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Global excellence in infectious diseases – surveillance, prevention and treatment: Central and south america

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Global excellence in infectious diseases – surveillance, prevention and treatment: Central and south america

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Editorial: Global excellence in infectious diseases – Surveillance, prevention and treatment: Central and South America

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KEYWORDS

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Editorial on the Research Topic
Global excellence in infectious diseases – Surveillance, prevention and treatment: Central and South America

Infectious diseases affecting humanity continue challenging the scientific community and the health sector worldwide; several bacteria, viruses, fungi, and parasites can be classified into this group. The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for coronavirus disease 2019 (COVID-19), demonstrated that control and prevention of infectious diseases require the sum of global efforts and community participation. Despite international efforts, COVID-19 has provoked over 200,000 deaths monthly globally in the last 2 years. Hospitalizations, long-term COVID-19 deaths, and work absences are the main COVID-19-associated factors responsible for economic depression. Low- and middle-income countries, such as Africa and Latin America, have been significantly affected. Therefore, it is important to maintain the molecular diagnosis of SARS-CoV-2 in the population, independent of symptom presentation. This strategy will provide an opportunity to identify new variants (Kuriyama et al.). In addition, this Research Topic presents preliminary data suggesting that nimotuzumab (anti-EGFR) is safe and can reduce mortality in cases of severe and critical COVID-19 (Diaz et al.). Influenza A virus is another important pathogen because it provokes respiratory infections worldwide and causes annual epidemics. In addition, the virus can potentially cause pandemics (such as H1N1, 2009). Influenza A produces an acute respiratory and febrile illness that is generally self-limiting in healthy people. However, a group of individuals is highly susceptible, such as children, pregnant women, elderly individuals, immunocompromised individuals, and underlying chronic disease patients. In this group of patients, influenza can cause death (das Chagas Sousa et al.). Obesity and its comorbidities, such as diabetes, have been classified as a non-infectious pandemic. Importantly, they generate the main complications in various infectious diseases, such as influenza.

Acquired immunodeficiency syndrome (AIDS) is another significant infectious disease affecting people worldwide. AIDS, caused by human immunodeficiency virus (HIV), provoking an estimated 1.5 million (1.0–2.0 million) new cases annually, and an esteemed 38 million people globally are infected with HIV. Exist risk factors associated with the HIV prevalence higher, include men who have sex with men, intravenous drug users, people in prisons, and sex workers. Due to antiretroviral therapy (ART), a considerable reduction in the morbidity and mortality associated with HIV has been noticed; however, many undiagnosed cases provoke a continued transmission of HIV and an increase in morbimortality. In the case of low-middle-income countries, other factors have been associated with this phenomenon, such as loss of clinical follow-up, treatment withdrawal, and rising resistance to some drugs (Quirola-Amores et al.).

Diseases transmitted by vectors affect many countries in Latin America, such as those transmitted by mosquitos (mainly by *Aedes aegypti*) and triatomines (family Reduviidae). Dengue virus (DENV) has four serotypes (DENV-1,−2,−3, and−4) and was considered among the "top 10 threats to global health" in 2019 by the World Health Organization. Dengue is the leading cause of death and illness among individuals infected with an arbovirus. It is estimated that almost half of the world's population is at risk of Dengue infection. Annually, 390 million new cases are reported, and nearly 100 million patients are affected with varying disease severity. In the case of Latin America and the Caribbean, 3.1 million cases were reported in 2019, the highest ever. Regarding the serotypes, the evidence showed that DENV-1, -2, -3, and -4 co-circulated in Brazil, Guatemala, and Mexico, and the number of severe dengue exceeded reports in the preceding 4 years. As occurs in other infections, children usually have the most severe presentations of dengue and the highest imputable morbidity and mortality (May Lue et al.). Guillain-Barré syndrome and its atypical variant, Miller-Fisher syndrome, can be associated with viral infections. The Research Topic presents a brief research report describing the association of these syndromes with Zika, Chikungunya, and Dengue viruses (Santana do Rosário et al.). Chagas disease, also known as American trypanosomiasis, was recognized by the World Health Organization as a neglected tropical disease (2005), provoked by a hemoflagellate protozoan, *Trypanosoma cruzi*. This parasite is transmitted predominantly through the feces or urine of triatomine insect vectors infected but also through congenital, transfusions, transplants, laboratory accidents, and oral routes. Is a potentially life-threatening illness, affecting over 6 million people in the Americas, and 7,500 deaths are associated with Chagas annually; Although endemic of 21 continental Latin American countries it has become a global disease. The control of Chagas is challenging mainly because the parasite has high genetic variability, as well as the great diversity of reservoirs of the parasite *T. cruzi* (wild animals such as pets) and a broad biodiversity of triatomine vectors. Unfortunately, there is no accurate standard assay for the serologic diagnosis of chronic *T. cruzi* infection. Given this situation, the recommendation of WHO and PAHO is to use two serologic tests to improve diagnosis. Another problem is that diagnosis procedures vary by location (endemic or non-endemic areas) and in the screening of blood/organ donors. In addition, the genetic polymorphism of this protozoan affects the test's performance and the geographic region where the screening tests are performed. A critical observation is that over 20% of new cases worldwide are provoked in blood banks, where people become infected with Chagas through contaminated blood transfusions. These results highlight the importance of universal donor screening to exclude Chagas disease. One alternative is using chimeric IBMP antigens to decrease the number of bags discarded due to false-positive results (Ferreira dos Santos et al.).

Leptospirosis, a reemerging disease, is a worldwide zoonosis caused by spirochetes of the genus Leptospira. This Research Topic includes a manuscript that evaluates the humoral response and the factors involved in the exposition of leptospirosis. This work assessed the antibody response of individuals with a natural and asymptomatic infection and compared the results of a biannual and quarterly serological survey (Cruz et al.).

Metagenomic next-generation sequencing is an essential diagnostic tool to identify uncommon pathogens. A case report on this Research Topic used metagenomic next-generation sequencing on cerebrospinal fluid analysis to diagnose anaerobic meningitis (Li et al.).

Many people living with infectious diseases are socioeconomically vulnerable and have limited access to medical care and limited access to diagnosis, treatment, and vaccines. Therefore, infectious disease surveillance (routine testing) must be a priority in all countries for the early detection of new infection outbreaks. The cost-utility analyses are essential because they provide crucial information and, according to Quirola-Amores et al., must be considered throughout the establishment, restructuring, implementation, or maintenance of a strategy for the control and prevention of diseases. In summary, the articles on this Research Topic discussed Surveillance, Prevention, and Treatment of important infectious diseases in Central and South America.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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

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CASE REPORT
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Polymicrobial Anaerobic Meningitis Detected by Next-Generation Sequencing: Case Report and Review of the Literature

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Background: Anaerobic meningitis is a severe central nervous system infection associated with significant neurological sequelae and high mortality. However, the precise detection of causative pathogen(s) remains difficult because anaerobic bacteria are difficult to culture. Next-generation sequencing is a technology that was developed recently and has been applied in many fields. To the best of our knowledge, the use of next-generation sequencing for cerebrospinal fluid analysis in the diagnosis of anaerobic meningitis has been rarely reported.

Case presentation: Here, we report a case of polymicrobial anaerobic meningitis diagnosed using next-generation sequencing of cerebrospinal fluid in a 16-year-old girl. Five species of anaerobic bacteria (*Porphyromonas gingivalis*, *Prevotella enoeca*, *Campylobacter rectus*, *Fusobacterium uncleatum*, and *Actinomyces israelii*) were detected by next-generation sequencing and treated with antibacterial agents (ceftriaxone, vancomycin, and metronidazole). The patient responded well to antibacterial treatment. Further inspection revealed bone destruction at the base of the skull, which further confirmed that these bacteria had originated from the oral cavity. One month later, the patient's condition improved significantly. At the same time, we performed a literature review on anaerobic meningitis using studies published in the last 20 years.

Conclusions: This case emphasizes the importance of applying metagenomic next-generation sequencing to clinch the clinical diagnosis for patients with central nervous system infection. Metagenomic next-generation sequencing has been reported to be an important diagnostic modality for identifying uncommon pathogens.

Keywords: metagenomic next-generation sequencing, anaerobic meningitis, polymicrobial infection, case report, nervous system infection

BACKGROUND

Anaerobic meningitis is an uncommon disease. However, its true incidence may be underestimated because anaerobic bacteria in cerebrospinal fluid are difficult to isolate and culture; therefore, the prognosis of anaerobic meningitis is usually poor (1, 2). Despite treatment, the mortality rate of patients with anaerobic meningitis may be as high as 30.8% (3). Reliable laboratory tests performed early in the disease course are essential for the diagnosis and treatment of anaerobic meningitis.

Anaerobic bacterial culture of cerebrospinal fluid (CSF) is not performed routinely because cases of meningitis caused by anaerobic pathogens are rarely encountered (4). In addition, anaerobic bacterial culture is difficult to perform. Instead, polymerase chain reaction (PCR) to amplify the 16S ribosomal RNA (rRNA) gene is often used to detect the anaerobic pathogens causing bacterial meningitis. However, PCR can only detect designated pathogens via specific probes and targeted primers, due to which many pathogens may be missed (5, 6).

Unlike traditional testing for specific pathogens, metagenomic next-generation sequencing (mNGS), an emerging and promising modality, can identify a wide variety of potential causes (bacterial, viral, tuberculosis, fungal, and parasitic) (7). Improving our ability to identify novel or unexpected pathogens (8, 9). It also has the advantages of low cost and rapid turnaround time (10).

Although anaerobic meningitis is being increasingly recognized and reported in recent years, rapid identification of the causative anaerobic pathogen using mNGS resulting is improved patient outcomes is rarely reported. Here, we present a case of a patient with anaerobic meningitis. Multiple CSF cultures performed initially remained negative; however, the patient was finally diagnosed with polymicrobial anaerobic meningitis secondary to sinusitis when five species of anaerobic bacteria were isolated using mNGS. In addition, we reviewed the main features of the reported cases of anaerobic meningitis published in recent years.

CASE PRESENTATION

A 16-year-old girl presented to the emergency department due to complaints of fever, severe headache. Her physical examination revealed a fever of 38.1°C; and neurological examination revealed a stiff neck and positive Brudzinski and Kernig signs. The Glasgow Coma Scale score was 10.

Head computed tomography (CT) showed brain swelling. A lumbar puncture was performed, which revealed a high opening pressure (310 mmH_2O). CSF analysis showed that the fluid was cloudy, having a high protein (544.1 mg/dL, reference range 15–45 mg/dL) and low glucose (0.88 mmol/L, reference range 3.3–4.5 mmol/L) content and an elevated white blood cell count with neutrophilic predominance (13,206 cells/mm3, polymorphs 80%). Meanwhile, her CSF sample was sent to laboratory for pathogen detection at low temperature. Briefly, the patient's parents had signed informed consent, the CSF sample was collected and stored at −20°C, and then sent to laboratory of BGI-Shenzhen within 12 h. DNA was extracted with a TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH, Beijing, China) following the manufacturers' instructions. DNA libraries were constructed via end-repaired adaptation added overnight, and application of polymerase chain reaction amplification to extracted DNA. A Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.) in combination with quantitative PCR was used to quantify DNA libraries. DNA sequencing was then performed on the BGISEQ-50 platform (BGI-Shenzhen, Shenzhen, China) (11). High-quality sequencing data were generated after filtering out low-quality, low-complexity, and shorter reads. Then, the remaining sequencing data were aligned to the Date of BGI, which contains 6,350 bacteria, 1,798 DNA viruses, 1,064 species of fungi and 234 parasites, to identify the pathogenic sequences. An advanced data analysis was then performed, as for the mapped data.

Our hospital laboratory test results showed leukocytosis with neutrophilia (total leukocyte count: 25.4 × 10^9/L; neutrophils: 88.7%) and elevated levels of interleukin-6 (200.8 pg/mL) and procalcitonin (49.9 ng/ml). CSF and blood cultures were performed multiple times. However, brain magnetic resonance imaging performed the following day was reported to be normal. Intravenous ceftriaxone 80 mg/kg once daily and vancomycin 50 mg/kg twice a day were administered. Mannitol 20% and steroids was also administered to lower the intracranial pressure.

Over the next 3 days, fever and headache resolved. However, the Kernig sign remained positive. Blood bacterial culture and gram staining of the CSF on admission was normal. We decided to perform further investigations (chest and abdominal CT and abdominal ultrasonography) to identify the source of infection. Unfortunately, these investigations did not yield positive results. Meanwhile, thorough oral cavity, dental, ear, nose, and throat examinations were performed to identify a potential source of infection; and no tooth decay or pathological changes were identified. mNGS results showed anaerobic bacteria, namely, *Porphyromonas gingivalis*, *Prevotella enoeca*, *Campylobacter rectus*, *Fusobacterium uncleatum*, and *Actinomyces israelii* (**Figure 1**), which are all oral bacteria. We thought it may be a mistake of the laboratory or contamination during CSF collection. And, repeat CSF cultures were negative.

Lumbar puncture was performed again to detect the pathogens by mNGS 20 days later, which showed that the anaerobic bacteria were the same as before (**Figure 1**). At this time, we reviewed the patient's head CT findings (**Figure 2**) again and found that the patient's clivus, bony part of the skull base, was eroded. Repeat head CT (**Figure 2**) was immediately performed, and the bone quality was significantly better than that before admission. This patient had no history of cancer or trauma, and we speculated that sphenoid sinusitis had led to the destruction of clivus with subsequent infection of the meninges by oral anaerobic bacteria. Due to persistent elevation of white blood cell count on CSF analysis, we added metronidazole, which targets anaerobes, to the regimen. At 3-month follow-up, the patient appeared well and had returned to normal

Abbreviations: CSF, cerebrospinal fluid; PCR, polymerase chain reaction; CT, computed tomography; mNGS, metagenomic next-generation sequencing; rRNA, ribosomal ribonucleic acid.

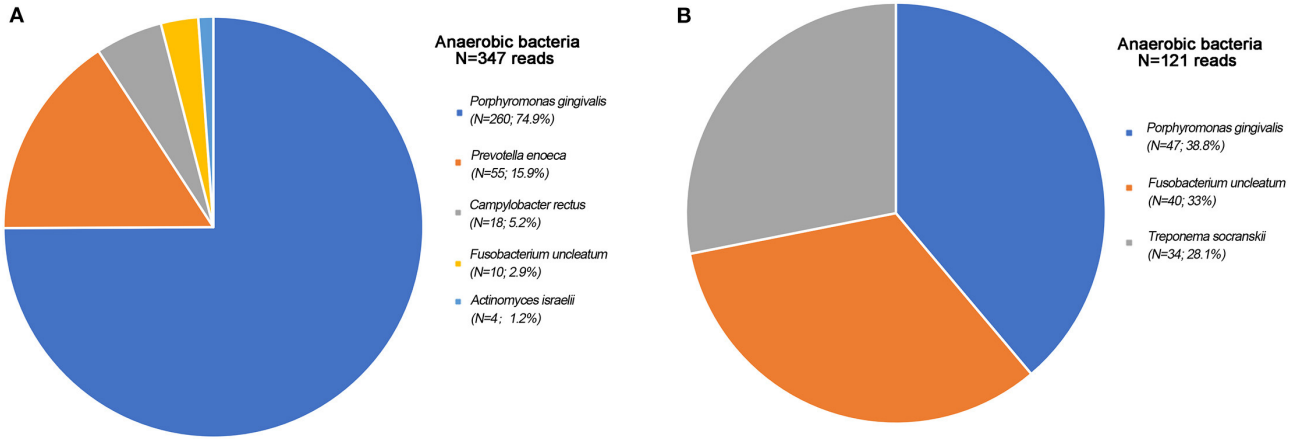

FIGURE 1 | Anaerobic bacteria found by mNGS. **(A)** Anaerobic bacteria found by mNGS first times. Porphyromonas gingivalis: 260 reads; Prevotella enoeca: 55 reads; Campylobacter rectus: 18 reads; Fusobacterium uncleatum: 10 reads; Actinomyces israelii: 4 reads; **(B)** Anaerobic bacteria found by mNGS second times. Porphyromonas gingivalis: 47 reads; Fusobacterium uncleatum: 40 reads; Treponema socranskii: 34 reads.

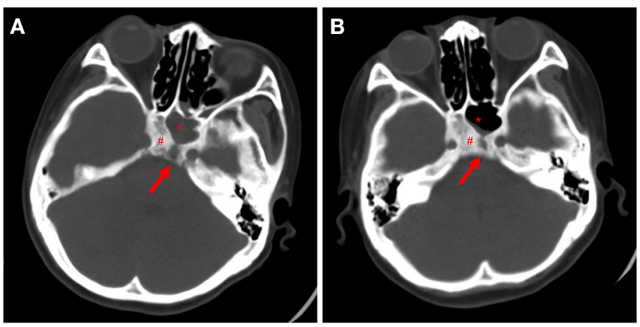

FIGURE 2 | Paranasal sinus caused significant bony erosion. **(A)** Cranial CT at admission. **(B)** Cranial CT 1 month later. Red arrows indicate bone destruction. #refer to the clivus, *for sphenoid sinus.

activity. Repeat head CT showed resolution of bone destruction (**Supplementary Information**).

DISCUSSION

Anaerobic meningitis is an uncommon disease occurring due to contiguous spread of infection from the head or neck (12, 13). In general, infections caused by anaerobic bacteria are usually devastating (14). Here, we report a case of polymicrobial anaerobic meningitis caused by oral anaerobic bacteria entering the intracranial cavity due to bone destruction of the base of the skull secondary to sinusitis. Moreover, mNGS identified the pathogen in time, and the patient received optimal antibiotics, which led to a good prognosis.

Currently, the diagnosis of bacterial infections relies on the isolation and culture of bacteria. Bacterial culture of CSF remains the gold standard for the diagnosis of bacterial meningitis. However, the diagnostic yield of CSF culture is low, and the process is time-consuming (requiring more than 72 h) (15–17). This may be the reason why the incidence of anaerobic meningitis is underestimated in clinical practice, since many of the pathogens causing intracranial infections are not identified (18, 19). In terms of treatment, when the causative pathogen cannot be identified, broad-spectrum empirical antibiotics are administered, which induces antibiotic resistance and increases the burden on the patients (20). Considering the difficulty in culturing anaerobes, PCR of CSF is employed to detect microbial DNA in patients with bacterial meningitis. The 16S rRNA gene is a DNA sequence encoding 16S rRNA. It is found in the bacterial chromosome, but does not exist in Non-prokaryotic organisms such as viruses and fungi. The 16S rRNA gene has a high degree of specificity and conservation. In the literature, most cases of anaerobic intracranial infections were definitively diagnosed using 16S rRNA PCR (14, 21, 22). However, this technique does not identify all bacteria and pathogens that are detected by CSF culture (21, 22). Moreover, the current PCR techniques used to detect bacterial meningitis are too expensive for patients in rural regions (23, 24). Thus, it is difficult to implement existing PCR tests in areas with the highest incidence of bacterial meningitis.

Therefore, rapid diagnosis of intracranial infections and preliminary classification of bacteria are clinical problems that need to be resolved urgently. mNGS is a novel and promising approach in diagnostic microbiology having the ability to detect many potential microorganisms using a single assay (7, 25). Previous studies show that mNGS of CSF obtained from patients with central nervous system infections improved the diagnostic rate and provided actionable information (26, 27). In this case, the mNGS detected the pathogens in time and provided a direction for us to identify the source of infection. Below, we review the relevant literature on anaerobic meningitis.

Recent studies on anaerobic meningitis that were published in the last 20 years were identified using an electronic search (**Table 1**). Our analysis showed that very few cases of anaerobic meningitis have been reported. However, the incidence of

TABLE 1 | Main features of reported cases of anaerobic meningitis.

Author	Bacteria	Methods of identification	Treatment	Type of pathogeny	Outcome	Sex/age (years)
Kalay et al. (4)	*Bacteroides fragilis*; *B. thetaiotaomicron* and *Fusobacterium necrophorum*; *Proteus mirabilis*.	MALDI-TOF MS; Left ear for culture.	Vancomycin; Metronidazole; Meropenem; Acyclovir metronidazole	Mastoiditis	Recovery	M/16
Llitjos et al. (18)	*Peptostreptococcus micros, Fusobacterium necrophorum*, and *Porphyromonas gingivalis*	16S rRNA sequencing; standard culture.	Meropenem; Aancomycin; Fosfomycin; Amoxicillin; Metronidazole	NA	Death (47 days)	W/69
Anusha et al. (19)	*Bacteroides fragilis*	16S rRNA sequencing; standard culture.	Ceftriaxone; Amoxicillin; Acyclovir,	A subdural empyema; Pre-sacral abscess.	Death	M/8-week
Yael et al. (12)	*Eubacterium multiforme*	16S rRNA sequencing;	Ceftriaxone; Vancomycin; Metronidazole; Ampicillin	Brain penetrating trauma	Neurological sequelae.	M/6
Joshua et al. (27)	*Anaerobic gram-negative bacillus.*	Blood culture	Benzylpenicillin;Metronidazole;	Rectothecal Fistula Arising from an Anterior Sacral Meningocele	Recovery	M/48
Juan at al. (28)	*Bacteroides fragilis, Staphylococcus aureus* and *Morganella morgagnii*	CSF culture	Vancomycin and meropenem	Colorectal surgery	Uneventful outcome	M/68

bacterial meningitis is very high worldwide (28, 29). The main reason for this may be that anaerobic meningitis is difficult to diagnose. The age at onset reported in these studies was variable, reflecting that anaerobic meningitis can affect a wide range of age groups. Moreover, the prognosis of the patients was very poor (21, 22), and only a few of the cases reported complete recovery of the patient (4, 30). However, the prognosis was poor compare to bacterial meningitis (31). Inappropriate antibiotic therapy administered due to the delay in diagnosis is the cause of increase in the sequelae and mortality of anaerobic meningitis (32).

Exact incidence of anaerobic meningitis is unclear and was presented in only a few case reports. We found that PCR can be an important method for the diagnosis of anaerobic meningitis but it does not detect all organisms and is expensive. Moreover, culturing anaerobic organisms may be difficult; therefore, it is challenging to promptly diagnose anaerobic meningitis. This case shows that mNGS may be more effective than traditional microbial detection methods. In addition, early diagnosis and timely administration of appropriate antibiotic treatment can be life-saving. Efforts should be made to ensure the widespread availability and use of mNGS.

CONCLUSIONS

We were able to correctly diagnose our patient with anaerobic meningitis, owing to the application of mNGS, due to which she was administered appropriate antimicrobial therapy. This case demonstrates that the process of diagnosing anaerobic meningitis is imprecise due to which its incidence may be higher than reported. Possibility of anaerobic meningitis should be kept in mind if the clinical course of the patient does not progress as expected and the mNGS technology may be a good tool to help establish the correct diagnosis.

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

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XLi reviewed the literature, analyzed the patient data, and wrote the manuscript. HD and XLi were responsible for data collection. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.840910/full#supplementary-material

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Performance of Chimeric *Trypanosoma cruzi* Antigens in Serological Screening for Chagas Disease in Blood Banks

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Chagas disease (CD) is among the top 10 causes of inability to blood donation. Blood donation centers screen for anti-*Trypanosoma cruzi* antibodies using highly sensitive immunoenzymatic (ELISA) or chemiluminescent methods, which can lead to false positive results. Since positive samples cannot be used, to avoid the loss of valuable blood donations, it is necessary to improve specificity without reducing the sensitivity of the tests used for blood screening. For this purpose, our group has developed four chimeric proteins (IBMP-8.1, IBMP-8.2, IBMP-8.3, and IBMP-8.4) that have been evaluated in phase I and II studies with high performance and low cross-reactivity rates. The study included a panel of 5,014 serum samples collected from volunteer blood donors at the Hematology and Hemotherapy Foundation of the State of Bahia (Brazil). They were subjected to the detection of anti-*T. cruzi* antibodies, using all four IBMP antigens individually and latent class analysis (LCA) as a reference test, since there is no gold standard test for this purpose. Considering the sample size analyzed, LCA classified 4,993 (99.6%) samples as *T. cruzi*-negative and 21 (0.42%) as *T. cruzi*-positive. Sensitivity values ranged from 85.71% for IBMP-8.1 and 90.48% for IBMP-8.2–95.24% for IBMP-8.3 and 100% for IBMP-8.4, while specificity ranged from 99.98% for IBMP-8.3 and IBMP-8.4–100% for IBMP-8.1 and IBMP-8.2. Accuracy values ranged from 99.4 to 99.98%. The pretest probability for the molecules was 0.42, whereas the positive posttest probability ranged from 95.24 to 99.95% and the negative posttest probability ranged from 0.00001 to 0.0006% for all antigens. The higher odds ratio diagnosis was found for IBMP-8.4, which has been shown to be a safe single antigen

for serological screening of CD in blood samples. The use of chimeric IBMP antigens is an alternative to reduce the number of bags discarded due to false-positive results. These molecules have high diagnostic performance and were shown to be suitable for use in screening CD in blood banks, isolated (IBMP-8.4) or in combination; and their use in blood banks could significantly reduce unnecessary disposal of blood bags or the risk of *T. cruzi* transmission.

Keywords: Chagas disease, blood bank, recombinant antigens, serological screening, diagnostic performance

INTRODUCTION

Human Chagas disease (CD) or American trypanosomiasis is a life-threatening, neglected tropical parasitic disease caused by the hemoflagellate protozoan *Trypanosoma cruzi*. According to recent estimates, approximately 6 million people in 21 Latin American countries are affected by CD and 7,500 CD-associated deaths are reported annually (1). *T. cruzi* is usually transmitted through contact with feces/urine from infected bloodsucking triatomines that harbor the parasite in their intestines. Due to constant presence of the vector, 65 million people in these regions are at risk of infection (1). In addition, other routes of transmission such as blood transfusion, organ donation, consumption of contaminated food or beverages, and mother-to-child transmission represent increasingly important alternative routes of infection (2, 3). Since the late 1990s, demographic shifts and migration flows have fueled the spread of *T. cruzi*-infected individuals worldwide, particularly in non-endemic countries in North America, Europe, and Oceania (4–6). Due to the lack of universal donor screening to exclude CD in blood banks, transmission through contaminated blood transfusions accounts for nearly 20% of new cases annually worldwide (7).

Laboratory diagnosis of CD depends on the stage of the disease. In the acute phase, which lasts about 2 months and is usually asymptomatic, the parasites are easily detected in the blood of infected individuals by direct parasitological tests, molecular biology methods, xenodiagnosis, or blood cultures (8). The chronic phase begins 8–10 weeks after the acute phase and may last for several years or even the entire life of the host. Due to intermittent or low parasitemia with high anti-*T. cruzi* antibody levels, CD diagnosis in the chronic phase requires the use of antigen-antibody detection techniques using *in vitro* diagnostic (IVD) techniques. These include indirect immunofluorescence (IIF), indirect hemagglutination (IHA), rapid diagnostic tests (RTDs), enzyme-linked immunosorbent assays (ELISA), and chemiluminescence-based immunoassays (CLIA) (8–11). Since there is no precise standard assay for serologic diagnosis of chronic *T. cruzi* infection, WHO and PAHO recommend the simultaneous use of two serologic tests based on different methods (e.g., RTD and ELISA or IHA and IIF) and/or antigens (e.g., recombinant antigens and whole parasite lysate) to improve diagnosis consistency (12, 13). Therefore, test algorithms vary by location (endemic or non-endemic areas) and application (screening of blood/organ donors or diagnosis) (14–17).

In blood banks, serologic screening for anti-*T. cruzi* antibodies should be performed using a high-sensitivity IVD (18, 19), which can be achieved by using purified, recombinant, or synthetic peptides as antigens mainly in ELISA or CLIA diagnostic platforms. Commercial tests for screening CD should be able to identify *T. cruzi* antibodies regardless of genetic variability, endemicity, and cross-reactivity with other infectious and parasitic diseases. The major challenge for blood banks in serological screening CD is to reduce both the number of blood bags that are incorrectly discarded due to false-positive results and the costs associated to assays used in the screening.

The Brazilian Health Regulatory Agency (ANVISA) reported serological inability for donation in 0.34% of all collections performed in Brazil due to non-negative results for CD in 2013, 0.16% in 2014, 0.21% in 2015, 0.16% in 2016, 0.26% in 2017, 0.17% in 2018 (20), and 0.15% in 2019 (21). Due to this high number of non-negative (and discarded) blood bags, the serological tests used for screening in blood banks must have high accuracy and low cross-reactivity. The Brazilian Ministry of Health has adopted only one test with high sensitivity (22), e.g., ELISA or chemiluminescence, because it is a high-throughput automated method that can analyze a large number of samples daily. On the other hand, high analytical sensitivity leads to a greater number of false-positive results, resulting in emotional distress to donors and improper disposal of blood bags (23). In addition, the high degree of genetic polymorphism of the parasite may have a direct impact on the performance of the test depending on the geographic region where the screening tests are performed (24).

To overcome these obstacles, assays with higher specificity and sensitivity are required. This can be achieved by using chimeric recombinant proteins as antigenic matrices for immunoassays, consisting of conserved and repeating regions of multiple *T. cruzi* proteins in a single molecule (25–27). This strategy allows maintaining high performance rates even when the assay is used in geographic regions where different genetic strains of the parasite circulate (28–30). To this end, four chimeric recombinant proteins (IBMP-8.1, IBMP-8.2, IBMP-8.3, and IBMP-8.4) were genetically engineered and tested in phase I (25) and II (30) studies using ELISA, liquid microarray (31), immunochromatographic (11), and impedimetric immunosensor (32) assays. These studies were performed with panels of previously characterized samples from different endemic settings in several Latin American countries and in their immigrants living in Barcelona/Spain. High accuracy and low cross-reactivity rates have been observed in several infectious and parasitic diseases, including leishmaniasis (30, 33). In addition, all antigens have been shown to maintain

their functional and structural stability under adverse conditions (34), making them robust and reliable candidates for future *in vitro* diagnostic assays that can be used for various models of point-of-care devices, including advanced biosensors. The performance of these antigens was evaluated using latent class analysis (LCA), a statistical tool used to evaluate new assays in the absence of a gold standard (35). Because the diagnostic potential of IBMP antigens has been extensively evaluated, the objective of this study was to evaluate the use of IBMP-8.1, IBMP-8.2, IBMP-8.3, and IBMP-8.4 chimeric *T. cruzi* antigens for serologic screening for Chagas disease in blood banks using a reference array of chimeric antigens as the gold standard.

MATERIALS AND METHODS

Synthesis of Recombinant Chimeric Antigens

Synthetic genes encoding *T. cruzi*-chimeric antigens were obtained from a commercial supplier (GenScript, Piscataway, NJ, USA), subcloned into the pET28a vector, and expressed in *Escherichia coli* BL21-Star DE3 (Thermo Fisher Scientific). Cells were grown in Lysogenic broth supplemented with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). *E. coli* lysates were prepared and His-labeled chimeric antigens were purified by affinity and ion exchange chromatography and then quantified using a fluorimetric assay (Qubit 2.0, Invitrogen Technologies, Carlsbad-CA, USA). Expression and purity of recombinant antigens were verified by SDS-PAGE (36). The plasmid constructs were described previously in Santos et al. (25). The antigenic composition of all four chimeric proteins is described in **Table 1**.

TABLE 1 | Constitution of the IBMP chimeric recombinant antigens.

Chimeric antigen	Sequence name	Amino acid range	Gene bank sequence ID
IBMP-8.1	Trans-sialidase	747–774	XP_820062.1
	60S ribosomal protein L19	218–238	XP_820995.1
	Trans-sialidase	1435–1449	XP_813586.1
	Surface antigen 2 (CA-2)	276–297	XP_813516.1
IBMP-8.2	Antigen, partial	13–73	ACM47959.1
	Surface antigen 2 (CA-2)	166–220	XP_818927.1
	Calpain cysteine peptidase	31-97	XP_804989.1
IBMP-8.3	Trans-sialidase	710–754	XP_813237.1
	Flagellar repetitive antigen protein	15–56	AAA30177.1
	60S ribosomal protein L19	236–284	XP_808122.1
	Surface antigen 2 (CA-2)	279–315	XP_813516.1
IBMP-8.4	Shed-acute-phase-antigen	681–704	CAA40511.1
	Kinetoplastid membrane protein KMP-11	76–92	XP_810488.1
	Trans-sialidase	1436–1449	XP_813586.1
	Flagellar repetitive antigen protein	20–47	AAA30177.1
	Trans-sialidase	740–759	XP_820062.1
	Surface antigen 2 (CA-2)	276–298	XP_813516.1
	Flagellar repetitive antigen protein	1–68	AAA30197.1
	60S ribosomal protein L19	218–238	XP_820995.1
	Microtubule-associated protein	421–458	XP_809567.1

Sample Collection

Samples were collected from volunteer blood donors at Hematology and Hemotherapy Foundation of the State of Bahia (HEMOBA Foundation) between December 2018 and August 2019 and stored in aliquots at −20°C. Because this is a prospective study (phase III), the results of screening tests performed by the HEMOBA Foundation for Chagas disease, syphilis, HIV-1/2, HTLV-1/2, hepatitis B (HBV), and hepatitis C (HCV), as well as the age, sex, and place of residence of blood donors, were kept confidential until the completion of the present study. The sample size was calculated with an expected sensitivity and specificity of 99%, an absolute error of 2%, a confidence interval of 95%, and a prevalence of chronic Chagas disease of 2% in the Brazilian population (37). Based on these parameters, the formula of Buderer (38) was used in the web version of the calculator (https://wnarifin.github.io/ssc/sssnsp.html) to estimate the minimum number of serum samples required to perform this study as 4,754. A total of 5,014 previously collected anonymized human serum samples were used to evaluate the individual performance of IBMP chimeras for *T. cruzi* by ELISA, using latent class analysis (LCA) as the reference test, as previously determined (33, 35, 39).

Immunoassays (IBMP-ELISA)

Anti-*T. cruzi* serology was performed by ELISA as described previously (30). Assays were performed on transparent 96-well flat-bottom microplates (UV-Star® Microplate, Greiner Bio-One, Kremsmünster, Austria) coated with one of the chimeric IBMP antigens at concentrations of 12.5 ng (IBMP-8.2) or 25 ng (IBMP-8.1, IBMP-8.3, and IBMP-8.4) per well in coating buffer (0.05 M carbonate bicarbonate, pH 9.6). Coating and blocking were performed simultaneously with a synthetic buffer (WellChampion; Kem-En-Tec Diagnostics A/S, Taastrup, Denmark) according to the manufacturer's instructions. Serum samples were added to the coated wells diluted 1:100 in 0.05 M phosphate-buffered saline (PBS; pH 7.4), and the microtiter plates were incubated at 37 °C for 60 min. Thereafter, all wells were washed with PBS-0.05% Tween-20 (PBS-T; pH 7.4) to remove non-adsorbed material and incubated again at 37 °C for 30 min with 100 μl of HRP-conjugated goat anti-human IgG (Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, Brazil) diluted 1:40,000 in PBS. After another wash cycle, 100 μl of TBM substrate (Kem-En-Tec Diagnostics A/S, Taastrup, Denmark) was added to the wells to detect the formation of immune complexes. Incubation was then performed for 10 min at room temperature in the dark. The colorimetric reactions were stopped by adding 50 μl of 0.3 M H_2SO_4 to each well. Optical density was determined in a SPECTRAmax 340PC microplate reader with a 450 nm filter (Molecular Devices, San Jose-CA, USA), and background values were subtracted from the measurement experiments.

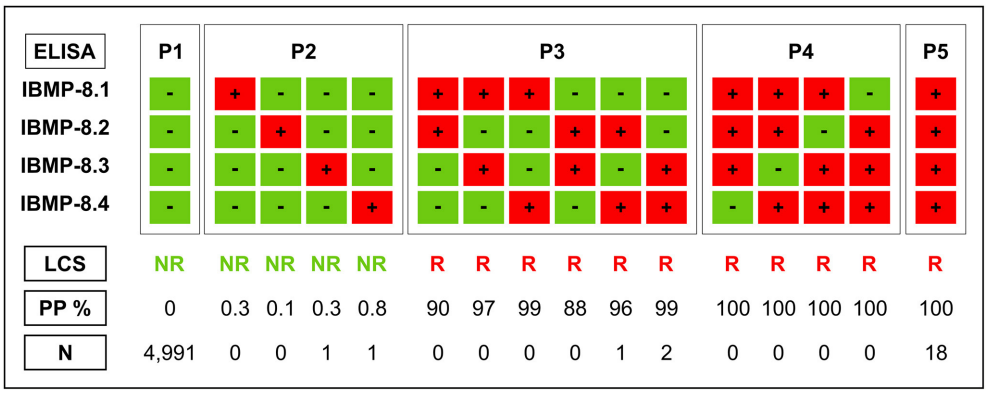


FIGURE 1 | Response patterns of chimeric antigens in latent class analysis (LCA) used in anti-*T. cruzi* ELISA tests in HEMOBA Foundation blood donor volunteers between December 2018 and August 2019. LCS, latent class status; NR, non-reactive; PP, a posteriori probability; R, reactive; P1, P2, P3, P4, and P5, reaction response; N, number of samples.

Latent Class Analysis as a Reference Test

Latent class analysis (LCA) was used for serological classification of *T. cruzi* as reactive or non-reactive for specific antibodies. This statistical model had been previously described and validated by our group in other studies (33, 35, 39). LCA is a multivariate statistical approach based on categorical indicators or latent variables. First, four indicators representing IBMP-8.1, IBMP-8.2, IBMP-8.3, and IBMP-8.4 were defined to characterize the latent variable that can correctly diagnose *T. cruzi* infection. Thus, the latent class response patterns defined a given sample as *T. cruzi* reactive if it showed positive results in at least two different chimera-based assays (*a posteriori* probability ranged from 87.9 to 100%). Conversely, a sample was considered non-reactive for *T. cruzi* if all four chimeric antigens gave a non-reactive result or if only one of the antigens was positive (*a posteriori* probability ranged from 0 to 0.8%) (**Figure 1**). A total of 16 response patterns were identified, which were divided into five categories (P1 to P5).

Statistical Analysis

Data were analyzed with Scatterplot software (Prism, version 8; GraphPad, San Diego-CA, USA). Descriptive statistics are presented as geometric means ± standard deviations. The Shapiro-Wilk test followed by Student's *t*-test was used to test normality of the data sets. Wilcoxon's signed-rank test was used when the assumed homogeneity could not be confirmed. A significance level of 5% was assumed for all statistical tests (p-value < 0.05). Threshold (cut-off) analysis was used to determine the optimal optical density value (OD) to discriminate between *T. cruzi*-negative and positive blood bags. The threshold was determined by calculating the area under the ROC curve (AUC). The AUC values were also used to assess the global accuracy for each antigen, which could be classified as low (0.51–0.61), moderate (0.62–0.81), elevated (0.82–0.99), or outstanding (1.0) (40). The performance of ELISA-IBMP was calculated using a dichotomous approach (2 × 2 contingency table), and the performance characteristics of each IBMP protein were compared in terms of sensitivity, specificity, accuracy, likelihood ratio (LR), diagnostic odds ratio (DOR), predictive values, and post-test probabilities (41, 42). To better assess the diagnostic performance of the four IBMP chimeras, multiple testing (serial and parallel approaches) was applied to individual test characteristics. Multiple tests can be ordered simultaneously (parallel tests), in which case a positive result in any of the tests is evidence of disease, or they can be ordered sequentially (serial tests), as new tests are requested depending on the result of the previous test. In this case, all results must be positive to establish a diagnosis of disease. (43). Confidence intervals (CI) with a 95% confidence level (95% CI) were used, and the absence of overlapping 95% CI bars was used to derive statistical significance (44). The results were expressed as an index representing the ratio between the OD of the samples and the OD of the cut-off. This index is called the reactivity index (RI) and all results >1.00 were considered positive. Samples were considered inconclusive (or in the gray zone) if the RI values fell in the indeterminate zone, which was assumed to be RI values of 1.0 ± 10%. Statistical analysis of RIs was performed based on the absence of overlapping 95% CI. The strength of agreement between the results of the screening tests IBMP-ELISA and the result of LCA was assessed with the Cohen's kappa coefficient (κ) (45) interpreted as follows: poor ($\kappa = 0$), slight ($0 < \kappa \leq 0.20$), fair ($0.21 < \kappa \leq 0.40$), moderate ($0.41 < \kappa \leq 0.60$), substantial ($0.61 < \kappa \leq 0.80$), and almost perfect ($0.81 < \kappa \leq 1.0$) agreement. The study workflow (**Figure 2**) was established according to STARD guidelines (46).

