EMERGING AND RE-EMERGING VECTOR-BORNE AND ZOONOTIC DISEASES

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EMERGING AND RE-EMERGING VECTOR-BORNE AND ZOONOTIC DISEASES

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EDITORIAL
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Editorial: Emerging and Re-emerging Vector-borne and Zoonotic Diseases

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Editorial on the Research Topic

Emerging and Re-emerging Vector-borne and Zoonotic Diseases

The Colombian Nobel laureate Gabriel Garcia Marquez stated in his last will, "*la muerte no llega con la vejez, sino con el olvido*" ("death does not come with old age, but with oblivion"). Indeed, how many deaths due to tropical diseases can be avoided? How can investment in these neglected diseases significantly change the course of the disease? Even in a macro vision, how could the socioeconomic condition of those affected by these diseases be changed to avoid transmission, morbidity, and mortality? We must rescue tropical and emerging global diseases from oblivion, and the rescue begins with us.

Despite significant advances in diagnostic tools, sequencing technologies (1–5), new drugs, and vaccine development using precision medicine (6–9), pharmacogenomics (10–12), computational and *in silico* models (13–16), and artificial intelligence (17–20); the benefits of these accomplishments have not been fully realized in the field of emerging and re-emerging vector-borne and zoonotic diseases (21). Emerging vector-borne and zoonotic diseases are a growing threat to global health and have caused hundreds of billions of US dollars of economic damage in the past 20 years (22). Together, these infections are responsible for a substantial disease burden, with endemic and enzootic zoonoses, and metaxenic diseases causing about a billion cases of illness in people and millions of deaths every year (22). Moreover, old foes with us for hundreds [like Chagas disease (23–25)] or thousands [like leprosy or Hansen's disease (26–28)] of years are yet to be eliminated or controlled in many countries. This is the tragedy of neglected tropical diseases. Disinterest and disincentive are monstrous impediments to the progress that could be made by governments, major pharmaceutical companies, and other actors in the development of new drugs, research initiatives, diagnostics, and vaccines for these diseases.

The rescue from oblivion is accomplished in multiple ways, including increasing visibility; generating, disseminating, and implementing new knowledge and evidence; elaborating strategies and tools for the benefit of communities and patients, and pursuing research in a more integrated and comprehensive form, but also looking for ways to translate that research into policies. After millennia, standing in the dawn of the XXI century, we are at a crossroads. We can reinvigorate the global commitment to confront these problems; otherwise, several of these conditions will persist, taking millions of lives, causing substantial disability, and increasing poverty in already impoverished populations.

This collection of articles endeavors to identify unique and essential challenges and opportunities for improving the management of vector-borne and zoonotic diseases. As

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clinician-investigators, we favor a future that is characterized by improved health metrics globally and a human population that seeks to be a good steward of the planet on which it depends. Of course, it is easy to pontificate about what must be done and how grand the scale should be. However, viable solutions will accept the arduous tasks that buttress their pursuit. Our world is one of limited capital (political, human, financial). Thus, the field of vector-borne and zoonotic diseases needs to include advocates, educators, and communicators to ensure that populations have awareness and understanding of what is at stake for their future and political leaders are held accountable to serve the best interest of their people through policy and resource allocation (29–31). Here, we outline vital ideas that will shape the future of the vector-borne and zoonotic diseases field. We draw on One Health and Planetary Health models—essentially recognizing the interdependence of human health, animal health, and environmental health; ideas endorsed by major international organizations such as WHO (32)—and include lessons learned from the contributors to this collection of articles.

The diagnostics underlying surveillance are a cornerstone for the public health management of infectious diseases, providing a means to monitor and model transmission and evaluate the impact of prevention and control activities (33). However, existing systems vary considerably in the organization, investment, and linkage to action or resource mobilization. Surveillance networks often focus on a specific pathogen but may also be centered on the syndromic presentation of illnesses such as acute respiratory illness or hemorrhagic fever. While serologic approaches remain important, molecular surveillance is the nexus of pathogen discovery and monitoring. Global surveillance must be strengthened at all levels. Bolstering existing vertical systems at local and national levels has advantages. These include investment in training and deployment of skilled field epidemiologists and maintenance of information systems for collecting, analyzing, and reporting data. Complimentary efforts by international entities such as the WHO to standardize practices and definitions help coordinate response efforts. Given the high volume of (pre-2020) international travel, unique surveillance networks that precisely monitor travel-related infections offer exciting opportunities to assess the spread of infectious diseases across borders (34–36). Of course, case definitions must be in place and diagnostic tools must be rapidly developed and deployed for novel pathogens to enable accurate surveillance. Making surveillance information robust and readily accessible accelerates research to understand and respond to existing and novel pathogens while informing public health decisions.

Treatment and prevention of infections are critical. Vaccination, which is not covered further here due to space limitations, is among the most celebrated achievements in medicine, and recent efforts in the Zika and COVID-19 pandemics have set an incredibly high bar for the rapidity with which safe and effective vaccine candidates can be developed (37). There are no licensed antivirals and a dearth of promising candidates for emerging diseases such as SARS, MERS, Ebola and Zika (38). The trend has been that intense research and publications follow on the heels of news of a new epidemic of potential global concern. However, interest and investment in these activities quickly subside. A greater armamentarium of treatments could have enormous benefits for combatting future vector-borne and zoonotic diseases, which is precisely the goal of innovative initiatives such as READDI (Rapidly Emerging Antiviral Drug Discovery Initiative) (38). Completing phase 1 trials of antivirals does not require a pre-known indication. We could start at phase 2, quickly screen *in vitro,* and test *in vivo* in parallel.

This Research Topic by *Frontiers in Medicine* and *Frontiers in Public Health* brings together a diversity of articles that focus on different pathogens, representing different points on the translational research spectrum, anchored in different disciplines. The goal was to provide a succinct sample of the vector-borne and zoonotic diseases affecting populations worldwide and some of the scientific methods involved in a public health response. The collection was finalized in 2019; but, the events of 2020 cannot be ignored. The COVID-19 pandemic is nothing less than a calamity. However, the emergence of SARS-CoV-2 is neither unique nor surprising; it is part of a pattern and process, and the palpable consequence of the close correlation of human, environmental, and animal health. Details of emergence are difficult to predict, but the reality that our global ecosystem holds countless potential pathogens, particularly RNA viruses, that could "spillover" under certain conditions and propagate among humans is well-known. The possible emergence of other CoV epidemics was predicted in the prescient work by Menachery et al., several years before the current pandemic (39). The COVID-19 pandemic has been difficult and tragic, but it also reveals the need for dedicated attention to comprehensive management plans for vector-borne and zoonotic diseases that could prevent future catastrophes.

This Research Topic has exceeded expectations in scope and content. The original article on dengue is an exciting paper, combining findings and experiences from two endemic countries in Latin America (Ecuador) and Asia (Thailand) Anderson et al. The Zika virus papers show the importance of surveillance and the broad spectrum of congenital disorders associated with this arbovirus, as is the case of arthrogryposis Contreras-Capetillo et al. and Lim et al. In addition to the epidemiological and clinical aspects of arboviruses, basic immunological aspects are also crucial in understanding these conditions, as shown in the article about T cell responses to Japanese encephalitis Pushpakumara et al. After the 2014 epidemics, Ebola persisted in countries such as the Democratic Republic of Congo, representing a significant burden of morbidity and mortality, as discussed in the article by Grimes et al. Myiasis, although forgotten by many, is still causing problems in different populations, including newborns, as discussed in the article by Ruiz-Zapata et al. The role of dogs in multiple zoonotic diseases still needs to be addressed regarding multiple pathogens, including *Echinococcus granulosus*, as presented by Khan et al., in their article. Coinfections represent a challenge in the context of tropical diseases related to other tropical diseases, but also other infectious diseases; this interesting aspect in the context of HIV is presented in the diagnosis of visceral leishmaniasis da Silva et al. In its vision of ecoepidemiology, the article of Krystosik

on the role of solid waste in breeding sites, burrows, and food for vectors and urban zoonotic reservoirs is fascinating Krystosik et al.

It seems clear that zoonotic and emerging infectious diseases must be confronted via a multifaceted approach, which includes integrating across disciplines (veterinary medicine, vector biology, immunology, epidemiology, among others) as well as across biological scales (molecules→pathogens→ecosystems). Among the best existing frameworks to improve integration in our concepts, health offers new collaborations and actions. We will undoubtedly be given additional opportunities to react to small outbreaks or new pandemic threats (32, 40–43). However, success in this field will be marked by an increasingly proactive and preemptive mode of operation—we need to know what is coming, have adequate tools and therapeutics poised for application and adaptation, and identify measures to prevent the emergence of novel pathogens (rather than the spread of emerging diseases)—particularly as a counter to myopic or destructive human activity, as these are a detrimental driver for the health of our species.

Many are hard at work, but more effort is required and the challenges are great. We want a world with equity, less disease, and more health, especially in those areas where the impact of these conditions has truncated the lives of millions. We hope readers will benefit from the insights of the experiences and findings in this Research Topic whilst also being motivated to take action in pulling these diseases, and those who suffer from them, out of oblivion.

AUTHOR CONTRIBUTIONS

All authors contributed to manuscript conception and design, literature review, manuscript preparation, critical review, and contributed to the article and approved the submitted version.

REFERENCES

1. Chen X, Kang Y, Luo J, Pang K, Xu X, Wu J, et al. Next-generation sequencing reveals the progression of COVID-19. *Front Cell Infect Microbiol.* (2021) 11:632490. doi: 10.3389/fcimb.2021.632490
2. Sehli S, Allali I, Chahboune R, Bakri Y, Al Idrissi N, Hamdi S, et al. Metagenomics approaches to investigate the gut microbiome of COVID-19 patients. *Bioinform Biol Insights.* (2021) 15:1177932221999428. doi: 10.1177/1177932221999428
3. Zhang X, Wu Z, Wang K. Diagnosis of *Streptococcus suis* Meningoencephalitis with metagenomic next-generation sequencing of the cerebrospinal fluid: a case report with literature review. *BMC Infect Dis.* (2020) 20:884. doi: 10.1186/s12879-020-05621-3
4. Callanan J, Stockdale SR, Shkoporov A, Draper LA, Ross RP, Hill C. Biases in viral metagenomics-based detection, cataloguing and quantification of bacteriophage genomes in human faeces, a review. *Microorganisms.* (2021) 9:524. doi: 10.3390/microorganisms9030524
5. Avila-Rios S, Parkin N, Swanstrom R, Paredes R, Shafer R, Ji H, et al. Next-generation sequencing for HIV drug resistance testing: laboratory, clinical, and implementation considerations. *Viruses.* (2020) 12:617. doi: 10.3390/v12060617
6. Martins Dos Santos VAP, Hardt C, Skrede S, Saccenti E. Systems and precision medicine in necrotizing soft tissue infections. *Adv Exp Med Biol.* (2020) 1294:187–207. doi: 10.1007/978-3-030-57616-5_12
7. Merker M, Tueffers L, Vallier M, Groth EE, Sonnenkalb L, Unterweger D, et al. Evolutionary approaches to combat antibiotic resistance: opportunities and challenges for precision medicine. *Front Immunol.* (2020) 11:1938. doi: 10.3389/fimmu.2020.01938
8. Rossi JF, Lu ZY, Massart C, Levon K. Dynamic immune/inflammation precision medicine: the good and the bad inflammation in infection and cancer. *Front Immunol.* (2021) 12:595722. doi: 10.3389/fimmu.2021.595722
9. Keij FM, Achten NB, Tramper-Stranders GA, Allegaert K, van Rossum AMC, Reiss IKM, et al. Stratified management for bacterial infections in late preterm and term neonates: current strategies and future opportunities toward precision medicine. *Front Pediatr.* (2021) 9:590969. doi: 10.3389/fped.2021.590969
10. Stocco G, Lucafo M, Decorti G. Pharmacogenomics of antibiotics. *Int J Mol Sci.* (2020) 21:5975. doi: 10.3390/ijms21175975
11. Takahashi T, Luzum JA, Nicol MR, Jacobson PA. Pharmacogenomics of COVID-19 therapies. *NPJ Genom Med.* (2020) 5:35. doi: 10.1038/s41525-020-00143-y
12. Al-Eitan LN, Alahmad SZ. Pharmacogenomics of genetic polymorphism within the genes responsible for SARS-CoV-2 susceptibility and the drug-metabolising genes used in treatment. *Rev Med Virol.* (2021) 31:e2194. doi: 10.1002/rmv.2194
13. Borba J, Silva AC, Lima MNN, Mendonca SS, Furnham N, Costa FTM, et al. Chemogenomics and bioinformatics approaches for prioritizing kinases as drug targets for neglected tropical diseases. *Adv Protein Chem Struct Biol.* (2021) 124:187–223. doi: 10.1016/bs.apcsb.2020.10.006
14. Dariolli R, Campana C, Gutierrez A, Sobie EA. *In vitro* and *in silico* models to study SARS-CoV-2 infection: integrating experimental and computational tools to mimic "COVID-19 Cardiomyocyte". *Front Physiol.* (2021) 12:624185. doi: 10.3389/fphys.2021.624185
15. Kinobe RT, Owens L. A systematic review of experimental evidence for antiviral effects of ivermectin and an *in silico* analysis of ivermectin's possible mode of action against SARS-CoV-2. *Fundam Clin Pharmacol.* (2021) 35:260–76. doi: 10.1111/fcp.12644
16. Mottaqi MS, Mohammadipanah F, Sajedi H. Contribution of machine learning approaches in response to SARS-CoV-2 infection. *Inform Med Unlocked.* (2021) 23:100526. doi: 10.1016/j.imu.2021.100526
17. Rasheed J, Jamil A, Hameed AA, Aftab U, Aftab J, Shah SA, et al. A survey on artificial intelligence approaches in supporting frontline workers and decision makers for the COVID-19 pandemic. *Chaos Solitons Fractals.* (2020) 141:110337. doi: 10.1016/j.chaos.2020.110337
18. Rosa V, Ho D, Sabino-Silva R, Siqueira WL, Silikas N. Fighting viruses with materials science: prospects for antivirus surfaces, drug delivery systems and artificial intelligence. *Dent Mater.* (2021) 37:496–507. doi: 10.1016/j.dental.2020.12.004
19. Alsharif MH, Alsharif YH, Albreem MA, Jahid A, Solyman AAA, Yahya K, et al. Application of machine intelligence technology in the detection of vaccines and medicines for SARS-CoV-2. *Eur Rev Med Pharmacol Sci.* (2020) 24:11977–81. doi: 10.26355/eurrev_202011_23860
20. Yoganandhan A, Rajesh Kanna G, Subhash SD, Hebinson Jothi J. Retrospective and prospective application of robots and artificial intelligence in global pandemic and epidemic diseases. *Vacunas.* (2021) 22:98–105. doi: 10.1016/j.vacun.2020.12.004
21. Rodriguez-Morales AJ, Paniz-Mondolfi AE, Faccini-Martínez ÁA, Henao-Martínez AF, Ruiz-Saenz J, Martinez-Gutierrez M, et al. The constant threat of zoonotic and vector-borne emerging tropical diseases: living on the edge. *Front Trop Dis.* (2021) 2:676905. doi: 10.3389/fitd.2021.676905
22. Karesh WB, Dobson A, Lloyd-Smith JO, Lubroth J, Dixon MA, Bennett M, et al. Ecology of zoonoses: natural and unnatural histories. *Lancet.* (2012) 380:1936–45. doi: 10.1016/S0140-6736(12)61678-X
23. Morel CM. Chagas disease, from discovery to control - and beyond: history, myths and lessons to take home. *Mem Inst Oswaldo Cruz.* (1999) 94(Suppl. 1):3–16. doi: 10.1590/S0074-02761999000700002
24. Von A, Zaragoza E, Jones D, Rodriguez-Morales AJ, Franco-Paredes C. New insights into Chagas disease: a neglected disease in Latin America. *J Infect Dev Ctries.* (2007) 1:99–111.

25. Franco-Paredes C, Von A, Hidron A, Rodriguez-Morales AJ, Tellez I, Barragan M, et al. Chagas disease: an impediment in achieving the Millennium Development Goals in Latin America. *BMC Int Health Hum Rights.* (2007) 7:7. doi: 10.1186/1472-698X-7-7
26. Franco-Paredes C, Rodriguez-Morales AJ. Unsolved matters in leprosy: a descriptive review and call for further research. *Ann Clin Microbiol Antimicrob.* (2016) 15:33. doi: 10.1186/s12941-016-0149-x
27. Franco-Paredes C, Marcos LA, Henao-Martinez AF, Rodriguez-Morales AJ, Villamil-Gomez WE, Gotuzzo E, et al. Cutaneous mycobacterial infections. *Clin Microbiol Rev.* (2018) 32:e00069-18. doi: 10.1128/CMR.00069-18
28. Hensler PG. Analysis of an important work, published a few years since in Denmark, and hitherto unknown in England, on the leprosy of the west in the middle ages, with a supplement relating to the history and knowledge of the disease. *Med Phys J.* (1803) 9:554–66.
29. Hess J, Boodram LG, Paz S, Stewart Ibarra AM, Wasserheit JN, Lowe R. Strengthening the global response to climate change and infectious disease threats. *BMJ.* (2020) 371:m3081. doi: 10.1136/bmj.m3081
30. Sands P, Mundaca-Shah C, Dzau VJ. The neglected dimension of global security–a framework for countering infectious-disease crises. *N Engl J Med.* (2016) 374:1281–7. doi: 10.1056/NEJMsr1600236
31. Zismer DK, Werner MJ. Managing the physics of the economics of integrated health care. *Physician Exec.* (2012) 38:38–45.
32. Emerging zoonoses: a one health challenge. *EClinicalMedicine.* (2020). 19:100300. doi: 10.1016/j.eclinm.2020.100300.
33. Martinez L. Global infectious disease surveillance. *Int J Infect Dis.* (2000) 4:222–8. doi: 10.1016/S1201-9712(00)90114-0
34. Freedman DO, Kozarsky PE, Weld LH, Cetron MS. GeoSentinel: the global emerging infections sentinel network of the International Society of Travel Medicine. *J Travel Med.* (1999) 6:94–8. doi: 10.1111/j.1708-8305.1999.tb00839.x
35. Gallego V, Berberian G, Lloveras S, Verbanaz S, Chaves TS, Orduna T, et al. The 2014. FIFA World Cup: communicable disease risks and advice for visitors to Brazil–a review from the Latin American Society for Travel Medicine (SLAMVI). *Travel Med Infect Dis.* (2014) 12:208–18. doi: 10.1016/j.tmaid.2014.04.004
36. Gallego V, Berberian G, Siu H, Verbanaz S, Rodriguez-Morales AJ, Gautret P, et al. The 2019 Pan American games: communicable disease risks and travel medicine advice for visitors to Peru - Recommendations from the Latin American Society for Travel Medicine (SLAMVI). *Travel Med Infect Dis.* (2019) 30:19–24. doi: 10.1016/j.tmaid.2019.06.011
37. Ball P. The lightning-fast quest for COVID vaccines - and what it means for other diseases. *Nature.* (2021) 589:16–8. doi: 10.1038/d41586-020-03626-1
38. Bobrowski T, Melo-Filho CC, Korn D, Alves VM, Popov KI, Auerbach S, et al. Learning from history: do not flatten the curve of antiviral research! *Drug Discov Today*. (2020). 25:1604–13. doi: 10.1016/j.drudis.2020.07.008
39. Menachery VD, Yount BL, Jr., Debbink K, Agnihothram S, Gralinski LE, et al. A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nat Med.* (2015) 21:1508–13. doi: 10.1038/nm.3985
40. Scott J. Proposed integrated control of zoonotic plasmodium knowlesi in southeast asia using themes of one health. *Trop Med Infect Dis.* (2020) 5:175. doi: 10.3390/tropicalmed5040175
41. Bonilla-Aldana DK, Holguin-Rivera Y, Perez-Vargas S, Trejos-Mendoza AE, Balbin-Ramon GJ, Dhama K, et al. Importance of the One Health approach to study the SARS-CoV-2 in Latin America. *One Health.* (2020) 10:100147. doi: 10.1016/j.onehlt.2020.100147
42. Rodriguez-Morales AJ, Patino-Cadavid LJ, Lozada-Riascos CO, Villamil-Gomez WE. Mapping Zika in municipalities of one coastal department of Colombia (Sucre) using geographic information systems during the 2015-2016 outbreak: implications for public health and travel advice. *Int J Infect Dis.* (2016) 48:70–2. doi: 10.1016/j.ijid.2016.05.012
43. Rodriguez-Morales AJ, Schlagenhauf P. Zoonoses and travel medicine: "one world–one health". *Travel Med Infect Dis.* (2014) 12:555–6. doi: 10.1016/j.tmaid.2014.11.003

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ORIGINAL RESEARCH
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Zika Virus Outbreak on Curaçao and Bonaire, a Report Based on Laboratory Diagnostics Data

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Background: Zika virus (ZIKV) emerged in May 2015 in Brazil, from which it spread to many other countries in Latin America. Cases of ZIKV infection were eventually also reported in Curaçao (January 2016) and Bonaire (February 2016).

Methods: In the period of 16 December 2015 until 26 April 2017, serum, EDTA-plasma or urine samples were taken at Medical Laboratory Services (MLS) from patients on Curaçao and tested in qRT-PCR at the Erasmus Medical Centre (EMC) in the Netherlands. Between 17 October 2016 until 26 April 2017 all samples of suspected ZIKV-patients collected on Curaçao, as well as on Bonaire, were tested at MLS. Paired urine and/or serum samples from patients were analyzed for ZIKV shedding kinetics, and compared in terms of sensitivity for ZIKV RNA detection. Furthermore, the age and gender of patients were used to determine ZIKV incidence rates, and their geozone location to determine the spatial distribution of ZIKV cases.

Results: In total, 781 patients of 2820 tested individuals were found qRT-PCR-positive for ZIKV on Curaçao. The first two ZIKV cases were diagnosed in December 2015. A total of 112 patients of 382 individuals tested qRT-PCR-positive for ZIKV on Bonaire. For both islands, the peak number of absolute cases occurred in November 2016, with 247 qRT-PCR confirmed cases on Curaçao and 66 qRT-PCR-positive cases on Bonaire. Overall, a higher proportion of women than men was diagnosed with ZIKV on both islands, as well as mostly individuals in the age category of 25–54 years old. Furthermore, ZIKV cases were mostly clustered in the east of the island, in Willemstad.

Conclusions: ZIKV cases confirmed by qRT-PCR indicate that the virus was circulating on Curaçao between at least December 2015 and March 2017, and on Bonaire between at least October 2016 and February 2017, with peak cases occurring in November 2016. The lack of preparedness of Curaçao for the ZIKV outbreak was compensated by shipping all samples to the EMC for diagnostic testing; however, both islands will need to put the right infrastructure in place to enable a rapid response to an outbreak of any new emergent virus in the future.

Keywords: Zika virus, outbreak, laboratory, qRT-PCR, epidemiology, Curaçao, Bonaire

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INTRODUCTION

Zika virus (ZIKV) is an arbovirus that belongs to the *Flaviviridae* family, genus Flavivirus, and is transmitted through the bite of infected *Aedes aegypti* mosquitoes, via sexual contact (1–3), or from mother to fetus (4). ZIKV infection is often asymptomatic or otherwise presents with mild symptoms such as fever, macopapular rash, conjunctivitis, myalgia, and headache (5). In a small number of cases, ZIKV infection can result in serious complications such as Guillain-Barré syndrome (6–10), maculopathy (11–13), or microcephaly in newborns when the mother is infected with the virus during pregnancy (14–18).

Historically, since its discovery in Uganda in 1947, ZIKV was confined to Africa resulting only in sporadic cases of mild disease. In 2007, however, this pattern changed when the first major outbreak of ZIKV occurred in Yap (Federal States of Micronesia) where ∼73% of the population was infected and symptomatic disease developed in ∼18% of infected persons (19). Since then, ZIKV spread rapidly across the Pacific Ocean, causing outbreaks in French Polynesia (20), Cook Islands (20), Easter Island (21), New Caledonia (22), until eventually emerging in the Americas (23). Here it was first reported in Brazil in continental South America in May 2015, after which the virus spread to other Latin American countries, such as Colombia (October 2015), Surinam, El Salvador, Mexico, Guatemala, Paraguay, Venezuela (November 2015), Panama, Honduras, French Guiana, Martinique, Puerto Rico (December 2015), Maldives, Guyana, Ecuador, Barbados, Bolivia, Haiti, Saint Martin, Dominican Republic, Nicaragua, Jamaica, Curaçao, Costa Rica (January 2016), Bonaire and Aruba (February 2016) (24, 25). In Brazil alone, it has been estimated that between 440,000 and 1.3 million persons were infected with ZIKV in 2015 (26), and around 2366 cases of ZIKV-associated microcephaly/CNS malformations have been reported (as of February 2017, www.statista.com). Since then, the epidemic continued to spread, and the total number of infected persons and children with congenital ZIKV syndrome still remains to be determined.

Curaçao, a nation of almost 150,000 inhabitants, is known for its circulation of *A. aegypti* transmitted viruses, such as dengue virus (DENV) and chikungunya virus (CHIKV). DENV has been endemic on Curaçao for decades and outbreaks of the virus occur here every few years. The most recent outbreak of dengue occurred in 2014, where Curaçao health authorities had reported 194 suspected and 20 confirmed cases of dengue at the end of August (27). In June of the same year, the first case of CHIKV was also reported, which was the start of a major outbreak on the island that lasted until February 2015. By the end of November 2014, 1,838 suspected and 835 confirmed cases of CHIKV had been reported (28). Dengue is also endemic on Bonaire, a nation with almost 19,000 inhabitants, but not many reports are available.

Due to the high degree of serological cross-reactivity between flaviviruses, confirmation of infection poses a challenge. As IgM is thought to be more specific than IgG, detection of IgM against ZIKV by ELISA represents a possibility; however, cross-reactivity of DENV and ZIKV IgM has been demonstrated (29). This means that confirmative neutralization assays would still be required. As a result, confirmation of flavivirus infections is mostly based on detection of viral RNA in serum by using quantitative real-time PCR (qRT-PCR). However, for several arboviruses such as DENV or ZIKV, the level of viremia present in the blood during the symptomatic phase is often very low, which makes detection problematic. The use of urine as an alternative matrix for detecting ZIKV RNA was investigated by several laboratories and was found to be a good alternative to serum, EDTA-plasma and saliva, due to the higher levels of RNA found, and the longer period of time that urine was found positive after the onset of symptoms (>10–20 days) (30, 31). In contrast, another study demonstrated detection of ZIKV in whole blood for a longer period of time compared to urine and serum (32). Based on these observations, official World Health Organization (WHO) interim recommendations included using either whole blood, serum, or urine for nucleic acid testing (NAT), and serum for IgM detection (33). The routine confirmation of serological results by virus neutralization assays was not recommended as it was considered unfeasible.

To define the scope of the ZIKV outbreak on Curaçao and Bonaire, we determined the number of confirmed ZIKV cases based on qRT-PCR diagnostics, the incidence rates of infection in patients in terms of age and gender, as well as the geospatial distribution of ZIKV cases on Curaçao. In addition, paired urine samples from Curaçao were assessed for ZIKV shedding kinetics, while paired urine and serum samples from Bonaire were compared for sensitivity of ZIKV RNA detection.

METHODS

In the period of 16 December 2015 until 26 April 2017, serum, EDTA-plasma or urine samples were taken at Medical Laboratory Services (MLS) from patients on Curaçao presenting with symptoms resembling ZIKV infection, such as fever, rash, headache or conjunctivitis. Between 16 December 2015 and 15 October 2016, the collected samples were inactivated and stabilized in MagnaPure lysis buffer (Roche Diagnostics, Almere, the Netherlands) and shipped to the diagnostic laboratory of the Erasmus Medical Centre (EMC) in Rotterdam, the Netherlands, where ZIKV RNA was tested by an ISO15189:2012 validated, internally controlled lab-developed real-time semi-quantitative qRT-PCR. In short, total nucleic acids were isolated using an external lysis protocol on the MagNA Pure LC robotics system (Roche Diagnostics) and subsequently tested in two independent qRT-PCRs using TaqMan® 1-Step Fast-Virus Master Mix (Thermo Fisher Scientific, Bleiswijk, the Netherlands) and primers targeting the envelope and the NS2A,

Abbreviations: ZIKV, Zika virus; DENV, dengue virus; CHIKV, chikungunya virus; IgM, immunoglobulin M; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; qRT-PCR, quantitative real-time polymerase chain reaction; RNA, ribonucleic acid; EDTA, ethylenediaminetetraacetic acid; WHO, World Health Organization; NAT, nucleic acid testing; MLS, Medical Laboratory Services; EMC, Erasmus Medical Center; NS2A, non-structural protein 2A; PDV, phocine distemper virus; C_T, cycle threshold; FTD, Fast Track Diagnostics; ADC, Analytisch Diagnostisch Centrum; HIV, human immunodeficiency virus.

TABLE 1 | Lab-developed qRT-PCR primers and probe used for diagnostics of ZIKV.

Name	Sequence (5′-3′)	Conc. (nM)	Target	PCR product (bp)	References
ZIKV_1086	CCGCTGCCCAACACAAG	600	Envelope	77	(29)
ZIKV_1107	FAM-AGCCTACCTTGACAAGCAGTCAGACACTCAA-BHQ1	100			
ZIKV_1162c	CCACTAACGTTCTTTTGCAGACAT	600			
Zika2_fwd	CTTGGAGTGCTTGTGATT	600	NS2A	187	(34)
Zika2_ probe	FAM-AGAAGAGAATGACCACAAAGATCA-BHQ1	100			
Zika2_rev	CTCCTCCAGTGTTCATTT	600			
PDV fwd	CGGGTGCCTTTTACAAGAAC	600	Heamagglutinine	78	(35)
PDV probe	Cy5-ATGCAAGGGCCAATT-MGB	200			
PDV rev	TTCTTTCCTCAACCTCGTCC	150			

PDV, phocine distemper virus (internal control); BHQ, black hole quencher.

TABLE 2 | Number of samples collected and tested, and the number of patients tested in qRT-PCR during the ZIKV outbreak on Curaçao and Bonaire.

	Curaçao	Bonaire
No. of samples collected	3,833	744
No. of patients	2,820	382
No. of samples tested in qRT-PCR	2,044	599
No. of patients tested in qRT-PCR	1,685	358
No. of qRT-PCR positive samples	815	129
No. of qRT-PCR positive patients	781	112
No. of patients submitting paired samples for qRT-PCR	324	–
No. of patients testing qRT-PCR positive for first sample	70	–
No. of patients testing qRT-PCR positive for second sample	32	–
No. of patients submitting paired urine and serum samples	–	262
No. of patients submitting paired urine and serum samples for qRT-PCR	–	183
No. of patients testing qRT-PCR positive for urine only	–	18
No. of patients testing qRT-PCR positive for serum only	–	17
No. of patients testing qRT-PCR positive for both urine and serum	–	13
No. of patients testing qRT-PCR negative for both urine and serum	–	135

in multiplex with an internal control (PDV), in a LC480-II cycler (Roche Life Science) (**Table 1**). The cut-off was set at <45 C_T values. Starting from 6 July 2016, only the primer pair targeting the envelope was used in the qRT-PCR for confirmation of ZIKV infection.

As the etiology of the clinical manifestations of patients was still uncertain during the first 2 months of the outbreak (December 2015 and January 2016), serum samples collected from patients were also tested for DENV and CHIKV RNA using FTD Dengue/Chik multiplex (Fast Track Diagnostics, Esch-sur-Alzette, Luxembourg). Between December 2015 and October 2016 paired urine samples (plasma if urine was not available) with a target interval of ∼2 weeks were submitted for testing. Starting from February 2016, either urine (matrix of choice) or EDTA-plasma samples (if urine was not available) were collected from patients.

In the period of 17 October 2016 until 26 April 2017, after the implementation of commercial ZIKV diagnostic assays at MLS, all samples of suspected ZIKV patients on Curaçao were collected and tested at MLS. In this period, samples were also collected from ZIKV-suspected patients on Bonaire by Fundashon Mariadal and sent to MLS for testing. In contrast to Curaçao, here it was chosen to collect paired serum and urine samples on the same day, from a large number of patients. The ZIKV diagnostic tests consisted of qRT-PCR and/or IgM/IgG ELISA (Euroimmun, Lübeck, Germany). For qRT-PCR, total nucleic acids were isolated using the MagNA Pure robotics system (Roche Diagnostis) and tested in a qRT-PCR using FTD Zika virus multiplex (Fast Track Diagnostics). Depending on the number of days after the onset of symptoms at which the patient was submitted for testing, the choice was made for either qRT-PCR alone (0–7 days), qRT-PCR and serology (7–14 days), or serology alone ($\geq$14 days). However, as neither an ELISA-positive IgM or IgG result for ZIKV in a DENV-endemic area can be considered reliable due to the cross-reactivity known to exist between DENV and ZIKV antibodies (36, 37), we only considered positive results obtained in the qRT-PCR for the analyses.

In the period of 17 October 2016 up to 8 November 2016, plasma samples were tested, but from 9 November 2016 onwards, serum was chosen over EDTA-plasma due to its superior practicality and durability in the lab, and recommendations made by the World Health Organization (33). Urine was no longer the matrix of choice as serum could be used in both qRT-PCR and ELISA.

Curaçao can be divided into 65 geozones, which consist of one or more neighborhoods. The patients' geozone of residence was used as a proxy for location and plotted on a map of Curaçao using www.mapcustomizer.com. The ZIKV incidence rates were determined for different age categories and the gender of patients.

Information such as presenting symptoms, day of onset, and pregnancy was not properly documented by the general practitioners on either Curaçao or Bonaire, and as a result, this data could not be included in the analyses. Written consent was provided by each individual submitting a urine, serum or plasma sample for testing, and written consent for children under 16 years of age was provided by their parent or guardian. As samples of patients were only collected for diagnostic purposes, no additional ethical clearance was required for this study.

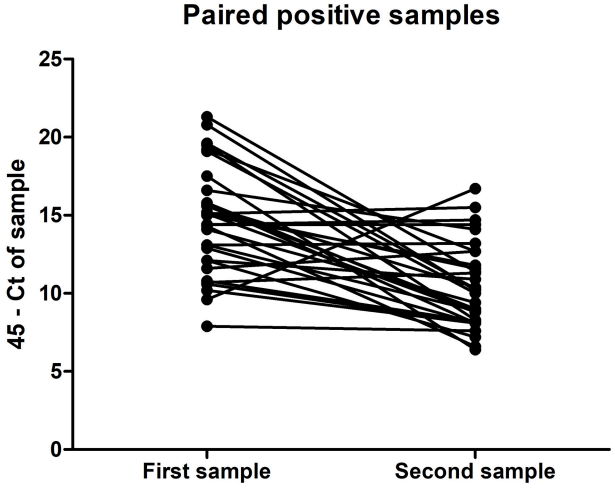

FIGURE 1 | Amount of ZIKV RNA detected in the first and second ZIKV-positive urine samples of the paired samples submitted for testing by 32 individuals, expressed in terms of C_T threshold 45 minus the C_T determined for the sample.

STATISTICAL ANALYSES

Paired samples were analyzed using a two-tailed paired *t*-test and *P*-values equal to or less than 0.5 were considered to be statistically significant.

RESULTS

Between 16 December 2015 and 26 April 2017, 3,833 samples of 2,820 individuals were collected by MLS on Curaçao. Of these, 2,044 samples belonging to 1,685 patients were tested in qRT-PCR, resulting in 815 qRT-PCR positive samples, consisting of 781 positive ZIKV patients (**Table 2**). Testing of serum samples of ZIKV-suspected patients on Curaçao was first initiated in December 2015, during which two patients tested positive for ZIKV using qRT-PCR. During the first 2 months of the outbreak (December 2015 and January 2016), when serum samples were also tested in DENV and CHIKV qRT-PCRs, four out of 87

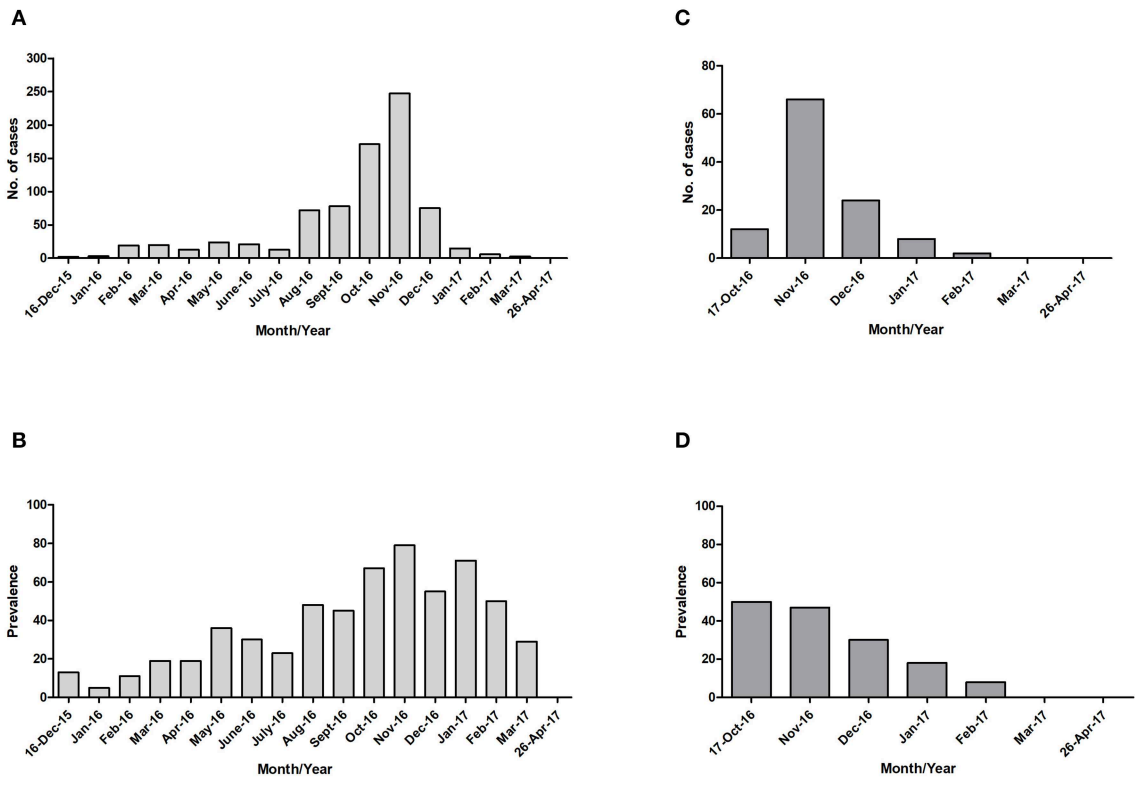

FIGURE 2 | Absolute number of cases and prevalence of ZIKV-positive patients confirmed by qRT-PCR on Curaçao from mid-December 2015 to late April 2017 **(A,B)**, and on Bonaire from mid-October 2016 to late April 2017 **(C,D)**.

