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Original article

Unexpected arboviruses found in an epidemiological surveillance of acute tropical febrile syndrome in the department of Meta, Eastern Colombia

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ABSTRACT

Background: Nonspecific acute tropical febrile illnesses (NEATFI) are common in the Latin American tropics. Dengue, Chikungunya, Zika, Mayaro, and Usutu, among others, can coexist in the American tropics. This study aimed to surveil the arboviruses that cause| acute febrile syndrome in patients in the Meta department, Colombia.

Methods: Between June 2021 and February 2023, an epidemiological surveillance study was conducted in the Llanos of the Meta department in Eastern Colombia.

Results: One hundred patients in the acute phase with typical prodromal symptoms of NEATFI infection who attended the emergency department of the Villavicencio Departmental Hospital were included. ELISA tests were performed for Dengue, Usutu, Chikungunya, and Mayaro. RT-qPCR was performed to detect the arboviruses Usutu, Dengue, Zika, Mayaro, and Oropouche. The seroprevalence for the Chikungunya, Mayaro, and Usutu viruses was 41 % (28/68), 40 % (27/67), and 62 % (47/75), respectively. Seroconversion for Chikungunya was observed in one patient; two seroconverted to Mayaro and one to Usutu. The NS5 gene fragment of the Usutu virus was detected in nine febrile patients. RT-qPCR of the remaining arboviruses was negative. The clinical symptoms of the nine Usutu-positive patients were very similar to those of Dengue, Chikungunya, Zika, and Mayaro infections.

Conclusions: The pervasive detection of unexpected viruses such as Usutu and Mayaro demonstrated the importance of searching for other viruses different from Dengue. Because Usutu infection and Mayaro fever have clinical features like Dengue, a new algorithm should be proposed to improve the accuracy of acute tropical fevers.

1. Introduction

Viruses, parasites, and bacteria can cause nonspecific acute tropical febrile illnesses (NEATFI). These diseases present a life-threatening clinical syndrome characterized by an insidious onset of nonspecific signs, sometimes followed by hemorrhagic manifestations and shock [1]. The intricate histopathological findings and clinical manifestations of NEATFIs, a complex puzzle, make clinical diagnosis and treatment a formidable challenge, especially concerning arbovirus infections. The urgency of this situation cannot be overstated.

South America has shown an incredible biodiversity of arboviral pathogens in studies that reveal the tropical region's high viral richness and propensity for emerging arboviral diseases. In the early