RESULTS

Diagnostic Performance

A total of 5,014 previously collected, anonymized human serum samples were included in the study. The mean age of the population studied was 40.4 years [interquartile range (IQR): 28.3–58.2 years] and the female-to-male ratio was 0.75/1. At least one blood donation was performed in each microregion of Bahia. This represents 232 of 417 (55.6%) municipalities and a total population of 11,448,009 inhabitants (∼77% of Bahia's population). Seventy-nine samples were from blood donors from

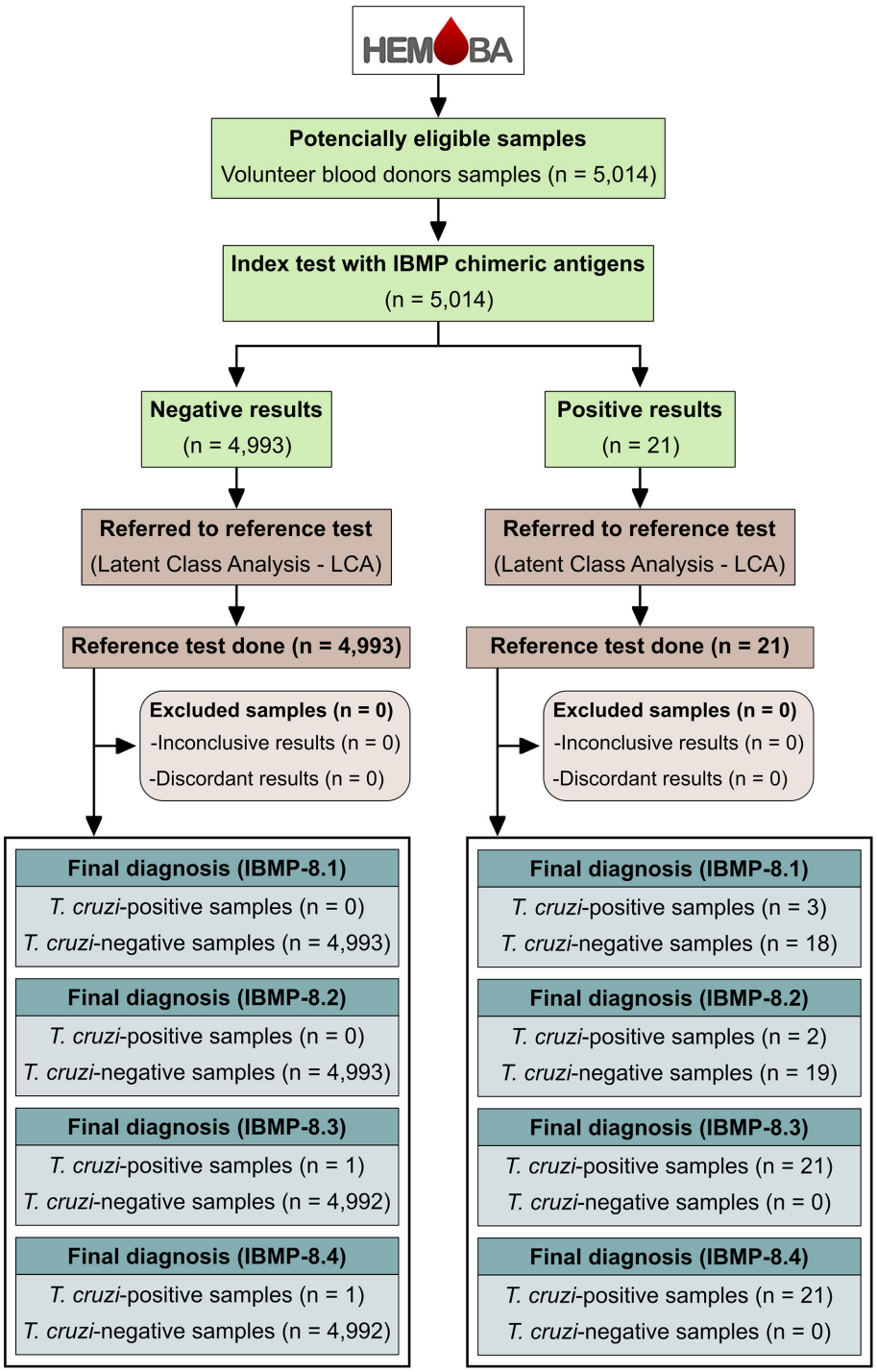


FIGURE 2 | Flowchart depicting study design in accordance with the Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines (46).

the Federal District ($n = 2$) and other Brazilian states: Rio Grande do Norte ($n = 1$), Pernambuco ($n = 58$), Alagoas ($n = 1$), Minas Gerais ($n = 5$), Goiás ($n = 2$), Espírito Santo ($n = 2$), Rio de Janeiro ($n = 1$), São Paulo ($n = 4$), Paraná ($n = 1$), and Rio Grande do Sul ($n = 2$). Information on the geographic origin of the blood donors was missing in 26 samples.

All human sera were employed to evaluate the individual performance of IBMP chimeras for *T. cruzi* by ELISA using latent class analysis (LCA) as the reference test. LCA classified

TABLE 2 | Data stratified by sociodemographic variables and reactivity indices for chimeric IBMP antigens from all 21 blood donors classified as *T. cruzi*-positive by latent class analysis.

Sample ID	Sex	Age	Microregion	RI 8.1	RI 8.2	RI 8.3	RI 8.4	LCA
3028	Male	52	Irecê	0.61	0.58	2.12	1.42	Pos
3295	Female	23	Jequié	1.46	1.38	1.22	1.93	Pos
4097	Male	27	Vitória da Conquista	0.32	0.29	1.99	2.60	Pos
4160	Male	49	Barreiras	1.74	1.84	1.33	1.44	Pos
4465	Female	40	Irecê	2.34	2.75	2.68	2.86	Pos
5231	Male	47	Barreiras	1.56	2.82	2.69	2.38	Pos
5617	Female	28	Salvador	1.59	2.78	2.21	2.20	Pos
5618	Male	39	Barreiras	0.44	1.14	0.79	1.15	Pos
5900	Male	29	Salvador	2.22	2.32	2.41	1.91	Pos
5901	Male	50	Sto Antônio de Jesus	2.55	2.65	2.93	2.46	Pos
5917	Male	41	Cotegipe	1.97	2.36	2.10	1.61	Pos
5918	Female	43	Feira de Santana	1.88	1.91	1.59	1.98	Pos
5936	Female	41	Salvador	1.69	1.29	1.14	1.81	Pos
6797	Female	30	Sto Antônio de Jesus	2.67	2.87	2.58	2.20	Pos
6802	Male	37	Cotegipe	1.54	1.41	2.00	1.31	Pos
6827	Male	41	Salvador	2.40	2.21	2.52	2.31	Pos
6840	Male	51	Salvador	3.35	3.71	2.80	2.68	Pos
6856	Male	51	Barreiras	2.73	2.48	2.02	2.33	Pos
6920	Female	63	Feira de Santana	2.89	1.93	3.24	2.67	Pos
7013	Male	30	Salvador	1.92	1.83	1.70	1.95	Pos
7087	Female	59	Brumado	2.28	2.21	1.58	2.07	Pos

RI, Reactivity Index; Sto Antônio de Jesus, Santo Antônio de Jesus; Cut-off = 1.0.

4,993 samples (99.58%) as *T. cruzi*-negative, of which 4,991 and two samples were categorized as P1 (negative result for all four IBMP proteins) and P2 (negative result for three IBMP proteins), respectively. The remaining 21 samples (0.42%) were categorized as *T. cruzi*-positive: 18 were categorized as P5 (positive for all four IBMP proteins) and three as P3 (positive result for 2 IBMP proteins). Sociodemographic variables for all 21 *T. cruzi*-positive samples and reactivity indices for all four chimeric IBMP antigens are summarized in **Table 2**. For these samples, the mean age of *T. cruzi*-positive blood donors was 41.0 (IQR: 30.0–50.5 years) and the female-to-male ratio was 0.62/1.

Following the serological definition of 5,014 samples as *T. cruzi*-positive or *T. cruzi*-negative by LCA, the performance parameters of chimeric IBMP proteins were determined (**Figure 3**; individual data points are available in the **Supplementary Table 1**). Area under the curve (AUC) values were extremely high for all chimeric proteins, ranging from 98.68 (IBMP-8.2) to 100% (IBMP-8.4), demonstrating excellent overall diagnostic accuracy for all chimeric proteins. Considering a 95% confidence interval, all IBMP antigens showed similar performance. For *T. cruzi*-positive samples, IBMP-8.4 provided the highest RI (reactivity index) values, while IBMP-8.1 had the lowest RI distribution. No significant differences were observed between the RIs of all four IBMP proteins. *T. cruzi*-negative samples yielded low mean RI values among all four chimeric antigens tested (<0.22). Global RI analysis showed a significant difference between *T. cruzi*-positive and negative samples for all four proteins ($p < 0.0001$).

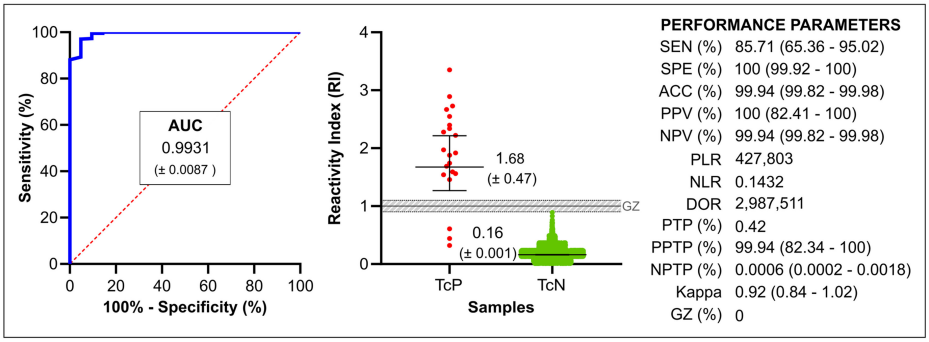
The diagnostic efficiency of antigens can also be assessed by the number of samples that fall into the gray zone. Considering a gray zone set as a cut-off value ± 10% (RI values of 1.0 ± 0.10), only one sample (0.02%) was found in the gray zone for the proteins IBMP-8.2 (*T. cruzi*-negative sample; sample ID 5245; RI 0.92), IBMP-8.3 (*T. cruzi*-negative sample; sample ID 7017; RI 0.97), and IBMP-8.4 (*T. cruzi*-negative sample; sample ID 6834; RI 0.92). No result was found in the gray zone when both *T. cruzi*-positive and *T. cruzi*-negative samples were tested with IBMP-8.1 antigen.

The IBMP-8.4 antigen yielded a sensitivity of 100%, followed by IBMP-8.3 (95.2%), IBMP-8.2 (90.5%), and IBMP-8.1 (87.7%). The differences in sensitivity were not statistically significant for the values obtained for all four proteins. The highest value for specificity was obtained for IBMP-8.1 and IBMP-8.2 proteins (100%), while IBMP-8.3 and IBMP-8.4 had values of 99.98%, with no differences between them. Regarding diagnostic accuracy, all chimeric proteins yielded values ≥ 99.94% (**Figure 3**). DOR Scores, based on positive and negative likelihood ratios, were 10,483,200 for IBMP-8.4, 4,722,244 for IBMP-8.2, 427,803 for IBMP-8.1, and 99,840 for IBMP-8.3. Qualitative assessment of the results showed near-perfect agreement between all chimeric IBMP proteins using the Cohens' kappa method ($\kappa \geq 0.92$), with particular emphasis on IBMP-8.4 ($\kappa = 0.98$).

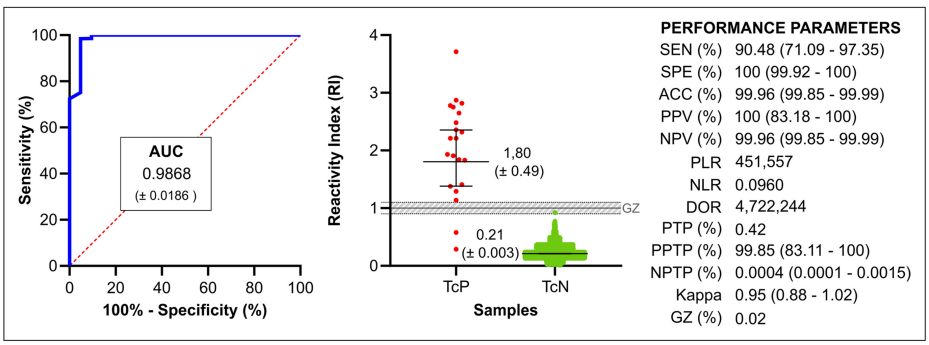
Predictive Values

Because this was a phase 3 study, it was possible for the first time to determine the positive and negative predictive values

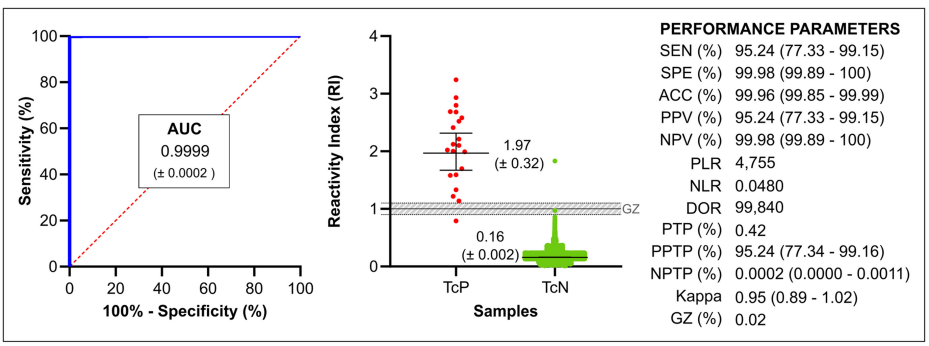
IBMP-8.1



IBMP-8.2



IBMP-8.3



IBMP-8.4

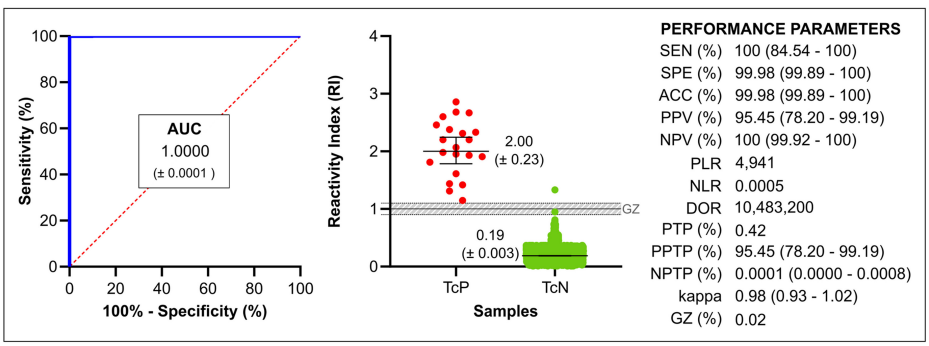


FIGURE 3 | Graphical analysis of areas under the curve (AUC) ROC (left). Reactivity index (middle) obtained with serum samples from *Trypanosoma cruzi*-positive (TcP) and *Trypanosoma cruzi*-negative (TcN) samples. The cut-off value is the reactivity index = 1.0 and the shaded area represents the gray zone (RI = 1.0 ± 0.10). The horizontal lines and numbers for each group of results represent the geometric means (± 95% CI). Performance parameters (right) obtained for all chimeric IBMP proteins. SEN, sensitivity; SPE, specificity; ACC, accuracy; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; DOR, diagnostic odds ratio; PTP, pre-test probability; PPTP, positive post-test probability; NPTP, negative post-test probability; *Kappa,* cohen's *Kappa* coefficient; GR, gray zone.

TABLE 3 | Analysis of the diagnostic performance of the pair of chimeric IBMP proteins using serial and parallel approaches.

Pair of tests Series	SEN % (95% CI)	SPE % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
IBMP-8.1/IBMP-8.2	77.6 (46.5–92.5)	100 (99.8–100)	100 (68.6–100)	99.9 (99.7–100)
IBMP-8.1/IBMP-8.3	81.6 (50.6–94.2)	100 (99.8–100)	95.2 (63.7–99.2)	99.9 (99.7–100)
IBMP-8.1/IBMP-8.4	85.7 (55.3–95.0)	99.9 (99.8–100)	95.5 (64.4–99.2)	99.9 (99.7–100)
IBMP-8.2/IBMP-8.3	86.2 (55.0–96.5)	99.9 (99.8–100)	95.2 (64.3–99.2)	99.9 (99.7–100)
IBMP-8.2/IBMP-8.4	90.5 (60.1–97.4)	99.9 (99.8–100)	95.5 (65.1–99.2)	100 (99.9–100)
IBMP-8.3/IBMP-8.4	95.2 (65.4–99.2)	99.9 (99. 8–100)	90.9 (60.5–98.6)	99.9 (99.8–100)
Parallel	**% (95% CI)**	**% (95% CI)**	**% (95% CI)**	**% (95% CI)**
IBMP-8.1/IBMP-8.2	98.6 (90.0–99.9)	100 (99.9–100)	100 (97.0–100)	100 (99.9–100)
IBMP-8.1/IBMP-8.3	99.3 (92.2–99.9)	100 (99.9–100)	100 (96.0–100)	100 (99.9–100)
IBMP-8.1/IBMP-8.4	100 (94.7–100)	100 (99.9–100)	100 (96.2–100)	100 (99.9–100)
IBMP-8.2/IBMP-8.3	100 (93.5–100)	100 (99.9–100)	100 (96.2–100)	100 (99.9–100)
IBMP-8.2/IBMP-8.4	100 (95.5–100)	100 (99.9–100)	100 (96.3–100)	100 (99.9–100)
IBMP-8.3/IBMP-8.4	100 (96.5–100)	100 (99.9–100)	99.8 (95.1–99.9)	100 (99.9–100)

CI, confidence interval; SEN, sensitivity; SPE, specificity; PPV, positive predictive value; NPV, negative predictive value.

of the IBMP proteins. The highest positive predictive value was obtained with IBMP-8.1 and IBMP-8.2 proteins (100%), followed by IBMP-8.4 (95.5%) and IBMP-8.3 (95.2%). All chimeric proteins yielded a negative predictive value >99.9%. Considering the 95% CI overlap, no statistical differences were observed in the positive and negative predictive values among the IBMP proteins. The pretest probability refers to the prevalence of the disease in the analyzed sample. It was estimated to be 0.42% of the samples regardless of the IBMP protein tested. IBMP-8.1 and IBMP-8.2 yielded the highest values for positive post-test probability: 99.94 and 99.85%, respectively. IBMP-8.3 provided the lowest value for positive post-test probability (95.24%), followed by IBMP-8.4 (99.45%). As for the negative post-test probability, all proteins yielded values ≤0.0006. At a confidence interval of 95%, all IBMP antigens showed similar positive and negative post-test probabilities. IBMP-8.4 offered the best performance among the chimeric recombinant proteins studied, as shown by the analysis of ROC and, most importantly, by the exceptionally high diagnostic odds ratio of this protein (DOR = 10,483,200; **Figure 3**).

Diagnostic Performance of IBMP Pairs

In addition to individual performance, the performance of pairs of all four chimeric IBMP proteins was also estimated in serial and parallel approaches (**Table 3**). In the serial scheme, sensitivity ranged from 77.6 to 95.2%, whereas minimum specificity and negative predictive values reached 99.9%. Positive predictive values ranged from 90.9 to 100%. Conversely, sensitivity values ranged from 98.3 to 100% with a parallel scheme. Interestingly, no false-negative result was obtained when the positive samples were tested with IBMP-8.1/IBMP-8.4, IBMP-8.2/IBMP-8.3, IBMP-8.2/IBMP-8.4, and IBMP-8.3/IBMP-8.4 pairs. Regardless of the IBMP pairs analyzed, no false-positive result was obtained using a parallel approach. Positive and negative predictive values were 100% for all IBMP pairs, except for the positive predictive value when IBMP-8.3/IBMP-8.4 was analyzed (99.8%).

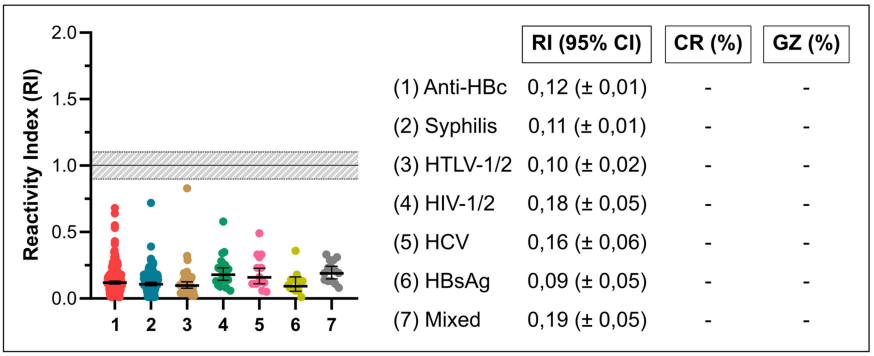
Cross Reaction Analysis

According to the serologic screening performed by the HEMOBA Foundation with the 4,993 *T. cruzi*-negative sera in the present study, 233 samples tested positive for anti-HBc, 150 for syphilis, 37 for HTLV-1/2, 20 for HIV-1/2, 15 for HCV, and 12 for HBsAg. Mixed infections were detected in 14 sera: anti-HBc + syphilis ($n = 4$), anti-HBc + HBsAg ($n = 2$), anti-HBc + HBV ($n = 2$), HBsAg + HBV ($n = 1$), HTLV-1/2 + syphilis ($n = 1$), anti-HBc + HTLV-1/2 ($n = 1$), HIV-1/2 + syphilis ($n = 1$), HCV + syphilis ($n = 1$), and HTLV-1/2 + syphilis ($n = 1$). All these positive samples were used to evaluate the potential cross-reactivity (RI ≥ 1.0) of the IBMP chimeric proteins. As shown in **Figure 4** (individual data points are available in the **Supplementary Table 2**), no cross-reactivity was found. In addition, only one sample that was anti-HBc positive (0.43%) was found to be inconclusive with the chimeric proteins IBMP-8.2 (sample ID 5245; RI 0.92) and IBMP-8.4 (sample ID 6834; RI 0.95). Among the 21 *T. cruzi*-positive samples, two were coinfected with HTLV-1/2, two with syphilis, one with both HCV and syphilis, and one was also positive for anti-HBc.

IBMP Antigens for Blood Screening

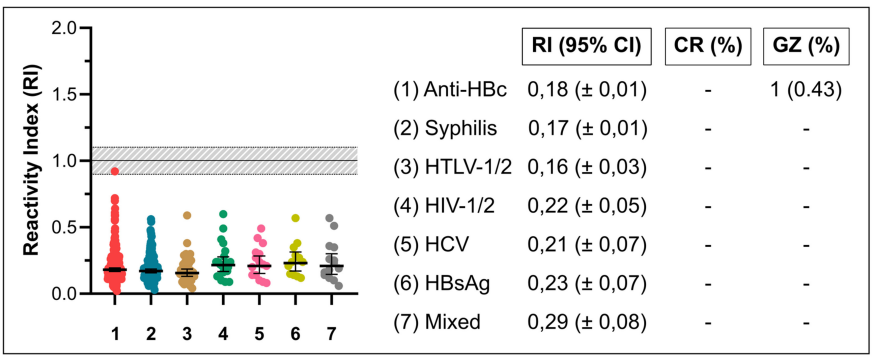
Individual use of chimeric IBMP proteins for CD serological screening was also analyzed (**Figure 5**). For this purpose, the criteria used by the HEMOBA Foundation were considered: (1) *T. cruzi*-positive samples: RI ≥ 1.00; (2) *T. cruzi*-negative samples: RI < 0.75; and (3) *T. cruzi*-inconclusive samples: 0.75 ≥ RI < 1.00. Both *T. cruzi*-positive and *T. cruzi*-inconclusive samples are considered unsuitable for blood donation; therefore,

IBMP-8.1



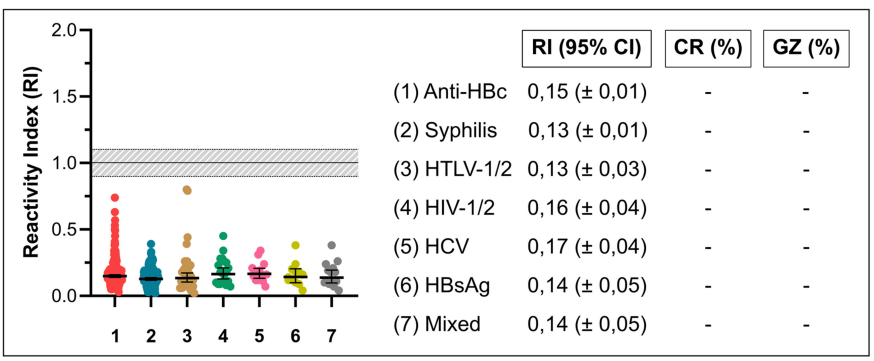
	RI (95% CI)	CR (%)	GZ (%)
(1) Anti-HBc	0,12 (± 0,01)	-	-
(2) Syphilis	0,11 (± 0,01)	-	-
(3) HTLV-1/2	0,10 (± 0,02)	-	-
(4) HIV-1/2	0,18 (± 0,05)	-	-
(5) HCV	0,16 (± 0,06)	-	-
(6) HBsAg	0,09 (± 0,05)	-	-
(7) Mixed	0,19 (± 0,05)	-	-

IBMP-8.2



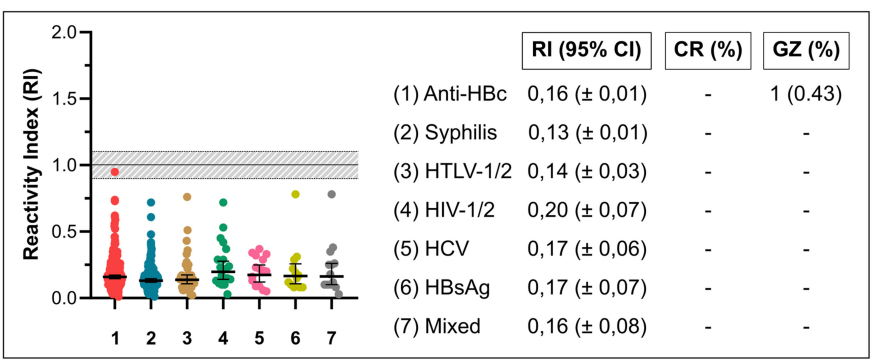
	RI (95% CI)	CR (%)	GZ (%)
(1) Anti-HBc	0,18 (± 0,01)	-	1 (0.43)
(2) Syphilis	0,17 (± 0,01)	-	-
(3) HTLV-1/2	0,16 (± 0,03)	-	-
(4) HIV-1/2	0,22 (± 0,05)	-	-
(5) HCV	0,21 (± 0,07)	-	-
(6) HBsAg	0,23 (± 0,07)	-	-
(7) Mixed	0,29 (± 0,08)	-	-

IBMP-8.3



	RI (95% CI)	CR (%)	GZ (%)
(1) Anti-HBc	0,15 (± 0,01)	-	-
(2) Syphilis	0,13 (± 0,01)	-	-
(3) HTLV-1/2	0,13 (± 0,03)	-	-
(4) HIV-1/2	0,16 (± 0,04)	-	-
(5) HCV	0,17 (± 0,04)	-	-
(6) HBsAg	0,14 (± 0,05)	-	-
(7) Mixed	0,14 (± 0,05)	-	-

IBMP-8.4



	RI (95% CI)	CR (%)	GZ (%)
(1) Anti-HBc	0,16 (± 0,01)	-	1 (0.43)
(2) Syphilis	0,13 (± 0,01)	-	-
(3) HTLV-1/2	0,14 (± 0,03)	-	-
(4) HIV-1/2	0,20 (± 0,07)	-	-
(5) HCV	0,17 (± 0,06)	-	-
(6) HBsAg	0,17 (± 0,07)	-	-
(7) Mixed	0,16 (± 0,08)	-	-

FIGURE 4 | Graphical analysis of cross-reactivity with IBMP antigens using non-negative samples screened by HEMOBA Foundation. 95% CI, 95% confidence interval; IR, reactivity index.

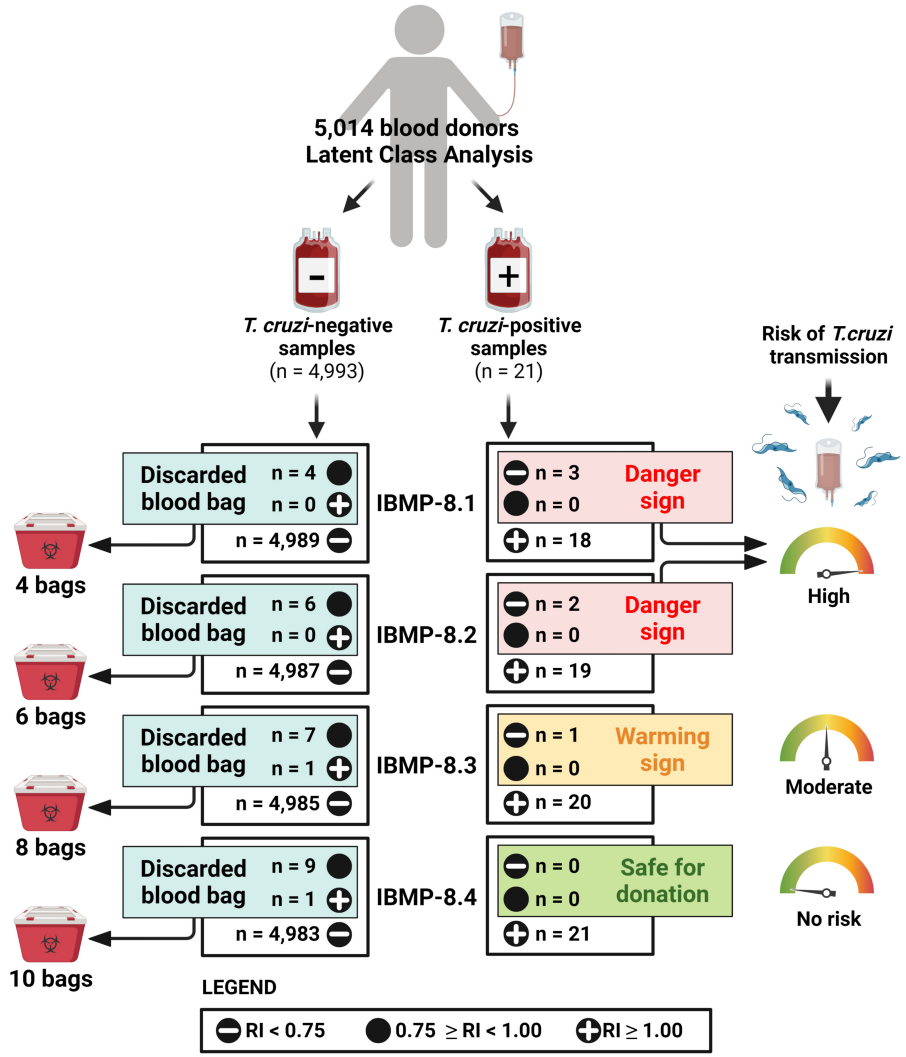


FIGURE 5 | Analysis of individual use of chimeric IBMP proteins for CD serological. RI, reactivity index.

the blood bags are discarded. Of the 4,993 *T. cruzi*-negative samples, IBMP-8.1, IBMP-8.2, IBMP-8.3, and IBMP-8.4 proteins classified four, six, seven, and nine blood bags, respectively, as *T. cruzi*-inconclusive samples, while IBMP-8.3 and IBMP-8.4 classified one sample each as *T. cruzi*-positive. Accordingly, screening with IBMP-8.4 would discard a total of ten *T. cruzi*-negative blood bags, followed by eight bags screened with IBMP-8.3, six bags screened with IBMP-8.2, and four bags screened with IBMP-8.1 protein. Conversely, all 21 *T. cruzi*-positive blood bags were correctly identified as positive when tested with IBMP-8.4 protein. One positive sample yielded an inconclusive result when tested with IBMP-8.3, which triggered a warning signal with a clear risk of *T. cruzi* transfusion transmission. A danger signal was triggered when IBMP-8.1 and IBMP-8.2 proteins gave a negative result in three and two bags, respectively, indicating a high risk of *T. cruzi* transmission.

DISCUSSION

In the present study, we evaluated the performance of the chimeric antigens for serological screening of CD and their potential use in blood banks. All four IBMP antigens showed high diagnostic capacity based on the AUC values found, which ranged from 98.68 to 100%, suggesting high discriminatory ability. These results are consistent with the phase I (11, 25, 32) and phase II (28–31) studies. Comparing the AUC values found here with those reported in the literature for other antigens, IBMP antigens showed higher values than those reported for mixtures of different synthetic epitopes (47), multiepitope antigens (48) and assays such as the Abbott Chagas Elisa (49). Conversely, some multiepitope antigens, such as CP1, CP3, and CP1 + CP3, showed similar AUC values to IBMP antigens (50).

The antigen IBMP-8.4 had the highest sensitivity value in the present study as well as in the previously performed studies, regardless of the population studied and the methodology used. This is due to the nature of the molecule, as it comprises a larger repertoire of epitopes compared to the others, making it responsive to a greater diversity of anti-*T. cruzi* antibodies (25, 30, 34, 35). In contrast to the IBMP-8.4 protein, identification of anti-*T. cruzi* was less efficient in blood donors with IBMP-8.1, probably due to its limited repertoire of antigens. This result contrasts with the results of other studies that have used this molecule as an antigenic matrix. In the sensitivity analysis, 21 samples were predicted by LCA to be positive. This small number of positive samples has a strong influence on the determination of sensitivity, since each false-negative result corresponds to a 4.76% reduction in the sensitivity value. The opposite is true for specificity, where a single false-positive sample would reduce the value by only 0.02%. Considering that this is a phase III study (blind study), it was not possible to control the sample size of each group (positive and negative). Nevertheless, the accuracy values for all antigens were $\geq$99.4%, thanks to the large number of negative samples and the high specificity of all antigens (99.98 to 100%).

Among the 21 *T. cruzi*-samples specimens positive at LCA classification, six were positive for coinfection with other diseases: HTLV, syphilis, HBV, and HCV. HTLV, HCV, and syphilis have similar characteristics: they are sexually transmitted, endemic, and considered a public health problem in Brazil. Their specific mode of transmission facilitates coinfection with other diseases (51–53). On the other hand, the prevalence rate for HBV in Brazil is low, most likely due to vaccination, which has been available for more than 20 years (54, 55), although there are still unvaccinated individuals at risk. HBV is transmitted through blood (parenterally and vertically), sexually, and by sharing contaminated objects (55), so it can be said that coinfections between HTLV, HCV, syphilis, and HBV are common because of their routes of transmission. In our study, there was no cross-reactivity to any of the four IBMP antigens. Only two samples were classified in the inconclusive zone (RI $\pm$ 10%) when analyzed with IBMP-8.2 and IBMP-8.4 antigens. These results confirm previous studies performed with the molecules for various infectious and parasitic diseases, such as dengue, HBV, HCV, HIV, HTLV, leishmaniasis, schistosomiasis, filariasis, leptospirosis, measles, rubella, and syphilis (30, 33).

Recommendations for serologic screening in blood banks vary according to the CD endemic area. In endemic countries, screening should be performed with a high-sensitivity IVD (18, 19), whereas in non-endemic countries, screening should take into account that (1) all donors with a history of CD should be permanently deferred; (2) if screening tests for CD are not available, all donors with a recognized risk for CD should be identified and permanently deferred; and (3) if screening tests for CD are available, all donors with an identified risk for CD should initially be deferred for 6 months after their last return from an endemic area. Their subsequent donations should then be screened for signs of infection using a highly sensitive IVD (18). In recent years, purified, recombinant, or synthetic peptide antigens have been used as solid phase in IVD for detection of anti-*T. cruzi* antibodies with acceptable sensitivity values for safe serological CD screening in blood banks.

After analyzing the performance parameters of the four all IBMP antigens, an analysis of the individual use of each molecule in serological screening for CD. Normally, according to safety criteria, blood banks set the cutoff point at 20 to 25% of the manufacturer's specified value to reduce the possibility of transmission of bloodborne pathogens as much as possible; HEMOBA Foundation lowers the cutoff value to 25%. Considering these cut-off values, four, six, seven, and nine bags were rejected as false positive for IBMP-8.1, IBMP-8.2, IBMP-8.3, and IBMP-8.4 antigens, respectively. The antigens IBMP-8.1 and IBMP-8.2 detected as false-negative four and two bags, respectively, indicating that their use alone in serological screening of CD in blood banks is not recommended because of the possibility of transmission during transfusion. However, the combined use of these molecules is safe because the false-negative or exclusion zone results are not the same when the four assays are compensated. Despite the greater number of bags discarded when the IBMP-8.4 molecule was used, this was the safest molecule to be used alone in serological screening in blood banks.

The disposal of 29 negative bags harms public health, not only because of the resources invested in collection, donor pickup, and serologic screening, but also because of the indirect costs of reducing the supply of blood available for transfusion. Overall, the prevalence of CD in Brazil is estimated at $\sim$2.16% (4.6 million people) (37), which is considered low. Therefore, a test with a specificity of $<$98.5% would lead to more false-positive than true-positive results (56). This gap could be easily closed by using the four antigens, especially with IBMP-8.1 and IBMP-8.2, since they have a specificity of 100%. The combined use of molecules, e.g., IBMP-8.4 in the first stage of diagnosis, would eliminate all false-negative results, then IBMP-8.1 or IBMP-8.2 can be used to exclude false-positive results. In this way, we would have a more effective and safer diagnosis, which would result in a lower number of blood bag disposals and reduce the cost of monitoring blood quality for transfusions.

In summary, this was a phase III study evaluating the four chimeric recombinant antigens IBMP-8.1, IBMP-8.2, IBMP-8.3, and IBMP-8.4 for serological diagnosis of chronic Chagas disease. The molecules exhibited high diagnostic performance and were shown to be suitable for screening CD in blood banks, isolated (IBMP-8.4) or in combination. Their use in blood banks could significantly reduce unnecessary disposal of blood bags or the risk of *T. cruzi* transmission.

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 studies involving human participants were reviewed and approved by Institutional Review Board (IRB) for

Human Research at the Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Bahia (BA), Brazil (CAAE 67809417.0.0000.0040). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

FS, MR, PC, MK, and NZ designed the experimental procedure. ES, ÂS, NF, LL, and RD performed the data acquisition, analysis, and interpretation. CS and MA determined the sample size. PC expressed and purified the chimeric recombinant antigens. ES performed the ELISA experiments. ES and FS wrote the paper. ÂS, NF, LL, and RD helped write the article. FS prepared the figures and supervised the work. FS, FA, MR, MK, and NZ provided laboratory space. FS, MR, MK, and NZ obtained funding for this study. All authors contributed substantially to the work described in this article, read, and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.852864/full#supplementary-material

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Biannual and Quarterly Comparison Analysis of Agglutinating Antibody Kinetics on a Subcohort of Individuals Exposed to *Leptospira interrogans* in Salvador, Brazil

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Introduction: Leptospirosis is a zoonosis with a worldwide spread that leads to clinical manifestations ranging from asymptomatic infection to a life-threatening disease. The immune response is predominantly humoral mediated limited to the infecting serovar. Individuals living in an area endemic for leptospirosis are often exposed to an environment contaminated with leptospires and there is a paucity of information on naturally acquired immunity. In the present study, we evaluated the kinetics of agglutinating antibodies in individuals from an endemic area for leptospirosis in Salvador, Brazil comparing two different intersample collection times.