TABLE 3 | Prevalence per month of qRT-PCR-confirmed ZIKV-positive patients on Curaçao and Bonaire during the outbreak.

Month	No. of patients tested	No. of positive patients	Prevalence (%)	No. of patients tested	No. of positive patients	Prevalence (%)
	Curaçao			Bonaire		
16-Dec-15	15	2	13			
Jan-16	66	3	5			
Feb-16	176	19	11			
Mar-16	104	20	19			
Apr-16	68	13	19			
May-16	66	24	36			
June-16	69	21	30			
July-16	56	13	23			
Aug-16	151	72	48			
Sept-16	172	78	45			
Oct-16*	254	171	67	24	12	50
Nov-16	311	247	79	141	66	47
Dec-16	136	75	55	79	24	30
Jan-17	21	15	71	45	8	18
Feb-17	12	6	50	26	2	8
Mar-17	7	2	29	31	0	0
26-Apr-17	1	0	0	12	0	0

**For Bonaire samples were collected starting from 17-Oct-16.*

ZIKV-suspected disease cases were confirmed as positive for DENV instead (C_T 26.6, 14.5, 29.5, and 34.4).

Of the 324 patients that submitted paired urine samples between December 2015 and October 2016, 70 patients tested positive for their first sample, while only 32 people still tested qRT-PCR-positive for their second sample (**Table 2**), taken between 11 and 17 days after the initial sample. This indicates that for some patients in this cohort, ZIKV RNA was still detectable in urine for up to 17 days. Furthermore, there was a significant trend in the decrease in the amount of virus shed in the urine over this time period ($P < 0.0001$, paired t-test) (**Figure 1**). No significant differences were found in the amount of virus shed in the urine between men and women (data not shown).

During the period of 17 October 2016 until 26 April 2017, a total of 744 samples were also collected from 382 individuals on Bonaire and tested at MLS Curaçao. Of these, 599 samples belonging to 358 patients were tested by qRT-PCR. A total of 129 samples consisting of 112 patients tested qRT-PCR positive for ZIKV. Of the 262 patients that had both a serum and urine sample taken on the same day, 183 had both samples concomitantly tested in qRT-PCR. Of these, 13 patients were positive for both serum and urine, while 17 patients tested positive for only serum, and 18 for only urine. One hundred 35 patients tested negative for both (**Table 2**).

For both islands, the peak number of absolute cases occurred in November 2016, with 247 qRT-PCR confirmed cases on Curaçao (**Figure 2A**) and 66 qRT-PCR-positive cases on Bonaire (**Figure 2C**; **Table 3**). In terms of prevalence, for Curaçao, the peak (79%) also occurred in November 2016 (**Figure 2B**), whereas for Bonaire the peak prevalence (50%) was in October 2016 (**Figure 2D**; **Table 3**). Overall, a higher proportion of women than men was diagnosed (∼73%) on both Curaçao and Bonaire (**Table 4**), with incidence rates of 737 and 875 per 100,000, respectively. Furthermore, ZIKV was diagnosed mostly in individuals in the age category of 25–54 years old on both Curaçao (61%; incidence rate of 863 per 100,000) and Bonaire (67%; incidence rate of 815 per 100,000) (**Table 4**).

TABLE 4 | Characteristics of the 781 patients confirmed by qRT-PCR for ZIKV infection on Curaçao between 16 December 2015 till 26 April 2017, and of the 112 patients confirmed on Bonaire between 17 October 2016 till 26 April 2017, according to sex and age [with use of population demographics data from July 2017 (www.indexmundi.com)].

Characteristics	*N*	%	Population (*N*)	Incidence per 100,000 population
Curaçao				
Total population	149,648			
ZIKV positive	781			
Sex				
Female	574	73.5	77,920	737
Male	207	26.5	71,728	289
Age group				
0-14	70	9.0	29,935	234
15-24	67	8.6	21,450	312
25-54	476	60.9	55,181	863
55-64	106	13.6	20,482	518
65+	62	7.9	22,600	274
Bonaire				
Total population	19,179			
ZIKV positive	112			
Sex				
Female	81	72.3	9261	875
Male	31	27.7	9918	313
Age group				
0-14	6	5.4	3,359	179
15-24	15	13.4	2,105	713
25-54	75	67.0	9,198	815
55-64	11	9.8	2,552	431
65+	5	4.5	2,194	228

To determine the distribution of ZIKV infections on Curaçao, the locations of patients that tested positive for ZIKV by qRT-PCR were plotted on a map of Curaçao. Locations of 197 patients could not be pinpointed on the map. The map shows that the majority of the ZIKV cases were clustered in the eastern part of the island, particularly in Willemstad (**Figure 3**). Geozones with a notable number of infections included Santa Rosa, Spaanse Water, St. Michiel, Dominguito, Brievengat, Berg Altena, Tera Cora, Stenen Koraal, and Groot Piscadera.

DISCUSSION

Despite the documented emergence of ZIKV into the Americas in Brazil in May 2015, phylogenetic analyses estimate the

FIGURE 3 | The locations of a selection of the patients on Curaçao that tested positive for ZIKV by qRT-PCR. The map was created by plotting the locations on www.mapcustomizer.com.

introduction of the virus to be earlier, either between August 2013 and July 2014 (38) or between May and December 2013 (39). On Curaçao, according to our analyses, the first cases of ZIKV were diagnosed in December 2015, a month before the first notification to the WHO on 28 January 2016 (24), which indicates that the virus, most likely introduced by travelers, emerged earlier than officially reported. Given the rapid spread of the virus throughout the Americas after its emergence in Brazil, Curaçao, and Bonaire were not prepared for an outbreak of ZIKV, and diagnostic assays had therefore not yet been implemented and validated at MLS. This problem was circumvented by shipping patient samples to the diagnostic laboratory of the EMC in the Netherlands, a WHO Collaborating Centre for arboviruses. Starting from October 2016, MLS Curaçao had implemented the necessary commercial diagnostic qRT-PCR assay and ELISAs in order to continue the diagnosis of ZIKV-suspected patients on Curaçao and start with the diagnostics for Bonaire. Of note, this study was not designed prospectively but performed in reaction to a dynamic outbreak situation.

During this period, a switch was also made from urine to serum for samples collected on Curaçao. Even though a few studies have shown that urine was more sensitive for detection of ZIKV by qRT-PCR compared to serum (30, 31), the data from the paired serum and urine samples from Bonaire suggest that in this cohort, these two matrices were required concomitantly to increase the chance of ZIKV detection. As a result, it is possible that many ZIKV cases on both Curaçao and Bonaire were missed as here, paired urine and serum samples were not consistently collected and/or tested in qRT-PCR. Even though many PCR-negative samples from Curaçao and Bonaire had also been tested in IgM/IgG ELISA, the cross-reactivity known to occur between ZIKV and DENV antibodies makes diagnosis based on serology difficult (36, 37) and could easily lead to false positives. As a result, serology data of samples from patients collected 14 days after onset of symptoms were not included in our analyses, and our results are therefore very likely an underrepresentation of the number of ZIKV cases on both islands. Another factor that may have led to an underrepresentation of the total number of cases is the fact that not all individuals that experienced symptoms went to the general practitioner to get tested. Furthermore, on Curaçao, three laboratories were involved in the diagnostic testing of ZIKV patients, namely MLS, Analytisch Diagnostisch Centrum (ADC) and Laboratorio de Medicos (LabdeMed). If all the data were to be combined, the total number of ZIKV cases would likely be much larger than presented in this article.

The peak of the ZIKV outbreak on Curaçao appeared to occur in November 2016, both in terms of the absolute number of cases and prevalence. For Bonaire, the peak in the absolute number of

cases seemed to occur in November 2016 as well, while in terms of prevalence it appeared to occur in October 2016. However, as no ZIKV diagnostics was carried out for Bonaire between mid-December 2015 and mid-October 2016, the data from October is not reliable for comparison with the other months, and it can also not be excluded that a larger number of people on Bonaire may have been infected in one of the months preceding November.

Interestingly, during the reported outbreak of ZIKV on Curaçao and Bonaire, no cases of microcephaly or fatalities due to ZIKV were reported. However, assuming a similar microcephaly risk of 0.02% for pregnant women as calculated for Brazil (40), and a fertility rate of ∼2.1 for Curaçao [based on data from 2011 (41)], which is equivalent to ∼2,100 live births per year, this would have given 0.42 cases of microcephaly during the outbreak on Curaçao (which lasted approximately a year). It is therefore not surprising that no cases of ZIKV-related microcephaly were observed in a population of only 150,000 and 19,000 people.

During the outbreak of ZIKV on both Curaçao and Bonaire, almost three times more women than men were infected with the virus. Infections occurred mostly in the age category of 25–54 years old for both men and women. This higher proportion of female infections during a ZIKV outbreak was also reported in Surinam (42) and Rio de Janeiro in Brazil (43). This disproportionate infection rate may be explained by the increased testing of pregnant women due to the concerns about microcephaly and other risks for the unborn babies. However, such a trend was also demonstrated in Rio de Janeiro during a DENV outbreak (43), where women were 30% more likely to be diagnosed with DENV than men. One explanation suggested by this study was that women are more conscientious about their health and therefore more likely to visit a general practitioner. Nonetheless, another possibility, as also speculated upon in the Coelho study (43), is that for ZIKV, a higher amount of male-to-female sexual transmissions occur in comparison to female-to-male transmissions. Infection of females by ZIKV via semen has already been demonstrated (1–3), and even though ZIKV has also been detected in the female genital tract and vaginal secretions (44–47), the ability of the virus to productively infect males via vaginal secretions during sexual intercourse has not yet been demonstrated. Furthermore, the influence of female reproductive hormones on ZIKV replication and transmission should also be investigated, as progestins have recently been shown to promote infection of HIV within the female reproductive tract of non-human primates (48).

In order to obtain an impression of the distribution of the number of ZIKV cases on Curaçao, the locations of the patients were plotted on a map. The majority of the cases were located in the east of the island, which may be the result of a reporting bias caused by a higher population density in the east (Willemstad) (41). Nonetheless, for geozones that contained the largest amount of ZIKV cases, no particular trend in terms of population density or average gross monthly income per household was identified (data not shown). It is possible that the geospatial distribution of ZIKV cases is a reflection of the presence of ZIKV-infected mosquitoes; however, as many inhabitants of Curaçao travel to different parts of the island on a daily basis, it is not possible to determine with certainty the location of transmission. Besides mosquito transmission, sexual transmission of ZIKV may also have influenced the geospatial distribution of cases on the island.

CONCLUSIONS

As Curaçao and Bonaire are (potential) hot-spots for emerging and re-emerging arbovirus infections, it is important that the islands are prepared for future outbreaks by implementing the appropriate diagnostic tools in advance. However, in addition to effective diagnostics, it is imperative that the right infrastructure is also put in place to allow communication during an outbreak setting and to facilitate the implementation of risk-reduction activities in order to deal with any infectious disease that may emerge in the future.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are not publicly available due to patient privacy rights but a selection of datasets are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Written consent was obtained from each individual that provided urine, serum or plasma samples. Consent for children under 16 years of age was provided by their parent or guardian. As MLS and the department of Viroscience are mandated to provide laboratory support for outbreak investigations, no additional ethical clearance was sought out.

AUTHOR CONTRIBUTIONS

RW, SP, and GB coordinated and supervised the laboratory diagnostics and logistics. SL, SP, and GB were involved with the analyses. SL, SP, MK, and BM wrote the manuscript.

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REFERENCES

1. Foy BD, Kobylinski KC, Chilson Foy JL, Blitvich BJ, Travassos da Rosa A, Haddow AD, et al. Probable non-vector-borne transmission of Zika virus, Colorado, USA. *Emerg Infect Dis*. (2011) 17:880–2. doi: 10.3201/eid1705.101939
2. Hills SL, Russell K, Hennessey M, Williams C, Oster AM, Fischer M, et al. Transmission of Zika virus through sexual contact with travelers to areas of ongoing transmission - continental United States, 2016. *MMWR Morb Mortal Wkly Rep*. (2016) 65:215–6. doi: 10.15585/mmwr.mm6508e2
3. D'Ortenzio E, Matheron S, Yazdanpanah Y, de Lamballerie X, Hubert B, Piorkowski G, et al. Evidence of sexual transmission of Zika virus. *N Engl J Med*. (2016) 374:2195–8. doi: 10.1056/NEJMc1604449
4. Calvet G, Aguiar RS, Melo ASO, Sampaio SA, de Filippis I, Fabri A, et al. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. *Lancet Infect Dis*. (2016) 16:653–60. doi: 10.1016/S1473-3099(16)00095-5
5. Galán-Huerta KA, Rivas-Estillaa AM, Martinez-Landerosb EA, Arellanos-Sotoab D, Ramos-Jiménez J. The Zika virus disease: an overview. *Med Univ*. (2016) 18:115–24. doi: 10.1016/j.rmu.2016.05.003
6. Oehler E, Watrin L, Larre P, Leparc-Goffart I, Lastere S, Valour F, et al. Zika virus infection complicated by Guillain-Barre syndrome–case report, French Polynesia, December 2013. *Euro Surveill*. (2014) 19:20720. doi: 10.2807/1560-7917.ES2014.19.9.20720
7. Cao-Lormeau VM, Blake A, Mons S, Lastère S, Roche C, Vanhomwegen J, et al. Guillain-Barre syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. *Lancet*. (2016) 387:1531–9. doi: 10.1016/S0140-6736(16)00562-6
8. Roze B, Najioullah F, Fergé JL, Dorléans F, Apetse K, Barnay JL, et al. Guillain-barre syndrome associated with Zika virus infection in Martinique in 2016: a prospective study. *Clin Infect Dis*. (2017) 65:1462–8. doi: 10.1093/cid/cix588
9. Nascimento OJM, da Silva IRF. Guillain-Barre syndrome and Zika virus outbreaks. *Curr Opin Neurol*. (2017) 30:500–7. doi: 10.1097/WCO.0000000000000471
10. Araujo LM, Ferreira ML, Nascimento OJ. Guillain-Barre syndrome associated with the Zika virus outbreak in Brazil. *Arq Neuropsiquiatr*. (2016) 74:253–5. doi: 10.1590/0004-282X20160035
11. Yepez JB, Murati FA, Pettito M, Peñaranda CF, de Yepez J, Maestre G, et al. Ophthalmic manifestations of congenital Zika syndrome in colombia and venezuela. *JAMA Ophthalmol*. (2017) 135:440–5. doi: 10.1001/jamaophthalmol.2017.0561
12. Kodati S, Palmore TN, Spellman FA, Cunningham D, Weistrop B, Sen HN. Bilateral posterior uveitis associated with Zika virus infection. *Lancet*. (2017) 389:125–6. doi: 10.1016/S0140-6736(16)32518-1
13. Wong CW, Ng SR, Cheung CMG, Wong TY, Mathur R. Zika-related maculopathy. *Retin Cases Brief Rep*. (2019) 13:171–3. doi: 10.1097/ICB.0000000000000552
14. Miranda-Filho DDB, Martelli CM, Ximenes RA, Araújo TV, Rocha MA, Ramos RC, et al. Initial description of the presumed congenital Zika syndrome. *Am J Public Health*. (2016) 106:598–600. doi: 10.2105/AJPH.2016.303115
15. de Araujo TVB, Rodrigues LC, de Alencar Ximenes RA, de Barros Miranda-Filho D, Montarroyos UR, de Melo APL, et al. Association between Zika virus infection and microcephaly in Brazil, January to May, 2016: preliminary report of a case-control study. *Lancet Infect Dis*. (2016) 16:1356–63. doi: 10.1016/S1473-3099(16)30318-8
16. Rubin EJ, Greene MF, Baden LR. Zika Virus and microcephaly. *N Engl J Med*. (2016) 374:984–5. doi: 10.1056/NEJMe1601862
17. Honein MA, Dawson AL, Petersen EE, Jones AM, Lee EH, Yazdy MM, et al. Birth defects among fetuses and infants of US women with evidence of possible Zika virus infection during pregnancy. *JAMA*. (2017) 317:59–68. doi: 10.1001/jama.2016.19006
18. Brasil P, Pereira JP Jr, Moreira ME, Ribeiro Nogueira RM, Damasceno L, Wakimoto M, et al. Zika virus infection in pregnant women in Rio de Janeiro. *N Engl J Med*. (2016) 375:2321–34. doi: 10.1056/NEJMoa1602412
19. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. *N Engl J Med*. (2009) 360:2536–43. doi: 10.1056/NEJMoa0805715
20. Roth A, Mercier A, Lepers C, Hoy D, Duituturaga S, Benyon E, et al. Concurrent outbreaks of dengue, chikungunya and Zika virus infections - an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. *Euro Surveill*. (2014) 19:20929. doi: 10.2807/1560-7917.ES2014.19.41.20929
21. Tognarelli J, Ulloa S, Villagra E, Lagos J, Aguayo C, Fasce R, et al. A report on the outbreak of Zika virus on Easter Island, South Pacific, 2014. *Arch Virol*. (2016) 161:665–8. doi: 10.1007/s00705-015-2695-5
22. Dupont-Rouzeyrol M, O'Connor O, Calvez E, Daurès M, John M, Grangeon JP, et al. Co-infection with Zika and dengue viruses in 2 patients, New Caledonia, 2014. *Emerg Infect Dis*. (2015) 21:381–2. doi: 10.3201/eid2102.141553
23. Fauci AS, Morens DM. Zika virus in the Americas–yet another arbovirus threat. *N Engl J Med*. (2016) 374:601–4. doi: 10.1056/NEJMp1600297
24. Kindhauser MK, Allen T, Frank V, Santhana RS, Dye C. Zika: the origin and spread of a mosquito-borne virus. *Bull World Health Organ. (*2016) 94:675–86C. doi: 10.2471/BLT.16.171082
25. Pan American Health Organization/World Health Organization. *Timeline of the emergence of Zika virus in the Americas*. Washington, DC: Pan American Health Organization/World Health Organization (2016).
26. Ministério da Saúde. *Protocolo de vigilância e resposta à ocorrência de microcefalia relacionada à infecção pelo vírus Zika*. Brasília: Departamento de Vigilância das Doenças Transmissíveis, Núcleo de Comunicação/SVS (2015).
27. Pan American Health Organization/World Health Organization. *PLISA: Health Information Platform for the Americas - Reported Cases of Dengue Fever in the Americas*. Pan American Health Organization/World Health Organization. Available at: http://www.paho.org/data/index.php/en/mnu-topics/indicadores-dengue-en/dengue-nacional-en/252-dengue-pais-ano-en.html (accessed July 19, 2018).
28. Pan American Health Organization/World Health Organization. *Number of Reported Cases of Chikungunya Fever in the Americas, by Country or Territory 2013-2014, Cumulative Cases, EW 49*. (2014). Available at: https://www.paho.org/hq/dmdocuments/2014/2014-dec-05-cha-chikungunya-cases-ew-49.pdf (accessed November 14, 2018).
29. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis*. (2008) 14:1232–9. doi: 10.3201/eid1408.080287
30. Gourinat AC, O'Connor O, Calvez E, Goarant C, Dupont-Rouzeyrol M. Detection of Zika virus in urine. *Emerg Infect Dis*. (2015) 21:84–6. doi: 10.3201/eid2101.140894
31. Lamb LE, Bartolone SN, Kutluay SB, Robledo D, Porras A, Plata M, et al. Advantage of urine based molecular diagnosis of Zika virus. *Int Urol Nephrol*. (2016) 48:1961–6. doi: 10.1007/s11255-016-1406-9
32. Lustig Y, Mendelson E, Paran N, Melamed S, Schwartz E. Detection of Zika virus RNA in whole blood of imported Zika virus disease cases up to 2 months after symptom onset, Israel, December 2015 to April 2016. *Euro Surveill*. (2016) 21:30269. doi: 10.2807/1560-7917.ES.2016.21.26.30269
33. World Health Organization. *Laboratory testing for Zika virus infection, WHO/ZIKV/LAB/16.1* (2016).
34. Ferreira-de-Brito A, Ribeiro IP, Miranda RM, Fernandes RS, Campos SS, Silva KA, et al. First detection of natural infection of *Aedes aegypti* with Zika virus in Brazil and throughout South America. *Mem Inst Oswaldo Cruz*. (2016) 111:655–8. doi: 10.1590/0074-02760160332
35. Hoek RA, Paats MS, Pas SD, Bakker M, Hoogsteden HC, Boucher CA, et al. Incidence of viral respiratory pathogens causing exacerbations in adult cystic fibrosis patients. *Scand J Infect Dis*. (2013) 45:65–9. doi: 10.3109/00365548.2012.708942
36. Priyamvada L, Quicke KM, Hudson WH, Onlamoon N, Sewatanon J, Edupuganti S, et al. Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus. *Proc Natl Acad Sci USA*. (2016) 113:7852–7. doi: 10.1073/pnas.1607931113
37. Felix AC, Souza NCS, Figueiredo WM, Costa AA, Inenami M, da Silva RMG, et al. *Cross reactivity of commercia*l anti-dengue immunoassays in patients with acute Zika virus infection. *J Med Virol*. (2017) 89:1477–9. doi: 10.1002/jmv.24789

38. Metsky HC, Matranga CB, Wohl S, Schaffner SF, Freije CA, Winnicki SM, et al. Zika virus evolution and spread in the Americas. *Nature*. (2017) 546:411–5. doi: 10.1038/nature22402
39. Faria NR, Azevedo RDSDS, Kraemer MUG, Souza R, Cunha MS, Hill SC, et al. Zika virus in the Americas: early epidemiological and genetic findings. *Science*. (2016) 352:345–9. doi: 10.1126/science.aaf5036
40. Jaenisch T, Rosenberger KD, Brito C, Brady O, Brasil P, Marques ETA. Risk of microcephaly after Zika virus infection in Brazil, 2015 to 2016. *Bull World Health Organ*. (2017) 95:191–8. doi: 10.2471/BLT.16.178608
41. Ter Bals M. *Demography of Curaçao - Publication Series Census 2011*. Willemstad: Central Bureau of Statistics (2014).
42. Codrington J, Roosblad J, Baidjoe A, Holband N, Adde A, Kazanji M, et al. Zika virus outbreak in Suriname, a report based on laboratory surveillance data. *PLoS Curr*. (2018) 10. doi: 10.1371/currents.outbreaks.ff0f6190d5431c2a2e824255eaeaf339
43. Coelho FC, Durovni B, Saraceni V, Lemos C, Codeco CT, Camargo S, et al. Higher incidence of Zika in adult women than adult men in Rio de Janeiro suggests a significant contribution of sexual transmission from men to women. *Int J Infect Dis*. (2016) 51:128–32. doi: 10.1016/j.ijid.2016.08.023
44. Penot P, Brichler S, Guilleminot J, Lascoux-Combe C, Taulera O, Gordien E, et al. Infectious Zika virus in vaginal secretions from an HIV-infected woman, France, August 2016. *Euro Surveill*. (2017) 22:30444. doi: 10.2807/1560-7917.ES.2017.22.3.30444
45. Murray KO, Gorchakov R, Carlson AR, Berry R, Lai L, Natrajan M, et al. Prolonged detection of Zika virus in vaginal secretions and whole blood. *Emerg Infect Dis*. (2017) 23:99–101. doi: 10.3201/eid2301.161394
46. Tang WW, Young MP, Mamidi A, Regla-Nava JA, Kim K, Shresta S. A mouse model of Zika virus sexual transmission and vaginal viral replication. *Cell Rep*. (2016) 17:3091–8. doi: 10.1016/j.celrep.2016.11.070
47. Sanchez-Montalva A, Pou D, Sulleiro E, Salvador F, Bocanegra C, Treviño B, et al. Zika virus dynamics in body fluids and risk of sexual transmission in a non-endemic area. *Trop Med Int Health*. (2018) 23:92–100. doi: 10.1111/tmi.13019
48. Carias AM, Allen SA, Fought AJ, Kotnik Halavaty K, Anderson MR, Jimenez ML, et al. Increases in endogenous or exogenous progestins promote virus-target cell interactions within the non-human primate female reproductive tract. *PLoS Pathog*. (2016) 12:e1005885. doi: 10.1371/journal.ppat.1005885

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REVIEW
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Solid Wastes Provide Breeding Sites, Burrows, and Food for Biological Disease Vectors, and Urban Zoonotic Reservoirs: A Call to Action for Solutions-Based Research

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Background: Infectious disease epidemiology and planetary health literature often cite solid waste and plastic pollution as risk factors for vector-borne diseases and urban zoonoses; however, no rigorous reviews of the risks to human health have been published since 1994. This paper aims to identify research gaps and outline potential solutions to interrupt the vicious cycle of solid wastes; disease vectors and reservoirs; infection and disease; and poverty.

Methods: We searched peer-reviewed publications from PubMed, Google Scholar, and Stanford Searchworks, and references from relevant articles using the search terms ("disease" OR "epidemiology") AND ("plastic pollution," "garbage," and "trash," "rubbish," "refuse," OR "solid waste"). Abstracts and reports from meetings were included only when they related directly to previously published work. Only articles published in English, Spanish, or Portuguese through 2018 were included, with a focus on post-1994, after the last comprehensive review was published. Cancer, diabetes, and food chain-specific articles were outside the scope and excluded. After completing the literature review, we further limited the literature to "urban zoonotic and biological vector-borne diseases" or to "zoonotic and biological vector-borne diseases of the urban environment."

Results: Urban biological vector-borne diseases, especially *Aedes*-borne diseases, are associated with solid waste accumulation but vector preferences vary over season and region. Urban zoonosis, especially rodent and canine disease reservoirs, are associated with solid waste in urban settings, especially when garbage accumulates over time, creating burrowing sites and food for reservoirs. Although evidence suggests the link between plastic pollution/solid waste and human disease, measurements are not standardized, confounders are not rigorously controlled, and the quality of evidence varies. Here we propose a framework for solutions-based research in three areas: innovation, education, and policy.

Conclusions: Disease epidemics are increasing in scope and scale with urban populations growing, climate change providing newly suitable vector climates, and immunologically naïve populations becoming newly exposed. Sustainable solid waste management is crucial to prevention, specifically in urban environments that favor urban vectors such as *Aedes* species. We propose that next steps should include more robust epidemiological measurements and propose a framework for solutions-based research.

Keywords: planetary health, infectious disease epidemiology, plastic pollution, vector-borne diseases, urban zoonoses, solid waste

INTRODUCTION

Rationale

The world is in a solid waste and plastic predicament (1–15)—single use-plastic packaging is increasing in an urbanized (11, 16–20) and globalized economy in which production of food happens farther from the consumer and packaging enables consumption far from the source; yet, plastics lack a circular economy (21–23) that would incentivize responsible management (3, 24–28), resulting in large accumulations of solid waste, specifically plastics which do not biodegrade (29).

The most common approach to eliminating accumulated trash in low- and middle- income countries is open burning. For example, in sub-Saharan Africa, more than 75% of waste is openly burned and worldwide an estimated 600 million tons are openly burned annually (30). Open burning of trash is dangerous for human health (6, 31–33) and the planet, as burning releases toxins into the air that pollute the environment and increase greenhouse gases which contribute to climate change (30).

Policies are slowly catching up to reduce single-use plastic supply, but these policies are only one part of a complete solution (34–37) to single-use plastic production, demand, and disposal (29) and these policies often face poor enforcement, especially in LMICs (29).

At the same time, the risk of zoonosis has increased with urbanization (38) and immunologically naïve populations are newly at risk for vector-borne disease transmission due to changing geographies of suitable vector climates (39–41). Vector-borne diseases such as dengue—transmitted by container breeding *Aedes* spp.—threaten about half a billion people in densely populated areas (42). One very important mosquito vector, *Aedes aegypti*, which spreads dengue, Zika, chikungunya, and yellow fever, prefers to breed in man-made containers (43, 44), such as recyclable plastic containers, tires, and trash. The 2,050 projections of over 6 billion people living in urban areas (45) suggest an impending increase in the risk of infectious disease transmission.

Objectives

Trash accumulation has been cited as a risk factor for infectious disease (46–50). Recent viewpoints discuss the subject (51–53), but analytical reviews are outdated (54–57). Other reviews exclude key references on trash and disease risk (19, 58–60), while others focus on urbanization or poverty (18, 19, 57). Some reviews take a narrow scope and are pathogen-specific [for example, we identified reviews on trash and dengue virus (61–63), protozoans (64), and leishmania (65)] or vector-specific [arthropods (51)], limited to landfills and incineration (66), microplastic-specific (67), or waste-specific (68). However, the potential risk of direct transmission of infectious diseases by any kind of solid waste depends on a multitude of inter-related factors including, but not limited to, the presence of an infectious agent, its viability in solid waste, and a susceptible host.

A holistic approach is needed to define the link between vector-borne diseases, urban zoonosis, and solid waste. Here, our objective is to identify research gaps through a review of current evidence on solid waste accumulation, in association with urban zoonosis and biological vector-borne disease risk, and to propose solutions that can interrupt the vicious cycle of solid waste accumulation and human health risks due to infectious diseases.

Research Question

We hypothesize that plastic pollution, including unused plastic bottles, containers, and tires, is a major environmental health risk and promotes vector-borne diseases (VBD) such as dengue, chikungunya, Zika, malaria, and other vectors of disease (triatomine, houseflies) and zoonotic reservoirs (rodents and canines).

METHODS

Search Strategy

We conducted a hypothesis-driven review from January to March 2019. Literature was identified by searches of PubMed, Google Scholar, and Stanford Searchworks, and references from relevant articles using the search terms ("disease" OR "epidemiology") AND ("plastic pollution," "garbage," and "trash," "rubbish," "refuse," OR "solid waste"). Abstracts and reports from meetings were included only when they related directly to previously published work. Only articles published in English, Spanish, or Portuguese (translated using https://www.deepl.com/translator) through 2018 were included, with a focus on post-1994 (the publication year of the last rigorous review on the topic). Cancer, diabetes, mechanical vectors, and food chain-specific articles were outside the scope of this review and excluded. The search was not constrained by geography. After completing the literature review, we further limited the literature to only biological vector-borne and zoonotic diseases (see general concepts defined in the **Supplementary Material**).

SYNTHESIZED FINDINGS: PUBLISHED LITERATURE ON VECTOR-BORNE DISEASES, URBAN ZOONOSIS, AND SOLID WASTE

One hundred and fifty three references were identified in the literature review, 73 of which discussed vector-borne and zoonotic diseases. We discuss vector-borne diseases and urban zoonosis in the context of solid waste and highlight major vectors, reservoirs, and diseases.

We identified 45 references related to vector-borne disease risk and solid waste. We categorized the results according to vector [*Aedes* species, *Phlebotomus* spp. (sand flies), triatominae, *Anopheles* species], pathogen (dengue, chikungunya, and Zika viruses, *Leishmania*, *Trypanosoma cruzi*, and *Plasmodium*) and type of evidence (case study, observational, intervention, policy, or review). We summarize the evidence in **Table 1** and details are available in **Supplementary Material 1**.

We identified 16 references related to urban zoonosis and solid waste. We categorized the results according to vector (rodent and canine), pathogen (*Orientia tsutsugamushi*, *Leptospira*, *Yersinia pestis*, *Toxoplasma gondii*, and rabies virus) and type of evidence (case study, observational, intervention, policy, or review). We summarize the evidence in **Table 2** and details are available in **Supplementary Material 2**.

Vector-Borne Diseases and Solid Waste

Vector-borne diseases, especially Aedes-borne diseases, are associated with solid waste accumulation in the urban environment, even small cups, and wrappers, but vector preferences vary over season and region. Other vectors are associated with trash as a burrow, source of food, and breeding site.

Aedes Species

Aedes species mosquitoes prefer to breed in man-made plastic containers (43, 44) and transmit dengue (DENV), Zika (ZIKV), and chikungunya (CHIKV) viruses. *Aedes albopictus* is reported to preferentially breed in solid waste (91), and tires (92), open coconut shells (92, 93) and small plastic containers (92, 93). *Aedes aegypti* prefers to breed in discarded tires (95, 98) and artificial water containers (95); plastic containers (96), solid waste (96, 98), buckets (97), drums (97), tires (97), pots (97), and garbage dumps (98). Both *Aedes albopictus* and *Aedes aegypti* breed in plastic teacups (100, 101), plastic containers (79–82, 102, 103, 128), tires (79, 82, 101), trash (96, 101), bottles (103), and cans (103). However, these associations change seasonally and regionally. During transmission season, *Aedes* prefers solid waste (96) in Delhi, India. During the rainy season in Brazil, *Aedes* prefers tires (92), open coconut shells and small plastic containers (92). In India, breeding preference ratio was highest for tires and container breeding during pre-monsoon (79). Human DENV transmission was strongly associated with irregular garbage collection during low transmission periods/interepidemic intervals (44).

At the household-level, the evidence shows an increase of dengue risk with the presence of cans, plastic containers, tires (70), a lack of consistent garbage collection (44, 71, 73, 74, 77), and with garbage accumulation (75).

CHIKV and ZIKV have also been associated with garbage accumulation in ecological models (88, 89). However, ecological models can be subject to biases and residual confounding (76). In a case study, Krystosik, Curtis (129) used spatial video and Google Street View in Cali, Colombia to create sub-neighborhood risk surfaces compared with routinely reported clinical cases of dengue, chikungunya, and Zika. Ministry of Health officials and Community Health Workers perceived proximity to unplanned urbanizations without solid waste management as a risk factor for dengue, chikungunya and Zika hotspots. Lack of sanitation can be systematic, for example, 80–90% of housing on Reunion Island was built by squatters resulting in the absence of adequate drainage systems for sewage and rainwater and the lack of properly organized garbage disposal and providing breeding grounds for vector-borne diseases, especially CHIKV (90).

Conversely, removing trash and stagnant water from around the residence is protective (78, 84–87, 94, 99), especially when the government acts with intention and the community is consistently mobilized (85, 86). However, results depend on the local ecology of vector breeding (83, 87).

Other Vectors

Other vectors use trash as a burrow, source of food, and breeding site. To prevent tick-borne diseases, The US Centers for Disease Control recommends removing old furniture, mattresses, or trash that may give ticks a place to hide (49); however, no other evidence of an association between trash and tick-borne disease was found. Abbasi et al. (111) identified 33 species of arthropods from a Municipal Solid Waste landfill in Urmia, Iran, including medically important species: *Periplaneta americana Linnaeus* (Blattodea: Blattidae) and *Shelfordella lateralis Walker* (Blattodea: Ectobiidae). Ahmad et al. (112) report that malaria was associated with low rates of solid waste collection system use. However, this association was based on geospatial analysis that did not control for potential confounders. Others report that *Anopheles stephensi* also breeds in manufactured containers (130, 131).

Community members in rural India report that visceral leishmaniasis-transmitting sand flies breed in trash (105). In two studies, the risk of visceral leishmaniasis increased in the absence of regular trash collection (104, 106).

In Yucatan Peninsula, Mexico, residents report triatomines, the vectors of *Trypanosoma cruzi,* burrow in accumulated trash, cardboard, and rocks (110). Strong entomological (109, 110) and clinical (107, 108) evidence supports this local perception. Dumonteil et al. (109) conducted entomological surveillance for one year in 38 randomly selected houses and created crude and adjusted models in which they observed a strong association between the practice of cleaning of trash from the peridomicile and house infestation by non-domiciliated *Triatoma dimidiate* (109). Fortunately, similar

TABLE 1 | Vector-borne disease evidence.

