018), which came in second place with a slightly lower citation intensity. This article highlights the difficulties in distinguishing OROF from other febrile diseases caused by arboviruses in the Americas. Warning that habitat loss could elevate the risk of OROV outbreaks in South America in the near future (Romero-Alvarez and Escobar, 2018). Third in terms of citation intensity was the study "*Molecular Epidemiology of OROV, Brazil*" by Vasconcelos HB et al. from the Instituto Evandro Chagas. This study provides insight into the molecular epidemiology of OROV, revealing that each RNA segment has a distinct evolutionary history. The importance of comprehensively considering the genetic information of all genetic segments when classifying genotypes is emphasized. In particular, genotype I (based on N-gene data) is responsible for the emergence and viral transmission of all other genotypes (Vasconcelos et al., 2011).

In terms of keywords, orthobunyavirus, oropouche virus, human, nonhuman, and Brazil are by far the most popular topics favoring further research. The main research hotspots in this field include Oropouche bunyavirus, virology, bunyavirus, priority journal, and nucleotide sequence. It is hoped that the work in this paper will provide new ideas to advance the scientific research and clinical application of OROV.

Recently the first confirmed case of OROF in Rio de Janeiro on February 29, 2024, marked a further spread of the virus to a wider region beyond the confines of the traditional outbreak areas of Amazonas, Acre, and Rondônia. This development poses a serious test for the public health system and requires us to be vigilant in order to ensure the accurate identification and effective control of diseases (Martins-Filho et al., 2024b). Currently, no vaccine approved for prophylaxis has been introduced.

The treatment strategy for OROV fever relies heavily on the prescription of antipyretic and analgesic medications to relieve the patient's symptoms. Given that the clinical manifestations of OROV infection are very similar to those of diseases caused by arboviruses, such as dengue fever, in their early stages; this has led to an underestimate of the actual number of cases of OROF. OROV, as a neglected virus, is still understudied in terms of its prevalence, transmission, and impact on the epidemiological landscape in Brazil and South America (Andreolla et al., 2024). Currently, OROV infection and its attack on the central nervous system can be recognized early by testing the blood and cerebrospinal fluid of suspected patients. Diagnostic methods include molecular techniques (e.g., RT-PCR or real-time RT-PCR) and traditional virologic assays (e.g., virus isolation, hemagglutination inhibition, and complement-binding assays), but these methods are primarily applicable during the period of viremia (Travassos da Rosa et al., 2017). There are no specific therapeutic drugs or preventive antiviral drugs for OROV infection. Therefore, control or eradication of arthropod vectors and personal protective measures have become the main means of preventing and controlling OROV infection. The risk of OROV infection is particularly significant in midge breeding sites near human settlements. Thus, it is particularly important to strengthen control and personal protection in these areas (Mohapatra et al., 2024). In light of this situation, the relevant authorities, medical experts, research teams, and the international community must come together and implement a proactive, coordinated response.

Our bibliometric analyses play a central role in highlighting the core values of research within a specific area. By comprehensively assessing and integrating research results, we provide insights into research dynamics, prominent contributors, and thematic clusters, thereby enhancing our overall understanding of the knowledge landscape. In the context of OROV research, bibliometric analysis has emerged as a crucial tool for assessing the breadth and impact of scholarly contributions. It not only offers a macroscopic view of the current body of knowledge but also helps identify gaps in knowledge and offers guidance for future research directions. This underscores the significance of bibliometric analysis as a driving force in scientific research and clinical practice, promoting scientific inquiry and enabling well-informed decision-making (Ellegaard and Wallin, 2015).

In an in-depth dissection of the bibliometric analysis of OROV, this study is based on the highly trusted Scopus database, which aims to guarantee the study's rigor. However, this strategy is also accompanied by some inherent limitations. There may be issues of omission of key literature from journals or databases other than SCI, as well as difficulties in fully assessing the quality of newly published literature. Notably, the number of citations is usually accumulated over time, which may result in older literature receiving more citations, while newer studies may be undervalued as a result. Although these limitations may have a subtle impact on the results of the study, they are not sufficient to reverse the major trends and findings of this study on OROV. In addition, this study was limited by the fact that only English language literature in the Scopus database was searched, which may have overlooked equally valuable studies in other languages. In conducting the bibliometric analysis, although we were able to construct quantitative descriptive maps of countries, journals, articles, authors, and keywords about OROV. However, there are still challenges to be faced, such as the difficulty of open access to scientific metrics data, possible incompleteness or duplication of data, and the complexity and diversity of bibliographic data itself. Therefore, researchers need to remain cautious when analyzing data. A deeper understanding of the various dimensions of the data and full consideration of the number of citations with time are required to avoid misjudging the results of recent research.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

JD: Data curation, Formal analysis, Investigation, Writing – original draft. ZL: Writing – review & editing. SG: Writing – review & editing. LZ: Conceptualization, Formal analysis, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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Evolving epidemiology, clinical features, and genotyping of dengue outbreaks in Bangladesh, 2000–2024: a systematic review

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Background: The 2023 dengue outbreak has proven that dengue is not only an endemic disease but also an emerging health threat in Bangladesh. Integrated studies on the epidemiology, clinical characteristics, seasonality, and genotype of dengue are limited. This study was conducted to determine recent trends in the molecular epidemiology, clinical features, and seasonality of dengue outbreaks.

Methods: We analyzed data from 41 original studies, extracting epidemiological information from all 41 articles, clinical symptoms from 30 articles, and genotypic diversity from 11 articles. The study adhered to the standards of the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) Statement and Cochrane Collaboration guidelines.

Results: A total of 565,438 dengue cases and 2,587 fatalities were documented from January 2000 to March 2024. Notably, 60% of cases during the 2019 and 2023 outbreaks were reported in regions previously considered non-endemic. Fatalities were more frequent among women (70%). The majority of the studies (95–100%) used the NS1Ag test, followed by IgG or IgM and RT-PCR tests. New hotspots of dengue transmission were identified in the southern (Khulna, 10.8% and Barishal, 11.8%) and southeastern (Chattogram, 13.8%) regions of Bangladesh. Serotyping was conducted on 92.4% (1,456 of 1,575) of isolates between 2012 and 2023. Of the four serotypes, DENV3 was the most prevalent (57%), followed by DENV2 (30%), DENV1 (11%), and DENV4 (<1%). Genotype DENV3-I (43 of 59 isolates) was the most prevalent, followed by DENV3-II (8 of 59). The highest frequency of dengue cases was observed in August (26.3%), followed by September (22.5%), October (20.2%), and November (13.08%). Fever (90.51, 95% CI 85–100%) was the most prevalent symptom, followed by headache (57.98, 95% CI 12–100%), vomiting (51.16, 95% CI 23–91%), abdominal pain (34.12, 95% CI 12–85%), and myalgia (25.53, 95% CI 13–85%), respectively.

Conclusion: This study provides integrated insights into the molecular epidemiology, clinical features, seasonality, and transmission of dengue in Bangladesh and highlights research gaps for future studies.

KEYWORDS

dengue, outbreak, epidemiology, seasonality, Bangladesh

Introduction

Dengue is an acute febrile disease caused by the dengue virus, which can now spread to more than 125 countries worldwide (WHO Dengue fact sheet, 2024). According to the World Health Organization (WHO), confirmed cases of dengue have increased from 505,430 in 2000 to 5. 2 million in 2019 (WHO Dengue fact sheet, 2024; Hadinegoro, 2012; Health bulletin on current Dengue situation published by DGHS, 2024; WHO, 2024; Deen et al., 2006; Prattay et al., 2022). It is estimated that 300 million cases of dengue occur, with 100 million cases reported clinically every year worldwide. Furthermore, 4 billion people are at risk of contracting dengue (WHO Dengue fact sheet, 2024; Health bulletin on current Dengue situation published by DGHS, 2024; Guo et al., 2017; Prattay et al., 2022). The perception that dengue is confined to tropical and subtropical areas is changing, as recent global expansion of cases has been documented (Health bulletin on current Dengue situation published by DGHS, 2024; WHO, 2024; Deen et al., 2006; Prattay et al., 2022; Muraduzzaman et al., 2018).

The dengue virus is transmitted from human to human by *Aedes* spp. mosquitoes. However, in the last decade, these vectors have rapidly spread to distant regions beyond the traditional endemic areas, becoming established in regions previously unexposed to dengue (WHO, 2024; Islam et al., 2022b; Ahmed et al., 2016). Rapid transportation, increased travel, and the mosquitoes' adaptation to new environments have significantly contributed to this widespread transmission.

Bangladesh is classified as an endemic region for dengue fever (Prattay et al., 2022; Muraduzzaman et al., 2018; Islam et al., 2022b; Ahmed et al., 2016; Islam et al., 2019; Islam et al., 2022a; Khan et al., 2021; Hasan et al., 2021a; Yang et al., 2023; Rafi et al., 2020). The first case of dengue was reported in early 2000, and outbreaks have been reported regularly in Bangladesh since 2000 (Health bulletin on current Dengue situation published by DGHS, 2024; WHO, 2024; Deen et al., 2006; Prattay et al., 2022; Islam et al., 2022a; Khan et al., 2021; Hasan et al., 2021a; Yang et al., 2023; Rafi et al., 2020; Shultana et al., 2019). However, the lack of strong and effective surveillance has likely led to an underestimation of the true number of cases and fatalities. Recent larger outbreaks, including 101,500 cases in 2019 and 321,073 cases in 2023, have spread across the majority of the non-endemic regions in Bangladesh, posing a major health threat (Hossain et al., 2023; Health bulletin on current Dengue situation published by DGHS, 2024; Rafi et al., 2020; Shultana et al., 2019; Mahmood et al., 2021; Yesmin et al., 2023b; Rahim et al., 2023). After COVID-19, dengue has emerged as a major public health issue, with 1,705 people dying from the outbreak in 2023 (Health bulletin on current Dengue situation published by DGHS, 2024; WHO, 2024). Many non-endemic regions have now become hotspots of dengue transmission. Without effective treatment and prevention measures, the ongoing spread of dengue has become an alarming global health concern (Ahmed et al., 2016; Hasan et al., 2021a).

The dengue virus is a single-stranded, positive-sense RNA virus with a genome of approximately 11,000 bases (Prattay et al., 2022; Muraduzzaman et al., 2018; Islam et al., 2022b). The genome encodes three structural proteins—capsid protein (C), membrane protein (M), and envelope protein (E)—as well as seven non-structural proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. Dengue virus has been classified into four serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. Based on the genetic and phylogenetic analysis, each serotype is further divided into different genotypes (WHO Dengue fact sheet, 2024; WHO, 2024; Islam et al., 2006; Aziz et al., 2002; Sultana et al., 2020; Hossain et al., 2003). However, genotypic surveillance data for the dengue virus is significantly lacking in Bangladesh (Health bulletin on current Dengue situation published by DGHS, 2024; Prattay et al., 2022; Amin et al., 2022; Kabir et al., 2020).

According to the WHO dengue case classification (WHO, 2009), cases of dengue can be classified as dengue without warning signs, dengue with warning signs, and severe dengue (Hadinegoro, 2012). Symptomatic infection can manifest as undifferentiated fever (viral syndrome), dengue fever syndrome (DFS), or dengue hemorrhagic fever (DHF). In many cases of DFS, unusual hemorrhaging may be present, while some DFS cases occur without hemorrhaging. DHF can be further classified into cases with or without shock, with dengue shock syndrome (DSS) representing the more severe form (Hadinegoro, 2012; Deen et al., 2006).

The majority of the cases are reported to be mild, with asymptomatic or mildly symptomatic infections. Common symptoms include fever (104°F), headache, muscle pain, pain behind the eyes, joint pain, nausea, vomiting, rash, and swollen glands (Hadinegoro, 2012; Deen et al., 2006). In severe cases, symptoms such as acute abdominal pain, continuous vomiting, breathing difficulties, bleeding from the gums or nose, blood in vomit or stool, restlessness, excessive thirst, pale and cold skin, and weakness are commonly reported (Hadinegoro, 2012; Deen et al., 2006). This study addresses the existing gap in integrated research on the prevalence, clinical symptoms, molecular epidemiology, and seasonality of dengue in Bangladesh. The main aim of this study was to evaluate the existing data on the molecular epidemiology and clinical characteristics of dengue outbreaks in Bangladesh.

Methods

Definition

The epidemiology of the dengue virus in this study was defined as the distribution and determinants of outbreak in various populations, as well as the steps taken to reduce the health effects on those

populations (Hadinegoro, 2012; WHO, 2024; Deen et al., 2006). Clinical symptoms included both the signs and symptoms observed during and after the confirmation of the dengue infection. Transmission was defined as the spread of the dengue virus from infected humans to healthy humans through mosquito vectors (Sharif et al., 2023a).

This study included previous studies that provided data on the epidemiology, clinical symptoms, genetic diversity, and seasonality of the dengue virus. The reported cases were confirmed using NS1 and/or IgM/IgG tests and/or RT-PCR, with molecular sequencing confirming dengue positivity (Hadinegoro, 2012; WHO, 2024; Deen et al., 2006). This study was conducted in accordance with the standards of the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) Statement and Cochrane Collaboration guidelines (Moher et al., 2015).

Study design

This study was conducted in accordance with the principles of systematic reviews, following the guidelines outlined in the Cochrane Handbook and the guidance document provided by the Center for Reviews and Dissemination (CRD) at York University, United Kingdom (Higgins and Green, 2020; Zeng et al., 2015). The study followed several key steps: identifying clear objectives, selecting data sources, developing search strategies, reviewing research articles, collecting and selecting data, minimizing bias, analyzing data, and summarizing the findings (Moher et al., 2015). This study included data and findings from original epidemiological studies, clinical and case studies, outbreak investigations, genotypic surveillance studies, and online databases. As strict assessment parameters were not available for these studies, the quality of the selected articles was evaluated based on the reports provided by their authors (Sharif et al., 2023a; Higgins and Green, 2020; Zeng et al., 2015; Sharif et al., 2021).

Data sources, search strategy, and selection criteria

Literature and data were collected from published original articles in databases such as MEDLINE (via PubMed), The New England Journal of Medicine (NEJM), Web of Science, EMBASE, Scopus, African Journals Online (AJOL), and The Lancet. Eligible articles and scientific studies published before 01 March 2024 were included in this study, with data extracted from sources written in the English language. A significant number of search terms included: "Dengue, Dengue virus, DENV, DENV-1, DENV-2, DENV-3, DENV-4, Bangladesh, Dengue outbreaks, Epidemiology of dengue, Epidemiology of dengue virus, Clinical features of dengue infection, Sign and Symptoms of dengue, Clinical characteristics of dengue, Cases of dengue, Prevalence, Molecular epidemiology, serotyping, genotyping, Transmission of dengue, Transmission of dengue virus, *Aedes* spp., Mosquito, mosquito-borne, vector-borne, arbovirus" and various combination of these terms. A single search was conducted for every term across different websites and databases.

We searched for data on dengue outbreaks across various databases, including the World Health Organization (WHO), the Centers for Disease Control and Prevention (CDC, United States), Epicenter, ProMed, the European Centers for Disease Control and Prevention (ECDC), and the Directorate General of Health Services (DGHS, Bangladesh). Daily and yearly updates on dengue outbreaks were monitored from these data sources. Additionally, preprint platforms such as SSRN, medRxiv, bioRxiv, and AAS Open Research were searched, though only data from peer-reviewed journals were included.

We also manually reviewed the first 10 pages of search results from Google Scholar to gather relevant information. Our focus was on the molecular epidemiology of dengue, covering areas such as prevalence, incidence, transmission dynamics, case reports, clinical history, genotypic variation, case fatality rate, and distribution of serotypes. We also included data on seasonality and the spatial distribution of dengue cases in non-endemic regions.

After conducting searches across the above-mentioned websites and databases, potential articles and information were selected by removing irrelevant data and conducting a thorough screening. Articles containing data on specific and relevant matters were selected, covering all districts, ethnicities, ages, sexes, seasonality, and clinical features. Studies focused on modeling and prediction, review articles, and meta-analysis, and those unrelated to the objectives of this study were excluded.

The quality of the selected articles was further evaluated by identifying and removing duplicates, as well as excluding letters to the editor, correspondence, or comments. The seasonal exclusion criteria were applied to studies with seasonality and environmental data. Additionally, we included only studies that provided data on specific serotypes and genotypes.

The risk of bias was assessed using the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE) assessment tool and the JBI critical appraisal checklist for studies reporting prevalence data (Moher et al., 2015; Higgins and Green, 2020; Zeng et al., 2015). The SYRCLE scale consists of 10 parameters to measure biases in studies, including attrition bias, reporting bias, detection bias, performance bias, selection bias, and other potential biases. Bias for each parameter was calculated using outcomes of *yes*, *no*, and *unclear*, corresponding to low, high, and undefined bias, respectively (Sharif et al., 2023a; Higgins and Green, 2020; Sharif et al., 2021). In the JBI tool, nine parameters were used to evaluate the studies, with each parameter evaluated using the outcomes *yes*, *no*, *unclear*, or not applicable.

Case definition

According to the WHO, a dengue case is defined differently depending on the situation. A *surveillance case* is defined as "A person who lived in, or traveled to, a dengue-endemic area with the onset of fever and two or more of the following: nausea/vomiting, rashes, aches and pains, positive tourniquet test, leukopenia, or any warning sign." A *confirmed dengue case* is defined as positive in polymerase chain reaction (PCR), virus culture, or positive IgM in a single sample, IgM seroconversion in paired sera, IgG seroconversion in paired sera, or a fourfold IgG titer increase in paired sera, or detection of viral antigen NS1+ in a single serum sample (WHO, 2024). According to the national guidelines for the clinical management of dengue syndrome in Bangladesh, depending on the time of testing after the onset of

symptoms, a positive result in any of the following tests—NS1 antigen, IgM /IgG test (MAC ELISA or Rapid ICT), RT-PCR, or virus isolation—can be considered diagnostic for dengue.

Protocol

This study followed the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) guidelines (Moher et al., 2015).

Statistical analysis

The total number of dengue cases and fatalities was calculated by summing the reported confirmed cases from the selected articles. The case fatality rate, defined as the proportion of dengue cases that result in death within a specified time period, was also determined. Pooled statistical analyses were conducted using Statistical Analysis System version 9.4 (North Carolina, United States).

Results

Studies included

We found 816 research articles on the epidemiology, genetic diversity, transmission, clinical characteristics, and seasonality of the dengue virus in Bangladesh using the previously mentioned search terms. Initially, 235 articles were selected for full-text analysis. The remaining 581 articles were excluded as they were reviews, mini-reviews, correspondence, or letters to the editor and did not meet inclusion criteria.

After a critical evaluation of the full texts, only 48 studies (20.5%, 48 of 235) were selected for further analysis. Based on the inclusion and exclusion criteria, 41 of 48 manuscripts (85.4%) were further analyzed. Among these 41 manuscripts, we extracted epidemiological data from all 41, clinical symptoms from 30 articles, genotypic diversity from 11 articles, and seasonality data from 6 articles (Figure 1). We also included data from two websites, including DGHS (Bangladesh) and Nextstrain.

Epidemiological features of dengue outbreaks

Published articles and databases were assessed to examine the temporal and spatial trends of dengue in Bangladesh. The first major outbreak, with over 5,000 confirmed cases and 93 fatalities reported in 2000. From 2000 to 2009, approximately 24,000 cases were documented, resulting in 234 deaths. During this period, dengue incidence was concentrated in the hotspot of Dhaka (Figure 2). However, the source of the first case in Dhaka could not be confirmed, and the history of dengue transmission before 2000 was not thoroughly investigated in Bangladesh. The case fatality rate was highest from 2000 to 2003, with no fatality reported from 2007 to 2010.

From 2010 to 2019, 130,000 cases and 250 deaths were recorded, with over 100,000 cases reported for the first time in 2019 (WHO Dengue fact sheet, 2024; Hadinegoro, 2012). Moreover, 179 deaths due to dengue infection were reported in 2019 alone. The massive outbreak in 2023 saw confirmed cases surpass 415,000, with 2,200 deaths reported from dengue infection over the four-year period from 2020 to 2024. After the onset of COVID-19, dengue cases were significantly underreported in 2021 and 2022 (Figure 2). The 2022 dengue outbreak, continuing into 2024, is particularly alarming. The highest number of cases (56.8%, 321,179 of 565,438) and fatalities (65.9%, 1,705 of 2,587) were reported from January 2023 to December 2023.

The majority of the studies included patients from all age groups, with a male-to-female case ratio of approximately 2:1 (Table 1). Cases of dengue were most prevalent among individuals aged 19–29 years (31%), followed by 0–18 years (26%), 40–59 years (19.2%), 30–39 years (15.8%), 60–79 years (7.6%), and > 80 years However, fatalities were more frequent among women (70%). All of the studies (100%) confirmed cases using the NS1Ag test, followed by anti-dengue IgM and IgG antibody testing. Molecular sequencing and confirmation by RT-PCR were found in fewer than 10% of studies (Table 1) (WHO, 2024; Deen et al., 2006; Prattay et al., 2022; Muraduzzaman et al., 2018; Islam et al., 2022b; Ahmed et al., 2016; Islam et al., 2019; Islam et al., 2022a; Khan et al., 2021; Hasan et al., 2021a; Yang et al., 2023; Rafi et al., 2020; Shultana et al., 2019; Mahmood et al., 2021; Yesmin et al., 2023b; Rahim et al., 2023; Islam et al., 2006; Aziz et al., 2002; Sultana et al., 2020; Zeng et al., 2015; Sharif et al., 2021; Titir et al., 2021; Rahman et al., 2002; Rouf et al., 2020; Siddiqua et al., 2018; Yasmin et al., 2020; Nahar et al., 2021; Islam et al., 2021; Parvin et al., 2022). The epidemiological trends indicate a rapid increase in the incidence and fatalities from dengue in recent years in Bangladesh.

Spatial distribution of dengue in Bangladesh

The spatial distribution and transmission of dengue cases in Bangladesh have shifted significantly over the past two decades. The majority of the cases between 2000 and 2011 were indigenous to Dhaka, with 100% of cases in Khulna, Rajshahi, Mymensingh, Barisal, and Sylhet being transported from Dhaka. More than 500 transported cases were reported in Khulna, Barisal, Chattogram, Mymensingh, Sylhet and Rajshahi. Before the larger outbreak in 2019, dengue was reported in only 30% of regions in Bangladesh. However, from 2012 to 2023, the distribution of dengue cases rapidly expanded to all 64 districts (100% regions) (WHO Dengue fact sheet, 2024; Health bulletin on current Dengue situation published by DGHS, 2024; Parvin et al., 2022; Mobarak et al., 2018; Sultana et al., 2019; Rahman et al., 2019; Pervin et al., 2017; Sultana et al., 2013; Datta et al., 2021; Hasan et al., 2021b; Sharmin et al., 2013; Rahman et al., 2007; Salma et al., 2021; Yesmin et al., 2023a).

During the 2019 outbreak, the dengue virus spread to the remaining 70% of regions in Bangladesh, with Dhaka being the focal point for the 2019, 2021, 2022, and 2023 outbreaks (Figure 3). However, during the 2023 outbreak, indigenous cases were locally transmitted in regions such as Chattogram, Rajshahi, Sylhet, Khulna, and Barisal. In 2023, 53% of cases were reported outside Dhaka, with the Chattogram division accounting for 14.2% of cases and the Barisal division for 11%, making them new major hotspots of dengue transmission after 2023 (Yesmin et al., 2023a; Uddin et al., 2014; Mutanabbi et al., 2022; Pervin et al., 2003; Baskey et al., 2024; Roy et al., 2023; Rahim et al., 2021).