Methods: Between 2017–2018, we carried out a biannual prospective cohort with 2,086 individuals living in an endemic area for leptospirosis in Salvador, Brazil. To compare agglutinating antibody kinetics using microscopic agglutination test (MAT) with different collection times, a subcohort of 72 individuals with quarterly follow-up was carried out in parallel.

Results: The results revealed that using a shorter time for intersample collection led to the detection of a higher number of infections and reinfection events. Furthermore, we observed a higher rate of titer decay indicating partial and short protection. However, there was no indication of major changes in risk factors for the disease.

Conclusions: We evaluated antibody kinetics among residents of an endemic area for leptospirosis comparing two sample collection times. The constant exposure to the contaminated environment increases the risk for leptospirosis infection with reinfection events being more common than expected. This indicates that the burden of leptospirosis might be underestimated by serological surveys, and further studies are necessary to better characterize the humoral response after infection.

Keywords: ***Leptospira*, leptospirosis, human, serosurvey, MAT, antibody kinetics**

INTRODUCTION

Leptospirosis is a zoonosis of worldwide distribution and an important reemerging disease caused by pathogenic spirochetes of the genus *Leptospira* (1). The disease is endemic in a diverse range of epidemiological settings given the high number of animal reservoirs that can harbor the bacteria in their kidneys and excrete in their urine (2, 3). The transmission in humans occurs mainly through contact with environmental sources contaminated with the urine of infected animals. Rodents are the main source of human infection and responsible for the maintenance of the bacteria in the urban environment (4, 5). The clinical manifestations of the disease vary from asymptomatic or mild to severe disease such as Weil syndrome and pulmonary hemorrhage syndrome, associated with a lethality rate of 10 and 50%, respectively (3, 6–8).

The World Health Organization (WHO) estimates the occurrence of more than one million human cases of leptospirosis worldwide, with more than 50,000 deaths each year, most of which occurs in developing countries and tropical and subtropical climate regions (9, 10). In Brazil, 13,000 severe cases are reported per year with a lethality rate of 10.8% (11). The occurrence of urban epidemics, in Brazil and similar regions, is associated with environmental, occupational, and recreational risk factors and the large population of reservoirs (4, 12). More than 300 serovars can cause disease in humans and animals (13), but serovars from *L. interrogans* species are the most pathogenic and common throughout the world (14). In Brazil, serovar Copenhageni is the leading cause of epidemics in urban environments representing more than 90% of infections in Salvador with *Rattus norvegicus* (rat or sewer rat) as the main carrier (11, 15).

During the course of leptospirosis infection, the immune response is predominantly humoral mediated by the production of circulating antibodies directed against the lipopolysaccharide (LPS) and limited to the infecting serovar (16). Individuals living in regions where leptospirosis is endemic, are frequently being exposed to *Leptospira* and there are reports of the presence of anti-*Leptospira* antibodies in patients recovering from severe disease and in individuals with no previous history of the disease, most likely resulting from asymptomatic infection (17). However, there is little information on whether these individuals develop a naturally acquired protective immunity against infection or severe disease (17). In the present study we evaluated the kinetics of the humoral response after leptospirosis infection on individuals living in an urban slum area to obtain relevant information that may contribute to the better understanding of the naturally acquired immunity against leptospirosis reinfection.

MATERIALS AND METHODS

Ethics Statement

This project was approved by the Research Ethics Committee of the Instituto Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), the National Research Ethics Council (CONEP) through the Certificate of Presentation for Ethical Appreciation (CAAE) #45217415.4.0000.0040 and the Yale University Institutional Review Board #1006006956. All participants received guidance on the objectives, procedures, and risks associated with participation during informed consent. Minors gave verbal consent and we obtained written consent by their parents or legal guardian. Collegiality of participation was assured. Laboratory results were made available to the cohort participants.

Study Site and Population

A prospective cohort study was carried out in the community of Pau da Lima (13° 32′53.47 "S: 38° 43′51.10" W), an urban slum community of Salvador, Bahia, Brazil (**Figure 1**). This community was described as an area of 0.24 km^2 made up of three valleys, with open sewage close to the residences, places of garbage accumulation, and risk of flooding mainly in the bottom areas of the valleys (**Figure 1**). Due to the irregular occupation of the land and the precarious urban infrastructure, the community of Pau da Lima presents similar conditions to other vulnerable communities in Brazil and tropical regions of the world (9, 18). Previous studies have shown that residents of this community are in contact with the contaminated environment throughout the year leading to a high risk of leptospirosis infection facilitated by rat infestation, contact with mud promoted by topographic factors such as home elevation and inadequate drainage (9, 19). The crude rate of *Leptospira* infection in this community was 37.8 per 1,000 person-years with a 2.3-fold higher rate of secondary infection when compared to the rate of primary infection (19).

Between September 2017 to December 2018, residents of Pau da Lima with ≥5 years and who slept at least three nights a week at home were enrolled in the biannual analysis study. During all visits (every 6 months), blood samples were collected for evaluation of anti-leptospire antibodies using the microscopic agglutination test (MAT). Previously validated epidemiological, exposure and sociodemographic questionnaires were applied annually for field data collection. At the moment of enrollment for the biannual study, individuals with a previous history of leptospirosis infection determined by MAT were enrolled for our subcohort, together with controls that had no history of infection. For this subcohort, a quarterly analysis follow-up was performed (every 3 months) for blood collection, with a total of five home visits to assess antibody kinetics. For the entire study, we considered as exclusion criteria the following: participant not found at the time of the team visit, refusal to participate in the study and inability to locate the participants after three attempts.

Serologic Evaluation for *Leptospira* Infection

The blood samples collected were sent to the Laboratory of Pathology and Molecular Biology at Instituto Gonçalo Moniz, Fiocruz, BA. Sera was obtained through centrifugation, and it was processed for the presence of agglutinating antibodies against *Leptospira* using the microscopic agglutination test (MAT). The MAT was performed with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, the most prevalent serovar in the region (9, 15, 18, 19). The screening was performed using 1:50 and 1:100 dilutions of serum. A positive sample was determined when agglutination was observed on more than 50% of leptospires compared to the control with no sera. If the sera were positive at the dilution of 1:100, the sample was titrated to

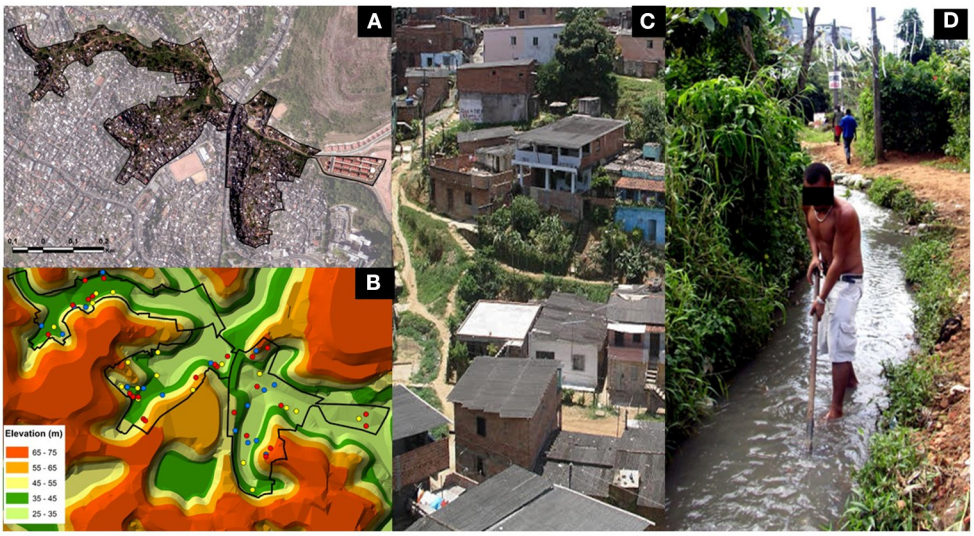

FIGURE 1 | Slum community site in the city of Salvador, Brazil. **(A)** Aerial photograph showing the study site boundary in the community of Pau da Lima, Brazil. **(B)** Topographic map highlighting the households participating in the subcohort study. In blue, households with uninfected individuals; in yellow, households with infected individuals and in red, households with reinfected individuals. Photographs showing social characteristics **(C)** and environmental risk factors **(D)** of the community.

determine the highest agglutination titer. The presence of anti-*Leptospira* agglutinating antibodies was used as a marker for previous infection. A case of *Leptospira* infection was defined as individuals with antibody titers ≥1:50 at any time-point, and/or with a seroconversion between consecutive time-points, defined as the absence of agglutination reaction in the first sample and the presence of agglutination with ≥1:50 in the following sample, and/or four-fold rise in titer between consecutive time-points. Reinfection was defined as participants who had two or more infections documented based on the MAT results during the follow-up period.

Statistical Analyses

Statistical analyses were performed using the RStudio package, version 1.2.5033. Descriptive analysis was performed to obtain absolute frequencies or means and medians for categorical variables and univariate analysis through Welch's two-sample *t*-test and Pearson's chi-square test, with 95% CI. Statistical significance was considered when the probability value $p \leq 0.05$. GraphPad Prism version 7 for Windows was used to evaluate the kappa coefficient of agreement between collection times and the kinetic data calculated in log10 and plotted against collection periods. A logistic regression model was used to determine the adjusted odds ratio (OR) (95% confidence intervals) to assess whether MAT titers protect against reinfection.

RESULTS

Enrollment and Follow-Up of Study Participants

During a cohort study for leptospirosis conducted from 2017–2018, a total of 2,086 individuals were enrolled for a biannual collection of blood for MAT assay and analysis of epidemiological data. Among them, 339 (16%) were serologically confirmed for leptospirosis infection. We enrolled 110 participants for a subcohort performing a quarterly analysis follow-up. Among those, 52 confirmed cases and 20 negative cases that had ≥4 blood samples at the end of the study were analyzed (**Figure 2**).

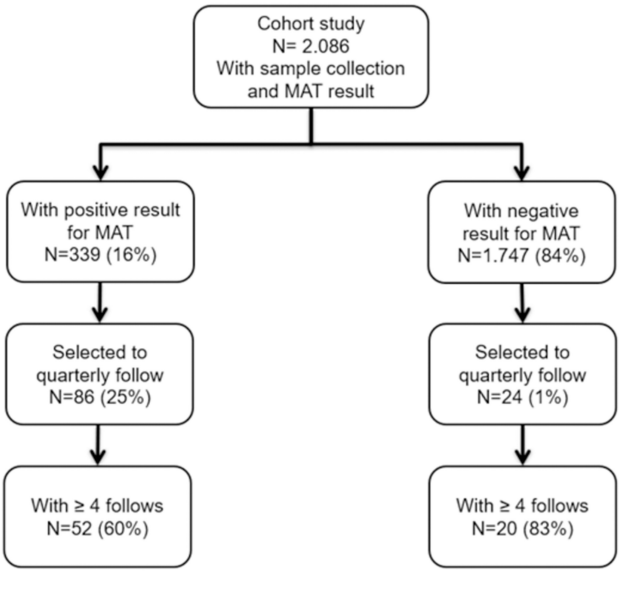

FIGURE 2 | Flowchart of the study participants enrollment.

Characteristic of the Participants

The demographic, socioeconomic and exposure information of all the cohort participants and the subcohort are shown

TABLE 1 | Sociodemographic and exposure characteristics of the participants enrolled in the study.

Characteristic	Cohort total ($N = 2,086$)[a]	Subcohort ($N = 72$)[a]
Median Age (years)	27 (17)	28 (16)
Age (years)		
05–14	631 (30.2%)	21 (29.2%)
15–24	404 (19.4%)	9 (12.5%)
25–34	399 (19.1%)	18 (25.0%)
35–44	292 (14.0%)	12 (16.7%)
> 44	360 (17.3%)	12 (16.7%)
Sex		
Female	1,206 (57.8%)	36 (50.0%)
Male	880 (42.2%)	36 (50.0%)
Ethnicity		
Black	944 (45.3%)	29 (40.3%)
Brown	969 (46.5%)	33 (45.8%)
White	154 (7.4%)	10 (13.9%)
Others	19 (0.9%)	0 (0.0%)
Education		
Up to 9th year	1,632 (78.2%)	56 (77.8%)
More than 9th year	454 (21.8%)	16 (22.2%)
Married or stable union	730 (35.0%)	22 (30.6%)
Informal employment	763 (36.6%)	36 (50.0%)
Per capita household income (US$/day)	4.7 (4.8)	4.3 (3.7)
Cleaned sewage	311 (14.9%)	10 (13.9%)
Open sewage at <10 m from home	1,464 (70.2%)	50 (69.4%)
Accumulated trash within <10 m of home	781 (37.4%)	24 (33.3%)
Sewage contact	777 (37.2%)	33 (45.8%)
Floodwater near home	822 (39.4%)	40 (55.6%)
Mud near home	949 (45.5%)	45 (62.5%)
Work in construction	179 (8.6%)	5 (6.9%)
Work related to hawker	47 (2.3%)	3 (4.2%)
Work related to garbage removal	74 (3.5%)	7 (9.7%)
Work involves contact with mud	60 (2.9%)	3 (4.2%)
Work involves contact with flood water	50 (2.4%)	3 (4.2%)
Work involves sewage contact	44 (2.1%)	3 (4.2%)
Fever	559 (26.8%)	16 (22.2%)

[a] *Median (IQR); n (%).*

in **Table 1**. Most of the participants were young adults with a median age between 27–28 years old, with 57.8% of women for the cohort and 50% for the subcohort, respectively. Regarding ethnicity, there was a predominance of brown race (46.5% for the cohort and 45.8% for the subcohort) and <9 years of education was reported in all groups (78.2 and 77.8%, respectively). Informal employment was described by 36.6% of the cohort and 50% of the subcohort participants. Construction work (8.6% for the cohort and 6.9% for the subcohort) and work related to garbage removal (3.5 and 9.7%, respectively) were most described activities in the groups. The median per capita household income was similar among groups, with US$ 4.7 daily for the cohort and US$ 4.3 daily for the subcohort. Among the exposure variables, in all groups open sewage was reported < 10 m from the house (70.2 and 69.4%), contact with sewage (37.2 and 45.8%), contact with floodwater near home (39.4 and 55.6%), contact with mud near home (45.5 and 62.5%) and 14.9% of the total cohort reported cleaning sewage followed by 13.9% of the subcohort group.

TABLE 2 | Concordance between the biannual and quarterly follow-up collections.

Quarterly follow-up		Biannual follow-up			
		Infection	Reinfection	No infection	Total
	Infection	12	2	8	22
	Reinfection	13	10	2	25
	No Infection	0	0	25	25
	Total	25	12	35	72

The kappa statistics for leptospirosis case classification at the different collection times was 0.48 (95% CI: 0.32–0.63), achieving moderate agreement (**Table 2**). In this evaluation we identified differences in leptospirosis case classifications when comparing biannual analysis and quarterly analysis collections, mainly between the infected vs. reinfected and no-infected vs. infected groups. When performing a biannual analysis, we identified 25 (34.7%) infections, 12 (16.6%) reinfections and 35 (48.6%) negative individuals, while in the quarterly analysis, we identified 22 (30.5%) infections, and 25 (34.7%) reinfections and non-infections, each. There are 13 (18%) individuals that would be classified as reinfection rather than infection when performing a quarterly analysis (**Table 2**, **Figure 3**). Furthermore, the quarterly analysis identified an extra 8 (11%) individuals as infection and 2 (2.8%) individuals as reinfection rather than no-infection determined by the biannual analysis (**Table 2**, **Supplementary Figure S1**). In contrast, there were only 2 individuals classified as reinfection by the biannual analysis that would be considered as infection by the quarterly evaluation (**Table 2**, **Supplementary Figure S1**). The multivariate analysis did not find an association between MAT titers and reinfection (**Table 3**). Taken together, those results indicate that the decay of agglutinating antibodies is shorter than expected in individuals exposed to leptospires without severe symptoms, and the time between assessment of those antibodies can influence the number of infections and reinfections. Further, our data suggests that agglutinating antibodies might not be the ideal correlates for naturally acquired immunity against reinfection.

Agreement Analysis

The distribution of MAT titers of samples positive for the Fiocruz L1-130 strain and their frequencies are listed in **Supplementary Table S1**. Of the total, 47 (65%) had anti-leptospire agglutinins in the quarterly analysis collection while 37 (51%) in the biannual analysis collection. Most participants in the group with quarterly analysis collection had low titers, with 62% ranging from 1:50 (28%) to 1:200 (21%). The highest agglutination titers in the biannual analysis collection period were observed at the 1:400 dilution (41%). Those results indicate that most of the exposures lead to low agglutinating titers.

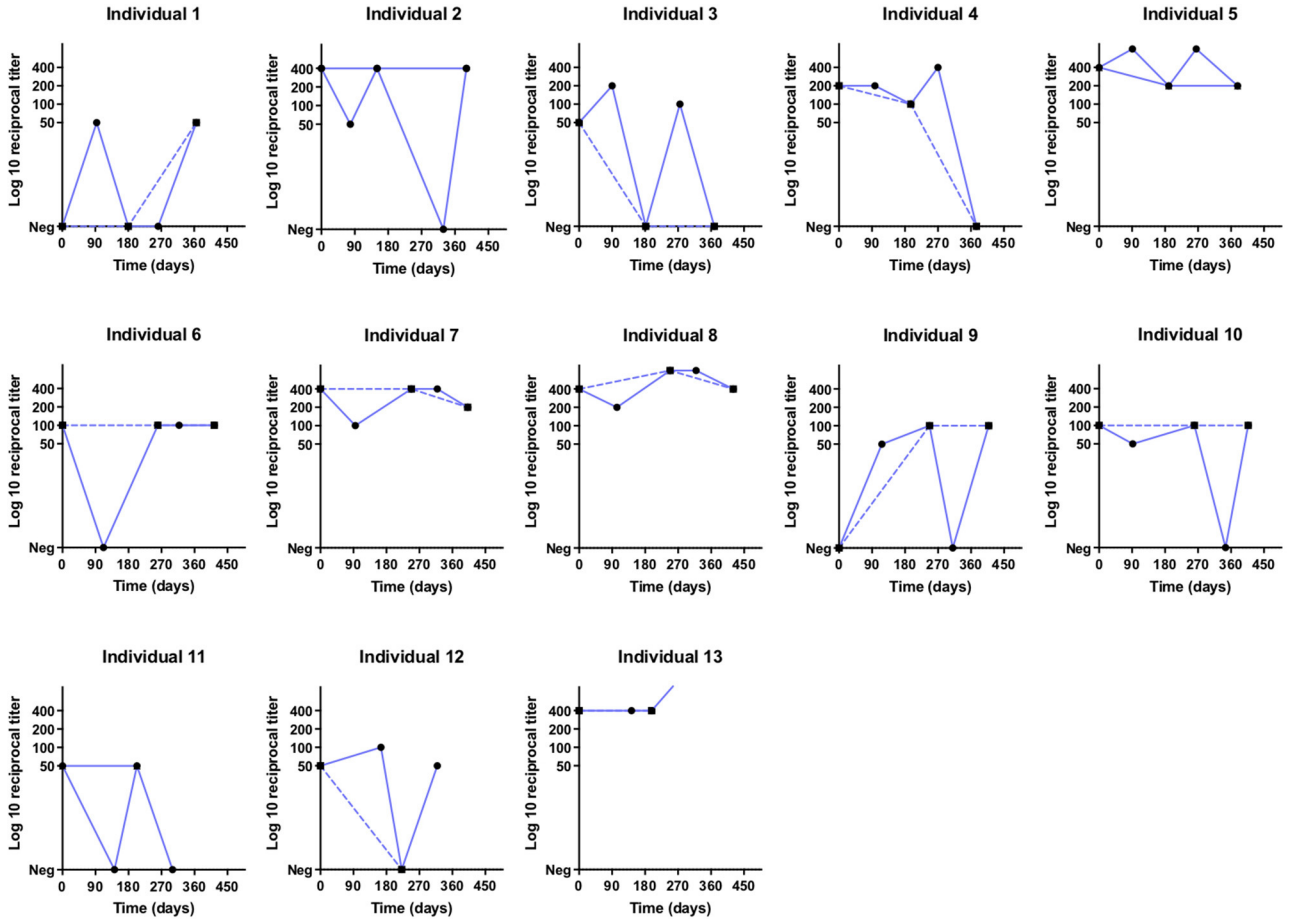

FIGURE 3 | Titration curves in log10 of individuals classified as reinfection in the quarterly analysis (•) and infection in the biannual analysis (•) at different times (days) of collection.

Biannual vs. Quarterly Follow-Up Analysis

We then evaluated the characteristics of the subcohort participants stratified by infection and non-infection based on different collection times (biannual vs. quarterly analysis) (**Table 4**). The variables age ($p < 0.001$ and $p = 0.005$) and cleaning sewage ($p = 0.003$ and $p = 0.033$) were associated with a higher chance of infection for both groups with different collection times. Furthermore, informal employment ($p = 0.013$) for the quarterly analysis collection group and being married or having a stable union ($p = 0.032$) for the biannual were also associated with a higher chance of infection when compared to the non-infection group (**Table 4**). A more detailed analysis of the participants per collection time stratifying the infection group by single infection and reinfection event showed that cleaning sewage was the only significant risk for infection ($p = 0.04$) on the quarterly analysis group (**Supplementary Table S2**). For reinfection, age ($p = 0.03$) and informal employment ($p = 0.01$) were both associated with a higher risk (**Supplementary Table S2**). A similar analysis using the biannual analysis data showed that variables age and cleaning sewage were both significant risks for infection and reinfection (**Supplementary Table S3**). Interestingly, having a married or stable union ($p = 0.047$) showed as a risk for infection on the biannual analysis (**Supplementary Table S3**). There was no statistically significant difference when comparing the infection and reinfection groups in both analyses with different collection times. Those results showed that despite the differences of infections and reinfections rates among the two different analyses, the risk for overall infection is similar in both groups, indicating that the time between serological evaluation might not have a major impact on the outcome of risk analysis.

DISCUSSION

The immunity against leptospirosis is based on a short-term humoral response for humans and animals (14). However, the few existing studies that report the kinetics of antibodies were performed in clinical patients associated with disease severity (20, 21) or in experimental animal models (14, 22). Evaluating antibody kinetics in individuals with natural *Leptospira* infection will help to better understand the duration of the immune response after infection, the course of infection, and the dynamics

TABLE 3 | Multivariate analysis to evaluate MAT titers as immune markers for reinfection on biannual and quarterly follow-up.

	Biannual analysis ($N = 47$)			Quarterly analysis ($N = 47$)		
Characteristic	**OR[a]**	**95% CI[b]**	***p*-value**	**OR[a]**	**95% CI[b]**	***p*-value**
(Intercept)	0.96	0.33 – 2.80	0.941	0.82	0.29 – 2.26	0.701
Sewage contact						
No	—	—		—	—	
Yes	1.08	0.33 – 3.54	0.894	1.26	0.37 – 4.43	0.713
Titers of MAT						
≤100	—	—		—	—	
≥200	2.33	0.49 – 13.35	0.303	4.26	0.52 – 90.63	0.226
400	0.8	0.16 – 3.94	0.786	1.69	0.45 – 6.72	0.438
≥800	1.01	0.19 – 5.44	0.991	0.34	0.02 – 3.21	0.386

[a]*OR = Odds Ratio.*
[b]*CI = Confidence Interval.*

of protective immunity. In this study, we had the opportunity to evaluate the kinetics of the antibody response and the factors associated with exposure to leptospirosis in naturally infected individuals with asymptomatic infection comparing a biannual and a quarterly serological analysis. Among the 72 individuals who participated in the subcohort with ≥4 quarterly collections, we found that 65% had circulating anti-*Leptospira* antibodies. The variables age ($p < 0.001$ and $p = 0.005$) and cleaning sewage ($p = 0.003$ and $p = 0.033$) were associated with a higher chance of infection in both analyses.

The time of blood collection between samples can affect the number of infection and reinfection of leptospirosis. A recent study applied a titer decay rate on the serological data of the same population in Salvador, Brazil and identified a higher number of mean infection rate on the biannual analysis and even higher when applying the decay to annual analysis (23). In agreement with this report, our quarterly serological analysis identified a higher number of leptospirosis infections and reinfections events in our population when compared with a biannual analysis, suggesting that exposures to the leptospirosis pathogen in this urban slum setting are frequent if not ubiquitous. Given the constant high risk for exposure to the pathogen observed in this community either by the environment (9, 18) or by the high mobility of its inhabitants (24) and the bias that a reexposure and potential boost of titers can do to titers decay (23), it might be impossible to calculate an accurate titer decay. This limitation can affect the correct incidence rate, data comparison among different longitudinal cohort studies and potentially risk assessment for exposure. Further considerations should be made on reducing the time of serological evaluation or applying decay rates estimations to take into account the differences observed here on titer decay.

Our results indicate that the humoral response detected by MAT is relatively short and provides partial protection against reinfection. Previous studies have reported that individuals with leptospirosis were protected against reinfection by the same *Leptospira* serovar or by related serovars for a short period (25, 26). However, a recent study from French Polynesia showed that individuals with a first infection might not be protected against subsequent reinfection (27). In our study, when performing a quarterly analysis, we observed that agglutinating antibodies have a short life span with titers up to 1:200 disappearing after 90 days. Also, our analysis showed that agglutinating antibodies don't seem to affect the risk for subsequent infection. These results are in agreement with previous data that showed that constant exposure and pre-existing anti-leptospire antibodies did not provide complete immunity (19). Of note, most of the titers observed in our quarterly analysis were low, with 62% ranging between 1:50 and 1:200. A recent study of an attenuated vaccine has shown that antibodies against proteins rather than agglutinating antibodies are correlated to protection (28). It is possible that individuals in this community, which are often being exposed to an environment contaminated with leptospires, have built an immune response similar to a live vaccine that reduces symptoms in case of reinfection and potentially providing cross-protection between unrelated *Leptospira* serovars (5, 29, 30). Further studies to better characterize the immune response after infection, focusing on B and T cell responses and memory, would provide valuable information about potential markers to protect against reinfection.

The time of sample collection and the higher infection rates don't seem to affect the major risk factors for leptospirosis infection. Despite the assumption that a more suitable analysis leading to higher infection rates could potentially affect the observed risk factors for the disease (23) our results indicate that regardless of the period of analysis the potential risks for infection are similar. Transmission dynamics and risk factors for *Leptospira* infection and reinfection are associated with environmental, demographic, and individual exposures. Our results show that risk factors for infection in this community corroborate previous studies (9, 18, 19). The chance of acquiring anti-leptospire antibodies was more frequent in young adults with <9 years of schooling, regardless of the time of collection. Although gender was not identified as a risk factor for acquiring infection in our study, several others consistently report that men in working age groups are at higher risk (9, 10, 15, 31, 32). In our study, being married or having a stable relationship was associated with a risk of infection. We also found that in this group 69% (11/16) of individuals with infection were women

TABLE 4 | Comparison of the sociodemographic and exposure characteristics of the individuals in the subcohort based on the biannual analysis and quarterly analysis follow-up.

Characteristic	Biannual ($N = 72$)			Quarterly ($N = 72$)		
	No infection ($N = 35$)[a]	Infection ($N = 37$)[a]	p-value[b]	No infection ($N = 25$)[a]	Infection ($N = 47$)[a]	p-value[b]
Age (years)			**<0.001**			**0, 005**
05–14	16 (45.7%)	5 (13.5%)		13 (52)	8 (17)	
15–24	4 (11.4%)	5 (13.5%)		2 (8.0)	7 (15)	
25–34	4 (11.4%)	14 (37.8%)		3 (12)	15 (32)	
35–44	2 (5.7%)	10 (27.0%)		1 (4.0)	11 (23)	
> 44	9 (25.7%)	3 (8.1%)		6 (24)	6 (13)	
Sex			>0.99			>0.99
Female	17 (48.6%)	19 (51.4%)		13 (52)	23 (49)	
Male	18 (51.4%)	18 (48.6%)		12 (48)	24 (51)	
Ethnicity			0, 34			0, 17
Black	13 (37.1%)	16 (43.2%)		8 (32)	21 (45)	
Brown	15 (42.9%)	18 (48.6%)		11 (44)	22 (47)	
White	7 (20.0%)	3 (8.1%)		6 (24)	4 (8.5)	
Others	0 (0.0%)	0 (0.0%)		0 (0)	0 (0)	
Education			0, 87			0, 53
Up to 9th year	28 (80.0%)	28 (75.7%)		21 (84)	35 (74)	
More than 9th year	7 (20.0%)	9 (24.3%)		4 (16)	12 (26)	
Married or stable union	6 (17.1%)	16 (43.2%)	**0, 032**	4 (16)	18 (38)	0, 092
Informal employment	14 (40.0%)	22 (59.5%)	0, 16	7 (28)	29 (62)	**0, 013**
Per capita household income (US$/day)	4.2 (4.1)	4.5 (3.3)	0, 67	3.9 (4.4)	4.6 (3.3)	0, 52
Cleaned sewage	0 (0.0%)	10 (27.0%)	**0, 003**	0 (0)	10 (21)	**0, 033**
Open sewage at <10 m from home	21 (60.0%)	29 (78.4%)	0, 15	15 (60)	35 (74)	0, 32
Accumulated trash within <10 m of home	11 (31.4%)	13 (35.1%)	0, 93	7 (28)	17 (36)	0, 66
Sewage contact	15 (42.9%)	18 (48.6%)	0, 8	12 (48)	21 (45)	0, 98
Floodwater near home	21 (60.0%)	19 (51.4%)	0, 62	15 (60)	25 (53)	0, 76
Mud near home	20 (57.1%)	25 (67.6%)	0, 5	15 (60)	30 (64)	0, 95
Work in construction	2 (5.7%)	3 (8.1%)	>0.99	1 (4.0)	4 (8.5)	0, 82
Work related to hawker	1 (2.9%)	2 (5.4%)	>0.99	1 (4.0)	2 (4.3)	>0.99
Work related to garbage removal	1 (2.9%)	6 (16.2%)	0, 13	1 (4.0)	6 (13)	0, 44
Work involves contact with mud	1 (2.9%)	2 (5.4%)	>0.99	1 (4.0)	2 (4.3)	>0.99
Work involves contact with flood water	1 (2.9%)	2 (5.4%)	>0.99	1 (4.0)	2 (4.3)	>0.99
Work involves sewage contact	1 (2.9%)	2 (5.4%)	>0.99	1 (4.0)	2 (4.3)	>0.99
Fever	5 (14.3%)	11 (29.7%)	0, 2	4 (16)	12 (26)	0, 53

[a] *Mean (SD) or Frequency (%).*

[b] *Welch Two Sample t-test; Pearson's Chi-squared test.*

The values in bold are the variables with statistical significance (≤ 0.05).

against 25% (4/16) of men. A study in Cali, Colombia, showed that the female gender was directly associated with the risk of *Leptospira* infection and that domestic factors may play an important role in transmission, particularly in urban slums (33, 34). Another possibility to explain this finding is related to factors such as age and exposure time. We observed that the general mean age in the group of married individuals with infection was 33 years, while for single individuals with infection it was 27 years. Our findings may be explained by the fact that being older resulted in longer exposure time and a greater risk of infection. Recently, a study on the same area showed that increasing age was associated with an increased risk of *Leptospira* infection, and that infections in this area can occur year-round (35). Infection is also often associated with occupational activity such as working in civil construction, working with garbage removal, and informal employment (9), the latter was also identified in our study. We also identified individual exposures related to the home environment such as contact with mud, standing water in the vicinity of the home, and especially sewage cleaning, which was associated with an increased risk of infection in both analysis and has been reported as a risk factor for *Leptospira* infection (9, 19).

This study has some limitations that should be considered. The sample size of the quarterly analysis was not ideal for some of our analysis. Longitudinal cohort analysis are logistically and financially troublesome, which is reflected on the few studies conducting such experiments on leptospirosis and the choice to make biannual or annual measurements (9, 18, 19). For that reason, we decided to select a sample of participants who had confirmed infection at the time the biannual survey was carried out. In addition, 35% of subjects in the subcohort did

not complete quarterly follow-up, primarily due to moving out of the study area, which is a common issue in longitudinal studies. Despite those limitations, we were able to identify significant differences in our analysis that agreed with previous studies, indicating the validity of our results. The MAT is the gold standard test recognized by the WHO, but it is a laborious test, subjective and requires experience from the reader. Further, the MAT does not differentiate past from current infection. Those limitations from the MAT are a common feature for several serological assays and always present on leptospirosis studies (30, 36, 37). To minimize impacts on MAT results in our study, only a well-trained and experienced technician was responsible for all readings. Our group has been working in Salvador, Brazil and in the community of Pau da Lima, where this study was conducted, for over 20 years. Since then, we have reduced our panel of MAT strains given the extensive knowledge of circulating strains and reservoirs (9, 18, 19). Our previous studies have shown that over 90% of severe cases of leptospirosis (15) and 90–98% of infections in the community are related to *L. interrogans* serovar Copenhageni. Furthermore, 80% of rats captured in the community (9, 18, 19, 38) were culture positive for leptospirosis, and the serovar Copenhageni was the only one isolated (39). The focus of our study was to understand the role of agglutinating antibodies on the naturally acquired immunity against reinfection, and to have statistical power our analysis were based on the most prevalent serovar in our study site, *L. interrogans* serovar Copenhageni. For those reasons we didn't evaluate agglutinating antibodies for other serovars, including *L. biflexa* serovar Patoc, commonly used as a control. Titers of 1:25 or 1:50, as well as higher titers, were directed against this serovar in our precious studies (9, 18, 19), indicating that this cutoff was a specific and more sensitive criteria for identifying prior infections in a region where a single serovar agent is circulating. Our study site has geographical and social-demographic features that are very similar to other regions of the world where leptospirosis is a problem. Furthermore, the *L. interrogans* is the most common species related to human cases of leptospirosis around the globe (14). Although our results can be generalized to the context of urban leptospirosis worldwide, considerations should be made given recent reports in a mice model that different strains can lead to different levels of immune responses (14).

In summary, we reported antibody kinetics in individuals from an endemic area for leptospirosis showing that frequent exposure to the contaminated environment is an important factor on the infection and reinfection rates of the disease, which are directly affected by the time of intersample collection. Our study also suggested a rapid decay of the humoral response related to agglutinating antibodies and a short-lived naturally acquired immunity against reinfections. Furthermore, our results indicated that serological surveys may be underestimating the burden of *Leptospira* infection and potentially the risk for disease. Further studies are needed to evaluate memory B cells and to assess the humoral response of individuals with previous leptospirosis infections, that could help better to understand the naturally acquired immunity of this important neglected disease and close the knowledge gap on correlates of immunity that can be used to improve prevention.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Yale University Institutional Review Board Research Ethics Committee of the Instituto Gonçalo Moniz Certificate of Presentation for Ethical Appreciation (CAAE). Written informed consent to participate in this study was provided by the participants or their legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JC: conception, methodology, and writing of the original draft. NN: data curation, data analysis, and review. GS, RV, AM, and JS: investigation and review. FC, AK, and MR: funding acquisition, investigation, methodology, and review. EW: funding acquisition, investigation, methodology, proofreading, supervision, writing-proofreading, and editing in English. All authors contributed to the revision and editing of the manuscript, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.862378/full#supplementary-material

Supplementary Figure S1 | Titration curves in log 10 of individuals classified in the quarterly analysis (•) as infection (Individual 14 to individual 21) and reinfection

(Individuals 22 and 23) at different times (days) of collection, compared to the bi-annual analysis (•).

Supplementary Table S1 | Anti-leptospira antibody titers distributed by classification and collection period.

Supplementary Table S2 | Characteristic of the subcohort group comparing the stratified analysis of infection based on the quarterly analysis follow-up.

Supplementary Table S3 | Characteristic of the subcohort group comparing the stratified analysis of infection based on the biannual analysis follow-up.

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SARS-CoV-2 Molecular Epidemiology Can Be Enhanced by Occupational Health: The Experience of Monitoring Variants of Concern in Workplaces in Rio de Janeiro, Brazil

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The emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to extra caution in workplaces to avoid the coronavirus disease 2019 (COVID-19). In the occupational environment, SARS-CoV-2 testing is a powerful approach in providing valuable information to detect, monitor, and mitigate the spread of the virus and preserve productivity. Here a centralized Occupational Health Center provided molecular diagnosis and genomic sequences for companies and industries in Rio de Janeiro, Brazil. From May to August 2021, around 20% of the SARS-CoV-2 positive nasopharyngeal swabs from routinely tested workers were sequenced and reproduced the replacement of Gamma with Delta variant observed in regular surveillance programs. Moreover, as a proof-of-concept on the sensibility of the occupational health genomic surveillance program described here, it was also found: i) the primo-identification of B.1.139 and A.2.5 viral genomes in Brazil and ii) an improved dating of Delta VoC evolution, by identifying earlier cases associated with AY-related genomes. We interpret that SARS-CoV-2 molecular testing of workers, independent of symptom presentation, provides an earlier opportunity to identify variants. Thus, considering the continuous monitoring of SARS-CoV-2 in workplaces, positive samples from occupation health programs should be regarded as essential to improve the knowledge on virus genetic diversity and VoC emergence.

Keywords: COVID, SARS-CoV-2, occupational medical care, surveillance, variants of concern (VOCs), molecular sequence, next generation (deep) sequencing (NGS)

INTRODUCTION

The emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of 2019 coronavirus disease (COVID-19), has led to over 200,000 death/month globally in approximately 2 years (1). Deaths, hospitalizations, long-term sequela, and work absence are among the COVID-19-associated factors that have led to economic depression (2). Especially in low- and middle-income countries, such as Africa and Latin America, where budget constraints are constant and reinforced under economic uncertainties, science, and public health funding may become at risk. Indeed, the percentage of sequenced SARS-CoV-2 genomes among confirmed cases is lower in these countries than in wealthier nations (3, 4).

SARS-CoV-2 surveillance has been built on influenza monitoring networks (5). In brief, State laboratories share samples from syndromic patients (under different levels of assistance, from immediate attention to deceased patients) with National Influenza Centers (NIC), which subsequently consolidate with World Health Organization (WHO) Collaborating Centers (CC) the variants to be prioritized for regular vaccine updates and pandemic preparedness. However, besides this standard surveillance component, SARS-CoV-2 has led to extra caution in workplaces to avoid COVID-19 (6), differently than any other emerging pathogens. In the occupational environment, SARS-CoV-2 testing is a powerful approach in providing valuable information to decision-makers to adopt measures to mitigate the spread of the virus, preserving a healthy workplace and productivity.

Thus, SARS-CoV-2 pandemics imposed a necessity that occupational health programs adapt to organize and implement the early identification of workers with SARS-CoV-2 positive diagnosis (6) and even vaccine advocacy. Nevertheless, the natural history of COVID-19 is challenging because asymptomatic and pre-symptomatic patients may impose a risk of virus spread among co-workers (7). In companies with an implemented occupational health system, routine systematic testing of workers, regardless of their symptoms, could provide recommendations for self-quarantine, avoiding SARS-CoV-2 spread, and better monitoring the clinical evolution of COVID-19.

As SARS-CoV-2 spilled over in the wet market in Wuhan, China, workers from there were among the very first patients with COVID-19, including the most likely patient "zero" (8). Apparently, in this market, two independent episodes posed the risk of SARS-CoV-2 spillover from animals to human (9), reinforcing the notion that this workplace was endowed with substantial risk for the emergence of new viruses. Although 'pneumonia of unknown origin' was the diagnosis assigned to the initial patients who underwent hospitalization, COVID-19 hospitalization was necessary in 5–7% of the initial cases, meaning that occupational health surveillance in Wuhan's wet market could have identified SARS-CoV-2 among the other 95–93% of persons with asymptomatic or mild symptoms (8) and allowed earlier contingency of the very first infected individuals. Although the wet market was most likely the epicenter of the virus spillover, other workplaces with lower risks of virus emergence also contribute to SARS-CoV-2 chains of transmission (10, 11).