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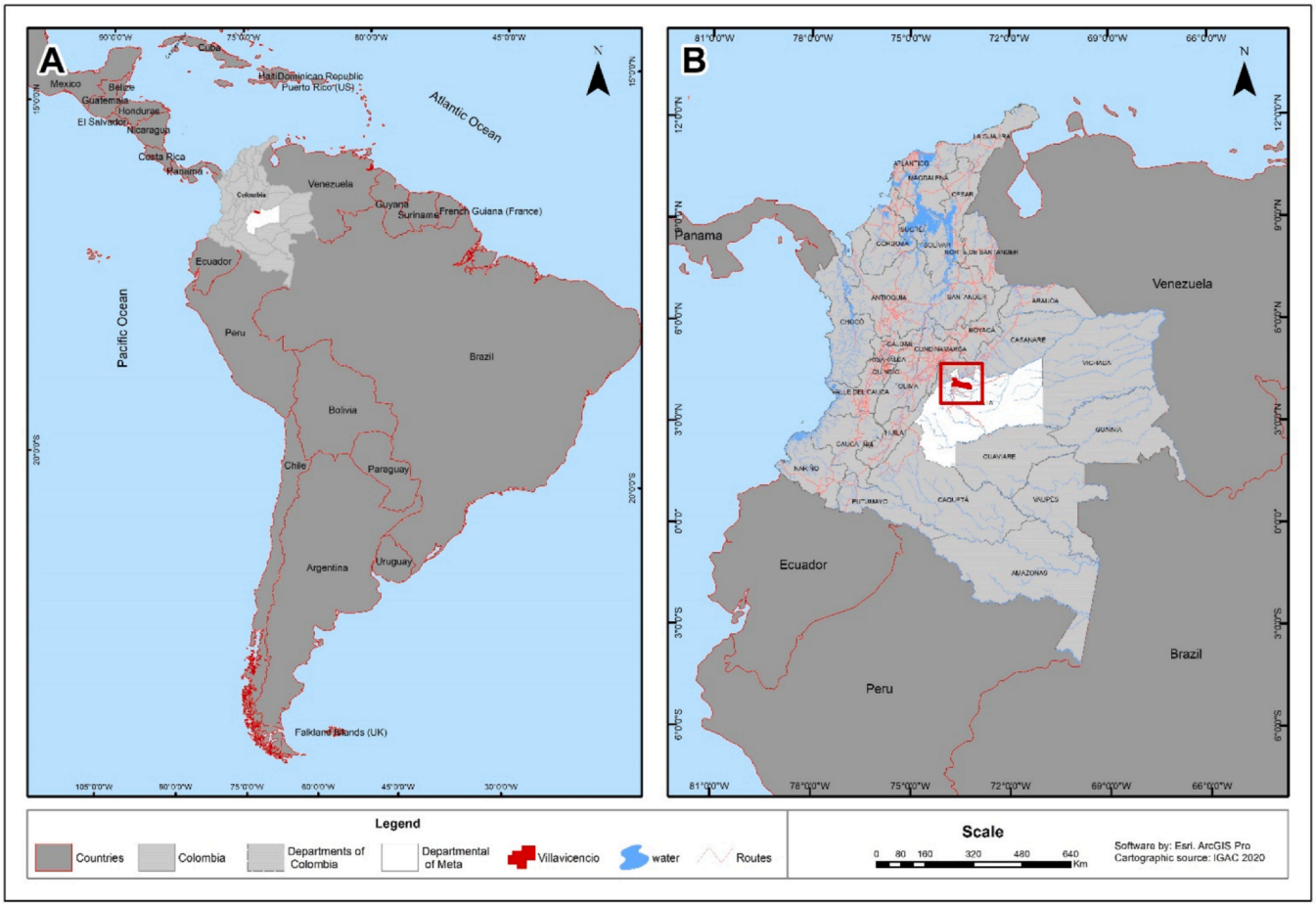

Fig. 1. Map of Colombia and the Department of Meta. A. South American map and location of Colombia. B. Department of Meta in Colombia. C. Location of Municipality of Villavicencio, the capital of Meta.

2010s, at least four epidemics in the Americas were caused by arboviruses. These include epidemics of dengue fever, chikungunya, Zika, and yellow fever [2]. Currently, the Mayaro and Oropouche viruses are also causing outbreaks, with the latter being responsible for cases in four countries: Brazil, Bolivia, Colombia, Cuba, and Peru [3]. This situation underscores the need for global collaboration in combating these diseases.

As a tropical country, Colombia has a large part of its population exposed to vector-borne infections. There are endemic areas where Dengue virus (DENV), Chikungunya virus (CHIKV), Zika virus (ZIKV), Mayaro virus (MAYV), and Oropouche virus (OROV), among others, can coexist in the prodromal phase. NEATFI presents similar clinical manifestations, such as fever, myalgia, malaise, headache, abdominal discomfort, chills, nausea, and vomiting. The coexistence of numerous tropical pathogens makes coinfections familiar [1,4]. The epidemiology and definition of NEATFI are changing in the tropics and worldwide. Population growth, disorderly urbanization, unplanned human activities, displacement of people due to internal violence, deforestation, illegal mining, and even climate variability contribute to the change in the epidemiology of NEATFI in the Colombian tropics.

Because there are many arboviruses in the tropics of developing countries, diagnosis is difficult due to the need for more resources in public health laboratories. Furthermore, due to the high endemicity of alphaviruses and flaviviruses, there are cross-serological reactions between arboviruses that circulate in Colombia, such as DENV, ZIKV, CHIKV, MAYV, and encephalitis viruses. Surveillance of reemerging arboviruses is necessary in Colombia and the tropics since dengue cannot be attributed to the entire NEATFI burden [4].