Study type	Sample size	Year	Study site	WHO Region (69)	Trash type/risk measured	References
Aedes: DENV						
Serosurveys						
	106 households (501 residents)	2000	El Salvador	Americas	Discarded cans, plastic containers, tire casings	(70)
	273 people	2008	Texas-Mexico border	Americas	Waste tires and buckets	(43)
	600 people	2004	Brownsville, Texas, and Matamoros, Tamaulipas, México	Americas	Water-holding containers, garbage collection	(71)
Focus groups	59 people	2003	San Juan, Puerto Rico	Americas	Insufficient garbage removal	(72)
Surveillance system studies						
Case-control study	34 cases and 34 controls	2001	Fortaleza (north-east Brazil)	Americas	No waste collection	(73)
Observational study	219 (139 with and 80 without infection)	2017	Machala, Ecuador	Americas	Daily garbage collection	(74)
Surveillance system modeling studies						
	4,165 households	2014	Thailand	S-E Asia	Outdoor solid waste disposal	(75)
	4,248 cases	2018	Guayaquil, Ecuador	Americas	Negative association: municipal garbage collection at the census block level	(76)
Population-based case-control study	538 clinical cases and 727 controls	2011	Campinas, São Paulo, Brazil	Americas	Frequency of garbage collection	(77)
Longitudinal models	165 cases; 492 controls	2018	Fortaleza, Brazil	Americas	Irregular garbage collection, scrapyards and sites associated with tires	(44)
Case-control study	165 cases; 492 controls	2014	Guangzhou, China	Western Pacific	Removing trash and stagnant water from around the residence	(78)
Entomological surveys						
Larval	70 clusters; 1,750 houses	2014	Thiruvananthapuram, Kerala, India	S-E Asia	Tires and containers	(79)
Larval	789 breeding habitats	2008–2009	Malaysia	Western Pacific	Plastic containers as breeding habitats	(80)
	205 households	September 2017	Five streets in urban Chidambaram, Cuddalore district, Tamil Nadu state, India	S-E Asia	Discarded plastic containers	(81)
Larval	347 DF/DHF cases in 120 study sites	July 2002–August 2003	Kandy District, Sri Lanka	S-E Asia	Tires, discarded plastic	(82)
Intervention studies						
Modeled a hypothetical sanitation program		1999	Montrose urbanization in Caroni County and Port Cumana in the St. Andrews/St. David district, Trinidad	Americas	No effect: tires and small miscellaneous discarded trash	(83)
Waste disposal act		1988–1993	Taiwan	Western Pacific	Discarded containers	(84)
Household level waste management intervention for vector control and community mobilization	200 houses	2012	Gampaha district of Sri Lanka	S-E Asia	Waste management at household level, the promotion of composting biodegradable household waste, raising awareness on the importance of solid waste management in dengue control and improving garbage collection bowls, tins, bottles	(85)

(Continued)

TABLE 1 | Continued

Study type	Sample size	Year	Study site	WHO Region (69)	Trash type/risk measured	References
Community-centered dengue-ecosystem management		2012	Yogyakarta city, Indonesia	S-E Asia	Solid waste management and recycling	(86)
		2006 and 2011	India, Sri Lanka, Indonesia, Myanmar, Philippines, Thailand	S-E Asia/Western Pacific	Solid waste management, composting and recycling schemes small discarded containers	(87)
Aedes: ZIKV/CHIKV						
Surveillance system modeling studies						
		2014–2016	Brazil	Americas	Man-made larval habitats and environmental management—water supply/storage and solid waste management as measured by the *Garbage accumulation index* (number of houses with accumulated and uncollected garbage)	(88)
		2018	Brazil	Americas	Reported garbage destination, type of sanitary installation	(89)
Aedes: CHIKV						
Policy brief		June 2012	Reunion Island	Africa	Garbage disposal	(90)
Aedes albopictus						
Entomological surveys						
Larval	3720 premises and 820 local inhabitants	2010	Sant Cugat, Spain	Europe	Premises with solid waste	(91)
Immatures	four city areas	2007	Fortaleza, Ceará, Brazil	Americas	Tires, opened coconuts and small plastic containers	(92)
Larvae	100 homes	2006–2009	Calicut, Kerala, India	S-E Asia	Coconut shells and plastic waste	(93)
Intervention studies						
Area-wide management	six 1000 parcel sites; 3 urban; 3 suburban areas	2013	New Jersey, United States	Americas	Tires and trash (plastic bags, soda cans, etc.)	(94)
Aedes aegypti						
Entomological surveys						
Larval	750 containers; 1,873 larvae	May-June to September-October 2014	Dire Dawa, East Ethiopia	Africa	Discarded tires and artificial water containers in houses and peridomestic areas	(95)
	18 localities	June 2013 to May 2014	Delhi, India	S-E Asia	Solid waste and plastic containers	(96)
Immature	20 sentinel houses in each of 4 study sites	June 2014 to May 2016	rural and urban sites in western and coastal Kenya	Africa	Buckets, drums, tires, and pots	(97)
Temporal dynamics and spatial patterns	17,815 fixed sites	2016	Tartagal, Salta Province, Argentina	Americas	Municipal garbage dump, tire repair shops, and small garbage accumulation sites	(98)
Intervention studies						
Community-based larval source reduction campaign		2003	Lautoka, Viti Levu, Fiji Islands	Western Pacific	Tires and drums	(99)
***Aedes* spp.**						
Entomological surveys						
Vector survey	175 discardable plastic teacups	2003	Coastal district, Ernakulam, in Kerala State, India	S-E Asia	Plastic teacups discarded at tea carts	(100)

(Continued)

TABLE 1 | Continued

Study type	Sample size	Year	Study site	WHO Region (69)	Trash type/risk measured	References
Immatures		2012	Delhi and Haryana, India	S-E Asia	Discarded trash, tires and plastic cups at roadside near tea stalls	(101)
Larval	26 types of wastes	2015	Kolkata, India	S-E Asia	Household wastes: earthen, porcelain, plastic, and coconut shells	(102)
Larval	262 containers	2009	University of Malaya, Kuala Lumpur	Western Pacific	Plastic containers, bottles, and cans	(103)
Sandflies: leishmaniasis						
case-control	Two large outbreaks of at least 1,000 newly reported cases	2005	Teresina, Brazil	Americas	Regular trash collection	(104)
KAP	3,968 heads of households	2006	Bihar state, India	S-E Asia	Garbage collection	(105)
Retrospective study	Five time periods; 3,252 cases	1990–2014	Rio Grande do Norte, Brazil	Americas	Lack of garbage collection	(106)
Triatomine: trypanosoma cruzi						
Seroprevalence						
	26 rural communities; 905 households, 2,156 humans, and 333 dogs	January 2005–December 2008	Parroquia San Miguel, Municipio Urdaneta, Estado Lara, Venezuela	Americas	Household disarray (measured as old and/or damaged artifacts accumulated, materials from construction, inadequate cleaning and free rubbish in the home)	(107)
	15 municipalities; 96 villages; 576 dwellings	2017	Sucre State, Venezuela	Americas	Accumulated garbage as measured by method of garbage disposal	(108)
Entomological surveys: mixed modeling approach	Three villages; 308 houses	2013	Yucatan, Mexico	Americas	Cleaning of trash from the peridomicile	(109)
KAP	Three villages; 570, 702, and 416 houses	2014	Yucatan Peninsula, Mexico	Americas	Trash, cardboard, yard cleaning (collecting trash, cutting down plants and grass, and burning trash)	(110)
Entomological surveys:	1,913 arthropod samples	2019	Urmia, Iran	Eastern Mediterranean	Municipal solid waste landfill	(111)
***Anopheles* spp.: Malaria**						
Geospatial analysis	450 water samples	2015	Rawalpindi, Pakistan	Eastern Mediterranean	Low rates of solid waste collection system use	(112)

KAP, Knowledge, attitude, and practice. One study found no effect (103) and one other found a negative association (93).

to *Aedes* interventions, environmental cleanup is associated with decreased risk of triatomine infestation (110). Clinical evidence also supports these findings. *Trypanosoma cruzi* infection seroprevalence in Venezuela was associated with the increase of accumulated garbage (108) and household disarray (measured as old and/or damaged artifacts accumulated, materials from construction, inadequate cleaning and free rubbish in the home) (107). Bonfante-Cabarcas et al. (107) speculate that accumulated garbage favors breeding of *T. cruzi* reservoirs (rats, mice, and opossum) and provides long-term refuge with immediate food sources for insects to reproduce and colonize the house for a long time, increasing the probability of intra-domiciliary vector transmission of *T. cruzi*.

Urban Zoonosis Associated With Solid Waste

Urban zoonoses, specifically those transmitted by rodent and canine reservoirs, are associated with solid waste, especially when garbage accumulates over time creating burrowing sites and food for reservoirs.

In a review of neglected tropical diseases and their impact on global health and development (50), Hotez states of zoonoses: "Of relevance to the NTDs, the poorest favelas do not benefit from regular garbage collection or sewage treatment, thereby creating excellent niches for rats and stray dogs." Rodents and canines directly transmit disease of importance to urban zoonosis (123, 125, 132). Solid waste accumulation is an important factor for urban rodent and canine feeding and sheltering strategies

TABLE 2 | Urban zoonosis evidence.

Study type	Sample size	Year	Study site	WHO Region (69)	Trash type/risk measured	References
Observational studies						
Surveillance		1984–2011	Marseille, France	Europe	Garbage collection strikes in which garbage is left on the street	(113)
	3,171 slum residents	April 2003 and May 2004	Slum in Salvador, Brazil	Americas	Residence <20 meters from accumulated refuse	(114)
Surveillance	79 autochthonous human cases	2011–2015	Federal District, Brazil	Americas	Public garbage collection service	(115)
Outbreak	87 leptospirosis cases	1996	Western Region of Rio de Janeiro	Americas	Lower access to solid waste collection –% households served by municipal solid waste collection (accumulation of organic wastes, promoting the proliferation of rodents)	(116)
Outbreak	87 leptospirosis cases	1996	Western Region of Rio de Janeiro	Americas	Waste accumulation	(117)
Cross-sectional KAP	257 residents	May and June 2007	Urban slum community in Salvador, Brazil	Americas	Improving trash collection	(118)
Outbreak & hospital-based surveillance	89 confirmed cases. 22 households with index cases and 52 control households located in the same slum communities	2001	Slum communities in Salvador, Brazil	Americas	Trash collections	(48)
Population based case-control study	66 lab-confirmed cases and 125 age and sex-matched healthy neighborhood controls	October 2000 and March 2001	Salvador, Brazil	Americas	no association: Peri-domiciliary trash accumulation (Visual inspection of accumulated trash & continuous presence of household trash within five meters of a residence—proximity to accumulated trash) and municipal waste collection	(119)
Rodent: scrub typhus (Orientia tsutsugamushi)						
Observational	2,002 adults		Vientiane City, Laos	S-E Asia	Poor sanitary conditions (presence of rubbish, animal excrement, etc.)	(120)
Rodent: bubonic plague						
Observational: case study		1900	Central Sydney, Australia	Western Pacific	Informal solid waste storage sites, solid waste management	(121)
Observational: outbreak study		1995–1998	Mahajanga, Madagascar	Africa	rubbish	(122)
Water studies	22 water samples		Southern Chile	Americas	Debris found around the household areas: buckets, pails, jars, barrels, and old tires	(123)
Water studies			Peruvian Amazon region of Iquitos	Americas	Clearing away garbage in urban areas	(124)
Observational	888 patients reported clinically	1975	Salvador	Americas	Sewage, rats, water, dogs, mud and garbage,	(125)
	236 households		Southern Chile	Americas	Open containers and debris presence of dogs and rodents	(123)
Canine: toxoplasmosis						
Observational: serosurvey of humans and dogs	564 households, which included 597 owners and 729 dogs		Urban areas of a major cities, Londrina, southern Brazil	Americas	Yard cleaning frequency, and having a dirty yard	(126)
Canine: rabies						
Observational		2005–2016	Lebanon	Eastern Mediterranean	Local garbage crisis: standing accumulated waste	(127)

One study found no association (119).

(126) and can be used as a proxy in the absence of reliable data on rodent distribution in the city (117, 126). Presence of rubbish increased risk of scrub typhus (120); Toxoplasma infection in owners and their domiciled dogs was associated with dirty yards (126); and the bubonic plague has historically been associated with solid waste (121, 122).

For example, Kassir et al. (127) conducted an observational study to investigate the risk of rabies and the neighboring Syrian war and the local garbage crisis, finding both were concomitant with a notable increase in the number of dog bites and thus possible rabies exposure. The evidence lies in a time-series of data from the Lebanese Ministry of Public Health (LMOPH) Epidemiological Surveillance Unit public database from 2005 to 2016. A sharp increase in reported animal bites was reported post-2013 (1,004 ± 272 vs. 355 ± 145 bites per year). The authors explain:

> "The accumulation of wastes in dumpsites led to the declaration of a severe problem in July 2015, and these open garbage dump sites have been previously shown to contribute to the rise in the number of stray dogs which amplifies the number of possible vectors. Garbage dumps are breeding areas of stray dogs, and if they are no longer around, dogs will migrate to other places. This is reflected by the peak in the stray to domestic dog ratio in October 2015, after heaps of garbage had been covering the Lebanese streets for several months. October, in fact, witnesses the beginning of the rain season in Lebanon, and the rainfall in the presence of open garbage dumps leads to the formation of leachate, a polluting by-product of organic matter. This poses both social and environmental problems such as nuisance, diseases and the spread of stray dogs and other harmful animals. This rise in stray dogs increases the possibility both of new vectors as well as new bites. It is noteworthy that this predominance of stray dog bites was only observed in October 2015, while it was not present in either 2013 or 2014. This further strengthens the correlation between the garbage crisis, a special circumstance of October 2015, and the increase in stray dog bites" (127).

Leptospirosis is associated with dogs (123, 125), accumulated refuse (114), garbage (113, 123) and open containers and debris in the peri-domestic area (123, 125). For example, leptospirosis emergence in Marseille, France is linked to garbage collection strikes that contribute to the expansion of the rat population (113). Among slum residents from Salvador, Brazil, residence <20 m from accumulated refuse was associated with increased odds of previous *Leptospira* infection (114). Residents of another urban slum in Salvador identified improving trash collection as necessary to control leptospirosis in their community and reported current payment for private trash collection service to avoid trash accumulation in their community or a willingness to pay for this service. Residents reported removing trash on a daily basis but identified that trash cans are >50 m from their homes (118). Leptospira interrogans and *L. icterohaemorrhagiae* are pathogens of severe diseases that may cluster in urban areas where trash accumulates (123) but are also found in rural households in peri-domestic open containers (debris found around the household areas including buckets, pails, jars, barrels, and old tires) (123). Evidence shows leptospirosis infection clusters at the household level (48). During a leptospirosis outbreak in Western Rio de Janeiro, Brazil, cases were associated with lower access to solid waste collection, measured as a percentage of households served by municipal solid waste collection (116), and waste accumulation was used as an indicator of probable rat presence (117).

Conversely, in Federal District Brazil, leptospirosis infection was negatively associated with population access to public services: sewage network, treated water network, and public garbage collection services (115); and in Salvador, Brazil, there was no association between leptospirosis infection and peri-domiciliary trash accumulation (119).

Framework for Solutions-Based Research

Here we propose a framework for solutions-based-research in three areas: innovation, education, and policy.

Lessons Learned From Previously Proposed Frameworks

Efforts to promote circular economies in plastics are gaining international attention (29, 133, 134). The United Nations Environment Programme published 'Single-Use Plastics: A Roadmap for Sustainability, 2018 (29). However, it noted policies and regulations have recently been established and lack monitoring and accountability and suffer from poor implementation. Hawken discusses the short and long term costs and benefits to multiple solutions to Reverse Global Warming (133). However, the solutions require significant investment from business and government to change without a focus on upstream education and innovation. Precious Plastics (134) focuses on the community engagement aspects of reusing plastics but fails to integrate with upstream policy. Examples of successful recycling exist in the metals industry (135–137)—aluminum (135, 136), and steel (137) are recycled and traded as commodities globally.

Perhaps the most common framework is "re-use, reduce, and recycle." Reusing and recycling receive ample attention given the technology involved, yet trends in the recycling industry are changing: China is no longer accepting foreign trash for recycling (138). Reusing is also challenging as few types of plastics are highly coveted and reusable. The poorer quality plastics are simply trash—unable to be reused or recycled. Therefore, while reusing/recycling/introducing plastic alternatives all have their place, reducing the consumption and sale of single-use plastics is key. Therefore, we are adapting the previously touted framework, emphasizing reduction, and encouraging a circular economy for re-use and recycle.

Building on previous frameworks (29, 133–137), we propose a framework (**Figure 1**) to reduce vector-borne disease risk and urban zoonoses from exposure to solid waste. Given the importance of intervening at the interface of solid-waste and disease-vectors-and-reservoirs, the framework creates a knowledge-to-action plan using policy and innovative plastic alternatives to decrease the upstream plastic supply, education and art to decrease the downstream global demand for plastic, and innovation to generate profitable uses for currently produced and consumed single-use plastics. The desired result is an action plan to create a circular economy of trash and reduce

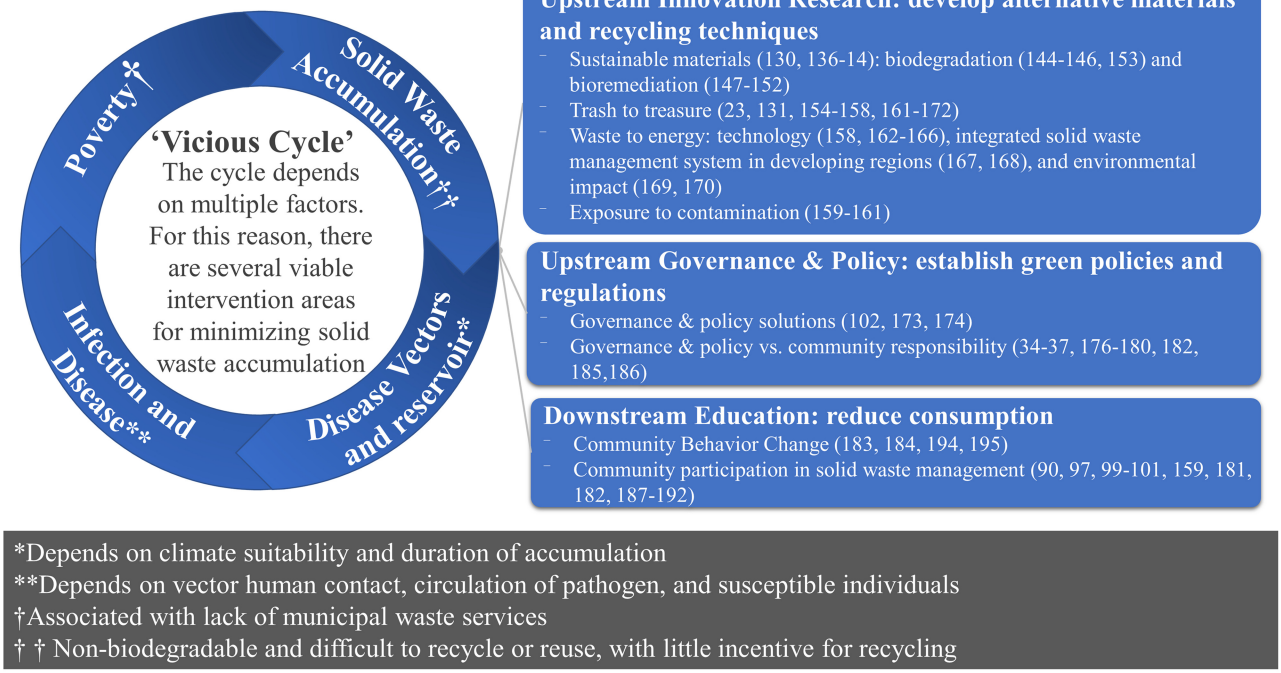

FIGURE 1 | Framework for solutions-based research: upstream innovation research, upstream governance and policy, and downstream education. Recognizing that the current practices and incentive structures that drive these practices are inadequate to address the volume of trash generated globally, different approaches are needed to manage the growing waste problem. Notes for the symbols in the circle. Disease vectors and reservoirs *Depends on climate suitability and duration of accumulation; Infection and Disease **Depends on vector human contact, circulation of pathogen, and susceptible individuals; Poverty is †Associated with lack of municipal waste services; Solid waste accumulation refers to †† Non-biodegradable materials which are not incentivized for recycling.

the supply and demand of single-use plastics and to cultivate empowered, educated, and healthy communities that resist trash accumulation to improve health via reduced vector-borne diseases and improved air quality. The expected impact relates to the critical need to understand how the complex system that generates and discards so much trash might be tweaked, so that less trash is produced or trash is put back into either the economic or ecological cycle. As current options are insufficient, we propose solution-oriented research to either better adapt these options or to create whole new options for plastics disposal, recycling, and reuse and discover possibilities for a future without disposable plastics through policy, education, and innovation. The evidence is summarized in **Figure 1** and details are available in **Supplementary Material 3**.

Upstream Innovation Research

Profitable upstream innovation research can decrease supply and improve the processing of solid wastes in an increasingly urbanized and market-based world. Immediate barriers are cost and scalability.

In his 2017 best-seller, Drawdown (133), Hawken discusses the possibility of converting up to 90% of current fossil-fuel based plastic production to bio-based production. However, he warns that the solution must include proper separation and processing to fulfill the goal of sustainable material. Innovation in this field is currently working to drop the price below that of current fossil-fuel-based production. According to a special report commission by the European Polysaccharide Network of Excellence and European Bioplastics, 90% of current plastics could be derived from plants (139). Zhang et al. (140) analyze sustainable materials, defined as a class of materials that are derived from renewable feedstocks and exhibit closed-loop life cycles including aliphatic polyesters and polycarbonates. They also discuss recent advancements that lower the technological barriers for developing more sustainable replacements for petroleum-based plastics including biopolymers (141–143) and agro polymers (144–146).

Two aspects of sustainable materials to consider are biodegradation (147–149) and bioremediation (150–155). Narancic and O'Connor (150–152, 156). We found bioremediation—whereby animals and bacteria can break down plastics into biodegradable products—to be particularly interesting. Narancic and O'Connor (156) review the advances and possibilities in the biotransformation and biodegradation of oil-based plastics, including bio-based and biodegradable polymers, end-of-life management of biodegradables, and a circular economy to reduce plastic waste pollution. New fungi species are biodegrading polyester polyurethane: *Pestalotiopsis* species (150) and *Aspergillus tubingensis* (151). *Ideonella sakaiensis* bacteria break PET (Polyethylene terephthalate) into

terephthalic acid and ethylene glycol in 2 weeks (152). Mealworm larvae can digest Styrofoam in <24 h with no cost to survival over 1 month, converting 47.7% of the ingested Styrofoam into CO_2 and biodegradable residue (153, 154). Wax moth *Galleria mellonella* caterpillars can biodegrade polyethylene bags (155). These methods are especially attractive as they require no behavior change and are sustainable and, in some cases, beneficial to the species performing the biodegradation. Yet, these pilot studies need to be studied at scale and adapted to local context to understand feasibility.

Repurposing trash for profit seems like a viable market-based solution (134, 157–161) but does carry some risk of exposure to contamination (162–164) for entrepreneurs and end-users depending on the type of materials and the processes used and this risk should be taken into consideration early in the process. One popular use case is waste-to-energy analyzes waste-to-energy strategies and concludes that for a net implementation cost of $36 billion, a net operational savings of $19.82 billion and 1.1 gigatons of CO_2 reduction could be gained. For example, Sweden currently converts 50% of household wastes to energy (161). Yet, Haken warns that this is only a transitional strategy, citing emissions of heavy metals and toxic compounds, even in state-of-the-art facilities. Several reviews discuss waste-to-energy regarding technological options and challenges (165–169), integrated solid waste management in developing countries (170, 171), and the environmental impact (172, 173).

These innovations must come equipped with a knowledge-to-action plan and pilots of these small-scale or theoretical solutions and engagement of external stakeholders such as existing companies, policymakers, and community groups.

Upstream Policy

Policymakers are uniquely positioned to make political and normative changes relatively quickly but struggle with enforcement, sustainability subject to elected officials, and community buy-in.

Policymakers are uniquely positioned to prevent and solve public health crises, in collaboration with public health officials and communities (84, 174–176). For example, Chen et al. (84) reported that discarded containers account for 25.4% of *Aedes* vector breeding sites in endemic regions of Taiwan pre-intervention. In 1988, the Waste Disposal Act was amended to make manufacturers, importers, and distributors responsible for the proper recovery, treatment, and recycling of packaging and containers which become an environmental menace. Non-compliance resulted in business suspension. A waste recycling system was established, and a breeding site reduction campaign was promoted for waste management. The authors reported a 98% decrease in dengue incidence reported to the Department of Health from 1988 to 1993. Several countries in Africa continue to implement bans to curb single-use plastic bags which clog drains, sewage systems, or hold rainwater, create breeding grounds for vectors (34).

Experts call for more policy solutions (177–179) and there is evidence that policy agendas can be influenced by popular norms (34, 180). Others argue that informal associations such as waste-picker cooperatives (35, 36, 181, 182) should be strengthened to improve solid waste systems. However, enforcement of such policies may be difficult, especially for nations with challenging processes or non-existent systems (37), and others call for a more community-based approach to increase participation in sustainable waste management (183–185). Businesses that use disposable packaging can also be engaged through social pressure and responsibility to adopt sustainable corporate practices (186, 187) and recoup disposable packaging for recycling.

Downstream Education to Decrease Demand

Community-based education and communication have the potential to change norms and create sustainable change but require greater initial investments to tailor and iterate community-based approaches.

Eagle et al. (188) argue that social marketing principles (183, 189, 190) paired with education (75, 85–87, 182, 183, 189–191) and policy (section upstream policy) can intervene to change behavior to positively impact plastic pollution using a transdisciplinary approach to identify barriers to and enablers of sustained behavior change.

Creating awareness about the crisis and health and environmental risks surrounding plastic pollution will not immediately decrease supply, but information may increase social pressure and responsibility to adopt sustainable practices at household (75, 85, 183, 192), community (75, 86, 87, 99, 183, 191, 193–196), and corporate levels (186, 187) that may decrease demand in the future (see details in **Supplementary Material 3**). For example, Sommerfeld et al. (87) summarize a 5-year research and capacity-building initiative conducted in South Asia and South-East Asia. The initiative developed community-based interventions aimed at reducing dengue vector breeding and viral transmission. Where small discarded containers presented the main problem, groups experimented with solid waste management, composting and recycling schemes. Many intervention tools were locally produced, and all tools were implemented through community partnership strategies. All sites developed socially- and culturally-appropriate health education materials. The study also mobilized and empowered women, students, and community groups and at several sites organized new volunteer groups for environmental health.

Tana et al. (86) built an innovative community-centered dengue-ecosystem management intervention in Yogyakarta city, Indonesia and assessed the process and results. The intervention results included: better community knowledge, attitude, and practices in dengue prevention; increased household and community participation; improved partnership including a variety of stakeholders with prospects for sustainability; vector control efforts refocused on environmental and health issues; increased community ownership of dengue vector management including broader community development activities such as solid waste management and recycling. Tana et al. (86) note, the community-centered approach needs a lot of effort at the beginning but has better prospects for sustainability than the vertical "top-down" approach.

DISCUSSION

Summary of Main Findings

Although evidence suggests the link between plastic pollution/solid waste and human disease, measurements are not standardized, confounders are not rigorously controlled, and the quality of evidence varies.

Here we have reviewed the available evidence for solid waste accumulation impact on biological vector-borne diseases. We hypothesized that plastic pollution, including unused plastic bottles, containers, plastic bags, and tires, is a major environmental health risk and promotes vector-borne diseases (VBD) such as dengue, chikungunya, Zika, malaria, and other VBD transmission. We conclude that solid waste accumulation is a risk factor for zoonotic and vector-borne disease transmission. However, measurements are not standardized, (107, 123, 197) and confounders are not rigorously controlled (106, 112, 123, 197, 198).

In the context of vicious cycles of solid waste accumulation, poor health, and poverty, policymakers use estimates of disease transmission, burden, and risk to inform the allocation of limited public health resources; thus, it is imperative epidemiological estimates control for known confounders and employ standardized measurement constructs (**Table 3**). Additionally, if surveillance data are used, hybrid surveillance (199, 200) should be employed to correct for known surveillance biases. A framework for solutions-based research is also critical to guide research priorities.

Of note, the landscape of single-use plastics innovations and policy is developing rapidly. For example, Christensen et al. described in April 2019 a next-generation plastic to incentivize recycling in closed-loop life cycles (201, 202). This new plastic can be disassembled and reassembled without loss of performance or quality, even in mixed waste streams (201). And the political trend is gaining momentum—in May 2019, 187 countries agreed to add plastics to the Basel Convention, a treaty that regulates the movement of hazardous materials from one country to another (202).

Limitations

We only included published literature and abstracts in English, Spanish, and Portuguese. We did not have access to primary data and relied on the interpretation of the publishing authors.

TABLE 3 | Standardized measurements to define and quantify exposure to solid waste.

Construct	Measurement	Unit	Covariates	Data source	References
Exposure	Distance to accumulated trash	Meters	Frequency of trash collection, size, and type of dump	Local mapping	(98, 114, 117, 119, 129, 182)
	Size of accumulated trash site	Meters	Frequency of trash collection, size, and type of dump	MOH/Local mapping	(117, 129, 182)
	Persistence of accumulated trash	Days	Types of trash	Local mapping	(119)
	Vector breeding in trash	Vector counts	Species, seasonality, infection rates, rainfall, temperature, trash type, trash persistence	Entomological surveys	(79–82, 91–93, 95–98, 100–103, 109, 111)
	Disease Reservoir associated with trash	Reservoir counts	Species, seasonality, infection rates, flooding, food sources, trash type, trash persistence	Animal Surveys	(113, 114, 123, 126, 127)
	Pathogen in trash	Species and concentration	Location, season, container type	Environmental studies	(123, 124)
Access to municipal trash collection	Method of trash disposal	Categorical	Frequency of trash collection, size and type of dump	MOH/Local mapping	(108)
	Population coverage	Percent by region	Distance to trash collection point, cost of service, types of trash accepted	MOH/Local mapping	(116, 117, 119)
	Frequency of collection	Days	Distance to trash collection point, cost of service, types of trash accepted	MOH/Local mapping	(77)
	Distance to trash collection point	Meters	Security of accessing trash collection point	MOH/Local mapping	(129)
	Cost of service	Local monetary unit	Frequency of collection	MOH/Local mapping	(118)
Access to municipal sewage system	Population coverage	Percent by region	Sewage system type (open, closed), distance, cost	MOH/Local mapping	(116)
	Distance to sewage system access	Meters	Rainfall, slope/terrain, manholes, sewage system type (open, closed)	MOH/Local mapping	(114, 129)

MOH, Ministry of Health.

Multiple studies included relied on surveillance data which did not correct for selection bias. Multiple studies included did not control for variables possibly associated with both exposure (trash) and outcome (disease), for example, socio-economic status (SES), access to health care, or climate. The data needed to understand the context-specific risk factors are not yet available; particularly, the authors noted a paucity of data from sub-Saharan Africa, where policies and regulations have recently been established (29). Interestingly, although geography was not constrained in the review, most studies identified were from low- and middle-income tropical countries.

After completing our search, we constrained the scope to only urban zoonosis associated with wild mammals and domesticated animals of non-agricultural interest such as dogs and cats. This may exclude some important research related to geographical areas where cows or other domestics animals can serve as crucial reservoirs of important etiological agents.

CONCLUSIONS

Despite gaps in the research base—lack of standardized measures and residual confounding—it is clear solid wastes breed vector-borne diseases and urban zoonoses.

Future populations are at increased risk—disease epidemics are increasing in scope and scale (42) with urban populations growing (38, 45), climate change providing newly suitable vector climates (39–41), and naïve populations becoming newly at risk, sustainable solid waste management is crucial to prevention, specifically in urban environments that favor urban vectors such as *Aedes* species and in poor urban and rural populations which lack access to municipal solid waste services.

We propose a framework for solutions-based research which includes upstream innovation research, upstream policy, and downstream education to decrease demand for single-use plastics.

AUTHOR CONTRIBUTIONS

JF, AK, FM, and AL conceived of the initial idea and secured funding. AK drafted the initial manuscript. AK and GN conducted the literature search. LO and AL provided editing on intellectual content. All authors contributed to manuscript revision, read, 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/fpubh.2019.00405/full#supplementary-material

REFERENCES

1. Hoornweg D, Bhada-Tata P. *What a Waste—A Global Review of Solid Waste Management*. Washington, DC: The World Bank (2012).
2. Kaza S, Yao LC, Bhada-Tata P, Van Woerden F. *What a Waste 2.0 : A Global Snapshot of Solid Waste Management to 2050*. Washington, DC: World Bank (2018). doi: 10.1596/978-1-4648-1329-0
3. Heacock M, Kelly CB, Suk WA. E-waste: the growing global problem and next steps. *Rev Environ Health.* (2016) 31:131–5. doi: 10.1515/reveh-2015-0045
4. Rhodes CJ. Plastic pollution and potential solutions. *Sci Progr.* (2018) 101:207–60. doi: 10.3184/003685018X15294876706211
5. Schmidt C, Krauth T, Wagner S. Export of plastic debris by rivers into the sea. *Environ Sci Technol.* (2017) 51:12246–53. doi: 10.1021/acs.est.7b02368
6. Ziraba AK, Haregu TN, Mberu B. A review and framework for understanding the potential impact of poor solid waste management on health in developing countries. *Arch Public Health.* (2016) 74:55. doi: 10.1186/s13690-016-0166-4
7. Taylor D. Talking trash: the economic and environmental issues of landfills. *Environ Health Perspect.* (1999) 107:A404–9. doi: 10.1289/ehp.99107a404
8. Dangi MB, Pretz CR, Urynowicz MA, Gerow KG, Reddy JM. Municipal solid waste generation in Kathmandu, Nepal. *J Environ Manage.* (2011) 92:240–9. doi: 10.1016/j.jenvman.2010.09.005
9. Suthar S, Singh P. Household solid waste generation and composition in different family size and socio-economic groups: a case study. *Sustain Cities Soc.* (2015) 14:56–63. doi: 10.1016/j.scs.2014.07.004
10. Ogwueleka TC. Survey of household waste composition and quantities in Abuja, Nigeria. *Resour Conserv Recy.* (2013) 77:52–60. doi: 10.1016/j.resconrec.2013.05.011
11. Boadi KO, Kuitunen M. Environment, wealth, inequality and the burden of disease in the Accra metropolitan area, Ghana. *Int J Environ Health Res.* (2005) 15:193–206. doi: 10.1080/09603120500105935
12. Qu XY, Li ZS, Xie XY, Sui YM, Yang L, Chen Y. Survey of composition and generation rate of household wastes in Beijing, China. *Waste Manag.* (2009) 29:2618–24. doi: 10.1016/j.wasman.2009.05.014
13. Boer E, Jedrczak A, Kowalski Z, Kulczycka J, Szpadt R. A review of municipal solid waste composition and quantities in Poland. *Waste Manag.* (2010) 30:369–77. doi: 10.1016/j.wasman.2009.09.018
14. Adeniran AE, Nubi AT, Adelopo AO. Solid waste generation and characterization in the University of Lagos for a sustainable waste management. *Waste Manag.* (2017) 67:3–10. doi: 10.1016/j.wasman.2017.05.002
15. Ostle C, Thompson RC, Broughton D, Gregory L, Wootton M, Johns DG. The rise in ocean plastics evidenced from a 60-year time series. *Nat Commun.* (2019) 10:1622. doi: 10.1038/s41467-019-09506-1
16. Tauil PL. Urbanization and dengue ecology. *Cad Saude Publica.* (2001) 17 (Suppl.):99–102. doi: 10.1590/S0102-311X2001000700018
17. LaDeau SL, Allan BF, Leisnham PT, Levy MZ. The ecological foundations of transmission potential and vector-borne disease in urban landscapes. *Funct Ecol.* (2015) 29:889–901. doi: 10.1111/1365-2435.12487
18. Moore M, Gould P, Keary BS. Global urbanization and impact on health. *Int J Hyg Environ Health.* (2003) 206:269–78. doi: 10.1078/1438-4639-00223

19. Alirol E, Getaz L, Stoll B, Chappuis F, Loutan L. Urbanisation and infectious diseases in a globalised world. *Lancet Infect Dis.* (2011) 11:131–41. doi: 10.1016/S1473-3099(10)70223-1
20. Puri A, Kumar M, Johal E. Solid-waste management in Jalandhar city and its impact on community health. *Indian J Occup Environ Med.* (2008) 12:76–81. doi: 10.4103/0019-5278.43265
21. Indrianti N. Community-based solid waste bank model for sustainable education. *Proc Soc Behav Sci.* (2016) 224:158–66. doi: 10.1016/j.sbspro.2016.05.431
22. Lohri CR, Camenzind EJ, Zurbrugg C. Financial sustainability in municipal solid waste management–costs and revenues in Bahir Dar, Ethiopia. *Waste Manag.* (2014) 34:542–52. doi: 10.1016/j.wasman.2013.10.014
23. Public Radio International. *The Plastic Bank Turns Plastic Waste into Money for the Poor. Waste360*. New York, NY: Informa (2018).
24. Kinnaman TC. The economics of municipal solid waste management. *Waste Manag.* (2009) 29:2615–7. doi: 10.1016/j.wasman.2009.06.031
25. Magalini F. Global challenges for e-waste management: the societal implications. *Rev Environ Health.* (2016) 31:137–40. doi: 10.1515/reveh-2015-0035
26. Oguntoyinbo OO. Informal waste management system in Nigeria and barriers to an inclusive modern waste management system: a review. *Public Health.* (2012) 126:441–7. doi: 10.1016/j.puhe.2012.01.030
27. Ezeah C, Roberts CL. Analysis of barriers and success factors affecting the adoption of sustainable management of municipal solid waste in Nigeria. *J Environ Manage.* (2012) 103:9–14. doi: 10.1016/j.jenvman.2012.02.027
28. Zhou Z, Tang Y, Dong J, Chi Y, Ni M, Li N, et al. Environmental performance evolution of municipal solid waste management by life cycle assessment in Hangzhou, China. *J Environ Manage.* (2018) 227:23–33. doi: 10.1016/j.jenvman.2018.08.083
29. United Nations Environment Programme. *Single-Use Plastics: A Roadmap for Sustainability*. Geneva: United Nations Environment Programme (2018).
30. Cogut A. *Open Burning of Waste: A Global Health Disaster. R20 Regions of Climate Action*. Geneva: R20 (2016).
31. Verma R, Vinoda KS, Papireddy M, Gowda ANS. Toxic pollutants from plastic waste—a review. *Proc Environ Sci.* (2016) 35:701–8. doi: 10.1016/j.proenv.2016.07.069
32. Akpinar-Elci M, Coomansingh K, Blando J, Mark L. Household bush burning practice and related respiratory symptoms in Grenada, the Caribbean. *J Air Waste Manage Assoc.* (2015) 65:1148–52. doi: 10.1080/10962247.2015.1070773
33. Franchini M, Rial M, Buiatti E, Bianchi F. Health effects of exposure to waste incinerator emissions:a review of epidemiological studies. *Ann Ist Super Sanita.* (2004) 40:101−15. Available online at: http://old.iss.it/publ/anna/2004/1/doi.pdf
34. Clapp J, Swanston L. Doing away with plastic shopping bags: international patterns of norm emergence and policy implementation. *Environ Polit.* (2009) 18:315–32. doi: 10.1080/09644010902823717
35. Tirado-Soto MM, Zamberlan FL. Networks of recyclable material waste-picker's cooperatives: an alternative for the solid waste management in the city of Rio de Janeiro. *Waste Manag.* (2013) 33:1004–12. doi: 10.1016/j.wasman.2012.09.025
36. Ferri GL, Chaves Gde L, Ribeiro GM. Reverse logistics network for municipal solid waste management: the inclusion of waste pickers as a Brazilian legal requirement. *Waste Manag.* (2015) 40:173–91. doi: 10.1016/j.wasman.2015.02.036
37. Ezeah C, Roberts CL. Waste governance agenda in Nigerian cities: a comparative analysis. *Habitat Int.* (2014) 41:121–8. doi: 10.1016/j.habitatint.2013.07.007
38. Hassell JM, Begon M, Ward MJ, Fèvre EM. Urbanization and disease emergence: dynamics at the wildlife-livestock-human interface. *Trends Ecol Evol.* (2017) 32:55–67. doi: 10.1016/j.tree.2016.09.012
39. Mordecai EA, Cohen JM, Evans V, Gudapati P, Johnson LR, Lippi CA, et al. Detecting the impact of temperature on transmission of Zika, Dengue, and Chikungunya using mechanistic models. *PLoS Negl Trop Dis.* (2017) 11:e0005568. doi: 10.1371/journal.pntd.0005568
40. Huber JH, Childs ML, Caldwell JM, Mordecai EA. Seasonal temperature variation influences climate suitability for dengue, chikungunya, and Zika transmission. *PLoS Negl Trop Dis.* (2018) 12:e0006451. doi: 10.1371/journal.pntd.0006451
41. Ali S, Gugliemini O, Harber S, Harrison A, Houle L, Ivory J, et al. Environmental and social change drive the explosive emergence of Zika virus in the Americas. *PLoS Negl Trop Dis.* (2017) 11:e0005135. doi: 10.1371/journal.pntd.0005135
42. Eder M, Cortes F, Teixeira de Siqueira Filha N, Vinícius Araújo de França G, Degroote S, Braga C, Ridde V, et al. Scoping review on vector-borne diseases in urban areas: transmission dynamics, vectorial capacity and co-infection. *Infect Dis Povert.* (2018) 7:90. doi: 10.1186/s40249-018-0475-7
43. M Ramos M, Mohammed H, Zielinski-Gutierrez E, Hayden MH, Lopez JL, Fournier M, et al. Epidemic dengue and dengue hemorrhagic fever at the Texas-Mexico border: results of a household-based Seroepidemiologic survey, December 2005. *Am J Trop Med Hyg.* (2008) 78:364–9. doi: 10.4269/ajtmh.2008.78.364
44. MacCormack-Gelles B, Lima Neto AS, Sousa GS, Nascimento OJ, Machado MMT, Wilson ME, et al. Epidemiological characteristics and determinants of dengue transmission during epidemic and non-epidemic years in Fortaleza, Brazil: 2011–2015. *PLoS Negl Trop Dis.* (2018) 12:e0006990. doi: 10.1371/journal.pntd.0006990
45. UN. *68% of the world population projected to live in urban areas by 2050, says UN | UN DESA | United Nations Department of Economic and Social Affairs. 2018-05-16*. Geneva: United Nations Department of Economic and Social Affairs (2018)
46. Smiley SL, Curtis A, Kiwango JP. Using spatial video to analyze and map the water-fetching path in challenging environments: a case study of Dar es Salaam, Tanzania. *Trop Med Infect Dis.* (2017) 2:8. doi: 10.3390/tropicalmed2020008
47. Curtis A, Blackburn JK, Widmer JM, Morris JG, Jr. A ubiquitous method for street scale spatial data collection and analysis in challenging urban environments: mapping health risks using spatial video in Haiti. *Int J Health Geograph.* (2013) 12:21. doi: 10.1186/1476-072X-12-21
48. Maciel EA, de Carvalho AL, Nascimento SF, de Matos RB, Gouveia EL, Reis MG, et al. Household transmission of leptospira infection in urban slum communities. *PLoS Negl Trop Dis.* (2008) 2:e154. doi: 10.1371/journal.pntd.0000154
49. CDC. *Preventing ticks in the yard*. (2019). Available online at: https://www.cdc.gov/ticks/avoid/in_the_yard.html (accessed February 14, 2019).
50. Hotez PJ. *Forgotten People, Forgotten Diseases : the Neglected Tropical Diseases and Their Impact on Global Health and Development*. Washington, DC: ASM Press (2013). doi: 10.1128/9781555818753
51. Lizzi KM, Qualls WA, Brown SC, Beier JC. Expanding integrated vector management to promote healthy environments. *Trends Parasitol.* (2014) 30:394–400. doi: 10.1016/j.pt.2014.06.001
52. Vethaak AD, Leslie HA. Plastic debris is a human health issue. *Environ Sci Technol.* (2016) 50:6825–6. doi: 10.1021/acs.est.6b02569
53. Hupert N. Who's invading whom? Zika and intergenerational public health. *Soc Res*. (2017) 84:83–105.
54. Knudsen AB, Slooff R. Vector-borne disease problems in rapid urbanization: new approaches to vector control. *Bull World Health Organ.* (1992) 70:1–6.
55. Ault SK. Environmental management: a re-emerging vector control strategy. *Am J Trop Med Hyg.* (1994) 50(Suppl. 6):35–49. doi: 10.4269/ajtmh.1994.50.35
56. Lee YS. Urban planning and vector control in Southeast Asian cities. *Gaoxiong Yi Xue Ke Xue Za Zhi.* (1994) 10 (Suppl.):S39–51.
57. Lines J, Harpham T, Leake C, Schofield C. Trends, priorities and policy directions in the control of vector-borne diseases in urban environments. *Health Policy Plan.* (1994) 9:113–29. doi: 10.1093/heapol/9.2.113
58. Stettler A. Changes in prevention of infection: a historic retrospect. *Ther Umsch.* (1991) 48:205–9.
59. Alam P, Ahmade K. Impact of solid waste on health and the environment. *Int J Sustain Dev Green Econ.* (2013) 2:165–8. Available online at: https://pdfs.semanticscholar.org/2ae9/675a58adb025fb799703750cd477ca838bab.pdf
60. World Health Organization. *Inheriting a Sustainable World? Atlas on Children's Health and the Environment*. Geneva, Switzerland: World Health Organization (2017).