SARS-CoV-2 surveillance must be a priority to properly represent viral genetic diversity within a community. Beyond regular surveillance, occupational health can readily provide opportunities for early detection. SARS-CoV-2 pathogenesis and ability to escape the humoral immune response may vary among the variants of concern (VoC) (12), making it necessary to effectively and efficiently identify COVID-19 clusters to categorize the viral lineages. Indeed, the Gamma, Delta, and Omicron VoCs emerged from uncertain origins (13–15). Thus, every opportunity to catalog SARS-CoV-2 genetic diversity should be taken to fulfill the phylogenetical and epidemiological puzzle imposed by this virus. Among these opportunities, the integration of occupational health and SARS-CoV-2 surveillance has been proposed (16). Still, it has never been tested whether programs in workplaces have the sensitivity to detect variants and lineages with similar trends as regular "influenza-like" surveillance programs.

In Rio de Janeiro, Brazil, centralized infrastructures support social and health programs for industries and other entrepreneurial activities (National Industrial Apprenticeship Service, SENAI; and Industry Social Service, SESI). From September 2020 to May 2021, the SESI Innovation Center for Occupational Health has supported industrial and service companies in this State to implement and perform the detection of SARS-CoV-2 through RT-PCR (17). From May 2021 forward, SARS-CoV-2 genome sequencing was implemented to reinforce the molecular surveillance associated with occupational health. Therefore, we evaluated the SARS-CoV-2 genetic diversity in workers from Rio de Janeiro and correlated these findings to those from regular surveillance programs.

MATERIALS AND METHODS

Population and Ethics

The mass testing program for COVID-19, proposed by SESI Innovation Center for Occupational Health (FIRJAN, Rio de Janeiro—Brazil), screened workers from industry service companies in Rio de Janeiro, Brazil, independently of any symptoms through RT-qPCR in nasopharyngeal swab specimens. Positive samples were randomly selected for genomic analyses through deep sequencing from May to August 2021.

The National Committee approved the present study of Research Ethics and the Ethics Committee of Hospital Universitário Clementino Fraga Filho under protocol number 4,317,270.

RNA Extraction

For the mass testing program, RNA was extracted from nasopharyngeal swabs transported in DMEM using the Total RNA Purification Kit (Ref.: 400793, Agilent Technologies) with the Bravo Automated Liquid Handling Platform (Agilent Technologies), according to the manufacturer's protocols.

For the sequencing analyses, we performed a new total viral RNA extraction from stored frozen stock using ReliaPrep™

Viral TNA Miniprep System (Ref #AX4820, Promega) following manufacturers' protocols with minor modifications. Briefly, we used 1,000 μl of the inoculated medium and adjusted proteinase K, cell lysis buffer, and isopropanol volumes accordingly.

RT-qPCR

RNA samples were screened through RT-qPCR following CDC protocols for SARS-CoV-2 detection, using viral targets N1 and N2, and the human gene for RNase P, using a primer-probe kit from IDT (Ref #10006713) and TaqPath™ 1-Step RT-qPCR Master Mix (Ref.: A15300, Applied Biosystems), following manufacturers' protocols for reaction volumes and cycling conditions (18). We considered positive samples those detectable for the three targets simultaneously with a cycle threshold value (CT) below 40.

SARS-CoV-2 Sequencing, Processing, and Analysis

RNA from the positive nasopharyngeal swabs with the highest viral loads (ct values below 30) were randomly chosen for sequencing. SARS-CoV-2-related reads were enriched with Atoplex (version 1.0; MGI Tech Co., China) and sequenced by 100-nt pair-ends DNA nanoball technology on a DNBSEQ-G50 apparatus (MGI Tech Co.) (19). Consensus FASTA genomes were generated by the GenomeDetective (20) (https://www.genomedetective.com/) online platform from raw sequencing data. Consensus genomes were aligned and assigned to pangolin lineages with the phylogenetic assignment of outbreak lineages (Pangolin, Galaxy Version 3.1.17+galaxy1) (21). Aligned FASTA files were also assigned to variants and quality checked by NextClade (https://clades.nextstrain.org/, version 1.10.0) (22). The output JSON file from NextClade was used to generate phylogeny through https://auspice.us/ (version 0.8.0) (23, 24).

Statistical Analysis

Standard descriptive statistics were used to describe the study population. Continuous variables were reported as appropriate as the mean ± standard deviation or median (range). Comparative analyses were performed using OpenEpi software (25). Statistical significance was reached when $p < 0.05$.

RESULTS

Clinical-Demographical Characteristics of the Occupational Health Cohort

In Rio de Janeiro, Brazil, we detected 292 positive cases of SARS-CoV-2 from May to August 2021, a period when the Delta variant was introduced in Brazil (26). Among these cases, 72 individuals presented samples with the highest virus loads, with CT values up to 29, prioritized for next-generation sequencing. We obtained 50 high-quality, full-length consensus genomes, with quality scores above 30 for base calling, at least 10x of depth, and adequate NextClade assignment. The distribution of the sequenced SARS-CoV-2 cases among economic activities is representative of the workers who tested positive during this period (**Table 1**). Interestingly, the occupational health cohort was younger than those patients assisted at regular health units (**Table 2**) (26, 27), consistent with an economically active population. Patients tended to be male, and no severe cases were observed in our cohort (**Table 2**).

TABLE 1 | The distribution of tested workers according to the industrial sector.

Industrial and entrepreneurial sectors	Subset subjected sequencing	Total positive cases
Services (health, realtor, informatics, administrative, commerce)	48%	36%
Construction	4%	7%
Processing industries	31%	37%
Extractive industries	17%	20%

The chi-square p-value for this table is 0.34693 and, therefore, not statistically significant at the $p < 0.05$ level.

TABLE 2 | Characteristics of the occupational health cohort.

	Occupational health cohort	Regular surveillance in Rio de Janeiro*
Age (years-old, median-IQR)	38 ± 11	63 ± 20
Males (%)	56	52
Mortality (%)	0	4.2
Hospitalization and other reported medical complication (%)	0	7.1

*Data obtained from regular surveillance bulletins (26, 27).

SARS-CoV-2 Variants in the Workers

The full-length SARS-CoV-2 genomes belonged to the Gamma and Delta clades (**Figure 1A**). The Delta variant started to replace Gamma from June to August (**Figure 1B**), consistent with public data on Brazilian molecular surveillance for SARS-CoV-2 from health care units (http://www.genomahcov.fiocruz.br/dashboard/). The distribution of the Gamma and Delta SARS-CoV-2 lineages per epidemiological week (**Figure 1C**) also indicates the timely identification of each variant (http://www.genomahcov.fiocruz.br/dashboard/) in the occupation health cohort.

We identified P.1 and P.1-related lineages in late May, consistent with virus circulation in Rio de Janeiro (http://www.genomahcov.fiocruz.br/dashboard/). The genomes added by this study increased the genetic diversity of the P.1-related lineages in Brazil and demonstrated the early identification of P.1.1, P1.1.9, and P.1.12 genomes from occupational health samples (**Figure 1C**) (https://outbreak.info/situation-reports/gamma?loc=BRA&loc=USA&loc=JPN&selected=BRA).

Similar to the Brazilian molecular surveillance program (Dashboard—Genomahcov—Fiocruz), we identified Delta AY-related lineages during late June/early July (**Figure 1C**). Through this study, we increased around 20% the number of high-quality genomes from this period in Rio de Janeiro, Brazil, and, more importantly, found that AY.42, AY.43, AY.46, AY.99, and AY.110

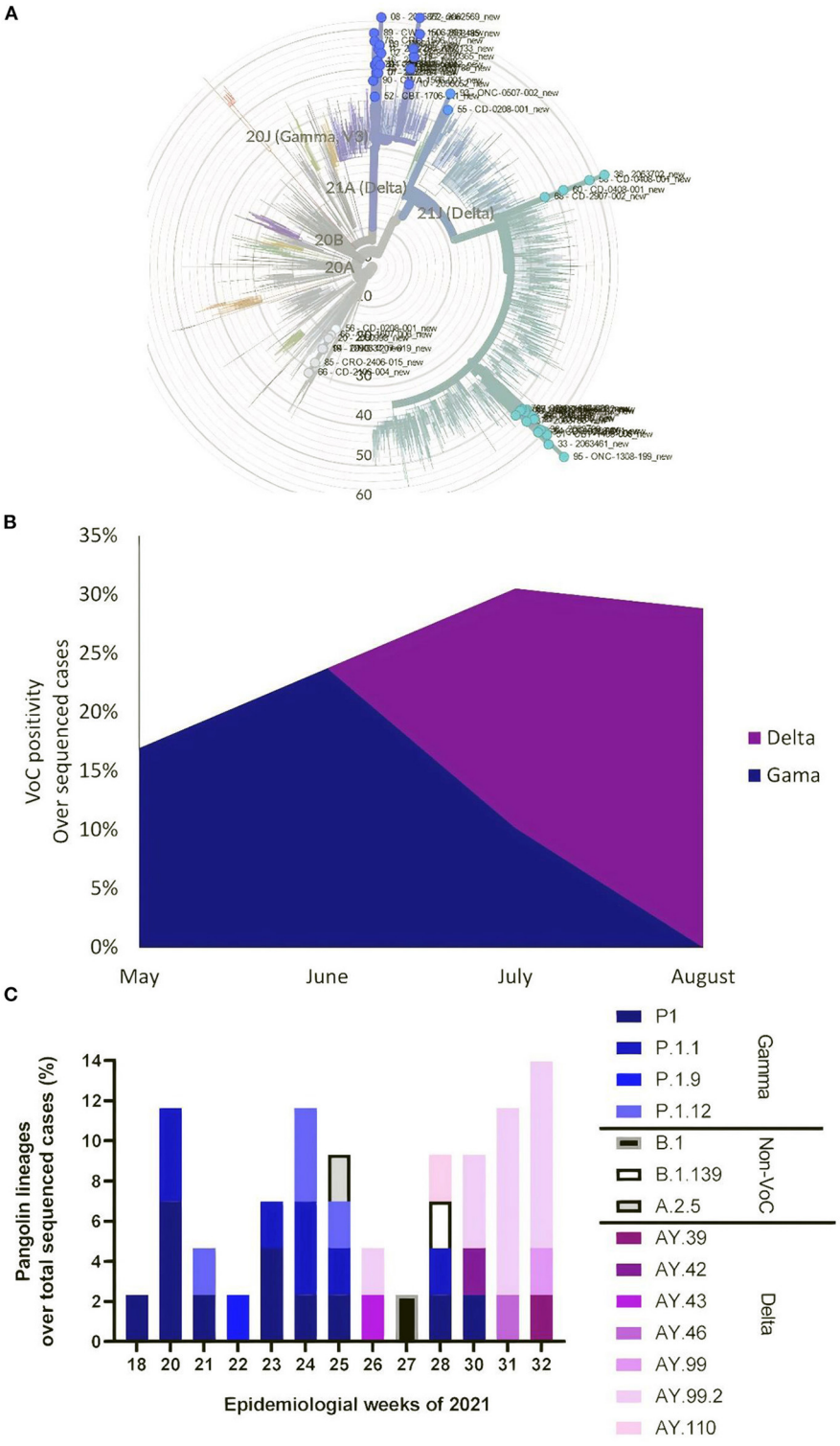

FIGURE 1 | Occupational health-associated SARS-CoV-2 molecular surveillance. Of the 292 positive nasopharyngeal swabs from a cohort of workers in Rio de Janeiro, Brazil collected between May and August, 72 samples were randomly chosen for SARS-CoV-2 sequencing (Atoplex version 1.0, using the pair-end of 100 nucleotides in the DNBSEQ-G50 sequencer). Fifty full-length consensus genomes were generated with10x depth and quality scores > 30. **(A)** Maximum-likelihood phylogeny showing mutation tree compared with 2265 genomes from **Supplementary File 1**. **(B)** Distribution of the Gamma and Delta variants, assigned by NextClade version 1.10.0, per month. **(C)** Distribution of Pangolin lineages, assigned by Phylogenetic Assignment of Outbreak Lineages (Galaxy Version 3.1.17 + galaxy1) per epidemiological week. The genomes generated in this study are deposited in GenBank under accession codes: OM188304-OM188353.

linages occurred contemporaneous or even a few weeks earlier than previously described (https://outbreak.info/situation-reports/delta?loc=IND&loc=GBR&loc=BRA&selected).

Occupational health samples also provide an opportunity to identify USA-related lineages, B.1.139 and A.2.5 (**Figure 1C**), not previously reported in Brazil (https://cov-lineages.org/lineage.html?lineage=B.1.139, https://cov-lineages.org/lineage.html?lineage=A.2.5).

Our results reinforce that molecular surveillance of infectious diseases could be enhanced by integrating occupational health initiatives that routinely monitor workers. For instance, during the COVID-19 pandemic and the emergence of SARS-CoV-2 variants, these samples could represent additional opportunities to observe viral genetic diversity.

DISCUSSION

Brazil and other Latin American countries struggle to combat COVID-19, including limitations to access diagnosis, intensive care units, and vaccines. Consequently, overcrowded megalopoleis, such as the City of Rio de Janeiro, have a case-fatality ratio of 7.1%, almost two times higher than its State and Country (26, 27). If the City or State of Rio de Janeiro were a country, it would fit among the three regions with the highest death rates in the world (1). Nevertheless, the percentage of sequenced SARS-CoV-2 genomes per confirmed case is low in Brazil, 0.3 genomes per 1,000 confirmed cases (4, 28). Given that Brazil has more representative sequences deposited in GISAID than any other South American country, other Latin American countries struggle to catalog the SARS-CoV-2 genetic diversity (4, 28). In Brazilian studies analyzing circulating SARS-CoV-2 variants using health surveillance programs, only a small subset of samples, around 1%, were sequenced (14, 29). In low- and middle-income countries, which are overwhelmed by the COVID-19 pandemic, most of the resources in public health are consumed by patient assistance, leaving a limited budget for disease prevention and prediction through continuous surveillance.

COVID-19 severely impacted Brazil (1), and, despite the limitation of cataloging only a subset of virus genomes, the pangolin lineages B.1.1.28 and B.1.1.33 are the most representative (Dashboard—Genomahcov—Fiocruz). The Gamma VoC also evolved from the B.1.1.28 lineage (14). The introduction of B.1.1.28 and B.1.1.33 likely occurred early in 2020, which overlaps with the Brazilian Carnival (30, 31), when no specific lockdown or self-quarantine measures were implemented. Stochastic events are thus not only associated with SARS-CoV-2 spillover from animal to humans (8) but also with virus dissemination. The SARS-CoV-2 spillover was associated with occupational risk for the workers at the wet market in Wuhan (8). When surveillance programs based on symptomatic patients identified the first cases of SARS-CoV-2, over 90% of asymptomatic or mildly affected individuals were likely circulating (8). Therefore, routine testing becomes an important tool to communicate public health surveillance systems early.

Our data points out that companies with implemented occupation health surveillance for COVID-19 may have an adequate and timely opportunity to increase awareness of the genetic diversity of circulating strains of SARS-CoV-2. Indeed, it has been proposed that occupational health should be an integral component of the response to COVID-19 (6, 16). Still, it is here that we document the experience from Rio de Janeiro, where a centralized Center for Occupational Health has reduced the dependence on governmental funding to catalog SARS-CoV-2 genetic diversity and find VoC with similar trends to a regular surveillance system. By sequencing representative samples, around 20%, from a cohort with characteristics than those on regular SARS-CoV-2 genomic surveillance networks, we even found: i) the primo-identification of the B.1.139 and A.2.5 viral genomes in Brazil; and ii) an improved dating of Delta VoC evolution, by identifying earlier cases of associated with AY-related genomes. We interpret that SARS-CoV-2 molecular testing of workers, independently of symptoms, has allowed an earlier opportunity to identify variants than regular health surveillance programs. These regular programs are primarily dependent on the spontaneous demand of syndromic patients and the cataloging of viral genetic diversity from hospitalized and deceased individuals, which may take weeks after the onset of illness to be identified.

CONCLUSION

With the emergence of variants that escape the humoral immune response (12), companies must continuously monitor their workers for SARS-CoV-2. Under the auspicious of this investigation, we interpret that SARS-CoV-2-positive samples from occupation health programs should be considered as necessary as those from regular health surveillance programs to expand our awareness knowledge on virus genetic diversity and VoC emergence. We propose continuously performing occupational health surveillance to screen for other communicable diseases and identify possible chains of transmission.

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 in the article in the legend of **Figure 1** and the **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Committee of Research Ethics and by the Ethics Committee of Hospital Universitário Clementino Fraga Filho under protocol number 4,317,270. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SK and AF-N coordinated the cohort. BF, BM, AC, and JG performed the sequencing. JA, JG, and TS analyzed the data. SK, AF-N, and TS conceptualized the study. All authors prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.862284/full#supplementary-material

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HIV Rapid Testing in the General Population and the Usefulness of PrEP in Ecuador: A Cost–Utility Analysis

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Introduction: HIV is considered one of the most important chronic transmitted diseases worldwide. The Joint United Nations Program on HIV/AIDS in 2020 proposed the strategy "95–95–95" which goals to achieve a 95% of cases identified, receives ART, and will have achieved suppression of the virus. In Ecuador by 2020, according to the Ministry of Public Health, 45,056 persons are living with HIV, principally men between 15 and 49 years, and a mortality rate of 4.8/100,000 habitats. This study aims to determine the cost–utility of applying an early screening to a sexually active population vs. only a high-risk population and if the use of PrEP is justified depending on different contexts.

Methods: For the cost–utility evaluation, it was compared: (a) HIV screening performed only in the high-risk population vs. HIV screening in all population sexually active; and (b) the use of ART only for HIV treatment vs. ART as a treatment in diagnosed cases and the use of PrEP (only at a high-risk population of acquiring HIV). Calculation and weight of DALYs for HIV/SIDA were obtained through WHO guidelines. To generate the Markov model for HIV/AIDS, subjects were classified as symptomatic or asymptomatic, as well as the HIV deaths.

Results: Cost–benefit analysis (CUA) showed that ICER for early diagnosis had a negative value which means a saving if the strategy will be implemented as a regular test (–$591, –$4,360) and −108 and −934 DALYs, in the case of ART and PrEP, ICER the $30,541–$59,410, which resulted in more than the GDP's threshold and health years between 2,511 and 10,635 in the general population. With a reduction of 70% in the assigned budget for the early diagnosis, Ecuadorian people could lose between 4 and 6 DALYs, while if the budget reduces more than 50% to ART, it will generate a loss of 10–12 years of healthy life.

Conclusion: CUA demonstrates that an early diagnosis in a sexually active population is cost-beneficial. This, combined with ART or PrEP, is ideal to add years of healthy life.

Keywords: HIV screening, HIV treatment, ART, PrEP implementation, DALYs, cost–utility analysis

INTRODUCTION

HIV is considered one of the most important chronic transmitted diseases worldwide. Many cases have been reported since its appearance. In 2020, there were 37.7 million (30.2–45.1 million) people living with HIV, and 1.5 million (1.0–2.0 million) new cases emerge every year worldwide (1). A couple of decades ago, the chances of surviving more than 10 years with HIV were slim. Today, a considerable decrease in HIV mortality was experienced thanks to antiretroviral therapy (ART) (2). The UNAIDS (the Joint United Nations Program on HIV/AIDS) in 2020 proposed the strategy knowing how "95–95–95" which goals to achieve by 2030 a 95% of cases identified, receives ART, and viral loads undetectable worldwide (3). Governments invest considerable amounts of their capital to prevent and control HIV, mainly for early detection by rapid screening, education, and treatment. Even though great results have been reached with these approaches, many undiagnosed cases are responsible for a continue transmission. The US Centers for Disease Control and Prevention since 2006 has recommended screening to all subjects between the ages of 13–64 because there is a trend to overlook some cases due to the misconception of only testing high-risk populations.

Consequently, undiagnosed patients have not received ART, and an increase in morbimortality has been observed (4). Other factors associated with this phenomenon have been identified (especially in low-middle income countries), like loss of clinical follow-up, treatment withdrawal, and rising resistance to some drugs. Also, a lack of adequate data collection tools makes HIV control a real challenge, suggesting that we will not probably reach the estimated goals soon (5). Thus, reinforcement of prevention and correct use of public health resources is necessary to optimize control strategy expenses. A constant evaluation of obtained results in different contexts is the cornerstones of effective management.

In Ecuador for 2020, according to the Ministry of Public Health (MSP), 45,056 persons are living with HIV, principally men between 15 and 49 years, and a mortality rate of 4.8/100,000 habitats (6). The implementation of basic worldwide recommendations is mainly focused on getting free access to ART. An opportune clinical management-led 79% of HIV carriers known their status, 73% were under ART, and 82% possessed undetectable viral loads in the last few decades (7). Undoubtedly, those results are encouraging and reflect a good approximation. Albeit, a considerable percentage of asymptomatic cases are not identified each year, basically because of the pathophysiology of the disease, its development, and an accurate screening on potential transmitters of HIV infection (8).

Cost–utility studies have been conducted for a long time. They are useful tools to decide, with evidence-based criteria, the best strategy/treatment to scale up in different contexts according to the health system's capacity. Studies usually use local or international data like prevalence, incidence, rates, loss of health, poverty, and economic and social determinants. The cost–benefit analysis by "top-down" considers general spending in a particular event and then calculates the cost (based on the potential use, time invested, personal, etc.), while the "bottom-up" manages a specific event considering their previous use (evidence and records) in different settings and unitary estimate costs (9). Usually, both are used in a mixed way, especially in low-middle income countries (LMIC), to create indexes and ratios and establish whether an intervention is a viable option, according to each local health assigned budget and the potential benefit to a specific population.

One of the most important is the incremental cost-effectiveness ratio (ICER), where variables like cost of each analyzed strategy denote if programs are economically efficient with the possibility to adapt according to each reality, and measurements of health improvement associated (like clinical outcomes and increasing of survival) through quality-adjusted life expectancy (QALY) that is the time spent in one state of health, in an attempt to reflected community perception (10). Disability-adjusted years (DALYs) is a measure that points out the amount of life in years lost due to unexpected death and the years of productive life lost due to disability (according to WHO) and estimates the benefit in health for a determinate population (11).

In a report from 2012, Ecuador mentions that the HIV government strategy allocates at least 50% of the annual budget only in prevention and ART (8). Still, it is not well understood, which are the criteria for this allocation of money. When national authorities decide that it is time to change or modify the HIV control policies, what are the parameters, results, and surveillance made for a clear assessment of capacity implementation? The recommendation of massive testing in subjects between 13 and 64 years was established by the Centers for Disease Control and Prevention (CDC) in 2006, without considering any risk factor (47), but since 2019 Ecuador made official this statement to the active sexual population and is pending how to perform it and measure the results of this intervention, calculate the spending, and corroborate if the suggested guidance is appropriate to our national reality.

On the contrary, pre-exposure prophylaxis (PrEP) has gained the field in clinical practice how prevention mechanism, especially at a high-risk population with different sexual behaviors with a proven beneficial effect, is still considered very expensive. An early HIV diagnosis has been demonstrated to reduce the emergence of new cases; however, some troubles have been experienced related to the adherence and follow-up once prophylaxis was initiated. This study aims to determine the cost–utility of applying an early screening to a sexually active population vs. only a high-risk population and if the use of PrEP is justified depending on different contexts (12).

METHODS

This study does not include human subjects or personal data; therefore, it did not require review or approval by an Independent Review Board (IRB). Data collected for this analysis were obtained between January and December 2020. The study was conducted in two groups: (1) people between 10 and 72 years at high risk of HIV infection (sex workers, GLBTI community, or pregnant women); and (2) general population between 10 and

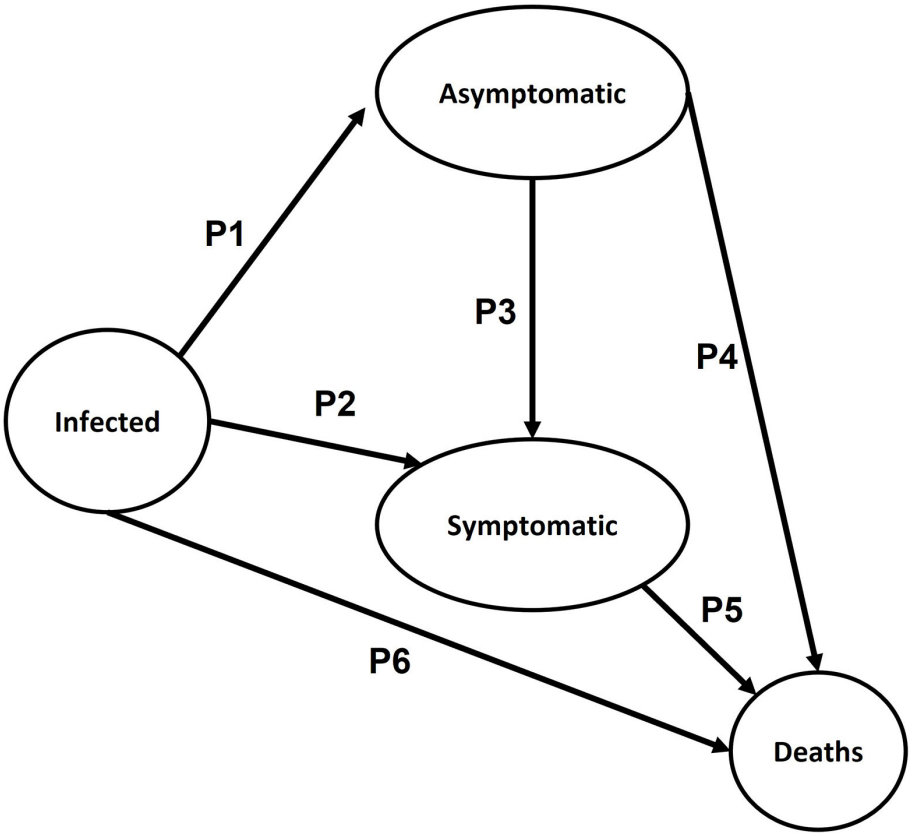

FIGURE 1 | Diagram of Markov's Model for cost–utility analysis (CUA) for general/early HIV screening vs. only to high-risk population and the use of TAR/PrEP.

72 years with a standard risk to acquire HIV. Also, for the cost–utility evaluation, it was compared: (a) HIV screening performed only in the high-risk population vs. HIV screening in all sexually active population; and (b) the use of ART alone for HIV-positive subjects vs. ART as a treatment in diagnosed cases plus the use of PrEP (only at a high-risk population of acquiring HIV).

Information was obtained from the HIV/AIDS National Bulletin of Ecuadorian Ministry of Public Health (13), while socio-demographic and health status variables required for the Markov model (14) were obtained from the National Health and Nutrition Survey (15), the Global AIDS Monitoring Report from Ecuador (16), other general info from the National Institute of Statistics and Censuses—INEC (17). ART/PrEP usage data were from the projection of the Pan-American Health Organization (18).

The Ministry of Public Health of Ecuador receives its budget for to each strategy or program (in this case, HIV/AIDS). It is calculated according to National resources/incomes, this information was extracted from the Global AIDS Monitoring (GAM), and some costs like budget handled in local prevention were obtained from PAHO reports of 2017, from the National costs list for the provision of services MSP 2012 (19), and the 2019 National Clinical Guidelines (48).

Calculation and weight of DALYs for HIV/SIDA were obtained through WHO reports (20), from the study of Murray et al. (21), and for the probabilities of change in health status (required in Markov's model), data from Haeussier et al., Sanni-Oba et al., and Vandewalle et al. were used (22–24).

To generate the Markov model for HIV/AIDS, the analysis was based on *infected* subjects (*I*) who are HIV-1 carriers and know about their status. They were classified as *symptomatic* (*S*) or *asymptomatic* (*AS*), as well as the *HIV deaths*, both related and unrelated (**Figure 1**). It was also considered the probability of an infected subject being asymptomatic (*P1*), an infected subject to be symptomatic (*P2*), an asymptomatic to become symptomatic (*P3*), and the probabilities (*P4, P5*, and *P6*) that each state has for death (14).

To model each scenario, two types of data were employed: deterministic (based on values entered in Microsoft Excel V16.40) and stochastic (initial values generated randomly in the simulation cycles). Some adopted parameters were as follows: the probabilities of changing status (25), age disability weighting of DALYs and disability weights for HIV (WHO), baseline costs for HIV testing, PrEP, and ART (26).

To calculate the costs, it was used the proportion of screening tests performed (third and fourth generation) for the risk

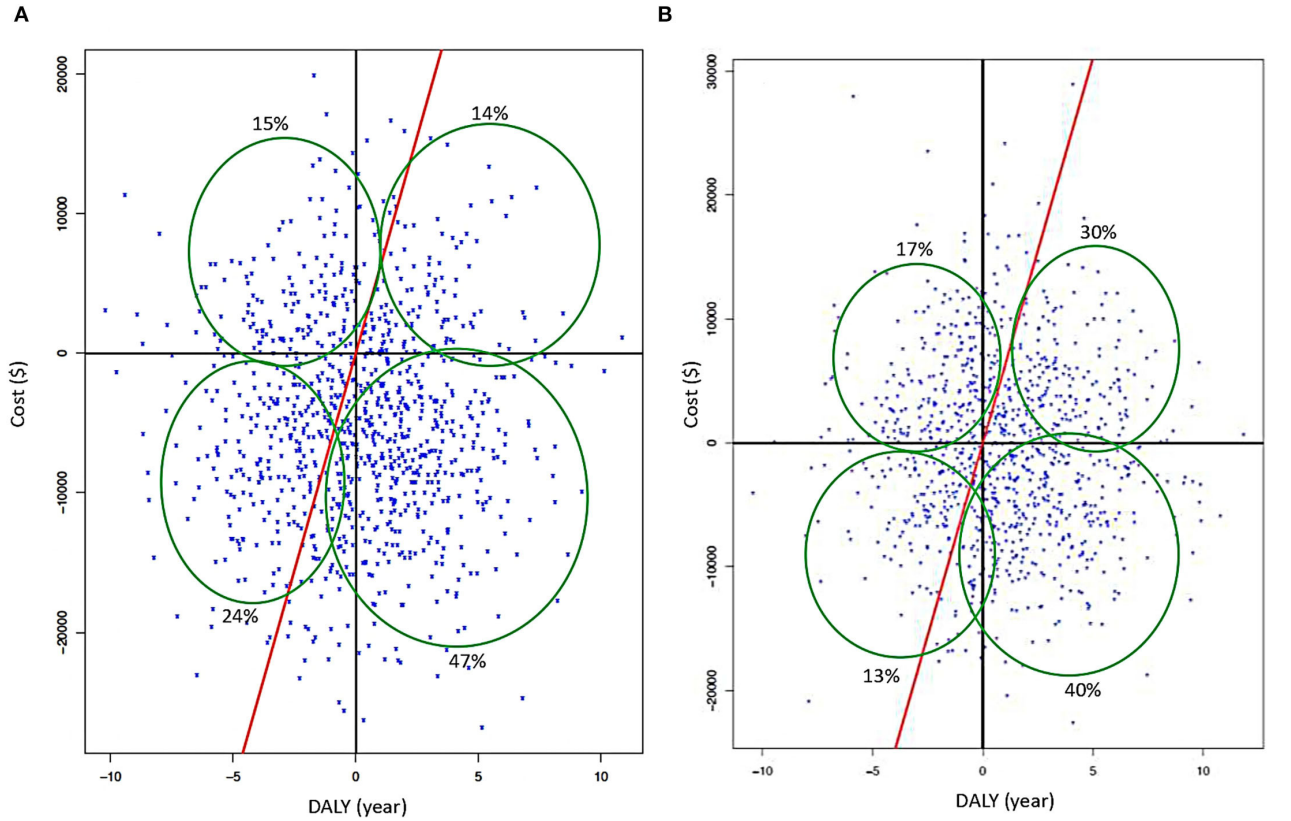

FIGURE 2 | (A) Cartesian plane from PSA when an early screening is applied with deterministic data. **(B)** Results of PSA for early screening using stochastic data (red continuous line represents GDP's threshold; cost is in American dollars and DALYs in years).

population (27), as well as the percentage of the national budget assigned for the purchase of ART in Ecuador (18). To estimate the variation that occurs with costs and DALYs in the simulated population, a matrix designed in Microsoft Excel was generated based on a previous design by Edlin et al. (14) (https://hta-modelling.leeds.ac.uk/downloads/) that recommends 100 cycles (simulations) for a better estimation of those values.

Models were generated with parameters for cost–utility estimation and considering both scenarios (the time of screening and the use of ART/PrEP). Then, it obtained the maximum and minimum cost for screening tests, ART/PrEP yields, and DALYs through the achieved simulations. All these variables were used in the probabilistic sensitivity analysis (PSA).

With the above results, two PSA scenarios were generated using R Studio (V1.2.5042) based on the principles by Edlin et al. (14) and the Monte Carlo model for screening the following hypotheses: random gamma distribution ($k = 3$; $\theta = 0.001$), DALYs with normal random distribution ($\mu = 3.46$, $\sigma^2 = 3.36$), and for ART/PrEP hypothesis costs. Hypotheses were considered as having a normal random distribution ($k = 5$, $\theta = 0.001$) (**Figure 2**), and 3D graphics of both scenarios were generated through a script in MATLAB (V:R2020b) (**Figure 3**).

Then, there were incorporated the results obtained by Markov's Model (minimum and maximum costs of screening, ART/PrEP, and their corresponding DALYs), with an iteration of 1,000 cycles, obtaining cost–utility indexes: NHB (net health benefit that represents the impact on population health when a new intervention is introduced) (Equation 4), NBM (a net monetary benefit that represents the value of an intervention in financial terms when a willingness to pay is known) (Equation 3), and incremental cost-effectiveness ratio (ICER that is the economic value of an intervention, compared with an alternative (comparator) (Equation 5) (**Figure 2**).

For the cost–utility analysis (CUA), the PSA indexes were generated before being used to develop a Cartesian plane divided into quadrants, based on the model by Ho et al. (28), and a threshold (λ) according to the growth domestic product (GDP) in the year of corresponding analysis (here 2017), to determine whether the ICER is lower or higher than λ (29) in the two proposed scenarios. At the same time, we generated other indexes of cost–utility, that is, average cost of the strategy (CEM, Equation 1) and amount of health lost (HL, Equation 2) in DALYs years; to determine whether the cost of intervention reduces years of unhealthy life (this value is intended to be near to zero)

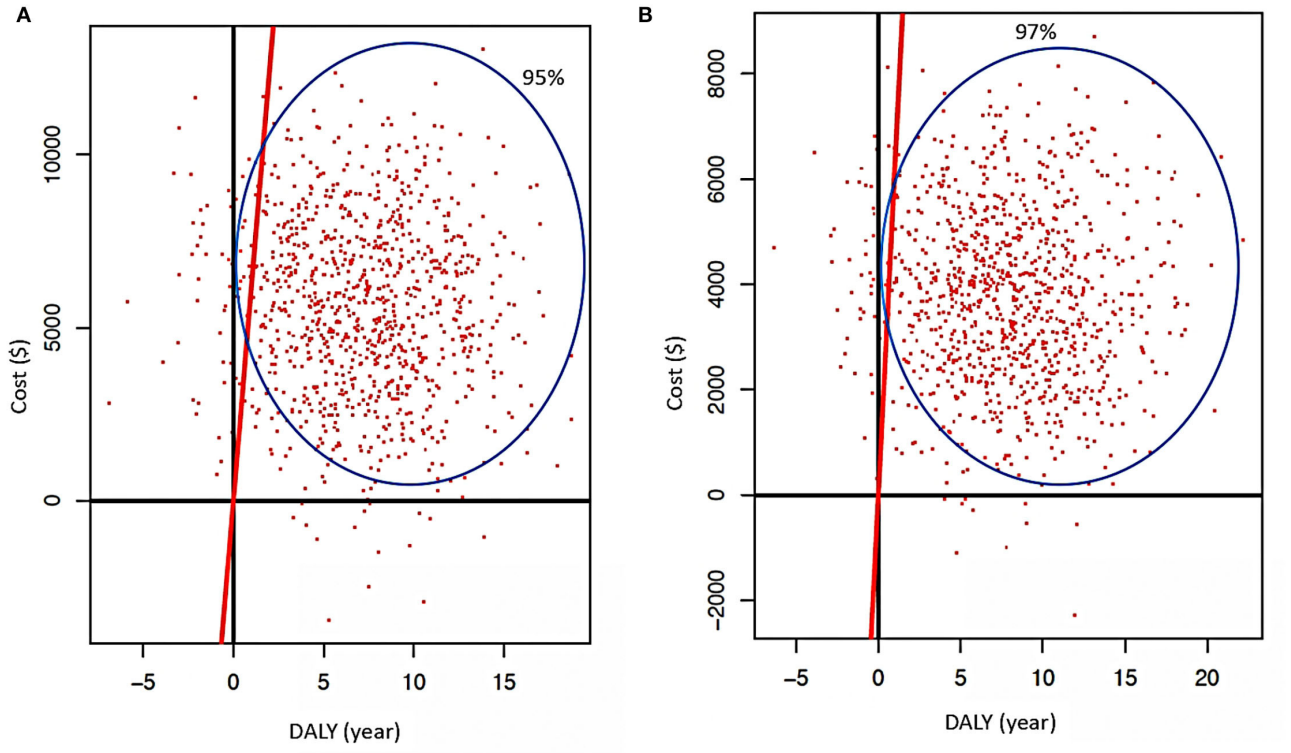

FIGURE 3 | (A) Cartesian plane representing PSA when ART is used as treatment without other intervention (traditional) is applied with deterministic data. **(B)** Results of PSA when ART is used (regular) using stochastic data (red continuous line represents GDP's threshold; cost is in American dollars and DALYs in years).

(29). Later, we calculate the probability of density (which means adopting the cost–utility measurements affect the DALYs).

$$CEM = \frac{anual\ treatment\ cost}{anual\ treatment\ DALYs}\ [DALYs] \quad (1)$$

$$Amount\ of\ health\ lost\ (HL) = \frac{NMB}{\lambda}\ [DALYs] \quad (2)$$

$$Net\ Benefit\ Money\ \ (NBM) = CostA - CostB \quad (3)$$

$$Net\ Health\ Benefit\ \ (NHB) = DALYsA - DALYsB\ \ [DALYs] \quad (4)$$

$$Incremental\ Cost\ Effectiveness\ Ratio\ (ICER) = \frac{NBM}{NHB}\ [DALYs] \quad (5)$$

RESULTS

In a scenario where all sexually active population is tested for HIV, simulations for CUA have a negative value for ICER, showing that investment between –USD 591 and –USD 4,360 could represent a future saving if this testing strategy is implemented. Screening for the high-risk population only generates a year's life between 108 and 934, which should be done to diminish one DALY year in the population with an early diagnosis. Both values are less than GDP's threshold (**Table 1**), and they are showing a correlation with the deterministic model, where is seven times less than the based threshold, and in the stochastic model, it was 0.095 times less than threshold.

Considering ART and PrEP use, in both scenarios, the ICER showed that, if we increase the investment in these strategies (using them together), they could increase health in 2.51–10.6 in the simulated population. Even though the investment is high, it is under the cost-effectiveness threshold. In addition, the ICER demonstrated that for a decline of one DALYs, we need to invest among USD 30,541–USD 59,410 per year, resulting in more than the GDP's threshold (**Table 1**), exhibiting a relation with the deterministic model regarding, which is five times more than the threshold, and nine times more than the stochastic.

Both the use of PrEP in a high-risk population and the early screening in the general population are more cost-beneficial than applying an early screening combined with ART in diagnostic patients as a strategy. In contrast, if ART is used only on diagnosed patients and PrEP in high-risk populations without an early screening, it would imply a reduction in costs but an increase in DALYs. Likewise, considering stochastic data, the use of PrEP at high-risk populations combined with an early screening at the general population is more cost-beneficial than the use of ART in diagnosed patients with an early screening from the general population. This combination of strategies showed that PrEP in a high-risk population with or without an early screening generates a lineal boost in DALYs. Finally, in the screening only to high-risk groups and the use of ART at infected

TABLE 1 | CUA results with deterministic and stochastic models applied to the different scenarios (early diagnosis and ART/PrEP) scenarios.