Colombia, like Latin America, has experienced recent Zika and Chikungunya epidemics. These became endemic quickly because, in most urban territories in Colombia, 50 million people live below 1200 m above sea level, facilitating the spread of the virus vector. Dengue continues to be the most prevalent arbovirus in the world. This is a reason for daily consultations in Colombia, especially when Latin America is experiencing a tremendous outbreak. Colombia, Brazil, and Mexico are the countries most affected [5].

Usutu virus (USUV) was discovered in 1959 in South Africa as a reemerging mosquito-borne neurotropic flavivirus. However, no infections in humans have been reported in the Americas. USUV belongs to the Japanese encephalitis virus (JEV) serocomplex and is phylogenetically close to several human and animal pathogens, such as West Nile virus (WNV) and Murray Valley encephalitis virus. In Europe, it has spread over the last twenty years. This underscores the importance of ongoing research in understanding and combating these diseases [6].

After the Chikungunya and Zika epidemics, since 2022, Dengue throughout South America has reemerged with great force, and currently, in Latin America, we are experiencing a large outbreak. Although it is known that other arboviruses can circulate, most doctors focus on dengue due to the endemic nature of the virus. However, in most cases, the diagnosis is based on symptoms.

Due to NEATFI's endemic nature in Colombia, clinical diagnosis is impossible because the symptoms are very similar [1]. Therefore, it is of the utmost importance to carry out epidemiological surveillance of NEATFI based primarily on molecular evidence to establish whether the disease burden is solely due to dengue or other reemerging circulating arboviruses.

This study, of significant importance, aimed to surveil the arboviruses of dengue, Zika, Chikungunya, Mayaro, and Usutu that cause acute febrile syndrome in febrile patients from the Meta department in Colombia.

2. Materials and methods

2.1. Design, type of study, and location

Between February 2021 and June 2023, a prospective descriptive cross-sectional study with non-probabilistic sampling of consecutive cases of febrile syndrome was carried out on the surveillance of acute febrile syndromes. The study was conducted in Eastern Colombia in the Meta department, an area known as Los Llanos, which has a population of 1,100,000 inhabitants (Fig. 1). The study was centralized in the departmental public Hospital of Meta, located in the capital, Villavicencio. The city has 550,000 inhabitants. The area is mainly dedicated to agricultural exploitation, with a tropical climate, an average annual temperature of 24 C°, humidity of 95 %, and a rainy season between April and November; Villavicencio, the capital, is 467 m above sea level.

2.2. Inclusion criteria

We included 100 consecutive cases with clinical suspicion of NEATFI according to the Centers for Disease Control and Prevention (CDC) case definition of viral hemorrhagic fevers [7] and the Ministry of Health of Colombia [8,9]. Patients in the acute phase who presented to the emergency room with prodromal symptoms typical of NEATFI infection were included. The samples were taken strictly in the acute phase of the disease between 1–5 days from the onset of symptoms. The most frequent symptoms were myalgia, arthralgia, headache, asthenia, chills, jaundice, dyspnea, abdominal pain, rash, nausea, and, in some cases, abdominal pain. Patients were enrolled in a NEATFI clinical trial at the Universidad de Cooperativa de Colombia-Villavicencio and the University of Córdoba. One hundred patients were recruited from whom paired acute and convalescent samples were taken between 15–20 days apart in each blood draw.

2.3. Exclusion criteria

Patients with malaria, leptospirosis, upper urinary tract infections, bronchitis, tonsillitis, otitis media, tuberculosis, liver abscesses, and diarrhea as primary symptoms were rigorously excluded. Patients with chronic liver disease, hemorrhagic syndrome of non-infectious etiology, acute poisoning, tumors, hematological diseases, and autoimmune diseases were also thoroughly excluded. Snake bites, bile duct diseases, patients under one year of age, and those with possible confusion with physiological jaundice were meticulously excluded.

2.4. Clinical, epidemiological, and demographic information

Clinical data collected from each patient during their hospital stay included name, age, sex, geographic origin, travel outside the residence, date of prodrome onset, admission date, symptoms, and physical findings. Data were collected on laboratory findings, such as blood cell counts, prothrombin time, and platelet count. In some cases, liver function and kidney function tests were performed. Clinical tests such as chest X-rays, electrocardiogram, and pulse oximetry were performed according to the patient's clinical condition.