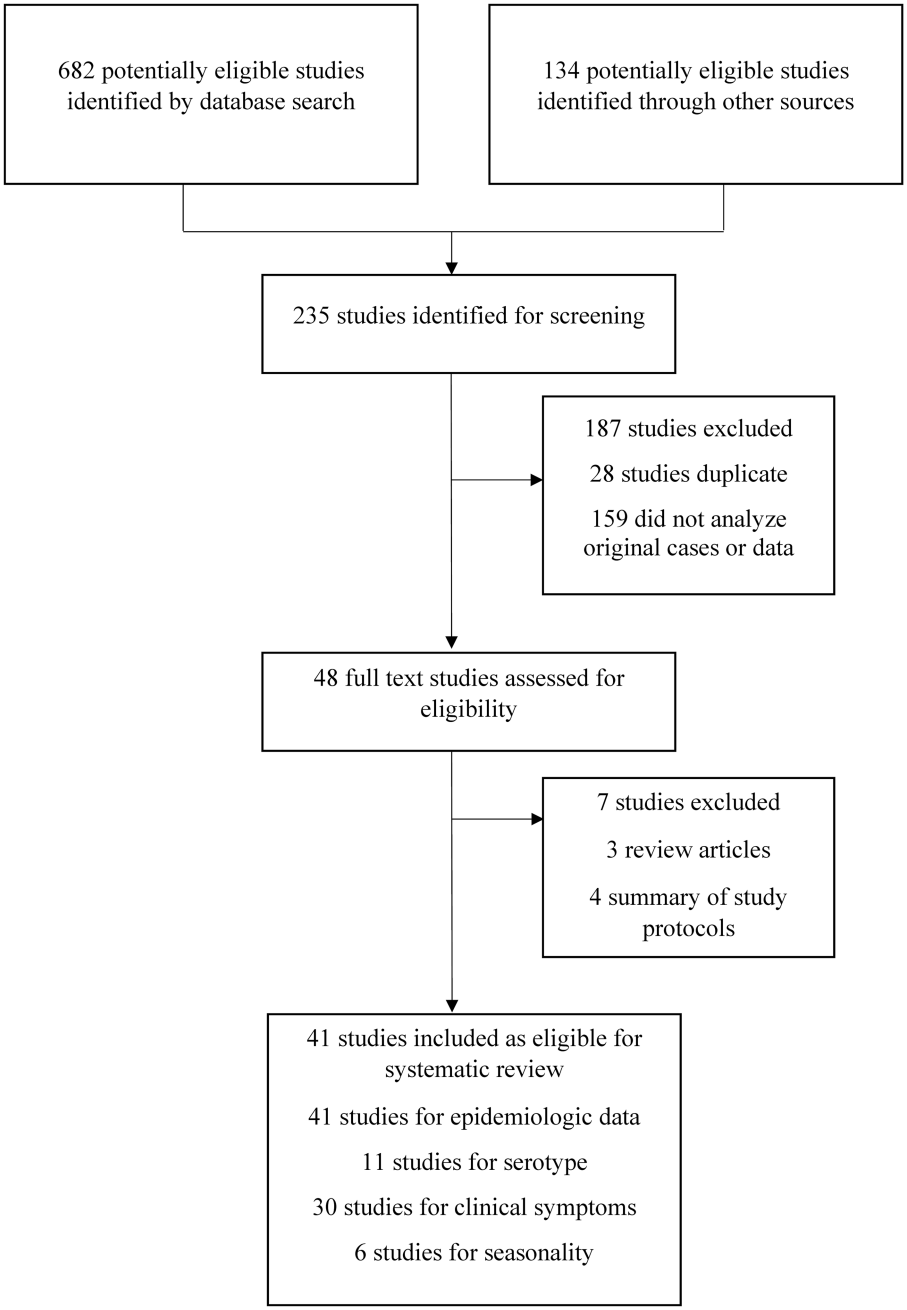

FIGURE 1
Selection and screening procedures of original studies. The excluded articles were irrelevant, duplicate, systematic reviews other than original studies and failed to meet inclusion criteria.

Serotype and genetic diversity of dengue viruses in Bangladesh

Data on the serotype diversity and genotypic characteristics of the dengue virus in Bangladesh are significantly lacking. We identified 11 studies that examined dengue virus serotype diversity in Bangladesh from 2000 to 2024. The majority of studies (100%) on the genotyping of dengue viruses were conducted in Dhaka, with a total of 1,575 samples analyzed. Serotyping was conducted on 92.4% (1,456 of 1,575) of samples during the period from 2012 to 2023, compared to only 5.4% (85 of 1,575) between 2000 and 2011 (Figure 4). Among the four serotypes, DENV3 was the most prevalent (57%, 868 of 1,541), followed by DENV2 (30%, 466 of 1,541), DENV1 (11%, 174 of 1,541), and mixed DENV2 and DENV3 infections (1%, 18 of 1,541). The diversity of serotypes and the occurrence of mixed infections increased during 2012–2023 (Figure 4).

DENV4 (4 of 1,541) and mixed infections of DENV3 and DENV4 were only reported in Dhaka in 2000. Additionally, mixed infections of DENV1 and DENV3 (7 cases) and DENV1, DENV2, and DENV3 (2 cases) were reported in Dhaka in 2018. The genotypic diversity of dengue viruses in Bangladesh remains poorly

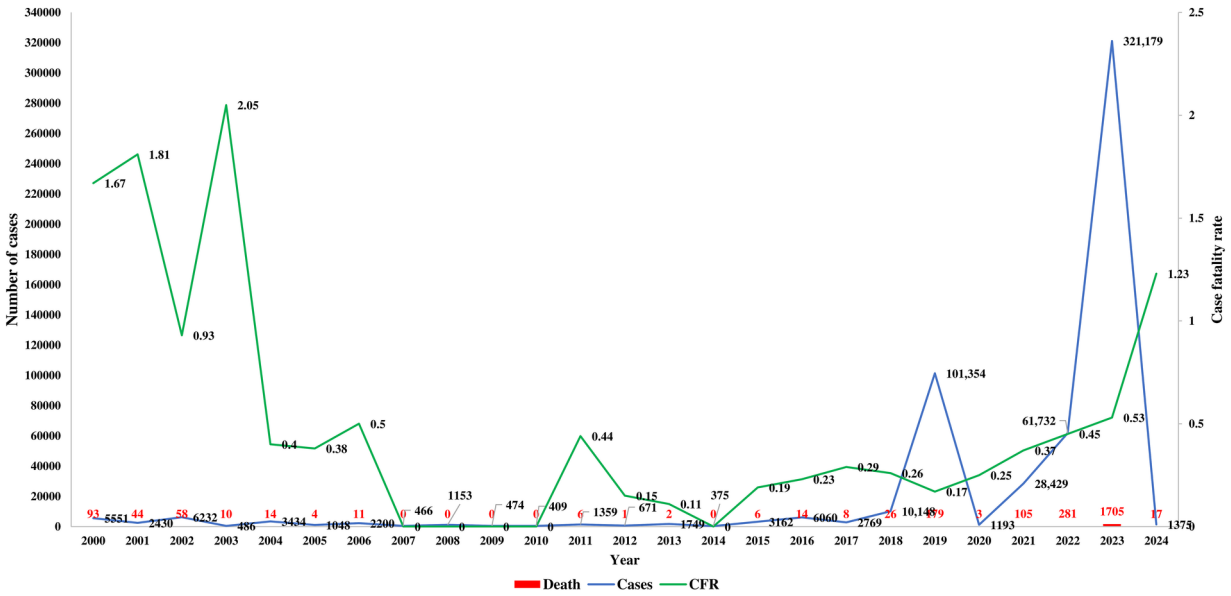

FIGURE 2
Trends of cases, fatalities, and CFR among the residents in Bangladesh.

studied. Among the genotypes, DENV3-I was the most prevalent (43 of 59), followed by DENV3-II (8 of 59), DENV3-III (2 of 59), and mixed genotype DENV3-I, III (6 of 59) (Supplementary Table S1). The cosmopolitan DENV2 genotype was reported in 2019, while the cosmopolitan DENV3 was identified in 2018 in Dhaka.

Phylogenomic analysis of dengue virus also confirmed the circulation of DENV2 and DENV3 genotypes (divergence 0.39) in Bangladesh. Furthermore, phylogenetic analysis of 12 isolates based on the E gene showed that 100% clustered with DENV3 and were closely related to isolates from Thailand, the Philippines, Indonesia, and Australia (Supplementary Figure S1). The sequence similarity of Bangladeshi DENV3-II strains was exceptionally higher (99.93%) than that of strains from India.

Clinical characteristics of patients with dengue virus infection

We extracted clinical data from 30 articles, 29 of which were conducted before the 2023 outbreak. The majority of the study (>90%) found fever, headache, rash, vomiting, abdominal pain, and diarrhea as the most prevalent symptoms, followed by arthralgia, myalgia, retro-orbital pain, nausea, fatigue, fluid leakage, and back pain (reported in 70–89% of studies) (Table 2). Body aches, malaise, petechiae, hemorrhage, gum bleeding, nasal bleeding, and hematuria were less commonly reported symptoms (<50% of the studies).

Approximately 6,788 cases with documented symptoms were reported across the 29 articles from dengue outbreaks between 2000 and 2022. Fever was the most prevalent symptom (90.51, 95% CI 85–100%), followed by headache (57.98, 95% CI 12–100%), vomiting (51.16, 95% CI 23–91%), abdominal pain (34.12, 95% CI 12–85%), myalgia (25.53, 95% CI 13–85%), arthralgia (24.29, 95% CI 8–91%), nausea (22.70, 95% CI 13–76%), rash (21.48, 95% CI 7–70%), fatigue (20.14, 95% CI 8–80%), retro-orbital pain (19.12, 95% CI 3–51%), back pain (17.31, 95% CI 3–73%), and diarrhea (15.44, 95% CI 7–66%). Furthermore, among the severe symptoms, fluid leakage (14.1, 95% CI 5–88%) was the most prevalent, followed by hemorrhage (11, 95% CI 3–51%), gum bleeding (10, 95% CI 1–41%), and hematuria (8, 95% CI 1–16%).

During the 2023 outbreak, changes in the frequency of symptoms were found. Fever was the most frequent symptom (99%), followed by myalgia (86%), anorexia (86%), fatigue (86%), headache (81%), malaise (81%), hemorrhage (74%), body ache (71%), diarrhea (65%), and vomiting (65%). The rash was found in fewer than 10% of patients during the 2023 outbreak (Table 2). Mild to moderate symptoms were reported in 85% of patients, with severe outcomes in 12% and death in fewer than 1% of cases.

Seasonality of dengue outbreaks in Bangladesh

Seasonal changes in dengue cases and outbreaks have been documented in Bangladesh. While the existing literature and databases offer limited information on the seasonality of dengue, we compiled all available data. Cases and fatalities increased sharply after June in Bangladesh, with a peak in outbreaks occurring between July and November. Among the documented 541,751 cases documented from 2008 to 2023, the highest frequency was recorded in August (26.3%), followed by September (22.5%), October (20.2%), November (13.08%), and July (12.4%) (Figure 5). The first 6 months of the year (January to June) contributed to less than 3% of total dengue cases in Bangladesh.

The seasonal pattern of the 2023 outbreak, which was the largest in Bangladesh's history, was similar to previous outbreaks with minor variations. In 2023, the highest number of cases was reported in September (79,598 cases), followed by August (71,976 cases), October

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TABLE 1 Prevalence and epidemiology of dengue outbreaks in Bangladesh during 2000–2024.

Study	Region/Time	Number of participants	Age	Sex ratio (Male: Female)	Prevalence	Diagnosis method
Prattay et al. (2022)	Dhaka, 2019	336	All age	2.2:1	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM and IgG antibody test
Muraduzzaman et al. (2018)	Dhaka, Chattogram, Khulna, 2013–2016	1,380	All age	N/A	42% (2013) 21% (2014) 16% (2015) 13% (2016)	ELISA, RT-PCR
Islam et al. (2022b)	Tangail, 2019	123	5 days to 17 years	62:38	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Ahmed et al. (2016)	Dhaka, 2004	198	All age	4:1	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Islam et al. (2019)	Dhaka, 2018	82	6 months to 15 years	1:1	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Islam et al. (2022a)	Dhaka, 2019–2020	478	All age	1.23:1	Only dengue-positive patients were included	NS1Ag test
Khan et al. (2021)	Dhaka, 2019	190	<15 years	1.22:1	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Hasan et al. (2021a)	Dhaka, 2019	747	All age	63:37	74% (553)	NS1Ag and anti-dengue IgM antibody test
Yang et al. (2023)	Dhaka, 2019	1,090	All age	60:40	Only dengue-positive patients were included	NS1Ag test
Rafi et al. (2020)	Bogra, 2019	319	All age	70:30	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Shultana et al. (2019)	Dhaka, 2018	89	< 15 years	1.2:1	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Mahmood et al. (2021)	Dhaka, 2019	542	All age	60:40	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Yesmin et al. (2023b)	Dhaka, 2019	369	>18 years	60:40	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Rahim et al. (2023)	Dhaka, 2018–2022	3,759	All age	60:40	839 (22.3%)	NS1Ag test, RT-PCR
Islam et al. (2006)	Dhaka, 2002	200	All age	2.7:1	100 (50%)	ELISA, RT-PCR
Aziz et al. (2002)	Dhaka, 2004–2005	45	All age	2:1	Only dengue-positive patients were included	ELISA, RT-PCR
Sultana et al. (2020)	Dhaka, 2018	316	N/A	N/A	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Hossain et al. (2003)	Dhaka, 1996–1997	409	All age	55:45	Only dengue-positive patients were included	Widal test, anti-dengue IgM, and IgG antibody test

(Continued)

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TABLE 1 (Continued)

Study	Region/Time	Number of participants	Age	Sex ratio (Male: Female)	Prevalence	Diagnosis method
Amin et al. (2022)	Dhaka, 2018	297	All age	60:40	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Kabir et al. (2020)	Noakhali, 2020	52	All age	80:20	Only dengue-positive patients were included	N/A
Titir et al. (2021)	Dhaka, Rangpur, Mymensingh, Sylhet, Chattogram, Barisal, Khulna, Jessore, Kustia, 2019	179	All age	61.5:38.5	162 (90.5%)	NS1Ag and anti-dengue IgM antibody test
Rahman et al. (2002)	Dhaka, 2000	336	All age	N/A	176 (73.3%)	ELISA, RT-PCR
Rouf et al. (2020)	Dhaka, 2018–2019	343	18 years and above	1.2:1	62 (18.1%)	NS1Ag and anti-dengue IgM antibody test, RT-PCR
Siddiqua et al. (2018)	Dhaka, 2015–2017	3,201	All age	N/A	1,037 (32.4%)	NS1Ag test, RT-PCR
Yasmin et al. (2020)	Dhaka, 2019	100	2 months–14 years	58:42	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Nahar et al. (2021)	Dhaka, 2019	213	All age	1.7:1	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM and IgG antibody test
Islam et al. (2021)	Dhaka, 2019	220	All age	53.6:46.4	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Parvin et al. (2022)	Dhaka, 2019	100	>12 years	1.04:1	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM and IgG antibody test
Mobarak et al. (2018)	Dhaka, 2016	56	1 year to 18 years	55:45	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM and IgG antibody test
Sultana et al. (2019)	Dhaka, 2018	899	All age	69.3:30.7	350 (38.93%)	NS1Ag and anti-dengue IgM antibody test
Rahman et al. (2019)	Dhaka, 2019	70	>12 years	79:21	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test
Pervin et al. (2017)	Dhaka, 2016	145	All age	62.5:37.5	40 (27.6%)	NS1Ag and anti-dengue IgM antibody test
Sultana et al. (2013)	Chattogram, 2009–2010	1,181	All age	N/A	533 (45.13%)	anti-dengue IgM and IgG antibody test
Datta et al. (2021)	Chattogram, 2019	192	< 12 years	59.4:40.6	Only dengue-positive patients were included	NS1Ag and anti-dengue IgM antibody test

(Continued)

TABLE 1 (Continued)

Study	Region/Time	Number of participants	Age	Sex ratio (Male: Female)	Prevalence	Diagnosis method
Hasan et al. (2021b)	Dhaka, 2019	747	27 ± 31 y (range 3–85 y)	62.7:37.3	74%	NS1 antigen or anti-dengue immunoglobulin M (IgM).
Sharmin et al. (2013)	Dhaka (2008)	201	All	72.5:27.5	137 (68.2)	Anti-dengue immunoglobulin M (IgM) and IgG
Rahman et al. (2007)	Dhaka (2006)	225	36.86+/−17.60 years		156 (69.3)	Anti-dengue immunoglobulin M (IgM) and IgG
Salma et al. (2021)	Dhaka (2019)	4,200	All	N/A	All	Anti-dengue immunoglobulin M (IgM) and IgG
Yesmin et al. (2023a)	Dhaka, Tangail (2019)	208	<18 years	N/A	All	Anti-dengue immunoglobulin M (IgM) and IgG
Uddin et al. (2014)	Dhaka (2008–2010)	262	All	N/A	All	Anti-dengue immunoglobulin M (IgM) and IgG
Mutanabbi et al. (2022)	Dhaka (2019)	50	<12 years old	62:38	All	NS1 antigen or dengue IgM or IgG antibodies

(67,769 cases), July (43,854 cases), and November (40,716 cases). The seasonal changes in the vector density have a strong impact on these outbreaks. Although Bangladesh lacks a strong vector surveillance program, recent studies from 2021 to 2023 have shown a high abundance of *Aedes aegypti* mosquitoes in Dhaka (Haque et al., 2023; Sim et al., 2020). Additionally, the rapid spread of dengue from 2019 to 2024 in non-endemic regions was driven not only by imported cases but also by indigenous transmissions. The vector has now spread to most districts in Bangladesh and has adapted to local climatic conditions.

Average temperatures in Bangladesh vary from 17°C (January) to 34°C (May). In the peak dengue months, the average temperature was 32°C in July, 31°C in August and September, 29°C in October, and 26°C in November. The highest average rainfall was recorded in July (378 mm), followed by June (345 mm), September (340 mm), August (305 mm), and May (275 mm) (Figure 5).

Future perspectives of dengue outbreaks in Bangladesh

The 2023 outbreak was the largest on record, with 400,000 confirmed cases, followed by the 2019 outbreak with 120,000 confirmed cases in Bangladesh. Massive transmission of dengue cases occurred in non-endemic regions during the 2019 and 2023 outbreaks, with all 64 districts affected. In 2023, 70% of cases were reported outside Dhaka, the traditional focal point. Without specific treatments or an approved vaccine, dengue poses a major health threat in Bangladesh.

The introduction of an effective dengue vaccine could mitigate this health risk, as evidenced by the recent randomized trial of the TV005 tetravalent vaccine in Bangladesh. Integrated vector management (IVM), along with community engagement and the introduction of innovative approaches such as *Wolbachia*-mediated biocontrol, could help reduce dengue transmission. Although Bangladesh's national dengue surveillance system has improved, active tracing and real-time monitoring are needed to assess the true burden of the disease.

Factors such as mixed and cross-infections, the circulation of DENV1 and DENV4 in neighboring countries, rapid communication, high population density, lack of awareness, insufficient genotypic characterization, a weak national vector management policy, changing climate conditions, and evolving vector characteristics will likely contribute to future major dengue outbreaks, potentially infecting millions of people in Bangladesh in the future.

Discussion

Dengue outbreaks have become a major health threat in Bangladesh (Health bulletin on current Dengue situation published by DGHS, 2024; Siddiqua et al., 2018; Nahar et al., 2021; Parvin et al., 2022; Mobarak et al., 2018; Sultana et al., 2019; Rahman et al., 2019; Pervin et al., 2017; Yesmin et al., 2023a; Rahim et al., 2021). The magnitude of recent dengue outbreaks has increased significantly, with countrywide transmission in 2019 and 2023 infecting approximately half a million (Health bulletin on current Dengue situation published by DGHS, 2024; Zeng et al., 2015; Sharif et al., 2021; Titir et al., 2021; Rahman et al., 2002; Rouf et al.,

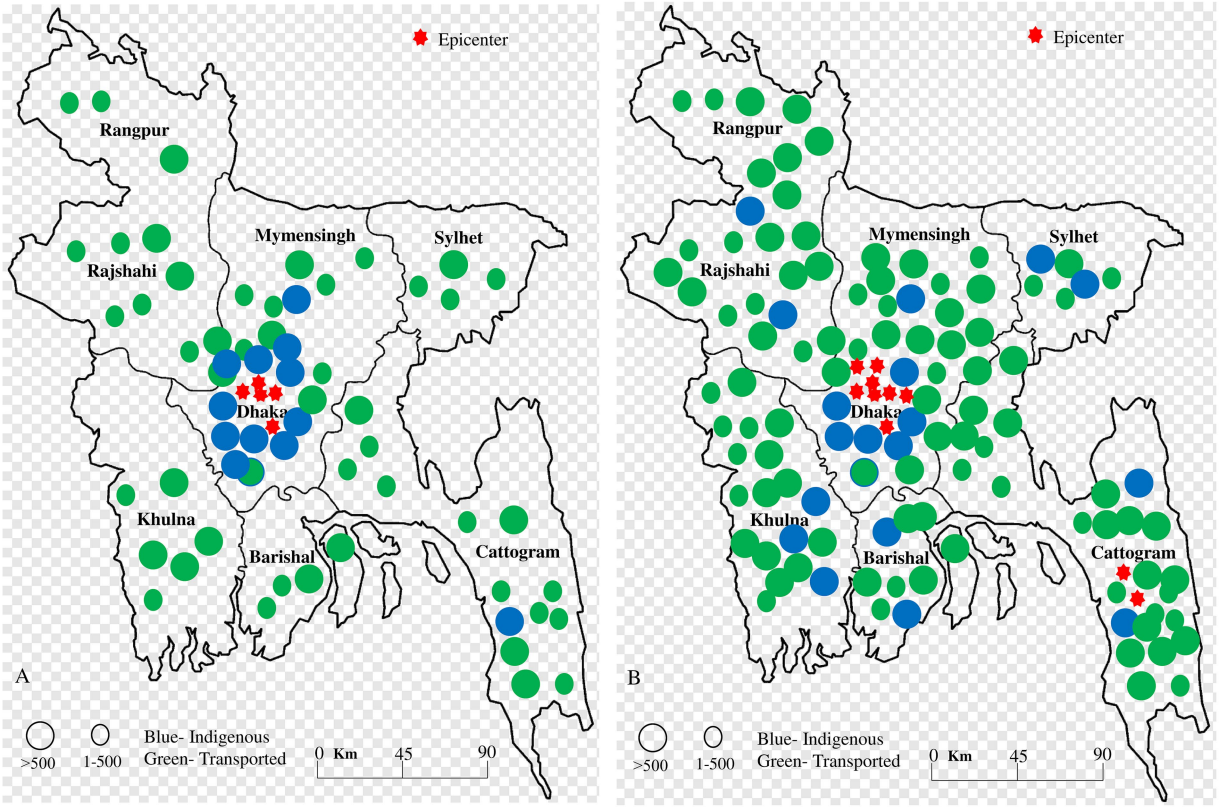

FIGURE 3
Spatial distribution of indigenous and transported cases (A) during 2000–2011 and (B) 2012–2023. The blue color indicated indigenous cases in specific regions, while the green color indicated transported cases. The red star indicated a hotspot of outbreaks. During 2000–2011, the majority of indigenous cases were confined to central regions in Bangladesh. However, from 2012 to 2023, the southern and southeastern regions became highly infected, and indigenous cases became more common throughout Bangladesh.

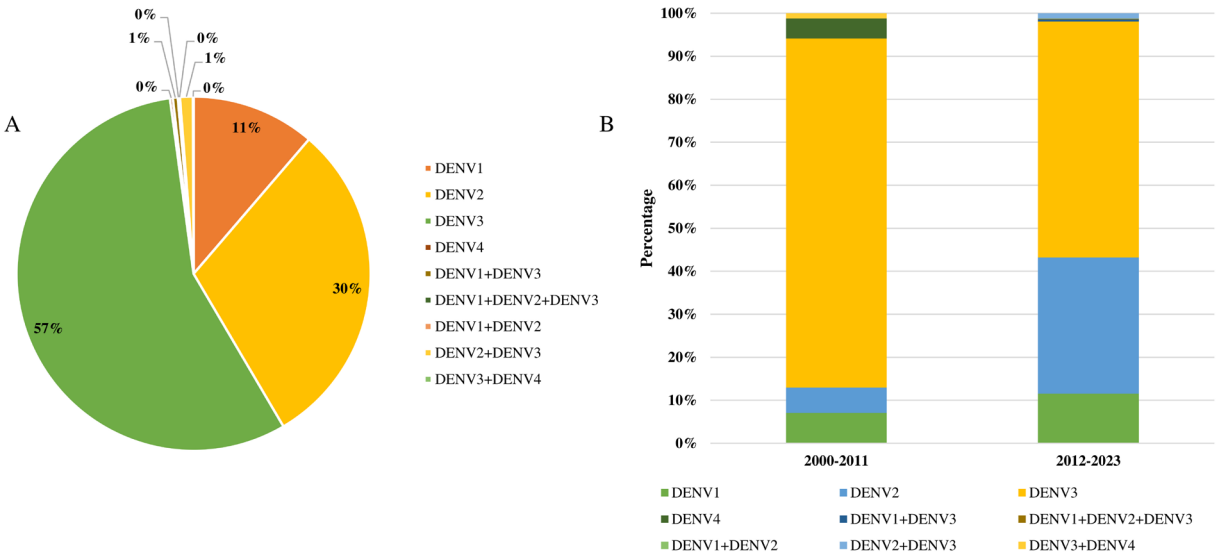

FIGURE 4
(A) Proportionate frequency of serotypes of dengue virus in Bangladesh during 2000–2024. (B) Temporal distributions of serotypes of dengue virus in Bangladesh.

TABLE 2 Clinical manifestations among persons infected with dengue virus in Bangladesh during 2000–2023.