61. Gubler DJ. Dengue, urbanization and globalization: the unholy trinity of the 21(st) century. *Trop Med Health.* (2011) 39 (Suppl. 4):3–11. doi: 10.2149/tmh.2011-S05
62. Kay BH. Intersectoral approaches to dengue vector control. *Gaoxiong Yi Xue Ke Xue Za Zhi.* (1994) 10 (Suppl.):S56–61.
63. Chang AY, Fuller DO, Carrasquillo O, Beier JC. Social justice, climate change, and dengue. *Health Hum Rights.* (2014) 16:93–104. Available online at: www.jstor.org/stable/healhumarigh.16.1.93
64. Graczyk TK, Knight R, Tamang L. Mechanical transmission of human protozoan parasites by insects. *Clin Microbiol Rev.* (2005) 18:128–32. doi: 10.1128/CMR.18.1.128-132.2005
65. Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. *Trends Parasitol.* (2006) 22:552–7. doi: 10.1016/j.pt.2006.09.004
66. Mattiello A, Chiodini P, Bianco E, Forgione N, Flammia I, Gallo C, et al. Health effects associated with the disposal of solid waste in landfills and incinerators in populations living in surrounding areas: a systematic review. *Int J Public Health.* (2013) 58:725–35. doi: 10.1007/s00038-013-0496-8
67. Karbalaei S, Hanachi P, Walker TR, Cole M. Occurrence, sources, human health impacts and mitigation of microplastic pollution. *Environ Sci Poll Res Int.* (2018) 25:36046–63. doi: 10.1007/s11356-018-3508-7
68. Hossain MS, Santhanam A, Norulaini NAN, Omar AKM. Clinical solid waste management practices and its impact on human health and environment–a review. *Waste Manag.* (2011) 31:754–66. doi: 10.1016/j.wasman.2010.11.008
69. World Health Organization. *Annex C: WHO Regional Groupings*. Geneva: WHO (2017).
70. Hayes JM, Garcia-Rivera E, Flores-Reyna R, Suárez-Rangel G, Rodríguez-Mata T, Coto-Portillo R, et al. Risk factors for infection during a severe dengue outbreak in El Salvador in 2000. *Am J Trop Med Hyg.* (2003) 69:629–33. doi: 10.4269/ajtmh.2003.69.629
71. Brunkard JM, Robles López JL, Ramirez J, Cifuentes E, Rothenberg SJ, Hunsperger, EA, et al. Dengue fever seroprevalence and risk factors, Texas-Mexico border, 2004. *Emerg Infect Dis.* (2007) 13:1477–83. doi: 10.3201/eid1310.061586
72. Perez-Guerra CL, Zielinski-Gutierrez E, Vargas-Torres D, Clark GG. Community beliefs and practices about dengue in Puerto Rico. *Revis Panam Salud Publica.* (2009) 25:218–26. doi: 10.1590/S1020-49892009000300005
73. Heukelbach J, de Oliveira FA, Kerr-Pontes LR, Feldmeier H. Risk factors associated with an outbreak of dengue fever in a favela in Fortaleza, north-east Brazil. *Trop Med Int Health.* (2001) 6:635–42. doi: 10.1046/j.1365-3156.2001.00762.x
74. Kenneson A, Beltran-Ayala E, Borbor-Cordova MJ, Polhemus ME, Ryan SJ, Endy TP, et al. Social-ecological factors and preventive actions decrease the risk of dengue infection at the household-level: results from a prospective dengue surveillance study in Machala, Ecuador. *PLoS Negl Trop Dis.* (2017) 11:e0006150. doi: 10.1371/journal.pntd.0006150
75. Suwannapong N, Tipayamongkholgul M, Bhumiratana A, Boonshuyar C, Howteerakul N, Poolthin S. Effect of community participation on household environment to mitigate dengue transmission in Thailand. *Trop Biomed.* (2014) 31:149–58.
76. Lippi CA, Stewart-Ibarra AM, Munoz AG, Borbor-Cordova MJ, Mejía R, Rivero K, et al. The social and spatial ecology of dengue presence and burden during an outbreak in Guayaquil, Ecuador, 2012. *Int J Environ Res Public Health.* (2018) 15:E827. doi: 10.3390/ijerph15040827
77. Cordeiro R, Donalisio MR, Andrade VR, Mafra ACN, Nucci LB, Brown JC, et al. Spatial distribution of the risk of dengue fever in southeast Brazil, 2006–2007. *BMC Public Health.* (2011) 11:355. doi: 10.1186/1471-2458-11-355
78. Chen B, Yang J, Luo L, Yang Z, Liu Q. Who is vulnerable to dengue fever? a community survey of the 2014 outbreak in Guangzhou, China. *Int J Environ Res Public Health.* (2016) 13:712. doi: 10.3390/ijerph13070712
79. Vijayakumar K, Sudheesh Kumar TK, Nujum ZT, Umarul F, Kuriakose A. A study on container breeding mosquitoes with special reference to *Aedes* (Stegomyia) aegypti and *Aedes* albopictus in Thiruvananthapuram district, India. *J Vector Borne Dis.* (2014) 51:27–32.
80. Rohani A, Aidil Azahary AR, Malinda M, Zurainee MN, Rozilawati H, Wan Najdah WM, et al. Eco-virological survey of Aedes mosquito larvae in selected dengue outbreak areas in Malaysia. *J Vector Borne Dis.* (2014) 51:327–32.
81. Boornema AR, Senthil Murugan TK. Breeding habitats of *Aedes aegypti* mosquitoes and awareness about prevention of dengue in urban Chidambaram: a cross sectional study. *Int J Commun Med Public Health.* (2018) 5:10. doi: 10.18203/2394-6040.ijcmph20184014
82. Kusumawathie PHD, Fernando WP. Breeding habitats of *Aedes aegypti* Linnaeus and Ae. albopictus Skuse in a dengue transmission area in Kandy, Sri Lanka. *Ceylon J Med Sci.* (2003) 46:51–9. doi: 10.4038/cjms.v46i2.4829
83. Focks DA, Brenner RJ, Chadee DD, Trosper JH. The use of spatial analysis in the control and risk assessment of vector-borne diseases. *Am Entomol.* (1999) 45: 173–83. doi: 10.1093/ae/45.3.173
84. Chen YR, Hwang JS, Guo YJ. Ecology and control of dengue vector mosquitoes in Taiwan. *Gaoxiong Yi Xue Ke Xue Za Zhi.* (1994) 10 (Suppl.):S78–87.
85. Abeyewickreme W, Wickremasinghe AR, Karunatilake K, Sommerfeld J, Axel K. Community mobilization and household level waste management for dengue vector control in Gampaha district of Sri Lanka; an intervention study. *Pathog Glob Health.* (2012) 106:479–87. doi: 10.1179/2047773212Y.0000000060
86. Tana S, Umniyati S, Petzold M, Kroeger A, Sommerfeld J. Building and analyzing an innovative community-centered dengue-ecosystem management intervention in Yogyakarta, Indonesia. *Pathog Glob Health.* (2012) 106:469–78. doi: 10.1179/2047773212Y.0000000062
87. Sommerfeld J, Kroeger A. Eco-bio-social research on dengue in Asia: a multicountry study on ecosystem and community-based approaches for the control of dengue vectors in urban and peri-urban Asia. *Pathog Glob Health.* (2012) 106:428–35. doi: 10.1179/2047773212Y.0000000055
88. Campos MC, Dombrowski JG, Phelan J, Marinho CRF, Hibberd M, Clark TG, et al. Zika might not be acting alone: using an ecological study approach to investigate potential co-acting risk factors for an unusual pattern of microcephaly in Brazil. *PLoS ONE.* (2018) 13:e0201452. doi: 10.1371/journal.pone.0201452
89. Aguiar BS, Lorenz C, Virginio F, Suesdek L, Chiaravalloti-Neto F. Potential risks of Zika and chikungunya outbreaks in Brazil: a modeling study. *Int J Infect Dis.* (2018) 70:20–9. doi: 10.1016/j.ijid.2018.02.007
90. Aoustin T. Chikungunya and urban sprawl on Reunion Island. *Med Trop.* (2012) 72:51–59. Available online at: https://www.jle.com/fr/MedSanteTrop/2012/72.1/051-059%20Chikungunya%20et%20habitat%20informel%20(Aoustin).pdf
91. Abramides GC, Roiz D, Guitart R, Quintana S, Gimenez N. Control of the Asian tiger mosquito (*Aedes* albopictus) in a firmly established area in Spain: risk factors and people's involvement. *Trans Royal Soc Trop Med Hygiene.* (2013) 107:706–14. doi: 10.1093/trstmh/trt093
92. Alencar CHM. Infestation by *Aedes* albopictus (skuse) in natural and artificial breeding sites found in green areas in the city of Fortaleza, Ceará. *J Venom Anim Toxin Includ Trop Dis.* (2009) 15:582. doi: 10.1590/S1678-91992009000300018
93. Rao BB, George B. Breeding patterns of *Aedes* stegomyia albopictus in periurban areas of Calicut, Kerala, India. *Southeast Asian J Trop Med Public Health.* (2010) 41:536–40.
94. Fonseca DM, Unlu I, Crepeau T, Farajollahi A, Healy SP, Bartlett-Healy K, et al. Area-wide management of Aedes albopictus. Part 2: gauging the efficacy of traditional integrated pest control measures against urban container mosquitoes. *Pest Manag Sci.* (2013) 69:1351–61. doi: 10.1002/ps.3511
95. Getachew D, Tekie H, Gebre-Michael T, Balkew M, Mesfin A. Breeding sites of *Aedes aegypti*: potential dengue vectors in Dire Dawa, East Ethiopia. *Interdiscip Perspect Infect Dis.* (2015) 2015:706276. doi: 10.1155/2015/706276
96. Vikram K, Nagpal BN, Pande V, Srivastava A, Gupta SK, Anushrita, et al. Comparison of Ae. aegypti breeding in localities of different socio-economic groups of Delhi, India. *Int J Mosquito Res.* (2015) 2:83—8.
97. Ngugi HN, Mutuku FM, Ndenga BA, Musunzaji PS, Mbakaya JO, Aswani P, et al. Characterization and productivity profiles of *Aedes aegypti* (L.) breeding habitats across rural and urban landscapes in western and coastal Kenya. *Parasit Vectors.* (2017) 10:331. doi: 10.1186/s13071-017-2271-9
98. Espinosa M, Weinberg D, Rotela CH, Polop F, Abril M, Scavuzzo CM. Temporal dynamics and spatial patterns of *Aedes aegypti* breeding sites, in the context of a dengue control program in Tartagal (Salta

Province, Argentina). *PLoS Negl Trop Dis.* (2016) 10:e0004621. doi: 10.1371/journal.pntd.0004621
99. Raju AK. Community mobilization in *Aedes aegypti.* control programme by source reduction in peri-urban district of Lautoka, Viti Levu, Fiji Islands. *Dengue Bull.* (2003) 27:149–155. Available online at: https://apps.who.int/iris/bitstream/handle/10665/163791/dbv27p149.pdf;sequence=1
100. Hiriyan J, Tewari SC, Tyagi BK. Aedes albopictus (Skuse) breeding in plastic cups around tea-vendor spots in Ernakulam City, Kerala State, India. *Dengue Bull.* (2003) 27:195–6.
101. Jain J, Kushwah RBS, Singh SS, Sharma A, Adak T, Singh OP, et al. Evidence for natural vertical transmission of chikungunya viruses in field populations of *Aedes aegypti* in Delhi and Haryana states in India-a preliminary report. *Acta Trop.* (2016) 162:46–55. doi: 10.1016/j.actatropica.2016.06.004
102. Banerjee S, Aditya G, Saha GK. Household disposables as breeding habitats of dengue vectors: linking wastes and public health. *Waste Manag.* (2013) 33:233–9. doi: 10.1016/j.wasman.2012.09.013
103. Chena CD, Leeb HL, Stella-Wonga SP, Laua KW, Sofian-Aziruna M. Container survey of mosquito breeding sites in a university campus in Kuala Lumpur, Malaysia. *Dengue Bull.* (2009) 33:187–93.
104. Werneck GL, Rodrigues L, Santos MV, Araújo IB, Moura LS, Moreira S, et al. Household structure and urban services: neglected targets in the control of visceral leishmaniasis. *Ann Trop Med Parasitol.* (2005) 99:229–36. doi: 10.1179/136485905X28018
105. Singh SP, Reddy DC, Mishra RN, Sundar S. Knowledge, attitude, and practices related to Kala-azar in a rural area of Bihar state, India. *Am J Trop Med Hyg.* (2006) 75:505–8. doi: 10.4269/ajtmh.2006.75.505
106. Lima ID, Lima ALM, Mendes-Aguiar CO, Coutinho JFV, Wilson ME, Pearson RD, et al. Changing demographics of visceral leishmaniasis in northeast Brazil: lessons for the future. *PLoS Negl Trop Dis.* (2018) 12:e0006164. doi: 10.1371/journal.pntd.0006164
107. Bonfante-Cabarcas R, Rodríguez-Bonfante C, Oviol Vielma B, García D, Saldivia AM, Aldana E, et al. Seroprevalence for Trypanosoma cruzi infection and associated factors in an endemic area of Venezuela. *Cad Saude Publica.* (2011) 27:1917–29. doi: 10.1590/s0102-311x2011001000005
108. Garcia-Jordan N, Berrizbeitia M, Rodriguez J, Concepcion JL, Caceres A, Quinones W. Seroprevalence of Trypanosoma cruzi infection in the rural population of Sucre State, Venezuela. *Cad Saude Publica.* (2017) 33:e00050216. doi: 10.1590/0102-311x00050216
109. Dumonteil E, Nouvellet P, Rosecrans K, Ramirez-Sierra MJ, Gamboa-León R, Cruz-Chan V, et al. Eco-bio-social determinants for house infestation by non-domiciliated Triatoma dimidiata in the Yucatan Peninsula, Mexico. *PLoS Negl Trop Dis.* (2013) 7:e2466. doi: 10.1371/journal.pntd.0002466
110. Rosecrans K, Cruz-Martin G, King A, Dumonteil E. Opportunities for improved chagas disease vector control based on knowledge, attitudes and practices of communities in the yucatan peninsula, Mexico. *PLoS Negl Trop Dis.* (2014) 8:e2763. doi: 10.1371/journal.pntd.0002763
111. Abbasi E, Rafinejad J, Hosseinpoor S, Gholami-Borujeni F, Gholizadeh S. Diversity of arthropods in municipal solid waste landfill of Urmia, Iran. *J Med Entomol.* (2019) 56:268–70. doi: 10.1093/jme/tjy187
112. Ahmad SS, Aziz N, Butt A, Shabbir R, Erum S. Spatio-temporal surveillance of water based infectious disease (malaria) in Rawalpindi, Pakistan using geostatistical modeling techniques. *Environ Monitor Assess.* (2015) 187:555. doi: 10.1007/s10661-015-4779-9
113. Socolovschi C, Angelakis E, Renvoise A, Fournier PE, Marié JL, Davoust B, et al. Strikes, flooding, rats, and leptospirosis in Marseille, France. *Int J Infect Dis.* (2011) 15:e710–5. doi: 10.1016/j.ijid.2011.05.017
114. Reis RB, Ribeiro GS, Felzemburgh RD, Santana FS, Mohr S, Melendez AXTO, et al. Impact of environment and social gradient on Leptospira infection in urban slums. *PLoS Negl Trop Dis.* (2008) 2:e228. doi: 10.1371/journal.pntd.0000228
115. Santos IOC, Landi MFA, Cruz LM, Bofill MIR, Santos DED, Lima EMM, et al. Human leptospirosis in the Federal District, Brazil, 2011–2015: eco-epidemiological characterization. *Rev Soc Bras Med Trop.* (2017) 50:777–82. doi: 10.1590/0037-8682-0234-2017
116. Barcellos C, Sabroza PC. Socio-environmental determinants of the leptospirosis outbreak of 1996 in western Rio de Janeiro: a geographical approach. *Int J Environ Health Res.* (2000) 10:301–13. doi: 10.1080/0960312002001500
117. Barcellos C, Sabroza PC. The place behind the case: leptospirosis risks and associated environmental conditions in a flood-related outbreak in Rio de Janeiro. *Cad Saude Publica.* (2001) 17 (Suppl.):59–67. doi: 10.1590/S0102-311X2001000700014
118. Navegantes de Araujo W, Finkmoore B, Ribeiro GS, Reis RB, Felzemburgh RD, Hagan JE, et al. Knowledge, attitudes, and practices related to Leptospirosis among urban slum residents in Brazil. *Am J Trop Med Hyg.* (2013) 88:359–63. doi: 10.4269/ajtmh.2012.12-0245
119. Sarkar U, Nascimento SF, Barbosa R, Martins R, Nuevo H, Kalofonos I, et al. Population-based case-control investigation of risk factors for leptospirosis during an urban epidemic. *Am J Trop Med Hyg.* (2002) 66:605–10. doi: 10.4269/ajtmh.2002.66.605
120. Vallee J, Thaojaikong T, Moore CE, Phetsouvanh R, Richards AL, Souris M, et al. Contrasting spatial distribution and risk factors for past infection with scrub typhus and murine typhus in Vientiane City, Lao PDR. *PLoS Negl Trop Dis.* (2010) 4:e909. doi: 10.1371/journal.pntd.0000909
121. Milke M. Plague in Sydney and its solid waste lessons. *Waste Manag.* (2004) 24:321–3. doi: 10.1016/j.wasman.2004.02.006
122. Boisier P, Rahalison L, Rasolomaharo M, Ratsitorahina M, Mahafaly M, Razafimahefa M, et al. Epidemiologic features of four successive annual outbreaks of bubonic plague in Mahajanga, Madagascar. *Emerg Infect Dis.* (2002) 8:311–6. doi: 10.3201/eid0803.010250
123. Munoz-Zanzi C, Mason MR, Encina C, Astroza A, Romero A. Leptospira contamination in household and environmental water in rural communities in southern Chile. *Int J Environ Res Public Health.* (2014) 11:6666–80. doi: 10.3390/ijerph110706666
124. Ganoza CA, Matthias MA, Collins-Richards D, Brouwer KC, Cunningham CB, Segura ER, et al. Determining risk for severe leptospirosis by molecular analysis of environmental surface waters for pathogenic Leptospira. *PLoS Med.* (2006) 3:e308. doi: 10.1371/journal.pmed.0030308
125. Caldas EM, Sampaio MB. Leptospirosis in the city of Salvador, Bahia, Brazil: a case-control seroepidemiologic study. *Int J Zoonoses.* (1979) 6:85–96.
126. Benitez ADN, Martins FDC, Mareze M, Santos NJR, Ferreira FP, Martins CM, et al. Spatial and simultaneous representative seroprevalence of anti-Toxoplasma gondii antibodies in owners and their domiciled dogs in a major city of southern Brazil. *PLoS ONE.* (2017) 12:e0180906. doi: 10.1371/journal.pone.0180906
127. Kassir MF, El Zarif T, Kassir G, Berry A, Musharrafieh U, Bizri AR. Human rabies control in Lebanon: a call for action. *Epidemiol Infect.* (2018):1–8. doi: 10.1017/S095026881800300X
128. Banerjee S, Aditya G, Saha GK. Household wastes as larval habitats of dengue vectors: comparison between urban and rural areas of Kolkata, India. *PLoS ONE.* (2015) 10:e0138082. doi: 10.1371/journal.pone.0138082
129. Krystosik AR, Curtis A, Buritica P, Ajayakumar J, Squires R, Dávalos D, et al. Community context and sub-neighborhood scale detail to explain dengue, chikungunya and Zika patterns in Cali, Colombia. *PLoS ONE.* (2017) 12:e0181208. doi: 10.1371/journal.pone.0181208
130. Gayan Dharmasiri AG, Perera AY, Harishchandra J, Herath H, Aravindan K, Jayasooriya HTR, et al. First record of *Anopheles stephensi* in Sri Lanka: a potential challenge for prevention of malaria reintroduction. *Malaria J.* (2017) 16:326. doi: 10.1186/s12936-017-1977-7
131. Thomas S, Ravishankaran S, Justin JA, Asokan A, Mathai MT, Valecha N, et al. Overhead tank is the potential breeding habitat of Anopheles stephensi in an urban transmission setting of Chennai, India. *Malar J.* (2016) 15:274. doi: 10.1186/s12936-016-1321-7
132. CDC. *Diseases Directly Transmitted by Rodents*. (2017). Available online at: https://www.cdc.gov/rodents/diseases/ (accessed February 28, 2019).
133. Hawken P. *Drawdown—The Most Comprehensive Plan Ever Proposed to Reverse Global Warming*. New York, NY: Penguin Books (2017).
134. Hakkens D. *Precious Plastics*. (2019). Available online at: https://preciousplastic.com/ (accessed February 1, 2019).
135. Schlesinger ME. *Aluminum Recycling*. Boca Raton, FL: CRC Press (2013). doi: 10.1201/b16192
136. Das SK, Yin W. The worldwide aluminum economy: the current state of the industry. *JOM.* (2007) 59:57–63. doi: 10.1007/s11837-007-0142-0
137. Pauliuk S, Wang T, Müller DB. Moving toward the circular economy: the role of stocks in the Chinese steel cycle. *Environ Sci Technol.* (2012) 46:148–54. doi: 10.1021/es201904c

138. Waste S. *SWANA Report: National Sword Impact and Solutions*. Waste360. (2019).
139. Shen L, Haufe J, Patel MK. Product overview and market Projection of emerging bio-based plastics copernicus institute for sustainable development and innovation, Utrecht University, The Netherlands: European polysaccharide network of excellence. *Eur Bioplast.* (2009) 1–243.
140. Zhang X, Fevre M, Jones GO, Waymouth RM. Catalysis as an enabling science for sustainable polymers. *Chem Rev.* (2018) 118:839–85. doi: 10.1021/acs.chemrev.7b00329
141. Pathak S, Sneha CLR, Mathew BB. Bioplastics: its timeline based scenario and challenges. *J Polym Biopolym Phys Chem.* (2014) 2:84–90.
142. Erich M, Hannes G, Maximilian L. PHB – bio based and biodegradable replacement for PP: a review. *Nov Tech Nutri Food Sci.* 2:1–4. doi: 10.31031/NTNF.2018.02.000546
143. Pena-Francesch A, Demirel MC. Squid-inspired tandem repeat proteins: functional fibers and films. *Front Chem.* (2019) 7:69. doi: 10.3389/fchem.2019.00069
144. Niranjana Prabhu T, Prashantha K. A review on present status and future challenges of starch based polymer films and their composites in food packaging applications. *Polym Compos.* (2018) 39:2499–522. doi: 10.1002/pc.24236
145. Banerjee A, Dick GR, Yoshino T, Kanan MW. Carbon dioxide utilization via carbonate-promoted C–H carboxylation. *Nature.* (2016) 531:215. doi: 10.1038/nature17185
146. Ottesen V, Kumar V, Toivakka M, Carrasco GC, Syverud K, Gregersen Ø. Viability and properties of roll-to-roll coating of cellulose nanofibrils on recycled paperboard. *Nordic Pulp Paper Res J.* 32:179–88. doi: 10.3183/npprj-2017-32-02-p179-188
147. Banerjee A, Chatterjee K, Madras G. Enzymatic degradation of polymers: a brief review. *Mater Sci Technol.* (2014) 30:567–73. doi: 10.1179/1743284713Y.0000000503
148. Zheng Y, Yanful EK, Bassi AS. A review of plastic waste biodegradation. *Crit Rev Biotechnol.* (2005) 25:243–50. doi: 10.1080/07388550500346359
149. Narancic T, Verstichel S, Reddy Chaganti S, Morales-Gamez L, Kenny ST, De Wilde, Bet al. Biodegradable plastic blends create new possibilities for end-of-life management of plastics but they are not a panacea for plastic pollution. *Environ Sci Technol.* (2018) 52:10441–52. doi: 10.1021/acs.est.8b02963
150. Russell JR, Huang J, Anand P, Kucera K, Sandoval AG, Dantzler KW, et al. Biodegradation of polyester polyurethane by endophytic fungi. *Appl Environ Microbiol.* (2011) 77:6076–84. doi: 10.1128/AEM.00521-11
151. Khan S, Nadir S, Shah ZU, Shah AA, Karunarathna SC, Xu J, et al. Biodegradation of polyester polyurethane by *Aspergillus tubingensis*. *Environ Pollut.* (2017) 225:469–80. doi: 10.1016/j.envpol.2017.03.012
152. Yoshida S, Hiraga K, Takehana T, Taniguchi I, Yamaji H, Maeda Y, et al. A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science.* (2016) 351:1196. doi: 10.1126/science.aad6359
153. Yang Y, Yang J, Wu W-M, Zhao J, Song Y, Gao L, et al. Biodegradation and mineralization of polystyrene by plastic-eating mealworms: part 2. Role of gut microorganisms. *Environ Sci Technol.* (2015) 49:12087–93. doi: 10.1021/acs.est.5b02663
154. Yang Y, Yang J, Wu W-M, Zhao J, Song Y, Gao L, et al. Biodegradation and mineralization of polystyrene by plastic-eating mealworms: part 1. Chemical and physical characterization and isotopic tests. *Environ Sci Technol.* (2015) 49:12080–6. doi: 10.1021/acs.est.5b02661
155. Bombelli P, Howe CJ, Bertocchini F. Polyethylene bio-degradation by caterpillars of the wax moth *Galleria mellonella*. *Curr Biol.* (2017) 27:R292–3. doi: 10.1016/j.cub.2017.02.060
156. Narancic T, O'Connor KE. Plastic waste as a global challenge: are biodegradable plastics the answer to the plastic waste problem? *Microbiology.* (2018). 165:129–37. doi: 10.1099/mic.0.000749
157. Temple EK, Rose E. Sustainable construction in rural Guatemala. *Archiv Dis Childhood.* (2011) 96:1048–51. doi: 10.1136/adc.2010.192641
158. Katz D. *Plastic Bank*. (2019). Available online at: https://www.plasticbank.com (accessed April 17, 2019).
159. PSF. *Plastic Soup Foundation*. (2019). Available online at: https://www.plasticsoupfoundation.org/2019 (accessed April 17, 2019).
160. Goodnet. *How Indonesia Turns Old Garbage into Free Healthcare*. (2015).
161. Hinde D. *The Swedish Recycling Revolution*. (2019). Available online at: https://sweden.se/nature/the-swedish-recycling-revolution/ (accessed April 29, 2019).
162. Spee T, Huizer D. Comparing REACH chemical safety assessment information with practice-a case-study of polymethylmethacrylate (PMMA) in floor coating in The Netherlands. *Int J Hyg Environ Health.* (2017) 220:1190–4. doi: 10.1016/j.ijheh.2017.05.012
163. He Z, Li G, Chen J, Huang Y, An T, Zhang C. Pollution characteristics and health risk assessment of volatile organic compounds emitted from different plastic solid waste recycling workshops. *Environ Int.* (2015) 77:85–94. doi: 10.1016/j.envint.2015.01.004
164. National Institute for Occupational Safety and Health. *Health and Safety Guide for Plastic Fabricators*. Cincinnati, OH: U.S. Dept. of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Division of Technical Services (1975).
165. Kumar A, Samadder SR. A review on technological options of waste to energy for effective management of municipal solid waste. *Waste Manag.* (2017) 69:407–22. doi: 10.1016/j.wasman.2017.08.046
166. Moya D, Aldas C, Lopez G, Kaparaju P. Municipal solid waste as a valuable renewable energy resource: a worldwide opportunity of energy recovery by using Waste-To-Energy Technologies. *Enrgy Proced.* (2017) 134:286–95. doi: 10.1016/j.egypro.2017.09.618
167. Brunner PH, Rechberger H. Waste to energy–key element for sustainable waste management. *Waste Manag.* (2015) 37:3–12. doi: 10.1016/j.wasman.2014.02.003
168. Bosmans A, Vanderreydt I, Geysen D, Helsen L. The crucial role of Waste-to-Energy technologies in enhanced landfill mining: a technology review. *J Clean Prod.* (2013) 55:10–23. doi: 10.1016/j.jclepro.2012.05.032
169. Stehlik P. Contribution to advances in waste-to-energy technologies. *J Clean Prod.* (2009) 17:919–31. doi: 10.1016/j.jclepro.2009.02.011
170. Marshall RE, Farahbakhsh K. Systems approaches to integrated solid waste management in developing countries. *Waste Manag.* (2013) 33:988–1003. doi: 10.1016/j.wasman.2012.12.023
171. Chattopadhyay S, Dutta A, Ray S. Municipal solid waste management in Kolkata, India—a review. *Waste Manag.* (2009) 29:1449–58. doi: 10.1016/j.wasman.2008.08.030
172. Tabasova A, Kropac J, Kermes V, Nemet A, Stehlik P. Waste-to-energy technologies: impact on environment. *Energy.* (2012) 44:146–55. doi: 10.1016/j.energy.2012.01.014
173. Kothari R, Tyagi VV, Pathak A. Waste-to-energy: a way from renewable energy sources to sustainable development. *Renew Sust Energ Rev.* (2010) 14:3164–70. doi: 10.1016/j.rser.2010.05.005
174. Novak RJ. A North American model to contain the spread of *Aedes* albopictus through tire legislation. *Parassitologia.* (1995) 37:129–39.
175. Forum WE. *India Will Abolish All Single-Use Plastic by 2022, vows Narendra Modi*. (2019). Available online at: https://www.weforum.org/agenda/2018/06/india-will-abolish-all-single-use-plastic-by-2022-vows-narendra-modi/ (accessed May 7, 2019).
176. Haregu TN, Ziraba AK, Aboderin I, Amugsi D, Muindi K, Mberu B. An assessment of the evolution of Kenya's solid waste management policies and their implementation in Nairobi and Mombasa: analysis of policies and practices. *Environ Urban.* (2017) 29:515–32. doi: 10.1177/0956247817700294
177. Fernandes JN, Moise IK, Maranto GL, Beier JC. Revamping mosquito-borne disease control to tackle future threats. *Trends Parasitol.* (2018) 34:359–68. doi: 10.1016/j.pt.2018.01.005
178. Gyawali N, Bradbury RS, Aaskov JG, Taylor-Robinson AW. Neglected Australian arboviruses and undifferentiated febrile illness: addressing public health challenges arising from the 'developing Northern Australia' government policy. *Front Microbiol.* (2017) 8:2150. doi: 10.3389/fmicb.2017.02150
179. Hotez PJ, Murray KO. Dengue, West Nile virus, chikungunya, Zika-and now Mayaro? *PLoS Negl Trop Dis.* (2017) 11:e0005462. doi: 10.1371/journal.pntd.0005462
180. Schnurr REJ, Alboiu V, Chaudhary M, Corbett RA, Quanz ME, Sankar K, et al. Reducing marine pollution from single-use plastics (SUPs): a review. *Mar Pollut Bull.* (2018) 137:157–71. doi: 10.1016/j.marpolbul.2018.10.001

181. Gutberlet J. Cooperative urban mining in Brazil: collective practices in selective household waste collection and recycling. *Waste Manag.* (2015) 45:22–31. doi: 10.1016/j.wasman.2015.06.023
182. I Got Garbage. *I Got Garbage*. (2019). Available online at: https://www.igotgarbage.com/what-we-do/2019 (accessed April 17, 2019).
183. Xiao LS, Zhang GQ, Zhu Y, Lin T. Promoting public participation in household waste management: a survey based method and case study in Xiamen city, China. *J Clean Prod.* (2017) 144:313–22. doi: 10.1016/j.jclepro.2017.01.022
184. Xanthos D, Walker TR. International policies to reduce plastic marine pollution from single-use plastics (plastic bags and microbeads): a review. *Mar Pollut Bull.* (2017) 118:17–26. doi: 10.1016/j.marpolbul.2017.02.048
185. Capolongo S, Rebecchi A, Dettori M, Appolloni L, Azara A, Buffoli M, et al. Healthy design and urban planning strategies, actions, and policy to achieve salutogenic cities. *Int J Environ Res Public Health.* (2018) 15:E2698. doi: 10.3390/ijerph15122698
186. Babiak K, Trendafilova S. CSR and environmental responsibility: motives and pressures to adopt green management practices. *Corp Soc Responsib Environ Manag.* (2011) 18:11–24. doi: 10.1002/csr.229
187. Adebanjo D, Pei-Lee T, Ahmed PK. The impact of external pressure and sustainable management practices on manufacturing performance and environmental outcomes. *Int J Operat Prod Manag.* (2016) 36:995–1013. doi: 10.1108/IJOPM-11-2014-0543
188. Eagle L, Hamann M, Low DR. The role of social marketing, marine turtles and sustainable tourism in reducing plastic pollution. *Mar Pollut Bull.* (2016) 107:324–32. doi: 10.1016/j.marpolbul.2016.03.040
189. Belontz SL, Corcoran PL, Davis H, Hill KA, Jazvac K, Robertson K, et al. Embracing an interdisciplinary approach to plastics pollution awareness and action. *Ambio.* (2018) 48:855–66. doi: 10.1007/s13280-018-1126-8
190. Hammami MBA, Mohammed EQ, Hashem AM, Al-Khafaji MA, Alqahtani F, Alzaabi S, et al. Survey on awareness and attitudes of secondary school students regarding plastic pollution: implications for environmental education and public health in Sharjah city, UAE. *Environ Sci Pollut Res Int.* (2017) 24:20626–33. doi: 10.1007/s11356-017-9625-x
191. Walther BA, Kunz A, Hu CS. Type and quantity of coastal debris pollution in Taiwan: a 12-year nationwide assessment using citizen science data. *Mar Pollut Bull.* (2018) 135:862–72. doi: 10.1016/j.marpolbul.2018.08.025
192. Dhokhikah Y, Trihadiningrum Y, Sunaryo S. Community participation in household solid waste reduction in Surabaya, Indonesia. *Resour Conserv Recy.* (2015) 102:153–62. doi: 10.1016/j.resconrec.2015.06.013
193. Ruckstuhl NA. Voluntary beach cleanups at famara beach, lanzarote—fighting marine litter invasion and accumulation locally. In: Baztan J, Jorgensen B, Pahl S, Thompson RC, Vanderlinden J-P, editors. *Fate and Impact of Microplastics in Marine Ecosystems*. Cambridge, MA: Elsevier (2017). p. 24. doi: 10.1016/B978-0-12-812271-6.00212-X
194. Konecny C, Fladmark V, De la Puente S. Towards cleaner shores: Assessing the Great Canadian Shoreline Cleanup's most recent data on volunteer engagement and litter removal along the coast of British Columbia, Canada. *Mar Pollut Bull.* (2018) 135:411–7. doi: 10.1016/j.marpolbul.2018.07.036
195. Sekito T, Prayogo TB, Dote Y, Yoshitake T, Bagus I. Influence of a community-based waste management system on people's behavior and waste reduction. *Resour Conserv Recy.* (2013) 72:84–90. doi: 10.1016/j.resconrec.2013.01.001
196. Tauil MC, de Azevedo AC. Community participation in health activities in an Amazon community of Brazil. *Bull Pan Am Health Organ.* (1978) 12:95–103.
197. Dung-Gwom JY, Yakubu Magaji J. The environmental health problems associated with solid waste management in Gwagwalada-Abuja. *Abuja J Geogr Dev.* (2007) 1:110–126.
198. Catapreta CA, Heller L. Association between household solid waste disposal and health, Belo Horizonte (MG), Brasil. *Rev Panam Salud Publica.* (1999) 5:88–96. doi: 10.1590/S1020-49891999000200003
199. Andrews JR, Barkume C, Yu AT, Saha SK, Qamar FN, Garrett D, et al. Integrating facility-based surveillance with healthcare utilization surveys to estimate enteric fever incidence: methods and challenges. *J Infect Dis.* (2018). doi: 10.1093/infdis/jiy494
200. Luby SP, Saha S, Andrews JR. Towards sustainable public health surveillance for enteric fever. *Vaccine.* (2015) 33 (Suppl. 3):C3–7. doi: 10.1016/j.vaccine.2015.02.054
201. Christensen PR, Scheuermann AM, Loeffler KE, Helms BA. Closed-loop recycling of plastics enabled by dynamic covalent diketoenamine bonds. *Nat Chem.* (2019) 11:442–8. doi: 10.1038/s41557-019-0249-2
202. Picheta R, Dean S. *Over 180 Countries—Not Including the US—Agree to Restrict Global Plastic Waste Trade. CNN, 9:39 AM ET, Sat May 11, 2019*. (2019). Available online at: https://www.cnn.com/2019/05/11/world/basel-convention-plastic-waste-trade-intl/index.html?no-st=1558034809 (accessed May 16, 2019).