Treatment/ intervention	Annual cost ($)	Annual DALYs	CEM (USD)	ΔCost (USD)	ΔDALY	NHB (years)	NMB (USD)	λ (USD)	Health gained	ICER
Deterministic model										
ART	71,673,908.3	9,245.31	7,752.45	−66,086,300	−2,163.795	66,086,299.6	2,163.795	6,213.5	−10,635.92	30,541.84
PrEP	5,587,608.75	7,081.51	789.04							
Stochastic model										
ART	19,364,060.5	8,133.177	2,380.87	−15,608,190	−262.716	15,608,189.9	262.716	6,213.5	−2,511.98	59,410.88
PrEP	3,755,870.54	7,870.461	477.21							
Deterministic model										
Late diagnostic	8,675,205.56	4,471.10	1,940.28	4,742,968.08	−108.63	−4,742,968.1	108.632	6,213.5	763.33	−43,660.87
Early diagnostic	13,418,173.6	4,362.47	3,075.82							
Stochastic model										
Late diagnostic	10,171,819.2	5,773.87	1,761.70	552,614.57	−934.36	−552,614.57	934.36	6,213.5	88.94	−591.43
Early diagnostic	10,724,433.7	4,839.50	2,216.02							

subjects, higher economic spending will occur (exponential trend) and an increase in DALYs (**Figures 4A,B**).

The early screening (first scenario), using stochastic and deterministic data, was a high-cost strategy with an enormous benefit, as the majority of simulated data are under the GDP's threshold. In the stochastic data, more than 85% fit under the mentioned threshold (**Figure 2A**). If the traditional screening continues, data of stochastic and deterministic models showed a high-cost–utility and under the GDP's threshold. However, the stochastic data showed a better cost-effective trend (**Figure 2B**).

For early diagnosis in both models, 60% of simulated data is under the GDP's threshold showing the strategies are highly beneficial, regarding low economic cost and loss of benefit lower than 30% for the combination of both (**Figures 2A,B**). When ART is employed like treatment (without other reinforcement strategies) while considering deterministic data, this strategy alone proves to be beneficial but represents a significant cost (finding 45% of simulated data under GDP's threshold). Considering only PrEP as the only approach at the high-risk population, simulations with stochastic and deterministic data demonstrate that the use of prophylaxis is beneficial but is extremely expensive (90% of these data are near the GDP's threshold), showing that the benefit could diminish if the cost increases (**Figures 5A,B**). When ART and PrEP are combined, 50% of stochastic and deterministic data were placed under GDP's threshold. As a result, both strategies are cost-beneficial when merged, representing an average economic expense, with a loss of benefit of at least 30% caused by their combination (**Figures 3A,B**).

PSA for Payment Capacity and DALYs

In an early screening scenario, a probabilistic sensibility analysis considering stochastic and deterministic data revealed that the investment for this intervention represents around 10,000 USD per person in the HIV/AIDS program per year. In contrast, with the regular screening and deterministic data, the investment would be 6,500 USD per person in the HIV/AIDS program per year (**Figures 6A,B**). Furthermore, considering the PSA DALYs with stochastic and deterministic data, in the hypothetical case that the National Authorities decide to decrease 50% of the budget that will be employed for early screening, there will be a loss of at least 5 years of healthy life. In the case of traditional screening with the same decreased percentage of the budget, the loss of years of health will be years of DALYs (**Figures 6C,D**).

Even more PSA simulations with stochastic and deterministic data indicate that the best cost-beneficial strategy of early screening includes general and high-risk populations. The investment to do this would be around 9,500 USD per person in the HIV/AIDS program per year. Without this strategy and a hypothetical reduction of 70% in the assigned budget, people could lose between 4 and 6 healthy years, but with the early screening of the general population, they could have 3–5 years of a healthy life, with a saving in resources of at least 20–30% (**Figures 6A,B**).

With PSA for ART and PrEP, and if these interventions are separated, their costs are high. For ART, using stochastic and deterministic data showed an investment between 20,000 and 50,000 USD per person in the simulated population, but a national strategy budget is required. In contrast, for PrEP, the value is less (2,000–5,000 USD per person in the simulation) and is under the GDP threshold (**Figure 7**). Considering DALYs with the same type of data, if the budget reduces more than 50% to ART, it will generate a loss of 10–12 years of healthy life. In PrEP, the loss could be between 6 and 8 years of healthy life (**Figure 8**). Finally, the strategy of using both ART and PrEP with stochastic and deterministic data would require an investment of 16,000–50,000 USD per person in the simulated population. This would also be under the GDP's threshold (**Figures 9A,B**). For DALYs, this combination strategy and the decrease of 50% in the local budget would lead to a loss of 4–6 years of healthy life (**Figures 9C,D**).

DISCUSSION

HIV is categorized as a chronic transmissible disease, which is still an issue for public health, generating a significant expense

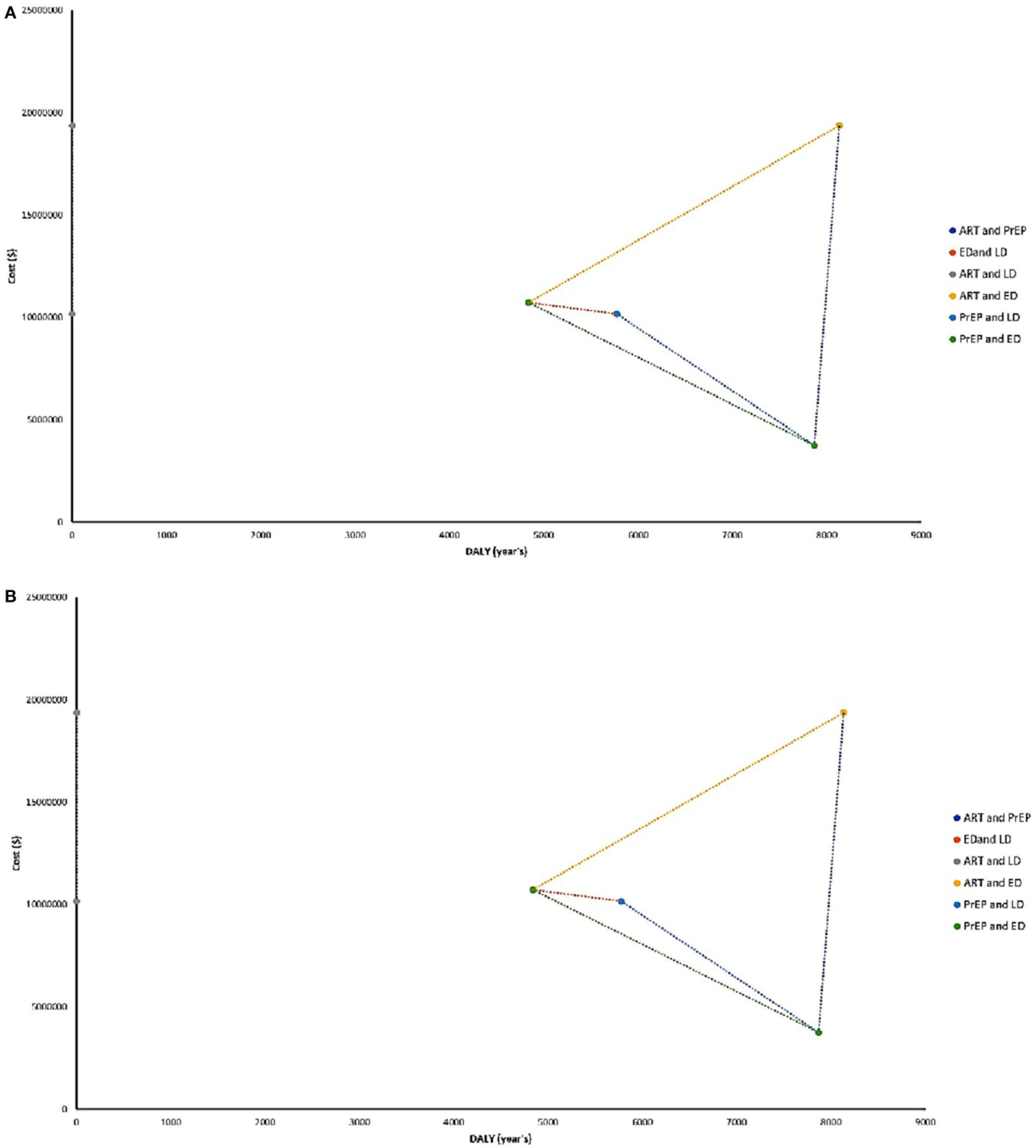

FIGURE 4 | (A) ICERs with both analyses using a deterministic data distribution. **(B)** ICERs with both analyses using stochastic data distribution. ART, antiretroviral therapy; PrEP, prophylactic treatment; ED, early diagnosis; LD, late diagnosis.

due to a delay in diagnosis and treatment. Although ART and optimal diagnosis strategies have reduced its morbimortality, there is no definitive cure or eradication of the virus from their hosts. Thus, local and international authorities should reinforce the prevention and early detection of HIV infections. According to WHO and MSP in 2019, Ecuador showed a decrease

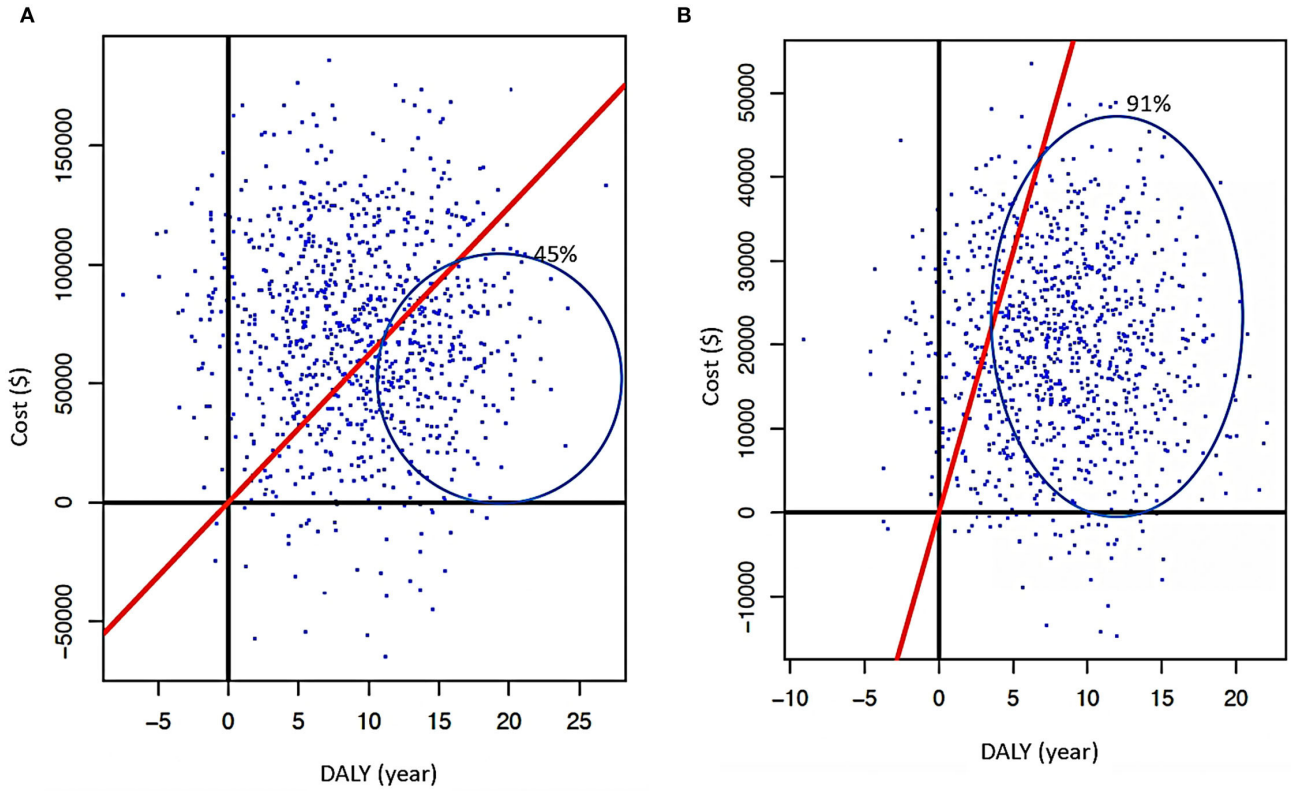

FIGURE 5 | (A) Cartesian plane representing PSA when PrEP is used without other intervention is applied with deterministic data. **(B)** Results of PSA when PrEP is used (at high-risk populations) using stochastic data (red continuous line represents GDP's threshold; cost is in American dollars and DALYs in years).

in HIV mortality (<4.55/10,000 inhabitants) and its incidence (<2.2 cases/1,000 inhabitants), indicating that the interventions implemented by the national authorities have been effective (8). However, they are not enough for controlling the HIV epidemic as some reevaluations and new implementations are required (13).

The cost–utility analyses (CUA) are highly significant since they provide essential information and should be considered during the establishment, restructuring, implementation, or maintenance of the strategy as a control and prevention mechanism in case of an event. There is a misconception that knowing the representative cost of any plan is the only factor required in formulating health budgets. A CUA helps to determine whether a country could invest in a specific strategy to improve the overall situation and to choose the best response against a health event, based on a GDP's threshold and the calculation of expenses with a correct budget in a non-arbitrary way.

CUA is also thoroughly recommended in diseases like HIV, where diagnosis, prevention, and treatment need a constant investment that should be continuously monitored or modified according to results. For example, an early HIV diagnosis depends entirely on a regular screening among critical populations to reach an effective infection control, but this only could be possible with well-conducted financial investment. During 2012–2020, the WHO spent 40 million dollars only on HIV/AIDS diagnosis (30). Countries like the United States in 2008 pointed out that an investment of 3 dollars per person was used for rapid HIV tests annually (31). In 2017, Ecuador invested 49% of its health budget only for HIV prevention. Nonetheless, the exact percentage used only in screening is still unknown (8).

The US Center for Control and Prevention of Diseases (CDC) suggests that an extra effort should be made on screening the population who maintains an active sex life (people between 13 and 64 years old) with or without traditional risk behaviors (sex workers, MSM, etc.). All of this is a part of continuous surveillance and routine medical checks (32). Ecuador has included this policy in its new HIV clinical practice guideline from 2019, along with the idea of gradual implementation. On the contrary, Spain served as a reference for successful implementation of this early screening for the target population mentioned before, as in 2018 reached a detection of 90% HIV-infected population (33) with a high incidence among MSM. Also, Murray et al. (34) mentioned that using this strategy reduces ART's cost significantly and the expenditure in morbimortality and healthy years gain (21).

With all these in mind, we applied a CUA and PSA using nationally available data to appreciate if a strategy where all the sex-active population could be screened is cost-beneficial. According to our results, the ICER showed that the mentioned

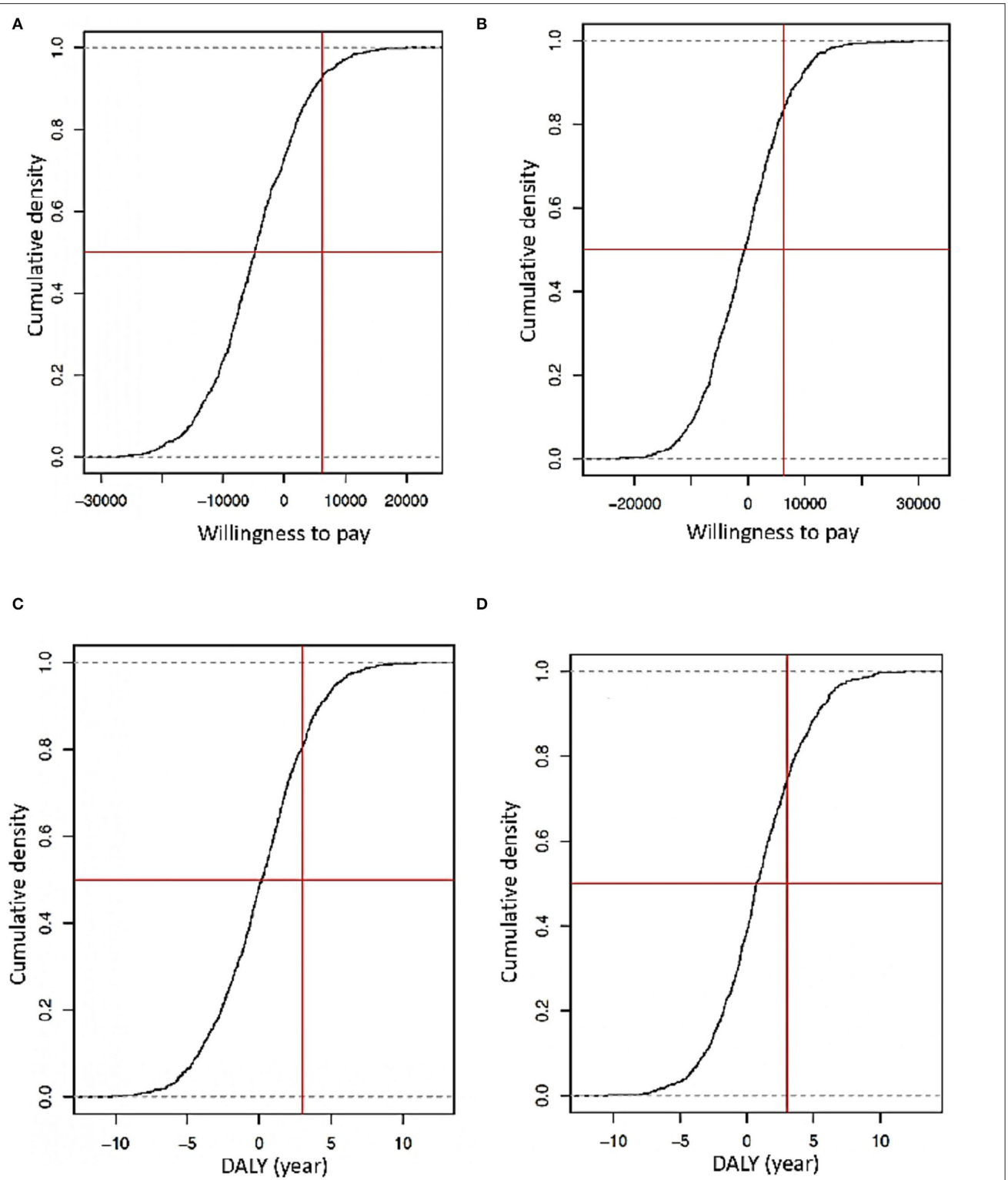

FIGURE 6 | (A) Simulation of the capacity of willingness considering an early screening scenario with deterministic data diagnosis. **(B)** Simulation of the capacity of willingness considering an early screening scenario with stochastic data diagnosis (the Y is the probability that a spend is accepted how a part of an implemented strategy, X is the capacity of the willingness of an indeterminate government approach concerning GDP that is the vertical red line, and the horizontal red line the threshold of logistic regression of 50%). **(C)** Simulation of DALYs loss or gained using deterministic data diagnosis. **(D)** Simulations of DALY's years gained or loss using stochastic data diagnosis (the Y-axis is the probability to lose or gain years of life by the strategy. The red vertical line represents standard DALYs lost by a late diagnosis, and the horizontal red line is the threshold of logistic regression in 50%).

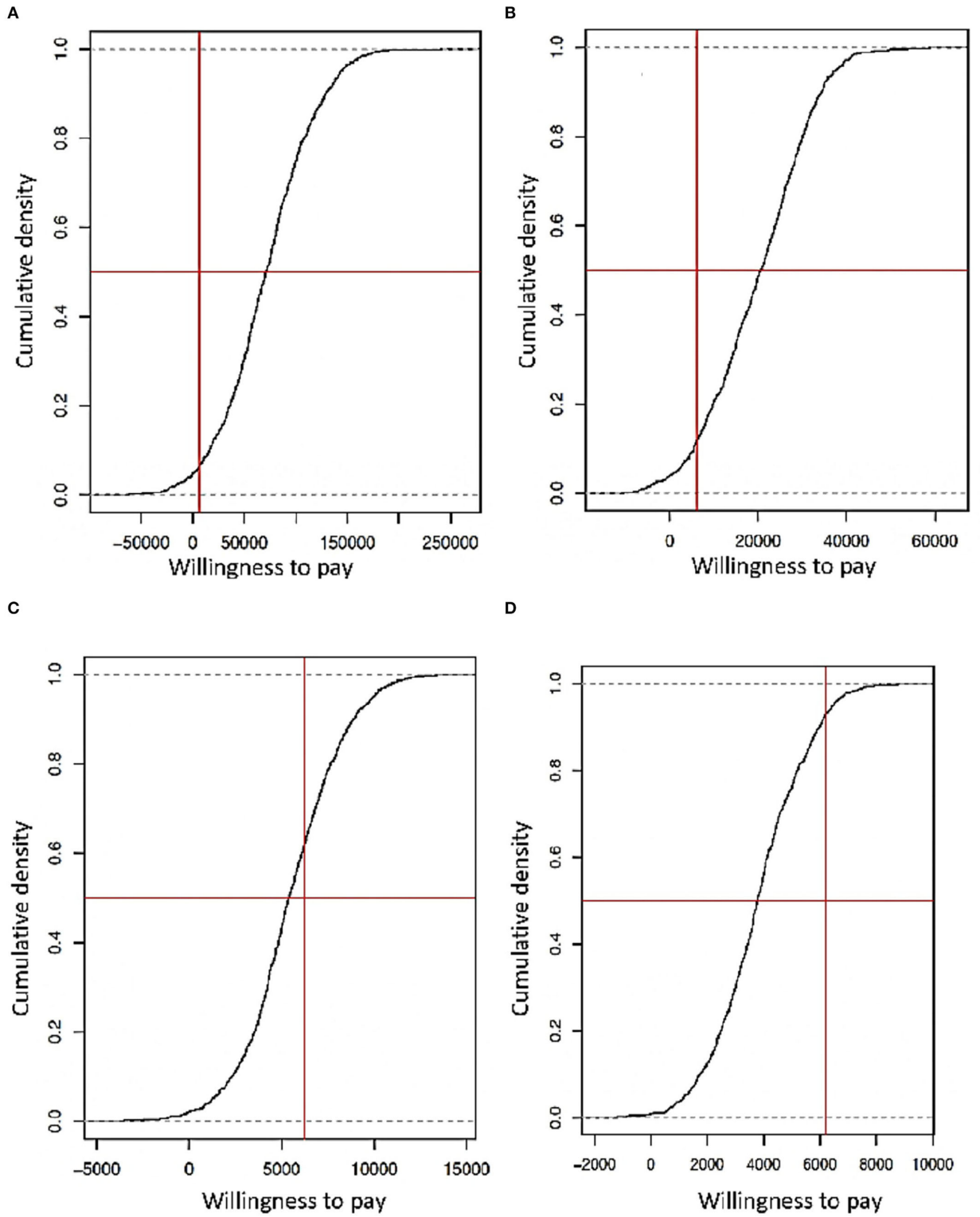

FIGURE 7 | (A) Simulation of the capacity of willingness considering the use of only ART with deterministic data. **(B)** Simulation of power of willingness viewing the using singular ART with stochastic data. **(C)** Simulation of the capacity of willingness considering the use of PrEP like prevention with deterministic data. **(D)** Simulation of the capacity of willingness considering only PrEP like prevention with stochastic data (the Y-axis is the probability that an expending would be accepted and forming part of the implementation strategy, X-axis represents how much a government can spend on a strategy according to its GDP, represented by a vertical red line, and the horizontal red line is the logistic regression threshold of 50%).

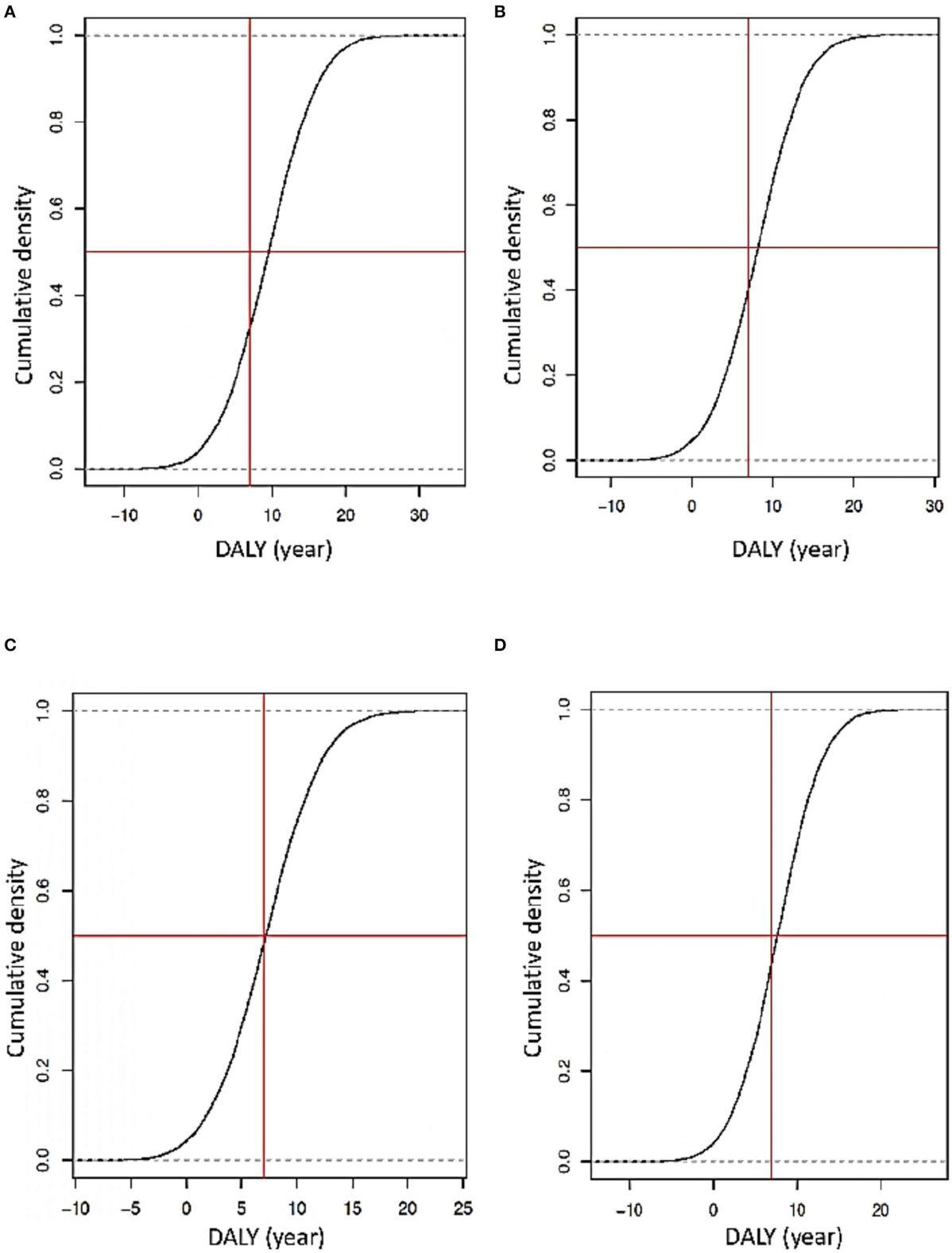

FIGURE 8 | (A) Simulation of DALY's years' loss or gain using deterministic data for the exclusive use of ART. **(B)** Simulations of DALY's years gained or loss using stochastic data for ART use alone. **(C)** Simulations of DALY's years gained or loss using deterministic data for PrEP like prevention. **(D)** Simulations of DALY's years gained or loss using stochastic data for PrEP like prevention (the Y-axis is the probability of loss or gain years of life due to an implemented strategy. The vertical red line represents the standard DALYs lost by a later diagnosis, and the horizontal red line is the logistic regression threshold of 50%).

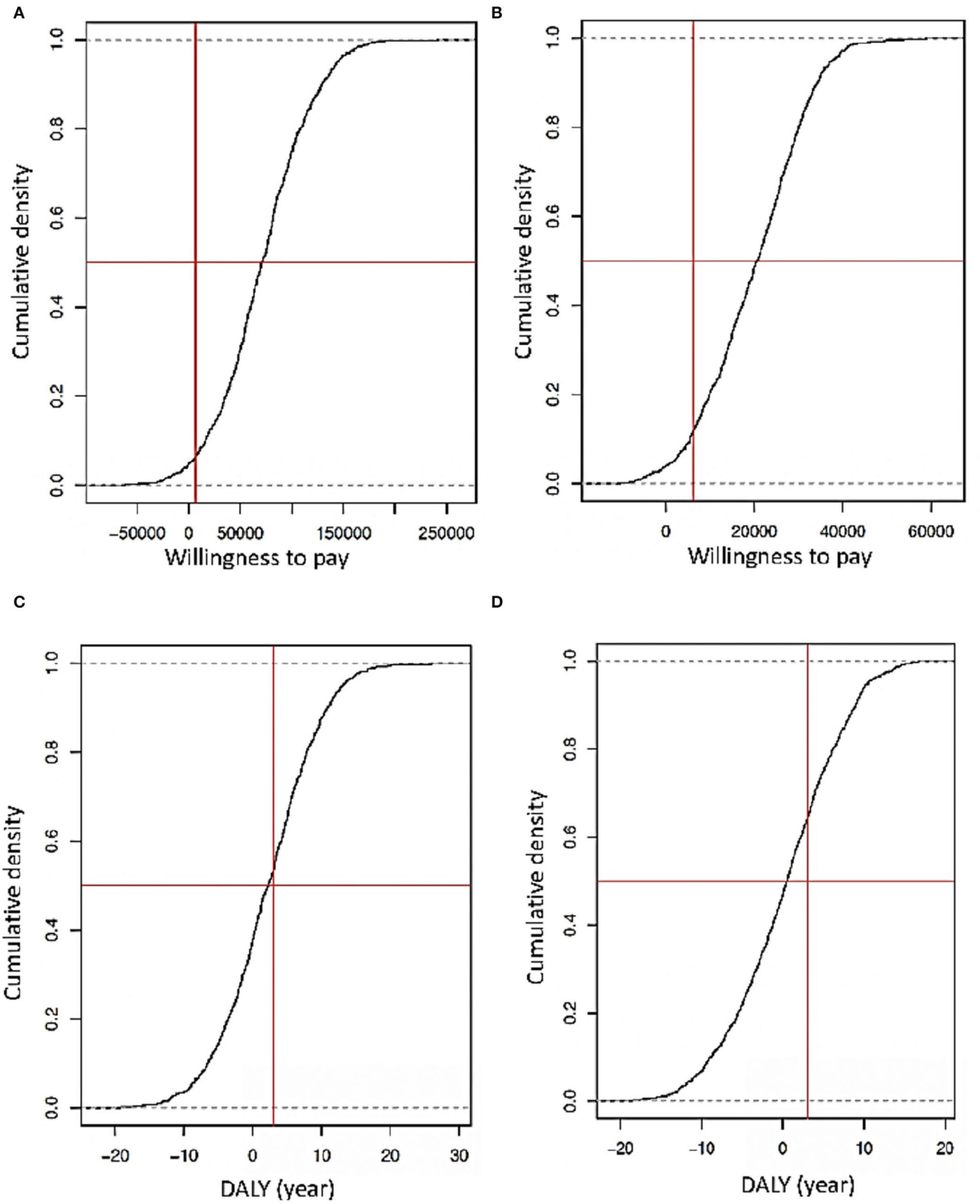

FIGURE 9 | (A) Simulation of DALY's years' loss or gain using deterministic data for the combination of ART and PrEP. **(B)** Simulations of DALY's years gained or loss using stochastic data for the combination of ART and PrEP (the Y-axis is the probability that an expending would be accepted and forming part of the implementation strategy, X-axis represents how much a government can spend on a plan according to its GDP, represented by a vertical red line, and the horizontal red line is the logistic regression threshold of 50%). **(C)** Simulations of DALY's years gained or loss using deterministic data for ART and PrEP. **(D)** Simulations of DALY's years gained or loss using stochastic data for the combination of ART and PrEP (* the Y-axis is the probability of loss or gain years of life due to an implemented strategy. The vertical red line represents the standard DALYs lost by a later diagnosis, and the horizontal red line is the logistic regression threshold of 50%).

strategy (using stochastic and deterministic data) is cost–utility. An increase of 15–35% of additional investment from the HIV national budget keeps being under our national spending capacity and the GDP's threshold. Thus, we will generate source savings in the medium-term future (the negative sign of ICER) (35, 36).

Moreover, we considered in the analysis DALY's values that demonstrate an early screening remains cost-beneficial by preventing the infection than an investment in ART for the long term. The same was concluded by Cylus et al. (37). Although a gain of healthy years for early HIV identification is observed, it also depends on each subject's age at the time of diagnosis, since people more than 70 years old lose 0.3 healthy years without ART, and people between 15 and 49 years old with the same condition can lose 21 DALY's years. So the profit of healthy years for an early diagnosis decrease as age increases (38, 39).

We simulated a scenario where 50% of the typically assigned budget to HIV diagnosis was reduced without any CUA analysis. It demonstrated a loss of years of healthy life (5 years/DALY's), meaning that HIV carriers, with this budget reduction, would lose up to 5 years of life, revealing the importance of continuing and improving a diagnosis strategy of this infection. Furthermore, with a combination of screening of the sexually active population and the early identification of cases and prompt use of ART, but 7% of budget reduction, a loss of 20 years/DALY's in the HIV population could occur (35, 40).

Ecuador health investment compared to its GDP increased in 2012 to 2.5% compared to only 0.6% in 2000. This only demonstrates that a slight decrease in health budget could be one explanation for the increase in HIV cases during those years and, in consequence, less healthy years and a 2.5 DALY's% of health budget (35, 40). Ecuador invests at least 40% of its HIV budget in ART than in prevention, but, in future, this strategy will generate more costs than source savings. The same pattern can be found in other countries.

The PAHO and other organizations have mentioned that a reduction in HIV/AIDS incidence was achieved by ART coverage (not an early diagnosis) (8, 41). However, in 2013, HIV/AIDS ranked number 10 in the causes of loss of years of healthy life (DALY's), and in 2017, it reached number two, showing that even though the use of ART and the adopted measurements are useful, they prove not to be enough to get an optimal control and management of HIV/AIDS. Also, ART may cause a loss of 1.4 healthy years by following our model results where drug treatment generates a loss of 2 to 2.5 of healthy years. It is important to emphasize that this loss does not support the idea that an HIV carrier should not receive treatment (21, 38), because it generates a loss of 60 years of health.

PrEP has been gaining considerable ground in the last decades, showing it is a good option for HIV prevention, especially at high-risk groups, but not on a large scale. A successful follow-up and control have been reached in small groups and specific locations. On the contrary, on an upper rate, it has been demonstrated that it does not work well if the cost increases and if it is not cost-beneficial (42, 43). In our analysis, we observed the same pattern in the simulations. PrEP had a lower cost compared to the costs generated by ART-like treatment. But considering that PrEP is not used in seropositive patients, and it is exclusive given to populations at high risk to acquire HIV, applying the early screening strategy (proposed above) to all sexually active people is not feasible, due to its high costs. Through ICER calculation, we exhibited that PrEP is less expensive than ART and with fewer DALYs dropped.

Nevertheless, the PSA was close to exceeding the amount (closely reaching a high cost and low-efficiency position). Comparing the PSA between ART and PrEP, regarding DALY's, a difference of 3–5 DALY's years was observed. This means that if PrEP is used among high-risk populations, a boost in healthy years could be reached, similar to what was observed in previous studies, where the loss of healthy years with ART and PrEP are 7 and 5 years, respectively (44).

We estimated the scenario at which early screening and use of ART occurred and observed that this combination could reduce up to three DALY's years. In addition, the ICER's consolidation is more profitable, following the WHO report, increasing healthy life years and diminishing costs. All of this, along with a constant investment, allow savings and infection control (45). On the contrary, if ART is used late, even though the diagnosis was early, a loss of nine DALYs could have experimented. The fusion of PrEP and an early diagnosis can generate a loss of up to two DALYs years, but if PrEP is not used in high-risk population and only early diagnosis is used, a decrease of eight DALYs years could be expected. Following HIV/AIDS reports regarding global economic issues, the coverage of pharmacological HIV treatments has decreased, leading to loss of 5 to 10 years/DALY's due to imperfect execution of strategies or lack of access to ART (38). Thus, there are cases in countries where PrEP or ART is intermittent without a timely diagnosis, provoking what a phenomenon called "islands of infection" where the accumulation of new cases is observed, and a spread of the HIV infection occurs (43, 46).

It is crucial to mention the weaknesses that this type of model has, which is calculated by GPD's thresholds in each country. According to the WHO, during the 90s, calculating a general threshold was complicated. For this reason, the GDP per country was proposed as a reference (37). Our models showed that according to the national context, it is cost-beneficial to apply an early screening. Even by considering our GDP's threshold, we should invest 49% of the national income, exclusively on the diagnosis and treatment of HIV. With this in mind, a solution could be to follow the English Health System that calculates a threshold based on the comparison of the total health gained by prevention against purchasing power parity of a new treatment. Usually used as an index, it portrays the real value to pay per habitant, for each government to be able to increase the population's healthy years. For England in 2013, this value was 4,260 USD (37). In Ecuador, it represents more than 80% of GDP, but we could find a real value with an accurate calculation.

CONCLUSION

Finally, the CUA demonstrates that an early diagnosis in a sexually active population is cost-beneficial. This, combined with ART or PrEP, is ideal to acquire more years of healthy life. If ART and PrEP were considered, this would be cost-beneficial but only aimed at the high-risk population; otherwise, its use is not justified. Maintaining a fixed budget for the HIV / AIDS programs, a thing that does not vary according to the government in office, will allow resource savings due to the decrease of DALY's. A thing that in future will allow the ART and PrEP costs are redefined. This may generate savings and improve the strategy toward early diagnosis, reach 90–100% ART coverage, meet the WHO target, and achieve a 15–30% budget savings with this strategy.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

PQ-A, PE, and ET conceptualized the study. SO, IH, and AH provided technical input for the methodology and analysis. PQ-A and PE collected and analyzed data. SO, IH, AH, and ET reviewed the analysis and validated the results. All authors contributed to writing the initial draft, reviewed, and approved the final version.

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Severity and Outcomes of Dengue in Hospitalized Jamaican Children in 2018–2019 During an Epidemic Surge in the Americas

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Objective: In 2019, dengue was among the "top-ten threats to global health," with 3.1 million cases reported from the Americas, the highest ever. Simultaneously, Jamaica reported its largest dengue outbreak in 40 years, following Chikungunya and Zika virus epidemics, in 2014 and 2016–2017, respectively. We describe dengue in children admitted to five hospitals in Jamaica during August 2018 through September 2019.

Methods: Hospitalized children and adolescents aged 0 to 15 years with dengue were managed using PAHO/WHO criteria. Data were extracted from questionnaires, entered into a dataset on Microsoft Excel version 2016, exported to SPSS version 20 and analyzed. Groups were compared using Student's *t*-test for normally distributed parametric data. Chi-square analysis, or Fisher's exact test was used for categorical variables. A p-value < 0.05 was considered statistically significant.

Results: There were 339 children, 245 (72.3%) aged 1–10 years, males:females 1:1. Classification was "dengue without warning signs" 53 (15.3%), "dengue with warning signs" 218 (64.3%) and "severe dengue" 68 (20%). Co-morbidities were reported in 88 (26%). Hemoglobin SC disease was associated with severe dengue with hemorrhage ($p = 0.005$). Organ-system involvement occurred in 334 (98.5%) including gastrointestinal 317 (93.5%), hematologic 311 (91.7%) and musculoskeletal 180 (53.1%). Thirty-nine (11.5%) had 5–7 organ-systems involved. Metabolomics emphasized increased hepatic transaminases 245 (72.3%), lactate dehydrogenase 164 (48.4%) and creatine phosphokinase 84 (24.8%) approaching the high thousands (121,560 u/L), both were markers for severe disease ($p < 0.002$). Thirteen (3.8%) received intensive care. Dengue was laboratory-confirmed in 220 (78.9%): NS1 antigen-positive (218); RT-PCR-positive (23), with an overlap of NS1 antigen and RT-PCR positive (21);

DENV-3 serotype (20). Seventeen (5%) died, 16 (94.1%) had severe dengue and 11 (64.7%) succumbed within 24 to 48 h of admission despite resuscitation and transfusion of blood products.