Study	Dengue-positive	Symptoms, *N* (%)															
		Fever	Headache	Body ache	Retro-orbital pain	Arthralgia	Myalgia	Anorexia	Rash	Nausea	Vomiting	Abdominal pain	Back pain	Diarrhea	Fluid leakages	Hemorrhage	Gum bleeding
Prattay et al. (2022)	336	301 (98)	71 (21)	132 (39)	N/A	7 (2)	N/A	60 (18)	18 (5)	45 (13)	99 (29)	41 (12)	8 (2)	26 (8)	N/A	N/A	3 (1)
Islam et al. (2022b)	123	123 (100)	70 (57)	71 (58)	26 (21)	N/A	N/A	N/A	68 (55)	N/A	N/A	36 (29)	N/A	24 (19)	N/A	37 (30)	N/A
Ahmed et al. (2016)	198	196 (99)	189 (96)	N/A	N/A	N/A	N/A	13 (6)	N/A	29 (15)	57 (29)	33 (17)	N/A	17 (9)	174 (88)	31 (15)	41 (21)
Islam et al. (2019)	82	82 (100)	15 (18)	N/A	10 (12)	13 (16)	39 (48)	N/A	57 (69)	N/A	53 (65)	49 (60)	N/A	9 (11)	48 (58)	N/A	10 (12)
Islam et al. (2022a)	478	452 (95)	234 (49)	41 (9)	260 (54)	324 (68)	N/A	N/A	280 (58)	N/A	153 (32)	N/A	N/A	57 (12)	N/A	N/A	49 (10)
Khan et al. (2021)	190	190 (100)	126 (68)	N/A	63 (34)	75 (41)	N/A	N/A	52 (28)	N/A	152 (80)	122 (65)	66 (37)	81 (43)	42 (24)	N/A	N/A
Hasan et al. (2021a)	553	553 (100)	347 (63)	N/A	216 (39)	25 (4)	N/A	210 (38)	25 (4)	385 (70)	385 (69)	230 (42)	58 (10)	145 (26)	N/A	N/A	20 (7)
Yang et al. (2023)	1,090	1,034 (95)	901 (83)	N/A	N/A	516 (47)	691 (63)	N/A	277 (25)	N/A	837 (77)	631 (58)	651 (60)	N/A	272 (25)	159 (15)	N/A
Rafi et al. (2020)	319	295 (93)	232 (73)	N/A	150 (47)	N/A	228 (71)	N/A	50 (16)	N/A	109 (34)	95 (30)	N/A	138 (43)	38 (12)	N/A	25 (9)
Shultana et al. (2019)	89	89 (100)	11 (12)	N/A	1 (1)	12 (13)	12 (13)	N/A	43 (48)	33 (37)	33 (37)	21 (24)	12 (13)	8 (67)	10 (11)	23 (26)	1 (1)
Mahmood et al. (2021)	542	505 (93)	249 (46)	N/A	27 (5)	N/A	146 (27)	37 (7)	137 (25)	331 (61)	331 (61)	160 (29)	-	107 (20)	N/A	N/A	N/A
Yesmin et al. (2023b)	369	369 (100)	223 (60)	N/A	132 (36)	75 (20)	73 (20)	162 (44)	64 (17)	249 (67)	249 (67)	136 (37)	104 (28)	134 (36)	91 (24)	83 (22)	22 (6)
Rahim et al. (2023)	67	N/A	16 (24)	23 (34)	N/A	N/A	N/A	12 (18)	9 (13)	36 (53)	36 (54)	37 (55)	N/A	6 (9)	10 (15)	11 (16)	N/A
Islam et al. (2006)	100	100 (100)	96 (96)	N/A	N/A	91 (91)	N/A	N/A	28 (28)	N/A	93 (93)	83 (83)	N/A	N/A	N/A	7 (7)	41 (41)
Aziz et al. (2002)	45	45 (100)	40 (89)	40 (89)	N/A	N/A	N/A	38 (84)	10 (22)	N/A	41 (91)	N/A	N/A	N/A	9 (20)	5 (11)	N/A
Amin et al. (2022)	297	287 (96.6)	269 (91)	79 (27)	151 (51)	87 (28)	79 (26)	239 (80)	98 (33)	227 (76)	227 (76)	100 (34)	218 (73)	115 (39)	43 (14)	10 (3)	6 (2)

(Continued)

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TABLE 2 (Continued)

Study	Dengue-positive	Symptoms, *N* (%)														
		Fever	Headache	Body ache	Retro-orbital pain	Arthralgia	Myalgia	Anorexia	Rash	Nausea	Vomiting	Abdominal pain	Back pain	Diarrhea	Fluid leakages	Hemorrhage	Gum bleeding
Kabir et al. (2020)	52	52 (100)	34 (65)	N/A	N/A	N/A	N/A	N/A	8 (15)	23 (44)	23 (44)	7 (13)	N/A	N/A	N/A	N/A	N/A
Rahman et al. (2002)	176	176 (100)	160 (91)	N/A	N/A	150 (85)	150 (85)	N/A	97 (55)	N/A	113 (64)	N/A	N/A	N/A	81 (46)	21 (12)	20 (11)
Rouf et al. (2020)	25	15 (60)	25 (100)	25 (100)	N/A	N/A	N/A	6 (24)	N/A	6 (24)	7 (28)	5 (20)	N/A	4 (16)	N/A	N/A	N/A
Siddiqua et al. (2018)	100	100 (100)	60 (60)	60 (60)	N/A	N/A	N/A	N/A	N/A	66 (66)	66 (66)	66 (66)	N/A	N/A	N/A	36 (36)	22 (22)
Yasmin et al. (2020)	213	213 (100)	24 (11)	23 (11)	23 (11)	23 (11)	N/A	N/A	N/A	51 (24)	51 (24)	46 (22)	N/A	28 (13)	N/A	104 (49)	N/A
Islam et al. (2021)	220	202 (92)	106 (48)	N/A	31 (14)	N/A	43 (19)	N/A	32 (14)	N/A	110 (50)	108 (49)	N/A	121 (55)	27 (12)	114 (52)	N/A
Parvin et al. (2022)	100	63 (63)	69 (69)	N/A	40 (40)	27 (27)	42 (42)	N/A	14 (14)	N/A	70 (70)	77 (77)	N/A	N/A	68 (68)	39 (39)	8 (8)
Mobarak et al. (2018)	56	56 (100)	38 (68)	N/A	41 (73)	35 (62)	43 (76)	N/A	31 (55)	N/A	29 (52)	26 (46)	N/A	N/A	31 (55)	N/A	N/A
Sultana et al. (2019)	350	350 (100)	214 (61)	N/A	71 (20)	81 (23)	154 (44)	N/A	21 (6)	N/A	N/A	N/A	11 (3)	18 (5)	N/A	N/A	N/A
Rahman et al. (2019)	70	64 (91)	62 (88)	N/A	36 (51)	45 (64)	N/A	59 (85)	12 (17)	49 (71)	46 (67)	19 (27)	47 (67)	N/A	3 (4)	N/A	N/A
Pervin et al. (2017)	40	40 (100)	10 (25)	N/A	20 (50)	35 (87)	5 (12)	11 (27)	5 (12)	11 (27)	11 (27)	12 (30)	N/A	10 (25)	11 (27)	N/A	N/A
Datta et al. (2021)	192	192 (100)	45 (23)	N/A	N/A	28 (14)	28 (14)	N/A	22 (11)	N/A	92 (48)	176 (92)	N/A	N/A	N/A	N/A	N/A
Sharif et al. (2024a)	47,854	(99)	(81)	(71)	N/A	(86)	(86)	N/A	(<10)	N/A	(62)	N/A	N/A	(65)	(65)	(74)	N/A

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2020; Siddiqua et al., 2018; Yasmin et al., 2020; Nahar et al., 2021; Mutanabbi et al., 2022; Pervin et al., 2003; Baskey et al., 2024; Roy et al., 2023; Rahim et al., 2021). Integrated studies on the prevalence, molecular epidemiology, clinical characteristics, and seasonality are crucial to provide a more accurate picture of dengue outbreaks in Bangladesh. The 2019 and 2023 outbreaks accounted for 78% of all dengue cases in the country. Our analysis reveals not only an increase in cases and fatalities but also a significant geographic expansion of cases into previously non-endemic regions.

The sharp rise in cases and fatalities during the 2019 and 2023 outbreaks is largely associated with the rapid increase in vector mosquitoes and the wide distribution of their habitats, along with the evolving genotypes of the dengue viruses. The frequently evolving genotypes of DENV may be contributing to this rapid spread, and further studies are needed to explore this connection.

These findings are similar to those reported by previous studies in Bangladesh (Rahim et al., 2021; Ahmad et al., 2020; Sharif et al., 2024a). However, the rapid expansion of dengue across the country contrasts with trends observed in nearby countries and other dengue-endemic regions globally. The reported number of dengue cases from 2020 to 2022 was lower during the COVID-19 pandemic (Bhowmik et al., 2023; Hasan et al., 2019; Sharif et al., 2023b). This low number was primarily due to underreporting. Additionally, strict lockdowns and reduced intercity travel, particularly in Dhaka, may have contributed to the reduced number of cases.

During the 2019 outbreak and afterward, *Aedes aegypti* and *Aedes albopictus* rapidly spread across Bangladesh. As a result, in the 2023 outbreak, the majority of the cases were documented outside the established hotspot of Dhaka (Health bulletin on current Dengue situation published by DGHS, 2024; Rahim et al., 2021; Ahmad et al., 2020). Moreover, cases in non-endemic regions were non-travel-related and indigenous, with transmission documented from the beginning to the end of the outbreak. These findings are alarming not only for Bangladesh but also for other dengue-endemic regions worldwide.

Among the various demographic characteristics reported in most data sources, age and sex were commonly mentioned. We found the majority of the cases (70%) between 2000 and 2024 occurred in men. However, the case fatality rate was significantly higher among women, with a women-to-men ratio of 3:2. Factors contributing to the higher death rate among females include extended time at home, delayed visits to healthcare providers, and the presence of anemia. Previous studies from Bangladesh, as well as from India, the Philippines, Indonesia, China, and Pakistan, have supported these findings (Khan et al., 2021; Hasan et al., 2021a; Yang et al., 2023; Rafi et al., 2020; Datta et al., 2021; Salma et al., 2021; Yesmin et al., 2023a; Uddin et al., 2014; Mutanabbi et al., 2022; Pervin et al., 2003; Baskey et al., 2024; Roy et al., 2023; Rahim et al., 2021; Ahmad et al., 2020; Sharif et al., 2024b).

Both the spatial and temporal distribution of dengue cases have shown a rapid increase in cases in recent times (2018–2024). While global data suggest some improvement in many endemic regions due to integrated management, the situation in Bangladesh has been markedly different (Sharif et al., 2024b). If the uncontrolled outbreak continues, millions of people could be infected, and many could die from dengue. The 2023 outbreak was the worst and longest in Bangladesh's history.

However, many cases were likely underreported due to a lack of advanced surveillance and limited diagnostic facilities (Rahim et al., 2021). Additionally, the dengue outbreak spread to rural areas where most residents had limited access to healthcare and low awareness of the disease. Consequently, asymptomatic individuals and those with mild symptoms were not included in the official documentation, making the actual health burden of recent dengue outbreaks much higher than documented. These observations are supported by previous studies in Bangladesh (WHO Dengue fact sheet, 2024; Health bulletin on current Dengue situation published by DGHS, 2024; Datta et al., 2021; Hasan et al., 2021b; Sharmin et al., 2013; Rahman et al., 2007; Salma et al., 2021; Rahim et al., 2021; Ahmad et al., 2020).

The highest frequency of cases in the 2023 outbreak was recorded in the Dhaka division (18.3%, 58,971 of 321,179 cases), followed by Chattogram (13.8%, 44,435 of 321,179 cases), Barishal (11.8%, 38,049 of 321,179), and Khulna (10.8%, 34,722 of 321,179 cases). The southern regions (Khulna and Barishal divisions) and the southeastern region (Chattogram division) emerged as new hotspots for dengue transmission. In the southern areas, both *Aedes aegypti* and *Aedes albopictus* mosquitoes are prevalent, while in the southeastern region, only *Aedes albopictus* has been reported. These findings are supported by previous studies in Bangladesh (WHO, 2024; Deen et al., 2006; Prattay et al., 2022; Muraduzzaman et al., 2018; Islam et al., 2022b; Ahmed et al., 2016; Islam et al., 2019; Islam et al., 2022a; Khan et al., 2021; Hasan et al., 2021a; Yang et al., 2023; Datta et al., 2021; Hasan et al., 2021b; Sharmin et al., 2013; Rahman et al., 2007; Salma et al., 2021; Yesmin et al., 2023a; Rahim et al., 2021; Ahmad et al., 2020; Sharif et al., 2024a; Bhowmik et al., 2023; Hasan et al., 2019; Sharif et al., 2023b).

The rapid spread of dengue in non-endemic regions and the larger outbreaks with uncontrolled cases in Bangladesh have been driven by the higher density and wider distribution of vector mosquitoes. Several factors contribute significantly to this situation, including environmental conditions, climate change, human activities, population density, poor management and policies, changes in vector characteristics, and the mosquitoes' adaptation capabilities (Rahim et al., 2021; Ahmad et al., 2020). Increased average temperature, seasonal rainfall, and flooding have notably supported the reproduction, survival, and spread of *Aedes* spp. in most regions of Bangladesh.

The majority of the study confirms a seasonal spike in cases of dengue in Bangladesh, with the highest frequency occurring between August and October, an increase in cases beginning in July and lasting through November (Ahmad et al., 2020). According to previous studies, we also found that the optimal temperature range for *Aedes* spp. reproduction and spread is between 23°C and 29°C, with higher rainfall further facilitating their growth. Similarly, during the period from August to November, the average temperature in Bangladesh ranged from 26°C to 31°C, further supporting the seasonal rise in dengue cases.

Serotype and genotype data highlight a significant research gap in Bangladesh. Among the isolates, DENV3 (57%) was the most common serotype, followed by DENV2 (30%) and DENV1 (11%). However, DENV4 has not been reported recently. Additionally, the prevalence of mixed infections among different serotypes, including DENV1-DENV3, was also significant (2–10%). This raises concerns about the high risk of secondary and post-secondary infections in individuals exposed to different serotypes, which could further complicate the situation. The risk is heightened by direct vector transmission and travelers' cases from nearby endemic countries, such as India, which could exacerbate the spread of dengue in Bangladesh.

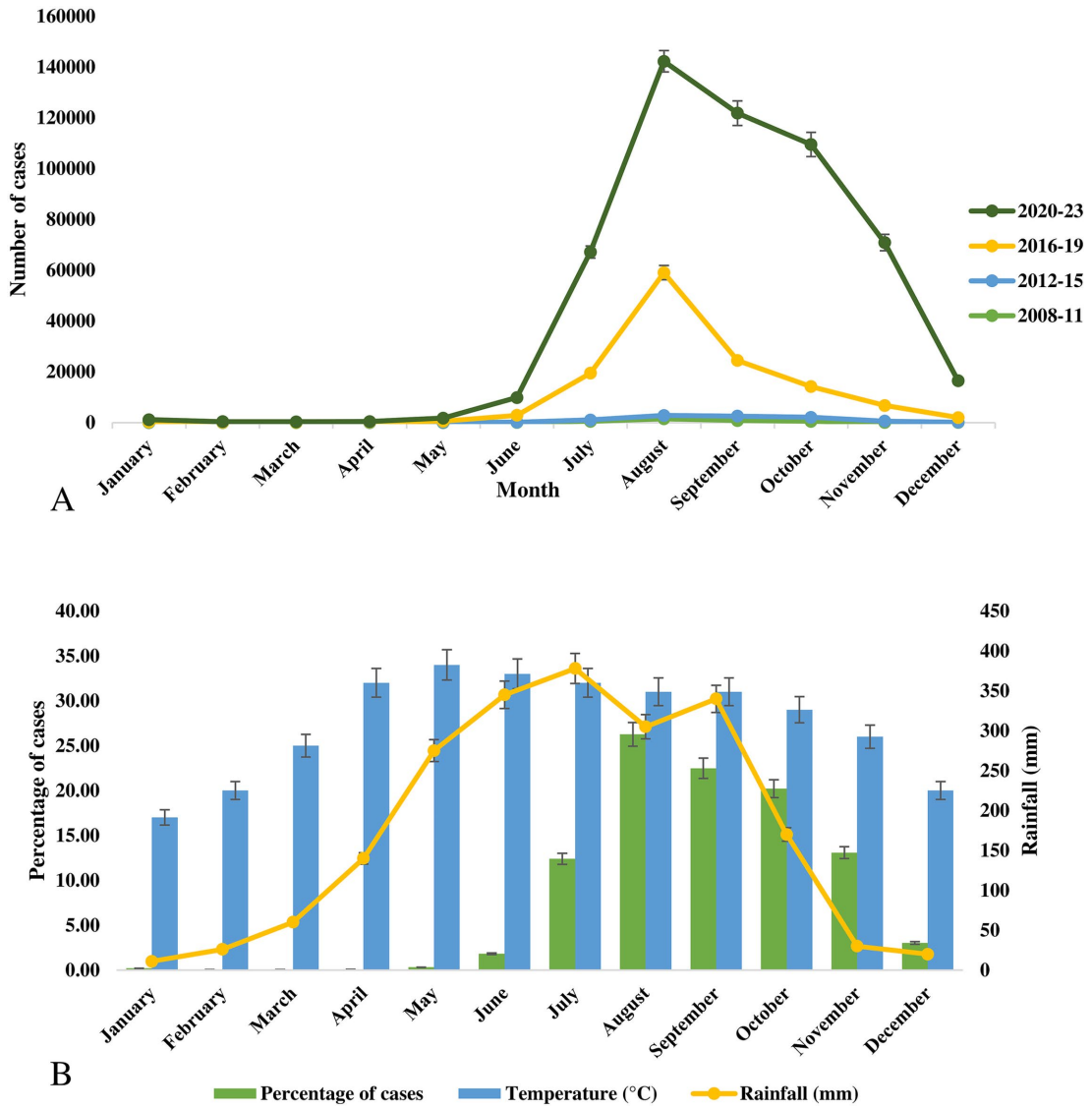

FIGURE 5
(A) Monthly distribution of cases of dengue outbreaks during 2008–2023 in Bangladesh. (B) Frequency distribution of cases of dengue virus, average temperature and rainfall in Bangladesh.

These findings are supported by previous studies (Health bulletin on current Dengue situation published by DGHS, 2024; WHO, 2024; Baskey et al., 2024; Rahim et al., 2021; Ahmad et al., 2020).

Genotype DENV3-I was the most prevalent (73%), followed by DENV3-II (13%). These genotypes show high similarity with isolates from India, the Philippines, Indonesia, and Australia (Hadinegoro, 2012). A recent study from Northern West Bengal, India, reported the prevalence of DENV1 and DENV3 of genotype III (Roy et al., 2023). Furthermore, a study in Kolkata found that the dominant serotypes of DENV evolved from DENV3 in 2015 to DENV1 in 2016 and DENV2 from 2017 to 2019 (Baskey et al., 2024). These studies support the idea that the importation of DENV from nearby countries could significantly affect the present dengue situation in Bangladesh. Our findings also suggest that studies on the diversity of genotypes in Bangladesh are still rare and require further attention.

Changes in the symptoms experienced by patients with dengue virus infection have been noted in existing studies. The number of patients presenting with critical health conditions has risen along with the increase in dengue cases from 2019 to 2023. Notably, there has been an increase in symptoms such as myalgia, anorexia, fatigue, headache, malaise, and hemorrhage, while the frequency of rashes has decreased. One possible explanation for these changing symptoms is the involvement of antibody-dependent enhancement (ADE). Secondary infections with different dengue serotypes can trigger immunoglobulin G (IgG)-mediated ADE, which may lead to greater disease severity in affected individuals. This theory aligns with recent studies conducted in Bangladesh (Sharif et al., 2021; Titir et al., 2021; Rahman et al., 2002; Rouf et al., 2020; Siddiqua et al., 2018; Yasmin et al., 2020; Nahar et al., 2021; Islam et al., 2021; Parvin et al., 2022; Mobarak et al., 2018; Sultana et al., 2019;

Rahman et al., 2019; Pervin et al., 2017; Sultana et al., 2013; Datta et al., 2021; Hasan et al., 2021b; Sharmin et al., 2013; Rahman et al., 2007; Salma et al., 2021; Rahim et al., 2021; Ahmad et al., 2020).

Dengue virus-specific antivirals are currently unavailable, and dengue vaccines are not approved for use in Bangladesh. The efficacy of existing dengue vaccines is moderate to low and recommended only for those who have been previously infected. The absence of targeted treatments or vaccine-mediated prevention elevates the risk of larger outbreaks. While integrated vector management has been successful in reducing the burden in many countries, Bangladesh faces significant gaps in this regard.

Key contributing factors to the outbreaks include the lack of active surveillance in rural areas, limited public awareness of vector control, gaps in integrated and nationwide management, and the absence of a clear roadmap for vector control. Additionally, the adaptability of vectors, rapid urbanization, deforestation, climate change, rising temperatures, prolonged monsoon seasons, and increased movement and transportation all play vital roles in fueling larger dengue outbreaks in Bangladesh.

This study has a number of limitations. Genomic data needed more in-depth analysis to predict mutations and evolutionary changes that could be linked to altered epidemiological characteristics. Additionally, since this study focuses on the dengue virus, detailed information on vector characterization could not be included. However, the main strength of this study lies in its comprehensive analysis, which incorporates the most up-to-date data and removes biases. Furthermore, it addresses a wide range of epidemiological aspects related to dengue outbreaks in Bangladesh.

Conclusion

This study concludes that dengue has recently become a major health burden in Bangladesh, affecting nearly 0.5 million people. Indigenous cases in non-endemic regions, particularly in the southern and southeastern parts of the country, have become more common than in the traditional hotspot of Dhaka since the 2019 outbreak. Serotypes DENV3 (57%) and DENV2 (30%) accounted for approximately 90% of characterized isolates in Bangladesh. The study highlights a significant gap in the genotypic and serotype characterization of the dengue virus in the country. Additionally, the lack of timely and effective policies for vector control has contributed to the rising number of cases. This study provides a valuable integrated dataset and serves as a guideline for future research, policymakers, and the scientific community.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

NS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RO: Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. TS: Data curation, Formal analysis, Writing – review & editing. AM: Data curation, Methodology, Writing – review & editing. JN: Data curation, Investigation, Methodology, Writing – review & editing. KAls: Data curation, Formal analysis, Writing – review & editing. KAlz: Conceptualization, Data curation, Formal analysis, Resources, Writing – review & editing. EA: Conceptualization, Data curation, Writing – review & editing. IN: Conceptualization, Data curation, Writing – review & editing. ID: Conceptualization, Data curation, Formal analysis, Writing – review & editing. SD: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1481418/full#supplementary-material

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Bioluminescent and fluorescent reporter-expressing recombinant Akabane virus (AKAV): an excellent tool to dissect viral replication

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Introduction: Akabane virus (AKAV) is a worldwide epidemic arbovirus belonging to the Bunyavirales order that predominantly infects livestock and causes severe congenital malformations. Reporter-expressing recombinant virus represents a powerful tool to characterize the viral biology *in vitro* and *in vivo*.

Methods: In this study, we have successfully established a reverse genetics system for AKAV. The recued virus possessed similar growth characteristics to the parental virus *in vitro*. Moreover, the recombinant AKAV reporter viruses expressing nanoluciferase (Nluc) or mWasabi were constructed by inserting into S segment, named rAKAV-Nluc and rAKAV-mWasabi, respectively.

Results: We investigated the virological characteristics of rAKAV-Nluc and rAKAV-mWasabi and found that rAKAV-Nluc displayed similar growth kinetics as the parental virus and could stably produce the nano-luciferase even after 10 rounds of serial passages. rAKAV-mWasabi also exhibited comparable growth kinetics and genetic stability as the parental virus. We further used the two reporter viruses to test the susceptibility of different cell lines to AKAV and found that cell lines derived from various host species, including human, swine, cattle, and monkey enables AKAV replication efficiently, accelerating our understanding of the AKAV cell tropism range.

Discussion: Taken together, our established reverse genetics system for AKAV provides more convenient screening tools and can be used to study AKAV virulence and tropism, and to elucidate the molecular biology of AKAV.

KEYWORDS

Akabane virus, reverse genetics system, reporter-expressing virus, nanoluciferase, mWasabi, cell tropism

1 Introduction

Akabane virus (AKAV) belongs to the *Orthobunyavirus* genus within the *Peribunyaviridae* family, serving as the etiological agent of arthrogryposis-hydranencephaly syndrome in cattle, sheep, and goats (Oya et al., 1961). AKAV exhibits teratogenic and lethal properties, resulting in substantial economic losses to the farming industry (Agerholm et al., 2015). Transmission of this virus primarily occurs

through biting midges of the genus *Culicoides*, with its widespread distribution spanning Australia, Southeast Asia, East Asia, the Middle East, and Africa (Zeller and Bouloy, 2000). Furthermore, serological studies showed that AKAV antibodies could be detected in bamboo rat, horses, and pigs (Tang et al., 2017; Tzeng et al., 2022; Yang et al., 2008), suggesting that AKAV is widely distributed across various animal hosts globally.