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CASE REPORT
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Umbilical Myiasis by *Cochliomyia hominivorax* in an Infant in Colombia

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Myasis is the infestation by fly larvae (Diptera) in live vertebrates including humans. Myasis has been reported most commonly in tropical and subtropical areas around the world with poor sanitation and presence of cattle. Neonatal umbilical myiasis is an important cause of death in bovines and produces major economic losses in the livestock industry. However, its presentation in humans is rare, with a few cases reported worldwide. Moreover, umbilical myasis can be life-treating due to the risk of larvae migration to deeper tissues of the abdomen, omphalitis, and sepsis. We describe the case of a 7-day-old infant admitted to the hospital due to umbilical cord myiasis. In total, 55 larvae were removed from the wound and identified as *Cochliomyia hominivorax.* The patient recovered satisfactorily after treatment with ivermectin and amoxicillin. A literature search was performed in Pubmed, Medline, Lilacs and Google Scholar, with 64 cases of myasis by *C. hominivorax* being reviewed. Oral cavity, wounds, scalp and natural orifices are the main affected anatomical areas. Risk factors include the extremes of age, male sex, poor hygiene, alcohol and drug use, cancer, and mental disability. Programs for human myiasis prevention and surveillance are needed in neotropical areas where living conditions make it difficult to implement control strategies.

Keywords: screwworm infection, newborn, umbilicus, myiasis, ivermectin, Colombia

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INTRODUCTION

Myiasis is infestation by fly larvae (Diptera) in live vertebrates, including humans. Fly larvae feed on wound tissue of their host, causing a disease whose severity may depend on the larva species and anatomical sites affected (1, 2). It is widespread in neotropical areas around the world, causing economic and public health problems in low-income populations. Human infection is facilitated by poor hygienic conditions and close contact with wild or domestic animals (2, 3). Umbilical cord myiasis is a common type of wound myiasis in animals but it has been described only rarely in humans (2). We present the first report of neonatal umbilical myiasis in Colombia and review the most relevant aspects of this disease. Recent case reports of myiasis by *C. hominivorax* are reviewed in the discussion.

CASE REPORT

The research procedures for this case were carried out in accordance with the recommendations of the guidelines of the Helsinki Committee. Written informed consent was obtained from the mother of the newborn for pictures and publication of this case.

A 7-day-old female neonate was taken to a primary health facility in June 2017 because something was coming out of her umbilicus. The patient was born full-term at the local hospital by vaginal delivery from a 17-year-old mother. At birth, the newborn had respiratory depression and mild perinatal asphyxia but without further complications. The umbilical cord was cut following standard care measures for in-hospital delivery. The patient lived on a farm with a cowshed next to the house in the rural area of the municipality of La Virginia (04°54′1.617″ N, 75°52′47.445″ W), in the state of Risaralda, located in the coffee region of Colombia. The mother practiced exclusive breastfeeding and used a fabric girdle, which is traditionally used in Colombia for protection of the umbilical stump during the first days of life.

The neonate was transferred to a tertiary care hospital. On admission, she was visibly irritated and jaundiced. On physical exam, weight was 3,300 g and vital signs (temperature, heart rate, respiratory rate, blood pressure, and blood oxygen levels) were normal. Umbilical stump inspection revealed numerous live larvae (**Supplementary Figure 1**) and foul-smelling serohaematic secretion. The rest of the examination was normal. Initial blood count showed 20,140 leukocytes/μl (52% neutrophils, 3% eosinophils, 4% lymphocytes, and 5% monocytes). Total serum bilirubin was 18.0 mg/dl (cutoff point to consider phototherapy: 20.5 mg/dl) (4). Wound and blood cultures on admission and 48 h later were negative.

Initial treatment included covering the umbilical stump with gauze soaked in ivermectin and nitrofurazone, followed by a single oral dose of ivermectin (0.15 mg/kg). To prevent late-onset sepsis, intravenous ampicillin (200 mg/kg/day) and gentamicin (4 mg/kg/day) were administered. On the second day, 39 live larvae were removed from the umbilical stump under aseptic conditions using a surgical clamp. One live and 15 dead larvae were extracted on the third day. A follow-up abdominal ultrasonography was normal and the patient was discharged 7 days after admission.

After extraction, seven larvae were preserved in a solution containing 80% alcohol. The specimens were sent to an entomologist and examined using a microscope with 10× magnification. Third instar larvae of *C. hominivorax* were identified by their smooth appearance with prominent spine bands and one body process in the last segments (**Supplementary Figure 2**). Pigmented dorsal tracheal trunks were present in two to three of the last segments. The posterior spiracular plates contained three oval-shaped slips pointing to the peritreme (5).

DISCUSSION

Umbilical myiasis is a rare type of wound myiasis in humans, but the occurrence of cases in widely distributed areas shows that this may be a latent risk in all neotropic zones were myiasis has been reported (2). A handful of case reports of umbilical myiasis have been made, mainly in India (3, 6–13). One case was reported in the United States (14) and another in Argentina (15), the latter associated with *C. hominivorax.* The largest case collection of umbilical myiasis was carried out in Nigeria, where active detection in a region of the Niger Delta resulted in 55 cases of omphalitis (16). Other anatomical sites of myiasis in human neonates include the nostrils (17), ear (18), skin (19), and genitals (20).

The warm and moist environment of the umbilical stump attracts the female flies to lay their eggs on it (11). In our case, the use of an umbilical girdle could have retained moisture around the stump and delayed the separation, creating ideal conditions for larvae growth and also hiding the disease. Umbilical girdles were used traditionally to secure the navel of newborns (21) and remain a common practice in Colombia that goes against current recommendations to keep the stump uncovered to help dry out the base. The girdle also facilitates omphalitis, which in turn increases the size of the wound and creates a proper environment for egg hatching (11). Traditional methods for stump care, such as application of cow dung or herb leaves on the umbilicus of neonates, have been described in previous reports as sources of cross-contamination (16, 22).

Clinical signs of umbilical myiasis are hardly recognized by the caregiver. The disease is usually detected once the larvae are visible or clinical signs of omphalitis appear (11). Imaging and biopsy are rarely necessary for diagnosis but may be useful in umbilical myiasis to determine the extent of the infestation and any organ involvement. Leukocytosis along with neutrophilia and eosinophilia are common clinical findings (2). Hyperbilirubinaemia that resolved after larvae extraction was reported in one case of cutaneous myiasis by Drosophila in a newborn (23), but not in prior cases of neonatal umbilical myiasis.

Neonatal myiasis has been consistently attributed to conditions related to low socioeconomic status, such as poor hygiene, contact with farm animals, home delivery using unsterilized instruments and the use of traditional methods to take care of the stump (8, 9, 11, 24). Nonetheless, wound myiasis can also be an indicator of neglect or self-neglect (24). Thus, social counseling should be considered in these cases and newborn care must be reinforced. Adequate wound care, keeping the umbilicus covered with clean dressings and adequate hygienic habits in general should all be included in the recommendations given to the mother or caretaker before discharge (2, 14, 15).

The New World screwworm (*C. hominivorax*), is the most common species causing myiasis in Central and South America. The incidence of human myiasis by this species has been declining progressively since 1958 due to the implementation of programmes using the sterile insect technique (SIT) that have led to the eradication of *C. hominivorax* in Curacao, North and Central America and North Africa (25). Sixty-five case reports of human disease have been published from 2000 up to 30 September 2019 according to a literature search performed in Pubmed, Medline, Google Scholar and Lilacs (**Table 1**). Sixty of the cases (92%) occurred in South America, mainly in Brazil ($n = 31$, 48%) and Argentina ($n = 7$, 11%). There was one case report in India, but the species could have been mistakenly identified. Common anatomical sites of infection were the oral cavity, chronic or traumatic wounds, scalp and natural orifices (ear, nose, vagina). Risk factors for infection include the extreme

TABLE 1 | Cases of myiasis by *Cochliomyia hominivorax* published since 2000 in Pubmed, Medline, Google Scholar, and Lilacs.

Country	Age and sex	Location	Risk factors	*n* larvae	References
Chile	37 M	Ear	Travel	22	(26)
Brazil	17 F	Vulva	Pregnancy, condilomatosis	67	(27)
Brazil	8 M	Oral cavity	Leukoderma, oral breathing	19	(28)
Brazil	66 F	Oral cavity	Alcohol abuse	40	(29)
Argentina	36 M	Scalp	Poor higiene conditions, pediculosis	>40	(30)
French Guiana	70 M; NA; 40 M; 72 M; NA	Oral cavity; wound in toe; thigh ulcer; low limb ulcer; scalp	NA; Alcoholism; Ulceration; Ulceration; Pediculosis	NA	(31)
Brazil	77 F	Vulva	Mental disability, lack of social support	50	(32)
Brazil	41 M	Wound in dorsal antebrachium	Wound, adventure sports	1	(33)
Argentina	10 M	Eye protesis	Hydroxyapatite implant	20	(34)
Surinam	51 M	Ankles	Ulcer	>100	(35)
Brazil	80 M	Eye	Alcohol and tabaco abuse, lack of social support	NA	(36)
Venezuela	40 F	Thigh ulcer	Bedridden, epilepsy	20	(37)
French Guiana	84 M	Nose wound	Hospitalized	9	(38)
Brazil	87 F	Vagina	Obese, diabetic, hypertensive, low socio-economic status	NA	(39)
Brazil	55 M	Rhino-orbital area	Ethmoidal sinus carcinoma	NA	(40)
Colombia	79 M	Skin carcinoma in the eye orbit	Skin carcinoma in the eye orbit	NA	(41)
Brazil	27 F	Eye	NA	1	(42)
Cuba	60 M	Nasal tumor	Nasal tumor	>200	(43)
Brazil	63 M	Pharynx and esophagus	Mouth-breather	100	(44)
Argentina	58 M	Scalp and brain cavity	Tuberculosis	NA	(45)
Brazil	7 F	Periorbital	Cerebral palsy	NA	(46)
Argentina	11-day old	Umbilical stump	Newborn	23	(15)
India	46 M	Facial wound	Poor higiene conditions, low IQ	NA	(47)
Colombia	12 F	Scalp	Psoriasis	142	(48)
Brazil	22 M; 70 M	Wound from dental extraction; Palate	Wound from dental extraction, mental disability; Senile	24; NA	(49)
Brazil	30 M	Scalp	Homeless, smoker, drug user	518	(50)
Brazil	5 F	Oral cavity	Poor oral hygiene	2	(51)
Brazil	89 F	Uterine prolapse	Dementia, poverty	NA	(52)
Venezuela	32 M	Pin-site	Alcohol and drug abuse, external metalic bone fixator	105	(53)
Brazil	9 NA	Oral cavity	Poor oral hygiene, malnutrition	NA	(54)
Colombia	80 F	Nose	Malnutrition, nasal septum perforation	NA	(55)
Brazil	80 M	Orbital region	Rural area, living alone	NA	(36)
Brazil	72 M; 35 F	Oral cavity; periodontal area	Hospitalized; Alcohol consumption	NA	(56)
Colombia	7 F	Scalp	Poor higiene conditions, pediculosis	NA	(57)
Argentina	32 M	Wound in scalp	Drug user	71	(58)
Peru	62 M	Oral cavity	Parkinson	75	(59)
Brazil	49 M	Tracheostomy site	Alcohol and tabaco abuse, larynx cancer, poor hygiene conditions	20	(60)
Cuba	83 F; 87 M	Facial skin carcinoma; facial skin carcinoma	Alzheimer's, rural residency, skin carcinoma; Skin carcinoma	NA; NA	(61)
Argentina	11 M; 9 F	Ear	NA; Malnutrition, intestinal parasitosis	NA	(62)
North India	80 M	Wound in eyelid skin	Squamous cell carcinoma	NA	(63)
Haiti	16 F; 10 M	Wound in eye; facial wound	Earthquake victims	3 7	(64)
Brazil	97 M	Oral cavity	Multiple diseases, Bedridden	110	(65)
Brazil	22 cases between 2007 and 2008	Mostly open wounds	Age group 41–50 years old, black race, low level of education, low hygiene conditions and poor urban infrastructure	NA	(1)
Brazil	49 M	Thoracic cavity	Hospitalized, tracheostomy	32	(66)
Brazil	54 M	Oral cavity	Aphasia	NA	(67)

(Continued)

TABLE 1 | Continued

Country	Age and sex	Location	Risk factors	*n* larvae	References
Colombia	50 M; 29 M; 20 M; 35 M; 6 M; 58 M	Oral cavity	Craniofascial trauma, altered conciousness	30; 60; 39; 126; 105;81	(68)
Brazil	10 cases between 2005 and 2011	Oral or maxillofacial	Diabetes, mental disease, AIDS, mental impairment, depression	NA	(69)
Brazil	95 M	Oral cavity	Hospitalized	103	(70)
Brazil	59 M	Wound in shoulder	Wound	287	(71)
Brazil	38 M	Mouth	Trauma	55	(72)
Argentina	54 M	Diabetic food ulcer	Diabetic food ulcer	NA	(73)
Brazil	36 M	Oral cavity	Leukoderma, rural residency	75	(74)
Colombia	26 M	Pin-site	External metalic bone fixator	80	(75)
Dominican Republic	26 F	Ear	Alcohol consumption, travel	NA	(76)
Ecuador	24 F	Oral cavity	Brain damage, prolonged mouth opening	NA	(77)
Peru	67 M	Tracheostomy site	Tracheostomy, gastrostomy, esophageal cancer	NA	(78)
Peru	9-F	Scalp	Pediculosis	42	(79)
Brazil	22 M; 50 F; 45 F; 33 M; 26 M; 57 M; 21 F; 24 M; 65 M	Head and neck	Poor oral hygiene, trauma	NA	(80)
Brazil	41 F	Breast	Breast cancer	NA	(81)
Chile	26-F	Scalp	Seborrheic dermatitis	29	(82)
Brazil	41 F	Finger	Necrosis and amputation	132	(83)
Brazil	27 M	Scalp	Mental disability	27	(84)
Peru	71 M; 71 F; 67 F; 85 M; 73 F	Foot; nose; nose; breast	Skin eruption; Cellulite; Necrosis; Ulcera	NA	(85)
Colombia	77 M	Pin-site	Prosthetic material, chronic wound	100	(86)

NA, not available; F, female; M, male.

ages, male gender, rural residency, poor hygienic conditions, cancer, alcohol and drug use, malnutrition, mental impairment, prolonged mouth opening, and prosthetic material. Myiasis in the scalp was facilitated by pediculosis or seborrheic dermatitis.

In Colombia, the geographic distribution and economic burden of *C. hominivorax*, as well as the epidemiology of myiasis in both animals and humans, is unknown but this species is recognized as in important cause of livestock loss (87). Human myiasis by this species has been reported in the states of Antioquia (88, 89), Atlantico (57), Cundinamarca (41), and Boyaca (90), however notification of cases is not mandatory. Research is needed on the biology, epidemiology and population dynamics of this species in order to assess the political, geographic and economic viability of the implementation of programs for insect control in the country (87). Thus, nationwide protocols and surveillance systems are urgently needed to control this ongoing threat to animal and human health.

During its larvae stage, *C. hominivorax* is an obligate parasite of warm-blooded animals, including humans. Once the female is gravid, it deposits an average of 200 eggs in open wounds or natural orifices (1). Egg hatching occurs in approximately 12 h and then it takes 5–7 days for larvae to reach the third instar of maturity inside bovine wounds. This means that the patient possibly was infected in the first 2 days of life. Larvae penetrate deeply into wounds, tearing tissue and making tunnels with their mouths to find a warm and moist place. Then, they hook and cause an extensive destruction of tissue known as traumatic myiasis, which provokes wound swelling that may facilitate bacterial infection (91). Umbilical myiasis is particularly dangerous because it might induce fistulation, penetration of deep layers of the abdomen wall and secondary sepsis associated with omphalitis (2, 92), although none of these were found in our patient.

As in our case, treatment of myiasis is based on the removal of all visible larvae, cleaning of the wound and debridement of remaining necrotic tissue. Irrigation is helpful if the lesions have holes and/or cavities. Local application of ivermectin paralyzes the parasite and kills the larvae, facilitating the extraction and relieving pain (31). Turpentine or ether is used to suffocate the larvae, but this practice is not recommended as it could lead to complications such as anaphylaxis and sepsis (22). Surgical treatment is required when larvae are dead, decomposing or laying in deep tissues (8). Topical anthelmintic medication, bactericides, tetanus toxoid vaccine and systemic antibiotics should also be considered to prevent secondary sepsis. In many reports, the use of systemic ivermectin showed positive results, but further studies are needed to consider this a standard therapy (2, 7, 8).

Correct identification by a trained entomologist is helpful to understand the infestation mechanism, to plan treatment

and to consider preventive actions. For etiological diagnosis, the larvae should be immersed in hot water for 30 s to retain length and morphology and then preserved in a 70–90% ethanol solution or isopropyl alcohol. The regions where the patient has been, the climatic conditions and the endemic species are also important for accurate identification (2). The peak period of infestation by *C. hominivorax* has been reported to be between June and August, in humid and warm locations (5), such as the city where the patient lived.

Livestock is an important economic source in neotropical regions where poverty and inadequate health conditions make it difficult to implement control and eradication programs. Therefore, myiasis will continue to be a sanitary problem in many countries of America, Africa and Asia. Furthermore, global warming and internationalization are likely to influence the migration of screwworm and other myiasis-causing species into new geographic areas that were previously unaffected by this problem. Naïve livestock host are more susceptible to insect replication, increasing the likelihood of outbreaks (93). Groups of individuals at high risk of myiasis should be targeted in prevention programs for *C. hominivorax* infection in areas were insect eradication programs are not available.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. A written informed consent was obtained from the mother of the newborn for pictures and publication of this case.

AUTHOR CONTRIBUTIONS

LF-G and JM-F contributed to the diagnosis and treatment of the patient. They also obtained informed consent and gathered clinical data. JR-Z and PM-G reviewed the literature and wrote the manuscript. All the authors discussed and analyzed the case.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2019.00292/full#supplementary-material

REFERENCES

1. Batista-da-Silva JA, Moya-Borja GE, Queiroz MM. Factors of susceptibility of human myiasis caused by the new world screw-worm, *Cochliomyia hominivorax* in Sao Goncalo, Rio de Janeiro, Brazil. *J Insect Sci*. (2011) 11:14. doi: 10.1673/031.011.0114
2. Francesconi F, Lupi O. Myiasis. *Clin Microbiol Rev*. (2012) 25:79–105. doi: 10.1128/CMR.00010-11
3. Mondal M. Umbilical myiasis with sepsis in a neonate. *Asian J Med Sci*. (2014) 5:106–7. doi: 10.3126/ajms.v5i4.10004
4. Rennie J, Burman-Roy S, Murphy MS. Neonatal jaundice: summary of NICE guidance. *BMJ*. (2010) 340:c2409. doi: 10.1136/bmj.c2409
5. The World Organisation for Animal Health (OIE). Chapter 3.1.13: New World screwworm (*Cochliomyia hominivorax*) and Old World screwworm (*Chrysomya bezziana*). In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019*. Paris (2019).
6. Ghosh T, Nayek K, Ghosh N, Ghosh MK. Umbilical myiasis in newborn. *Indian Pediatr*. (2011) 48:321–3.
7. Patra S, Purkait R, Basu R, Konar MC, Sarkar D. Umbilical myiasis associated with *Staphylococcus aureus* sepsis in a neonate. *J Clin Neonatol*. (2012) 1:42–3. doi: 10.4103/2249-4847.92229
8. Ambey R, Singh A. Umbilical myiasis in a healthy newborn. *Paediatr Int Child Health*. (2012) 32:56–7. doi: 10.1179/1465328111Y.0000000043
9. Kumar V, Gupta S. Umbilical myiasis in a neonate. *Paediatr Int Child Health*. (2012) 32:58–9. doi: 10.1179/1465328111Y.0000000022
10. Dey P, Bhattacharya T, Pal S, Das S, Pal S. Umbilical myiasis in a newborn: a case report. *JCMS Nepal*. (2013) 8:42–5. doi: 10.3126/jcmsn.v8i4.8700
11. Kumar M, Thakur KC, Chib R, Gupta G. Neonatal umbilical myiasis. *J Clin Neonatol*. (2017) 6:121. doi: 10.4103/jcn.JCN_122_16
12. Kotha R, Pandala P, Singh H, Reddy BS, Reddy ST, Rathod M, et al. Neonatal umbilical myiasis. *Int J Contemp Pediatr*. (2019) 6:1. doi: 10.18203/2349-3291.ijcp20190004
13. Jauhari S, Nautiyal S. Umblical myiasis in a newborn: a case report. *Int J Commun Med Public Health*. (2017) 4:872–4. doi: 10.18203/2394-6040.ijcmph20170776
14. Puvabanditsin S, Malik I, Weidner LM, Jadhav S, Sanderman J, Mehta R. Neonatal umbilical cord myiasis in New Jersey. *J Perinatol*. (2014) 34:718–9. doi: 10.1038/jp.2014.69
15. Duro E, Mariluis JC, Mulieri PR. Umbilical myiasis in a human newborn. *J Perinatol*. (2007) 27:250–1. doi: 10.1038/sj.jp.7211654
16. Ogbalu O, Eze C, Manuelrb B. A new trend of Omphalitis complicated with myiasis in neonates of the Niger Delta, Nigeria. *Epidemiology*. (2016) 6:231. doi: 10.4172/2161-1165.1000231
17. Martínez-Rojano H, Noguez JC, Huerta H. Nosocomial myiasis caused by *Lucilia sericata* (Diptera: Calliphoridae) and neonatal myiasis by *Sarcophaga* spp. (Diptera: Sarcophagidae) in Mexico. *Case Rep Infect Dis*. (2018) 2018:5067569. doi: 10.1155/2018/5067569
18. Singh A, Singh Z. Incidence of myiasis among humans—a review. *Parasitol Res*. (2015) 114:3183–99. doi: 10.1007/s00436-015-4620-y
19. Dedeke IOF, Agbo DD, Soile BH, Alao SO, Evwibovwe E, Akinseinde JA. Neonatal cutaneous myiasis: a mistaken identity for impetigo. *Open J Pediat*. (2019) 9:133–8. doi: 10.4236/ojped.2019.92014
20. Ogbalu OK, Achufusi TG, Orlu EE, Bawo DS, Adibe CH, Kumbe L, et al. Human myiasis in neonates and children of the Niger Delta Wetlands and South-East Nigeria. *J Cosmet Dermatol Sci Appl*. (2011) 1:171–6. doi: 10.4236/jcdsa.2011.14026
21. Myrtha G, inventor; Google Patents, assignee. Umbilical binder (1968).
22. Burgess I. Umbilical myiasis. *Paediatr Int Child Health*. (2012) 32:1–2. doi: 10.1179/204690512X13296079158208
23. Clark J, Weeks W, Tatton J. Drosophila myiasis mimicking sepsis in a newborn. *West J Med*. (1982) 136:443–4.
24. Hall MJR, Wall RL, Stevens JR. Traumatic myiasis: a neglected disease in a changing world. *Annu Rev Entomol*. (2016) 61:159–76. doi: 10.1146/annurev-ento-010715-023655
25. Skoda S, Chen H, Chaudhurry M, Sagel A, Phillips P. Artificial diets used in mass production of the New World screwworm, *Cochliomyia hominivorax*. *J Appl Entomol*. (2014) 138:708–14. doi: 10.1111/jen.12112
26. Neira O P, Muñoz S N, Cantero C D. Miasis auricular por *Cochliomyia hominivorax* (Diptera: Calliphoridae) (Coquerel, 1858). *Revista Médica de Chile*. (2002) 130:907–9. doi: 10.4067/S0034-98872002000800011

27. Passos MRL, Varella RQ, Tavares RR, Barreto NA, Santos CC, Pinheiro V, et al. Vulvar myiasis during pregnancy. *Infect Dis Obstet Gynecol*. (2002) 10:153–8. doi: 10.1155/S1064744902000157
28. Chicarelli M, Daniel AN, Santoro MA, Teodoro U. Miíase humana bucal por *Cochliomyia hominivorax* (Coquerel, 1858) em Nova Esperança, estado do Paraná, Brasil. *Revista da Faculda de de Odontologia-UPF*. (2002) 7. doi: 10.5335/rfo.v7i2.1217
29. Gomez RS, Perdigão PF, Pimenta FJGS, Rios Leite AC, Tanos de Lacerda JC, Custódio Neto AL. Oral myiasis by screwworm *Cochliomyia hominivorax*. *Br J Oral Maxillofac Surg*. (2003) 41:115–6. doi: 10.1016/S0266-4356(02)00302-9
30. Visciarelli EC, García SH, Salomón C, Jofré C, Costamagna SR. Un caso de miasis humana por *Cochliomyia hominivorax* (Díptera: Calliphoridae) asociado a pediculosis en Mendoza, Argentina. *Parasitología Latinonot*. (2003) 58:166–8. doi: 10.4067/S0717-77122003000300014
31. Clyti E, Couppie P, Cazanave C, Fouque F, Sainte-Marie D, Pradinaud R. Traitement des myiases dues à *Cochliomyia hominivorax* par application locale d'ivermectine. *Bull Soc Pathol Exot*. (2003) 96:410–1.
32. Martinez CAR, Romani G, Prioli D. Miíase vulvar: relato de caso. *RBGO*. (2003) 25. doi: 10.1590/S0100-72032003000400011
33. Seppänen M, Virolainen-Julkunen A, Kakko I, Vilkamaa P, Meri S. Myiasis during adventure sports race. *Emerg Infect Dis*. (2004) 10:137–9. doi: 10.3201/eid1001.020825
34. Devoto MH, Zaffaroni MC. Orbital myiasis in a patient with a chronically exposed hydroxyapatite implant. *Ophthalmic Plast Reconstr Surg*. (2004) 20:395–6. doi: 10.1097/01.IOP.0000139526.01850.D1
35. Zupan-Kajcovski B, Simonian H, Keller JJ, Faber WR. [*Cutaneous myiasis* caused by a double infestation with larvae of *Dermatobia hominis* and *Cochliomyia hominivorax*]. *Ned Tijdschr Geneeskund*. (2004) 148:2086–9.
36. Pierre-filho PDTP, Minguini N, Pierre LM, Pierre AM. Use of ivermectin in the treatment of orbital myiasis caused by *Cochliomyia hominivorax*. *Scand J Infect Dis*. (2004) 36:503–5. doi: 10.1080/00365540410020136
37. Moissant de Román E, García ME, Quijada J, Simoes D, Marcial T. Miasis cutánea humana. Un caso clínico. *Kasmera*. (2004) 32:12–5.
38. Couppié P, Roussel M, Rabarison P, Sockeel M-J, Sainte-Marie D, Marty C, et al. Nosocomial nasal myiasis owing to *Cochliomyia hominivorax*: a case in French Guiana. *Int J Dermatol*. (2005) 44:302–3. doi: 10.1111/j.1365-4632.2004.02547.x
39. da Silva BB, Borges US, Pimentel ICC. Human vaginal myiasis caused by *Cochliomyia hominivorax*. *Int J Gynecol Obstet*. (2005) 89:152–3. doi: 10.1016/j.ijgo.2004.12.046
40. Costa D, Pierre-Filho PdTP, Medina FMC, Mota R, Carrera C. Use of oral ivermectin in a patient with destructive rhino-orbital myiasis. *Eye*. (2005) 19:1018–20. doi: 10.1038/sj.eye.6701713
41. Osorio J, Moncada L, Molano A, Valderrama S, Gualtero S, Franco-Paredes C. Role of ivermectin in the treatment of severe orbital myiasis due to *Cochliomyia hominivorax*. *Clin Infect Dis*. (2006) 43:e57–9. doi: 10.1086/507038
42. Saraiva VdS, Amaro MH, Belfort R Jr, Burnier MN Jr. A case of anterior internal ophthalmomyiasis: case report. *Arquivos Brasileiros de Oftalmologia*. (2006) 69:741–3. doi: 10.1590/S0004-27492006000500023
43. Rodríguez Diego JG, Córdova Ramos G, Arozarena R. First notification of the cattle screw worm (*Cochliomyia hominivorax*) in a human case in Cuba. *Revista de Salud Animal*. (2007) 29:193.
44. Pasternak J, Joo SH, Ganc AJ, Junior MdSD, Morsh RD, Pinto TH. A case of throat *Cochliomyia hominovorax* infestation. *Einstein*. (2007) 5:170–2.
45. Oliva A, Ramos NL, Bosio L. Fatal scalp myiasis: autopsy finding of *Cochliomyia hominivorax* (Diptera: Calliphoridae) in the brain cavity. *Can Soc Forensic Sci J*. (2007) 40:183.
46. Takahagi RU, Gonçalves FP, Madeira NG, Schellini SA. Oftalmomiíase externa causada por *Cochliomyia hominivorax*. *Revista Brasileira de Oftalmologia*. (2007) 66:58–62.
47. Baskaran M, Jagan Kumar B, Geeverghese A. Cutaneous myiasis of face. *J Oral Maxillofac Pathol*. (2007) 11:70–2. doi: 10.4103/0973-029X.37386
48. Mariwalla K, Langhan M, Welch KA, Kaplan DH. Cutaneous myiasis associated with scalp psoriasis. *J Am Acad Dermatol*. (2007) 57:S51–2. doi: 10.1016/j.jaad.2006.10.022
49. Gealh WC, Ferreira GM, Farah GJ, Teodoro U, Camarini ET. Treatment of oral myiasis caused by *Cochliomyia hominivorax*: two cases treated with ivermectin. *Br J Oral Maxillofac Surg*. (2009) 47:23–6. doi: 10.1016/j.bjoms.2008.04.009
50. Ferraz AC, Nunes RV, Gadelha BQ, Nascimento BP, Meirelles P, Coelho VM, et al. Raro caso de miíase por *Cochliomyia hominivorax* (Diptera: Calliphoridae) e *Dermatobia hominis* (Diptera: Oestridae) em paciente humano. *Arquivos de Ciências da Saúde da UNIPAR*. (2008) 15:142–4.
51. de Souza Barbosa T, Salvitti Sa Rocha RA, Guirado CG, Rocha FJ, student G, Duarte Gavião MB. Oral infection by Diptera larvae in children: a case report. *Int J Dermatol*. (2008) 47:696–9. doi: 10.1111/j.1365-4632.2008.03725.x
52. Lopes-Costa PV, dos Santos AR, Pereira-Filho JD, da Silva BB. Myiasis in the uterine cavity of an elderly woman with a complete uterine prolapse. *Trans Royal Soc Trop Med Hyg*. (2008) 102:1058–60. doi: 10.1016/j.trstmh.2008.04.004
53. Paris LA, Viscarret M, Uban C, Vargas J, AJ R-M. Pin-site myiasis: a rare complication of a treated open fracture of tibia. *Surg Infect*. (2008) 9:403–6. doi: 10.1089/sur.2007.045
54. de Araújo RJG, Corrêa AM, Santos WRA, Júnior MTM. Advanced stage of oral myiasis in children: a clinical case report. *Quintessence Int*. (2008) 39:39–43.
55. González C, Salamanca JC, Olano V, Pérez CE. Miasis cavitaria. Reporte de un caso. *Rev Med*. (2008) 16:95–8.
56. Lima Júnior SM, Asprino L, Prado ÂP, Moreira RWF, de Moraes M. Oral myiasis caused by *Cochliomyia hominivorax* treated nonsurgically with nitrofurazone: report of 2 cases. *Oral Surg Oral Med Oral Pathol Oral Radiol Endodontol*. (2010) 109:e70–3. doi: 10.1016/j.tripleo.2009.11.014
57. de la Ossa N, Castro LE, Visbal L, Santos AM, Díaz E, Romero-Vivas CM. Cutaneous myiasis by *Cochliomyia hominivorax* (Coquerel)(Diptera Calliphoridae) in Hospital Universidad del Norte, Soledad, Atlántico. *Biomédica*. (2009) 29:12–7. doi: 10.7705/biomedica.v29i1.36
58. Trombetta L, Oliva A, Galache V, Bava J, Troncoso A. Cutaneous myiasis due to *Cochliomyia hominivorax* in a drug user. *J Infect Dev Ctries*. (2009) 3:873–6. doi: 10.3855/jidc.170
59. Espinoza A, Quiñones-Silva J, Garay O. Miasis en cavidad oral por *Cochliomyia Hominivorax*: reporte de un caso. *Revista Peruana de Medicina Experimental y Salud Publica*. (2009) 26:573–6.
60. de Carvalho DC, Camargo RPM, Menegali TT, Gehlen D, Klaus MZB. Relato de caso: infestação da cânula de traqueostomia por miíase. *Arquivos Catarinenses de Medicina*. (2009) 38:96–9.
61. Delys Fernández DRE, Borges García DT, Valdés Borroto DAC, Rivas de Armas DRA. Miiasis facial por gusano barrenador del ganado asociado a un carcinoma. *Presentación de dos Pacientes*. (2011) 2011.
62. Menghi C, Gatta C, Oliva A. Otomiasis por *Cochliomyia hominivorax* en dos niños del conurbano bonaerense, Argentina. *Revista Argentina de Microbiología*. (2010) 42:176–8.
63. Khurana S, Biswal M, Bhatti H, Pandav S, Gupta A, Chatterjee S, et al. Ophthalmomyiasis: three cases from North India. *Indian J Med Microbiol*. (2010) 28:257–61. doi: 10.4103/0255-0857.66490
64. Lindsay R, Stancil J, Ray JM. Myiasis of facial wounds by *Cochliomyia hominivorax* sustained in a natural disaster in Haiti. *Otolaryngol Head Neck Surg*. (2010) 143:595–6. doi: 10.1016/j.otohns.2010.04.273
65. Ribeiro MC, De Oliveira Pepato A, De Matos FP, Sverzut CE, Abrahão AAC, Trivellato AE. Oral myiasis in an elderly patient. *Gerodontology*. (2012) 29:e1136–9. doi: 10.1111/j.1741-2358.2010.00432.x
66. Batista-da-Silva JA, Borja GEM, Queiroz MMC. Patient with tracheostomy parasitized in hospital by larvae of the screwworm, *Cochliomyia hominivorax*. *J Insect Sci*. (2011) 11:163. doi: 10.1673/031.011.16301
67. Vale DS, Cavalieri I, Araujo MM, Santos MBP, dos Santos Canellas JV, Espínola LVP, et al. Myiasis in palate by *Cochliomyia hominivorax*. *J Craniofac Surg*. (2011) 22:e57–9. doi: 10.1097/SCS.0b013e318231e1f3
68. Duque FL, Ardila CM. Oral myiasis caused by the screwworm *Cochliomyia hominivorax* treated with subcutaneous ivermectin and creolin: report of six cases after trauma. *Dental Traumatology*. (2011) 27:404–7. doi: 10.1111/j.1600-9657.2011.01004.x
69. Antunes AA, de Santana Santos T, Avelar RL, Neto ECM, Macedo Neres B, Laureano Filho JR. Oral and maxillofacial myiasis: a case series and literature

review. *Oral Surg Oral Med Oral Pathol Oral Radiol Endodontol*. (2011) 112:e81–5. doi: 10.1016/j.tripleo.2011.05.026
70. Thyssen PJ, Nassu MP, Costella AMU, Costella ML. Record of oral myiasis by *Cochliomyia hominivorax* (Diptera: Calliphoridae): case evidencing negligence in the treatment of incapable. *Parasitol Res*. (2012) 111:957–9. doi: 10.1007/s00436-012-2856-3
71. Batista-da-Silva JA, Borja G, Queiroz M. A severe case of cutaneous myiasis in São Gonçalo, Brazil, and a simple technique to extract New World screw-worm *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae). *Neotropical entomol*. (2012) 41:341–2. doi: 10.1007/s13744-012-0038-8
72. Costa FS, Bellotti A, Farah GJ, Camarini ET. Treatment of myiasis resulting from complex facial trauma. *Revista de Cirurgia e Traumatologia Buco-Maxilo-Facial*. (2012) 12:25–30.
73. Olea MS, Centeno N, Aybar CAV, Ortega ES, Galante GB, Olea L, et al. First report of myiasis caused by *Cochliomyia hominivorax* (Diptera: Calliphoridae) in a diabetic foot ulcer patient in Argentina. *Korean J Parasitol*. (2014) 52:89–92. doi: 10.3347/kjp.2014.52.1.89
74. Novo-Neto JP, Santos FdSAd, Pontes AEF, Ribeiro FS, Scannavino FLF, Martins AT. Oral myiasis caused by *Cochliomyia hominivorax* in a disabled person. *Case Rep Pathol*. (2015) 2015:904658. doi: 10.1155/2015/904658
75. Africano FJ, Faccini-Martinez AA, Perez CE, Espinal A, Bravo JS, Morales C. Pin-site myiasis caused by screwworm fly, Colombia. *Emerg Infect Dis*. (2015) 21:905–6. doi: 10.3201/eid2105.141680
76. LaCourse SM, Martinez RM, Spach DH, Fang FC. Pain and bloody ear discharge in a returning traveler. *Am J Trop Med Hyg*. (2015) 92:599–600. doi: 10.4269/ajtmh.14-0617
77. Reinoso-Quezada S, Alemán-Iñiguez JM. Rara miasis maxilar por *Cochliomyia hominivorax*: reporte de caso, actualidad y entomología. *Revista Española de Cirugía Oral y Maxilofacial*. (2016) 38:111–6. doi: 10.1016/j.maxilo.2014.04.005
78. Failoc-Rojas VE, Silva-Díaz H. Review of cases and a patient report of myiasis with tracheostomy, peru. *Emerg Infect Dis*. (2016) 22:563–5. doi: 10.3201/eid2203.151631
79. Calderón-Castrat X, Idrogo-Bustamante JL, Peceros-Escalante J, Ballona R. Wound myiasis caused by *Cochliomyia hominivorax*: the role of entodermoscopy. *Int J Dermatol*. (2017) 56:330–2. doi: 10.1111/ijd.13432
80. de Arruda JAA, de Oliveira Silva LV, Silva PUJ, de Figueiredo EL, Callou G, Mesquita RA, et al. Head and neck myiasis: a case series and review of the literature. *Oral Surg Oral Med Oral Pathol Oral Radiol*. (2017) 124:e249–56. doi: 10.1016/j.oooo.2017.06.120
81. Rodrigues FT, Klemig LR, Cardozo MRP, Alves PC, Aguiar VM, Lessa CS. Myiasis associated with an invasive ductal carcinoma of the left breast: case study. *Revista do Instituto de Medicina Tropical de São Paulo*. (2017) 59:e35. doi: 10.1590/s1678-9946201759035
82. Calderon HP, Rojas EC, Apt BW, Castillo OD. [Cutaneous myiasis due to *Cochliomyia hominivorax* associated with seborrheic dermatitis]. *Rev Med Chil*. (2017) 145:250–4. doi: 10.4067/S0034-98872017000200013
83. Durão C, Barros A, Campos P. A rare case of digital myiasis. *J Infect Public Health*. (2017) 10:886–7. doi: 10.1016/j.jiph.2016.11.002
84. Vianna Gontijo JR, Vasques Bittencourt F. Wound myiasis: the role of entodermoscopy. *Anais Brasileiros de Dermatologia*. (2018) 93:746–8. doi: 10.1590/abd1806-4841.20188043
85. Failoc-Rojas VE, Molina-Ayasta C, Salazar-Zuloeta J, Samamé A, Silva-Díaz H. Case report: Myiasis due to *Cochliomyia hominivorax* and *Dermatobia hominis*: clinical and pathological differences between two species in Northern Peru. *Am J Trop Med Hyg*. (2018) 98:150–3. doi: 10.4269/ajtmh.16-0437
86. Villamil-Gómez WE, Cardona-Ospina JA, Prado-Ojeda JS, Hernández-Prado H, Figueroa M, Causil-Morales PN, et al. Pin-Site myiasis caused by screwworm fly in nonhealed wound, Colombia. *Emerg Infect Dis*. (2019) 25:379–80. doi: 10.3201/eid2502.181053
87. Forero E, Cortés J, Villamil L. The problem of screwworm, *Cochliomyia hominivorax* (Coquerel, 1858), in Colombia. *Rev MVZ Córdoba*. (2008) 13:1400–14.
88. Pape T, Wolff M, Amat E. Los califóridos, éstridos, rinofóridos y sarcofágidos (Diptera: Calliphoridae, Oestridae, Rhinophoridae, Sarcophagidae) de Colombia. *Biota Colomb*. (2004) 5:201–8.
89. Maxwell M, Subia J, Abrego J, Garabed R, Xiao N, Toribio R. Temporal and spatial analysis of the new world screwworm (*Cochliomyia hominivorax*) in Darien and Embera, Panama (2001–2011). *Transb Emerg Dis*. (2017) 64:899–905. doi: 10.1111/tbed.12457
90. Forero-Becerra G, Cortés-Vecino J, Villamil-Jiménez L. Associated risk factors to myiasis by *Cochliomyia hominivorax* on cattle farms in Puerto Boyacá (Colombia). *Rev Científica Fac de Cienc Vet Univ del Zulia*. (2009) 19:460–5.
91. Adams TS, Reinecke JP. The reproductive physiology of the screwworm, *Cochliomyia hominivorax* (Diptera: Calliphoridae). I. oogenesis. *J Med Entomol*. (1979) 15:472–83. doi: 10.1093/jmedent/15.5-6.472
92. Thomas DB, Mangan RL. Oviposition and wound-visiting behavior of the screwworm fly, *Cochliomyia hominivorax* (Diptera: Calliphoridae). *Ann Entomol Soc Am*. (1989) 82:526–34. doi: 10.1093/aesa/82.4.526
93. French NP. Impacts of non-native species on livestock. In: Vilà M, Hulme P, editors. *Impact of Biological Invasions on Ecosystem Services. Invading Nature - Springer Series in Invasion Ecology*. Vol. 12. Cham: Springer (2017). p. 139–54. doi: 10.1007/978-3-319-45121-3_9

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Spread of Cystic Echinococcosis in Pakistan Due to Stray Dogs and Livestock Slaughtering Habits: Research Priorities and Public Health Importance

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Background: Cystic echinococcosis (CE) is a global zoonotic parasitic disease caused by the larval stage of *Echinococcus granulosus* and it has been reported from both livestock and humans in Pakistan. The definitive host of *E. granulosus* is the dog, and the large number of stray dogs in Pakistan contributes to the spread of CE. However, there is little information between stray dogs and CE relation in the country.