Conclusion: Severe dengue with increased attributable mortality occurred in hospitalized children after Jamaica's maiden Zika epidemic.

Keywords: dengue, severe dengue, dengue serotype, antibody-dependent enhancement, children, immunity, Jamaica

INTRODUCTION

Dengue was designated one of the "top ten threats to global health" in 2019 by the World Health Organization (1). It is a leading cause of death and illness among persons with arbovirus infections (2). Over half the world's population is at risk, over 390 million cases are reported annually and 96 million present clinically with varying disease severity (3). Dengue is caused by the arthropod-borne flavivirus with four dengue virus (DENV) serotypes DENV-1, 2, 3 and 4 (1). Transmission is mainly by the *Aedes aegypti* mosquito (4). In 2009, the WHO re-classified dengue infection: "dengue without warning signs," "dengue with warning signs," and "severe dengue with and without hemorrhage" (5). In Latin America and the Caribbean, over 3.1 million cases were reported in 2019, the highest ever historically, with all four serotypes (1–4) co-circulating in Brazil, Guatemala and Mexico (1, 6). The proportion of severe dengue, 0.9%, exceeded reports in the preceding 4 years (6). Children typically have the most severe presentations of dengue and the highest attributable-morbidity and mortality (7).

In Jamaica, an upper middle-developing Caribbean island-nation, with population 2.97 million, national surveillance data are available from 1977 for dengue epidemics, where sero-prevalence rates approach 99–100% in pregnancy, which is a readily-accessible representative Jamaican subpopulation (8). In 2014, an epidemic with several thousands of suspected cases of chikungunya virus (CHIKV) infections were reported with an 83.6% seropositivity (9–12). In 2016, Jamaica experienced its maiden Zika virus (ZIKV) epidemic, when all three arboviruses co-circulated endemically (13–16). In Cuba, two *Ae aegypti* mosquito populations became infected with and transmitted DENV-1, ZIKV and CHIKV together, albeit at low rates (17).

Dengue outbreaks have increased in frequency and intensity over 40 years of active dengue surveillance in Jamaica, with increased intensity, severity and reported cases in recent years. According to the national surveillance data, there were 5,461 dengue-reported cases in 2007 and 5,903 in 2012 (16). Jamaica experienced an upsurge in cases late 2018 with an epidemic declared on January 3, 2019 (18). In Jamaica, during 2012, among 134 children who were admitted with dengue to the academic referral center, the University Hospital of the West Indies (UHWI), 10.45% were severe with a case fatality rate (CFR) of 3.73% (19). The circulating serotype was DENV-3 (13). In the 2018-2019 dengue epidemic, children were also significantly affected (6, 16). There were 10,411 suspected and confirmed total dengue cases in adults and children in the 2018–2019 epidemic of which 22% required hospitalization (16). Among adults and children, there were 86 suspected and confirmed dengue-related deaths island-wide in 2018–2019 with an overall national case fatality rate (CFR) for the combined ambulatory and hospitalized cases who died of 0.83%. This has increased from 0.39% in 2012 and 0.46% in 2007 (16). Jamaica's dengue mortality rates therefore exceed PAHO's dengue-attributable mortality threshold of 0.05% for the Americas (20).

There is a paucity of reports describing the clinical severity of dengue in children in the aftermath of the ZIKV "epidemic of international concern" that was declared in the Americas. Initial reports by the pediatricians and national surveillance of high attributable-morbidity and mortality in children in the 2018–2019 dengue epidemic in Jamaica, were of major importance. Information obtained about severe dengue in this setting can be used to further inform other regions and further elucidate the patho-physiology of "severe dengue" in similar populations of children who are hospitalized with dengue fever.

The primary objective of the study was to evaluate and describe clinical and laboratory-confirmed cases of dengue in hospitalized Jamaican children ages 0–15 years at five hospitals during the 2018–2019 epidemic using WHO diagnostic criteria. Additionally, we identified the associated clinical factors and laboratory markers of severe disease.

MATERIALS AND METHODS

This ambispective cohort study was conducted for suspected and confirmed dengue cases identified before February 2019 and prospectively thereafter. Cases were included and enrolled from August 2018 to September 2019 during the epidemic. DENV, CHIKV and ZIKV infections are all reportable Class 1 Notifiable Diseases to the Jamaican Ministry of Health and Wellness (MoHW) and immediately, within 24 h of suspicion. Five hospitals across Jamaica, serving about half the island's pediatric population were included: Bustamante Hospital for Children, providing services for children aged 0–11 years; University

Abbreviations: ADE, Antibody-dependent enhancement; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CARPHA, Caribbean Public Health Agency; CFR, Case fatality rate; CPK, Creatine phosphokinase; DENV, Dengue virus; DHF, Dengue hemorrhagic fever; ELISA, Enzyme-linked immunosorbent assay; ICU, Intensive Care Unit; IgG, Immunoglobulin G; IgM, Immunoglobulin M; LDH, Lactate dehydrogenase; MoHW, Ministry of Health and Wellness; NS1, Non-structural protein 1; PAHO, Pan American Health Organization; PCR, Polymerase chain reaction; PT, Prothrombin time; PTT, Partial thromboplastin time; UHWI, University Hospital of the West Indies; WHO, World Health Organization; ZIKV, Zika virus.

Hospital of the West Indies, Mandeville Regional Hospital, May Pen Hospital and Spanish Town Hospital, all providing services for children and adolescents aged 0 to 15 years. The UHWI and Bustamante Hospital for Children accepted referrals island-wide for further care, including intensive care services. Hospitalized children and adolescents aged 0 to 15 years with pediatrician-diagnosed and/or laboratory-confirmed dengue were included. Cases were ascertained from the ward admission registers and MoHW Class 1 Notifiable Disease registers of all hospitals. A data extraction sheet was used to collect data after review of their hospital files. We also collaborated with the national surveillance records for dengue.

Samples at the UHWI were tested for dengue NS1 antigen using enzyme-linked immunosorbent assay (ELISA). While samples sent to the private laboratories were tested using rapid dengue NS1 antigen, dengue IgM and IgG serology. A subset of samples was tested using reverse transcriptase polymerase chain reaction (RT-PCR) at the UHWI and the Caribbean Public Health Agency (CARPHA) laboratories with serotypes identified. CARPHA also tested a subset for acute ZIKV and CHIKV infections by RT-PCR. Hematologic and biochemical tests were performed on blood samples routinely collected during hospital care.

Primary data collected included demographics, hospitalization, intensive care unit (ICU) admission, anthropometry, exposure to ill-contacts, previous arbovirus infections, co-morbidities, drug history, clinical presentation, complications, laboratory investigations including dengue RT-PCR and NS1 antigen, ZIKV infections (IgG), ZIKV and CHIKV RT-PCR, hematology, chemistry, radiology, treatment modalities including transfusion of blood products, organ-system involvement and outcomes.

Case definitions for "dengue" from WHO's revised criteria in 2009 were included: dengue without warning signs, dengue with warning signs, severe dengue without hemorrhagic features and severe dengue with hemorrhagic features. Cases of "pediatrician-diagnosed" dengue fever in hospitalized children were included. Laboratory-confirmed dengue was diagnosed by a positive dengue NS1 antigen and/or dengue RT-PCR. Children were managed according to PAHO criteria which were modified and national training done for their appropriate implementation and use (21).

Data were extracted from questionnaires, unlinked to patient identifiers, entered into a dataset on Microsoft Excel version 2016 and then exported to Statistical Package for the Social Sciences (SPSS) version 20 and analyzed. Comparisons were made between groups using Student's *t*-test for normally distributed parametric data. Chi-square analysis, or Fisher's exact test was used for categorical variables. Continuous variables were analyzed using Student's *t*-test. A p-value < 0.05 was considered statistically significant.

Ethics approval was formally obtained to perform this study from the Medical Research Ethics Committee of the University Hospital of the West Indies Mona Campus and also the Ethics **(ECP 218 18/19; September 16, 2019)** and also the Medico-legal Affairs Committee of the Ministry of Health and Wellness, Jamaica **(2019/18; November 11, 2019).** Dengue fever is a Class 1 Notifiable Disease in Jamaica. Parents were informed of their child's diagnosis and its mandatory notification to the National Health Authorities within 24 h. Only medical records were extracted and there was no study intervention other than routine medical care, so there was no need for informed consent to be obtained from the parents.

RESULTS

A total of 339 children and adolescents fitting the criteria for dengue were enrolled. Of this total, 279 had testing for dengue NS1 antigen and/ or dengue RT-PCR. Of these, 220 (78.9%) had a positive laboratory confirmation: 218 (71.8%) were dengue NS1 antigen positive and 23 (6.8%) were RT-PCR positive, with overlap of 21 (7.5%), both dengue RT-PCR and NS1 antigen positive. DENV-3 serotype was confirmed in a subset of 20. No other arbovirus was co-circulating. Cases were reported from the Bustamante Hospital for Children (159), UHWI (130), Mandeville Regional Hospital (19), May Pen Hospital (16) and Spanish Town Hospital (15).

Aggregate results for 339 children were compared against the subset of 17 (5%) who died (**Tables 1**–**5**). Dengue classification, overall, comprized "severe dengue" in 68 (20.1%), those without hemorrhage in 28 (8.3%) and those with hemorrhage in 40 (11.8%). Among the 68 with severe dengue, more than half, 59% had hemorrhage. "Dengue with warning signs" were 218 (64.3%) and "dengue without warning signs" in 53 (15.6%). The children were aged 4 months to 15 years with a mean age 6 years. The majority of those affected were 6–10 years-126 (37.2%), followed by 1–5 years-119 (35.1%) (**Table 1**). The male:female ratio was 1:1.

Co-morbidities were present in 88 (26%). The most commonly reported were: asthma 46 (52.3%), sickle cell disease 19 (21.6%) specifically Hb SC in 10 (11.4%) and epilepsy 8 (9.1%) (**Table 1**). Hb SC was associated with severe dengue with hemorrhage ($p = 0.005$). Non-steroidal anti-inflammatory drug use was reported in 36 (10.6%).

The time between onset of illness and hospitalization was 0–21 days (**Table 2**). There were 13 (3.8%) ICU admissions, all had severe dengue with hemorrhage of which 7 (53.8%) died. Duration of ICU admissions and hospitalizations ranged from 1 to 26 days and 1–46 days, respectively. Those with severe dengue with hemorrhage had the longest hospitalization (**Table 2**).

Three hundred and thirty-six (99.1%) had fever (**Table 3A**). Organ-system involvement occurred in 334 subjects (98.5%) (**Table 4**). More organ-systems were involved with increasing disease severity, with 39 (11.5%) having 5–7 organ-systems involved. The gastrointestinal system was most commonly affected in 317 (93.5%) (**Table 4**), including vomiting-223 (65.8%), abdominal pain/ loss of appetite-(53%), diarrhea-123 (36.3%) and hepatomegaly only-70 (20.6%) (**Table 3A**). Hematologic involvement occurred in 311 (91.7%) and musculoskeletal in 180 (53.1%) (**Table 4**). Other frequent clinical presentations included headache (54.9%), lethargy (53%), cough (34.5%), rash (28.9%) and eye pain (22.7%). Those who died had higher rates of lethargy (82.4%), loss of appetite and diarrhea

TABLE 1 | Demographics and co-morbidities in pediatric patients hospitalized with suspected and confirmed dengue in the 2018–2019 epidemic.

	Dengue without warning signs ($n = 53$)	Dengue with warning signs ($n = 218$)	Severe dengue without haemorrhagic features ($n = 28$)	Severe dengue with haemorrhagic features ($n = 40$)	Total ($n = 339$)	Mortality cases ($n = 17$)
Age group	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
<1 year	7 (2.1)	24 (7.1)	4 (1.2)	3 (0.9)	**38 (11.2)**	1 (5.9)
1–5 years	20 (5.9)	69 (20.4)	13 (3.8)	17 (5.0)	**119 (35.1)**	9 (52.9)
6–10 years	14 (4.1)	91 (26.8)	7 (2.1)	14 (4.1)	**126 (37.2)**	6 (35.3)
11–15 years	10 (2.9)	33 (9.7)	4 (1.2)	6 (1.8)	**53 (15.6)**	1 (5.9)
Missing data	2 (0.6)	1 (0.3)	0	0	**3 (0.9)**	0
Sex	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Male	29 (8.6)	108 (31.9)	15 (4.4)	16 (4.7)	**168 (49.6)**	5 (29.4)
Female	22 (6.5)	109 (32.2)	13 (3.8)	23 (6.8)	**167 (49.3)**	12 (70.6)
Missing data	2 (0.6)	1 (0.3)	0	1 (0.3)	**4 (1.2)**	0
Co-morbid illnesses						
Hb SC	1 (0.3)	3 (0.9)	2 (0.6)	4 (1.2)*	**10 (2.9)**	3 (17.6)
Hb SS	0	6 (1.8)	2 (0.6)	0	**8 (2.4)**	0
Hb SBthal	0	0	0	1 (0.3)	**1 (0.3)**	0
Hb AS	1 (0.3)	4 (1.2)	0	0	**5 (1.5)**	0
Asthma	5 (1.5)	35 (10.3)	1 (0.3)	5 (1.5)	**46 (13.6)**	4 (23.5)
Cardiac	1 (0.3)	3 (0.9)	0	1 (0.3)	**5 (1.5)**	0
Epilepsy	0	4 (1.2)	2 (0.6)	2 (0.6)	**8 (2.4)**	2 (11.8)
Cerebral palsy	0	1 (0.3)	0	0	**1 (0.3)**	1 (5.9)
Others:	4 (1.2)	8 (2.4)	2 (0.6)	1 (0.3)	**15 (4.4)**	1 (5.9)

*$p < 0.05$.

TABLE 2 | Time of presentation and duration of hospitalization in pediatric patients hospitalized with suspected and confirmed dengue in the 2018–2019 epidemic.

	Dengue without warning signs ($n = 53$)	Dengue with warning signs ($n = 218$)	Severe dengue without haemorrhagic features ($n = 28$)	Severe Dengue with haemorrhagic features ($n = 40$)	Mortality cases ($n = 17$)
Time between onset and presentation to hospital	**Days**	**Days**	**Days**	**Days**	**Days**
Minimum-maximum	0–14	0–21	0–14	0–21	1–7
Median	4	4	4	3	3
Mean	4.44	4.67	4.43	4.08	3.12
CI (95%)	3.493–5.391	4.195–5.136	3.051–5.806	2.913–5.237	2.267–3.968
Duration of hospitalization	**Days**	**Days**	**Days**	**Days**	**Days**
Minimum-maximum	1–14	3–21	4–14	3–46	
Median	4	6	9	8	
Mean	5.07	6.06	8.63	12.04	
CI (95%)	4.35–5.80	5.72–6.39	7.46–9.80	7.64–16.44	

(76.5%), vomiting (70.6%), hypotension (64.7%) and shock (58.8%) (**Tables 3A–D**).

Thrombocytopenia was the most common hematologic manifestation affecting 283 (83.4%) and was severe in 125 (36.9%) with platelet count <50 x 10^9/L (**Tables 5A,B**). Leukopenia occurred in 169 (49.9%) (**Tables 5A,B**). The majority of those who died had severe thrombocytopenia (76.5%) ($p = 0.003$), deranged partial thromboplastin time (PTT) (70.6%), deranged prothrombin time (PT) (58.8%) and higher hematocrit concentrations >45% (47.1%) (**Tables 5A,B**). Elevated aspartate aminotransferase (AST) occurred in 245 (72.3%), where 3-10 times the upper limit for age was statistically significant in those with dengue with warning signs ($p = <$ 0.001). Elevated alanine aminotransferase (ALT) was seen in 147 (43.4%), elevated lactate dehydrogenase (LDH) in 164 (48.4%) and elevated creatine phosphokinase (CPK) in 84 (24.8%) with levels as high as 121,560 u/L. Higher levels of biochemical markers were observed in severe disease and those

TABLE 3A | Clinical manifestations in pediatric patients hospitalized with suspected and confirmed dengue in the 2018-2019 epidemic.

	Dengue without warning signs (*n* = 53)	**Dengue with warning signs (*n* = 218)**	**Severe dengue without haemorrhagic features (*n* = 28)**	**Severe dengue with haemorrhagic features (*n* = 40)**	**Total (*n* = 339)**	**Mortality cases (*n* = 17)**
	Number (%)	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Fever	51 (15.0)	217 (64.0)	28 (8.3)	40 (11.8)	336 (99.1)	17 (100)
Height of fever (°C)						
Minimum-maximum	38–40.6	38–41.3	38–42.1	38–40.2	-	38.0–39.7
Median	38.9	39	38.95	39.05	-	39.15
Mean	38.98	39.13	39.13	39.09	-	39.03
CI (95%)	38.72–39.24	39.01–39.25	38.69–39.58	38.83–39.34	-	38.66–39.40
Duration of fever (days)	**Days**	**Days**	**Days**	**Days**	**Days**	**Days**
Minimum-maximum	1–14	1–38	1–14	1–12	-	1–7
Median	4.5	4	5	4	-	3.5
Mean	4.43	5.03	5.5	5	-	3.5
CI (95%)	3.47–5.39	4.36–5.71	4.01–6.99	3.91–6.09	-	2.23–4.77
Hypothermia	0	0	1 (0.3)	5 (1.5)	**6 (1.8)**	**6 (35.3)**
Gastro-intestinal	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Vomiting	30 (8.8)	145 (42.8)	17 (5.0)	31(9.1)	**223 (65.8)**	12 (70.6)
Diarrhea	15 (4.4)	76 (22.4)	9 (2.7)	23 (6.8)	**123 (36.3)**	13 (76.5)
Abdominal pain	10 (2.9)	124 (36.6)	21 (6.2)	27 (8.0)	**182 (53.7)**	10 (58.8)
Abdominal tenderness	4 (1.2)	86 (25.4)	21 (6.2)	23(6.8)	**134 (39.5)**	7 (41.2)
Hepatomegaly	0	38 (11.2)	12 (3.5)	20 (5.9)	**70 (20.6)**	10 (58.8)
Splenomegaly	0	2 (0.6)	0	1 (0.3)	**3 (0.9)**	1 (5.9)
Hepato-splenomegaly	0	4 (1.2)	4 (1.2)	2 (0.6)	**10 (2.9)**	1 (5.9)
Ascites	0	7 (2.1)	15 (4.4)	15 (4.4)	**37 (10.9)**	7 (41.2)

TABLE 3B | Clinical manifestations in pediatric patients hospitalized with suspected and confirmed dengue in the 2018–2019 epidemic.

	Dengue without warning signs (*n* = 53)	**Dengue with warning signs (*n* = 218)**	**Severe dengue without haemorrhagic features (*n* = 28)**	**Severe Dengue with haemorrhagic features (*n* = 40)**	**Total (*n* = 339)**	**Mortality cases (*n* = 17)**
Musculoskeletal	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Arthralgia	20 (5.9)	65 (19.2)	13 (3.8)	15 (4.4)	**113 (33.3)**	4 (23.5)
Myalgia	9 (2.7)	47 (13.9)	5 (1.5)	9 (2.7)	**70 (20.6)**	2 (11.8)
Skin						
Rash	19 (5.6)	61 (18.0)	7 (2.1)	11(3.2)	**98 (28.9)**	2 (11.8)
Petechiae	2 (0.6)	29 (8.6)	8 (2.4)	16 (4.7)	**55 (16.2)**	6 (35.3)
Ecchymoses	0	7 (2.1)	4 (1.2)	10 (2.9)	**21(6.2)**	5 (29.4)
Tourniquet test done	1 (0.3)	11(3.2)	3 (0.9)	0	**15 (4.4)**	0
Positive tourniquet test	1 (0.3)	3 (0.9)	3 (0.9)	0	**7 (2.1)**	0
Bleeding from lips/ mouth	0	3 (0.9)	0	2 (0.6)	**5 (1.5)**	0
Cardiovascular system						
Documented shock	0	0	6 (1.8)	13 (3.8)	**19 (5.6)**	10 (58.8)
Narrow pulse pressure	0	1 (0.3)	0	4 (1.2)	**5 (1.5)**	3 (17.6)
Hypotension	0	42 (12.4)	15 (4.4)	21 (6.2)	**78 (23)**	11 (64.7)
Postural changes	0	6 (1.8)	9 (2.7)	3 (0.9)	**18 (5.3)**	1 (5.9)
Pericardial effusion	0	1 (0.3)	0	4 (1.2)	**5 (1.5)**	3 (17.6)
Myocarditis	0	2 (0.6)	0	4 (1.2)	**6 (1.8)**	2 (11.8)
Oedema	1 (0.3)	25 (7.4)	16 (4.7)	14 (4.1)	**56 (16.5)**	4 (23.5)
Palpitation	0	7 (2.1)	0	3 (0.9)	**10 (2.9)**	0

TABLE 3C | Clinical manifestations in pediatric patients hospitalized with suspected and confirmed dengue in the 2018–2019 epidemic.

	Dengue without warning signs (*n* = 53)	Dengue with warning signs (*n* = 218)	Severe dengue without haemorrhagic features (*n* = 28)	Severe dengue with haemorrhagic features (*n* = 40)	Total (*n* = 339)	Mortality cases (*n* = 17)
Respiratory/ Ear, Nose and Throat (for ENT)	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Cough	11 (3.2)	76 (22.4)	10 (2.9)	20 (5.9)	**117 (34.5)**	8 (47.1)
Post-tussive vomiting	0	5 (1.5)	0	0	**5 (1.5)**	0
Chest Pain	1 (0.3)	11 (3.2)	2 (0.6)	5 (1.5)	**19 (5.6)**	2 (11.8)
Wheeze	0	6 (1.8)	1 (0.3)	1 (0.3)	**8 (2.4)**	0
Pleural effusion	0	5 (1.5)	17 (5.0)	17 (5.0)	**39 (11.5)**	8 (47.1)
Ear pain	2 (0.6)	4 (1.2)	0	2 (0.6)	**8 (2.4)**	1 (5.9)
Runny nose	7 (2.1)	41 (12.1)	4 (1.2)	10 (2.9)	**62 (18.3)**	5 (29.4)
Stuffy	4 (1.2)	23 (6.8)	4 (1.2)	5 (1.5)	**36 (10.6)**	2 (11.8)
Sore throat	4 (1.2)	16 (4.7)	2 (0.6)	3 (0.9)	**25 (7.4)**	2 (11.8)
Central nervous system (for CNS)	25 (7.4)	124 (36.6)	15 (4.4)	22 (6.5)	**186 (54.9)**	0
Headache						
Confusional state	0	6 (1.8)	0	2 (0.6)	**8 (2.4)**	2 (11.8)
Seizure	2 (0.6)	4 (1.2)	2 (0.6)	6 (1.8)	**14 (4.1)**	3 (17.6)
Seizure with fever	2 (0.6)	4 (1.2)	0	3 (0.9)	**9 (2.7)**	2 (11.8)
Complex febrile seizure	1 (0.3)	1 (0.3)	0	2 (0.6)	**4 (1.2)**	1 (5.9)
Syncope	0	6 (1.8)	1 (0.3)	2 (0.6)	**9 (2.7)**	2 (11.8)
Encephalitis	0	0	1 (0.3)	7 (2.1)	**8 (2.4)**	3 (17.6)
Encephalopathy	0	0	1 (0.3)	9 (2.7)	**10 (2.9)**	6 (35.3)
Phonophobia	0	9 (2.7)	2 (0.6)	2 (0.6)	**13 (3.8)**	0
Photophobia	1 (0.3)	8 (2.4)	0	2 (0.6)	**11 (3.2)**	0

TABLE 3D | Clinical manifestations in pediatric patients hospitalized with suspected and confirmed dengue in the 2018–2019 epidemic.

	Dengue without warning signs (*n* = 53)	Dengue with warning signs (*n* = 218)	Severe dengue without haemorrhagic features (*n* = 28)	Severe dengue with haemorrhagic features (*n* = 40)	Total (*n* = 339)	Mortality cases (*n* = 17)
Eye	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Conjunctivitis	4 (1.2)	24 (7.1)	0	9 (2.7)	**37 (10.9)**	3 (17.6)
Pain	10 (2.9)	48 (14.2)	8 (2.4)	11 (3.2)	**77 (22.7)**	2 (11.8)
Sub-conjunctival hemorrhage	0	2 (0.6)	0	3 (0.9)	**5 (1.5)**	0
Genitourinary (for GU)						
Increased urinary frequency	0	6 (1.8)	0	2 (0.6)	**8 (2.4)**	1 (5.9)
Decreased urine output	1 (0.3)	6 (1.8)	3 (0.9)	2 (0.6)	**12 (3.5)**	1 (5.9)
Other symptoms						
Lethargy/ decreased activity	0	137 (40.4)	17 (5.0)	26 (7.7)	**180 (53.1)**	14 (82.4)
Restless	0	5 (1.5)	3 (0.9)	2 (0.6)	**10 (2.9)**	2 (11.8)
Irritable	10 (2.9)	30 (8.8)	6 (1.8)	9 (2.7)	**55 (16.2)**	3 (17.6)
Weight loss	1 (0.3)	9 (2.7)	3 (0.9)	1 (0.3)	**14 (4.1)**	1 (5.9)
Decreased/ loss of appetite	15 (4.4)	124 (36.6)	16 (4.7)	25 (7.4)	**180 (53.1)**	13 (76.5)
Malaise	0	2 (0.6)	0	3 (0.9)	**5 (1.5)**	1 (5.9)
Chills	4 (1.2)	9 (2.7)	2 (0.6)	3 (0.9)	**18 (5.3)**	0
Rigors	2 (0.6)	8 (2.4)	2 (0.6)	2 (0.6)	**14 (4.1)**	0
Fatigue	1 (0.3)	6 (1.8)	1 (0.3)	3 (0.9)	**11 (3.2)**	1 (5.9)
Weakness	4 (1.2)	38 (11.2)	2 (0.6)	12 (3.5)	**56 (16.5)**	5 (29.4)
Dizziness	4 (1.2)	11 (3.2)	1 (0.3)	3 (0.9)	**19 (5.6)**	0

TABLE 4 | Organ-system involvement in pediatric patients hospitalized with suspected dengue in 2018–2019 epidemic.

	Dengue without warning signs (*n* = 53)	Dengue with warning signs (*n* = 218)	Severe dengue without haemorrhagic features (*n* = 28)	Severe Dengue with haemorrhagic features (*n* = 40)	Total (*n* = 339)	Mortality cases (*n* = 17)
Outcome	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Alive	53 (15.6)	217(64.0)	27 (8.0)	25 (7.4)	**322 (95)**	-
Dead	0	1 (0.3)	1 (0.3)	15 (4.4)	**17 (5.0)**	17 (100)
Organ-system involvement # of cases (%)	48 (14.2)	218 (64.3)	28 (8.3)	40 (11.8)	334 (98.5)	17 (100)
# of organs involved						
Range	0–4	1–5	2–6	2–7	-	3–7
Median	2	3	4	5	-	5
Mean	2	2.72	4.25	4.55	-	5.294
CI (95%)	1.71–2.29	2.61–2.82	3.89–4.61	4.05–5.05	-	4.73–5.86
Type of organ-system involved	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Gastrointestinal	37 (10.9)	213 (62.8)	28 (8.3)	39 (11.5)	**317 (93.5)**	17 (100)
Haematologic	39 (11.5)	204 (60.2)	28 (8.3)	40 (11.8)	**311 (91.7)**	17 (100)
Musculoskeletal	26 (7.7)	108 (31.9)	19 (5.6)	27 (8.0)	**180 (53.1)**	12 (70.6)
Cardiac	0	44 (13.0)	19 (5.6)	24 (7.1)	**87 (25.7)**	13 (76.5)
Respiratory	0	5 (1.5)	18 (5.3)	18 (5.3)	**41 (12.1)**	9 (52.9)
Renal	1 (0.3)	15 (4.4)	5 (1.5)	20 (5.9)	**41 (12.1)**	14 (82.4)
Central nervous system	3 (0.9)	3 (0.9)	2 (0.6)	13 (3.8)	**21 (6.2)**	7 (41.2)

TABLE 5A | Laboratory investigations in pediatric patients hospitalized with suspected and confirmed dengue in the 2018–2019 epidemic.

	Dengue without warning signs (*n* = 53)	Dengue with warning signs (*n* = 218)	Severe dengue without haemorrhagic features (*n* = 28)	Severe Dengue with haemorrhagic features (*n* = 40)	Total	Mortality cases (*n* = 17)
Haematocrit	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
<30%	0	4 (1.2)	3 (0.9)	2 (0.6)	**9 (2.7)**	3 (17.6)
30–35%	7 (2.1)	28 (8.3)	6 (1.8)	5 (1.5)	**46 (13.6)**	1 (5.9)
35.1–40%	26 (7.7)	90 (26.5)	7 (2.1)	13 (3.8)	**136 (40.1)**	4 (23.5)
40.1–45%	11 (3.2)	69 (20.4)	8 (2.4)	7 (2.1)	**95 (28.0)**	1 (5.9)
>45%	1 (0.3)	16 (4.7)	4 (1.2)	10 (2.9)	**31 (9.1)**	8 (47.1)
Htc. ≥ 20% for age	2 (0.6)	6 (1.8)	4 (1.2)	7 (2.1)	**19 (5.6)**	5 (29.4)
WBC (<4 cell/mm3)						
Leukopenia (# of cases)	22 (6.5)	124 (36.6)	14 (4.1)	9 (2.7)	169 (49.9)	3 (17.6)
Range	1.05–3.90	0.51–3.98	1.38–3.90	1.00–3.96	-	1.81–2.60
Median	2.54	2.68	3.5	2.6	-	1.90
Mean	2.58	2.57	3.22	2.66	-	2.10
CI (95%)	2.24–2.93	2.43–2.72	2.77–3.68	1.92–3.40	-	1.03–3.18
Platelet (10^9/L)						
Thrombocytopenia (# of cases)	34 (10.0)	187 (55.2)	26 (7.7)	36 (10.6)	283 (83.5)	15 (88.2)**
≤50	6 (1.8)	75 (22.1)	20 (5.9)	24 (7.1)	**125 (36.9)**	13 (76.5)
51–100	19 (5.6)	82 (24.2)	3 (0.9)	10 (2.9)	**114 (33.6)**	2 (11.8)
101–149	9 (2.7)	30 (8.8)	3 (0.9)	2 (0.6)	**44 (13.0)**	0
Range	15–141	3–147	6–139	8–136	-	8–74
Median	83	61	28	33.5	-	33
Mean	82.29	65.79	42.96	44.19	-	31.13
CI (95%)	71.02–93.57	60.69–70.90	27.75–58.17	33.66–54.73	-	20.79–41.48

***p < 0.01. The highest haematocrit, lowest WBC and lowest platelet count during hospitalization were used.*

TABLE 5B | Laboratory investigations in pediatric patients hospitalized with suspected and confirmed dengue in the 2018–2019 epidemic.

	Dengue without warning signs (*n* = 53)	**Dengue with warning signs (*n* = 218)**	**Severe dengue without haemorrhagic features (*n* = 28)**	**Severe dengue with haemorrhagic features (*n* = 40)**	**Total (*n* = 339)**	**Mortality cases (*n* = 17)**
Coagulopathy (# of cases)	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Prolonged PT	0	2 (0.6)	1 (0.3)	11 (3.2)	**14 (4.1)**	10 (58.8)
Prolonged PTT	9 (2.7)	78 (23.0)	18 (5.3)	26 (7.7)	**131 (38.6)**	12 (70.6)
Biochemical	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Elevated AST	**25 (7.4)**	**163 (48.1)**	**27 (8.0)**	**30 (8.8)**	**245 (72.3)**	**15 (88.2)**
(<3 xULN)	18 (5.3)	71 (20.9)	4 (1.2)	3 (0.9)	**96 (28.3)**	0
(3-10 xULN)	7 (2.1)	77 (22.7)	10 (2.9)	7 (2.1)	**101 (29.8)**	2 (11.8)
(>10 xULN)	0	15 (4.4)	13 (3.8)	20 (5.9)	**48 (14.2)**	13 (76.5)
Range (u/L)	75–181	56–773	133–5,525	112–18,703	-	222–18,703
Median (u/L)	154	222	1341.50	1118.50	-	2,387
Mean (u/L)	137.91	255.28	1,814	2887.69	-	3729.69
CI (95%)	113.26–162.56	222.98–287.57	939.77–2688.23	1026.02–4749.36	-	749.38–6710.01
	Number (%)	**Number (%)**	**Number (%)**	**Number (%)**	**Total (%)**	**Number (%)**
Elevated ALT	**11 (3.2)**	**89 (26.3)**	**20 (5.9)**	**27 (8.0)**	**147 (43.4)**	**14 (82.4)**
(<3 xULN)	10 (2.9)	48 (14.2)	4 (1.2)	6 (1.8)	**68 (20.1)**	1 (5.9)
(3-10 xULN)	1 (0.3)	39 (11.5)	4 (1.2)	8 (2.4)	**52 (15.3)**	3 (17.6)
(>10 xULN)	0	2 (0.6)	12 (3.5)	13 (3.8)	**27 (8.0)**	10 (58.8)
Range (u/L)	43–134	31–452	48–4,552	34–4,369	-	55–4,369
Median (u/L)	59	99	395	305	-	809
Mean (u/L)	67.27	124.84	790.05	810.19	-	956.46
CI (95%)	48.18–86.36	105.77–143.91	283.37–1296.73	356.10–1264.29	-	265.33–1647.60

TABLE 5C | Biochemical investigations in pediatric patients hospitalized with suspected and confirmed dengue in the 2018–2019 epidemic.

	Dengue without warning signs (*n* = 53)	**Dengue with warning signs (*n* = 218)**	**Severe dengue without haemorrhagic features (*n* = 28)**	**Severe dengue with haemorrhagic features (*n* = 40)**	**Total (*n* = 339)**	**Mortality cases (*n* = 17)**
Elevated LDH - # of cases (%)	15 (4.4)	106 (31.3)	18 (5.3)	25 (7.4)***	164 (48.4)	10 (58.8)
Range (u/L)	382–1,299	293–3,123	373–5090	419–11708	-	1,640–9,997
Median (u/L)	674	660.5	888.5	2162	-	4385.5
Mean	672.60	777.64	1843.61	3345.04	-	5045.10
CI (95%)	543.71–801.49	695.38–859.90	1038.38–2648.84	2021.50–4668.58		2911.30–7178.90
Elevated CPK - # of cases (%)	7 (2.1)	49 (14.5)	10 (2.9)	18 (5.3)**	84 (24.8)	10 (58.8)
Range (u/L)	226–1,601	202–10,248	243–4,113	305–121560	-	343–63,211
Median (u/L)	303	449	1,031	1360.50	-	1360.50
Mean (u/L)	519.14	1279.71	1,297	13255.56	-	7535.90
Elevated GGT - # of cases (%)	1 (0.3)	44 (13.0)	13 (3.8)	20 (5.9)	78 (23)	10 (58.8)
Range (u/L)		31–598	41–861	34–515	-	34–515
Median (u/L)		64.5	141	87.50	-	78.50
Mean (u/L)		98.71	183.62	146.5	-	164.40
CI (95%)		66.47–130.94	53.13–314.10	74.41–218.59		40.81–287.99
Other:	Number (%)	Number (%)	Number (%)	Number (%)	Total (%)	Number (%)
Hyponatramia (<135 mmol/l)	2 (0.6)	23 (6.8)	4 (1.2)	10 (2.9)	**39 (11.5)**	6 (35.3)
Hypernatraemia (>145 mmol/L)	5 (1.5)	26 (7.7)	11 (3.2)	8 (2.4)	**50 (14.7)**	6 (35.3)
Elevated urea	2 (0.6)	17 (5)	4 (1.2)	19 (5.6)	**42 (12.4)**	14 (82.4)
Elevated creatinine	0	3 (0.9)	1 (0.3)	10 (2.9)	**14 (4.1)**	10 (58.8)

$^{**}p < 0.01$; $^{***}p < 0.001$.

who died (**Tables 5B,C**). Elevated LDH and CPK levels were significantly associated with severe dengue with hemorrhage, p values = <0.001 and 0.002, respectively. A subset of NS1 positive samples was assessed for serotyping at the UHWI and CARPHA with all (20/20) identified as DENV-3.

Seventeen (5%) patients died, 16 (94.1%) with severe dengue, of which 15 (88.2%) had hemorrhage. The ages ranged from 7 months to 15 years, mean age 5 years, subset 1–5 years - 9 (52.9%). The male:female ratio was 1:2.4. Eight (47.1%) had co-morbidities: 4-asthma, 3-sickle cell disease Hb SC, 2-epilepsy and one with cerebral palsy (**Table 1**). All had hematologic and gastrointestinal manifestations with more organ-system involvement of 5–7 organs in 11 (64.7%). Eleven (64.7%) died within 24–48 h of presentation despite resuscitation and transfusion of blood products.

DISCUSSION

According to PAHO, in this "new dengue epidemic cycle in the Americas" in 2019, children under 15 years of age were predominantly affected, representing 64% of severe dengue cases in Guatemala and 58% of all confirmed deaths in Honduras (6). In this study from Jamaica, severe cases of dengue in hospitalized children approached 20.1% and CFR was 5% in 2018 to 2019. Of the 339 cases in this study, ages 1–10 years were most affected. Hb SC, elevated LDH and CPK were all statistically significant laboratory markers for severe dengue with hemorrhage. Of the 17 who died, the most affected included those aged 1–5 years, females more than males and those with more organ-system involvement. An alarming 64.7% died within 24 to 48 h of presentation.

This severity of illness and attributable-mortality in hospitalized children, synchronized with the national surveillance data for all reported cases in the 2018–2019 epidemic (16). The 2018 to 2019 dengue outbreak in Jamaica was more intense, atypical and severe as compared to previous epidemics, with 5,903 reported cases in 2012 and 10,411 cases in 2018–2019 after its maiden ZIKV epidemic in 2016 (16). No other arboviruses were circulating. Those under 15 years of age represented 41% of the total number of 10,411 reported cases and 42% of those who were hospitalized. The overall increase in the number of severe dengue cases in 2019 was also observed in Honduras (20).

There is a paucity of detailed reports describing the clinical severity of dengue infection in pediatric populations in the "new epidemic cycle" which occurred after the maiden ZIKV epidemic in the Americas (6, 22). Our study was first contrasted to others in ZIKV-naïve populations. The proportion of severe dengue cases in 2018–2019 in our study of Jamaican children (20.1%) was greater than the 10.5% reported from the UHWI, Jamaica in 2012 and 17.4% from Queen Elizabeth Hospital, Barbados in 2009 (18, 23). However, it was less than the 26% reported in Dominican Republic in 2008-2009 (24). Severe cases reported in the Caribbean region of the Americas were significantly less than the Asia-Pacific: Philippines 76.5% (2009–2010) and Indonesia 73% (2009–2013) (25, 26).

Jamaica observed an increase in the number of dengue-related deaths in children. Of the 86 dengue-related deaths that were reported nationally, 53 (61.6%) occurred in children under 15 years of age. Our CFR of 5% was higher than the 3.7% reported in hospitalized children from the UHWI in 2012, the 1.7% for the Queen Elizabeth Hospital in Barbados in 2009 and <1% mortality rates reported in the Philippines and Indonesia (19, 23, 25, 26). However, our child-mortality rate was similar to the 5.1% reported for children in Dominican Republic in 2008-2009 (24).

In our study, sickle cell disease Hb SC was significantly associated with severe dengue with hemorrhage ($p = 0.005$) and comprised 17.6% of those who died. In Jamaica, during 2010–2012, those with genotypes Hb SC, or SS had significantly higher dengue-attributable CFR of 12.5 vs. 0.4% in the general population (27). During 2005–2013, in the French Americas, children under 15 years with Hb SC had a higher proportion of severe disease ($p < 0.001$) and more deaths ($p = 0.02$) (28). Hemoglobin concentration at presentation and change in hemoglobin concentration from steady state were also independent predictors of mortality, other than SS genotype (27).