AKAV is a single-stranded, negative-sense RNA virus. Its genome is composed of three distinctive segments: large (L), medium (M), and small (S) (Bishop, 1996). The M RNA segment encodes two envelope glycoproteins including Gn and Gc, as well as the non-structural protein (NSm). Gn and Gc form hetero-multimers that create spike-like projections up to 20 nm in length. These projections facilitate virus attachment to the surface of target cells (Ludwig et al., 1991). The non-structural protein M (NSm) plays a pivotal role in suppressing the host immune response (Barker et al., 2023). The L fragment encodes the viral RNA-dependent RNA polymerase (RdRp), while the S RNA encodes the viral nucleocapsid (N) proteins and non-structural proteins (NSs). RdRp is encapsulated into the virions, which plays roles in both viral transcription and genome replication. While, N proteins bind to the viral RNA segments, forming ribonucleoprotein structures. Each segment exhibits partially complementary 5′ and 3′ ends, potentially promoting the formation of a "panhandle" secondary structure, which could efficiently interact with RdRp (Barr et al., 2003).

The advent of viral reverse genetics has greatly advanced our ability to generate recombinant or modified viruses, offering invaluable insights into various facets of viral biology. Notably, recombinant viruses equipped with reporter proteins have emerged as powerful tools for probing the virus life cycle, discerning modes of viral spread *in vivo*, and screening antiviral agents. One pivotal milestone in developing the reverse genetical system for *Orthobunyavirus* genus occurred in 1996, when a reverse genetics system based on Bunyamwera virus was pioneered (Bridgen and Elliott, 1996). Given the frequent occurrence of AKAV in Japan, research on its reverse genetics has been actively pursued to aid in the development of vaccines and treatments. In 2007, the first reverse genetics system for AKAV based on the TS-C2 strain were established, subsequently, recombinant AKAV expressing enhanced green fluorescent protein (eGFP-AKAV) were developed as well (Ogawa et al., 2007; Takenaka-Uema et al., 2015, 2016). Since then, Takenaka-Uema et al.'s (2019) team has further optimized this reverse genetic system.

In this study, we developed a reverse genetic system using a clinical isolate AKAV/GX/2016, serving as a valuable tool to explore the fundamental mechanisms of AKAV pathogenesis. We further generated rAKAV strains expressing either fluorescent (mWasabi) or bioluminescent (Nluc) reporters. These reporter-expressing viruses showed similar replication kinetics compared to the parental virus. Consequently, we used these reporter viruses for the assessment of AKAV capability to infect various host cells and found that AKAV could infect the cell lines originated from different species. Coral-derived mWasabi is a relatively bright green monomeric fluorescent protein that performs well in protein chimeras, providing a bright and stable fluorescent signal that does not significantly interfere with the localization or function of the target protein. The protein is also tolerant to a wide range of protein fusions and subcellular microenvironments and is virtually harmless to live cells and easy to detect. In addition, Nluc has a high degree of physical stability and is much more tolerant to temperature, pH and urea. In cells, Nluc exists as a single molecular species with no post-translational modifications and is uniformly distributed without significant regional variations. The novel substrate furimazine produces higher light intensities than natural coelenterazine and is more stable with lower background autofluorescence. The combination of these properties allows Nluc to be widely used as a cellular reporter gene to produce highly sensitive signals. The stability of Nluc is not dependent on disulfide bonds, so the enzyme can be efficiently expressed intracellularly or extracellularly. In addition, the small size of Nluc makes it ideally suited for use as a protein fusion tag, allowing its own luminescence to be correlated with the dynamics of specific intracellular proteins. Nluc may also provide a unique opportunity for the development of protein complementation assays, where the small size, high luminescence, and structural stability are more advantageous. Overall, our optimized system enhances AKAV recovery efficiency, offering valuable tools for studying AKAV molecular virology and advancing next-generation AKAV vaccines and expression vectors.

2 Materials and methods

2.1 Cell, viruses, and antibodies

BSR-T7/5 cells (a BHK-21 derivative cell that stably expresses T7 RNA polymerase), African green monkey kidney cells (Vero E6), Madin-Darby ovine kidney cells (MDOK), Madin-Darby bovine kidney cells (MDBK), Madin-Darby Canine Kidney cells (MDCK) and human embryonic kidney 293T cells (HEK293T cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified 5% CO_2 atmosphere. The Aedes albopictus mosquito cells (C6/36) was cultured in Minimum Essential Medium (MEM), supplemented with 10% FBS and 1% penicillin/streptomycin, at 28°C in a 5% CO_2 atmosphere. The AKAV strain AKAV/GX/2016 was the parental virus for generating the reporter virus below. The anti-AKAV N mAb was generated in our laboratory. Furthermore, Alexa Fluor™ 568 goat anti-mouse IgG (H + L) was purchased from Invitrogen.

2.2 Construction of recombinant full-length cDNA clones

To construct a full-length cDNA clone from the S, M and L segments of AKAV/GX/2016, a set of primers was designed for amplifying the viral RNA genome using RT-PCR (Table 1). The complete cDNAs were amplified by PCR and cloned into the pCI-T7-HDVrz plasmid, which contains a T7 RNA polymerase promoter and HDV Ribozyme. Subsequently, the products were sequenced using Sanger sequencing. Three plasmids expressing

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TABLE 1 Nucleotide sequence of primers used in this study.

RNA segment	Primer	Sequence (5′-3′)
S	SF	TAATACGACTCACTATAAGTAGTGAACTCCACT
	SL	GATGCCATGCCGACCCTAAGTAGTGTGCTCC
M	MF	TAATACGACTCACTATAAGTAGTGAACTACCAC
	ML	GATGCCATGCCGACCCAGTAGTGTTCTACCAC
L	LF	TAATACGACTCACTATAAGTAGTGTACCCCT
	LL	GATGCCATGCCGACCCAGTAGTGTGCCCCT
L	L3590U	ATTCCGGGTAGTAACTTGTGC
	L3725L	CAGGGGAAGAAACTATCC
	L2085L	CAGATTAGCCATAATC
mWasabi	mWasabi-F	GGGCAGCCTTAACCTTTACTTGTACAGCTC
	mWasabi-L	CGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGA TGCCATGCCGACCCTAAGTAGTGTGCTCCACTAATT AACTATAAACAATAAAATCCGAGCAGCTGAACAAAG TGTGCACCACATAGACATGGTGCACTTAGAAATAGA AGTAAGAAAACTGGAGAATCAGCAGAGAATGGTGA GCAAGGGCGAGG
Nluc	Nluc-F	GGGCAGCCTTAACCTCGCCAGAATGCGTTC
	Nluc-L	CGAGTGTGAAGACCATTCTCTGCTGATTCTCCAGTTT TCTTACTTCTATTTCTAAGTGCACCATGTCTATGTGGT GCACACCTTTGTTCAGCTGCTCGGATTTTATTGTTTAT AGTTAATTAGTGGAGCACACTACTTAGGGTCGGCAT GGCATCTCCACCTCCTCGCGGTCCGACCTGGGCAT CCGAAG
IGR	IGR-F	CTCAGTTGCCCAGATATCCTGGGCCAAATCTGGCTT CTCACCTGCAGCTAGAGCTTTCTTGGCTCAATTTGG TATTCAGATCTAAGTGGCTGCCCAGGGG
	IGR-L	AGGTTAAGGCTGCCCCAC

viral RNAs were generated, namely pCI-S-*Sal* I, pCI-M, and pCI-L (Figure 1A). The pCI-S-*Sal* I plasmid was employed to generate pCI-S-Nluc and pCI-S-mWasabi, where the Nluc or mWasabi gene was fused with a Rift Valley fever virus-derived intergenic region (IGR) and inserted in the opposite orientation to the N gene (Figure 2A). The coding region for Nluc was amplified from pNL1.1 (NovoPro), while the coding region for mWasabi was amplified from pT7-mWasabi (Solarbio). This process resulted in the generation of an ambisense S-segment, designated S-Nluc and S-mWasabi, containing the N/NSs gene, IGR sequence, and either the Nluc or mWasabi gene.

2.3 *In vitro* transfection

Recovery of Recombinant AKAV from Cloned cDNAs. Monolayers of BSR-T7/5 cells (8×10^5) in 6-well plates were co-transfected with plasmids using 1.5 μL of Lipofectamine™ 3000 transfection reagent per microgram of plasmid DNA, as follows: 2 μg of each strain AKAV/GX/2016 rescue plasmids pCI-S-*Sal* I, pCI-M, and pCI-L (Figure 1B). After 120 h of incubation in FBS-free medium, transfected cells were lysed by freeze/thaw. The lysates were then transferred to fresh BSR-T7/5 cells. After adsorption at 37°C for 5 h, the lysate-adsorbed BSR-T7/5 cells were washed and cultured in FBS-free DMEM supplemented and incubated at 37°C for 48 h. When evident cytopathic effects (CPE) emerged, supernatants containing the recombinant viruses were harvested and named rAKAV.

For the rescue of the reporter virus, either pCI-S-Nluc or pCI-S-mWasabi, replacing pCI-S-*Sal* I, was included in the transfection process (Figure 2B). When CPE emerged and Nanoluciferase signal can be detected, supernatants containing the recombinant viruses were harvested and named rAKAV-Nluc. By the following day post-transfection, green fluorescence could be observed under a fluorescent microscope. The supernatants from the cell culture were collected at 120 h post-transfection, designated as rAKAV-mWasabi, and stored at −80°C.

2.4 Reverse transcription-PCR and sequencing

Viral RNAs were extracted from the supernatant of rAKAV-infected BSR-T7/5 cells using the SimplyP Total RNA Extraction Kit (BioFlux). Subsequently, the viral genome RNA was converted to cDNA using SuperScript II reverse transcriptase (TaKaRa). The S segments of the recombinant viruses were then amplified by PCR employing KOD FX Neo. Following amplification, the RT-PCR products underwent direct sequencing. The Nluc and mWasabi

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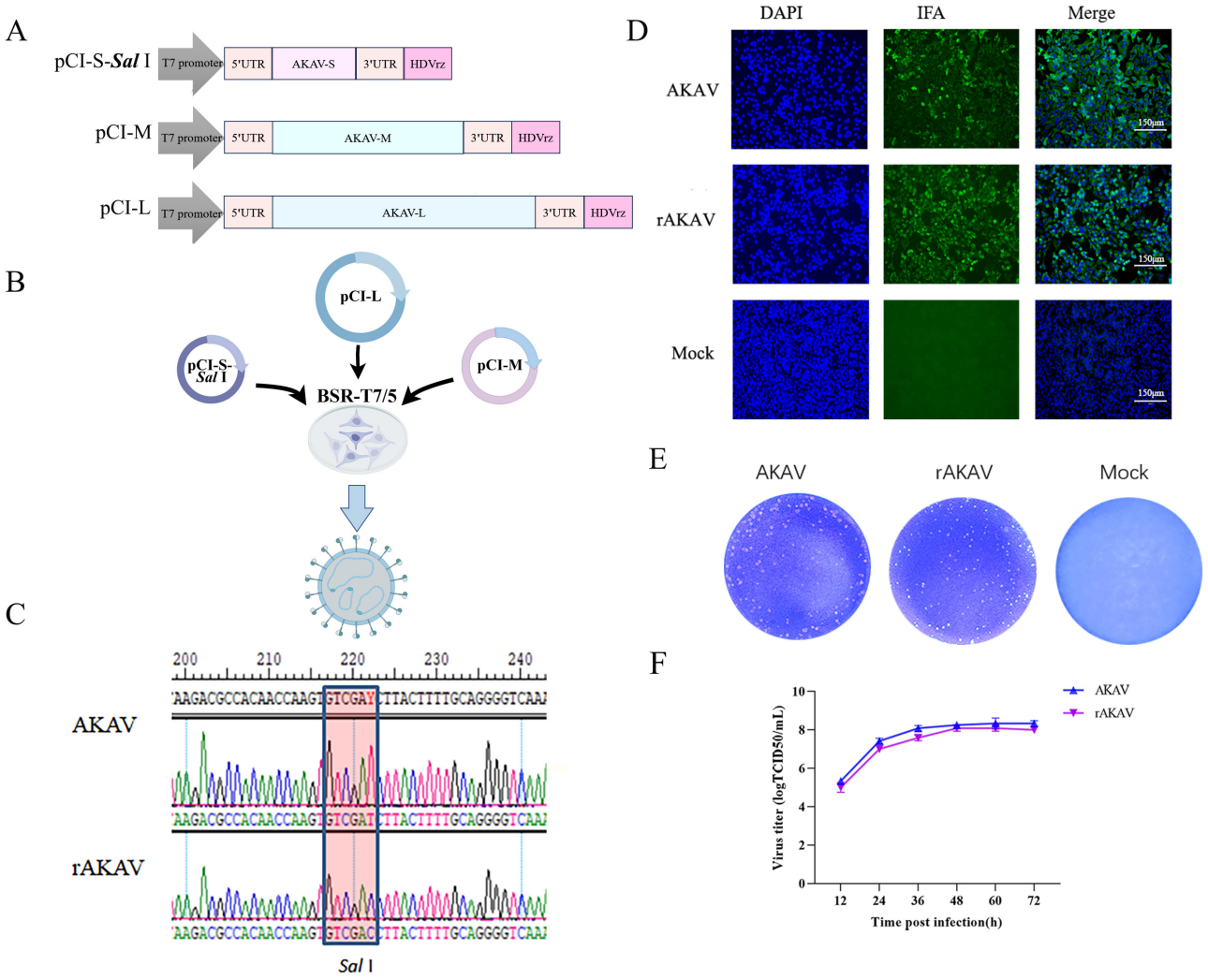

FIGURE 1
Construction, identification, and characterization of AKAV-WT and rAKAV. **(A)** The recombinant pCI plasmid of S segment, named pCI-S-*Sal* I. The recombinant plasmid of M segment, named pCI-M. The recombinant pCI plasmid of L segment named pCI-L. **(B)** Transfection strategy for rAKAV. **(C)** Sequence analysis of the amplified S products of AKAV. **(D)** Immunofluorescence assays of polyclonal antibody in the BSR-T7/5 cells at 24 hpi. **(E)** Plaque morphology of the two viruses in BSR-T7/5 cells at 72 hpi. **(F)** Growth kinetics curves of AKAV-WT, rAKAV in the BSR-T7/5 cells at MOI = 0.01. All the experiments were repeated three times.

fragments were obtained using RT-PCR from the rescued reporter viruses and sequenced accordingly.

2.5 Indirect immunofluorescence assay (IFA)

The AKAV was diluted 50-fold with DMEM medium and 100 μL of the diluted virus was inoculated onto a 96-well plate covered with a single layer of BSR-T7/5, incubated for 1 h, then the inoculum was discarded and replaced with serum-free DMEM for further culture. 36 h post infection, AKAV-infected BSR-T7/5 cells were harvested, fixed and permeabilized using a mixture of methyl alcohol and acetone (1:1) and then washed with PBS once. The cells were blocked with 10% goat serum made in 1% BSA in PBS for 1 h, 100 μL/well, and then incubated with primary antibody (Mouse-derived positive sera; 1:500 dilutions made in 1% BSA in PBS), 100 μL/well. After 1 h inoculation at 37°C. Following three washes with PBS (5 min each, 100 μL/well), cells were incubated with secondary antibody (Alexa Fluor™ 488 goat anti-mouse IgG, diluted 1:2,000 in 1% BSA/PBS) for 1 h at 37°C (100 μL/well). Afterwards, the cells were stained with 4′6-diamidino-2-phenylindole (DAPI) for 15 min. After a final round of three washes with PBS (5 min each, 100 μL/well), results were observed using an inverted fluorescence microscope.

2.6 Growth kinetics and plaque morphology

A monolayer of BSR-T7/5 cells was infected with recombinant virus at an MOI of 0.1 PFU/cell for multi-step growth curves. After

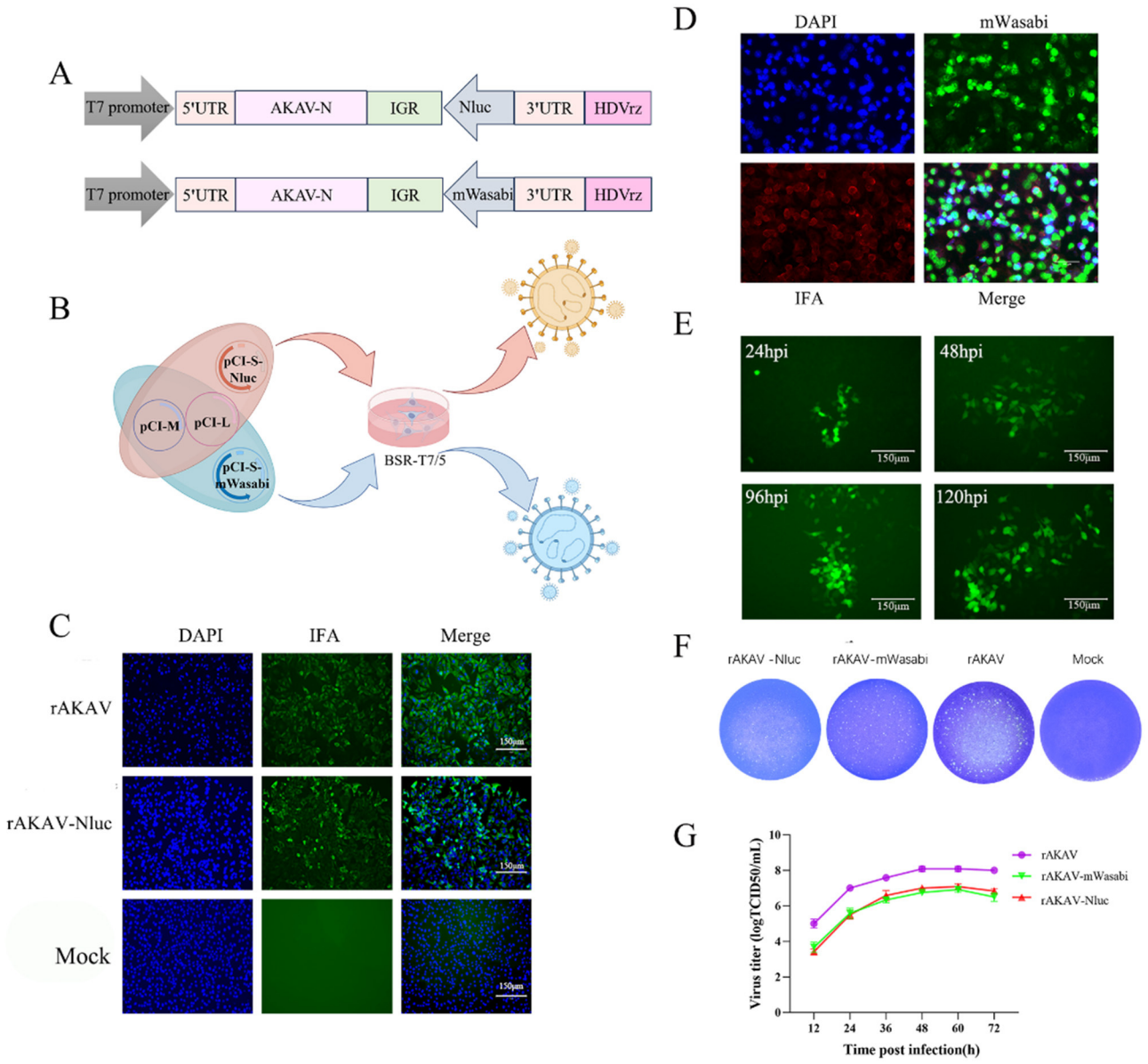

FIGURE 2
Construction, identification, and characterization of rAKAV-Nluc and rAKAV-mWasabi. **(A)** Schematic diagram of the intermediate plasmid pCI-S-Nluc and pCI-S-mWasabi. Based on pCI-S-*Sal* I, Nluc or mWasabi and IGR gene were added between 3′ UTR and N protein ORF, named pCI-S-Nluc and pCI-S-mWasabi. **(B)** Transfection strategy for rAKAV-Nluc and rAKAV-mWasabi. **(C)** Indirect immunofluorescence analysis of BSR-T7/5 cells infected with rAKAV and rAKAV-Nluc at 24 hpi using anti-AKAV N mAb. **(D)** Indirect immunofluorescence analysis of BSR-T7/5 cells infected with rAKAV-mWasabi at 24 hpi using anti-AKAV N mAb. **(E)** Changes in fluorescence at different times of rAKAV-mWasabi infection of BSR-T7/5 cells. **(F)** Plaque morphology and size of the rAKAV-Nluc and rAKAV-mWasabi cells are similar to that of the rAKAV. **(G)** Multiple-step growth curves of the rAKAV, rAKAV-Nluc and rAKAV-mWasabi on BSR-T7/5 cells. Cells were infected with rAKAV, rAKAV-Nluc and rAKAV-mWasabi at an MOI of 0.01, and cell supernatants were collected at the various time points post-infection, followed by $TCID_{50}$ assays on BSR-T7/5 cells.

adsorption for 1 h at 37°C, cells were washed twice with PBS, and the medium was replaced with DMEM containing 0.9% agar and 5% FCS. Cells were frozen at 80°C at 12, 24, 36, 48, 60, and 72 h post-infection. Virus titers were determined in a $TCID_{50}$ using BSR-T7/5 cells. Kinetics were evaluated based on data from three independently replicated experiments.

For the plaque assay, the BSR-T7/5 cells were infected with recombinant virus at a MOI of 0.001. The virus was allowed to adsorb to BSR-T7/5 cells for 1 h at 37°C, followed by two washes. Subsequently, the cells were covered with DMEM containing 0.9% agar and 5% FCS. After 3 days of incubation, the overlay was stained with 0.75% crystal violet, 10% formaldehyde and 5% ethanol.

2.7 Nanoluciferase and mWasabi stability during passaging

The rescued rAKAV-Nluc and rAKAV-mWasabi were serially passaged in BSR-T7/5 cells for 10 passages. The cell culture

supernatants from passages F1 to F10 were harvested to extract viral RNA for RT-PCR analysis, assessing the stability of the foreign sequence in the rAKAV-Nluc and rAKAV-mWasabi genomes. The amplified products were analyzed using agarose gel electrophoresis and samples from passages F1, F5, F10, and F15 were subjected to Sanger sequencing.

2.8 Nanoluciferase activity assay and fluorescent protein observation

HEK293T, BSR-T7/5, Vero E6, MDOK, MDBK, MDCK and C6/36 cells were seeded in 96-well plates and infected with rAKAV-Nluc at a MOI of 0.01. After 48 h post-infection, the infected cells were lysed using an integrated lysis buffer and subjected to luciferase activity assay using the Nano-Glo Luciferase Assay System (Promega), following the manufacturer's instructions. Similarly, fluorescence in cells infected with the rAKAV-mWasabi virus was observed using inverted fluorescence microscopy after 48 h of incubation under similar conditions.

2.9 Statistical analysis

GraphPad Prism 9.5 software and Excel were employed for statistical analysis. The nano-luciferase activity data obtained from experiments were presented in graphics or a table as the mean and standard deviation (SD). Abbreviations for technical terms were provided upon their first usage. Each sample underwent luciferase activity measurement in triplicate, with the activity value at 0 h post-transfection or infection subtracted during calculations. For viral titration, whether through luciferase or endpoint dilution assays, we conducted three separate experiments, each in triplicate, for each recombinant virus, as well as the parental virus. All calculations were performed using Microsoft Excel.

3 Results

3.1 Establishment of reverse genetics systems for AKAV/GX/2016

To successfully rescue AKAV/GX/2016 using a T7 RNA polymerase-based reverse genetic system, we constructed a full-length cDNA clone from the S, M and L segments of AKAV/GX/2016. All these three plasmids expressing viral RNAs were confirmed by Sanger sequencing, namely pCI-S-*Sal* I, pCI-M and pCI-L. We then used two different transfection reagents, PEI (plasmid: PEI = 1:2, 1:3, 1:4) and Lipofectamine™ 3000 (plasmid: Lipofectamine™ 3000 = 1:1.5) for the recovery of AKAV in BSR-T7/5 cells that is permissive to AKAV infection. To further validate the efficiency of the rescue system, we also tried different transfection ratios and total amount of different plasmids [pCI-S-*Sal* I, pCI-M, pCI-L = 1:3:2.5 (total amount of plasmid = 0.65 μg) (Lowen et al., 2004), 1:1:1 (total amount of plasmid = 3 μg, 6 μg, 9 μg) (Chen et al., 2021), 2:1:2 (total plasmid amount = 3 μg) (Takenaka-Uema et al., 2016)]. Despite varying plasmid ratios and total amounts, we failed to rescue AKAV/GX/2016 by using PEI transfection reagent. Compared to other transfection reagents, Lipofectamine™ 3000 proved most efficient in transfecting larger plasmids into cells (Alencar et al., 2021). Surprisingly, we could successfully rescue AKAV/GX/2016 across all plasmid ratios and total amounts tested followed by Lipofectamine™ 3000 transfection. This suggests that while the plasmid proportions are not severely restrictive, transfection efficiency can be influenced by transfection reagents.