2.5. Serology and seroconversion

The following IgM and IgG ELISA tests of Euroimun (Lubeck, Germany) were performed on the acute and convalescent sera. Dengue IgM (EI 266a-9601-1M), Chikungunya IgG (CHIKV EI 293a-9601), Usutu virus IgG (EI 2667-9601G), and Mayaro IgG (MAYV EI 295c-9601). The cut-offs were added for the ELISA tests. All ELISAs are standardized with the same cut-off points: < 0.8 negative, > 0.8–1.1 gray zone and > 1.1 positive. Seroconversion, defined as the process of developing detectable antibodies in the blood due to an infection, is critical in diagnostic testing. It occurs when a person transitions from having negative antibodies to having positive antibodies for a specific pathogen. This process's precision is evident in its ability to confirm recent infection or exposure to specific pathogens. A four-fold increase in IgG titers in convalescent-phase samples demonstrated a seroconversion.

2.6. Molecular detection of the DENV, USUV, MAYV, ZIKV, CHIKV, OROV (Oropouche)

Once the sera were obtained, they were frozen at −80 C°. Subsequently, the RNA was extracted using the COVID-19 Viral RNA extraction kit with magnetic beads (Bioland Scientific), and the RNA was kept at −80 C. RT-qPCR for DENV, CHIKV, ZIKV, MAYV, and USUV was performed on all acute-phase sera. For the Usutu virus, the protocol of Cavrini et al. [9]. was followed. The primers were designed based on USUV genomic sequences from GenBank, and the nucleotide sequences are from the NS5 gene of USUV isolates from Austria and Hungary [9]. The real-time RT-qPCR assay was conducted using the qScript One-Step RT-qPCR Kit with primers and a probe described by Cavrini et al. [9] to amplify a partial sequence of the NS5 gene detected by a dual-labeled probe. Each reaction had a final volume of 25 µl, containing 0.9 µM of each USUV primer and 0.25 µM of the probe. The reverse-transcription reaction was carried out at 50 °C for 10 min, followed by a 5-minute incubation at 95 °C, and then 40 amplification cycles (15 s at 95 °C, 1 min at 60 °C). A synthetic sequence from the NS5 gene was used as a positive control. For DENV, CHIKV, and ZIKV, a multiplex RT-qPCR was performed that amplified the regions for NS1, CHIKV NSP2, and ZIKV polyprotein (E) described in the protocol of Wagoner et al. [10]. To detect MAYV, an RT-qPCR that amplifies the NSP-1 gene followed the protocol described by Wagoner et al. [10]. To detect OROV, an RT-qPCR amplification of the Nucleoprotein N gene followed the protocol Rojas et al. [11] described. **(See supplement 1)**. A cut-off (Ct=37) for all RT-qPCR validation was used.

2.7. Ethics

The low-risk work involving only blood samples was approved by the ethics committee of the Tropical Biological Research Institute of the University of Córdoba Number 1209–2019. We ensured that written informed consent was obtained from all patients and, in the case of pediatric patients, from their accompanying family members. The study was conducted strictly to the principles of the Declaration of Helsinki and resolution number 008430 of 1993 of the Colombian Ministry of Health, which regulates health studies, ensuring the highest ethical standards were met.

2.8. Analysis of data

Because this is a descriptive study, frequency tables were used as descriptive statistical tools.

Table 1
Main clinical and epidemiological findings of nine patients with USUV infection.