Methods: During the study, total 123 butcher's shops and abattoirs were included for collection of data relating to the hydatid cyst prevalence in slaughtered animals (sheep, goat, cattle, and buffaloes). The number of animals slaughtered in each butcher's shop during sampling period was also recorded, and the association of the shop environment with dogs was inspected.

Results: Data was collected for CE from 123 butcher's shops in Rawalpindi and Islamabad, Pakistan. The slaughtering rate the in the butcher's shops was 2–10 animals/day including sheep/goat/cattle and buffaloes. The overall prevalence of CE in all examined animals was 2.77%. In buffaloes the higher prevalence was recorded as compared to other hosts. The findings showed that lung and liver were most affected organs and majority (59%) of the cysts were fertile in infected animals. The presence of a large number of stray dogs were an important factor in the spread of CE. They were rarely vaccinated, have easy access to infected offal at slaughtering site and had insufficient or inappropriate anthelmintic treatment.

Conclusions: The most pressing need is to raise public awareness of this huge problem by considering CE a major ailment and promoting the collection and mapping of epidemiological data. Efficient CE control is required, especially treating dogs with antiparasitic drugs, for which government support and affiliation with the veterinary sector is essential.

Keywords: cystic echinococcosis, *Echinococcus granulosus*, livestock, dog, public health, Pakistan

INTRODUCTION

Echinococcosis is one of the 20 neglected zoonotic diseases (NZD) prioritized by the World Health Organization (WHO) (1). Cystic echinococcosis (CE) is a globally NZD caused by the dog tapeworm *Echinococcus granulosus*. The global annual infection rate is 1.2 million, the annual death rate is about 2.2%, and an estimated 3.6 million disability-adjusted life years (DALYs) are lost because of this disease per annum (2). In addition, CE is responsible for over US$ 3 billion expenses every year (3). CE is more prevalent in areas where people survive on animal husbandry and agricultural activities (4), and the rate is higher in nomadic and semi-nomadic populations due to this lifestyle (5).

Conditions such as poor hygiene and failure to wash contaminated food facilitate the spread of CE infection in the human population (6). CE transmission from food to humans is common in areas where people usually consume raw vegetables; most are cultivated in open fields where stray dogs roam freely and contaminate the vegetables by dropping feces containing *E. granulosus* eggs (7). One of the major risk factors for CE infection is open slaughtering of livestock without veterinary supervision. Due to lack of supervision, infected offal is ingested by dogs, which, as the intermediate host, spread infected eggs to the environment. The study aimed to analyze CE prevalence, presence of stray dogs and their association with slaughtering habits in abattoirs /butcher shops in the study area.

MATERIALS AND METHODS

Study Area

A study was conducted in Islamabad and Rawalpindi districts of Pakistan.

Topography

Islamabad, the capital city of Pakistan, is located in Pothohar Plateau (33.43°N 73.04°E) at 540 m (1,770 ft.) above the sea level. 505 km^2 of this area is urban whereas 401 km^2 of is rural (8). Adjoining Islamabad is the city of Rawalpindi and both the cities are often referred to as the twin cities, 84% of the population here is Punjabi, 9% Pashto and 7% others. Rawalpindi is located at an elevation of 508 m and spans over an area of 259 km^2 (9).

Study Duration

The data was collected from January to July, 2017(for 6 months). Butcher shops in different areas of Rawalpindi and Islamabad were visited twice per month to collect the data on prevalence and presence of stray dogs in the slaughterhouses.

Study Design

A cross-sectional survey was designed to get the recent data hydatid cyst incidence. The data was collected from butcher shops of the twin cities. Questionnaire was designed for butcher shops/slaughterhouses among urban and rural areas which was descriptive in nature. The information about stray dogs present along the territory of slaughterhouses/butcher shops were recorded.

Data Collection Methods

The data on presence of stray dogs, CE prevalence in animals, as well as on socio-demographic characteristics was collected using questionnaires. Moreover, data was analyzed to determine the factors associated with the risk of CE. As there is no local specific name of this disease, pictures of cysts in animal organs and of infected humans were shown to the participants to identify the disease better. The knowledge of the participants was measured as binary outcomes (10, 11).

Laboratory Investigations

In order to examine hydatid cysts properly, following parameters were carried out: Types of cysts (sterile, fertile, calcified, or underdeveloped), organ specificity (lungs and liver), and prevalence of hydatidosis. Presence of cysts in different organs was analyzed by routine post-mortem of the carcass. The cysts were dissected and collected into sterile containers separately on organ basis for further description.

Cyst Characterization

Sterile scalpel blades were used for cyst incision. The fluid present inside these cysts was used to check the existence of protoscoleces either in the form of brood capsule (closes to the germinal layer) or in the cyst fluid considering as a fertility indicative. Viability test was performed on fertile cysts. In viability test a drop of fluid from cyst containing the protoscoleces was observed under microscope to check amoeboid like peristaltic movements. For clear microscopic observations equal volume of 0.1% aqueous eosin solution was also mixed with equal volume of fluid containing the protoscoleces. Sterile hydatid cysts were characterized on the basis of inner lining, generally smooth with a slight turbid enclosed fluid otherwise rough calcified cyst with no or less fluid (12). Calcified cysts were coarse and nodular having an internal chamber with calcified or chalky deposits in the cyst wall. Underdeveloped cysts were small 1–2 mm in size, defined germinal layer are firm in texture with very little fluid but presence of protoscoleces was not observed (13).

Morphology of Protoscoleces

Polyvinyl-lactophenol was used for mounting protoscoleces cysts. Hooks damage was prevented by applying gentle pressure on cover slip. A calibrated eye-piece micrometer was used for all measurements under oil immersion. Morphometric analysis was done as described by Hobbs et al. (14).

Data Analysis

Data was analyzed as described previously (15).

RESULTS

Data was collected for CE from 123 butcher's shops in Rawalpindi and Islamabad, Pakistan. The slaughtering rate in the butcher's shops was 2–10 animals/day including cattle, goat, sheep, and buffaloes. Overall prevalence of CE in

Abbreviations: WHO, World Health Organization; NZDs, Neglected Zoonotic Diseases; CE, Cystic Echinococcosis; DALYs, Disability Adjusted Life Years

TABLE 1 | Overall prevalence (%) of hydatidosis in various organs of slaughtered Cattle, Buffalo, Goat, and Sheep.

Host	Overall prevalence			Site of infection			No. of cysts (%)		Kind of cysts (%)			
	N	Infected	Frequency (%)	Lung	Liver	Others	Single	Multiple	Fertile	Sterile	Calcified	Undeveloped
Cattle	3,845	132	3.43	✓	✓	✓	103 (78)	29(22)	73 (55.3)	31 (23.48)	19 (14.39)	9 (6.81)
Buffalo	1,103	58	5.25	✓	✓	✓	47 (81)	11(19)	48(82)	5(8.62)	4(6.89)	1(1.72)
Goat	4,307	76	1.76	✓	✓		68 (89)	08 (11)	37 (48.68)	21 (27.63)	15 (19.73)	3 (3.94)
Sheep	1,545	34	2.20	✓	✓		34 (100)	–	19 (55.88)	3(8.82)	7(20.58)	5(14.7)
Total	10,800	300	2.77				252 (84)	48 (16)	177 (59)	60 (20)	45(15)	18(6)

TABLE 2 | Rostellar hooks morphology of protoscoleces in infected animals.

Parameters	Mean + S.E			
	Cattle	Buffalo	Goat	Sheep
Total No. of Hooks (NH)	29.21 ± 1.13	26.03 ± 1.17	21.00 ± 1.06	27.80 ± 1.11
Large Hook Length (LTL) (μm)	24.02 ± 1.03	18.37 ± 0.96	27.12 ± 0.91	19.78 ± 1.02
Large Hook Blade Length (LBL) (μm)	15.21 ± 0.44	16.02 ± 0.54	9.77 ± 0.57	10.06 ± 0.38
Small Hook Length (STL) (μm)	19.54 ± 1.03	17.97 ± 1.00	11.22 ± 0.77	13.15 ± 0.72
Small Hook Blade Length (SBL) (μm)	8.9 ± 0.56	6.9 ± 0.30	9.30 ± 0.38	7.2 ± 0.37

the slaughterhouses/butcher shops was 2.77% (300/10,800) according to this survey. Prevalence was higher in buffaloes followed by cattle, sheep, and goat, respectively. The site of infection, number of cysts and kind of cysts are shown in **Table 1**.

Rostellar Hook Morphology

The parameters which were observed to check protoscolex rostellar hook morphology in infected animals were total hooks number, their total length (μm) of hooks and blade length (μm) as shown in **Table 2**.

Total Number of Hooks (NH)

Protoscoleces hooks number was observed and it was found that total number was 29.21 ± 1.13 in cattle origin, 26.03 ± 1.17 in buffalo origin, 21.0 ± 1.06 in goat origin, and 27.80 ± 1.11 in sheep origin as shown in **Table 1**. The study results indicated that the maximum number of hooks were observed on protoscoleces of sheep origin and minimum on those of goat origin.

Large Hook Total Length (LTL) (μm)

Protoscoleces large hooks was observed for total length (micrometers, μm) and it was found that it was 24.02 ± 1.03 in cattle origin, 18.37 ± 0.96 in buffalo origin, 27.12 ± 0.91 in goat origin, and 19.78 ± 1.02 in sheep origin. In goat origin large hook length was maximum (27.12 ± 0.91) and in case of buffalo origin it was minimum (18.37 ± 0.96).

Large Hook Blade Length (LBL)(μm)

Protoscoleces blade length of large hooks on was observed as 15.21 ± 0.44 in cattle originated infections, 16.02 ± 0.54 in buffalo originated infections, 9.77 ± 0.57 in goat origin, and 10.06 ± 0.38 in sheep origin as shown in **Table 1**. It is clear from these values that buffalo originated infection LBL was found maximum (16.02 ± 0.54) and in goat originated it was minimum (9.77 ± 0.57).

Small Hook Total Length (STL) (μm)

Protoscoleces of small hooks total length was observed as 19.54 ± 1.03 in cattle, 17.97 ± 1.00 in buffalo, 11.22 ± 0.77 in goat, and 13.15 ± 0.72 in sheep origin as shown in **Table 1**. In cattle originated STL was maximum (19.54 ± 1.03) and in case of goat origin it was minimum (11.22 ± 0.77).

Small Hook Blade Length (SBL)(μm)

Protoscoleces small hooks blade length on was recorded as 8.9 ± 0.56 in cattle origin, 6.9 ± 0.30 in buffalo origin, 9.30 ± 0.38 in goat origin, and 7.2 ± 0.37 in sheep origin. In goat origin SBL was maximum (9.30 ± 0.38), while in case of buffalo origin it was minimum (6.9 ± 0.30).

In the present study the number of stray dogs were recorded in all 123 slaughterhouse/butcher shops. It ranged from 1 to 5 dogs/site. The main contributing factor to the spread of CE was the large number of stray dogs (**Table 3**); they were rarely vaccinated, have easy access to infected offal in rural areas (**Figures 1A–C**), and had insufficient or inappropriate anthelmintic treatment.

In addition, there were few municipal slaughterhouses, limited veterinary supervision and inspection of slaughterhouses, few facilities for the disposal of infected offal, and there was home or illegal livestock slaughtering, and lack of health education. It was observed that stray dogs have a close association with the slaughtering sites and increase the chances to get infected with CE. The finding of this study has showed that stray dogs (range 1–5) were present in the territories of all the butcher shops/slaughter houses that has an open access to infected offal of the slaughtered livestock. These stray dogs are not treated with any antiparasitic drug.

DISCUSSION

Cystic echinococcosis (CE) is a chronic larval cestode infection caused by *E. granulosus* in humans and domestic livestock,

TABLE 3 | Potential risk factors analysis of CE.

S. No	Risk Factors	Responses	
		Yes	No
1	Ever heard about Zoonoses	04	119
2	Presence of stray dogs inside the slaughter house/butcher shop	107	16
3	Proper facilities to dispose animals offals in slaughter house/butcher shop	04	119
4	Discard of infected organs (Lungs/Liver) at the site of slaughtering	121	02
5	Access of stray dogs to the infected organs	121	02
6	Stray dogs were fed with useless meat (Infected)	112	11
7	Stray dogs are ever vaccinated	02	122
8	Cystic Echinococcosis is spreaded from dogs?	01	23
9	Veterinary supervision of slaughtered animals	04	119
10	Health education to butchers	0	123
11	Anthelminthic treatment of stray dogs	0	123

FIGURE 1 | (A-C) Showing the association and access of stray dogs to infected offal's at butcher shops.

principally transmitted by an intermediate host (16). CE is recognized as a neglected disease of public health significance worldwide, particularly in low-income countries (17). Pakistan is a country with low socioeconomic development and the hygiene conditions are poor. Poor hygiene conditions such as no proper hand washing, no water boiling, lack of proper cleanliness of shops and surrounding areas, eating of contaminated food and raw vegetables, and feeding dogs meat infected with cysts are involved in the prevalence of CE in humans. The epidemiological studies showed that CE is highly prevalent r in third world countries (18). The higher prevalence of CE in Pakistan might be due to inappropriate waste dumping, poor social-economic condition of the country, very poor sanitary system, and unorganized slaughtering. In addition to these factors personal unhygienic situation is also playing a crucial role (19).

The findings showed that overall prevalence of CE was 2.77%. The prevalence was higher in buffaloes followed by cattle, sheep, and goat, respectively. In Pakistan the first incidence of CE in intermediate hosts was explored in 1968. The prevalence of *E. granulosus* was 35% (52/148) in buffaloes and 27% (17/62) in cattle (10).

In current study, lung and liver was most affected organ as compared to others. The lung wise prevalence was 30.9, 22.8, and 58.8%, in cattle, buffaloes, and camels, respectively while in liver it was 21.42, 17.47, and 26.4% in cattle, buffaloes, and camels, respectively (11). The prevalence of hydatid cyst in liver, lung, spleen, heart, and kidneys was 25.31, 47.31, 1.83, 0.06, and 0.51%, respectively (15). In sheep and goat, the prevalence was 8.25 and 8.05%, respectively (20). In a comprehensive survey, the overall prevalence of hydatidosis was 6.67% in livestocks (21). Mustafa et al. (22), reported that the prevalence of hydatid cysts as 3.24, 2.44, and 2.44% in sheep, goats and cattle, respectively while Tasawar et al. (23) reported the prevalence of 7.39% in sheep and 10.69% in buffaloes of Multan, Punjab, Pakistan. Previously it was shown that hydatid cyst prevalence was between 5 and 46% in livestock species (24).

A report from Lahore showed that hydatidosis is prevalent in sheep (8.85%) and in goats (6.21%). This survey was conducted to determine the organ specificity of hydatidosis, organ wise distribution of hydatidosis showed that in goats 40.56% in liver, followed by 34.38% in lungs, 16.95% in lungs and liver together, and 0.49% in spleen. In sheep, highly infected organ was lungs whereas liver was most infected organ in goats (20). Sheep and goat liver hydatid cyst prevalences were 46.74 and 23.28% and the rates in lungs were 17.37 and 13.68%, respectively (25).

Similarly, frequency of fertile cysts was higher as compared to sterile, calcified, and underdeveloped cysts, respectively. Hydatid cysts can be categorized as non-viable, viable, and fertile (26). Only the fertile cysts carry the active form of the parasite protoscoleces (27). The cysts diameter was 2–30 cm and it is as the inner layer from where larvae grow (28).

Zoonotic helminthes (*Toxocara* spp. and *Echinococcus* spp.) can transmit to humans by dogs and cats (29). Globally, human and dog interaction cause significant social, economic and public health issue mainly the zoonotic diseases (30). Dogs play crucial role in spread of many zoonotic infectious diseases (31). Higher population of stray dogs is one of the main contributing factors in spread of CE in Pakistan. They are infrequently vaccinated and easy access to infected offal. Poor hygienic conditions, lack of veterinary supervision and inspection of slaughterhouses, home or illegal livestock slaughtering occurs, and there is a lack of health education due to poverty (6).

The dog population depends on the accessibility of resources (for example, shelter, food, and water) (32). Although the actual number of stray dogs worldwide is not known, of the 500 million

dogs in the world, around 75% are thought to be stray (33). Stray dogs survive consists of edible debris and contributions from human beings (34). Dog populations is directly linked with the size of the local human population (35).

Stray dogs are one of the important reservoirs for the transmission of zoonotic helminthes that are of public health concern especially *Echinococcus* specie. A study from Karachi (the biggest city of Pakistan), shows that among selected dogs presence of intestinal helminthes was confirmed 99% dogs and 7% carried *E. granulosus* (36).

To attain effective control of CE, it is essential to raise knowledge and awareness regarding hazardous practices and defensive measures against the disease within the community. Pakistan, being a developing country, is densely populated and socioeconomically poor. Overall poor sanitary system in Pakistan is very poor and majority of the inhabitants lives in crowded area. Rural inhabitants mainly survive on small-scale agriculture and farming. Laborers working in the fields often interact with animals and, due to illiteracy, have limited knowledge of health and hygiene and therefore are often infected by *Echinococcus* spp. (37).

In the early years of the twenty-first century, CE contributed a major global disease burden; it is one of the 12 commonest NZDs (38). It is very difficult to regulate the control of NZDs, particularly when curing humans does not prevent transmission; moreover, treatment of livestock is perceived as a low priority because the livestock hosts are usually asymptomatic (39). Since 1960, several intervention programs have demonstrated effective control of *E. granulosus* transmission, leading to a significant reduction of CE and improved public health (40). Despite these intervention programs, further work is still necessary. Thus, at present, we recommend increasing the awareness of the seriousness of CE and promoting the collection and mapping of epidemiological data. Efficient CE control requires government support and affiliation with the veterinary sector.

CONCLUSION

In countries with a high number of stray dogs, such as Pakistan, and where the public education level is low, the first task for CE control should be to raise public awareness and try to prevent infected offal from being fed to dogs. Field studies should be conducted on this subject, training seminars should be given, information should be given to children in primary schools, butchers should be trained, the community should be informed by imams in mosques, and informative TV and radio programs should be broadcast.

DATA AVAILABILITY STATEMENT

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Departmental Ethics Review Board (ERB) at the COMSATS University Islamabad (CUI), Pakistan, under ERB/18/72. This study was carried out in strict accordance with the recommendations of the guide for the care and use of laboratory animals.

AUTHOR CONTRIBUTIONS

AK and MA collected the data and wrote the paper following discussions with HA and SS. SS and JC also revised the paper and improved the technical quality of the manuscript. AK and JC contributed reagents and materials. All authors approved the final version of the paper.

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REFERENCES

1. The World Health Organization. *Working to Overcome the Global Impact of Neglected Tropical Diseases: First WHO Report on Neglected Tropical Diseases*. Geneva: World Health Organization (2010).
2. Craig PS, Budke CM, Schantz PM, Tiaoyin L, Jiamin Q, Yurong Y, et al. Human echinococcosis: a neglected disease. *Trop Med Health*. (2007) 35:283–92. doi: 10.2149/tmh.35.283
3. Agudelo Higuita NI, Brunetti E, Mccloskey C. Cystic echinococcosis. *J Clin Microbiol*. (2006) 54:518–23. doi: 10.1128/JCM.02420-15
4. Romig T, Deplazes P, Jenkins D, Giraudoux P, Massolo A, Craig PS, et al. Ecology and life cycle patterns of echinococcus species. *Adv Parasitol*. (2017) 95:213–314. doi: 10.1016/bs.apar.2016.11.002
5. Harandi MF, Moazezi SS, Saba M, Grimm F, Kamyabi H, Sheikhzadeh F, et al. Sonographical and serological survey of human cystic echinococcosis and analysis of risk factors associated with seroconversion in rural communities of Kerman, Iran. *Zoonoses Public Health*. (2011) 58:582–8. doi: 10.1111/j.1863-2378.2011.01407.x
6. Possenti A, Manzano-Romá R, Sánchez-Ovejero C, Boufana B, La Torre G, Siles-LucasM, et al. Potential risk factors associated with human cystic echinococcosis: systematic review and meta-analysis. *PLoS Neglect Trop Dis*. (2016) 10:e0005114. doi: 10.1371/journal.pntd.0005114
7. Moshfe A, Sarkari B, Arefkhah N, Nikbakht R, Shahriarirad R, Rezae, Z, et al. Seroepidemiological study of cystic echinococcosis in nomadic communities in the southwest of Iran: a population-based study. *J Immunoassay Immunochem*. (2018) 40:183–92. doi: 10.1080/15321819.2018.1547974
8. Rahman T. Islamabad, Pakistan. p. 1–15 (2003).
9. Sheikh IS, Pasha MK, Williams VS, Raza SQ, Khan KSA. Environmental geology of the Islamabad-Rawalpindi Area, Northern Pakistan. In: Warwick PD, Wardlaw BR, editors. *Regional Studies of the Potwar Plateau Area, Northern Pakistan*. U.S. Geological Survey (2007). p. 1–8. doi: 10.3133/b2078

10. Shiekh SA, Hussain MZ. Incidence of hydatidosis in livestock in Lahore. *Pak J Sci*. (1968) 19:239–42.
11. Khan MQ, Afzal M, Ali S. Prevalence and serology of hydatidosis in large ruminants of Pakistan. *Vet Parasitol*. (1990) 37:163–8. doi: 10.1016/0304-4017(90)90071-I
12. Parija SC. *Medical Parasitology, Protozoology and Helminthology Text and Atlas, Vol.* 2. New Delhi: Chennai Medical Book Publisher (2004). p. 221–9.
13. Simsek S, Koroglu E. Evaluation of enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunoelectrotransfer blot (EITB) for immunodiagnosis of hydatid diseases in sheep. *Acta Trop*. (2004) 92:17–24. doi: 10.1016/j.actatropica.2004.04.006
14. Hobbs RP, Lymbery AJ, Thompson RCA. Rostellar host morphology of *Echinococcus granulosus* (Batsch, 1786) from natural and experimental Australian hosts, and its implication for strain recognition. *Parasitology*. (1990) 101:273–81. doi: 10.1017/S0031182000063332
15. Anwar AH, Shamim H, Rana MN, Khan A. Qudoos. Hydatidosis: prevalence and biometrical studies in cattle *(Bob indicub)* in Pakistan. *J Agric Sci*. (2000) 37:29–32.
16. Larrieu E, Gavidia CM, Lightowlers MW. Control of cystic echinococcosis: background and prospects. *Zoonoses Public Health*. (2019) 66:889–99. doi: 10.1111/zph.12649
17. Abdulhameed MF, Robertson ID, Al-Azizz SA, Habib I. Neglected zoonoses and the missing opportunities for one health education: the case of cystic echinococcosis among surgically operated patients in Basrah, Southern Iraq. *MDPI*. (2019) 7:4. doi: 10.3390/diseases7010004
18. Fatimi SH, Sajjad N, Muzaffar M. Ruptured hydatid cyst presenting as pneumothorax. *J Infect Dis Dev Countries*. (2010) 4:256–8. doi: 10.3855/jidc.538
19. Hussain A, Maqbool A, Tanveer A, Anees A. Studies on morphology of *Echinococcus granulosus* from different animal-dog origin. *Punjab Univ J Zool*. (2005) 20:151–7.
20. Iqbal HJ, Maqbool A, Lateef M, Khan MA, Riaz A, Mahmood A, et al. Studies on hydatidosis in sheep and goats at Lahore, Pakistan. *J Anim Plant Sci*. (2012) 22:894–7.
21. Latif AA, Tanveer A, Maqbool A, Siddiqi N, Kyaw-tanner M, Traub RJ. Morphological and molecular characterization of *Echinococcus granulosus* in livestock and humans in Punjab, Pakistan. *Vet Parasitol*. (2010) 170:44–49. doi: 10.1016/j.vetpar.2010.02.003
22. Mustafa I, Shahbaz M, Asif S, Khan MR, Saeed U, Sadiq F, et al. Availability, cyst characteristics and hook morphology of *Echinococcus granulosus* isolates from livestock (cattle, sheep and goats) in Central Punjab, Pakistan. *Kafkas Univ Vet Fak Derg*. (2015) 21:849–54.
23. Tasawar Z, Naz F, Lashari MH. The prevalence of hydatidosis in sheep and buffaloes at Multan, Punjab, Pakistan. *Global Vet*. (2014) 12:332–5. doi: 10.5829/idosi.gv.2014.12.03.82272
24. Shafiq M, Tanveer A, Athar M. *Epidemiology and economical aspects of hydatidosis/echinococcosis in different animals and man* (Ph.D. Thesis). University of the Punjab, Lahore, Pakistan (2005). p. 415.
25. Ahmed S, Nawaz M, Gul R, Zakir M, Razzaq A. Some epidemiological aspects of hydatidosis of lungs and livers of sheep and goats in Quetta, Pakistan. *Pak J Zool*. (2006) 38:1.
26. Larrieu EJ, Frider B. Human cystic echinococcosis: contributions to the natural history of the disease. *Ann Trop Med Parasitol*. (2001) 95:679–87. doi: 10.1080/00034980120094730
27. Kamenetzky L, Gutierrez AM, Canova SG, Haag KL, Guarnera EA, Parra A, et al. Several strains of *Echinococcus granulosus* infect livestock and humans in Argentina. *Infect Genet Evol*. (2002) 2:129–36. doi: 10.1016/S1567-1348(02)00131-4
28. Harandi MF, Hobbs RP, Adams PJ, Mobedi I, Morgan-Ryan UM, Thompson RCA. Molecular and morphological characterization of *Echinococcus granulosus* of human and animal origin in Iran. *Parasitology*. (2002) 125:367–73. doi: 10.1017/S0031182002002172
29. Kardjadj M, Ben-Mahdi MH. Epidemiology of dog-mediated zoonotic diseases in Algeria: a One Health control approach. *New Microbes New Infect*. (2019) 28:17–20. doi: 10.1016/j.nmni.2019.01.001
30. Strube C, Neubert A, Springer A, von Samson-Himmelstjerna G. Survey of German pet owners quantifying endoparasitic infection risk and implications for deworming recommendations. *Parasit Vectors*. (2019) 12:203. doi: 10.1186/s13071-019-3410-2
31. Anonymous. *Leading Edge. Rabies Has Its Day*. Available online at: http://infection.thelancet.com; www.rabiescontrol.net/Lancet.pdf (accessed September 20, 2010).
32. Wandeler AI, Matter HC, Kappeler A, Budde A. The ecology of dogs and canine rabies: a selective review. *Rev Sci Tech*. (1993) 12:51–71. doi: 10.20506/rst.12.1.663
33. WSPA. *Suffering in Slums: The Global Stray Dog Problem*. (2010). Available online at: www.wspa-usa.org (accessed September 22, 2010).
34. Smith R. *How to Solve Romanian Street Dog Problem Effectively, Humanely and Forever*. (2005). Available online at: www.actionagainstpoisoning.com (accessed August 2, 2013).
35. Butler JRA, Du Toit JT, Bingham J. Free-ranging domestic dogs (*Canis familiaris*) as predators and prey in rural Zimbabwe: threats of competition and disease to large wild carnivores. *Biol Conserv*. (2004) 115:369–78. doi: 10.1016/S0006-3207(03)00152-6
36. Ahmed N, Riaz A, Zubair Z, Saqib M, Ijaz S, Nawaz-Ul-Rehman MS, et al. Molecular analysis of partial VP-2 gene amplified from rectal swab samples of diarrheic dogs in Pakistan confirms the circulation of canine parvovirus genetic variant CPV-2a and detects sequences of feline panleukopenia virus (FPV). *Virol J*. (2018) 15:45. doi: 10.1186/s12985-018-0958-y
37. Ahmed H, Ali S, Afzal MS, Khan AA, Raza H, Shah ZH, et al. Why more research needs to be done on echinococcosis in Pakistan. *Infect Dis Poverty*. (2017) 6:90. doi: 10.1186/s40249-017-0309-z
38. WHO. *The Control of Neglected Zoonotic Diseases: Community-based Interventions for Prevention and Control*. Geneva: World Health Organization (2010).
39. Craig PS, McManus DP, Lightowlers MW, Chabalgoity JA, Garcia HH, Gavidia CM, et al. Prevention and control of cystic echinococcosis. *Lancet Infect Dis*. (2007) 7:385–94. doi: 10.1016/S1473-3099(07)70134-2
40. Gemmell MA, Roberts MG, Beard TC, Campano Diaz S, Lawson JR, Nonnemaker JM. Chapter 6: Control of echinococcosis. In: Eckert J, Gemmell MA, Meslin FX, Pawlowski ZS, editors. *WHO/OIE Manual on Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern* (Paris: WHO/OIE) (2001).

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Identification of Immune Responses to Japanese Encephalitis Virus Specific T Cell Epitopes

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Background: Due to the similarity between the dengue (DENV) and the Japanese encephalitis virus (JEV) there is potential for immune cross-reaction. We sought to identify T cell epitopes that are specific to JEV and do not cross react with DENV.

Methodology: 20mer peptides were synthesized from regions which showed >90% conservation. Using IFNγ cultured ELISpot assays, we investigated JEV-specific T cell responses in $DENV^-$ and JEV^- non-immune individuals ($DENV^-JEV^- = 21$), JEV seronegative and had not received the JE vaccine, but who were DENV seropositive ($DENV^+JEV^- = 22$), JEV^+(seropositive for JEV and had received the JE vaccine), but seronegative for DENV ($DENV^-JEV^+ = 23$). We further assessed the responses to these peptides by undertaking *ex vivo* IFNγ assays and flow cytometry.

Results: None of $DENV^-JEV^-$ individuals responded to any of the 20 JEV-specific peptides. High frequency of responses was seen to 6/20 peptides by individuals who were JEV^+ but $DENV^-$, where over 75% of the individuals responded to at least one peptide. P34 was the most immunogenic peptide, recognized by 20/23 (86.9%) individuals who were $DENV^-JEV^+$, followed by peptide 3 and peptide 7 recognized by 19/23 (82.6%). Peptide 34 from the NS2a region, showed <25% homology with any flaviviruses, and <20% homology with any DENV serotype. Peptide 20 and 32, which were also from the non-structural protein regions, showed <25% homology with DENV. *Ex vivo* responses to these peptides were less frequent, with only 40% of individuals responding to peptide 34 and 16–28% to other peptides, probably as 5/6 peptides were recognized by CD4+ T cells.

Discussion: We identified six highly conserved, T cell epitopes which are highly specific for JEV, in the Sri Lankan population. Since both JEV and DENV co-circulate in the same regions and since both JE and dengue vaccines are likely to be co-administered in the same geographical regions in future, these JEV-specific T cell epitopes would be useful to study JEV-specific T cell responses, in order to further understand how DENV and JEV-specific cellular immune responses influence each other.

Keywords: Japanese encephalitis virus, dengue virus, cross reactive T cell responses, cultured ELISpot assays, highly conserved regions

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INTRODUCTION

Mosquito borne viral infections are one of the leading emerging infectious diseases and represent a major public health problem in many tropical and subtropical countries. Among the rapidly emerging flaviviruses, infections due to the dengue viruses (DENV) are the most common, with the incidence increasing from 285.3 per 100,000 individuals in 1990–1371.1 in 2013 (1). Although case fatality rates due to dengue are declining in many countries including Sri Lanka, the rates are still significantly high in countries such as India, where the case fatality rates are estimated to be 2.6% (2). Other flavivirus infections such as the Japanese Encephalitis virus (JEV) and the West Nile virus (WNV), co-circulate in the same geographical regions such as DENV (3, 4), and due to the similarity between these viruses, have a potential to modulate the immune responses to each other. Natural infection with JEV has shown to generate highly cross reactive T cell responses that has a potential to lead to either milder or more severe disease when infected with DENV (5).

The studies which describe the effect of pre-existing JEV immunity on the outcome of DENV infection have shown varied results. A large prospective study carried out in Thailand showed that individuals with neutralizing antibodies to JEV, had a significantly increased risk of developing symptomatic dengue (6). In contrast another study in Thailand showed that those who received the inactivated JEV vaccine were less likely to get severe dengue (7). In a previous study, we observed that those who were seropositive for JEV were more likely to have been hospitalized due to dengue, compared to those who were seronegative for JEV (8). However, due to the cross-reactive nature of DENV-specific antibodies with JEV, it could not be ascertained if JEV positivity was due to the presence of highly cross reactive DENV-specific antibodies, or due to actual infection with JEV. Therefore, currently it is still not clear if DENV or JEV-specific antibody and T cell responses influence the immune responses to each other virus during subsequent infection and thus influence the disease outcome.

Both CD8+ and CD4+ T cells have been shown to play an important role in protection against DENV, JEV and Zika virus (5, 9–12). Individuals who were naturally exposed to JEV were shown to have antibody and T cell responses, that showed high cross-reactivity with DENV (5, 13). In our previous studies we showed that T cell responses of 20–30% of individuals who were naturally infected with DENV were cross-reactive with JEV (14). Apart from the magnitude of the T cell response, the functionality of T cell responses, specific to either JEV or DENV, have been shown to associate with the clinical disease outcome in both infections (5, 15). Virus-specific T cells of patients who had a milder clinical disease (either JEV or DENV), had different polyfunctional T cell signatures compared to those who had more severe disease (5, 12, 15). Due to the similarity of JEV and DENV, infection or immunization with either virus has a potential to influence both the magnitude and the functionality of T cell responses to each other. Although flavivirus cross-reactive T cells are likely to be cross protective, it is difficult to speculate on such protection in the absence of data regarding either reduced or enhanced disease severity following sequential infection with flaviviruses.