3.2 Characterization of rAKAV

By following the described protocol above, a significant CPE was observed in the transfected cells on day 3 post-transfection. The rAKAV was subjected to an immunofluorescence assay (IFA) to assess infectivity in BSR-T7/5 cells using anti-AKAV N mAbs. As expected, AKAV-specific green fluorescence was observed in cells infected with AKAV or rescued viruses (Figure 1D). Meanwhile the RT-PCR and DNA sequencing analyses of rAKAV both confirm the persistent presence of the *Sal* I molecular marker (Figure 1C). The plaques formed by rAKAV in BSR-T7/5 cells exhibited a similar size to the wild type (Figure 1E). Virus titer detection at 12, 24, 36, 48, 60, and 72 h, at the MOI = 0.01, revealed that the growth curves of rAKAV and AKAV followed similar trends, with nearly identical titers (Figure 1F). The viral titer showed the most rapid increase occurred between 12 and 24 h. It continued to rise steadily from 24 to 60 h, peaking at 60 h, and subsequently declined from 60 to 72 h. All these results demonstrated that the reverse genetical system of AKAV/GX/2016 was successfully established.

3.3 Characterization of rAKAV-Nluc and rAKAV-mWasabi

Recombinant AKAV engineered to express eGFP genes have been developed for monitoring viral distribution in mice (Takenaka-Uema et al., 2015). However, the eGFP signal produced from viral replication is too weak for detection *in vivo* imaging study (Takenaka-Uema et al., 2019). Therefore, in this study, we introduced mWasabi reporter that was 1.6-fold brighter than eGFP into AKAV genome (Kaishima et al., 2016). In addition, nano-luciferase gene was introduced to allow more efficient and convenient real-time visualization and quantification of AKAV replication. The pCI-S-*Sal* I plasmid in the three-plasmid system was replaced with a plasmid containing a reporter gene, leading to the successful rescue of reporter viruses rAKAV-Nluc and rAKAV-mWasabi (Figures 2A, B). Immunofluorescence assay (IFA) results revealed the rAKAV-Nluc and rAKAV-mWasabi could produce AKAV N proteins (Figures 2C, D), meanwhile rAKAV-mWasabi-positive cells could be directly identified under fluorescence microscope (Figure 2E). Plaques formed by rAKAV-Nluc and rAKAV-mWasabi in BSR-T7/5 cells closely resembled those of the wild-type virus, exhibiting uniform shapes similar to rAKAV (Figure 2F). To assess the replication kinetics of the rescued reporter viruses, multiple-step growth curves for rAKAV-Nluc and rAKAV-mWasabi were determined after infection of BSR-T7/5 cells at a low multiplicity of infection (MOI) of 0.01 PFU/cell.

Growth curves of the rAKAV-Nluc and rAKAV-mWasabi had no significant difference, while their titers were lower than that of the parental rAKAV (Figure 2G). This indicated that a possible effect of the reporter gene on the replication characteristics of this strain. Overall, we generated rAKAV-Nluc and rAKAV-mWasabi which can efficiently express the reporter gene.

3.4 Stability of reporter genes from recombinant reporter viruses during delivery in BSR-T7/5 cells

To evaluate the stability of rAKAV-Nluc in BSR-T7/5 cells, the recombinant virus underwent 10 serial passages. From F1 to F15, the luciferase signal intensity of rAKAV-Nluc virus was maintained above 10^5 (Figure 3A). The culture supernatant of passages 1–15 virus was harvested for detecting the rAKAV-Nluc fusion gene by RT-PCR to determine the stability of the Nluc gene at the insertion site. The RT-PCR products with an expected 513 bp were detected from the supernatant of the infected BSR-T7/5 cells (Figure 3B). After nucleotide sequencing of the exogenous luciferase gene inserted region, the sequence alignment showed no mutant sites in the region of interest. The results confirmed the existence of intact Nluc in rAKAV-Nluc. Similarly, the stability of the reporter virus rAKAV-mWasabi was assessed through ten consecutive passages in BSR-T7/5 cells. Strong signals were observed from F1, F3, F5, F7, F9, F11, F13, and F15, indicating stable expression of the mWasabi gene during passaging (Figure 3D). Furthermore, Green fluorescence and cytopathic effects co-existed in BSR-T7/5 cells infected with rAKAV-mWasabi. RNA extracted from the infected cells at each passage stage and subjected to RT-PCR revealed a 711 bp band was consistently detected in rAKAV-mWasabi (Figure 3C). After nucleotide sequencing of the exogenous fluorescent protein gene inserted region, the sequence alignment showed no mutant sites in the region of interest. The data suggest that the reporter viruses remain stable and transmissible in BSR-T7/5 cells, with the fluorescence signal of rAKAV-Nluc virus was mildly maintained at a high level from F1 to F15. And the rAKAV-mWasabi maintained strong green fluorescence for 15 generations.

3.5 Application of Nluc/mWasabi reporter virus in identifying permissive cell lines

To explore the cellular tropism of AKAV, various cell lines derived from different animal species and humans were infected with rAKAV-Nluc and rAKAV-mWasabi at MOI of 0.01 (Figure 4A). Notably, MDOK, Vero E6 and BSR-T7/5 cells showed high susceptibility to the rAKAV-Nluc and rAKAV-mWasabi (Figures 4B, C), suggesting these cells were suitable for AKAV propagation, but lower infectivity in MDBK (bovine) and C6/36 (mosquito) cells. MDCK cells, which are not susceptible to AKAV, were used as negative controls, and we verified that the reported virus did not infect MDCK cells. Surprisingly, AKAV could efficiently replicate in HEK293T cells. Furthermore, MDBK cells displayed lower susceptibility to AKAV compared to HEK293T and Vero E6 cells, consistent with the infection pattern of the closely related SBV virus in MDBK cells (Elliott et al., 2013). In summary, the AKAV reporter viruses could be used for assessment of the replication pattern of AKAV in different cell lines.

4 Discussion

The Bunyavirales order is the largest group of RNA viruses. Among the 12 families within this order, Arenaviridae, Hantaviridae, Nairoviridae, Peribunyaviridae and Phenuiviridae are known to contain important pathogens that cause severe diseases in both humans and animals (Endalew et al., 2019; Oya et al., 1961). Understanding the life cycle of highly pathogenic viruses is often a prerequisite for developing effective vaccines and antiviral treatments. Therefore, there is an urgent need to develop robust tools to elucidate the molecular basis of pathogenesis. Reverse genetics technology has been extensively developed for bunyaviruses and is widely employed by researchers to dissect various aspects of the viral life cycle. As of now, reverse genetics systems for many members of the families Arenaviridae, Hantaviridae, Nairoviridae, Peribunyaviridae, and Phenuiviridae have been successfully established and applied to study a range of bunyaviruses (Caì et al., 2018; Ren et al., 2021, 2020; Tercero and Makino, 2020).

AKAV has shown its ability to cause stillbirths, abortions, and premature births in pregnant ruminants. The rising incidence of AKAV infection in ruminants is expected to lead to unpredictable and significant outbreaks (Oem et al., 2012). AKAV has been detected across various ruminant species worldwide. Moreover, epidemiological investigations conducted between 2005 and 2016 have found the virus was spreading in China (Wang et al., 2017). While some progress has been made in AKAV field, significant gaps remain, particularly in areas such as available vaccines and the host's antiviral innate immunity. To address this, we developed two reporter virus platforms: rAKAV-Nluc and rAKAV-mWasabi. These two-reporter gene-containing viruses serve as valuable tools for detecting and quantifying viral replication.

The utilization of AKAV reverse genetic systems remains somewhat restricted in constructing recombinant viruses for expressing foreign genes. Initially, fluorescence imaging was employed to localize the eGFP-based recombinant AKAV reporter virus in mice. To further optimize the detection method for eGFP, we explored the application of the mWasabi fluorescent protein in this study. Compared to eGFP, mWasabi fluorescent protein demonstrates superior fluorescence properties, boasting a relative brightness 167% greater than that of eGFP (Kaishima et al., 2016). Moreover, mWasabi exhibits higher sensitivity to acidic environments compared to eGFP, owing to its pK value (6.5:5.9). This implies that the green fluorescence of mWasabi is more susceptible to quenching in acidic lysosomes than that of eGFP (Chudakov et al., 2010). As a result, the fluorescence signal emitted by mWasabi proves to be more sensitive and accurate in assessing the localization of AKAV. Additionally, its low sequence similarity to eGFP may mitigate the potential impact of eGFP-mediated disulfide bond formation on virus rescue.

Additionally, we engineered a recombinant reporter virus (rAKAV-Nluc). Nano luciferase, being a novel luciferase, presents numerous advantages over Fluc and Rluc. Notably, it boasts

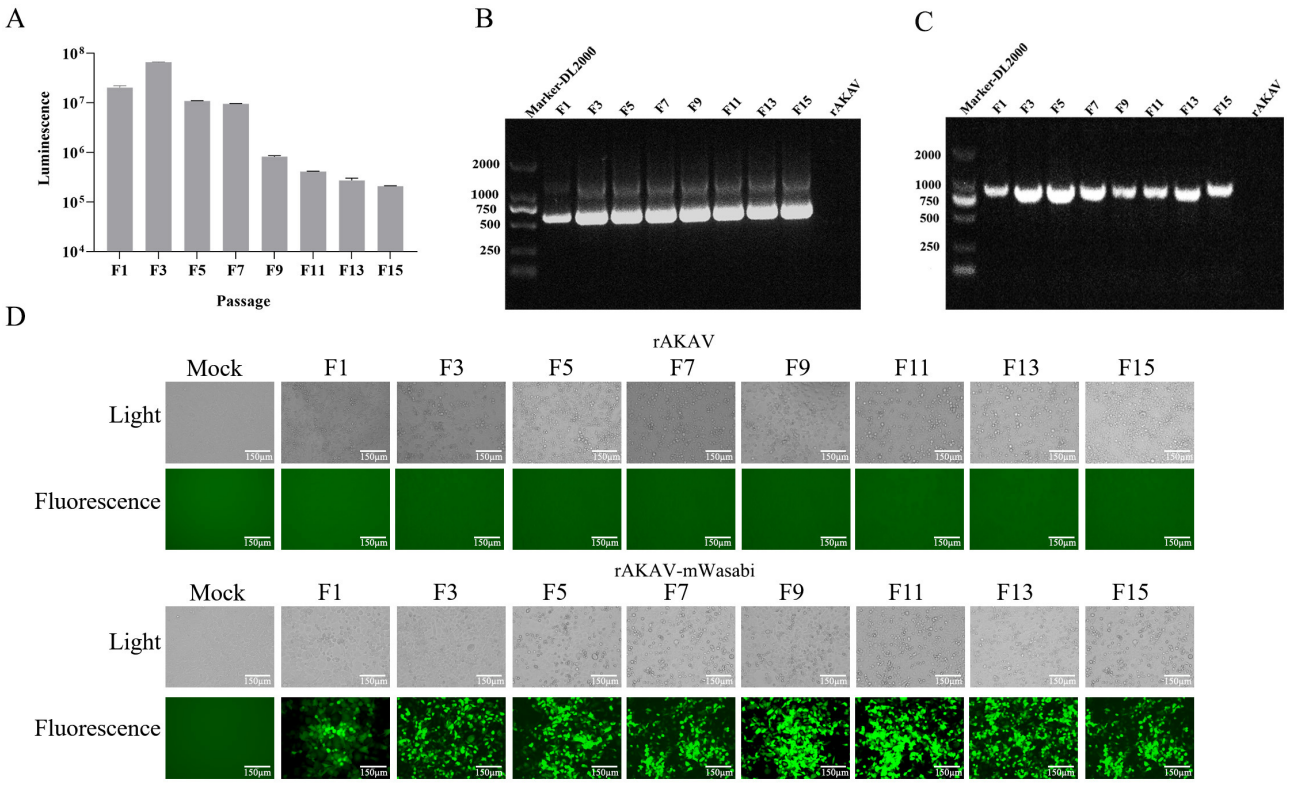

FIGURE 3
The reporter genes stability in rAKAV-mWasabi and rAKAV-Nluc during passaging in BSR-T7/5 cells. **(A)** The Nluc expression in BSR-T7/5 cells after infection with rAKAV-Nluc of F1-F10 generation. The expression of mWasabi was detected under a fluorescent signal at 48 hpi. **(B)** The RT-PCR detection of the Nluc gene in BSR-T7/5 cells after infection with rAKAV-Nluc of F1–F10. The resulting RT-PCR products were resolved by 1% agarose gel electrophoresis. **(C)** The RT-PCR detection of the mWasabi gene in BSR-T7/5 cells after infection with rAKAV-mWasabi of F1–F10. **(D)** The mWasabi expression in BSR-T7/5 cells after infection with rAKAV-mWasabi of F1, F3, F5, F7, F9, F11, F13, and F15 generation. The expression of mWasabi was detected under a fluorescent microscope at 48 hpi.

enhanced stability, a reduced size, and a luminescence intensity surpassing 150 times that of the original luciferase (England et al., 2016). Secondly, rAKAV-Nluc offers time-saving benefits and increased sensitivity, making it suitable for neutralizing antibody assays (Yao et al., 2021). Furthermore, owing to the amplifying nature of the Nluc enzyme, rAKAV-Nluc exhibits a broader dynamic range and enhanced sensitivity compared to the rAKAV-eGFP viral assay. Additionally, reporter viruses have been extensively employed in screening interferon-stimulated genes (ISGs) and siRNAs to identify host factors that could impact viral replication (Karlas et al., 2010). Nluc has been employed in the study of viruses belonging to the Bunyaviridae family (Arenaviridae e.g.), including the thrombocytopenia syndrome virus (SFTSV) (Xu et al., 2024), among others (Wen et al., 2022). Leveraging *in vivo* imaging facilitates the visualization of virus distribution in diverse organs throughout the body, along with virus replication. Viruses harboring reporter genes integrated into their genomes serve as invaluable tools for detecting and quantifying viral replication, as well as for vaccine development.

One crucial application of the reverse genetic system is using the viral backbone for the expression of foreign genes. In a previous study with the Iriki strain, a recombinant virus containing the eGFP reporter gene was constructed, and it was inserted between the N and 3′UTR (Takenaka-Uema et al., 2016). Nonetheless, the reporter virus was exclusively employed to scrutinize AKAV tropism in mice. Previous studies on other bunyaviruses have suggested that the expression of foreign proteins, with varying insertion sizes, could potentially affect virus replication. In this study, we inserted reporter protein gene sequences between the N and 3′UTR regions in AKAV-S. Comparing the replication of rAKAV-mWasabi and rAKAV-Nluc with wild-type parental rAKAV, our findings illustrate that rAKAV-mWasabi and rAKAV-Nluc displayed comparable growth kinetics to the parental virus. In this study, we conducted cytophilic assays employing rAKAV-mWasabi and rAKAV-Nluc to evaluate AKAV capacity to infect various cells. AKAV enters non-bovine-derived cell lines (Vero and BHK cells) in the manner indicated Grid protein endocytosis. In contrast, AKAV infection in bovine-derived cell lines (MDBK cells) depends on dynamin (Bangphoomi et al., 2014). It may be that it is the two different mechanisms that contribute to the difference between AKAV-infected bovine and MDBK. The findings from these studies indicate that the reporter viruses exhibited diverse tropism properties across various cell lines of diverse origins. This offers valuable insights into the viral interaction with diverse host cells and may contribute to elucidating its pathogenesis. The discovery that AKAV infected

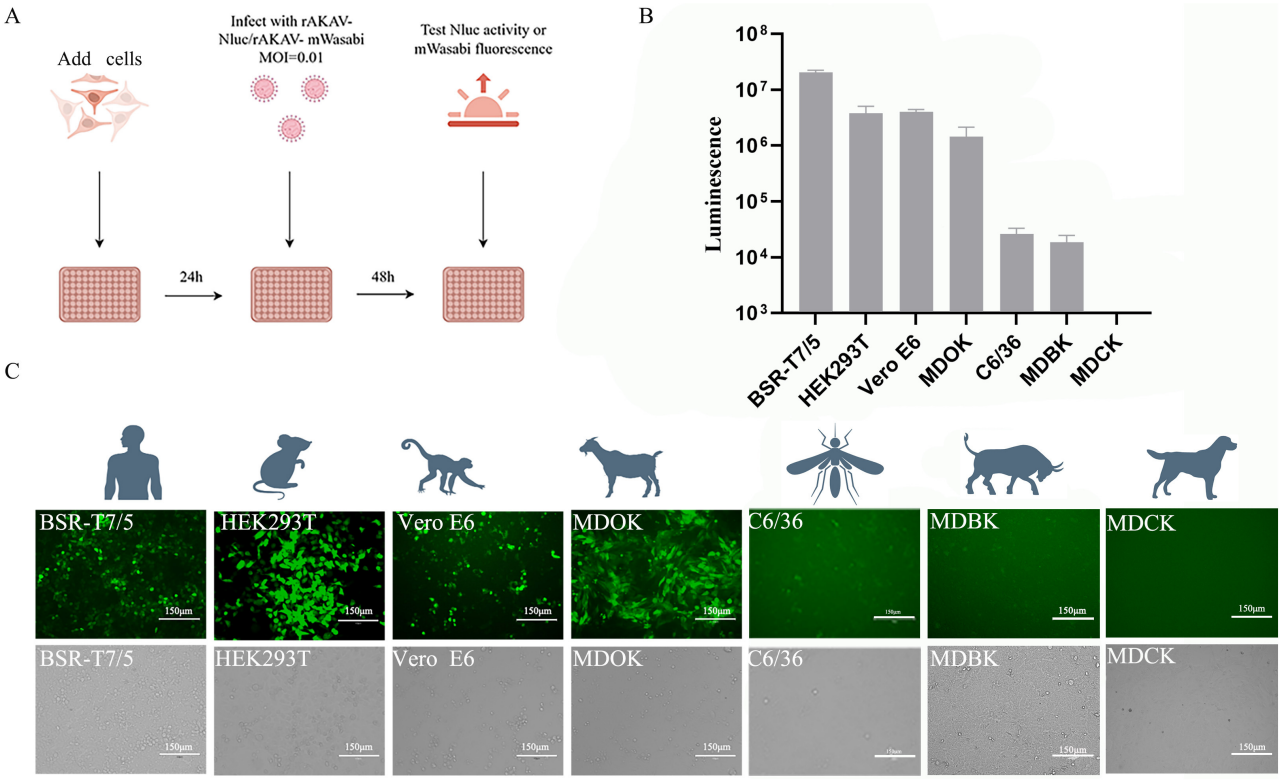

FIGURE 4
The infection of rAKAV-Nluc and rAKAV-mWasabi in cell lines derived from human, mouse, monkey, sheep, bovine and mosquito. **(A)** Strategies for reporting virus infection of different cell lines. **(B)** The Nluc signal in different cell lines infected rAKAV-Nluc at an MOI = 0.01 at 48 hpi. **(C)** The mWasabi expression in different cell lines infected rAKAV-mWasabi at an MOI = 0.01 at 48 hpi. All the experiments were repeated three times.

HEK293T cells underscores the potential zoonotic risk linked to AKAV and underscores the importance of implementing suitable safety measures. Additionally, the establishment of AKAV reverse genetics systems has facilitated the generation of recombinant infectious viruses, which serve as powerful tools for understanding AKAV biology and vaccine development. While Akabane disease is widely prevalent in China, no vaccines are currently available. Therefore, AKAV reverse genetics systems provide a platform for the development of live-attenuated vaccine candidates.

5 Conclusion

Our study has successfully devised a reverse genetics system for AKAV and rescued two reporter viruses, serving as valuable tools for further AKAV research. These reporter viruses have demonstrated stability in cell culture and exhibited distinct tropism properties. While rAKAV-mWasabi facilitates qualitative virus analysis, rAKAV-Nluc is optimized for quantitative analysis. Together, these platforms complement each other. The reverse genetics platform we've established lays the groundwork for investigating the structure and function of AKAV proteins and can bolster the development of a live attenuated vaccine against AKAV. Leveraging reporter viruses enables the visualization and tracking of virus movement in infected animals, thereby enriching our comprehension of AKAV structure, function, replication, and pathogenesis. Consequently, this holds the potential to propel vaccine development and preventive strategies against AKAV infection forward.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The cell lines present in this study were obtained from American Type Culture Collection [ATCC].

Author contributions

JL: Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. FW: Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. JZ: Data curation, Software, Writing – review & editing. YQ: Formal analysis, Resources, Writing – review & editing. JC: Methodology, Resources, Writing –

review & editing. CS: Formal analysis, Funding acquisition, Writing – review & editing. ZJ: Investigation, Resources, Writing – review & editing. JG: Conceptualization, Supervision, Writing – review & editing. XY: Conceptualization, Data curation, Funding acquisition, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Seroprevalence and risk factors of Chikungunya in Ethiopia: a systematic review and meta-analysis

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Introduction: The resurgence of the Chikungunya virus has led to public health concerns due to frequent epidemics worldwide. Chikungunya was first detected in Ethiopia in 2016, and it has been identified in various regions. However, the current status of the disease in Ethiopia remains unknown, underscoring the need for updated information.

Objective: To provide up-to-date epidemiological data on the status of Chikungunya in Ethiopia.

Methods: A systematic review and meta-analysis were conducted using the PubMed, Scopus, and Google Scholar databases in accordance with the PRISMA guidelines, the literature search was conducted from September to October 2024. The search terms used included 'Chikungunya,' 'Chikungunya Virus,' 'Prevalence,' 'Seroprevalence,' 'Risk Factor,' and 'Ethiopia.' The inclusion criteria covered online articles published between 2016 and 2024 in English and published in Ethiopia. The quality assessment involved independent expert evaluations, and publication bias was assessed using Begg's and Egger's tests. The analysis was performed using STATA 17 software.

Results: A total of five articles met the eligibility criteria and were included in the data extraction. The pooled seroprevalence of Chikungunya in Ethiopia was 24.0%. The highest seroprevalence was reported in the Southern Nations, Nationalities, and Peoples' Region (SNNPR), at 43.6%, while the lowest seroprevalence was in Dire Dawa, at approximately 12.0%. Factors such as occupation, education, age, and sex contributed to the variation in seroprevalence of the disease. Subgroup meta-analysis revealed heterogeneity across the types of studies included. No indications of publication bias or small-study effects were found according to Begg's test or Egger's test.

Conclusion and relevance: The pooled prevalence of Chikungunya underscores its significance in Ethiopia, necessitating proactive monitoring, active viral disease surveillance, and robust health system enforcement.

KEYWORDS

Chikungunya, Ethiopia, meta-analysis and systematic review, risk factors, seroprevalence

Highlights

- Chikungunya is a neglected tropical disease.
- The Chikungunya virus was first detected in Ethiopia in 2016.
- The pooled seroprevalence of Chikungunya in Ethiopia is 24.0%.
- The seroprevalence of Chikungunya varies with different factors.
- Seroprevalence is higher in males and farmers.
- ELISA is commonly used for detecting the virus in Ethiopia.

1 Introduction

Chikungunya, a mosquito-borne viral disease, is caused by an RNA virus belonging to the Alphavirus genus of the family Togaviridae known as Chikungunya virus (CHIKV). It is responsible for millions of documented cases worldwide (1). The disease is characterized by clinical signs such as fever, debilitating severe joint pain, joint swelling, muscle pain, headache, nausea, fatigue and rash (2).

Chikungunya virus was first isolated during a 1952–53 outbreak in southern Tanzania (3), although clinical descriptions suggest its presence as far back as the 1600S (4). Today, CHIKV has become widespread globally, has been identified in more than 110 countries and represents a significant global public health concern (5). Factors such as climate change, vector adaptations, urbanization, and human migration have contributed to the spread of the virus to new areas (6). In addition, studies have shown that the incidence of Chikungunya varies with factors such as occupation, age, sex, and education. Farmers and older people are associated with higher prevalence rates. Gender disparities also play a role in influencing exposure and transmission patterns, as the occurrence of the disease is higher in female (7–12).

Chikungunya in Ethiopia is becoming a significant public health concern, as it has caused considerable morbidity since it was first detected (13). The virus was first documented in Ethiopia in June 2016, with the confirmation of its first case in the Suuf kebele, Dollo Ado district of the Somalia regional state (14), which is assumed to have originated from Kenya. The Somalia regional state shares a border with the Mandera region of Kenya, where a Chikungunya outbreak was ongoing (15). Since then, Chikungunya has spread rapidly and has been reported in different districts of Ethiopia (16, 17).

At present, there are no approved vaccines or antiviral therapies available for Chikungunya (18). Meanwhile, nucleic acid therapeutics are emerging as transformative agents in antiviral treatment, leveraging precise genetic interventions to combat viral infections (19). However, the main approach for treating and controlling and preventing this disease is through alleviating symptoms or supportive treatment and eliminating the mosquitoes that transmit the virus (18, 20). In addition, public awareness creation through community education and training about the outbreak of emerging and reemerging vector-borne diseases, methods of transmission, and control and prevention methods remain important mechanisms for managing Chikungunya (18).

Like many developing nations, Ethiopia cases involve struggles with a range of public health challenges that contribute to the outbreak of disease. Limited healthcare infrastructure and uneven distribution of resources hinder effective prevention, detection, and response to health crises (21). Despite these limitations, Ethiopia has made an effort to limit the spread of CHIKV across the country and to prevent the potential transmission of the disease in affected regions (13). The government was taking vector control measures such as indoor residual spraying, distributing insecticide-treated bed nets and encouraging the population to eliminate a breeding ground for mosquitoes such as stagnant water (22). However, the effectiveness of these measures may vary, and there are reports of the disease from various locations in Ethiopia. The disease's current status in Ethiopia is unclear. Knowing the current status of the disease in the country is important. The outcome provides insights for health professionals and concerned authorities to develop effective control and prevention strategies. Therefore, this systematic review and meta-analysis aimed to provide up-to-date epidemiological data on the status of Chikungunya in Ethiopia.