Hematologic and biochemical changes can assist in the classification of disease severity, identify complications and initiate management with the aim to decrease morbidity and mortality. A higher proportion of those with severe disease and deaths had hematocrits >40%, severe thrombocytopenia (platelets $< 50 \times 10^9$/L) and deranged clotting indices. This is congruent with findings found in Sri Lankan children aged 5–12 years with dengue in 2013–2014 (29). The rise in the hematocrit was rapid and higher in those with dengue hemorrhagic fever who also had significantly lower platelet counts. WHO recognizes this as a sign of severe disease and need for aggressive treatment (21).

Higher levels of AST and ALT were observed in those with severe disease, particularly with hemorrhage and was an indicator for identifying severe disease. Those who died in our study, had severe hepatic transaminitis with high levels of AST (18,703 u/L) and ALT (4,369 u/L). These findings are supported by studies from Sri Lanka (2013–2014), Malaysia (2014–2015) and India (2014–2015) (29–31). Higher levels of LDH and CPK were both significantly associated with severe dengue with hemorrhage ($p =< 0.002$). Our findings are congruent with Indian reports using CPK and LDH as biomarkers of disease severity (32). WHO reclassified dengue in 2009 to include: "dengue without warning signs," "dengue with warning signs" and "severe dengue." This classification to identify severe cases had a sensitivity of 59–98% and specificity 41–99% (33). This classification may require further revision as it lacks specific parameters that define the signs and measurable clinical/laboratory parameters. It can also be expanded to include laboratory investigations such as elevated LDH and CPK, as predictors of disease severity.

We identified DENV-3 as the sole serotype in those patients who were tested in this study. This is consistent with MoHW data indicating that this serotype was nearly the sole serotype (97%) circulating in Jamaica during 2018-2019 along with a few cases of DENV-2 (3%). A meta-analysis from the South East and non-South East Asia showed that primary DENV-3 infection and secondary infection with DENV-2, 3 and 4

increased the risk of severe dengue infections (34). During the 2016 ZIKV epidemic in Jamaica, DENV was co-circulating with ZIKV and was predominantly of the DENV-3 serotype (12, 13). Yet, severe disease and case fatality rates in DENV-infected Jamaican children during 2019 were much higher than in 2016, suggesting that secondary DENV infection after a primary ZIKV infection could have impacted disease severity.

Antibody-dependent enhancement (ADE) is characteristic of dengue and other flaviviruses (22, 35–37). Dengue and ZIKV viruses are flaviviruses whose envelope proteins share ∼50% sequence homology (22, 37). In Nicaragua, a 12.1% probability of symptomatic DENV-2 infection in children with a previous ZIKV infection vs. 3.5% in flavivirus-naïve children, suggested ADE (22). A prior history of ZIKV infection was also associated with severe dengue in 5.4 vs. 0.7% in flavivirus-naïve children (22). Enhanced infection of heterologous DENV 1,3,4 serotypes and a live tetravalent vaccine are linked to ADE (22, 38, 39). The ZIKV and DENV ADE correlation is still being debated.

Laboratory-confirmation of dengue infection was present in 79% (220) of our cases. "Pediatrician-diagnosed" clinical cases in hospitalized children and adolescents were accepted in the remainder. Some who had "dengue without warning signs" were hospitalized, if pediatricians were unsure whether cases with fever would evolve into "severe dengue," septicemia, meningitis, or other illnesses. Others were hospitalized over an abundance of caution, if family support was questionable for monitoring and supporting the child, should there be sudden deterioration and inability to return promptly for treatment and care, in the context of an evolving epidemic with increased attributable pediatric morbidity and mortality. The surveillance systems, protocols and training were similar across the five hospitals, the Childrens' Hospital and UHWI had 85% of cases with the highest burden of disease severity, as would be expected for these referral hospitals.

A new wave of dengue emerged after the ZIKV epidemic with increased disease severity and attributable-mortality in children and adolescents, as was observed in Jamaica. This occurred *in tandem* with the highest ever reported incidence and severity of epidemic dengue fever in Jamaica and the Americas and in the aftermath of the ZIKV "epidemic of international concern" in Latin America and the Caribbean. This unique increase in severe dengue in PAHO's "new epidemic cycle" may be an isolated event, may represent the peak in the cyclical nature of epidemics, or may become the new norm. It also raises the important point for continued surveillance for arboviruses in general as the ZIKV "epidemic of international concern" caught the entire Americas off-guard and then unexpectedly may have impacted dengue disease severity subsequently. Notwithstanding, recent statistical modeling has shown that since March 2020, the COVID-19 pandemic with the associated public health interventions and social disruptions, including closure of schools, has reduced dengue transmission by 0.72 million cases, or 44%, in 23 countries within Latin America and South East Asia, including Jamaica, which experienced a 95% decrease in reported dengue cases in 2020 (40).

CONFERENCE PRESENTATION

https://www.zikaconference.com/scientific-program. This was the first, oral platform abstracted presentation, by Dr. Aileen Lue, in the First Scientific Session of the "Third International Conference on ZIKA and *Aedes*-related Infections," at the Metro Marriott Hotel, in Washington D.C., Feb 13-16, 2020. Abstract—8815. This was the subject of the doctoral thesis in support of Dr. Aileen Lue's Doctorate in Pediatrics degree, from the University of the West Indies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Research Ethics Committee of the University Hospital of the West Indies Mona Campus and also the Ethics (ECP 218 18/19; September 16, 2019) and also the Medico-legal Affairs Committee of the Ministry of Health and Wellness, Jamaica (2019/18; November 11, 2019). Written informed consent from the 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

AL, M-AR-D, and CC conceptualized and designed the work. AL, CC, SK, JD-T, TJ-P, CP, CM, JA, and KW-K acquired the data. AL and GG-S managed the dataset, exported, and performed data analysis. AL and CC drafted and critically revised the work and continually provided intellectual input, all other co-authors contributed to this process throughout. All co-authors commented on the proposal, the work, data interpretation throughout, approved the final version submitted herein for consideration for publication, and agreed to be responsible for all aspects of the work reported herein.

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study. The works reported herein are entirely the responsibility of the coauthors.

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BRIEF RESEARCH REPORT
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Guillain-Barré Syndrome and Miller Fisher Syndrome in Association With an Arboviral Outbreak: A Brazilian Case Series

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Introduction: Guillain-Barré syndrome (GBS) in association with arboviruses, such as Zika, chikungunya, and dengue, has been previously documented; however, Miller-Fisher Syndrome (MFS) and other GBS subtypes are rarely reported.

Methods: We identified a series of GBS and MFS cases that were followed during the Zika virus outbreak in Salvador, Brazil (2015–2016). Blood and CSF samples were collected for virus diagnosis. In addition, serological studies to verify previous arboviral infection and electromyography (EMG) were performed.

Results: Of the 14 patients enrolled, 10 were diagnosed with GBS, including three GBS subtypes (two cases of bifacial weakness with paresthesia and one case of paraparetic GBS), and four as MFS. IgM antibodies against one or more of three arboviruses were present in 11 (78.6%) patients: anti-zika IgM positivity in eight (57%), anti-Chikungunya IgM in three (21%), and anti-Dengue in one (7%) individual. A single case was positive for both anti-Dengue IgM and anti-Chikungunya IgM, suggesting co-infection. EMG revealed an AIDP pattern in all nine patients analyzed.

Conclusion: The current case series contributes to our knowledge on the clinical presentation of arbovirus-associated GBS and its subtypes, including MFS, and serves as an alert to clinicians and other healthcare professionals in regions affected by arbovirus outbreaks. We highlight the importance of recognizing arboviruses in diagnosing GBS and its subtypes.

Keywords: Guillain-Barré syndrome, Miller Fisher syndrome, Zika virus, dengue, chikungunya

INTRODUCTION

Guillain-Barré syndrome (GBS) is an acute, immune-mediated polyradiculoneuropathy typically occurring 2–8 weeks after viral or bacterial infection. Motor function is usually affected, beginning distally and progressing proximally over an up to 4-week period. Areflexia, sensory disturbances, and cranial nerve involvement may also occur. Diverse clinical subtypes with different neurological features have been reported, such as Miller-Fisher syndrome (MFS), Bickerstaff syndrome, and others (1).

About two-thirds of GBS patients report a prior acute infectious illness episode. Moreover, numerous infectious agents have been associated with GBS, more commonly *Campylobacter jejuni*, as well as Cytomegalovirus, Epstein-Barr virus, varicella-zoster virus, and *Mycoplasma pneumoniae* (2, 3). In addition, in regions that have experienced arboviruses outbreaks, some studies have reported GBS followed by arboviral infection, including Dengue virus (DENV), chikungunya virus (CHIKV) or Zika virus (ZIKV) (4, 5).

In Brazil, concomitant or subsequent outbreaks of these three arboviruses have imposed significant challenges to national and local health systems. DENV has circulated in the country since 1845 and is one of the main diseases that causes a relevant public health impact. Since 1981, several outbreaks have occurred (6). CHIKV, introduced in 2014, rapidly disseminated throughout the country (7). In 2015, the first cases of Zika infection were reported (8), subsequently followed by a large-scale epidemic, and clusters of newborns with congenital Zika infection resulted in the declaration of a state of public health emergency in the country.

Human ZIKV infection was considered a benign and self-limited exanthematic illness (9). However, identifying neurological complications arising from ZIKV infections, mainly related to Guillain–Barré syndrome (10) and congenital malformations (11) raised new public health concerns. The association between GBS and ZIKV infection was first reported in French Polynesia in 2013 (12) and then subsequently confirmed in a case-control study (10). In Brazil, a rising incidence of GBS has also been linked to ZIKV, mainly in the northeast region, with several cases reported in the literature (13, 14).

Chikungunya virus infection is an acute febrile illness that may cause chronic arthropathy. Neurological complications associated with CHIKV are believed to be unusual; however, reports of CHIKV-associated encephalitis increased during the 2005–2006 CHIKV outbreak on the island of La Réunion (15). Following a 2014 outbreak of CHIKV in French Polynesia, increased numbers of cases of GBS were also reported (16). Rare associations between GBS and DENV infection have been described in case reports (17). While MFS in association with arbovirus infection is rarely reported, the literature does contain some instances of cases associated with Dengue and Zika infection (18, 19).

Herein, we present a case series of patients with GBS and its subtypes, including MFS, arising from the 2015–2016 ZIKV outbreak in Salvador, Brazil. Our study aims to characterize relevant clinical and neurological features in GBS and its subtypes occurring in association with ZIKV, CHIKV, or DENV infection.

METHODS

A hospital surveillance study was performed at two reference general hospitals in Salvador, Bahia-Brazil, from May 2015 to April 2016.

Inclusion Criteria

Patients with symptoms compatible with GBS or its subtypes were included following admission to a neurology ward at one of the participating hospitals. A single trained neurologist established a clinical diagnosis of GBS, Miller-Fisher syndrome (MFS), or other subtypes in accordance with criteria described by the GBS classification group (1).

Exclusion Criteria

Patients or their legal guardians who did not consent to participation or patients exhibiting symptoms likely related to other plausible causes, such as cancer, bacterial infection, trauma, intoxication, metabolic disease, and other medical conditions, were excluded from the study.

Data Collection

The collection of clinical, epidemiological, and laboratory data was performed. All participants were evaluated by a single trained neurologist (MSR) who recorded clinical data on a standardized case report form. Clinical data were recorded both during the hospital stay and after hospital discharge in an outpatient setting.

The Guillain-Barré syndrome disability scale (GBS-DS) was used to evaluate patient impairment and the severity of neurological symptoms. Scores on this scale range from 0 to6, with higher values corresponding to a greater degree of neurological dysfunction (20). The House-Brackmann scale (HBS) was also used to evaluate the severity of facial paralysis in patients affected by this manifestation, with higher values (grades 1–6) indicating greater dysfunction (21).

Biological Samples

Serum and cerebrospinal fluid (CSF) samples were collected by the neurologist researcher for laboratory analysis; an aliquot was marked with patient identification, processed, and sent to the Gonçalo Moniz Institute (IGM-Fiocruz) for arbovirus diagnosis. All samples were conditioned and transported under refrigeration.

Serological Diagnosis

Serological arbovirus diagnosis was performed in all samples collected for the detection of anti-DENV, anti-CHIKV and anti-ZIKV IgM antibodies by enzyme-linked immunosorbent assay (ELISA). The commercial kits Euroimmun® Dengue IgM and Chikungunya IgM (Euroimmun, Lübeck, Germany) were used for antibody detection in accordance with the manufacturer's instructions. The detection of anti-Zika IgM antibodies was performed by in-house MAC-ELISA following protocols established by the Centers for Disease and Control (CDC-Atlanta, USA) (22).

To perform differential diagnosis, serological analysis to detect toxoplasma, rubella, cytomegalovirus, Herpes, Syphilis and HIV and HTLV was performed. Indirect ELISA and/or capture ELISA

were used for the detection of specific IgG and/or IgM pertaining to each infectious agent. All diagnostic tests were performed, following manufacturer protocols, on an automated or semi-automated apparatus.

Molecular Diagnosis

Viral RNA was extracted from CSF samples using a QIAmp viral RNA mini kit (Qiagen; Hilden, Germany). Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) was performed for Zika, Dengue and Chikungunya viruses using a multiplex PCR kit (ZDC Bio-Manguinhos- Instituto de Tecnologia em Imunobiológicos, Brazil), following manufacturer protocols, on an ABI 7500 Real-time PCR system (Thermo Fisher Scientific).

Electrodiagnostic Testing

Electromyography (EMG) and nerve conduction studies (NCS) were performed in most of the included patients. These examinations were performed by an expert neurologist trained in electrophysiology as part of patient clinical follow-up. The Nihon Kohden Neuropack® M1 MEB-9200 EMG/EP/IOM 4-channel system was used to measure electromyography and evoked potential. Electrophysiological assessments were performed using standard electromyography techniques, including motor nerve conduction studies of the median nerve (recording of the abductor pollicis brevis), the ulnar nerve (recording of the abductor digiti minimi), and the peroneal nerve (recording of the extensor digitorum brevis), as well as sensory nerve conduction studies in radial and sural nerves.

Ethical Considerations

All participants agreed to participate in the study and signed a term of informed consent, detailing explicit information about the nature and objectives of the research undertaken in language appropriate to the educational level of the study population.

This project was submitted to and approved by the institutional review board of the Gonçalo Moniz Institute (CEP-IGM-Fiocruz; protocol no. 1184454). Risks to volunteers were considered minimal, as routine procedures were employed, i.e., the obtainment of peripheral blood or CSF collection. Refusal to participate did not affect patient treatment. Both CSF analysis and EMG examination were previously part of these patients' diagnostic routine.

Statistical Analysis

Data entry and data management were performed using REDCap© software v.6.14.0 (Vanderbilt University, 2016). All statistical analyses were performed using the IBM-SPSS version 21 program. A descriptive analysis of the study population was initially performed. Categorical data are described as proportions with 95% confidence intervals, while numerical data are described as means ± standard deviation, or as medians with interquartile range (IQR). For all patients who underwent subsequent evaluations ∼30 days after admission, mean values corresponding to GBS-DS scores and House-Brackmann (HBS) grades were calculated at each timepoint. The results obtained from these two scales were categorized to reflect the level of patient function as follows: mild (GBS-DS 0–2) or severe disability (GBS-DS 3–5); mild (HBS 1–3) or severe facial nerve palsy (HBS 4–6).

Categorical data were compared using the Chi-squared (χ^2) or Fisher's exact tests.

RESULTS

During the study period, a total of 14 patients diagnosed with GBS or its subtypes were included. All but one resided in Salvador. The median age of the participants was 40.5 years (IQR: 28–52), with a slightly higher prevalence of females (57%).

Most participants (92%) reported symptoms characteristic of viral infection prior to the onset of neurological symptoms. The most prevalent symptoms were polymyalgia, skin rash, arthralgia and/or pruritus, each reported by 8 (57%) patients, while headache and/or fever were reported by 6 (42%) participants. Conjunctivitis and edema in the extremities were uncommon (7 and 14%, respectively). It is important to highlight that 8 (57%) participants reported paresthesia during the acute phase of viral infection. The median duration of viral symptoms was 4 days (IQR: 3–6.5) and the median time until onset of neurological symptoms was 10 days (IQR: 9–14) (**Table 1**).

Ten participants were diagnosed with GBS; most (7, 70%) presented classical GBS, while three presented GBS subtypes: Two had a bifacial weakness with paresthesias and one had paraparetic GBS. In most GBS cases, symptoms compatible with acute polyneuropathy were predominant: 9 (90%) experienced paresthesia (socks and gloves pattern), muscle weakness and hyporeflexia, while 8 (80%) suffered facial paralysis. Other

TABLE 1 | Demographic and clinical characteristics of 14 patients with GBS or subtypes in Salvador, Brazil.

Characteristics	*n* (%)
Mean age (years)*	40.5 (28–52)
Females	8 (57)
Acute viral symptoms	
Polymyalgia	8 (57)
Rash	8 (57)
Arthralgia	8 (57)
Pruritus	8 (57)
Paresthesia at the onset of symptoms	8 (57)
Headache	6 (42)
Fever	6 (42)
Edema in limbs	2 (14)
Conjunctivitis	1 (7)
Duration of viral symptoms (days)*	4 (3–6.5)
Time elapsed between viral and neurological symptoms (days)*	10 (9–14)
Time elapsed between neurological symptoms and hospital admission (days)*	4 (1–10)

*Median (IQR 25–75%).

clinical manifestations, including dysarthria, dysphagia and ataxia were also reported (**Table 2**).

Four participants were diagnosed as MFS; only one presented classical MFS, while the other three presented the acute ataxic neuropathy subtype. All had symptoms of ataxia, paresthesia in the hands and feet, and sensory disturbance. A pharyngeal-cervical-brachial pattern of muscle weakness was reported in two cases. Just one out of four individuals respectively had ocular motor abnormalities, hyporeflexia or dysphagia, and none experienced facial paralysis or the inability to walk (**Table 2**).

Regarding serological diagnosis, IgM antibodies against one or more of the three arboviruses were detected in 11 (78.6%) participants. Eight (57%) tested positive for anti-ZIKV IgM, 1 (7.1%) for anti-DENV IgM and 3 (21.4%) for anti-CHIKV IgM. Positivity for both anti-ZIKV IgM and anti-DENV IgM was not evidenced. One case was positive for both anti-DENV IgM and anti-CHIKV IgM, suggestive of co-infection (**Table 3**).

All of the patients tested negative for HIV, HTLV, CMV, EBV, HCV, HBV, and syphilis.

TABLE 2 | Neurological symptoms and clinical characteristics of 14 patients with Guillain-Barré syndrome or Miller Fisher syndrome in Salvador, Brazil.

	Guillain-Barré syndrome (*n*=10) *n* (%)	Miller Fisher syndrome (*n*=4) *n* (%)
Symptoms upon admission		
Paresthesia	9 (90)	4 (100)
Sensory disturbance	9 (90)	3 (75)
Muscle weakness	9 (90)	2 (50)
Areflexia/hyporeflexia	9 (90)	1 (25)
Facial nerve palsy	8 (80)	0
Dysarthria	7 (70)	0
Inability to walk	5 (50)	0
Dysphagia	5 (50)	1 (25)
Ocular abnormality	0	1 (25)
Ataxia	3 (30)	4 (100)
CSF fluid analysis		
WBC/mm^3 (mean ± SD)	10 ± 21	30 ± 42
Albumin mg/dl (mean ± SD)	117 ± 77	217 ± 284
Increased protein (>40 mg/dl)	9 (90)	3 (75)
GBS-DS (0–2)—mild disease	3 (30)	3 (75)
GBS-DS (3–5)—severe disease	7 (70)	1 (25)
HBS (1–3)—mild facial palsy	0	0
HBS (4–6)-severe facial palsy	7 (70%)	0
Treatment with IVIG	8 (80)	0
Days between admission and IVIG*	3 (1–10)	N/A
Days between neurological symptoms and IVIG*	9 (1–25)	N/A
Admission to ICU	7 (70)	3 (75)
Need for orotracheal intubation	0	0
Death	0	0
Median length of hospital stay*	9 (5–18.5)	7 (3–15.5)

Median(IQR 25%-75%); GBS, Guillain-Barré syndrome; MFS, Miller Fisher syndrome; GBS-DS, Guillain-Barré syndrome disability scale; HBS, House-Brackmann scale; IVIG, intravenous immunoglobulin; ICU, intensive Care Unit; N/A, not applicable.

Testing by multiplex qPCR for Zika, Dengue and Chikungunya in all CSF samples returned negative results for all three arboviruses.

Cerebrospinal fluid analysis revealed albuminocytological dissociation in 9 (90%) patients with GBS and 3 (75%) with MFS. Eight (80%) GBS patients were treated with intravenous immunoglobulin (IVIG) therapy, with the onset of treatment occurring on average 3 days (range: 1–10 days) after hospital admission (**Table 2**). Biological samples were collected prior to IVIG infusion.

Electromyography was performed in 9/14 (64%) patients (8 GBS cases and 1 MFS). Motor NCS revealed similar patterns in most cases, with prolonged distal latencies and slowed conduction, but without any reduction in the distal compound muscle action potential (CMAP). Sensory nerve action potential amplitude and sensory nerve conduction velocity were mildly altered in the radial and sural nerves, which is compatible with acute inflammatory demyelinating polyneuropathy (AIDP) with conduction block. Facial nerve demyelination was observed in 87%.

With regard to clinical severity, the majority (70%) of GBS patients presented scores between 3 and 5 on GBS-DS, denoting greater disease severity. Only one of the MFS cases presented severe disease (GBS-DS >2). In all, 10/14 (71%) were admitted to an intensive care unit, but none required mechanical ventilation support or died. The mean length of hospital stay was 11 days for patients with GBS versus 12 days for those with MFS (**Table 2**).

Ten patients (71%) were reevaluated 30 days after discharge (eight GBS, two MFS cases). All patients exhibited GBS-DS scores <2. Overall mean GBS-DS on admission was 2.87 compared to 0.25 upon reevaluation; a 2-point drop was observed ($P = 0.0001$). Seven of these patients presented severe dysfunction of the facial nerve upon admission (HBS > 4), yet all showed a drastic recovery when examined 30 days after discharge (HBS < 3). The mean difference between HBS on admission and reevaluation was 3.43 points ($P < 0.001$).

DISCUSSION

Guillain-Barré syndrome is a term used to describe a broad spectrum of acute autoimmune neuropathies. GBS presentation varies widely, as several subtypes have been identified. Some subtypes are rare, e.g., acute ptosis or acute mydriasis. Others are more common, yet usually go unrecognized by clinicians, such as paraparetic GBS, bifacial weakness with paraesthesias, pharyngeal-cervical-brachial weakness (1). MFS, an acute idiopathic polyneuritis, is characterized by ophthalmoplegia, areflexia, and ataxia. This subtype is likely a midbrain form of GBS. In contrast to other GBS case series (10), GBS subtypes were identified in half of the patients followed in this study, including paraparetic GBS and bifacial weakness with paraesthesias or acute ataxic neuropathy. Importantly, GBS subtypes may be misdiagnosed as other diseases (e.g., Bell's palsy or cerebellar disfunction) by physicians unfamiliar with the GBS clinical spectrum.

TABLE 3 | Neurological syndrome and arbovirus diagnosis profiles in 14 patients with GBS or its subtypes; Salvador, Brazil, 2015–2016.

ID number	Neurological syndrome	Anti-DENV IgM	Anti-CHIKV IgM	Anti-ZIKV IgM	ZDC multiplex PCR
1	Classical GBS	Negative	Negative	Positive	Negative
2	Classical GBS	Negative	Negative	Positive	Negative
3	Classical GBS	Negative	Negative	Positive	Negative
4	Classical GBS	Negative	Negative	Negative	Negative
5	Classical GBS	Negative	Negative	Positive	Negative
6	Classical GBS	Negative	Negative	Negative	Negative
7	Classical GBS	Negative	Positive	Negative	Negative
8	Bifacial weakness and paresthesia GBS	Negative	Positive	Negative	Negative
9	Bifacial weakness and paresthesia GBS	Negative	Negative	Positive	Negative
10	Paraparetic GBS	Negative	Negative	Negative	Negative
11	MFS—acute ataxic neuropathy	Negative	Negative	Positive	Negative
12	MFS—acute ataxic neuropathy	Negative	Negative	Positive	Negative
13	MFS—acute ataxic neuropathy	Negative	Negative	Positive	Negative
14	MFS—pharyngeal-cervical-brachial weakness	Positive	Positive	Negative	Negative

GBS, Guillain-Barré syndrome; MFS, Miller-Fisher syndrome; ZDC, Zika, Dengue, and Chikungunya; ZIKV, Zika virus; CHIKV, chikungunya virus; DENV, dengue virus.

Previous infection by ZIKV was inferred by the presence of anti-ZIKV IgM antibodies in the majority of cases herein. Many studies suggest that GBS and other neurological syndromes are strongly associated with ZIKV infection (10). Even though this study was conducted during the ZIKV outbreak in our region, not all cases could be definitely attributed to ZIKV. Three (21%) patients presented positivity for anti-CHIKV IgM and one (7%) for anti-Dengue IgM. This finding was expected, since the other co-circulating arboviruses (CHIKV and DENV) in this area have also been linked to neurological disorders, including GBS (23).

One case of MFS was positive for both anti-CHIKV IgM and anti-DENV IgM, suggesting the occurrence of co-infection. Arboviral co-infection has been reported in acute neurological syndromes and could be responsible for atypical clinical presentations (24).

Importantly, none of the patients tested positive for both anti ZIKV IgM and anti-DENV IgM antibodies, which indicates that anti-ZIKV IgM positivity did occur due to cross-reactivity. In all 11 cases presenting IgM antibodies against arboviruses, diagnosis can only be inferred as all individuals were negative under molecular testing by Multiplex qPCR and viral isolation was not performed. However, since GBS is an immune-mediated syndrome and the onset of neurological symptoms typically occurs days to weeks after infection, the etiological diagnosis of GBS is mostly based on serological testing, and few studies have described positivity for DENV, CHIKV or ZIKV by PCR (10).

Cerebrospinal fluid analysis demonstrated the presence of albuminocytological dissociation in 90% of the GBS patients and 75% of MFS cases, which is similar to two other case-control studies previously published in French Polynesia and Colombia (10, 25).

Prolonged distal latencies and reduced distal CMAP on EMG studies can be interpreted as facial nerve demyelination and conduction slowing and blockage, leading to the classification of patients as AIDP with axonal degeneration, which is consistent with a pattern of GBS. Most of our patients (87%) presented demyelinating polyneuropathy and secondary axonal degeneration of the facial nerves. The single MFS patient submitted to EMG also presented a pattern consistent with AIDP in the absence of facial nerve dysfunction. The pattern of demyelination found in the present study is similar to that seen in a case-control study conducted in Colombia (25), yet differs from another case-control study in French-Polynesia that observed an axonal polyneuropathy pattern on EMG (10).

GBS-DS and HBS evaluations are rarely reported in other GBS cohorts associated with arbovirus infection (10, 25). Most (70%) of the GBS patients in this study presented with GBS-DS scores of 3 or higher on admission, denoting severe disease. This was also reflected by a high frequency of facial nerve palsy (80%) and HBS scores >4 (88%). Patients with MFS presented with milder disease, with a predominance of GBS-DS scores <3 (75%). As expected, none of the MFS patients had facial nerve palsy. Despite severe GBS-DS presentation upon hospitalization and a high frequency of ICU admission in these patients, all cases recovered.

All patients re-evaluated 30 days after hospital discharge presented markedly less severe neurological symptoms (GBS-DS <2 and HBS <3). This stands in contrast to other GBS studies indicating that patients with higher scores on these scales tend to exhibit low rates of recovery (26). We hypothesize that the favorable rates of recovery seen herein may be due to prompt treatment with IVIG, which was administered at a median time of 3 days following admission. It is also possible that this was due to the result of GBS syndrome in association with arbovirus (27).

The clinical picture of ZIKV-associated GBS, as well as GBS occurring in association with other arboviruses, is generally similar to GBS arising from other causes. However, a recently published review indicated that cranial nerve palsy and autonomic dysfunction are more frequently associated with GBS following arbovirus infection, and that the clinical course of the disease is shorter, with a brief plateau phase and a high proportion of facial nerve involvement (23). Nonetheless, more consistent studies need to be conducted to define whether clinical

peculiarities are related to arbovirus-associated GBS. Herein, we found a high proportion of GBS subtypes and facial nerve palsy, with EMG findings compatible with AIDP. In addition, all patients had favorable outcomes, with clinical improvement noted after 30 days of reevaluation.

The concept that Classical GBS could be associated with emergent arboviruses has been well-established. However, the diagnosis of MFS and other GBS subtypes still poses significant challenges, consequently leading to misdiagnosis or the underreporting of GBS cases. Our case series offers additional information on clinical presentation and follow-up of arbovirus-associated GBS and its subtypes and serves as an alert to clinicians and other healthcare professionals in regions affected by arbovirus outbreaks. Furthermore, recognizing GBS and its subtypes is essential to providing prompt treatment and supportive care for patients to prevent mortality and long-term sequelae. Accordingly, we would like to emphasize the need to carry out the diagnosis for arboviruses in patients with GBS and its subtypes, including MFS.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitê de Etica e Pesquisas do Centro de Pesquisas Gonçalo Moniz- Fiocruz. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IS and LA contributed to the study design. MR and IS contributed to data analysis and writing of the manuscript. MR, PJ, DF, and MN contributed to participant enrolment, review of medical records, and the collection of samples and data. MF, CS, DM, and FL contributed to laboratory analysis. All authors contributed to the article and approved the submitted version.

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Nimotuzumab Increases the Recovery Rate of Severe and Critical COVID-19 Patients: Evaluation in the Real-World Scenario

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EGFR signaling is an important regulator of SARS-CoV induced lung damage, inflammation and fibrosis. Nimotuzumab is a humanized anti-EGFR antibody registered for several cancer indications. An expanded access study was conducted to evaluate the safety and recovery rate of severe and critical patients with confirmed SARS-CoV-2 infection, treated with nimotuzumab in combination with the standard of care in the real-world scenario. The antibody was administered as an intravenous infusions every 72 h, up to 5 doses. In order to assess the impact of nimotuzumab, the recovery rate was compared with a paired retrospective cohort. Control patients received standard treatment according the national protocol but not nimotuzumab. Overall, 1,151 severe or critical patients received nimotuzumab in 21 hospitals of Cuba. Median age was 65 and 773 patients had at least one comorbidity. Nimotuzumab was very well-tolerated and mild or moderate adverse events were detected in 19 patients. 1,009 controls matching with the nimotuzumab patients, were selected using a "propensity score" method. The 14-day recovery rate of the nimotuzumab cohort was 72 vs. 42% in the control group. Controls had a higher mortality risk (RR 2.08, 95% CI: 1.79, 2.38) than the nimotuzumab treated patients. The attributable fraction was 0.52 (95% CI: 0.44%; 0.58), and indicates the proportion of deaths that were prevented with nimotuzumab. Our preliminary results suggest that nimotuzumab is a safe antibody that can reduce the mortality of severe and critical COVID-19 patients.

Keywords: brief research report nimotuzumab, COVID-19, SARS-CoV-2, inflammation, fibrosis, EGFR, monoclonal antibody

INTRODUCTION

Growth factor receptors can be altered after a viral infection (1). Remarkably, the overexpression of some receptors may promote viral replication and immune response evasion (1). Representative altered receptor pathways include fibroblast growth factor (FGF), transforming growth factor beta (TGF beta), and epidermal growth factor receptor (EGFR) (2). Particularly, upon a viral infection, EGFR overactivation could have a major role in the inflammatory response and mucus production (1, 3). According to Hirano et al., EGFR participates in a positive feedback loop that enhances the inflammatory responses by preventing the inhibition through the negative regulator SOCS3 (suppressor of cytokine signaling-3) (4). Furthermore, a crucial regulatory role of the EGFR in thrombin-mediated inflammation was recently reported (4, 5).

EGFR can also be overexpressed after a lung injury (6). Alveolar type II cells in fibrotic lung tissues may express high levels of EGFR resulting in hyperplasia of the alveolar epithelial cells (4, 5). Preclinical studies of SARS-CoV-1 infections indicated that EGFR is upregulated and its overexpression contributes to enhanced lung disease (2). Particularly, in SARS-CoV-2 infected lungs, EGFR would be overexpressed after the acute lung injury or by the reduced Signal Transducer and Activator of Transcription 1 (STAT1) activity (7).

The blockade of EGFR emerges as a novel strategy for COVID-19 patients, provided its role in inflammation, immune-thrombosis and fibrosis (8, 9). Nimotuzumab is a humanized anti-EGFR monoclonal antibody, which is approved for the treatment of several epithelial tumors (10–14). The antibody efficiently inhibits the EGFR associated signal transduction, prevents proliferation, arrests the cell cycle in the G1 phase and decreases interleukin 6 (IL-6) secretion by the cancer cells (15, 16).

A phase I clinical trial evaluating nimotuzumab was conducted in moderate and severe COVID-19 patients. Forty-one patients were included in the trial. Seven patients received one dose of nimotuzumab, 29 received 2 infusions while 5 subjects required 3 doses. The antibody was very safe. Recovery rate was 80.64% in severe patients. Inflammatory markers decreased overtime and interleukin-6 concentration diminished from 46.5–14.51 pg/ml at day 7 (9).

Then, an expanded access study was launched nation-wide to evaluate the safety and recovery rate of severe or critical COVID-19 patients in comparison with matched controls in the real-world scenario.

MATERIALS AND METHODS

An expanded access study was conducted to evaluate the safety and recovery rate of patients with confirmed SARS-CoV-2 infection by reverse transcription polymerase chain reaction (RT-PCR), in the conditions of the standard medical practice.

Subjects older than 18 of any gender or skin color with severe or critical illness were enrolled. Patients with severe disease were those individuals who have oxygen saturation (SpO2) <94% on room air at sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO2/FiO2) <300 mm Hg, a respiratory rate of 30 breaths/min or more, or lung infiltrates >50% while subjects with critical illness were defined as those with respiratory failure, septic shock, and/or multiple organ dysfunction (17). In addition, patients received other drugs included in the national protocol for COVID-19, including steroids, anticoagulants and antibiotics. Patients did not receive any other immunoregulatory compound and were not vaccinated at the moment of enrollment. At the moment of the retrospective study (July–October 2021), the delta variant of concern was the dominant strain nation-wide (18).

Nimotuzumab was administered as an intravenous (IV) infusions every 72 h, up to 5 doses. The recommended number of doses was 3. The loading dose was 200 mg, followed by 100 mg in the next infusions. The antibody was diluted in 250 mL of saline solution (0.9%), administered over 2 h.

In order to assess the impact of nimotuzumab in the recovery rate, information from patients with severe or critical disease was collected from the national database of the Cuban Ministry of Health (9,027 patients). Control patients received standard treatment according the state COVID-19 guideline but not nimotuzumab or any other immunomodulatory drug. In order to guarantee that both cohorts were balanced, controls matching with the nimotuzumab patients were selected with a "propensity score" method, using demographics, province of residence as well as the most important comorbidities associated with COVID-19 prognosis. Briefly, a logistic regression score was assigned to each patient, considering the nimotuzumab treatment variable as the dependent variable and age, sex, comorbidities and province of residence as independent. Overall, 1,009 patients not treated with nimotuzumab or immunomodulatory drugs were selected with an exactly coincident score (0 tolerance), as compared to the patients treated with the antibody. The homogeneity of the groups was verified using the Pearson's chi-square and the *t*-student tests. The recovery rate from COVID-19 was estimated for treated and control patients at 14 days. Then, the relative risks of death from COVID-19 between the studied cohorts were estimated by using chi-square association test. The nimotuzumab and population attributable fractions, representing the number of deaths that could be prevented with nimotuzumab administration, were also estimated. Finally, a logistic regression analysis was performed in each subgroup defined by the control variables to estimate the mortality risk ratio, i.e., the relative increase in the probability of death rather than recovery of patients not receiving nimotuzumab. The risk ratio estimates and their 95% CI in each subgroup were displayed in a forest plot. Descriptive statistic was used to analyze the data set. The analyses were made with the SPSS version 25.0. The study was conducted according to the Helsinki ethical principles for medical research involving human subjects. The study was funded by the Cuban Ministry of Health and the Center of Molecular Immunology. The trial was approved by the ethical review board of the leading institutions and the protocol was listed in the public registry of clinical trials (https://rpcec.sld.cu/ensayos/RPCEC00000369-En).

TABLE 1 | Predictive value measures of the logistic regression model before and after matching.

	Before matching	After matching
Omnibus tests of model chi-square	1,510.890	0.000
−2 log likelihood	5,094.6	2,797.5
Cox & snell R square	0.156	0.000
Nagelkerke R square	0.298	0.000

RESULTS

From July 8 to October 27, 2021, 1,151 severe or critical patients received treatment with nimotuzumab in 21 hospitals from 9 provinces of Cuba. Median age was 65 (18–99) and 773 patients (67.2%) had at least one associated primary condition. Most common comorbidities were hypertension (57.5%), diabetes mellitus (20.8%), cardiovascular disease (14.2%), bronchial asthma (8.3%) chronic obstructive pulmonary disease (COPD) (3.9%), chronic kidney disease (0.8%) and cancer (0.2%).

Patients received nimotuzumab concomitantly with the standard of care including low molecular weight heparin, steroids and antibiotics. All patients were treated in the intensive care unit (ICU). The mean time between hospitalization and nimotuzumab was 2.7 days. Overall, 552 patients (48%) received a single infusion of the antibody, 245 subjects (21.3%) received 2 doses, 313 patients (27.2%) needed 3 nimotuzumab doses, while 28 (2.4%) and 13 patients (1.1%) required 4 or 5 antibody doses, respectively, at the discretion of the treating physicians.

Nimotuzumab was very well-tolerated and mild or moderate adverse events were detected in 19 patients (1.65%). Adverse events consisted mainly on chills, tremors, fever, headache, dyspnea and hypotension. Most adverse events were detected after the first antibody infusion. The 14-day lethality rate was 20.8%. Severe and critical patients without comorbidities had significantly less mortality as compared with those with 1 associated condition or more. The lethality rate was 15.8% in subjects without any chronic disease and 22.3% in subjects with one or more previous condition. The lethality rate decreased in relation with the number of doses. The l4-day lethality rate was 24.3%, 21.6%, 13.4%, 10.7% and 0, for patients receiving from 1 to 5 nimotuzumab doses, in that order.

To preliminary assess the impact of nimotuzumab, a paired retrospective cohort study was done. Globally, 1,009 controls matching with the nimotuzumab patients, were selected using a "propensity score" method. Control patients received standard treatment according the national COVID-19 protocol but not nimotuzumab. As a measure of the success of the balance between the cohorts achieved with the matching, the reduction obtained in the R squares (R^2), the likelihood ratio statistic and the omnibus tests of the logistic regression performed before and after matching are shown in **Table 1**.

Both groups were homogeneous in terms of demographics and number of comorbidities (**Table 2**). As described in other series, the most frequent comorbidities of these severe and critical patients were hypertension, diabetes, cardiovascular disease and obesity. **Table 2** describes the characteristics of both cohorts.

The 14-day recovery rate for the nimotuzumab cohort was 72 vs. 42% in the control group. Controls had higher mortality risk (RR 2.08, 95% CI: 1.79, 2.38) than the nimotuzumab treated patients (Pearson's Chi-square $p = 0.000$).

In our data set, the nimotuzumab attributable fraction was 0.52 (95% CI: 0.44; 0.58), and represents the proportion of deaths that was prevented with the antibody. The population attributable fraction, which is a measure of the potential impact that nimotuzumab would have on the recovery of severe or critical patients, was also estimated. The population attributable fraction was 0.26 (95% CI: 0.22%; 0.29), and indicates that in a prospective scenario, 26% of the deaths of severe and critical patients would be avoided with nimotuzumab administration.