The occurrence of mild/asymptomatic illness in the majority of DENV infected individuals, and severe dengue and death in some individuals, has been attributed to many risk factors such as a secondary dengue infection, the time interval between two dengue infections (16), the incidence of dengue infection in a particular year and preceding years (17) and the presence of co-morbid illnesses (18, 19). Although disease enhancement due to the presence of non-neutralizing antibodies and possibly cross-reactive T cells is thought to lead to severe disease (20), DHF and fatalities have also been reported in primary dengue infection in the absence of DENV specific antibodies or T cells (21, 22). The presence of T cell responses that cross-react with other flaviviruses such as JEV, has a potential to be protective or to be involved in disease pathogenesis leading to severe clinical disease when individuals are naturally infected with DENV and have the potential to modulate immune responses to dengue vaccines (23). As the incidence of dengue and other flaviviruses are on the rise and as several dengue vaccine candidates are currently undergoing clinical trials, it would be important to investigate how the immune response to one of these co-circulating flaviviruses, influence the disease outcome during subsequent infections with other flaviviruses.

In order to determine if JEV-specific T cell responses are indeed cross protective when infected with DENV, it would be initially important to differentiate JEV-specific T cell responses from those which are broadly cross-reactive with DENV. This would be important especially in order to understand how sequential infection with different flaviviruses or immune responses induced by vaccination against JEV, would subsequently influence the disease outcome when naturally infected or vaccinated with DENV. It was recently shown that infection with JEV was far commoner than previously thought in DENV endemic countries and interpretation of natural infection with JEV was difficult especially following secondary dengue infections, due to the presence of more cross-reactive heterotypic antibodies (13). Therefore, as an initial step it would be important to identify T cell epitopes that are specific to JEV and do not cross-react with DENV in order to identify individuals who have had natural JEV infection, and also to further investigate T cell responses to JEV, independent of DENV-specific T cell responses. In this study, we identified JEV-specific, DENV non cross-reactive T cell epitopes and we proceeded to determine the immunogenetic JEV-specific T cell responses both *ex vivo* and by cultured ELISpot assays in individuals who received JEV vaccine and those who were naturally infected with DENV but were non-immune to JEV.

Abbreviations: JEV, Japanese encephalitis virus; DENV, Dengue virus; WNV, West nile virus.

MATERIALS AND METHODS

Identification of JEV-Specific Highly Conserved Regions Within JEV

One hundred and twelve JEV polyprotein sequences, which were isolated within a period of 50 years from the South Asian and South East Asian regions were retrieved from National Center

for Biotechnology information. These sequences were aligned using ClustalW, on Mega 7 software (www.megasoftware.net/) to identify the degree of conservation. Regions which showed >90% conservation were identified and sectioned into 20mer peptides overlapping by 5 or 10 amino acids. The specificity of these JEV peptides was determined by using Clustal Omega of European Bioinformatics Institute (EBI) (www.ebi.ac.uk) to confirm that they did not significantly cross-react with DENV (**Supplementary Table 1**). Out of these 36 peptides, only 20 peptides were successful in the synthesis with 90% purity (GENEscript USA) and were used for further analysis.

Recruitment of Healthy Individuals to Identify JEV Peptide Specific T Cell Responses

In order to identify the immunogenic JEV peptides from the 20 JEV-specific peptides identified above, we recruited 66 individuals, through the Family Practice Center, University of Sri Jayewardenepura, which is the primary health care facility of the University. These 66 individuals comprised of 21 individuals who were seronegative for both JEV and DENV, 22 were seronegative for JEV (and had not received the JE vaccine) but were DENV seropositive, 23 individuals were seronegative for DENV and had received the JE vaccine (**Table 1**). These individuals were initially recruited in year 2013 as a part of a large longitudinal community cohort study (n = 1,689) (14) and were invited to provide an additional sample of blood in year 2018, to re-evaluate their serostatus to DENV and JEV. In order to re-evaluate their serostatus at the time of donating a blood sample to this study, a serum sample was also obtained for detection of JEV and DENV IgG at the time of obtaining PBMCs (see below for details regarding the assay). These cohort of individuals have been followed by us through 2013, and all cases of febrile episodes for reported to the Family Practice Center.

Due to the limitations of the PBMC samples of the above 66 individuals, we recruited an additional cohort of 95 individuals for further assessment of the *ex vivo* IFNγ ELISpot responses to JEV-specific, immunodomoninant peptides. These 95 individuals too were initially recruited in 2013 as a part of the large community study (14). However, as 6 years had elapsed since 2013, we re-evaluated their serostatus for JEV and DENV IgG at the time of obtaining blood samples in 2019 for *ex vivo* ELISpot assays. PBMCs were extracted from these fresh blood samples and the *ex vivo* ELISpot assays we carried out using the fresh PBMCs. The time elapsed between JEV vaccination in these two cohorts of individuals was a mean of 17.15 years (SD ± 2.4 years).

Of the 95 individuals recruited for the *ex vivo* ELISpot assays, 20 were seronegative for JEV and DENV ($DENV^-JEV^-$), 25 were seropositive for DENV and were seronegative for JEV (and had not received the JE vaccine) ($DENV^+JEV^-$), 25 were seropositive for JEV (and had received the JE vaccine), but seronegative for DENV ($DENV^-JEV^-$), 25 were seropositive for both JEV and DENV ($DENV^+JEV^+$) (**Table 1**). Fresh PBMC samples were used for both culture and *ex vivo* ELISpot assays.

Ethical approval for this study was granted by Ethics Review Committee of the University of Sri Jayewardenepura.

TABLE 1 | Number of individuals recruited for culture and *ex vivo* ELISpot assays.

Group	Number of individuals
RECRUITED INDIVIDUALS FOR CULTURED ELISpot ASSAYS	
$DENV^-JEV^-$	21
$DENV^+JEV^-$	22
$DENV^-JEV^+$ (JE vaccinated)	23
Total	66
RECRUITED INDIVIDUALS FOR *EX VIVO* ELISpot ASSAYS	
$DENV^-JEV^-$	20
$DENV^+JEV^-$	25
$DENV^-JEV^+$ (JE vaccinated)	25
$DENV^+JEV^+$ (JE vaccinated)	25
Total	95
Total number of recruits	161

Determining DENV and JEV Serostatus in Healthy Individuals

The seropositivity of individuals to DENV was assessed using the indirect dengue IgG capture ELISA (Panbio, Australia) (8) and for JEV by JE direct IgG ELISA (InBios International, USA). Immune status to JEV was calculated using the immune status ratio (ISR) according to the manufacturers' instructions. An ISR of >5 was considered positive; an ISR of 2–5 equivocal and an ISR of <2 was considered negative.

Of these individuals, those who had not received the JE vaccine and were also seronegative for JEV IgG antibodies by a commercial ELISA (Inbios, USA), were considered as JEV seronegative (JEV^-). Those who had received the JE vaccine and who had detectable JEV IgG antibodies by a commercial ELISA were considered as JEV seropositive (JEV^+). Individuals who had received the JE vaccine and were seronegative by the commercial JEV IgG ELISA or those who had not received the JE vaccine and were seropositive based on the commercial JEV IgG ELISA were not considered in the analysis. DENV seropositivity of these individuals were identified by using commercially available dengue IgG panbio ELISA kit (Australia).

Cultured ELISpot Assays

Cultured ELISpot assays were performed to identify JEV-specific peptides recognized by memory T cells of JEV immune individuals, as previously described (14, 24). Cultured ELISpot assays have been previously used to detect antigen specific memory T cells, especially present in low frequency in HIV infection, Epstein Barr virus infection, malaria, hepatitis C infection and memory T cell responses to the DENV in acute dengue and in healthy DENV seropositive individuals (14, 25–27).

The responses to these 20 JEV-specific peptides were assessed in DENV and JEV seronegative individuals ($DENV^-JEV^-$, n = 21), DENV seronegative individuals who were vaccinated for JEV ($DENV^-$ JEV^+, $n = 23$), and DENV seropositive individuals who were not vaccinated for JEV ($DENV^+$ JEV^-, $n = 22$). Briefly, 5.0

$\times 10^6$ PBMCs were incubated for 10 days with 20 μl of the JE vaccine (SA 14-14-2 live attenuated) in a 24 well plate. The SA 14-14-2 is a mouse brain derived, live attenuated JE vaccine has been attenuated for neurovirulence with changes in 57 nucleotides resulting in changes in 24 amino acids compared to the live virus (28). IL-2 was added on day 3 and 7 at a concentration of 100 units/ml. All cell lines were routinely maintained in RPMI 1,640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml plus 10% human serum at 37°C, in 5% CO_2. T cell lines were tested individually after 10 days culture for responses to the 20 JEV-specific 20mer peptides. Briefly, ELISpot plates (Millipore Corp., Bedford, USA) were coated with anti-human IFNγ antibody overnight (Mabtech, Sweden). For cultured ELISpot assays, 4×10^5 cultures cells were added to a final volume of 200 μl. JEV-specific peptides were added at a final concentration of 10 μM as previously described (29, 30). All peptides were tested in duplicate. PHA was always included as a positive control and media alone with the cells alone was included as a negative control. The plates were incubated overnight at 37°C and 5% CO_2. The cells were removed, and the plates developed with a second biotinylated Ab to human IFNγ and washed a further six times. The plates were developed with streptavidin-alkaline phosphatase (Mabtech AB) and colorimetric substrate, and the spots enumerated using an automated ELISpot reader. Background (cells plus media) was subtracted and data expressed as number of spot-forming units (SFU) per 10^6 PBMC.

Ex vivo ELISpot Assays

As the cultured ELISpot responses predominantly assess central memory T cells, in order to assess the *ex vivo* effector memory T cell responses to these peptides, we assessed *ex vivo* IFNγ ELISpot responses in 95 individuals to 6/20 peptides, which were identified as being immunogenic with the cultured ELISpot assays (P2, P3, P7, P20, P32, and P34). The *ex vivo* IFNγ ELISpot responses were assessed in DENV and JEV seronegative individuals ($DENV^-JEV^-$, $n = 20$), DENV seronegative individuals who were vaccinated for JEV ($DENV^-JEV^+$, $n = 25$), DENV seropositive individuals who were not vaccinated for JEV ($DENV^+ JEV^-$, $n = 25$), and DENV seropositive individuals who were vaccinated for JEV ($DENV^+ JEV^+$, $n = 25$).

Ex vivo ELISpot assays were performed as previously described [see detailed description under cultured ELISpot assays (14, 31, 32)]. In *ex vivo* ELISpot assays PBMCs 1×10^5 were added to each well and JEV-specific, conserved peptides were added at a final concentration of 10 μM as previously described and tested in duplicate (32) The spots were enumerated using an automated ELISpot reader (AID, Germany). Background (cells with media) was subtracted and data expressed as number of spot-forming units (SFU) per 10^6 PBMC. All peptides that induced an IFN-γ response of more than mean ± 3 standard deviations of the negative controls were considered positive.

Flow Cytometry

To identify the subtype of T cells that were responding to JEV specific peptides, intracellular cytokine staining of PBMCs were performed. As we assessed the IFNγ production by *ex vivo* and cultured ELISpot assays, we assessed the degranulation capacity of JEV-specific T cells by carrying out CD107a expression in responses to these peptides *ex vivo*.

Briefly, the PBMCs were stimulated at 2×10^6/ml in RPMI-1640 plus 10% heat inactivated human serum with the relevant peptides (10 μM) for 16 h according to the manufacturer's instructions in the presence of Monensin (2 μM) (Biolegend, USA). The following monoclonal antibodies from Biolegend, USA, were used in this study after optimization by serial dilutions: anti CD3-APC Cy7 (clone OKT3), anti CD8-PE™ (clone SK1), anti CD4 Pacific blue (clone OKT4), CD107a FITC (clone H4A3) and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit were used. Intracellular staining was carried out as previously described (27, 33). To determine CD107a expression, PBMCs were stained with anti CD107a-FITC monoclonal antibodies for 30 min at 1–2 $\times 10^6$/ml in RPMI 1640 plus 10% FCS, prior to stimulation with the antigen (15). PBMCs were stained with anti CD3, anti CD4 and CD8, permeabilized and fixed with Cytofix/Cytoperm (Biolegend, USA) and acquired using a Guava-easy Cyte 12 HT Flowcytometer (Merck, Germany) and analyzed with FCS express 6 Flow Research Edition. A hierarchical gating strategy was used to gate live, single, CD3+, CD4+, and CD8+ T cells. Each antibody was titrated to determine the optimum concentration to use by comparing it with Fluorescence Minus One (FMO) controls.

Statistical Analysis

PRISM version 8.1 was used in statistical analysis, which was used to analyse the responses to individual JEV peptides. As the data were not normally distributed, differences in means were compared using the Mann–Whitney *U*-test (two tailed).

RESULTS

Identification and Specificity of JEV Peptides

Using bioinformatic tools, although we identified 36 JEVspecific, highly conserved regions within JEV, only 20/36 20mer JEV specific peptides representing these regions were successfully synthesized. The region within JEV where these peptides were identified and the homology of these regions with other flaviviruses and the 4 DENV serotypes is shown in **Table 2**. Although the structural proteins represent <20% of the whole JEV polyprotein, the 14/20 JEV-specific peptides were identified within the structural regions and 11/14 peptides within the envelope region. Only 6/20 identified JEV-specific peptides were located within the regions representing the non-structural proteins. The SA 14-14-2, live attenuated JE vaccine which was used as the antigen to stimulate PBMCs in this study, has changes in 57 nucleotides resulting in changes in 24 amino acids. These changes in the amino acids between the wild type virus and the JE vaccine virus was only seen in peptide 11. None of the other peptides, were within the regions where the changes in the amino acids were seen between the wild type viruses and the vaccine virus.

TABLE 2 | The homology of JEV-specific peptides with four dengue serotypes, WNV, YFV, and Zika virus.

No.	Peptide sequence	Protein	Peptide ID	DENV1%	DENV2%	DENV3%	DENV4%	WNV%	YFV%	Zika%
1	^{20}GLPRVFPLVGVKRVVMSLLDG^{39}	Capsid	P1	30	25	30	30	55	50	40
2	^{155}YSAQVGASQAAKFTVTPNAP^{174}	Envelope	P2	30	15	35	15	55	20	25
3	^{149}SENHGNYSAQVGASQAAKFT^{168}	Envelope	P3	25	25	25	25	55	35	25
4	^{331}SDGPCKIPIVSVASLNDMTP^{350}	Envelope	P5	35	30	30	25	75	35	35
5	^{341}SVASLNDMTPVGRLVTVNPF^{360}	Envelope	P6	35	35	25	25	90	35	40
6	^{351}VGRLVTVNPFVATSSANSKV^{370}	Envelope	P7	30	35	35	30	75	30	40
7	^{53}LAEVRSYCYHASVTDISTVA^{72}	Envelope	P8	20	30	25	35	70	30	45
8	^{77}TGEAHNKKRADSSYVCKQG^{95}	Envelope	P9	30	25	35	25	60	30	40
9	^{194}SGLNTEAFYVMTVGSKSFLV^{213}	Envelope	P10	25	20	25	25	65	30	35
10	^{261}GLHQALAGAIVVEYSSSVKL^{280}	Envelope	P11	30	35	25	30	75	35	30
11	^{471}MGVNARDRSIALAFLATGGV^{490}	Envelope	P12	30	25	20	35	80	15	45
12	^{481}ALAFLATGGVLVFLATNVHA^{500}	Envelope	P13	25	25	25	35	70	20	50
13	^{121}LQIGVHGILNAAAIAWMIVR^{140}	NS2A	P14	15	15	15	20	40	25	25
14	^{108}NESSIMWLASLAIVTACAG^{126}	Capsid	P16	15	15	15	20	20	30	25
15	^{73}SSQAGSLFVLPRGVPFTDLD^{92}	NS4B	P18	30	30	35	30	50	25	40
16	^{11}ADLKSMFAGKTQASGLTGLP^{33}	NS4B	P19	20	20	20	20	40	25	30
17	^{21}TQASGLTGLPSMALDLRPAT^{40}	NS4B	P20	20	25	20	20	50	30	35
18	^{100}KQNKRGGNEGSIMWLACLAV^{119}	Capsid	P32	10	15	10	25	40	25	25
19	^{105}QITLTTFLTAMVLATLHYGY^{124}	NS4B	P33	25	25	25	30	60	25	35
20	^{111}AAFFQLASADLQIGVHGILN^{130}	NS2A	P34	20	10	10	20	40	25	25

Protein sequence in the table showed as 'N' terminal to 'C' terminal, and superscript number showed the peptide position in the relevant protein.

Identification of JEV Specific Immunogenic Peptides in JEV Immune Individuals Through Cultured ELISpot Assays

Cultured ELISpot assays have widely used as a sensitive assay that measures central memory T cells that are even present at low frequency (25, 34, 35). Therefore, we used this approach to identify JEV-specific memory T cell responses, that could be even be present low frequency and therefore, be missed by using *ex vivo* ELISpot assays. Cultured ELISpot responses to the 20 JEV-specific 20mer peptides in the JE vaccinated, DENV seronegative individuals ($DENV^-JEV^+$, $n = 23$), DENV seropositive but JEV non-vaccinated individuals ($DENV^+$ JEV^-, $n = 22$), and both DENV and JEV seronegative individuals ($DENV^-$ JEV^-, $n = 21$) are shown in **Figure 1**. Cutoff value for a positive T cell response was considered as the mean ± 3SD of all negative controls, and in this study, it was ± 1,930 SFU/10^6 PBMCs. Although quite a few responses were considered negative based on these criteria, we wished to have a more stringent assessment criteria to only select the T cell responses which displayed a high magnitude. This was to avoid selection of any responses that would be false positive. An example of a plate layout and responses to the JEV specific peptides, the negative and positive control is shown in **Supplementary Figure 1**.

None of JEV and DENV seronegative individuals ($DENV^-JEV^-$) responded to any of these peptides, while three individuals who were DENV seropositive but JEV seronegative responded to P11, P12, and P18 (**Figure 1**). Responses to other JEV-specific peptides were not detected in any of $DENV^+JEV^-$ individuals. P34 was the most immunogenic JEV-specific peptide, recognized by 20/23 (86.9%) individuals who were $DENV^-JEV^+$ (**Figure 1**). P3 and P7 were recognized by 19/23 (82.6%) of $DENV^-JEV^+$ group of individuals and P2, P32, and P20 recognized by 18/23 (78.3%) individuals (**Table 3**). The alignment of each of these peptides with DENV, ZIKV, YFV and WNV is shown in **Supplementary Figure 2**. Only 14 (61%) of individuals responded to peptide 11 (aa261–280), where there is a one amino acid difference between the vaccine virus and the wild type JEV.

Ex vivo IFNγ ELISpot Responses to JEV Specific Peptides

Based on the results of cultured ELISpot responses, 18/23 (78.3%) of $DENV^-JEV^+$ individuals responded to 6/20 peptides tested and we wished to determine their immunogenicity using *ex vivo* ELISpot assays as we sought to investigate if the frequency of JEV-peptide specific T cells were present at frequency that they can be detected *ex vivo*.

Ex vivo T cell responses to the six 20mer peptides (P2, P3, P7, P20, P32, and P34) were evaluated in a second cohort of JE vaccinated, DENV seronegative individuals (JEV^+DENV^-, $n = 25$), JEV non-vaccinated (JEV seronegative) but DENV seropositive individuals (JEV^-DENV^+, $n = 25$), JEV vaccinated, DENV seropositive individuals (JEV^+DENV^-, $n = 25$), and both DENV and JEV seronegative (JEV^-DENV^-, $n = 20$). We also pooled all these peptides together and evaluated the *ex vivo* IFNγ ELISpot responses to this pool of peptides in the above 4 groups. The cutoff value for a positive IFNγ T cell response was considered as the mean ± 3SD of all negative controls, and in this study, it was 220 SFU/10^6 PBMCs. As seen with the cultured ELISpot assays, none of the JEV^-DENV^- and JEV^-DENV^+

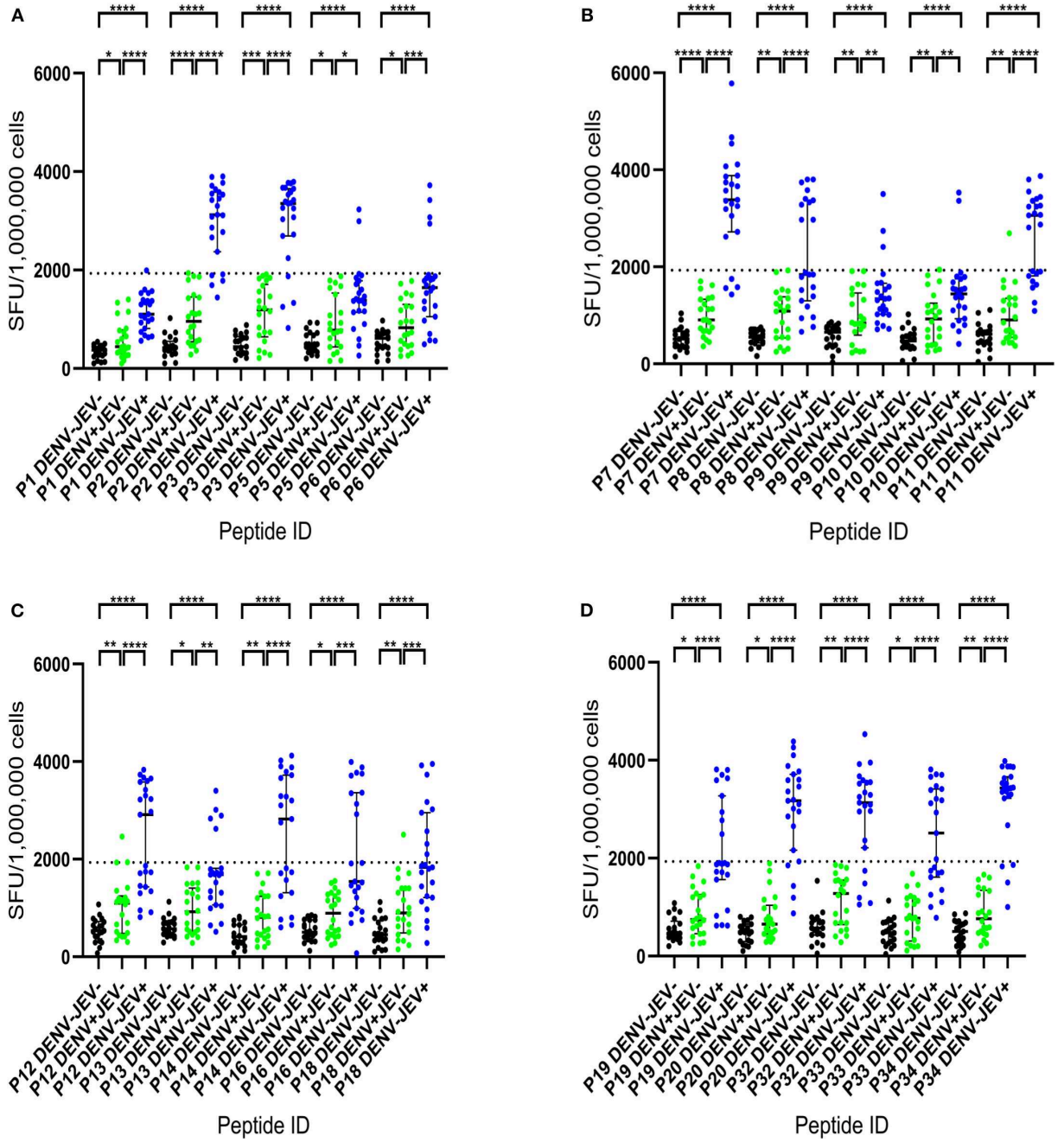

FIGURE 1 | IFNγ cultured ELISpot responses to 20 JEV-specific peptides in individuals with varied DENV and JEV seropositivity. T cell responses to twenty 20mer JEV-specific peptides were measured by following short term culture with IFNγ ELISpot in those who were JEV seropositive but seronegative for DENV (JEV^+DEV^-, $n = 23$), DENV seropositive but JEV seronegative (JEV^-DENV^+, $n = 22$) and seronegative for both (JEV^-DENV^-, $n = 21$). Error bars indicate the median and the interquartile range. The horizontal dotted line represents the cut-off value of 1,930 SFU/10^6, which was considered as the mean, ± 3SD of the negative controls given spot count for all three groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. The background has been subtracted from the responses displayed. Black color- JEV^-DENV^-; Green color- JEV^-DENV^+; Blue color- JEV^+DEV^-. **(A)** IFNγ cultured ELISpot responses of P1 to P6. **(B)** IFNγ cultured ELISpot responses of P7 to P11. **(C)** IFNγ cultured ELISpot responses of P12 to P18. **(D)** IFNγ cultured ELISpot responses of P19 to P34.

individuals responded to any of the six JEV-specific peptides (**Figure 2**). The number of individuals who responded to each of the peptides is shown in **Table 4**. Again, quite a few individuals responded to these peptides, which were below the cut off value stimulated by us. However, we wished to have stringent assessment criteria to only select the T cell responses which displayed a high magnitude, so that false positive responses are not selected.

Although none of $DENV^+JEV^-$ or $DENV^-JEV^-$ individuals responded to any of these peptides, in the *ex vivo* IFNγ

TABLE 3 | IFNγ cultured ELISpot responses to JEV-specific peptides.

Peptide ID	Peptide sequence	Protein	Number of individuals who responded ($n = 23$)	Median (IQR) SFU/10^6 cells
P1	^{20}GLPRVFPLVGVKRVVMSLLDG^{39}	Capsid	1 (4%)	1,100 (810–1,390)
P2	^{155}YSAQVGASQAAKFTVTPNAP^{174}	Envelope	18 (78%)	3,120 (2,370–3,580)
P3	^{149}SENHGNYSAQVGASQAAKFT^{168}	Envelope	19 (83%)	3,350 (2,690–3,650)
P5	^{331}SDGPCKIPIVSVASLNDMTP^{350}	Envelope	2 (9%)	1,380 (1,140–1,780)
P6	^{341}SVASLNDMTPVGRLVTVNPF^{360}	Envelope	4 (17%)	1,640 (1,050–1,870)
P7	^{351}VGRLVTVNPFVATSSANSKV^{370}	Envelope	19 (83%)	3,380 (2,720–3,880)
P8	^{53}LAEVRSYCYHASVTDISTVA^{72}	Envelope	10 (43%)	1,840 (1,300–3,370)
P9	^{77}TGEAHNKKRADSSYVCKQG^{95}	Envelope	3 (13%)	1,340 (1,020–1,660)
P10	^{194}SGLNTEAFYVMTVGSKSFLV^{213}	Envelope	2 (9%)	1,440 (930–1,750)
P11	^{261}GLHQALAGAIVVEYSSSVKL^{280}	Envelope	14 (61%)	3,060 (1,810–3,360)
P12	^{471}MGVNARDRSIALAFLATGGV^{490}	Envelope	12 (52%)	2,910 (14,300–3,580)
P13	^{481}ALAFLATGGVLVFLATNVHA^{500}	Envelope	5 (22%)	1,680 (1,060–1,810)
P14	^{121}LQIGVHGILNAAAIAWMIVR^{140}	NS2A	13 (57%)	2,820 (1,310–3,720)
P16	^{108}NESSIMWLASLAIVTACAG^{126}	Capsid	9 (39%)	1,540 (990–3,360)
P18	^{73}SSQAGSLFVLPRGVPFTDLD^{92}	NS4B	9 (39%)	1,830 (1,210– 2,950)
P19	^{11}ADLKSMFAGKTQASGLTGLP^{33}	NS4B	9 (39%)	1,880 (1,560–3,270)
P20	^{21}TQASGLTGLPSMALDLRPAT^{40}	NS4B	18 (78%)	3,170 (2,160–3,710)
P32	^{100}KQNKRGGNEGSIMWLACLAV^{119}	Capsid	18 (78%)	3,130 (2,210–3,550)
P33	^{105}QITLTTFLTAMVLATLHYGY^{124}	NS4B	(57%)	2,510 (1,610–3,410)
P34	^{111}AAFFQLASADLQIGVHGILN^{130}	NS2A	20 (87%)	3,430 (3,220–3,660)

ELISpot assays, the number of individuals of DENV-JEV+ and $DENV^+JEV^+$ groups who responded were also low. For instance, only 5 (20%)−10 (40%) individuals of each of the two groups responded any of these peptides *ex vivo*. Again, the most immunogenic peptide was P34, with 8–10 (32–40%) individuals responding to it.

Investigating if JEV-Peptide Specific T Cell Responses Were of the CD4 ± or the CD8 ± Subtype

Following identification of six JEV-specific, highly conserved peptides, we further proceeded to determine if the T cells recognizing these peptides were predominantly CD4+ or CD8+ T cell subtype. As we determined the IFNγ-producing capacity using *ex vivo* and cultured ELISpot assays, in ICS assays we instead determined the degranulating capacity by assessing CD107a/CD4/CD8 expression *ex vivo* when stimulated with these peptides. In order to carry out these assays we re-recruited 4 individuals who were $DENV^+JEV^+$ and were found to respond to these peptides. We found that in these individuals, peptide 2, 7, and 20 were predominantly recognized by CD4+ T cells whereas the subset responding to peptide 34 was inconclusive. The CD107a expression to these peptides in these 4 individuals varied from 1.4 to 4.21% of the proportion of the CD4+ T cells. Very low CD1017a expression was induced by peptide 3 and 32 such that it was difficult to determine if the responding cells were CD4+ or CD8+. The gating strategy and an example CD107a expression for a JEV specific peptide is shown in **Supplementary Figure 3**.

As mentioned above, P34 was the most immunogenic JEV-specific peptide, recognized by 86.9% individuals who were $DENV^-JEV^+$, P3 and P7 were recognized by 82.6% of $DENV^-JEV^+$ group of individuals and P2, P32, and P20 recognized by 78.3% individuals. Apart from P34, P3, and P32, which the T cell subtype could not be determined, all other peptides were recognized by CD4+ T cells, thus likely presented by MHC Class II molecules. Although, we did not HLA type the 66 donors, we used the IEDB analysis resource to predict binding of these peptides to MHC class II alleles (36). The most immunodominant peptide P34 gave very high binding scores for many DQB1 alleles suggesting that it was likely to be presented by many different alleles (**Supplementary Materials**). For instance, it gave extremely high binding scores to different alleles of DQB1*02, DQB1*03, DQB*05, and DQB1*06 which are present in the 17.6, 20.6, 28.15, and 29.4%, respectively (37). The other JEV-specific peptides were also shown to bind to multiple MHC class II alleles, although at a lower frequency than P34 (**Supplementary Materials**).

DISCUSSION

In this study, we have identified highly conserved regions, specific to JEV which are recognized by JEV-specific T cells and were not recognized by any of DENV seropositive individuals. Identification of JEV-specific T cells that do not cross react with the T cells specific to the DENV, would be important to further understand the protective or pathogenic role of JEV specific T cells in acute JE infection and to find out how sequential infection

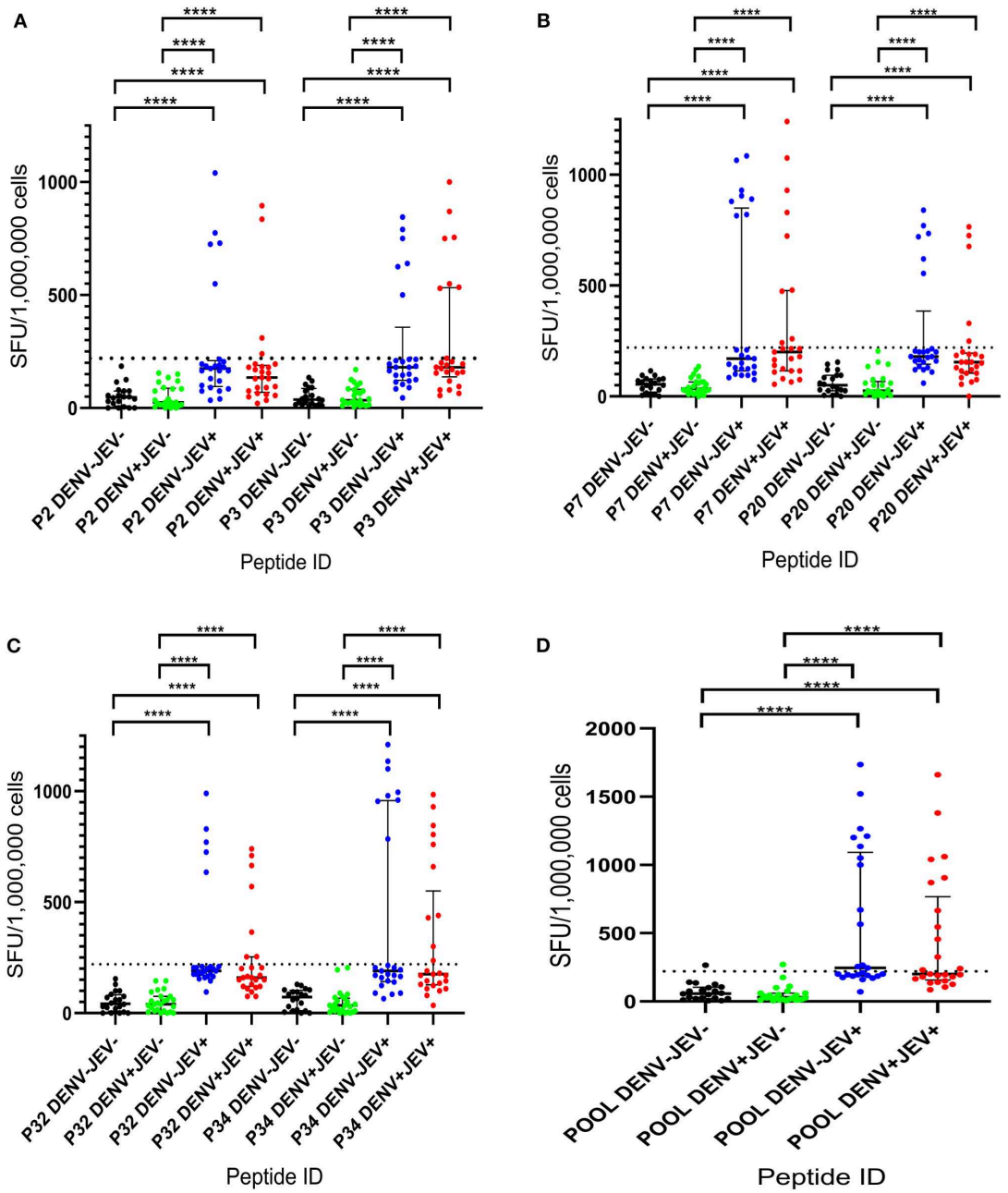

FIGURE 2 | *Ex vivo* T cell immune responses to six JEV-specific peptides in individuals with varied DENV and JEV seropositivity. JEV-specific T cell responses were measured by ELISpot assay to 6 JEV-specific peptides (which were given higher T cell immune responses in culture ELISpot assays) in those who were both JEV and DENV seropositive (JEV^+DEV^+, $n = 25$), JEV seropositive but seronegative for DENV (JEV^+DEV^-, $n = 25$), DENV seropositive but JEV-seronegative (JEV^-DENV^+, $n = 25$), and seronegative for both (JEV^-DENV^-, $n = 20$). Error bars indicate the median and the interquartile range. The horizontal dotted line represents the cut-off value of 220 SPU/10^6, which was considered as the mean, ± 3SD of the negative controls given spot count for all four groups. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. The background has been subtracted from the responses displayed. Black color- JEV^-DENV^-; Green color- JEV^-DENV^+; Blue color- JEV^+DEV^-; Red color- JEV^+DEV^+. **(A)** *Ex vivo* T cell immune responses of P2 and P3. **(B)** *Ex vivo* T cell immune responses of P7 and P20. **(C)** *Ex vivo* T cell immune responses of P32 and P34. **(D)** *Ex vivo* T cell immune responses of pool.

with the DENV would affect the development of JEV-specific T cell immunity on subsequent exposure.

Of the six JEV-specific peptides which gave a high frequency of responses, three of the serotype specific regions identified were within the envelope of JEV (peptide 2, 3, and 7), and the other three regions were located in capsid (peptide 32), NS4B (peptide 20) and NS2a (peptide 34). T cell responses to these peptides were assessed in individuals who had received the JE vaccine and not in those who were naturally infected with the virus, as the JE vaccine was included in the National

TABLE 4 | *Ex vivo* IFNγ ELISPot responses to six of the immunodominant JEV-specific peptides.

Peptide ID	Peptide sequence	Protein	Number of individuals who responded		Median (IQR) SFU/10^6		CD107a expression (%)	
			$DENV^-JEV^+$ ($n = 25$)	$DENV^+JEV^+$ ($n = 25$)	$DENV^-JEV^+$	$DENV^+JEV^+$	CD4	CD8
P2	^{155}YSAQVGASQAAKFTVTPNAP^{174}	Envelope	5 (20%)	4 (16%)	175 (95–210)	135 (70–189)	4.21	2.46
P3	^{149}SENHGNYSAQVGASQAAKFT^{168}	Envelope	6 (24%)	7 (28%)	180 (122.5–357.5)	180 (137.5–532.5)	-	-
P7	^{351}VGRLVTVNPFVATSSANSKV^{370}	Envelope	8 (32%)	9 (36%)	170 (115–850)	200 (115–477.5)	3.61	2.08
P20	^{21}TQASGLTGLPSMALDLRPAT^{40}	NS4B	6 (24%)	5 (20%)	180 (145–385)	155 (105–195)	2.55	1.44
P32	^{100}KQNKRGGNEGSIMWLACLAV^{119}	Capsid	5 (20%)	7 (28%)	190 (170–210)	160 (120–253.8)	-	-
P34	^{111}AAFFQLASADLQIGVHGILN^{130}	NS2A	8 (32%)	10 (40%)	190 (142.5–957.5)	175 (127.5–550)	-	-

Immunization schedule in Sri Lanka since 1988, in a stepwise manner (38). Therefore, the incidence of natural JEV infection has been very low during the past two decades in Sri Lanka and it was not possible to find individuals naturally infected with the JEV recently. The live JE vaccine has shown to induce highly cross-reactive CD4+ and CD8+ T cell responses, which cross-react with DENV, and predominantly targeted the PrM, NS1 and NS3 regions (39). These regions, which are preferentially targeted by T cells following JE immunization, show a high degree of homology with DENV and many other flaviviruses (39).