Patient	Age/gender	Living area	Clinic presentation and symptons	Hematologic results	Presuntive diagnosis	Associated diseases
4	44/F	Urbana	arthralgia, rash with scaly and pruritic lesions, general malaise, myalgia, nausea and retrocular pain	Platelet count 333.000 mcL	Prolonged febrile syndrome	Sjogren syndrome
37	9/M	Urbana	Fever 40 °C, headache, dizziness, vomiting, abdominal pain and diarrhea	Mild leukocytosis and neutrophilia.		None
72	27/M	Urbana	Fever, headache, chills, asthenia, respiratory distress and cough	ND		None
75	7/F	Urbana	Fever 38 °C, headache, arthralgia, asthenia and Rash	Platelet count 190.000 mcL, no leukocytosis no neutrophilia	Unspeciefied fever	None
82	8/F	Urbana	Fever, headache, arthralgia, dizziness, nausea, vomiting, abdominal pain and diarrhea with traces of blood	Platelet count 278.000 mcL y neutrophilia	Amoebic dysentery and gastroenteritis	None
84	9/F	Urbana	Fever, headache, arthralgia, generalized rash, chills, general malaise, myalgia, nausea abdominal pain, cough, pulmonary infiltrate, pneumonia and appearance of white spots in red sea on legs	Platelet count 6.000 mcL no leukopenia.	Dengue with warning signs	Down síndrome
94	20/F	Urbana	Fever and Myalgia	ND		None
97	10/F	Rural	Fever, abdominal pain and shortness of breath	ND		None
100	13/F	Urbana	Fever, Headache, generalized arthralgia, Rash, Chills, General malaise, Myalgia, Dizziness, Asthenia, Nausea, vomiting, respiratory distress, retro-ocular pain and abdominal pain	Platelet count 95.000 mcL and leukopenia.	Dengue with warning signs; Unspeciefied fever	None

3. Results

One hundred patients voluntarily participated in the study; 84 % were from urban areas and 16 % from rural areas. No differences were observed concerning gender; 51 % were female, and 49 % were male. The age range of the patients was 5 to 65 years, of which 55 % were between 5 to 15 years old. The main occupation of the study population was that of students; all participants had water service, and 77 % lived with pets, mainly dogs and cats. Twenty-seven (27 %) reported having been diagnosed with dengue years ago; none reported having suffered from Zika or Chikungunya.

The seroprevalence for the CHIKV and MAYV viruses was 41 % (28/68) and 40 % (27/67), respectively, while the seroprevalence of the USUV virus was 62 % (47/75) (Table 1). Our study, however, had its challenges. The need for serum samples prevented tests from being performed on 100 patients, posing a significant hurdle. Despite this, we were able to identify one patient seroconverting to CHIKV (patient 36), two patients to MAYV (patients 4 and 57), two patients (4 and 75) seroconverting to USUV, and one of them (patient 4) seroconverting simultaneously to MAYV and USUV (Table 1). The ZIKV serology was not performed, but molecular detection was done.

Concerning molecular detection, nine sera were amplified for the USUV NS5 gene, and NGS was performed on all of them. The Ct of the nine patients with USUV was high between 26 and 33, and although sequencing was attempted several times, conclusive results could not be obtained. RT-qPCR for RNA the DENV, CHIKV, ZIKV, MAYV, and OROV viruses were negatives. Table 1, summarizes the clinical findings of the nine patients with USUV RT-qPCR positive; Table 2 shows the serological information of the patients with RNA USUV and other serology seroconversion of others arboviruses.

4. Discussion

This study is a significant milestone, as it is the first to report positive RT-qPCR for the Usutu Virus NS5 gene fragment in the Americas. This finding suggests the potential circulation of USUV in human infections, a phenomenon that has not been extensively studied in this region. The findings reported in this study are of significant national and international importance, as they represent the first detection of the Usutu virus as a cause of acute febrile syndrome in the department of Meta, in Colombia, and the Americas. Our results suggest that the Usutu virus is actively circulating in the region, leading to acute febrile syndromes. This study marks the first report of positive RT-qPCR for the Usutu Virus NS5 gene fragment in the Americas, hinting at the potential circulation of USUV in human infections. Future investigations should focus on active surveillance of USUV in the Americas, followed by genome sequencing, to confirm the presence of this emerging virus. We acknowledge the limitations of our study, which could be attributed to factors such as low viremia in the samples, multiple thaw and freeze cycles during attempted genome sequencing, and potential degradation of viral RNA. The potential implications for public health are significant, underscoring the urgency and importance of this research.