Abbreviations: CHIKV, Chikungunya virus; ELISA, Enzyme-linked immunosorbent assay; IgG, Immunoglobulin G; IgM, Immunoglobulin M; RT–qPCR, quantitative reverse transcription polymerase chain reaction; WHO, World Health Organization.

2 Methods

2.1 Systematic review protocols

The guidelines and procedures of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (23) were followed in this systematic review and meta-analysis (Figure 1) and registered in the database of the Prospective Register of Systematic Reviews (PROSPERO) under the reference number CRD42023271579.

2.2 Literature search strategy

The data were extracted from published public articles available from different electronic databases, including Medline/PubMed, Science Direct/Scopus, Google Scholar and Web of Science. The literature search was conducted from September to October 2024, applying language restrictions to English published between 2016 and 2024. Mendeley version 1.19.8 (Mendeley Ltd) was used for search, collection and removal of duplicates of articles. A set of keywords, such as "Chikungunya," "Chikungunya Virus," "Chikungunya Fever," "Vector-borne," "Arbovirus," "Incidence," "Prevalence," "Seroprevalence," "Seroepidemiology," "Risk Factors," "Potential Factors" and "Ethiopia," were used in the search. The search queries were configured using Medical Subject Headings (MeSH), and the "OR and "AND" Boolean operators were used to identify studies with any of the keywords in their titles, abstracts and full texts that might be included in this systematic and meta-analysis review. Moreover, unpublished thesis manuscripts were also accessed from various Ethiopian universities and research centers.

2.3 Inclusion and exclusion criteria

Regardless of the research population type, articles reporting CHIKV infection or disease conducted in Ethiopia and involving the general population or a specific age group were included in this study. The study's objectives served as the basis for defining the inclusion and exclusion criteria. The inclusion criteria include articles that focused on CHIKV infection or disease, written in English, published between

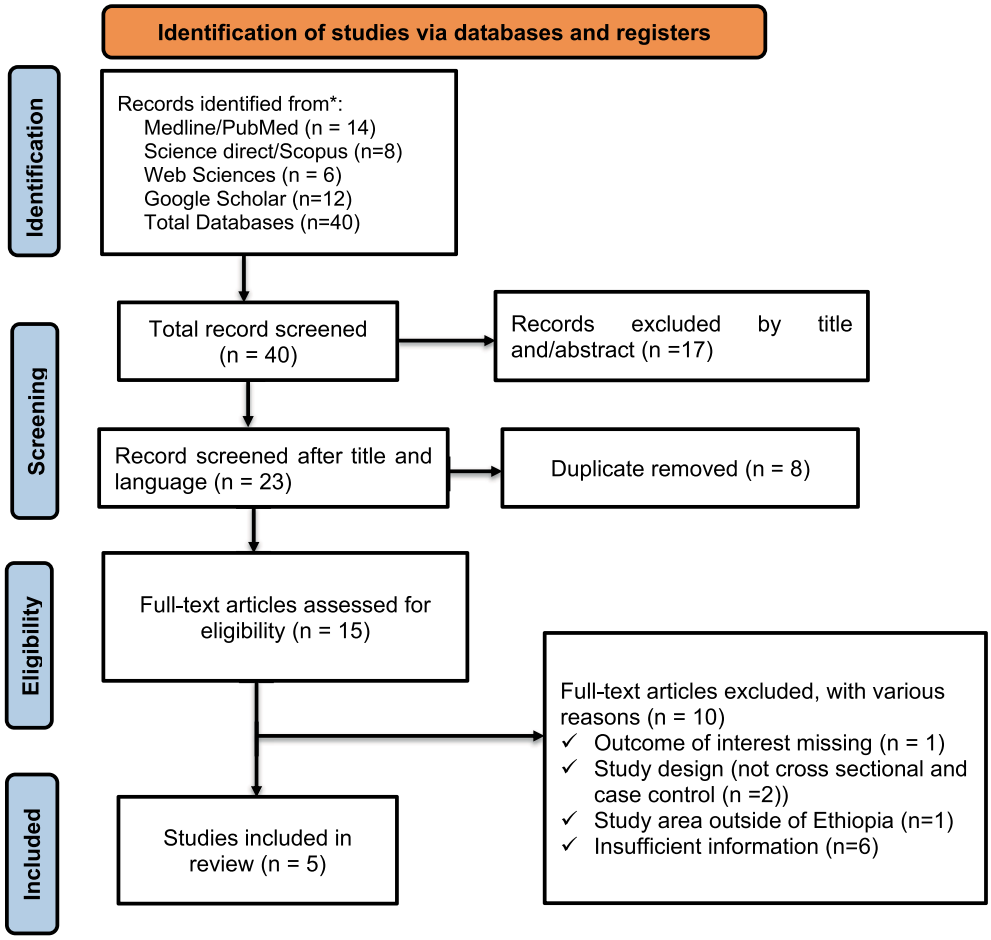

FIGURE 1
PRISMA flowchart for the selection of studies on the CHIKV in Ethiopia.

2016 and 2024, using case–control, cross-sectional and cohort studies in which the seroprevalence of Chikungunya was confirmed via laboratory tests, encompassing serologic or virological antibody detection methods such as ELISA IgG, ELISA IgG + IgM and/or molecular diagnosis, and full-text articles included in the systematic review and meta-analysis. Papers are rejected based on the exclusion criteria. These criteria include articles that were unpublished, systematic reviews and meta-analysis, did not align with the specified emphasis outlined in the systematic review, exclude important things about seroprevalence, not studied in Ethiopia and not written in English. The analysis did not take into account the specificity or sensitivity of the tests.

2.4 Data extraction

Two experts (ATG and SLA) independently identified articles from the search engines using key terminologies and subsequently screened them based on their titles and abstracts. The selected publications were then imported into Mendeley, after which the full texts were retrieved. The eligibility of the full texts was assessed by checking whether they addressed the main outcomes of interest. The proportion of seropositive Chikungunya individuals was considered the main outcome of the study. Data extraction took place independently by two experts (GGD) from November 1, 2024, to November 21, 2024, and cross-verification was performed by another two experts (ATG & HD). If discrepancies arose, the data were re-extracted, even if there were no initial discrepancies.

2.5 Quality and risk-of-bias assessment

A comprehensive search for all possibly relevant articles and the application of precise, repeatable criteria for article selection were two of the tactics we used to reduce bias and random error. An established systematic methodology that complies with evidence-based methodological standards was followed in evaluating research designs and study characteristics, the synthesis of data, and the interpretation of the findings. To choose which papers to include and omit from the review, HD and GGD examined the titles, abstracts, and full-text publications. After that, the articles are evaluated to see if they meet the specified eligibility requirements. BK assessed quality using the appraisal tool for cross-sectional studies (AXIS tool) (Supplementary File 1). There are 20 items on this checklist (24, 25). In addition, the presence of publication bias or small-study effects was assessed by using Begg's test and regression-based Egger test, which examine the correlation between the effect size and the standard error of the effect size across studies (26).

2.6 Data synthesis and meta-analysis

The extracted data were entered into an Excel spreadsheet (2019). The seroprevalence of Chikungunya from each study was recorded, and the individual study weight, standard error, and 95% confidence interval (CI) were calculated based on the inverse variance method and the binomial equation (27). The logit transformation of the proportional prevalence with its variance and standard error was calculated. Subgroup analyses for the primary outcome (seroprevalence of Chikungunya) were performed using the DerSimonian and Laird model by considering geographical locations and laboratory techniques employed (PCR or ELISA) (28). The heterogeneity among and within the studies was estimated from the inverse variance of the random effects model (29). The parameters tau-squared (τ^2), I-squared (I^2) and H-squared (H^2) were calculated to measure the variance in the true effect sizes between-study variance, interstudy heterogeneity, and total variability, respectively (30). The pooled prevalence and standard error with 95% confidence intervals (CIs) were calculated (31). STATA version 17 software was used to perform the statistical tasks. A p-value less than 0.05 ($p < 0.05$) was considered significant in all analyses (32).

3 Results

3.1 Literature search results

This systematic review and meta-analysis focused on published studies regarding Chikungunya in Ethiopia. A total of 40 articles published between 2016 and 2024 were identified using search engines. Of these, 17 articles were rejected based on their titles and abstracts, which indicated irrelevance to our review. The remaining 23 studies underwent further evaluation, resulting in the exclusion of eight duplicates or inappropriate articles. A total of 15 full-text papers were accessed and assessed for eligibility using pre-set criteria, leading to the inclusion of five studies in the systematic review and meta-analysis. The remaining 10 articles were excluded based on study area and other factors. Ultimately, five relevant studies were included in this systematic review and meta-analysis (Figure 1).

3.2 Study characteristics

The selected articles encompassed research conducted in five regional states—South Nations and Nationalities Region, Amhara, Tigray, Gambella, and Southeast Ethiopia—as well as one city administration (Dire Dawa) (Figure 2). The included studies were cross-sectional (80%, 4/5) and one case–control study (20%, 1/5). There were two types of assays used for diagnosis across the studies, namely, the enzyme-linked immunosorbent assay (ELISA; 80%, 4/5) and quantitative reverse transcription polymerase chain reaction (RT–qPCR; 20%, 1/5) (Table 1).

3.3 Seroprevalence of Chikungunya

The pooled seroprevalence of Chikungunya in Ethiopia was 12.4% (95% CI: 12.24, 12.46%). The highest prevalence was reported in the Southern Nations, Nationalities, and Peoples' Region (SNNPR) at 43.6%, while the lowest seroprevalence was found in Dire Dawa, at approximately 12%. At the district level, the highest prevalence of Chikungunya infection occurred in the Bebub Ari district (51.58%; SE (logit) = 0.15, 95% CI = −0.22, 0.35), while the lowest prevalence was recorded in the Lare district (6.25%; SE (logit) = 0.73, 95% CI = −4.14, −1.28) (Table 1).

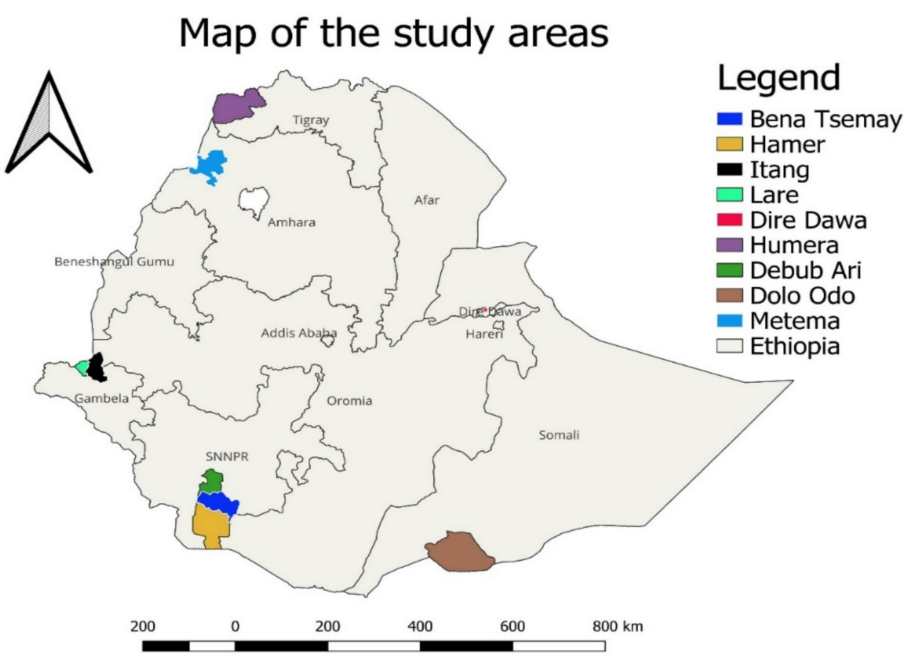

FIGURE 2
Map of Ethiopia showing Chikungunya report (Drawn by QGIS 3.34.1).

TABLE 1 Seroprevalence of Chikungunya virus in Ethiopia from selected publications between 2016 and 2020.

References	Study area		Study design	Sample size	Test type	Sero-prevalence (%)	Logit (95% CI)
	Region	District					
Endale et al. (33)	SNNPR	Debub Ari	Cross-sectional	190	ELISA	51.58	0.15 (−0.22, 0.35)
	SNNPR	BenaTsemay		35	ELISA	25.71	0.39 (−1.82, −0.30)
	SNNPR	Hamer		135	ELISA	37.04	0.18 (−0.88, −0.18)
Ferede et al. (34)	Amhara	Metema	Cross-sectional	274	ELISA	30.66	0.13 (−1.07, −0.56)
	Tigray	Humera		312	ELISA	16.35	0.15 (−1.93, −1.33)
Asebe et al. (35)	Gambella	Itang	Cross-sectional	58	ELISA	20.69	0.32 (−1.98, −0.71)
	Gambella	Lare		32	ELISA	6.25	0.73 (−4.14, −1.28)
Takele (36)	South–Eastern Ethiopia	Dolo ado	Case–control	99	RT–qPCR	14.14	0.29 (−2.37, −1.24)
Geleta et al. (17)	Dire Dawa	Dire Dawa	Cross-sectional	334686	RT–qPCR	12.30	0.01 (−1.97, −1.95)

CI, Confidence interval.

3.4 Meta-analysis

The overall pooled effect size of Chikungunya was 24.0% (95% CI: 15.0, 32.0%) across all eligible studies. There was significant heterogeneity in the reports of Chikungunya seroprevalence between studies ($tau^2 = 0.02$, H^2, 27.8, $I^2 = 96.35\%$, Q-test = 216.6, df = 8, $p \leq 0.001$) (Figure 3).

3.5 Quality assessment result

In this review, a range of studies, with quality ratings from low to moderate, was evaluated. In the current meta-analysis, eight articles used the random sampling method. Additionally, all eight studies (88.9%) obtained a sample frame from a population closely resembling the target or reference population. Of these, 9 studies (100%) met six of the 20 key criteria, including aims/objectives, definition of the target/reference population, internal consistency of results, justification of findings, sample size justification, and appropriate methodological techniques. Conflicts of interest and descriptions of statistical methods were also addressed.

3.6 Subgroup meta-analysis

The results of a subgroup analysis based on type of test conducted are shown in Figure 4. Accordingly, the highest pooled effect size of Chikungunya was found in ELISA technique [27.0%% (95% CI: −16.0, 39.0%)]. In addition, a high degree of heterogeneity between the various studies was shown by the subgroup analysis's heterogeneity results ($I^2 = 96.31\%$, $p \leq 0.001$) (Figure 4).

3.7 Risk factors associated with Chikungunya infection

Risk factors, including occupation, education, sex, and age, were identified in four of the included articles. The Chikungunya incidence exhibited variation based on these factors.

The prevalence of Chikungunya significantly varied with occupation, irrespective of geographical location. According to Endale et al. (33), farmers had a 49.7% higher seroprevalence of Chikungunya than did pastoralists (34.9%). Similar findings were reported in northwest Ethiopia by Ferede et al. (34) in 2021. Additionally, Asebe et al. (35) reported that pastoralists had the lowest Chikungunya infection rate, 4.1% (Figure 5).

The prevalence of chikungunya was greater among individuals who had received formal education than among those who had not attended formal education or were illiterate (Figure 6).

The seroprevalence of Chikungunya was greater in the adult age group, while the lowest prevalence was recorded in children. Endale et al. (33) reported that the highest Chikungunya incidence in individuals aged 36–55 years was 53.5%, and the lowest was approximately 17.7% in individuals aged 5–10 years. Similar results were reported by Ferede et al. (34). However, Geleta et al. (17) reported a higher Chikungunya infection rate in individuals aged 5–14 years (17.1%), with the lowest prevalence in those aged ≤5 years (3.6%) (Figure 7).

Notably, the seroprevalence of Chikungunya across different sex groups was not consistent. Asebe et al. (35) reported a 22.6% higher rate of Chikungunya infection in males than in females (5.4%). Similarly, Ferede et al. (34) reported a higher prevalence of NAFLD in males (26.8%) than in females (14.1%). However, Endale et al. (33) reported a higher incidence of cancer among females (47.7%) than among males (39.8%). The approximate seroprevalence of

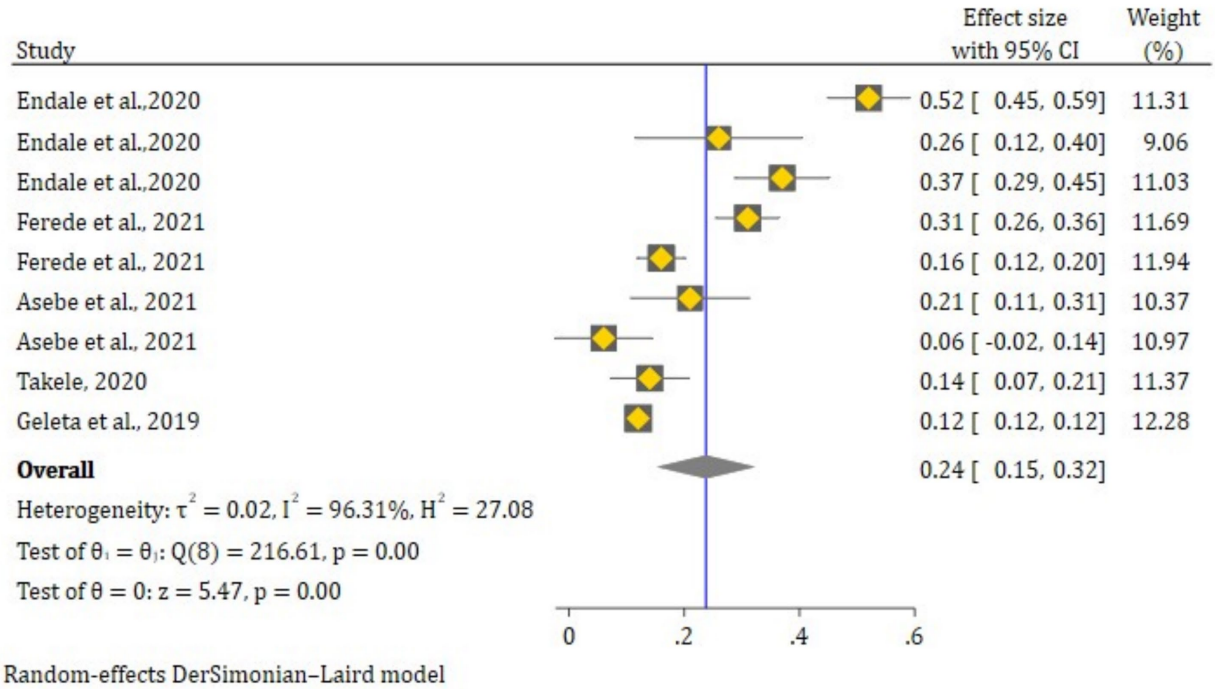

FIGURE 3
The random effect estimates of the effect size in different districts of Ethiopia.

Chikungunya in both sexes was reported by Geleta et al. (17) (Figure 8).

3.8 Publication bias

The statistical tests for small-study effects in a meta-analysis resulted in nonsignificant results. Begg's test showed the absence of significant small-study effects (Kendall's score = −8.00, SE = 9.592, *z* value = −0.94, $p = 0.47$). Similarly, the regression-based Egger test detected funnel plot asymmetry and revealed no indication of publication bias or small-study effects in the meta-analysis (beta1 = −1.74, SE of beta1 = 1.766, *z* value = −0.99, $p = 0.32$).

4 Discussion

Chikungunya in Ethiopia is becoming a significant public health concern, as it has caused considerable morbidity since it was first detected (13). Like many developing nations, Ethiopia struggles with a range of public health challenges that contribute to the outbreak of disease. Limited healthcare infrastructure and uneven distribution of resources hinder effective prevention, detection, and response to health crises (21). The aim of this systematic review and meta-analysis to estimate the pooled seroprevalence and identify potential risk factors of Chikungunya disease in Ethiopia.

In the current systematic review and meta-analysis five studies on Chikungunya conducted in Ethiopia and published in English between 2016 and 2024 were deemed eligible and were included in this systematic review and meta-analysis. The meta-analysis revealed that the pooled seroprevalence of Chikungunya was 24.0%. Factors such as geographical location, occupation, age, sex, and education contributed to the variation in the Chikungunya seroprevalence. Subgroup analysis based on the study area and the type of tests performed revealed significant heterogeneity.

The pooled seroprevalence of Chikungunya highlights the significance of the disease in Ethiopia. This prevalence was relatively lower than that reported in neighboring countries. For instance, Sudan seroprevalence has a median of 12% among the general population, with a range of 0–43% (8), while Kenya has prevalence rates ranging from 0.97 to 42% (15). Moreover, according to the results of this systematic review and meta-analysis, the highest seroprevalence of Chikungunya was reported in the Debub Ari district of the SNNPR, while the lowest was recorded in the Lare district of the Gambella regional state. In addition, seroprevalences differed among districts within the same region. For instance, the prevalence of Chikungunya in Itang (Gambella) was approximately triple that in Lare (Gambella). This result is in line with previous studies revealing that geographical region contributes to the inconsistency in Chikungunya incidence (37). This disparity prompted an exploration of potential contributing factors. Geographical nuances, including climate and ecosystems, may impact the abundance and behavior of Aedes mosquitoes, which are the vectors responsible for CHIKV transmission (22). Furthermore, variations in sanitation and hygiene practices, as well as the effectiveness of vector control measures, could influence the prevalence of Chikungunya. Another aspect to consider is diagnostic capacity; differences in the accuracy and sensitivity of disease detection methods could lead to variations in reported prevalence. The difference in the seroprevalence observed between different regions is likely due to the extensive distribution and high population density of vectors, particularly in urban centers, which are factors that favor their occurrence. The prevalence of this pest was

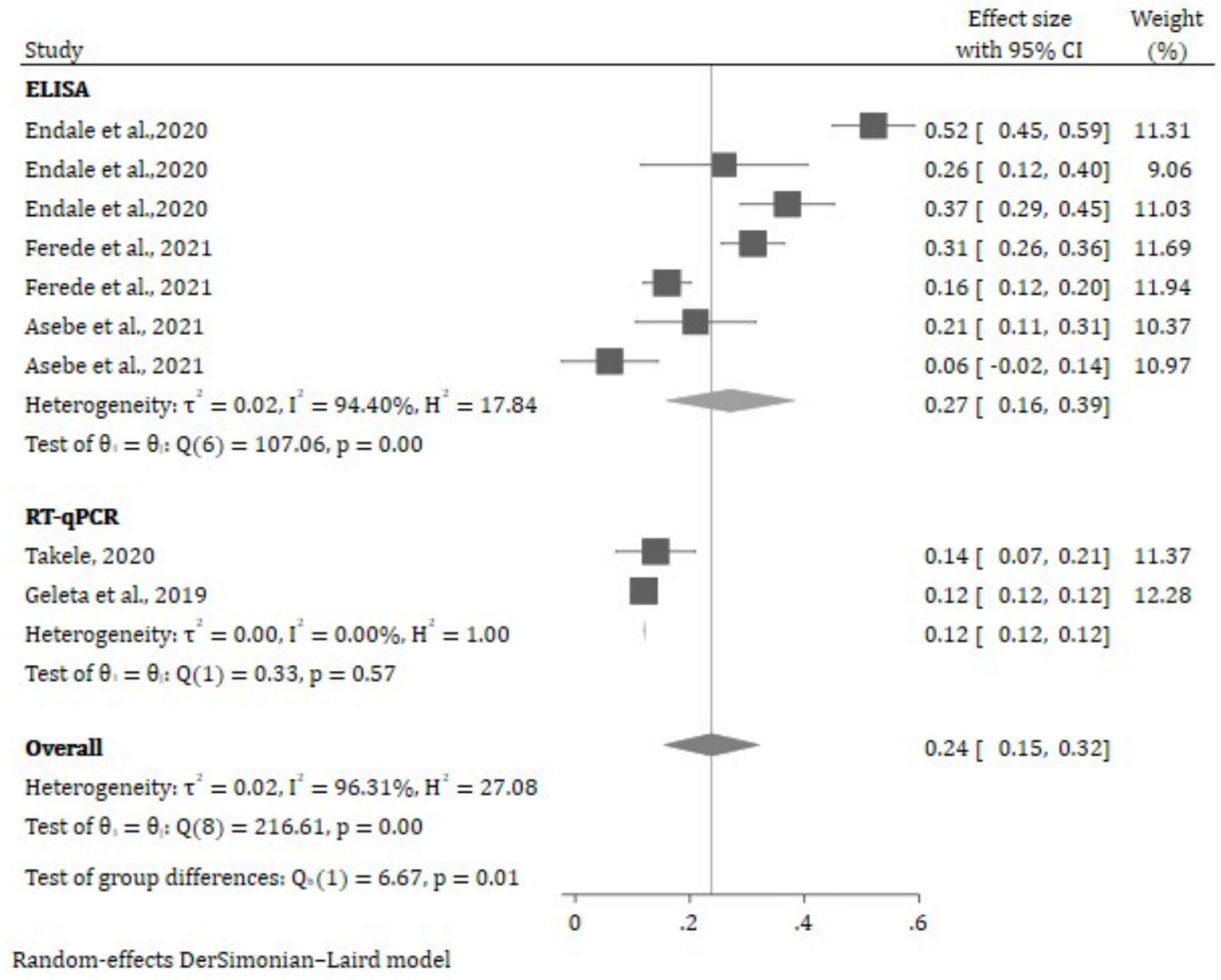

FIGURE 4
The random effect estimates of the effect size across the type of test performed.