A subgroup analysis of the mortality risk of the control vs. nimotuzumab treated patients was done. The forest plot is shown in **Figure 1**. In all subgroups, the probability of death was significantly higher in non-nimotuzumab treated subjects. The largest treatment benefit was seen in patients older than 90 and in patients with COPD. For the subgroup of subjects older than 90, the mortality risk was 11 times higher in the control vs. nimotuzumab patients and in patients with COPD, the risk of death was 9 times higher in the control vs. nimotuzumab group.

DISCUSSION

EGFR is implicated in inflammation through NF-kB, angiogenesis and profibrotic events (19). Multiple pieces of evidences support the role of the EGFR in the COVID-19 pathogeny (1, 7, 20, 21). Martinez et al., found higher levels of EGFR in COVID-19 individuals vs. community associated pneumonia subjects (19) and osimertinib, a well-known EGFR antagonist, showed *in vitro* anti-SARS-CoV-2 action (22) and prevented the virus cytopathic effect (23). In addition, several phosphoproteomic studies of SARS-CoV-2- infected cells disclosed that the virus activates EGFR (24). According Camara and Brandao, EGFR is the main influential receptor involved in COVID-19 (25). In spite of the multiple theoretical and *in vitro* evidences of the key role of the EGFR in COVID-19, this is the first proof of concept that blocking EGFR can have a positive impact in decreasing COVID-19 mortality. EGFR is a very well-validated target in oncology (26) but not in COVID-19. Moreover, the use of EGFR inhibitors in the setting of COVID-19 can be controversial, due to the previous reports of interstitial lung disease in patients with lung adenocarcinoma treated with EGFR tyrosine kinase inhibitors (27). The first clinical trial in hospitalized COVID-19 patients, demonstrated that nimotuzumab was very safe and the 14-day recovery rate was 82.9% (9). Only 8 patients (19.5%) of 41 required invasive mechanical ventilation. After 7 days, 76.2% of the subjects with a severe condition, improved the PO2/FiO2 ratio and there was a significant reduction of the affected lung areas. Inflammatory markers including C-reactive protein, ferritin, lactate dehydrogenase (LDH), neutrophil to lymphocyte ratio (NLR), D-dimer, interleukin 6 and plasminogen activator inhibitor-1 (PAI-1) decreased over time (9). This manuscript

TABLE 2 | Baseline characteristics of patients treated with nimotuzumab vs. controls not receiving immunomodulatory drugs.

		Nimotuzumab $N = 1{,}009$		Control $N = 1{,}009$		χ^2 *p*-value
		Freq	%	Freq	%	
Sex	F	464	46.0%	423	41.9%	0.07
	M	545	54.0%	586	58.1%	
Age	Median (SD)	64.56 ± 14.9		64.67 ± 14.8		0.87*
Age groups	19–29	9	0.9%	9	0.9%	1.00
	30–39	41	4.1%	41	4.1%	
	40–49	120	11.9%	120	11.9%	
	50–59	227	22.5%	227	22.5%	
	60–69	192	19%	192	19%	
	70–79	238	23.6%	238	23.6%	
	80–89	154	15.3%	154	15.3%	
	90–100	28	2.8%	28	2.8%	
Comorbidities	Hypertension	644	63.8%	644	63.8%	1.00
	Diabetes	215	21.3%	215	21.3%	1.00
	COPD	32	3.2%	32	3.2%	1.00
	Cardiovascular disease	156	15.5%	184	18.2%	0.10
	Cancer	3	0.3%	3	0.3%	1.00
	Asthma	85	8.4%	68	6.7%	0.15
	Chronic kidney Disease	5	0.5%	5	0.5%	1.00
	Obesity	118	11.7%	128	12.7%	0.50

**Student's t-test for comparison of means.*

reports for the first time the safety and recovery rate of patients treated with an anti-EGFR drug plus the standard of care vs. the standard of care alone, in a relatively large population in the conditions of the usual medical practice. Apart from other EGFR antibodies or small tyrosine kinase inhibitors, nimotuzumab exhibit an intermediate affinity against its target (10^{-9} M) (28). Previously, several authors have demonstrated that nimotuzumab requires bivalent binding for stable attachment to the cellular surface, leading to selectively targeting cells with high EGFR expression (29, 30). The biodistribution study in patients with epithelial tumors found that the percent of the injected dose of nimotuzumab per gram of tissue decreased 24 h post-treatment for normal organs, while the uptake in the tumor remained relatively constant (31). As a result, according its intrinsic properties, nimotuzumab would only recognize tissues with an aberrant EGFR overexpression like tumors or respiratory cells affected by infections leading to diffuse alveolar degeneration like in COVID-19 (32).

In our data set, the antibody was very safe. These results are compatible with previous findings in cancer, and represent a large advantage as compared to other biologics (11, 15, 28). Anti-inflammatory drugs, including steroids and anti-IL-6 receptor (IL-6R) or anti-TNFα antibodies can reduce tissue harm but augment the risk of sepsis (33–35). The majority of the patients required from 1 to 3 nimotuzumab doses and there was an association between the number of doses and the recovery rate. The optimal number of nimotuzumab doses needs further studies.

Under multiple circumstances, conducting traditional randomized clinical trials is not feasible, and real-world data and real-world evidence play a crucial role in taking the best medical decisions (36, 37). High-quality observational studies provide a useful platform for all clinical evaluations (38). Its greatest limitations consist of the biases induced by possible confounding factors (39, 40). The propensity score, defined as the conditional probability of receiving treatment given the covariates, can be used to balance the covariates and thus, reduce bias (41, 42). To estimate the propensity score, the distribution of the treatment variable is modeled given the observed covariates (41, 42).

In the present study, information was collected from all severe or critical patients (1,151) treated with nimotuzumab in 21 hospitals from July to October 2021. Then, patients were matched with other severe or critical COVID-19 subjects from the Cuban national database (9,027 cases) not receiving nimotuzumab or any other immunomodulatory drug. The propensity score included age, sex, eight different comorbidities, the province of residence as well as the treatment period as independent variables. After applying the propensity score method, the sample size obtained in each cohort (1,009 patients), granted a statistical power of 100% to detect differences in proportions, using the Mantel-Haenszel Chi-Square Test, at a significance level of 0.01.

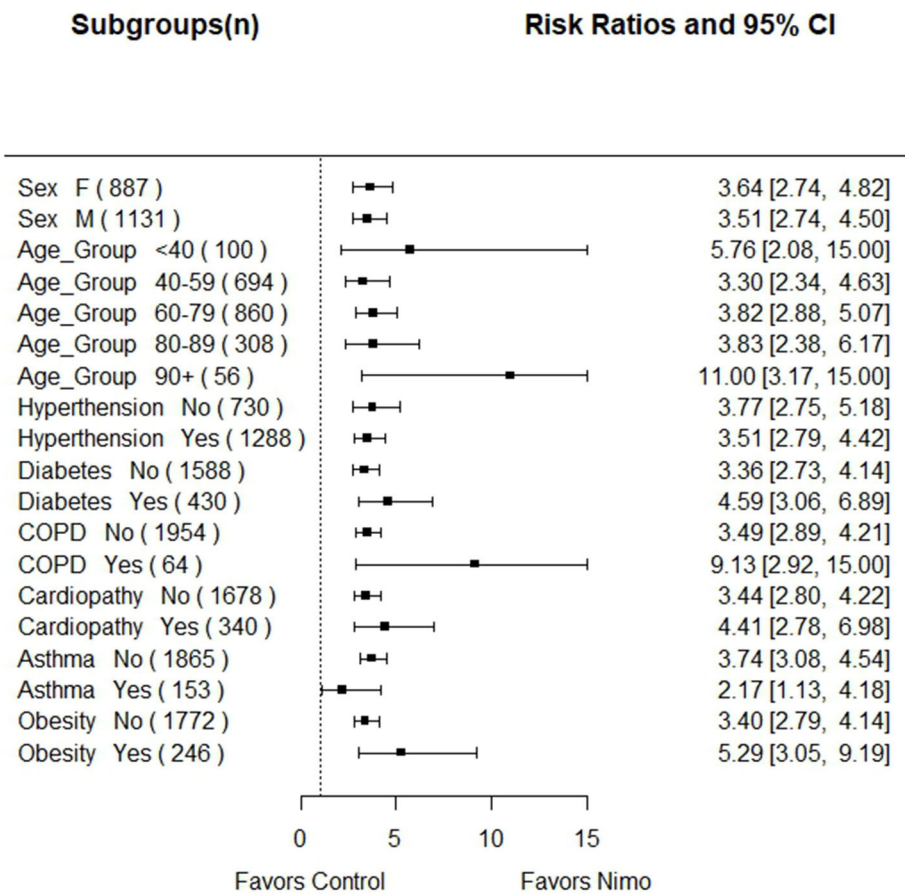

FIGURE 1 | Forest plot showing the mortality risk of the control vs. nimotuzumab treated patients according demographics and comorbidities. In all subgroups, the probability of death was significantly higher in non-nimotuzumab treated subjects.

Although this was not a randomized study, our preliminary data suggest that nimotuzumab reduced the relative risk of death in comparison to the matched controls. The 14-day recovery rate of the nimotuzumab cohort was 30% higher than the control group (RR 72 vs. 42%), and controls had twice the mortality risk than the nimotuzumab treated patients. The observational study concluded that 52% of the deaths among the ICU patients were prevented with nimotuzumab treatment. All patient subgroups benefitted from therapy and particularly, nimotuzumab seems to be very efficacious for vulnerable patients like those older than 90 or with COPD associated comorbidity.

Other drugs devoted to decrease the hyperinflammatory response have been evaluated. For patients treated at the intensive care units (severe or critical), the addition of one or two doses of tocilizumab, an anti-IL6R antibody, to usual care significantly reduced the mortality rate compared with usual care alone (31 vs. 35%) (relative risk 0.85; 95% CI, 0.76–0.94) (33). In another controlled trial in 3,924 severe or critical patients, 40.6% of patients who did not receive therapy with the anti-IL-6R antibody died while the mortality rate was 28.9% after the use of tocilizumab, respectively (43). The case fatality rate of severe and critical patients treated with nimotuzumab plus the standard of care (28%) compares very favorably with the rates reached after tocilizumab.

In summary, our results suggest that nimotuzumab is a safe antibody that can reduce the mortality of severe and critical COVID-19 patients. These results should be confirmed in a prospective randomized study.

DATA AVAILABILITY STATEMENT

Data will be available after article publication and will end 36 months following publication. The information will be shared with researchers whose proposed use of the data has been approved by an independent review committee identified for this purpose.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Julio Trigo Hospital and Salvador Allende Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TC, MR, DS, and PL-L designed the clinical trial and CRFs of the clinical trial. HD, JJ, AH, LV, AM, LPo, RH, NT, and JHJ administered the experimental drug plus the SOC and followed the COVID-19 patients at the hospital intensive care unit. LM, MT, YS, AG, DB, AV, LC, MD, and LPé were responsible of monitoring and data management. PL-L and LS did data processing. DS, MR, and TC interpreted the final results. All authors reviewed and approved the final manuscript.

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Low prevalence of influenza A strains with resistance markers in Brazil during 2017–2019 seasons

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The influenza A virus (IAV) is of a major public health concern as it causes annual epidemics and has the potential to cause pandemics. At present, the neuraminidase inhibitors (NAIs) are the most widely used anti-influenza drugs, but, more recently, the drug baloxavir marboxil (BXM), a polymerase inhibitor, has also been licensed in some countries. Mutations in the viral genes that encode the antiviral targets can lead to treatment resistance. Worldwide, a low prevalence of antiviral resistant strains has been reported. Despite that, this situation can change rapidly, and resistant strain surveillance is a priority. Thus, the aim of this was to evaluate Brazilian IAVs antiviral resistance from 2017 to 2019 through the identification of viral mutations associated with reduced inhibition of the drugs and by testing the susceptibility of IAV isolates to oseltamivir (OST), the most widely used NAI drug in the country. Initially, we

analyzed 282 influenza A(H1N1)pdm09 and 455 A(H3N2) genetic sequences available on GISAID. The amino acid substitution (AAS) NA:S247N was detected in one A(H1N1)pdm09 strain. We also identified NA:I222V ($n = 6$) and NA:N329K ($n = 1$) in A(H3N2) strains. In addition, we performed a molecular screening for NA:H275Y in 437 A(H1N1)pdm09 samples, by pyrosequencing, which revealed a single virus harboring this mutation. Furthermore, the determination of OST IC_{50} values for 222 A(H1N1)pdm09 and 83 A(H3N2) isolates revealed that all isolates presented a normal susceptibility profile to the drug. Interestingly, we detected one A(H3N2) virus presenting with PA:E119D AAS. Moreover, the majority of the IAV sequences had the M2:S31N adamantanes resistant marker. In conclusion, we show a low prevalence of Brazilian IAV strains with NAI resistance markers, in accordance with what is reported worldwide, indicating that NAIs still remain an option for the treatment of influenza infections in Brazil. However, surveillance of influenza resistance should be strengthened in the country for improving the representativeness of investigated viruses and the robustness of the analysis.

KEYWORDS

influenza A virus, resistance, neuraminidase (NA) inhibitors, oseltamivir, baloxavir marboxil, adamantane

Introduction

Influenza virus (IV) is an important pathogen that causes respiratory infections and a high disease burden worldwide, often presenting as annual epidemics. The acute respiratory and febrile illness is normally self-limiting in healthy people, but it can be especially threatening in high-risk individuals such as elderly people, children, pregnant women, immunocompromised individuals, and patients with underlying chronic diseases. Per year, influenza infections are reported in 1 billion cases and up to 650,000 deaths worldwide (1). Belonging to the *Orthomyxoviridae* family, IVs are enveloped and contain a segmented genome composed of negative-sense single-stranded RNA (2). Importantly, influenza A virus (IAV) has the potential for causing pandemics, and its strains are classified according to their hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, with the subtypes A(H1N1)pdm09 and A(H3N2) currently being the most relevant to human health.

Influenza viruses have a well-characterized seasonal circulation pattern in some parts of the world, with peaks during the coldest seasons (autumn-winter). However, Brazil does not have a homogeneous seasonality pattern, due to its continental dimensions, and each country region can have specific patterns for IVs circulation over the year, which is influenced by the different climates present in the country (3). There are three distinct climatic patterns in Brazil, namely, equatorial, in the north and part of the northeast; tropical, in the northeast, midwest, and southeast; and subtropical (temperate), in the south of the country. In view of this, the influenza detection in the country is distinct by comparing the northern equatorial regions and the southern temperate regions. According to the Brazilian Ministry of Health, in 2019, 1,122 fatalities due to IVs infection were reported (4).

To control the high morbidity and mortality caused by IAV infections, two main strategies are currently employed, namely, the annual vaccination for target high-risk populations and the treatment with antiviral drugs. Even though the vaccination against influenza is the most effective form of prevention, it still faces some challenges, such as the absence of a universal influenza vaccine and the need to update vaccine strains included in the vaccines regularly (5). In contrast, antiviral drugs are used for the prophylaxis and control of IAV infections. These compounds act directly on some of the IV's essential proteins, interrupting its replicative cycle and presenting benefits, for instance, shortening the duration of the disease symptoms, and improving clinical outcomes if administered early in the infection (6, 7). Antiviral drugs are also important because they can act as the first line of defense for the population at particular risk in the event of the emergence of a new IAV strain, since the mass production of an effective vaccine can take months. At present, there are several pharmacological classes of drugs with anti-IV activity approved for use. The adamantanes, such as amantadine and rimantadine, act by blocking the viral M2 ion channel (8). Neuraminidase inhibitors (NAIs), represented by oseltamivir (OST), zanamivir (ZAN), peramivir (PER), and laninamivir (LAN), bind to NA and prevent the release of the newly generated virions (9). The drug baloxavir marboxil (BXM),

which is a cap-dependent endonuclease inhibitor, targets the polymerase acid (PA) protein, one of the three subunits that constitute the IAV RNA polymerase, inhibiting its cap-snatching mechanism, hence inhibiting mRNA transcription and ending viral replication (10). BXM was initially released for use in 2018 and has been approved in over 30 countries around the world, such as Japan, the USA, and the European Union (11).

However, due to the constant evolution of IAVs, through genetic drift, mutations may arise in the genes that encode viral proteins targeted by antiviral drugs, leading to amino acid substitutions (AAS), which may reduce the drug's effectiveness. This affected the adamantanes, which have not been recommended for the treatment of IAV infections since the 2005–2006 seasons. AASs such as M2:S31N, which remains fixed in IAVs, led to viral resistance to this pharmacological drug class and showed good viral fitness (12). Despite that, in 2017, a cluster of IAV sensitive to the adamantanes was detected in Australia, but their dissemination has not been observed (13).

In relation to NAIs resistance, some AASs in NA correlating with reduced inhibition (RI) to these drugs have been identified. The NA:H275Y was previously detected in high prevalence in the former A(H1N1) viruses that circulated until the end of the 2000s, causing high RI to OST, a fact that impaired the value of this drug (14) at that time. However, the emergence of the pandemic strain A(H1N1)pdm09, which did not have NA:H275Y, allowed the return of OST use to treat those infections. Since then, surveillance of NAIs-resistant strains has been intensified, with the detection of clusters of viruses carrying resistance markers in the USA, Australia, and Japan (15–20). Despite that, further dissemination of these mutated viruses has not been observed. Thus, in the most recent global reports, the frequency of resistant strains has remained low (<1%) (18).

Regarding BXM, which has not yet been approved for use in Brazil, resistance markers in the PA, associated with RI, have been identified during the drug initial trials and in IAVs circulating in some countries, with PA:I38T being the most relevant one (21–24).

Brazilian surveillance of NAIs-resistant IAVs has identified A(H1N1)pdm09 strains carrying the markers NA:H275Y, NA:S247N, and NA:I223K, with a low frequency (25–27). IVs antiviral susceptibility surveillance is conducted by the World Health Organization (WHO) through the Global Influenza Surveillance and Response System (GISRS), which comprises six WHO Collaborating Centers (CCs) and 141 institutions in 111 WHO member states. Brazil is part of the GISRS and has an influenza surveillance system (ISS) with 3 national influenza centers (NICs), which are located at the Respiratory Virus and Measles Laboratory of the Oswaldo Cruz Foundation in Rio de Janeiro (Fiocruz/RJ), the Virology Laboratory of the Evandro Chagas Institute in Pará (IEC/PA), and the Respiratory Virus Laboratory of the Adolfo Lutz Institute in São Paulo (IAL/SP). Brazilian ISS comprises sentinel units for influenza-like illness (ILI) and severe acute respiratory infection (SARI) that are spread across the country collect the samples, which are further sent to the Central State Laboratories (LACENs) located in each of the 27 Brazilian states. Then, a subset of the samples received by the LACENs is sent to the NICs (28).

This study presents the analysis of Brazilian IAVs susceptibility to antivirals in Brazil between 2017 and 2019. We evaluated viral genetic sequences of circulating viruses and performed functional phenotypic analysis by determining the OST concentration that inhibited 50% of NA activity (IC_{50}) of Brazilian IAV isolates from the period.

Materials and methods

IAV genetic sequences

The Brazilian IAV sequences that were available in the EpiFlu platform in the Global Initiative on Sharing Influenza database (GISAID) (https://www.gisaid.org/) were downloaded and further evaluated. For that, we used the following screening criteria: type: A, host: human, region: South America/Brazil, and collection date: from 1 January 2017 to 31 December 2019. Duplicate sequences were removed. The analysis of the presence of antivirals resistance markers was performed by using the FluSurver tool (https://www.gisaid.org/epiflu-applications/flusurver-app/).

Samples and data collection

Our laboratory is an NIC for WHO and the Brazilian Ministry of Health and, therefore, continuously receives a subset of samples from ISS collected in 9 of 27 Brazilian states. Respiratory secretion samples, previously characterized as positive for IAV in their respective LACENs, collected from 2017 to 2019, were included in this study. These samples were collected through nasopharyngeal swabs or aspirates from patients displaying ILI or SARI. A case of ILI is defined as the presence of fever, even if reported, accompanied by cough or sore throat and, at least, one of the following symptoms: headache, myalgia, or arthralgia, in the absence of another specific diagnosis. In children aged <2 years, it is defined as the presence of fever (even if reported) and symptoms (cough, coryza, and nasal obstruction), in the absence of another specific diagnosis. SARI cases were defined as cases requiring hospitalization and presenting dyspnea or one of the following signs of severity: peripheral capillary oxygen saturation <95%, respiratory distress, or acute respiratory insufficiency.

Ethics

This study was approved by the FIOCRUZ—Oswaldo Cruz Institute (IOC) Ethics Committee under the number 68118417.6.0000.5248.

Pyrosequencing

A molecular screening was performed in influenza A(H1N1)pdm09 positive samples in order to identify the most relevant and frequently detected NAIs resistance marker, the NA:H275Y. We selected representative samples by using the following criteria: epidemiological week, Brazilian State, severe and fatal cases, OST administration, and availability of reagents. For that, the RNA of the clinical samples was extracted using a viral RNA mini kit (QIAGEN, USA), according to the manufacturer's instructions, and then an RT-PCR reaction was performed, followed by a pyrosequencing reaction and analysis using the PyroMark™ Q96 ID Platform. The run was performed in single-nucleotide polymorphism (SNP) mode, and analysis of results was performed by both SNP and allele quantification (AQ) modes, as previously described (29). The NA:H275Y substitution is characterized by the punctual exchange of the guanine (G) nucleotide by the adenine (A) nucleotide, where the GTG triplet would be changed to ATG triplet in the analyzed sequence, which was 7 bases long and is contained into NA catalytic site.

Cell culture and viral isolation

Virus isolation was performed for all IAV-positive clinical samples received at the laboratory. Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM™) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin (Gibco, USA), and 100 µg/ml streptomycin (Gibco, USA). After removing the culture medium, 100 µl of the clinical sample was added and incubated at 37°C in 5% CO^2 in the presence of 2 µg/ml TPCK-treated trypsin (Sigma, USA). Visualization of the cytopathic effect (CPE) for up to 72–96 h post-infection was performed. After observing CPE, the viral isolation was confirmed by using NA activity assay with the chemiluminescent kit NA-STAR (Thermofisher, USA). For the viruses with antivirals resistance markers, we also tried additional passages in cultured cells, even if they do not show any CPE after the first inoculation.

Functional antiviral assay

Determination of OST IC_{50} (drug concentration to inhibit 50% of NA activity) was done by measuring the NA activity, in the presence of increasing concentrations of OST, by using the chemiluminescent kit NA-STAR™ (Thermofisher™). To this end, NA activity was measured in relative light units (RLUs) on the FLUOstar™ Optima equipment (BMG Labtech™). Classification of the susceptibility profile of the isolates was performed according to the WHO criteria for the OST IC_{50} values for the IAVs, by comparing the IC_{50} median to previously tested viruses (30). Thus, the classification was performed as follows: normal susceptibility, when the IC_{50} value is up to 10 times the median value; RI, when the IC_{50} value corresponds to an increase of 10–100 times in the median value; and high reduced inhibition (HRI), when the IC_{50} value corresponds to an increase in more than 100 times of the median value.

Statistical analysis

The median IC_{50} of the isolates, 95% confidence interval (95% C.I.), and the analysis of the variance of the medians between isolates, clinical samples, and collection year were calculated using Kruskal–Wallis's test, followed by the Dunn's test for multiple comparisons. All analyses were carried out by using the GraphPad Prism software version 8.0.

Results

Identification of NA mutations associated with NAIs RI

We analyzed the sequences of the NA, the gene of interest for NAIs resistance monitoring, from IAVs that circulated in Brazil in the period of 2017–2019 that were available on GISAID (Supplementary Table 1). We evaluated 280 complete NA segments from A(H1N1)pdm09 viruses as well as 450 complete NA segments from A(H3N2) (Table 1). We identified one A(H1N1)pdm09 strain (A/Ceara/152545-IEC/2018) with the NA:S247N AAS collected from a patient who was infected in 2018. In addition, we detected six A(H3N2) strains harboring the NA:I222V AAS (A/Parana/99/2017, A/Parana/152/2017, A/Parana/235/2017, A/Parana/340/2017, A/Brazil/399/2017, and A/Parana/490/2017) and one with the N329K AAS (A/Espirito Santo/174/2017), which were collected in 2017. The clinical and epidemiological data of these cases are summarized in Table 2.

To complement the surveillance of Brazilian viruses that would pose a threat to the efficiency of the anti-influenza treatment, a pyrosequencing molecular screening was

TABLE 1 Brazilian IAVs genetic sequences NA, PA, and M genes available at the EpiFlu database on GISAID by gene and year and amount of AAS found in each year.

Year	A(H1N1)pdm09			A(H3N2)		
	NA	M2	PA	NA	M2	PA
2017	10	7	7	236	111	108
2018	137	115	108	108	89	86
2019	132	86	84	106	75	71
Total	280	208	199	450	275	265

NA, Neuraminidase; AAS, Amino acids substitution, M2, Matrix 2; PA, Polymerase Acid.

TABLE 2 Clinical and epidemiological characteristics of the cases that were infected by the IAVs carrying AAS associated with antivirals RI.

Virus	IAV subtype	Gene	AAS	ID GISAID	Brazilian state	Gender	Age (years)	Hospitalization	SARI	Fatal	Collection date	OST initiation
A/Espirito Santo/174/2017	A(H3N2)	NA	N329K	EPI_ISL_274162	ES	M	34	No	No	No	03/10/2017	NT
A/Parana/99/2017	A(H3N2)	NA	I222V	EPI_ISL_268344	PR	M	42	No	No	No	02/15/2017	03/07/2017
A/Parana/152/2017	A(H3N2)	NA	I222V	EPI_ISL_268348	PR	M	86	Yes	NI	No	03/07/2017	03/10/2017
A/Parana/235/2017	A(H3N2)	NA	I222V	EPI_ISL_274170	PR	M	50	Yes	Yes	No	04/14/2017	04/14/2017
A/Parana/340/2017	A(H3N2)	NA	I222V	EPI_ISL_274655	PR	M	8	Yes	Yes	No	05/12/2017	05/11/2017
A/Brazil/399/2017	A(H3N2)	NA	I222V	EPI_ISL_275869	SC	M	28	NI	Yes	Yes	21/05/2017	NT
A/Parana/490/2017	A(H3N2)	NA	I222V	EPI_ISL_300393	PR	F	15	No	No	No	06/26/2017	NT
A/Brazil/339/2017	A(H3N2)	PA	E199D	EPI_ISL_275866	PR	M	21	No	No	No	05/02/2017	NT
A/Ceara/152545-IEC/2018	A(H1N1) pdm09	NA	S247N	EPI_ISL_320232	CE	F	3	NI	NI	NI	04/06/2018	NI
A/Espirito Santo/974/2019	A(H1N1) pdm09	NA	H275Y	ND	ES	M	41	No	No	No	09/04/2019	09/04/2019

IAV, Influenza A virus; AAS, Amino Acid Substitution; ID GISAID, Identification on GISAID; SARI, Severe Acute Respiratory Infection; OST, Oseltamivir; ES, Espírito Santo; PR, Paraná; SC, Santa Catarina; CE, Ceará; NA, Neuraminidase; PA, Polymerase Acid; M, Male; F, Female; ND, Not deposited; NI, Not Informed; NT, Not treated.

performed specifically to identify the NA:H275Y AAS, as it is the most frequently and importantly detected NAI marker in A(H1N1)pdm09 viruses. In the study period, the laboratory received 1,112 A(H1N1)pdm09 samples, from which 437 were successfully analyzed by this methodology. We identified one strain presenting the NA:H275Y marker (A/Espírito Santo/974/2017), which was collected in September 2019 from a 41-year-old male patient from Espírito Santo State located in the Brazilian Southeastern region. The infected patient presented common ILI symptoms in addition to dyspnea, initiating OST treatment on the same day of sample collection, but did not require hospitalization (Table 2). Allele quantification analysis showed that the sample had 100% of its AA residues at position 275 of NA mutated, which shows the predominance of the mutation in the patient's viral populations. With the aim to verify the spread of H275Y mutated A(H1N1)pdm09 variants in the region where the sample A/Espirito Santo/974/2019 was detected, molecular screening was performed in further 18 samples from the same Brazilian State and period (from August to October 2019). However, no additional samples were detected showing the NA:H275Y AAS.

Susceptibility of Brazilian IAV isolates to OST

The gold standard for the determination of the susceptibility of IAVs to antivirals is through the functional analyses of the isolates in direct contact with the drug to determine OST IC_{50}. Over the study period, we successfully isolated 222 A(H1N1)pdm09 and 83 A(H3N2) strains from the 1,987 IAV samples received at the NIC. The A(H1N1)pdm09 isolates the presented IC_{50} values ranging from 0.02 to 0.35 nM in the 2017 (median of 0.13 nM); 0.01 to 1.89 nM in 2018 (median of 0.35 nM); and 0.01 to 0.58 nM in 2019 (median of 0.11 nM). Moreover, the A(H3N2) isolates the presented IC_{50} values ranging from 0.10 to 1.39 nM (median of 0.27 nM) in 2017; 0.04–1.76 nM (median of 0.29 nM) in 2018; and 0.05–0.34 nM (median of 0.13 nM) in 2019 (Table 3). As a result, all the tested isolates showed a normal susceptibility profile to OST, according to WHO criteria (Figure 1). It is noteworthy that none of the viruses that presented any NAI RI mutation were successfully isolated as they did not show any CPE during isolation protocols nor had any NA activity detected after cell culture passages.

Identification of M2 and PA mutations associated with adamantanes and BXM

Further complementing the genetic analysis of the IAVs that circulated in Brazil during the proposed period, we reviewed their M2 and PA gene sequences available in the GISAID database with the objective to identify markers associated with RI to the adamantanes and BXM. A total of 483 M2 gene sequences were evaluated (208 from H1N1pdm09 and 275 from H3N2 viruses) and, as expected, M2:S31N AAS was predominantly found in Brazilian IAV sequences. However, one sample collected from an A(H3N2) strain (A/Brazil/358/2017) had no adamantane resistance marker. This virus was recovered in May 2017 from an 82-year-old female patient who was hospitalized with SARI in Espírito Santo State (Table 2).

TABLE 3 IAVs isolates OST IC_{50} median by year.

Season	A(H1N1)pdm09			A(H3N2)		
	N	Median (nM)	95% C.I.	*N*	Median (nM)	95% C.I.
2017	6	0.13	(0.02, 0.35)	24	0.27	(0.14, 0.8)
2018	94	0.35	(0.31, 0.43)	45	0.29	(0.16, 0.39)
2019	122	0.11	(0.08, 0.13)	14	0.13	(0.07, 0.23)

CI, Confidence interval.

In addition, a review of available Brazilian IAV complete PA sequences deposited at GISAID retrieved 464 results (199 from H1N1pdm09 and 265 from H3N2 viruses). Despite not identifying any A(H1N1)pdm09 virus harboring BXM RI-associated mutations, we detected one A(H3N2) PA sequence presenting the PA:E199D AAS (A/Brazil/339/2017) that was collected in May 2017 from a 21-year-old female patient who lived in Paraná State, Brazilian Southern region, and showed mild ILI symptoms (Table 2).

Discussion

The availability of antiviral treatments to any emerging and reemerging infectious disease, such as caused by IVs, is critical for public health responses and control of the morbidity and mortality of these infections. In Brazil, influenza treatment is based on the NAIs OST and ZAN, of which oral OST is the most widely available drug to treat these infections, followed by the inhaled ZAN (31). BXM treatment, which has already been approved in countries including USA, Europe, and Asia, is still not authorized for use in Brazil (10, 22, 32).

This study evaluated the circulation of IAV strains with genetic markers associated with RI to the antivirals NAIs, adamantanes, and BXM, in the period between 2017 and 2019, which circulated in Brazil. Among the mutations detected in this study, the NA:H275Y is the most frequent and relevant marker associated with RI to NAIs. The sample identified with this substitution showed 100% of its amino acid residues altered. Interestingly, this sample was collected on the same day that the individual started treatment with OST, which allows us to speculate that the emergence of AAS was not due to antiviral pressure. Moreover, allele quantification analysis showed that the sample had 100% of its nucleotides mutated at the NA position of AAS, which would suggest that the individual may have been initially infected with a variant virus that was spreading in the community. Despite that, we analyzed additional A(H1N1)pdm09 samples that were collected by the Brazilian ISI from the same region and period and identified no additional mutated strain. When it is present in A(H1N1)pdm09 viruses, NA:H275Y causes a high decrease in the binding affinity between NAIs and NA, which leads to a significant reduction in the effectiveness of OST and PER drugs (33). It is known that this mutation affects viral fitness and may influence the ability of viral transmission and cell replication in cell culture, as we and others demonstrated (34, 35). Despite that, permissive mutations, such as NA:V241I and NA:N369K, can act to compensate for this loss of fitness and are already incorporated in the circulating IAVs around the world, including Brazil (25). Before the 2009 pandemic, the NA:H275Y was incorporated into the genome of seasonal influenza A(H1N1) strains circulating at that time, leading to their resistance to OST. However, the pandemic strain A(H1N1)pdm09, which did not contain this mutation and was sensitive to OST treatment, emerged, spread, and replaced the previous seasonal A(H1N1) strain. Since then, the frequency of NA:H275Y identification in these viruses has remained low (18). In addition, we have identified an A(H1N1)pdm09 virus that harbored the NA:S247N marker, which exclusively causes a significant RI effect when it is associated with the NA:H275Y mutation, causing a synergistic effect, increasing the reduction in OST and PER susceptibility (36). Even though they were not jointly detected in our sample, NA:S247N should be closely monitored.

In relation to the evaluated A(H3N2) viruses, the AAS NA:I222V, which is associated with the reduction of OST active site hydrophobicity, therefore, decreasing favorable drug interactions (37), was identified in six samples. However, similar to NA:S247N, this AAS has a relevant effect on the NAIs susceptibility strictly when it is associated with NA:E119V in A(H3N2) or NA:H275Y in A(H1N1)pdm09 viruses (38). Notably, sample A/Parana/340/2017, identified as carrying AAS NA:I222V, was collected 1 day after starting OST treatment, which cannot exclude antiviral pressure for the emergence of this mutation. This sample came from an 8-year-old individual with SARI. Furthermore, we show the identification of NA:N329K, already reported to cause RI to OST and ZAN (15, 20).

The functional analysis of OST IC_{50} showed that all Brazilian isolates from the period remained sensitive to the drug. We observed that median OST IC_{50} was reduced in 2019, in comparison with the previous years, for both influenza A subtypes, which would be an indicator of the stabilization of the OST sensitivity of the IAVs and a further support on its use for the treatment of IAV infections. Future investigations will confirm this hypothesis.

Our analyses of the M2 IAV gene sequences showed that most of them had the adamantanes resistance marker M2:S31N, as has globally been reported since the 2005–2006 season (39). For this reason, the adamantane drugs are no longer recommended for the treatment of IAV infections in Brazil and elsewhere for more than a decade. Despite that, one A(H3N2) strain (A/Brazil/358/2017) did not present any known adamantanes resistance markers. This sample was collected from a 2017 case, when A(H3N2) was the predominant IAV subtype circulating (40). Interestingly, in the same year,

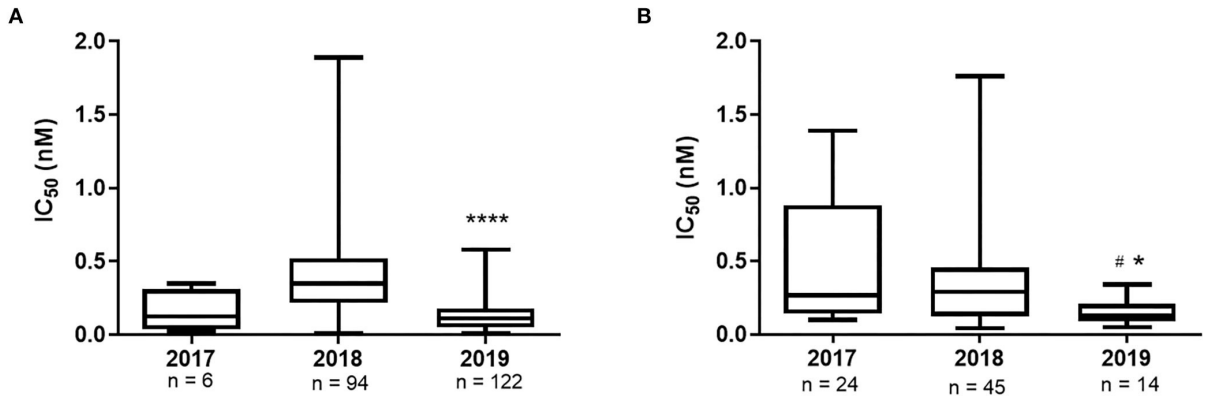

FIGURE 1
OST IC50 values of Brazilian IAV isolates A(H1N1)pdm09 **(A)** and A(H3N2) **(B)**, by year. ****p value <0.0001 2019 versus 2018, *p value <0.05 2019 versus 2017, #p value <0.05 2019 versus 2018. The median variance was obtained using the Kruskal-Wallis test.

A(H3N2) clusters without any adamantanes resistance markers were also detected in Australia, despite this, their spread was not observed (13). During the emergence of IAVs with M2:231M AAS, it was suggested that their dissemination was less related to the selective pressure exerted by the use of adamantane drugs. Instead, they would have emerged spontaneously in viral variants and were fixed through their interaction with additional advantageous mutations located in other parts of the viral genome, probably through hitch-hiking effect (41).

Herein, we also revealed that BXM susceptibility AASs in PA were detected in a unique A(H3N2) virus that contained PA:E199D. This substitution is in the same position of a distinct one (PA:E199G) that has been reported in association with a discrete (4.5-fold) RI to BXM (22). The position 199 of PA is important for this drug interaction. Nonetheless, further studies need to be performed to confirm whether PA:E199D AAS also affects the effectiveness of BXM. It is worth mentioning that this marker was detected 1 year before the first approval of BXM in the world and until now, no PAI has been approved for use in Brazil, suggesting that this AAS has emerged spontaneously in the PA gene. PA is one of the 3 subunits that make up the IAV polymerase, playing a crucial role in the replication cycle of the virus. The gene encoding the PA protein is highly conserved and the emergence of mutations is a rare event. However, some markers of RI to BXM have already been identified, especially at position PA:I38X (T/F/M/S/L/V), the most frequently found (18).

The monitoring of IAVs to detect reduced susceptibility to antivirals is an essential activity of the surveillance networks for providing information regarding the continuous use of these compounds. However, this study presents some limitations, such as the number of available virus sequences and isolates, which limited a robust assessment of the prevalence of the mutated viruses. In addition, there is a need for a closer monitoring of individuals in the country who are receiving treatment with these antivirals, especially among the immunocompromised groups. Therefore, there is a need to strengthen the Brazilian ISS to address these limitations. Moreover, there is less consistency in the quantity of data and numbers of samples that are collected and evaluated from distinct Brazilian regions, making it important to increase the representativeness of strain identification in some states to strengthen the planning for national vaccination campaigns.

Conclusion

This study, covering IAVs that circulated in Brazil in the period of 2017–2019, reveals a low prevalence of IAVs with genetic markers associated with resistance to the anti-influenza drugs NAIs and BXM. Our data further demonstrate that the available IAVs isolates from Brazil were sensitive to OST. In addition, the majority of IAVs from the country have the adamantanes resistance markers. Therefore, NAIs remain an option for the control of influenza infections in the country. Monitoring the susceptibility of the viruses to the available treatments is crucial for the guidance of the medical interventions.

Data availability statement

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

Ethics statement

This study was approved by the FIOCRUZ—Oswaldo Cruz Institute (IOC) Ethics Committee under the number 68118417.6.0000.5248. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

TS, MS, and AM: conceptualization. TS, JM, CG, MM, PR, CS, MD, RR, AC, TG, FI, FP, SF, JF, KS, FM, MS, and AM: methodology, analysis, and investigation. MS, AM, and WA: resources. TS, DB, MS, and AM: writing. All authors contributed to the article and approved the submitted version.

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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

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.944277/full#supplementary-material

SUPPLEMENTARY TABLE 1
Brazilian IAVs gene sequences data that were included in this study from GISAID EpiFlu platform.

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