The clinical presentation of the nine patients with USUV highlights the significant challenge in diagnosing the infection based solely on clinical signs. As shown in Table 1, most patients exhibited classic symptoms resembling those of a Dengue infection, leading clinicians to classify it as such in their diagnostic impressions initially. Only the patient 84 tested positive for IgM; the remaining patients did not have IgM antibodies, suggesting that the cause of the disease may not have been Dengue. Unfortunately, due to resource constraints typical in many public hospitals in Colombia, the NS1 test, which is more sensitive than IgM in the acute phase, was not administered to the patients. While the RT-qPCR of the sera

Table 2
Serological information of arboviruses in patients with positive RT-PCR for USUV.

Patient	Usutu (IgG)*				Chikungunya (IgG)**	Mayaro (IgG)**	Dengue (IgM) ***
	Acute serum	Convalescent serum	Seroconversion	CtRT-qPCR	Convalescent serum	Convalescent serum	Acute serum
4	Neg	Pos	Yes	32.4	Posit (3,8 > 5,8) ◆	Posit (0,6 > 5,2) ◆	Neg
37	Neg	Neg	No	30.3	Neg	Pos	Neg
72	Neg	Neg	No	26.8	Neg	Neg	Neg
75	Neg	Pos	Yes	32.0	Neg	Neg	Neg
82	Neg	Neg	No	31.0	Neg	Neg	Neg
84	Pos	Pos	Yes	34.3	Pos	Neg	Pos
94	Neg	Neg	No	33.1	Neg	Neg	Neg
97	Neg	Neg	No	32.4	Neg	Pos	Neg
100	Pos	ND	ND	29.8	Neg	ND	Neg

Neg: negative
Pos: Positive
ND: Not done
*All patients were RT-qPCR positive for USUV.
**PCR Neg, except for patient 4, serology was only done on convalescent serum.
***PCR Neg, was not determined in convalescent serum.
◆patients with high seroconversion (acute serum < convalescent serum).

returned negative for Dengue virus RNA, it is plausible that the serum was collected early, and potential Dengue viremia went undetected. Coinfections are common in the Colombian tropics. The high seroprevalence of USUV might be attributed to cross-reactions with DENV and ZIKV. ELISA may not be an ideal marker for alphaviruses and flaviviruses, but seroconversion can provide valuable diagnostic insights.

There are also similarities in symptoms with Chikungunya and Mayaro, mainly in patients 4 and 37 who were seroconverted. However, the RNA of these viruses could not be detected in the serum, leaving doubts about whether it was a coinfection with USUV. The USUV symptomatology described here is like DENV, CHIKV, ZIKV, VEEV, and yellow fever, among other etiologies [1]. Thrombocytopenia (55.5 %), arthralgia (44 %), and rash (44 %) were present in patients with USUV. These notable clinical findings usually guide the diagnosis of the main arboviruses such as DENV, CHIKV, and ZIKV. However, as expected, cross-reactions between flaviviruses, possibly with DENV, ZIKV, and yellow fever, are noted.

Similar challenges are observed with *Togaviridae* members, such as CHIKV, MAYV, and encephalitis viruses. In line with the outcomes of our study (Table 2), it is evident that serology is not an effective method for diagnosing USUV in Colombia. However, for the first time in Colombia and the Americas, RT-qPCR targeting the NS5 gene of USUV has been confirmed as a positive diagnostic method for USUV infection. This pivotal discovery poses a new challenge for healthcare providers and public health authorities in the Americas, urging them to unravel novel etiologies of NEATFI and be prepared for potential outbreaks.

Alongside Dengue, Zika, and Chikungunya, emerging arboviruses like Oropouche, Mayaro, and Usutu are a growing concern. Mayaro and Oropouche, already prevalent in many Latin American countries, cause fever and joint pain, potentially leading to chronic arthritis. Usutu, originating in Africa but now found in European countries like Croatia, Italy, Bosnia, Germany, and France, affects birds and can cause encephalitis in humans. This global spread of arboviruses through trade and migration of humans and animals underscores the importance of epidemiological surveillance of febrile patients and mosquito control. As a global community, it is our collective responsibility to prevent the spread of these emerging viruses.