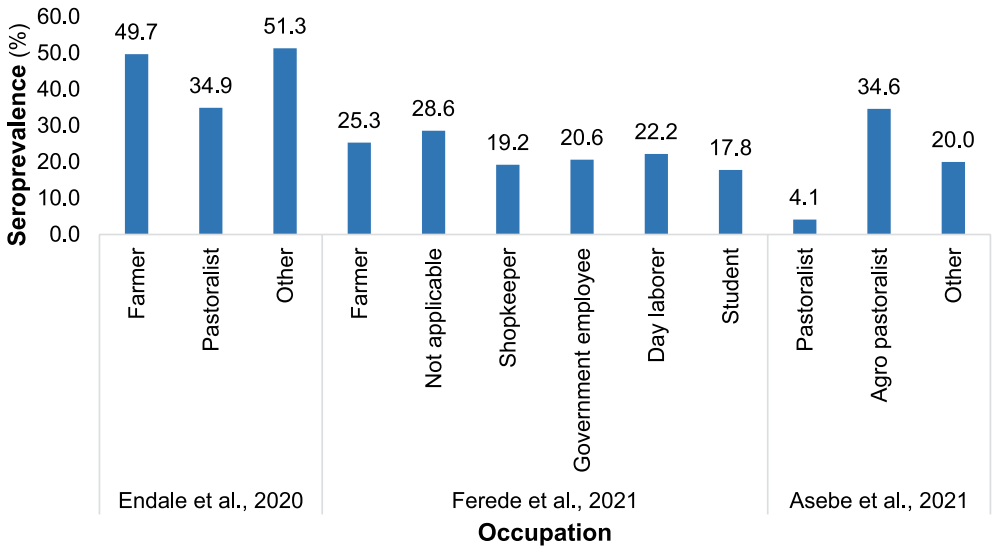

FIGURE 5
Distribution of Chikungunya among various occupations in selected publications from 2016 to 2024.

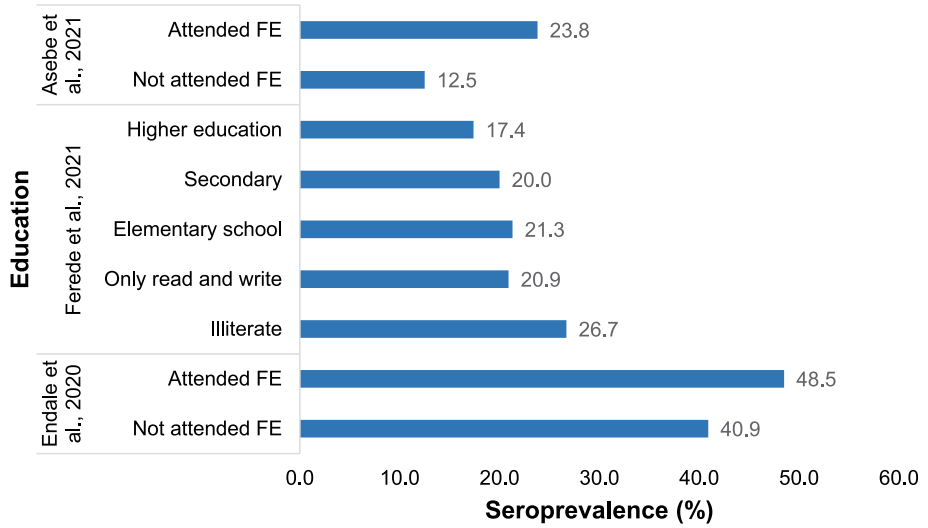

FIGURE 6
Distribution of Chikungunya according to education status in selected publications between 2016 and 2024.

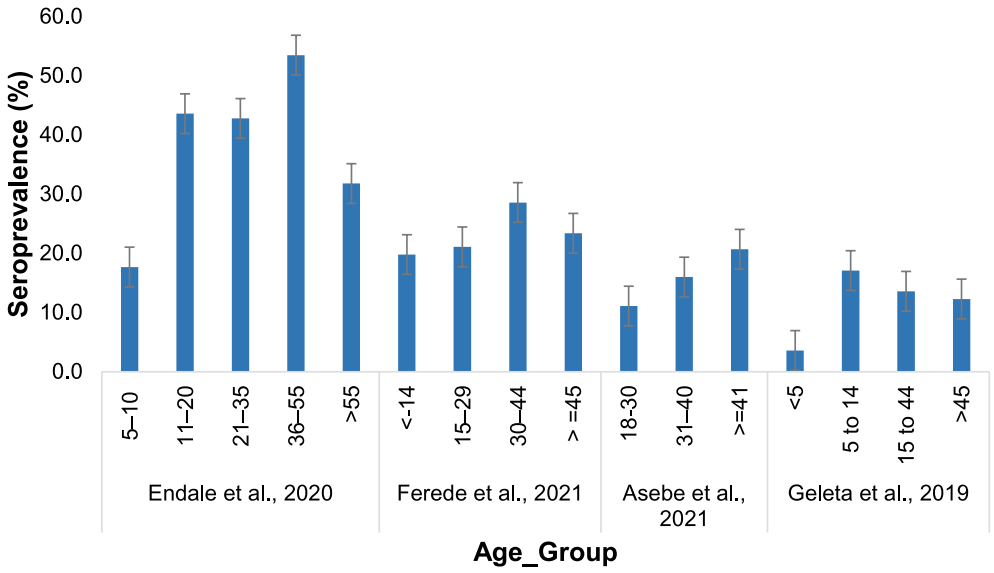

FIGURE 7
Distribution of Chikungunya among participants in different age groups in the selected publications from 2016 to 2024.

greater in the town, especially at internally displaced population sites, where various water containers, such as tyres, clay pots, barrels, plastic water tanks, flower vases, and old cars, are widely present as potential breeding grounds for Aedes mosquitoes (38). Additionally, the remoteness of certain areas may also play a role, as there are inequalities in the distribution of health facilities. These findings underscore the importance of considering the geographical context when implementing control and prevention strategies.

The current systematic review and meta-analysis demonstrated considerable variability in the seroprevalence of Chikungunya among occupations in Ethiopia. The highest seroprevalence of Chikungunya was found among farmers compared to individuals in other occupations (33). These findings are consistent with those of a study conducted in northwest Ethiopia by Ferede et al. (34). The variation may be associated with the degree of exposure to vectors transmitting CHIKV. Seropositivity for Chikungunya is greater in individuals who regularly move in forests, engage in agricultural activities, or have documented incidents of mosquito bites (6). Arboviruses typically circulate in forested areas through a sylvatic cycle involving primates as reservoir hosts (6). A study by Thiberville et al. (6), who reported that most seropositive individuals were engaged in farming activities, supported the higher prevalence of Chikungunya among farmers.

The prevalence of CHIKV in the reports included in this systematic review and meta-analysis was greater in the 31–40 years age group. Similar findings were reported from Tanzania (39). The higher infection rate in this age group may suggest that people in those

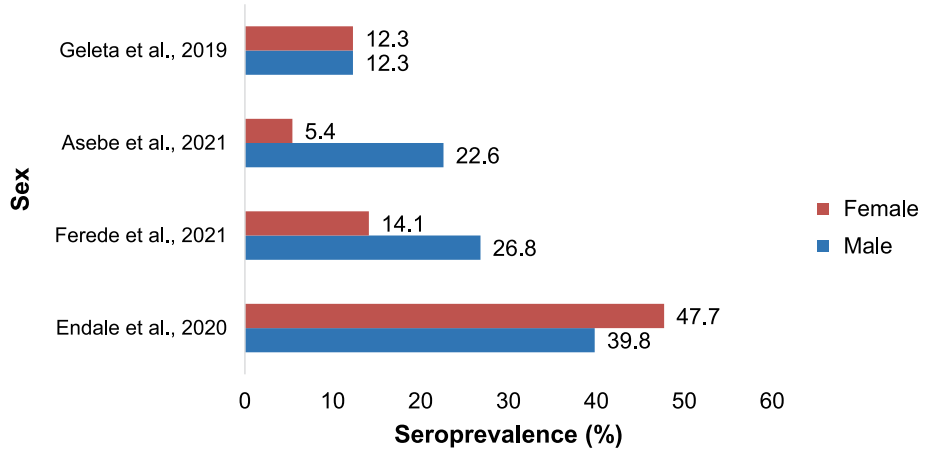

FIGURE 8
The prevalence of Chikungunya in Ethiopia in males and females in selected publications between 2016 and 2024.

age groups are active workers and exposed to the bites of the vector that transmits the disease. However, one study by Geleta et al. (17) reported a higher prevalence of Chikungunya in the 5–14 years age group and the lowest prevalence in the ≤5 years age group. This difference may be a result of the sample size difference, as the number of people aged 5–14 years who underwent diagnosis was almost double that of people aged ≥30 years.

According to the results of this systematic review and meta-analysis, education was associated with the seroprevalence of Chikungunya in Ethiopia. People who attended formal education were more strongly affected than people who did not attend formal education. This may be associated with educated people might be living in cities or towns where favorable environments for vectors, such as containers with water, are found. Educated people might have a more frequent migration history than non-educated people (6). Moreover, a higher prevalence of COVID-19 in educated individuals might be associated with better access to healthcare facilities as they live in cities where better health infrastructures are present, leading to increased detection and reporting of Chikungunya cases.

The results of these studies were inconsistent with those of sex. The prevalence of Chikungunya in males was greater than that in females according to Asebe et al. (35) and Ferede et al. (34). These findings are in line with other studies revealing that men are more susceptible than women are (40, 41). This may affirm the argument that males face a greater likelihood of encountering mosquito bites in the course of agricultural work or other comparable travel and occupational activities. In contrast, Endale et al. (33) reported that there were more CHIKV IgG+ females than males, indicating that females were more susceptible than males were. This opposite trend is supported by other seroprevalence studies (42, 43), in which females were more susceptible than males were. These conflicting reports highlight the necessity of further exploration of the associations between arbovirus infection and sex.

The results of subgroup analysis based on districts and test types were inconsistent across studies. The subgroup analysis, categorized by the type of tests conducted, revealed differences in study outcomes. Districts employing ELISA demonstrated significant variability among the studies compared to districts that utilized RT–qPCR, which exhibited a lack of variability among the studies, although this difference was not statistically significant. The overall heterogeneity was found to be high. These findings underscore the importance of accounting for the type of test in the analysis, as it appears to be a significant factor contributing to the observed heterogeneity. Further exploration of the sources of variability and careful consideration of the clinical implications are essential for a comprehensive interpretation of these results.

4.1 Study strengths and limitations

The strengths of this systematic review and meta-analysis are that the study included published data since the first detection of Chikungunya, and it is the first to report the pooled seroprevalence of Chikungunya in Ethiopia. This study has several limitations. The number of included studies was limited due to the restricted pool of available research, and the pooled prevalence may not accurately reflect the current reported rates. Almost all the included studies were cross-sectional. No molecular studies were carried out at the country level, except for RT–qPCR tests for confirmation. This limitation makes it challenging to predict circulating strains of the Chikungunya virus.

5 Conclusion and recommendations

In the current systematic review and meta-analysis, the pooled seroprevalence of Chikungunya was 24.0%. In addition, geographical location, occupation, age, sex, and education contributed to the variation in the Chikungunya seroprevalence. The subgroup analysis based on the study area and the type of tests performed revealed significant heterogeneity. The observed seroprevalence of Chikungunya reveals that the disease is remain persistent public health concerns in Ethiopia. Therefore, recognizing the significance of proactive operational readiness in mitigating the spread of infectious

diseases during an outbreak is recommended. Furthermore, it is crucial for the Ministry of Health and other concerned bodies to emphasize collaboration and public awareness campaigns to better respond to similar outbreaks.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

GD: Conceptualization, Data curation, Formal analysis, Software, Writing – original draft, Writing – review & editing. AG: Data curation, Writing – review & editing. SA: Data curation, Formal analysis, Writing – review & editing. AB: Data curation, Writing – review & editing. KB: Data curation, Writing – review & editing. HD: Conceptualization, Data curation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2025.1538911/full#supplementary-material

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Evidence of WNV infection in migratory birds passing through Xinjiang, China, using viral genome amplicon approach

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The West Nile virus (WNV) is a mosquito-borne virus of the Flaviviridae family that is transmitted through the mosquito-migratory bird-mosquito cycle. Currently, WNV infection is widespread in the Americas, Europe, and Africa, and is one of the most important global epidemic infectious diseases. Although migratory birds play an important role in the spread of WNV, monitoring of migratory birds carrying the WNV remains limited. Here, we developed a new nucleic acid test for detecting migratory birds carrying WNV, which uses amplicons of WNV to test fecal samples from migratory birds. This new method was validated by using full-length WNV genomic plasmid. With this amplicon method, we tested the migratory bird droppings collected in different locations. The results indicated that the positive rate of WNV nucleic acid in migratory bird droppings was over 39%, which provides clues to the fact that migratory birds may carry the WNV in Xinjiang, China.

KEYWORDS

West Nile virus, migratory bird, amplicon, fecal samples, epidemiology

1 Introduction

WNV is primarily transmitted through the bite of infected mosquitoes (Olufemi et al., 2021; Ronca et al., 2021). Mosquito become infected by feeding on birds that carry the virus. In nature, WNV is maintained in transmission cycle between birds and ornithophilic mosquitoes (Ferraguti et al., 2024). Mosquito species such as members of the *Culex pipiens* complex and *Aedes* spp. can act as bridge vectors and transmit WNV to vertebrate host species, including humans and horses. Virus-carrying birds migrate to various areas seasonally, and mosquitoes in the local area may be infected with the WNV by biting and sucking blood of the birds. Subsequently, infected mosquitoes may transmit the virus to other hosts by biting humans or other animals. Thus, the migratory bird-mosquito cycle is the limiting factor in the occurrence of regional epidemics of West Nile fever virus, which may result in regional epidemics when virus-carrying mosquitoes bite people or animals (Seidowski et al., 2010; Li et al., 2013).

Human infection with WNV is usually asymptomatic (>80%), and of those infected, ~20% will develop to West Nile fever, which manifests as fever, diarrhea, and respiratory symptoms, easily confused with a cold, and lasts for 3–6 days. Less than 1% of these infected individuals develop severe neuroinvasive disease, which is characterized by three clinical signs: West Nile meningitis, West Nile encephalitis, and acute flaccid paralysis (Klingelhöfer et al., 2023). The number of neuroinvasive cases and deaths increases with age, especially in people aged 65–89 years. The risk of neuroinvasive disease is nearly 1/50 in the elderly population aged 65 years or older, and is 16 times higher than that of people aged 16–24 years, with a median age of 64 years for neuroinvasive cases and 49 years for West Nile fever cases. Like humans, mammals such as horses and cattle are terminal hosts for WNV, and infection results in neuroinvasive diseases including encephalitis, fatal encephalomyelitis, and abortion in pregnant horses. In horses, for example, ~8% of horses infected with WNV develop severe neurological signs, with ataxia as the primary clinical manifestation and progression to encephalomyelitis. Other common signs include weakness of the limbs, lateral recumbency, and muscle tremors. Susceptible birds are storage and amplification hosts for WNV. Migratory birds infected with WNV mainly show neurological and respiratory symptoms including weakness, paralysis, visual disturbances, respiratory distress and runny nose, etc. Infected birds develop viraemia in their bodies and lead to death, with a lethality rate of up to 90%. According to national infectious disease surveillance information, WNV infects a significant number of cases globally each year and the mortality rate after infection (~6%) ranks among the highest for infectious disease mortality (Yeung et al., 2017).

Environmental factors such temperature, rainfall patterns, and availability play a crucial role in determining distribution and abundance of mosquito vectors (Ain-Najwa et al., 2020; Fayet, 2020; García-Carrasco et al., 2022). The presence of susceptible bird populations and suitable breeding sites also contribute to WNV transmission. In recent years, there has an increase in WNV in certain regions. This can be attributed to various factors such climate change, urbanization, globalization. Climate change can alter mosquito vectors and bird host geographic range and seasonal activity, potentially expanding the areas risk for WNV transmission. Urbanization can create favorable conditions for mosquito breeding and increase human-mosquito interactions. Globalization facilitates the movement infected individuals or animals across borders, potentially introducing new WNV to different regions (Seidowski et al., 2010; Lu et al., 2014; Fayet, 2020).

Current migratory bird carriage of WNV is difficult to monitor: migratory birds are wild animals and cannot be easily killed. It is difficult to collect tissue samples from migratory birds on a large scale for WNV isolation or detection. Most of the current reports are based on tissue organ sampling of abnormal birds, which makes it difficult to monitor and analyze the scale of WNV infection in detail. In other studies, fecal samples from pigs and penguins can be used for virus detection (Ogrzewalska et al., 2022; Liu et al., 2024). This is all because animals are difficult to sample *in vivo*. Although the virus can be detected in feces (Kipp et al., 2006; Dawson et al., 2007), it is difficult to use feces in actual monitoring.

WNV remains a significant global health concern with its current distribution spanning multiple continents. Looking ahead, it is essential to continue monitoring and studying the epidemiology of WNV to understand its current spread better and predict future trends. This includes surveillance programs detect virus activity in mosquitoes birds, animals, and humans. Additionally, research efforts should be on developing effective vector control strategies and vaccines to prevent mitigate WNV outbreaks. So far, there is no high-throughput detection method applicable to migratory birds for monitoring WNV epidemiology. Traditional detection methods require capturing and dissecting migratory birds. In this study, we established a new WNV monitor method for the detection of viruses carried by migratory birds. This study showed a new perspectives and monitoring methods on the carriage and transmission of WNV by migratory birds, which will be beneficial for WNV prevention and control measures and new vaccine research globally.

2 Materials and methods

2.1 Sampling and route

The sample collection sites in this study were waypoints located along the migratory routes of migratory birds in the Xinjiang Uygur Autonomous Region of China, namely, Manas Lake, Bosten Lake, Qinggda Lake, and Wulapo Reservoir (Figure 1). Samples were collected in October 2023 and May 2024: 25 samples were collected from Manas Lake and 101 samples were collected from Bosten Lake, totaling 126 samples; and 48 samples were collected from Qinggda Lake and Wulapo Reservoir. One hundred and sixty-one samples were collected from Wulapo Reservoir, and 280 samples were collected from Bosten Lake, totaling 489 samples. The total number of samples collected at the two time points was 515. Samples were collected by placing fresh sticky or solid feces of migratory birds in RNA-free EP tubes, adding RNA preservation solution (Coolabor) in the ratio of 1:1, and storing them for a short period of time at −70°C to −80°C.

2.2 RNA extraction

After sampling was completed, the sample RNA was extracted uniformly with the Viral RNA Extraction Kit (QIAGEN Inc.). RNA was isolated according to the manufacturer's instructions with minor adjustments: take 560 μL of the Buffer AVL that had been prepared in a 1.5 mL centrifuge tube. Add 140 μL of sample and vortex for about 15 s. Incubate at room temperature for 10 min. Incubate at room temperature for 10 min. centrifuge briefly to remove the solution from the cap of the tube. Add 560 μL of anhydrous ethanol, vortex for about 15 s, and then centrifuge briefly to remove the solution from the cap. Gently transfer 630 μL of solution from step e to the adsorbent column (in a 2 mL collection tube), centrifuge at 8,000 rpm for 1 min, and transfer the column to a new 2 mL collection tube. Carefully open the adsorbent column and repeat step f until all solutions are filtered by the adsorbent column. Add 500 μL of Buffer AW1 to the column, centrifuge

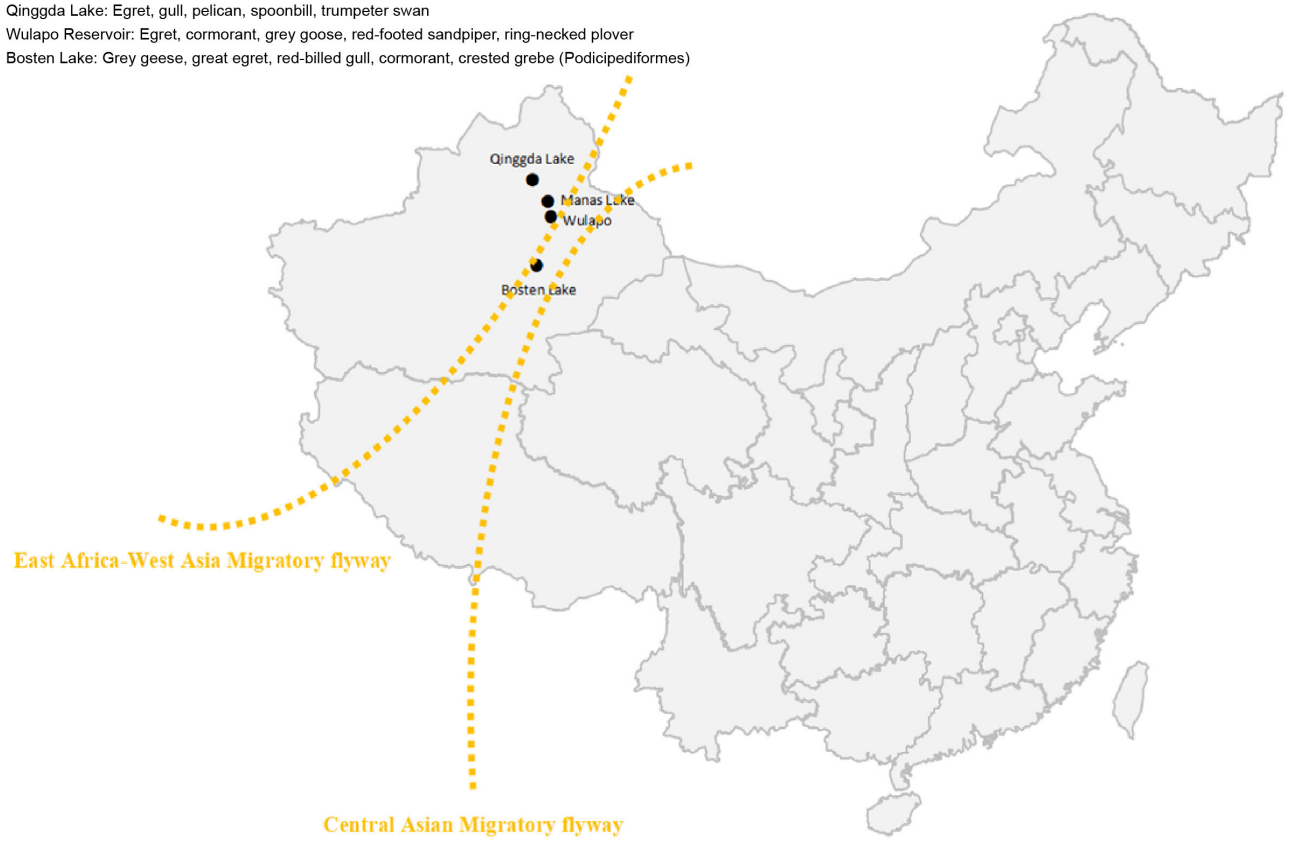

FIGURE 1
Sampling map. Manas Lake, Bosten Lake, Qinggda Lake, and Wulapo Reservoir in Xinjiang, China were selected as sampling sites. Yellow lines represent the East Africa-West Asia flyway of migratory birds.

at 8,000 rpm for 1 min, and transfer the column to a new 2 mL collection tube. Add 500 μL of Buffer AW2 to the adsorbent column, centrifuge at the highest speed (14,000 rpm) for 3 min, and transfer the adsorbent column to a new 2 mL collection tube. Centrifuge the column at the highest speed (14,000 rpm) for 1 min, transfer the column to a new 1.5 mL tube, add 60 μL of Buffer AVE, and incubate at room temperature for 1 min. elute RNA: centrifuge at 8,000 rpm for 1 min, and the eluted solution will be the solution containing the sample RNA.

2.3 Amplicon design and PCR detection

For WNV RNA amplication, amplicon methods were employed (Song et al., 2022). By using the NY99 strain WNV genome (DQ211652) as the reference sequence, a total of 63 amplicons of viral genes were designed (Figure 2). Based on the full-length genome sequence of reference virus, we designed a total of 63 pairs of primers for the amplification of the viral nucleic acid. Each amplification covered a viral fragment of about 200 bp, and each individual fragment has an overlap of about 30 bp in length. The 63 pairs of primers cover the full length of the WNV virus genome. After sequencing the amplified fragments, the residual components of the WNV nucleic acid in the sample can be determined.