MAYV has been previously reported in Colombia; a study carried out between 2001 and 2004 showed seropositivity of 15.6 % (10/64). However, cross-reactions between alphaviruses make it challenging to confirm the diagnosis [12]. Currently, only 2 % of the patients showed IgG antibodies against MAYV; however, it is the first evidence of a circulation of MAYV in this department of Colombia. Coinfections are not strange in Colombia, and in a department where the leading cause of acute febrile syndrome is Dengue, other etiologies are dismissed. MAYV, OROV, and USUV are not arboviruses routinely searched for in febrile syndromes in Colombia. The cases of USUV and MAYV found in the present study are rare, and their clinical presentation further confuses the epidemiological panorama and poses an exciting challenge for tropical medicine in the country. The clinical presentation of USUV and MAYV requires further investigation and alerting the scientific community to initiate exhaustive surveillance throughout Colombia.

Significantly, of the 596 municipalities in Colombia, 373 (63 %) are below 1.200 m above sea level, posing a high risk for the population to contract arbovirus diseases. Eighty-four percent of the arbovirus cases were from urban areas. This alarming trend is exacerbated by the country's urban growth, which has driven cities towards rural areas. This invasion of human populations could potentially lead to a surge in emerging arboviral infections, a matter of grave concern that necessitates immediate attention.

Regarding USUV, in Europe, patients had neurological manifestations such as encephalitis and meningitis [13–15]. A similar fact happened with WNV in Colombia in 2001 when it is believed that the virus entered the country, and horses were found infected by WNV; however, they were asymptomatic [16]. High flavivirus endemicity is thought to produce protective heterologous antibodies against flaviviruses [14]. However, the reduced virulence of some flaviviruses in Caribbean basin countries remains a topic of debate and speculation [16].

USUV is maintained in the environment through a typical enzootic cycle involving mosquitoes and birds. Recent studies have shown that 13 USUVs have spread to much of the European continent over two decades, mainly causing significant bird mortality, with a significant upsurge in bird infections recorded across Europe in recent years [13]. This caused epizootics with significant bird mortality in Europe in 2016 and 2018. Occasionally, it can infect other mammals, including horses and bats, which act as incidental hosts [13].

Unlike Europe, where the arrival of WNV in 2002 was marked by alarming mortality of wild birds, Colombia has not experienced such a phenomenon. However, it is crucial to remember that Colombia boasts a rich bird biodiversity and a large population of mosquito vectors, *Culex* spp. Therefore, the findings of this work underscore the urgent need to initiate surveillance of the vectors and intermediate hosts of USUV in Colombia. This task is not just a scientific endeavor, but a crucial step towards protecting public health and must be completed on time.

One of the study's limitations was the sample size, which was relatively small and may need to be fully representative of the

population. Another limitation was the access to the second convalescent serum sample, especially for those people from rural areas who, due to economic situations, had difficulties attending the hospital. However, despite these limitations, the present work provides a vital motivation to continue searching for emerging viruses, emphasizing MAYV and USUV. This motivation stems from the potential public health impact of these viruses and the need for further research to understand their epidemiology and develop effective control measures.

The unexpected presence of Usutu (USUV) and Mayaro (MAYV) viruses in patients with acute febrile syndrome necessitates immediate and further research and action. This highlights the importance of expanding the search for other arboviruses beyond Dengue and Chikungunya. Given that USUV and MAYV present clinical characteristics similar to Dengue, it is essential to consider these and other arboviruses in the differential diagnosis of acute febrile infections. This consideration can lead to more accurate diagnoses and better patient outcomes. In conclusion, the findings of this study underscore the need to strengthen epidemiological surveillance and molecular diagnosis of arboviruses in Colombia, calling for immediate action and further research in this area.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.On behalf of the authors, I declare that this paper's disclosure will not generate or constitute any conflict of interest. I also declare that this material has not been and will not be submitted for publication elsewhere as long as it stays under consideration by Journal of Infection and Public Health. Furthermore, we affirm that the work is an original study, and the manuscript has not been published elsewhere.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jiph.2024.102510.

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