In order to test all samples, every 25–30 fecal samples were combined into one test group. From a total of 615 fecal samples of migratory birds collected in Xinjiang, China, of which 126 were collected in 2023 and 489 in 2024, total RNA was extracted using microsample extraction method and then analyzed via amplification and sequencing. The collected migratory bird fecal samples included undried fresh guano and dried guano (presumed to be no more than 2 weeks old based on the degree of drying and weathering). The fragments obtained via amplification were purified, cloned to the T vector, and sequenced, and the sequencing results were compared and analyzed in NCBI database. Finally, 11 amplicons of the viral genes were detected, and these 11 amplicons with serial number designations and sequence information are listed in Tables 1, 2.

The micro-extracted sample RNA was prepared according to the following table as a reverse transcription system, gently mixed, and then reacted in a PCR instrument through the reverse transcription program: 37°C for 15 min, 85°C for 5 s. After the end of the reaction, the reaction product was temporarily placed at 4°C for storage. After reverse transcription, the PCR system was prepared according to the following table, gently mixed, and then the reaction program in the PCR instrument was

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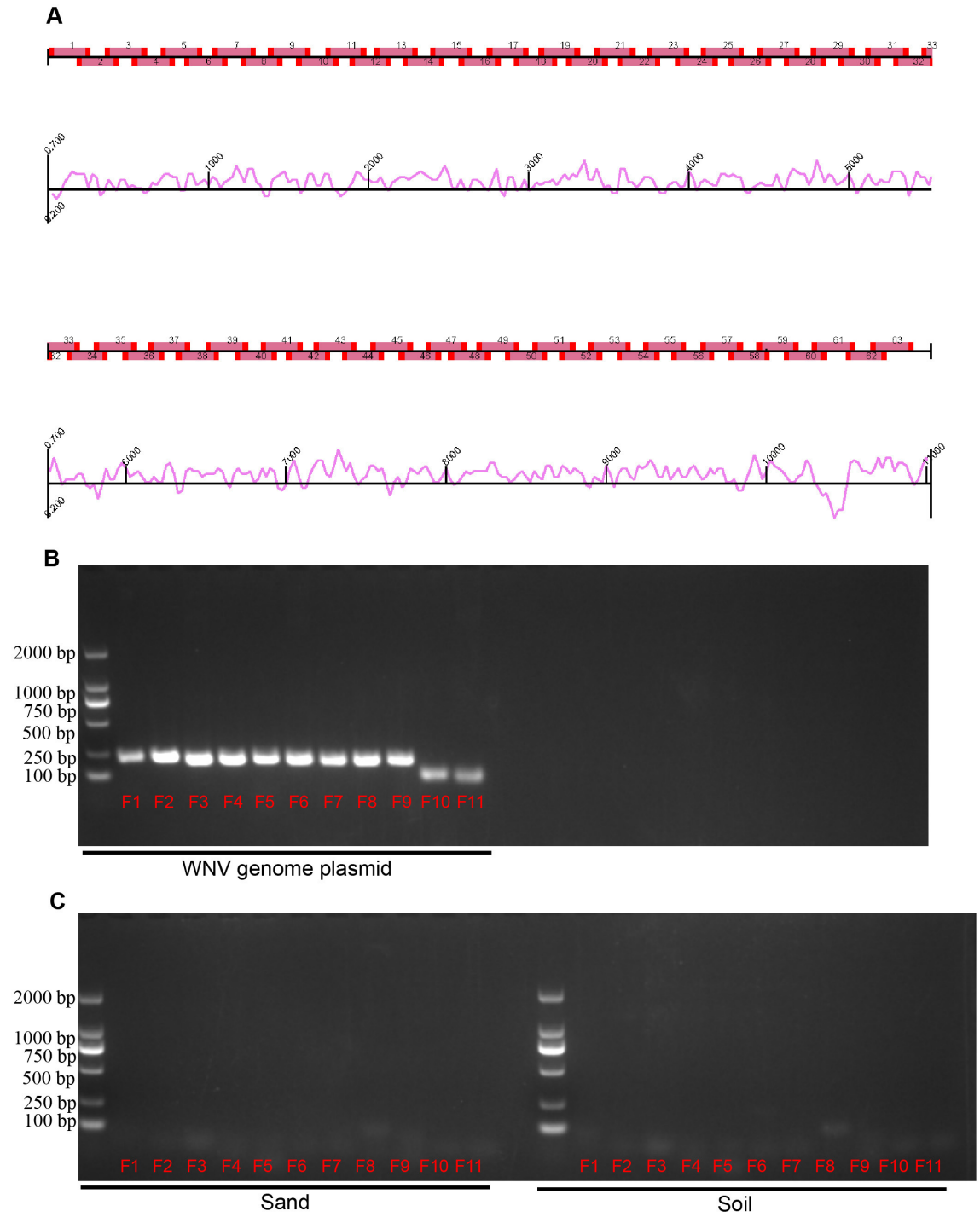

FIGURE 2
Design and validation of West nile virus amplicons. **(A)** Map of WNV amplicons. Sixty-three amplicons covering the WNV genome, with curves indicating the potential efficiency of amplification. **(B)** Validation of amplicon primers by WNV genome plasmid. **(C)** Validation of amplicon primers by environmental factors. Sand and soil in sampling sites were detected. Lane 1 (from left to right) is DNA marker, lanes 2–12 (F1–F11) are fragments of the amplicon.

as follows: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 15 s, and final extension at 72°C for 10 min, with denaturation-annealing for 30 cycles. The full-length WNV genomic (DQ211652) was synthesized by Synbio company and cloned in pACYC177 plasmid in our laboratory. Purified plasmids were used as positive controls. Soil and sand in the sampling environment were used as negative controls.

PCR products were validated using electrophoresis by running 20 μL of PCR product with 10X loading buffer for 30 min. Bands

TABLE 1 Sequences of WNV amplicon.

No.	Sequences	Length
1	GGTGGGAAAACCCCTGCTCAACTCAGACACCAGTAAAATCAAGAACAGGATTGAACGACTCAGGCGTGAGTACAGTTCGACGTG GCACCACGATGAGAACCACCCATATAGAACCTGGAACTATCACGGCAGTTATGATGTGAAGCCCACAGGCTCCGCCAGTTCGCTGGTC AATGGAGTGGTCAGGCTCCTCTCAAAACCAT	203
2	CACTCGCACCACCACAGAGAGCGGAAAGTTGATAACAGATTGGTGCTGCAGGAGCTGCACCTTACCACCACTGCGCTACCAAACT GACAGCGGCTGTTGGTATGGTATGGAGATCAGACCACAGAGACATGATGAAAAGACCCTCGTGCAGTCACAAGTGAATGCTTA TAATGCTGATATGATTGACCCTTTTCAGTTGGGC	205
3	GAACAACAGATCAATCACCATTGGCACAAGTCTGGAAGCAGCATTGGCAAAGCCTTTACAACCACCCTCAAAGGAGCGCAGAGAC TAGCCGCTCTAGGAGACACAGCTTGGGACTTTGGATCAGTTGGAGGGGTGTTCACCTCAGTTGGGAAGGCTGTCCATCAAGTGTT CGGAGGAGCATTCCGC	186
4	CTCGTGGGCTGCTCGGCAGTTATCAAGCAGGAGCGGGCGTGATGGTTGAAGGTGTTTTCCACACCCTTTGGCATACAACAAAAGG AGCCGCTTTGATGAGCGGAGAGGGCCGCCTGGACCCATACTGGGGCAGTGTCAAGGAGGATCGACTTTGTTACGGAGGACCCT GGAAATTGCAGCACAAG	185
5	ATCACAGAATACACCGGGAAGACGGTTTGGTTTGTGCCTAGTGTCAAGATGGGGAATGAGATTGCCCTTTGCCTACAACGTGCTG GAAAGAAAGTAGTCCAATTGAACAGAAAGTCGTACGAGACGGAGTACCCAAAATGTAAGAACGATGATTGGGACTTTGTTATCA CAACAGACATATCTGA	185
6	CCTGATCGACGGCAAGGGGCCAATACGATTTGTGTTGGCTCTCTTGGCGTTCTTCAGGTTCACAGCAATTGCTCCGACCCGAGCA GTGCTGGATCGATGGAGAGGTGTGAACAAACAAACAGCGATGAAACACCTTCTGAGTTTTAAGAAGGAACTAGGGACCTTGACCA GTGCTATCAATCGG	184
7	ACCTGGCAAGAACGTTAAGAACGTCCAGACGAAACCAGGGGTGTTCAAAACACCTGAAGGAGAAATCGGGGCCGTGACTTTGGA CTTCCCCACTGGAACATCAGGCTCACCAATAGTGGACAAAAACGGTGNNGTGATTGGGCTTTANGGCAATGGAGTCATAATGCC CAACGGCTCAT	179
8	GAAAGTCATAGAGAAGATGGAGCTGCTCCAACGCCGGTATGGGGGGGGACTGGTCAGAAACCCACTCTCACGGAATTCCACGCA CGAGATGTATTGGGTGAGTCGAGCTTCAGGCAATGTGGTACATTCAGTGAATATGACCAGCCAGGTGCTCCTAGGAAGAATGGAA AAAAGGACC	178
9	CTGAGGAAAAAACAGATCACTGTACTGGATCTCCATCCCGGCGCCGGTAAAACAAGGAGGATTCTGCCACAGATCATCAAAGAG GCCATAAACAGAAGACTGAGAACAGCCGTGCTAGCGCCAACCAGGGTTGTGGCTGCTGAGATGGCTGAAGCACTGAGAGGAC TGCCCATCC	175
10	GTAAAACAAGGAGGATTCTGCCACAGATCATCAAAGAGGCCATAAACAGAAGACTGAGAACAGCCGTGCTAGCGCCAACCAG	82
11	AAAGCTGCGTGCCCGACCATGGGAGAAGCTCACAATGACAAACGTGCTGACCCAGCTTTTGTGTGCAGACAAGGA	75

of each amplicons were evaluated by comparing with DL2000 DNA marker. Correctly sized bands were cut and sequenced.

3 Results

Effective monitoring of WNV carriage by migratory birds is difficult. In this study, we developed a method for detecting WNV nucleic acids in bird dropping samples via amplification and analysis of WNV genome fragments and detected WNV in fecal samples of migratory birds collected on different years (2023 and 2024) in Xinjiang, China, which proved that the source of WNV infection risk in Xinjiang, China, is related to migratory birds (Figure 3). Based on the genome sequence of the NY99 strain of WNV (National Center for Biotechnology Information accession no. DQ211652.1), we divided the WNV genome sequence into 63 amplicons (Figure 3). Each amplicon was ~200 nt in length, with ~50 nt of overlapping sequences between adjacent amplicons. The primers for each amplicon were 20 nt in length (primers available upon request).

Analysis of the migratory bird fecal samples showed that 101 of the 126 and 200 of the 489 migratory bird fecal samples collected in 2023 and 2024, respectively, were WNV nucleic acids positive, with 11 and 5 WNV gene fragments amplified via amplicon sequencing, respectively (Table 3). The detection rate of WNV nucleic acids in migratory bird feces ranges from 39% to 41%. The five gene fragments in the 2024 samples were included in the 11 gene fragments in the 2023 samples and ranged from 80 to 200 nt in length. Sequencing data proved that the 11 gene fragments obtained belonged to WNV.

Comparison of these 11 gene fragments revealed that five of them were from NS3, two were from NS5, two were from E, and one each was contained in NS1 and the capsid. Nucleic acid fragments from NS3 had the highest abundance and detection rates and were detected in both the 2023 and 2024 samples. In order to analyze the genetic relationships of WNV fragments, phylogenetic analysis was performed (Figure 3D). The results showed that the fragments from Manas Lake were closely related to HM147823.1 and FJ159131.1. Only one fragment was detected in Ulanbuy, which was closely related to FJ159131.1. All fragments except Fragment seven were detected in Bosten Lake, with relatively loose genetic relationships. This indicates that the viruses carried by migratory birds do not have obvious strain—specific characteristics. By comparing the strains from East Africa and Russia, it can be seen that the WNV strains in the countries along these migratory bird routes do not show obvious regional distribution characteristics, and all contain strains closed to Lineage 1 (AF202541.1) and Lineage 2 (DQ116961.1).

The sampling sites were different lakes in Xinjiang: Manas Lake and Bosten Lake for the 2023 samples, and Wulapo Reservoir,

TABLE 2 Primers of WNV genome amplicon.

Number of amplicons	Sequences of primers
1	F: GGTGGGAAAACCCCTGCTCA
	R: ATGGTTTTGAGAGGAGCCTG
2	F: CACTCGCACCACCACAGAGA
	R: GCCCAACTGAAAAGGGTCAA
3	F: GAACAACAGATCAATCACCA
	R: GCGGAATGCTCCTCCGAACA
4	F: CTCGTGGGCTGCTCGGCAGT
	R: CTTGTGCTGCAATTTCCAGG
5	F: ATCACAGAATACACCGGGAA
	R: CAGATATGTCTGTTGTGATA
6	F: CCTGATCGACGGCAAGGG
	R: CCGATTGATAGCACTGGT
7	F: ACCTGGCAAGAACGTTA
	R: ATGAGCCGTTGGGCATTA
8	F: GAAAGTCATAGAGAAGATGG
	R: GGTCCTTTTTTCCATTCTTC
9	F: CTGAGGAAAAAACAGATCAC
	R: GGATGGGCAGTCCTCTC
10	F: GTAAAACAAGGAGGATTCTG
	R: CTGGTTGGCGCTAGCA
11	F: AAAGCTGCGTGCCCGACC
	R: TCCTTGTCTGCACACAAA

Qinggeda Lake and Bosten Lake, which are the most active sites for migratory birds, for the 2024 samples. WNV nucleic acids were detected in samples from all locations, except Qinggeda Lake. The sampling time was when migratory birds in Xinjiang concentrated their activities: the 2023 sampling was in October, and the 2024 sampling was in May. These were the most active times for migratory birds in Xinjiang and the time when mosquitoes were most active. The interior of Xinjiang is covered by the East Africa-West Asia migratory flyway and the Central Asian migratory flyway, both of which pass through regions where WNV is prevalent lakes such as Manas Lake and Bosten Lake in Xinjiang are stopover or roosting sites for migratory birds, and a large number of migratory birds and bird dropping samples can be found and collected at these sites. Therefore, collecting fecal samples of birds from these locations can help determine the risk of carrying the WNV in the East Africa-West Asia migratory flyway and the Central Asian migratory flyway.

4 Discussion

A small number of WNV infections were reported in Xinjiang, China, between 2005 and 2014, and some cases may have been unaccounted for or undetected (Li et al., 2013; Lu et al., 2014; Cao et al., 2017; Zhang et al., 2021). The reason is WNV pathogens are not conventional measured in the non-endemic region. A clue to the discovery of these infections was the presence of patients with fever or viral encephalitis in local hospitals, and these infections were experimentally diagnosed via detection of WNV IgM antibodies or WNV-neutralizing antibodies in the serum. WNV nucleic acids have also been isolated from mosquitoes in Xinjiang, and primers designed for the WNV E and NS5 genes have been used to detect WNV nucleic acids in a subset of mosquitoes (Lu et al., 2014; Cao et al., 2017). These data suggest that mosquitoes in the Xinjiang region carry the WNV and can cause human infections. Five strains of WNV were isolated from mosquitoes that tested positive, and sequencing of these strains showed that they belong to lineage 1, which is highly homologous to the WNV strains prevalent in Russia (Cao et al., 2017). This suggests that the WNV cases in Xinjiang, China, may be related to bird migratory, as migratory routes connect WNV-endemic regions in Eastern Europe and Africa and passes through Xinjiang, China, and Russia.

Effective monitoring of WNV carriage by migratory birds using current technological methods is difficult (Seidowski et al., 2010; Ain-Najwa et al., 2020), which makes infection risk analysis and prediction of WNV epidemics difficult. A new convenient and rapid assay is important for WNV nucleic acid surveillance and risk assessment. Based on our new test method, it is possible to determine whether migratory birds carry or have carried the WNV virus, and to determine the regional transmission and epidemiological risk of WNV based on the carriage of viral nucleic acids by migratory birds.

In our experiment, the amplicon detection method was introduced, and 63 pairs of primers were designed to cover the full-length genome of the West Nile virus (WNV). Compared with the conventional PCR detection, this method will not miss the viral nucleic acid in the samples. It can avoid the problem of false negatives caused by RNA degradation and is suitable for the detection of fecal samples. It should be noted that this method is more likely to produce false positives during the amplification process, so it is necessary to sequence and verify the amplified gene fragments. Considering the difficulty of experiments on migratory birds, using the amplicon method to detect viral nucleic acid in feces is a good choice for monitoring viral nucleic acid in migratory birds. Detection of WNV nucleic acids in the feces of migratory birds allows speculation on whether the bird is carrying WNV virus (Liu et al., 2024), but does not provide valid information on the extent of carriage and tissue and organ deficiencies. Further experimental studies are needed to obtain detail infection information and bird species information from feces.

China has not historically been an endemic area for WNV, and only occasional cases have been reported in Xinjiang. Based on the flight routes of migratory birds, the occurrence of WNV in Xinjiang may be related to the migration of birds on the East Africa-West Asia migratory flyway and the Central Asian migratory flyway; however, no relevant study has confirmed this hypothesis (Fayet, 2020; Ferraguti et al., 2024). Migratory bird feces are easy to collect and do not harm migratory birds; however, their RNA is easily degraded (Kagzi et al., 2022). Despite this, RNA degrades over time, and some are not degraded quickly or completely because of their structure

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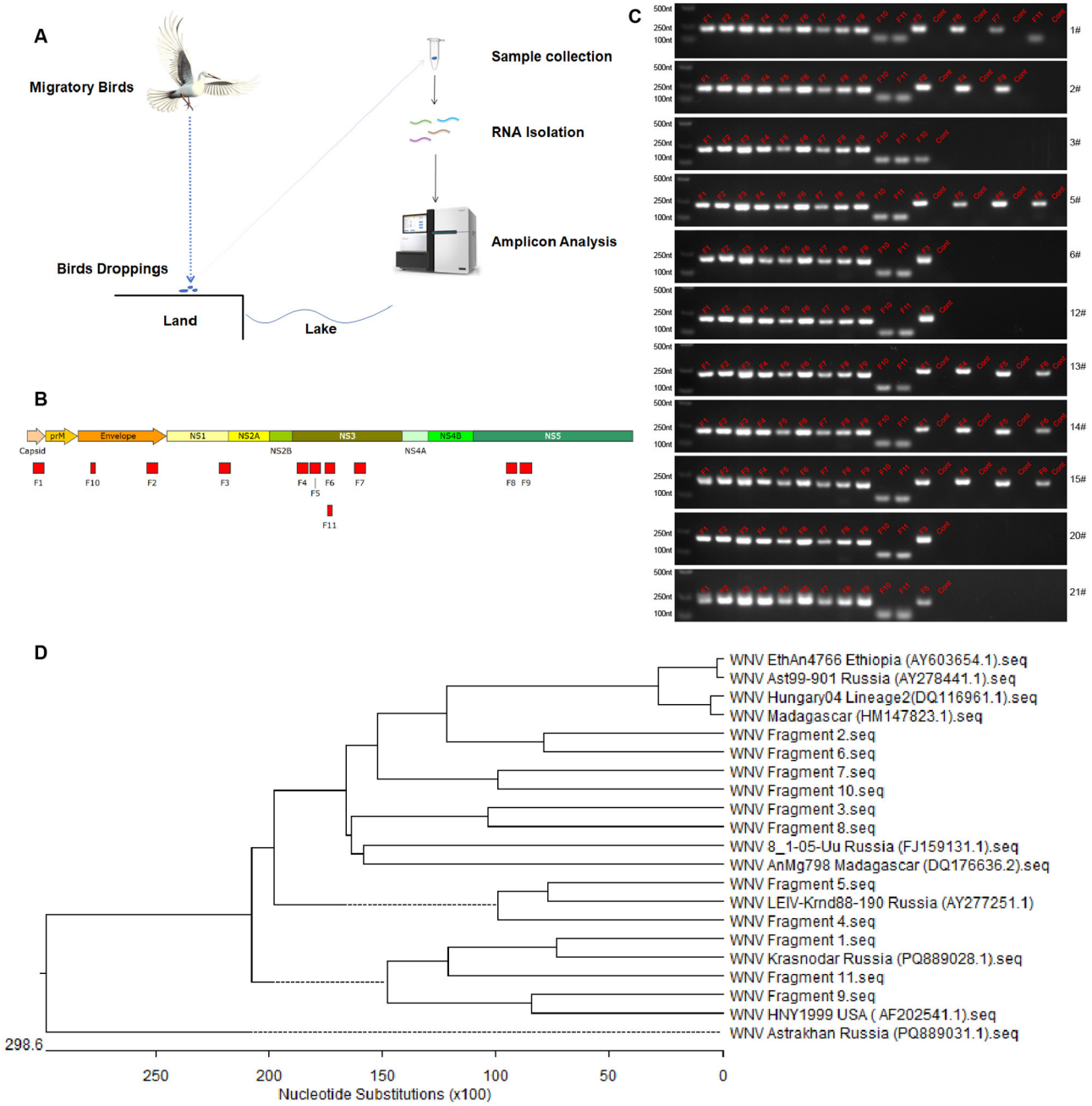

FIGURE 3
Detection of WNV fragments in feces of migratory birds. **(A)** Schematic diagram of sample collection and viral fragments detection. **(B)** Amplicons obtained in the whole viral genome amplification. Eleven amplicons from viral gene were determined by amplification and sequencing. **(C)** Electrophoretic analysis of WNV fragments. Lane 1 (from left to right) is DNA marker, lanes 2–12 are fragments of the amplicon obtained from WNV plasmid as positive control, other lanes are amplicons obtained from feces samples or negative controls (cont). The numbers of samples are presented on the right of each electrophoretic image. **(D)** Phylogenetic analysis of WNV fragments. The phylogenetic analysis was performed using the R code.

and external environment. To the best of our knowledge, this study is the first to determine that migratory birds in Xinjiang carry the WNV via microextraction of RNA from feces of migratory birds and subsequent detection of WNV amplicons. The detection rate of WNV nucleic acids in the feces of fresh and 2-week-old migratory birds ranged from 39% to 41%, suggesting that the WNV carriage rate of migratory birds was not <39%. The results of this study are important for WNV epidemic prediction, prevention, and control, and provide a new method for the global monitoring of WNV carriage in migratory birds.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

TABLE 3 WNV fragments detection in fecal samples.

Groups	Fecal samples in each groups[a]	Fragment of viral RNA	Number of RNA fragments	Sampling point[b]	Sampling time
1#	25	3, 6, 7, 11	4	Manas Lake	2023.10
2#	25	2, 4, 9	3	Bosten Lake	2023.10
3#	25	10	1	Bosten Lake	2023.10
4#	25	None	None	Bosten Lake	2023.10
5#	26	1, 5, 6, 8	4	Bosten Lake	2023.10
6#	50	3	1	Ulanbuy	2024.05
7#	50	None	None	Ulanbuy	2024.05
8#	30	None	None	Ulanbuy	2024.05
9#	31	None	None	Ulanbuy	2024.05
10#	24	None	None	Qinggeda Lake	2024.05
11#	24	None	None	Qinggeda Lake	2024.05
12#	25	3	1	Bosten Lake	2024.05
13#	25	1, 2, 4, 5	4	Bosten Lake	2024.05
14#	25	1, 2, 4, 5	4	Bosten Lake	2024.05
15#	25	1, 2, 4, 5	4	Bosten Lake	2024.05
16#	25	None	None	Bosten Lake	2024.05
17#	25	None	None	Bosten Lake	2024.05
18#	25	None	None	Bosten Lake	2024.05
19#	25	None	None	Bosten Lake	2024.05
20#	25	3	1	Bosten Lake	2024.05
21#	25	5	1	Bosten Lake	2024.05
22#	30	None	None	Bosten Lake	2024.05

[a]25–50 fecal samples were combined into one group.
[b]All samples were collected in Xinjiang, China.

Author contributions

KT: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – review & editing. CH: Investigation, Software, Writing – original draft. TZ: Formal analysis, Validation, Writing – review & editing. CX: Data curation, Writing – original draft. LD: Data curation, Writing – original draft. ZL: Data curation, Writing – original draft. DS: Data curation, Writing – review & editing. JW: Writing – original draft. BL: Writing – original draft. YQ: Writing – original draft. ZM: Funding acquisition, Writing – review & editing. KL: Funding acquisition, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Correction Note

A correction has been made to this article. Details can be found at: 10.3389/fmicb.2025.1638113.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1468530/full#supplementary-material

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Correction: Evidence of WNV infection in migratory birds passing through Xinjiang, china, using viral genome amplicon approach

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Evidence of WNV infection in migratory birds passing through Xinjiang, China, using viral genome amplicon approach

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In the published article, there was an error in the legend for Figure 3D as published. [**(D)** Phylogenetic analysis of WNV fragments. The phylogenetic analysis was performed using the DNAstar software.]. The corrected legend appears below.

[**(D)** Phylogenetic analysis of WNV fragments. The phylogenetic analysis was performed using the R code.].

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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