The peptides identified within the envelope region had <35% homology with the envelope proteins of all DENV serotypes and the other peptides had 25% homology with the regions of all DENV serotypes. Peptide 34, which was the most immunogenic peptide recognized by 86.9% of individuals who had received the JE vaccine (and none of DENV immune individuals) showed <20% homology with any of DENV serotypes and 25% homology with yellow fever virus and Zika virus. Infection with either Zika or yellow fever virus has not been reported in Sri Lanka so far. However, sporadic cases of West Nile virus (WNV) have been reported (3, 40) and JEV-peptide 34 gives a 40% homology with the WNV, which could induce cross-reactive T cells. Two of the peptides (peptide 2 and 3) which were identified within envelope region of JEV and which did not induce any responses in DENV seropositive individuals, showed 55% homology with WNV. Peptide 7, again within the envelope region of JEV, had a homology of 75%, which may induce WNV cross-reactive T cell responses due to the degree of homology.

Although recognition of antigens by T cells is HLA-restricted and therefore, responses to these JEV specific peptides would depend on an individual's HLA type, we wished to identify responses that are recognized by a large proportion of JEV immune individuals irrespective of their HLA type. For instance, in acute DENV infection, although recent studies show that DENV-specific T cells are likely to be protective (9, 12, 15, 41), studies have also shown that DENV-specific T cells are highly cross reactive and possibly contribute to disease pathogenesis by producing pro-inflammatory cytokines (29, 42). However, identification of JEV-specific T cell epitopes that do not cross react with DENV, would also enable us to better understand T cell immunity to the DENV, in the context of background immunity to the JEV, especially following vaccination. Furthermore, it would also be useful to investigate if the magnitude and the phenotype of JEV-specific T cell responses influence the strength and breadth of the DENV-specific T cell response following natural infection or following immunization with the DENV. Therefore, we wished to identify JEV-specific T cell epitopes that could be used for this purpose. Knowing the HLA restriction of these epitopes would be important to characterize the phenotype of these T cells. Such experiments were beyond the scope of this study. However, since the JEV-specific epitopes identified in this study were not investigated in relation to the donor HLA types, these findings are broadly relevant to the population studied.

The responding T cell subset was not clear for peptide 34, 32, and 3, while responses to peptide 2, 7, and 20 were predominantly from CD4+ T cells. The low frequency of CD107a expression from P34, P32, and P3 was probably due to them being predominantly been recognized by CD4+ T cells too, which have a poor degranulation capacity. Furthermore, although there was detectable CD107a expression for peptide 2, 7, and 20 the responses were of relatively low frequency (between 1.4 and 4.2%), suggesting that these JEV-peptide specific T cells have overall poor degranulation capacity. However, in these assays we measured the capacity of these JEV-peptide specific T cells to degranulate and it is possible that IFNγ production could be by a completely different subset of T cells. The dominance of CD4+ T cell epitopes to JEV specific peptides, could be due to several reasons. Firstly, we assessed JEV-specific T cell responses in those who received the JE vaccine and not those who were naturally exposed. It was shown that those who received the JE vaccine are more likely to have a higher frequency of a CD4+ T cell response compared to those who were naturally infected with JEV (39). Secondly, we used cultured ELISpot responses to identify JEV-specific memory T cell responses. Although cultured ELISpot responses are a valuable tool in investigating memory T cell responses, it has been shown this process results in reduced proliferation of CD8+ T cells compared to CD4+ T cells (34). Therefore, our approach would have biased the memory JEV-specific responses toward finding memory CD4+ T cell responses. The high frequency of recognition of these peptides could also be due to their presentation by MHC class II molecules. For instance, 78% responded to peptide 2 while 83% responded to peptide 7. The CD107a expression for peptide 34 was generated by both CD4+ and CD8+ T cells. Epitopes presented by MHC class II alleles are shown to be highly promiscuous. The same epitope has shown to be presented by

multiple T cell alleles in breast cancer (HER2) (43), Ag85B T-cell epitope in Mycobacterium tuberculosis (44), T cell epitopes in Mycobacterium leprae (45) and in many other instances.

Although we found that >75% of individuals responded to these 6 peptides by cultured ELIspot assays, the responses detected by *ex vivo* IFNγ ELISpot assays were less frequent. For instance, only 40% of individuals responded to peptide 34, by *ex vivo* assays, whereas 86.9% of individuals responded in the cultured ELISpot assays. Again, since the frequency of virus specific CD4+ T cells are known to be lower than the frequency of virus specific CD8+ T cells, is likely to be the reason for the limited responses detected by us through *ex vivo* ELISpot assays. In addition, as individuals with certain HLA types are only likely to present these peptides, these epitopes might not be presented by the individuals who showed negative responses. However, due to the low frequency of responses to these peptides *ex vivo,* the use of these peptides to evaluate JEV-specific T cell responses in a community or as a diagnostic test would not be suitable. Although a high frequency of responses was seen in cultured ELISpot assays, such assays would not be practical to be used as a diagnostic assay as they are labor intensive and expensive. Currently, one of the major challenges is distinguishing JEV or DENV T cell responses, when individuals are immune to both viruses. Since we have identified several JEV-specific peptides, they could be used to further understand the pathogenic or protective role of JEV-specific T cell responses.

One of the limitations of our study is the use of the JE Inbios IgG ELISA to define JEV-specific seropositivity. It has been shown that this assay has poor sensitivity and detected the presence of JEV specific antibodies in only 20% of those who were found to have JEV specific IgG by neutralization assays (46). In order to recruit JEV seronegatives, we only recruited those who had never received the JEV vaccine and none of the $JEV^{-}DENV^{-}$ responded to any of the peptides. Furthermore, we had a very high cutoff value in our cultured ELISpot assay, so that we only pickup responses of high magnitude so that low frequency possible non-specific responses are not taken into account.

In summary, both JEV and DENV co-circulate in the same regions and since JEV and DENV vaccines are likely to be co-administered in the same geographical regions in future. We had previously identified DENV serotype-specific T cell epitopes in conserved regions of all four DENV serotypes (24). Therefore, identification of these JEV-specific, conserved, immunogenic regions are likely to help in understanding T cell responses to both JEV and DENV independently of each other.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Review Committee of the University of Sri Jayewardenapura. Written informed consent to participate in this study was provided by the participants and their legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PP carried out the bioinformatics analysis, cultured, and *ex vivo* ELISpot assays. CJ recruited all individuals to the study and carried out the JEV ELISA. AW helped with both the cultured and *ex vivo* ELISpot assays and ICS assays. LG helped with the DENV and JEV ELISA. GO helped in designing the study and writing the paper. CG helped in the bioinformatic analysis, planning the study, and obtaining funding. GM helped in designing the study, data analysis, obtaining funding, and writing 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/fpubh.2020.00019/full#supplementary-material

REFERENCES

1. Stanaway JD, Shepard DS, Undurraga EA, Halasa YA, Coffeng LE, Brady OJ, et al. The global burden of dengue: an analysis from the global burden of disease study 2013. *Lancet Infect Dis.* (2016) 16:712–23. doi: 10.1016/S1473-3099(16)00026-8
2. Murhekar MV, Kamaraj P, Kumar MS, Khan SA, Allam RR, Barde P, et al. Burden of dengue infection in India, 2017: a cross-sectional population based serosurvey. *Lancet Glob Health.* (2019) 7:e1065-73. doi: 10.1016/S2214-109X(19)30250-5
3. Lohitharajah J, Malavige GN, Chua AJ, Ng ML, Arambepola C, Chang T. Emergence of human west nile virus infection in Sri Lanka. *BMC Infect Dis.* (2015) 15:305. doi: 10.1186/s12879-015-1040-7
4. Baruah A, Hazarika RA, Barman NN, Islam S, Gulati BR. Mosquito abundance and pig seropositivity as a correlate of Japanese encephalitis in human population in Assam, India. *J Vector Borne Dis.* (2018) 55:291–6. doi: 10.4103/0972-9062.256564
5. Turtle L, Bali T, Buxton G, Chib S, Chan S, Soni M, et al. Human T cell responses to Japanese encephalitis virus in health and disease. *J Exp Med.* (2016) 213:1331–52. doi: 10.1084/jem.20151517
6. Anderson KB, Gibbons RV, Thomas SJ, Rothman AL, Nisalak A, Berkelman RL, et al. Preexisting Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a school-based cohort in Thailand. *PLoS Negl Trop Dis.* (2011) 5:e1311. doi: 10.1371/journal.pntd.0001311
7. Hoke CH, Nisalak A, Sangawhipa N, Jatanasen S, Laorakapongse T, Innis BL, et al. Protection against Japanese encephalitis by inactivated vaccines. *N Engl J Med.* (1988) 319:608–14. doi: 10.1056/NEJM198809083191004
8. Jeewandara C, Gomes L, Paranavitane SA, Tantirimudalige M, Panapitiya SS, Jayewardene A, et al. Change in dengue and Japanese encephalitis

seroprevalence rates in Sri Lanka. *PLoS ONE*. (2015) 10:e0144799. doi: 10.1371/journal.pone.0144799
9. Weiskopf D, Angelo MA, de Azeredo EL, Sidney J, Greenbaum JA, Fernando AN, et al. Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. *Proc Natl Acad Sci USA*. (2013) 110:E2046–53. doi: 10.1073/pnas.1305227110
10. Weiskopf D, Angelo MA, Sidney J, Peters B, Shresta S, Sette A. Immunodominance changes as a function of the infecting dengue virus serotype and primary versus secondary infection. *J Virol.* (2014) 88:11383–94. doi: 10.1128/JVI.01108-14
11. Weiskopf D, Bangs DJ, Sidney J, Kolla RV, De Silva AD, de Silva AM, et al. Dengue virus infection elicits highly polarized CX3CR1+ cytotoxic CD4+ T cells associated with protective immunity. *Proc Natl Acad Sci USA*. (2015) 112:E4256–63. doi: 10.1073/pnas.1505956112
12. Wijeratne DT, Fernando S, Gomes L, Jeewandara C, Ginneliya A, Samarasekara S, et al. Quantification of dengue virus specific T cell responses and correlation with viral load and clinical disease severity in acute dengue infection. *PLoS Negl Trop Dis.* (2018) 12:e0006540. doi: 10.1371/journal.pntd.0006540
13. Nealon J, Taurel AF, Yoksan S, Moureau A, Bonaparte M, Quang LC, et al. Serological evidence of Japanese encephalitis virus circulation in asian children from dengue-endemic countries. *J Infect Dis*. (2019) 219:375–81. doi: 10.1093/infdis/jiy513
14. Jeewandara C, Adikari TN, Gomes L, Fernando S, Fernando RH, Perera MK, et al. Functionality of dengue virus specific memory T cell responses in individuals who were hospitalized or who had mild or subclinical dengue infection. *PLoS Negl Trop Dis.* (2015) 9:e0003673. doi: 10.1371/journal.pntd.0003673
15. Wijeratne DT, Fernando S, Gomes L, Jeewandara C, Jayarathna G, Perera Y, et al. Association of dengue virus-specific polyfunctional T-cell responses with clinical disease severity in acute dengue infection. *Immun Inflamm Dis.* (2019) 7:276–85. doi: 10.1002/iid3.271
16. Anderson KB, Gibbons RV, Cummings DA, Nisalak A, Green S, Libraty DH, et al. A shorter time interval between first and second dengue infections is associated with protection from clinical illness in a school-based cohort in Thailand. *J Infect Dis.* (2014) 209:360–8. doi: 10.1093/infdis/jit436
17. Endy TP, Anderson KB, Nisalak A, Yoon IK, Green S, Rothman AL, et al. Determinants of inapparent and symptomatic dengue infection in a prospective study of primary school children in Kamphaeng Phet, Thailand. *PLoS Negl Trop Dis.* (2011) 5:e975. doi: 10.1371/journal.pntd.0000975
18. Figueiredo MA, Rodrigues LC, Barreto ML, Lima JW, Costa MC, Morato V, et al. Allergies and diabetes as risk factors for dengue hemorrhagic fever: results of a case control study. *PLoS Negl Trop Dis.* (2010) 4:e699. doi: 10.1371/journal.pntd.0000699
19. Pang J, Hsu JP, Yeo TW, Leo YS, Lye DC. Diabetes, cardiac disorders and asthma as risk factors for severe organ involvement among adult dengue patients: a matched case-control study. *Sci Rep*. (2017) 7:39872. doi: 10.1038/srep39872
20. Guzman MG, Alvarez M, Halstead SB. Secondary infection as a risk factor for dengue hemorrhagic fever/dengue shock syndrome: an historical perspective and role of antibody-dependent enhancement of infection. *Arch Virol.* (2013) 158:1445–59. doi: 10.1007/s00705-013-1645-3
21. Ong A, Sandar M, Chen MI, Sin LY. Fatal dengue hemorrhagic fever in adults during a dengue epidemic in Singapore. *Int J Infect Dis.* (2007) 11:263–7. doi: 10.1016/j.ijid.2006.02.012
22. Singla M, Kar M, Sethi T, Kabra SK, Lodha R, Chandele A, et al. Immune response to dengue virus infection in pediatric patients in New Delhi, India—association of viremia, inflammatory mediators and monocytes with disease severity. *PLoS Negl Trop Dis.* (2016) 10:e0004497. doi: 10.1371/journal.pntd.0004497
23. Elong Ngono A, Shresta S. Cross-reactive T cell immunity to dengue and zika viruses: new insights into vaccine development. *Front Immunol.* (2019) 10:1316. doi: 10.3389/fimmu.2019.01316
24. Malavige GN, McGowan S, Atukorale V, Salimi M, Peelawatta M, Fernando N, et al. Identification of serotype-specific T cell responses to highly conserved regions of the dengue viruses. *Clin Exp Immunol.* (2012) 168:215–23. doi: 10.1111/j.1365-2249.2012.04566.x
25. Keating SM, Bejon P, Berthoud T, Vuola JM, Todryk S, Webster DP, et al. Durable human memory T cells quantifiable by cultured enzyme-linked immunospot assays are induced by heterologous prime boost immunization and correlate with protection against malaria. *J Immunol.* (2005) 175:5675–80. doi: 10.4049/jimmunol.175.9.5675
26. Calarota SA, Baldanti F. Enumeration and characterization of human memory T cells by enzyme-linked immunospot assays. *Clin Dev Immunol.* (2013) 2013:637649. doi: 10.1155/2013/637649
27. Malavige GN, Jeewandara C, Alles KM, Salimi M, Gomes L, Kamaladasa A, et al. Suppression of virus specific immune responses by IL-10 in acute dengue infection. *PLoS Negl Trop Dis.* (2013) 7:e2409. doi: 10.1371/journal.pntd.0002409
28. World Health Organization (2005). *Weekly Epidemiological Record*. Geneva: World Health Organization.
29. Mongkolsapaya J, Duangchinda T, Dejnirattisai W, Vasanawathana S, Avirutnan P, Jairungsri A, et al. T cell responses in dengue hemorrhagic fever: are cross-reactive T cells suboptimal? *J Immunol.* (2006) 176:3821–9. doi: 10.4049/jimmunol.176.6.3821
30. Malavige GN, Jones L, Kamaladasa SD, Wijewickrama A, Seneviratne SL, Black AP, et al. Viral load, clinical disease severity and cellular immune responses in primary varicella zoster virus infection in Sri Lanka. *PLoS ONE.* (2008) 3:e3789. doi: 10.1371/journal.pone.0003789
31. Simmons CP, Dong T, Chau NV, Dung NT, Chau TN, Thao le TT, et al. Early T-cell responses to dengue virus epitopes in vietnamese adults with secondary dengue virus infections. *J Virol.* (2005) 79:5665–75. doi: 10.1128/JVI.79.9.5665-5675.2005
32. Malavige GN. *Ex Vivo* ELISpot assay to investigate dengue virus specific T-cell responses. *Methods Mol Biol.* (2018) 1808:173–9. doi: 10.1007/978-1-4939-8567-8_15
33. Park SH, Shin EC. Direct *ex vivo* functional analysis of HCV-specific T cells. *Methods Mol. Biol.* (2019) 1911:349–61. doi: 10.1007/978-1-4939-8976-8_24
34. Todryk SM, Pathan AA, Keating S, Porter DW, Berthoud T, Thompson F, et al. The relationship between human effector and memory T cells measured by *ex vivo* and cultured ELISPOT following recent and distal priming. *Immunology*. (2009) 128:83–91. doi: 10.1111/j.1365-2567.2009.03073.x
35. Jeewandara C, Ogg GS, Malavige GN. Cultured ELISpot assay to investigate dengue virus specific T-cell responses. *Methods Mol. Biol.* (2018) 1808:165–71. doi: 10.1007/978-1-4939-8567-8_14
36. Vita R, Mahajan S, Overton JA, Dhanda SK, Martini S, Cantrell JR, et al. The immune epitope database (IEDB): 2018 update. *Nucleic Acids Res.* (2019) 47:D339–43. doi: 10.1093/nar/gky1006
37. Malavige GN, Rostron T, Rohanachandra LT, Jayaratne SD, Fernando N, De Silva AD, et al. HLA class I and class II associations in dengue viral infections in a Sri Lankan population. *PLoS ONE.* (2011) 6:e20581. doi: 10.1371/journal.pone.0020581
38. Epidemiology Unit of the Ministry of Health Sri Lanka (2012). *Japanese Encephalitis: a manual for Medical Officers of Health*. Colombo: Epidemiology Unit of the Ministry of Health.
39. Turtle L, Tatullo F, Bali T, Ravi V, Soni M, Chan S, et al. Cellular immune responses to live attenuated Japanese Encephalitis (JE) vaccine SA14-14-2 in adults in a JE/dengue co-endemic area. *PLoS Negl Trop Dis.* (2017) 11:e0005263. doi: 10.1371/journal.pntd.0005263
40. Lohitharajah J, Malavige N, Arambepola C, Wanigasinghe J, Gamage R, Gunaratne P, et al. Viral aetiologies of acute encephalitis in a hospital-based South Asian population. *BMC Infect Dis.* (2017) 17:303. doi: 10.1186/s12879-017-2403-z
41. Weiskopf D, Sette A. T-cell immunity to infection with dengue virus in humans. *Front Immunol.* (2014) 5:93. doi: 10.3389/fimmu.2014.00093
42. Dong T, Moran E, Vinh Chau N, Simmons C, Luhn K, Peng Y, et al. High pro-inflammatory cytokine secretion and loss of high avidity cross-reactive cytotoxic T-cells during the course of secondary dengue virus infection. *PLoS ONE.* (2007) 2:e1192. doi: 10.1371/journal.pone.0001192
43. Kobayashi H, Wood M, Song Y, Appella E, Celis E. Defining promiscuous MHC class II helper T-cell epitopes for the HER2/neu tumor antigen. *Cancer Res.* (2000) 60:5228–36.
44. Hossain MS, Azad AK, Chowdhury PA, Wakayama M. Computational identification and characterization of a promiscuous T-cell epitope on the extracellular protein 85B of *Mycobacterium* spp. for peptide-based subunit vaccine design. *Biomed Res Int.* (2017) 2017:4826030. doi: 10.1155/2017/4826030

45. Mustafa AS, Lundin KE, Meloen RH, Shinnick TM, Oftung F. Identification of promiscuous epitopes from the Mycobacterial 65-kilodalton heat shock protein recognized by human CD4(+) T cells of the *Mycobacterium leprae* memory repertoire. *Infect Immun.* (1999) 67:5683–9. doi: 10.1128/IAI.67.11.5683-5689.1999
46. Turtle L, Brindle HE, Schluter WW, Faragher B, Rayamajhi A, Bohara R, et al. Low population Japanese encephalitis virus (JEV) seroprevalence in Udayapur district, Nepal, three years after a JE vaccination programme: a case for further catch up campaigns? *PLoS Negl Trop Dis.* (2019) 13:e0007269. doi: 10.1371/journal.pntd.0007269

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Key Findings and Comparisons From Analogous Case-Cluster Studies for Dengue Virus Infection Conducted in Machala, Ecuador, and Kamphaeng Phet, Thailand

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Dengue viruses (DENV) pose a significant and increasing threat to human health across broad regions of the globe. Currently, prevention, control, and treatment strategies are limited. Promising interventions are on the horizon, including multiple vaccine candidates under development and a renewed and innovative focus on controlling the vector, *Aedes aegypti*. However, significant gaps persist in our understanding of the similarities and differences in DENV epidemiology across regions of potential implementation and evaluation. In this manuscript, we highlight and compare findings from two analogous cluster-based studies for DENV transmission and pathogenesis conducted in Thailand and Ecuador to identify key features and questions for further pursuit. Despite a remarkably similar incidence of DENV infection among enrolled neighborhood contacts at the two sites, we note a higher occurrence of secondary infection and severe illness in Thailand compared to Ecuador. A higher force of infection in Thailand, defined as the incidence of infection among susceptible individuals, is suggested by the higher number of captured *Aedes* mosquitoes per household, the increasing proportion of asymptomatic infections with advancing age, and the high proportion of infections identified as secondary-type infections by serology. These observations should be confirmed in long-term, parallel prospective cohort studies conducted across regions, which would advantageously permit characterization of baseline immune status (susceptibility) and contemporaneous assessment of risks and risk factors for dengue illness.

Keywords: dengue, epidemiology, observational study, Thailand, Ecuador

INTRODUCTION

Infection with dengue viruses (DENV) is responsible for a significant burden of disease across tropical and subtropical regions of the globe. However, the history and epidemiology of DENV clearly differ between Asia and the Americas. Asia has been hyperendemic for all four DENV serotypes for decades and consistently demonstrates one of the highest burdens of dengue-related disease in the world (1). In contrast, in the Americas, following the abandonment of successful mosquito control programs in the 1960s, DENV serotypes were sequentially reintroduced into circulation. In Ecuador, DENV re-emerged in 1988, co-circulation of all four DENV serotypes was documented in 2000, and the first cases of severe dengue were seen in 2001 (2, 3).

The nature and extent of differences in DENV transmission intensity and clinical manifestations of disease between Asia and the Americas remain poorly understood. Observational cohort studies conducted in multiple regions of the globe have contributed significantly to our understanding of DENV transmission and pathogenesis (4–7), however, differences in study-specific aims and methodologies have largely precluded direct comparisons across regions. Limited regionally-comparative analyses of DENV seroprevalence (8) and mean ages of infection (9) suggest that the force of infection (incidence among susceptible individuals) may be higher, on average, in Asia than in the Americas. Relatedly, presumed higher levels of susceptibility to DENV serotypes in the Americas compared to Asia may indicate the potential for DENV epidemics for more explosive epidemics of greater magnitude in the Americas.

There is currently no licensed antiviral for DENV infection and the only DENV vaccine currently licensed for use in multiple countries has recently generated safety concerns due to an increased risk of hospitalized illness observed in DENV-naïve vaccine recipients (10). Currently-available vector control measures have been ineffective in stopping the transmission of Aedes-transmitted pathogens (including DENV) (11), however, pioneering methods of innovative vector control such as the field release of Wolbachia-infected *Aedes* mosquitoes (12) and transgenic mosquitoes (13) offer promise. The effective evaluation and implementation of novel DENV vaccines and vector control measures would benefit from an improved understanding of the similarities and differences in DENV transmission and pathogenesis across continents.

Multiple recent epidemics of DENV and other arboviruses spread by *Aedes* vectors across diverse regions of the globe underscore the urgent need to better understand the patterns and drivers of arboviral disease transmission in order to prepare for the epidemics to come. In this manuscript, we summarize and contrast findings from two analogous cluster investigation studies conducted in Machala, Ecuador, and Kamphaeng Phet, Thailand, to highlight possible distinguishing features in DENV epidemiology between the two regions and to identify important avenues for future research.

MATERIALS AND METHODS

Summary

Analogous case-cluster surveillance studies were conducted in Kamphaeng Phet (KPP), Thailand, from 2009 to 2012, and Machala, Ecuador, from 2014 to 2015. Both studies utilized enhanced passive surveillance to identify and recruit patients with suspected dengue illnesses presenting to local participating clinics and hospitals (**Figure 1**). Subsequent confirmation of a DENV infection prompted the further study of individuals residing within and around the home of the infected individual. Active surveillance was then used to identify symptomatic and asymptomatic infections occurring within enrolled neighborhood contacts. The detailed methods for both studies have been published previously (14, 15). Detailed comparisons of the surveillance and diagnostic methods are presented in the text below and **Table 1**.

Study Sites

Kamphaeng Phet province is located in northern Thailand, with a moderately dense urban center (Muang) surrounded by agricultural zones. Machala is the capital of El Oro province, located in southern coastal Ecuador; the town is densely populated and surrounded by agriculture and aquaculture areas. The study sites are comparable in total population, elevation, gross domestic product (GDP), and the co-circulation of all four DENV types (**Table 2**). Both regions experience the co-circulation of multiple other arboviruses in addition to DENV. Chikungunya virus (CHIKV) emerged in Ecuador at the end of 2014, and the first confirmed instances of autochthonous Zika virus (ZIKV) transmission in Ecuador were reported in January 2016 (15). Neither ZIKV nor CHIKV were known to be in circulation in northern Thailand at the time of the study, however there is increasing evidence for long-standing endemicity of ZIKV in the region (19). Both regions practice routine immunization of pediatric populations for non-DENV flaviviruses: Japanese encephalitis vaccine (JEV) is part of the Expanded Program on Immunization (EPI) in Thailand, with rates of coverage estimated to be 92% or higher (20), and yellow fever vaccine (YFV) vaccine is part of the EPI in Ecuador, with rates of coverage approaching 80% in certain high-risk areas (21).

Definitions

Initiates are individuals who presented to participating clinical sites with suspected dengue illnesses, who were subsequently confirmed to have DENV infection. A subset of Initiates were randomly selected for participation in community-based cluster investigations. Associates are individuals residing within the Initiate's household or within a 200-m radius of the Initiate's household, who met study-specific enrollment criteria. Together, the Associate homes plus the Initiate's home made up a cluster.

Recruitment and Surveillance of Initiates

- Thailand. Initiates were recruited from among individuals admitted to the public referral hospital, Kamphaeng Phet

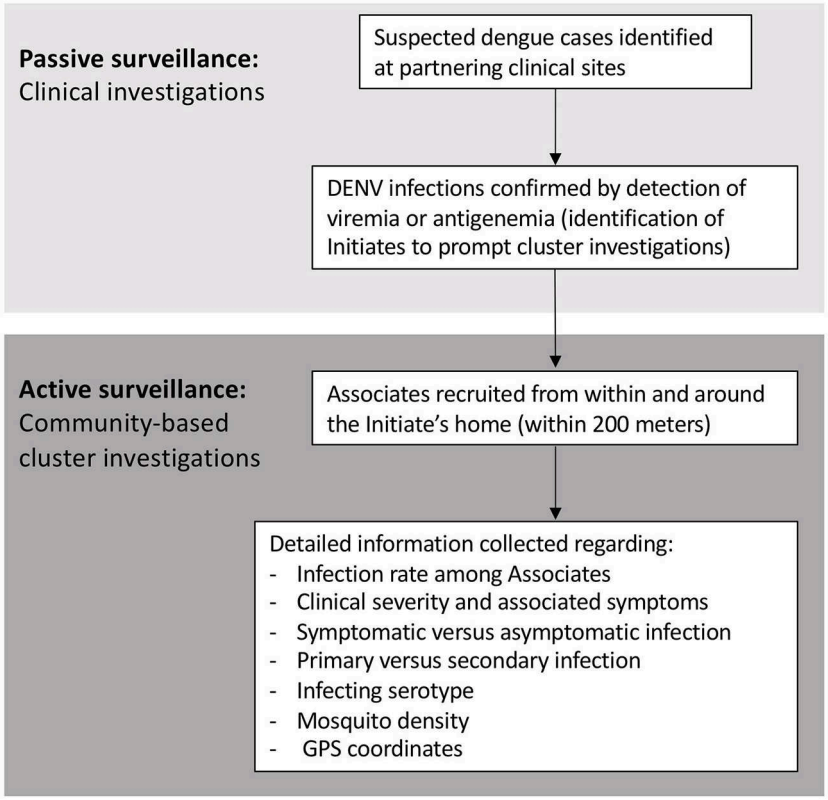

FIGURE 1 | Summary of study activities for the Thai and Ecuadorian studies.

TABLE 1 | Study-specific methods in KPP, Thailand, and Machala, Ecuador.

	KPP, Thailand	Machala, Ecuador
Identification of initiates		
Locations/s of recruitment	Kamphaeng Phet Provincial Hospital (inpatients)	Inpatients and outpatients presenting to MOH clinics and hospitals
Inclusion criteria	• Age >6 months • DENV infection confirmed by RT-PCR	• Age >6 months • Clinical diagnosis of suspected dengue
Identification of associates		
Eligible homes	All homes located within 200m of Initiate's home, with reported fever in preceding 7 days	5 homes: the Initiate's home and one each located in the four cardinal directions (N, S, E, W)
Inclusion criteria	• Age >6 months • Residing in a house with a history of reported fever in the prior week	• Age >6 months • Residing in the Initiate's home or in the four houses located N, S, E, W
Follow-up	Specimens and data collected on days 0 and 15	Specimens and data collected on day 0 only
Laboratory diagnostic methods		
Molecular	• DENV RT-PCR (16)	• DENV NS1 rapid test (PanBio) • Qualitative DENV rtRT-PCR (17) • CHIKV and ZIKV RT-PCR
Serological	• DENV and JEV IgM and IgG ELISA (paired specimens) (18)	• DENV IgM and IgG ELISA (PanBio)

Provincial Hospital (KPPPH) with suspected dengue infection. Inclusion criteria were: age >6 months and blood drawn and RT-PCR performed to confirm DENV infection within 24 h of hospital admission. Acute and convalescent blood specimens were collected on enrollment and 15 (±5 days) thereafter.

- Ecuador. Initiates were recruited from among inpatients and outpatients presenting to four clinics operated by the Ministry of Health and the associated public referral hospital, Teófilo Dávila Hospital. Inclusion criteria were: age ≥6 months and a clinical diagnosis of suspected dengue. Acute blood specimens were collected on enrollment. A maximum of

TABLE 2 | Comparison of key features of the field sites in Muang Kamphaeng Phet (KPP), Thailand, and Machala, Ecuador.

Variables	KPP, Thailand	Machala, Ecuador
Population	213,228	280,000
Location (lat, long)	Southeast Asia (16°28′ N, 99°31′ E)	Pacific coast of South America (3°15′ S, 79°57′ W)
Elevation	80 m	9 m
Land use	Moderately dense urban area surrounded by agricultural areas (rice)	Dense urban area surrounded by coastal mangroves, farming (bananas) and aquaculture (shrimp)
Climate	Tropical climate with marked rainy season: May to Oct (dengue season); avg max temp 33.5°C; avg min temp: 22.9°C	Tropical climate with marked rainy season: Feb to May (dengue season); avg max temp 29.1°C; avg min temp 22.1°C
Annual per capita GDP (2017 USD)	$6,594	$6,199
Dengue transmission	Endemic seasonal transmission, interannual outbreaks	
Arbovirus context	DENV is a top public health concern; ZIKV likely with long standing endemicity; JEV vaccination widespread	DENV is a top public health concern; CHIKV/ZIKV are new; YFV vaccination widespread
Dengue vectors	*A. aegypti and A. albopictus*	*A. aegypti*

4 Initiates were randomly selected each week to initiate cluster investigations.

Recruitment and Surveillance of Associates

- Thailand. All individuals aged >6 months residing within the Initiate's household were invited to enroll. Further, all homes located within a 200-m radius of the Initiate's household were visited by the study team. If anyone in a given household reported fever within the previous 7 days, all residents of that household aged >6 months were invited to participate in the study, to a maximum to 25 Associates enrolled per cluster. Blood specimens were collected on the day of enrollment ("day 0") and roughly 15 ± 5 days thereafter. If an Associate developed fever during the 15-day follow-up period, a second acute blood specimen was drawn and the period of follow-up shifted by an additional 15 days for that individual. Adult *Aedes* mosquitoes were collected from all homes within 200 m of the Initiate home using backpack aspirators.
- Ecuador. All individuals aged ≥6 months residing within the Initiate's household were invited to enroll. Further, all individuals aged ≥6 months and residing in households located in the cardinal directions from the Initiate household at a maximum distance of 200-m were invited to enroll. Thus, there was an imposed limit of five households per cluster (the Initiate's home, plus one home each located to the north, south, east, and west). Blood specimens were collected on the day of enrollment only ("day 0"). Adult *Aedes* mosquitoes were collected from the five enrolled homes (the Initiate's home and the four neighboring homes) using backpack aspirators.
- Geospatial data collection. For both sites, the locations (latitude, longitude) of all Initiate homes and all homes within 200-m of the Initiate home were recorded using handheld GPS devices.

Laboratory Diagnostics

- Thailand. DENV RT-PCR and DENV and JEV IgM/IgG ELISA were used to identify DENV infections occurring in Initiates and Associates. The nested RT-PCR method described by Lanciotti et al. was used to detect DENV RNA and to identify the infecting serotype (16). AFRIMS in-house IgM and IgG ELISAs were used to serologically diagnose DENV infections and to discern DENV and JEV as described previously (18). RT-PCR and IgM/IgG ELISA were performed on the day 0 and 15 specimens for all enrolled Associates.
- Ecuador. DENV NS1 rapid strip tests (PanBio Dengue Early Rapid Test) were used to identify confirmed DENV infections (Initiates) from among ill patients at clinical sites. Qualitative real-time RT-PCR assays for DENV1-4 were performed as per the CDC DENV1-4 Real Time RT-PCR Assay (CDC, Catalog number KK0128) (17). Commercial ELISA kits (PanBio) were used to detect DENV IgM (Dengue Capture IgM) and IgG (Dengue Capture IgG). All Initiates and Associates from Ecuador also underwent testing for ZIKV and CHIKV by RT-PCR; these results have been previously presented and are not discussed here (15).

Classification of DENV Infections

- Thailand. For the purposes of this analysis an acute or recent DENV infection in an Associate was defined as: (1) detection of DENV RNA in a specimen collected at any time point (e.g., from the day 0 and 15 visits as well as acute specimens collected in the setting of incident fever), or (2) detection of DENV IgM in any specimen, or (3) DENV IgM not detected but DENV IgG >100 and rising (acute infection) or decreasing (recent infection) in paired specimens. A primary infection was defined as an IgM/IgG ratio ≥1.8, a secondary infection as a ratio <1.8. A symptomatic DENV infection was defined as (1) an acute laboratory-confirmed DENV infection plus (2) the presence of one or more classical dengue symptom/s (e.g., fever, headache, muscle/joint pain, retro-orbital pain, abdominal pain, drowsiness/lethargy, rash). Clinical data were collected for Initiates and Associates during the day 0 and 15 visits, as well as during unscheduled visits prompted by reported fever, inquiring about any current and recent symptoms since the last study visit. An asymptomatic DENV

infection was defined as (1) an acute laboratory-confirmed DENV infection plus (2) the absence of all of these symptoms during the entire period of follow-up (typically 15 ± 5 days).

- Ecuador. An acute DENV infection in an Associate was defined as the detection of DENV RNA by RT-PCR in the enrollment specimen (only a single specimen collected). A recent infection was defined as the detection of IgM in the enrollment specimen (and RT-PCR negative). A primary infection was defined as an IgM/IgG ratio ≥1.8, a secondary infection as a ratio <1.8. A symptomatic DENV infection was defined as (1) an acute laboratory-confirmed DENV infection plus (2) the presence of one or more classical dengue symptom/s (e.g., fever, headache, muscle/joint pain, retro-orbital pain, abdominal pain, drowsiness/lethargy, rash). Clinical data were collected for Associates at the time of enrollment only and reflected symptoms present at the time of enrollment or at any point during the preceding 7 days. An asymptomatic DENV infection was defined as (1) an acute laboratory-confirmed DENV infection plus (2) the absence of all of these symptoms at the time of interview and during the preceding 7 days.

Ethics Statement

For the Ecuador study, the protocol was reviewed and approval by Institutional Review Boards (IRBs) at SUNY Upstate Medical University, the Human Research Protection Office (HRPO) of the U.S. Department of Defense, the Luis Vernaza Hospital in Guayaquil, Ecuador, and the Ecuadorean Ministry of Health. For the Thai study, the protocol was approved by the IRBs of the Thai Ministry of Public Health (MOPH), Walter Reed Army Institute of Research (WRAIR, protocol number 1526), and SUNY Upstate Medical University. The IRBs of the University of California, Davis (UCD), University of Rhode Island (URI), and University at Buffalo established relying agreements with WRAIR IRB. Prior to the start of the study, all participants engaged in a written informed consent or assent process as previously described (14, 15).

RESULTS

Characteristics of Enrolled Initiates and Associates

Three hundred twenty-three Initiates were enrolled in Thailand between November 2009 and November 2012 (**Table 3**), with enrollment thus capturing three peak periods for DENV transmission (i.e., the rainy season), which variably reaches its maximum in July-August and wanes in October–November each year. Forty-four Initiates were enrolled in Ecuador between January 2014 and June 2015, with enrollment thus spanning two peak periods for DENV transmission, which typically reaches its maximum in March-May and wanes in June–July each year (22). All four DENV serotypes were detected in Initiates in Thailand, during the study period, while only DENV-1 and DENV-2 were detected among Initiates in Ecuador. 26.4% of Initiates in Ecuador were RT-PCR negative, with DENV infection confirmed by NS1 rapid test or NS1 ELISA. The median ages of Initiates in Ecuador and Thailand were similar (16 and 14.5 years, respectively). Only 1.9% of Initiates in Thailand had primary DENV infections by serology vs. 25.0% in Ecuador. By definition, 100% of Initiates were derived from hospitalized illnesses in Thailand, while 25.0% were hospitalized in Ecuador.

One thousand two hundred forty-two Associates were enrolled in Thailand and 384 in Ecuador, with a median of 3 and 8 Associates per Initiate in each country, respectively (**Table 4**). Overall, relatively few Associates reported a history

TABLE 3 | Features of Initiates in Thailand and Ecuador.

	KPP	Machala
Number of initiates	323	44
Median age in years (range)	16 (2–72)	14.5 (1–67)
% female	48.6%	38.6%
DENV serotype		
DENV-1	23.2%	25.0%
DENV-2	60.4%	40.9%
DENV-3	11.5%	0%
DENV-4	5.0%	0%
Not detected*	0%	26.4%
% primary	1.9%	25.0%**
% hospitalized	100.0%	25.0%

**NS1 rapid tests were used to identify Initiate cases in Ecuador; thus, not all were RT-PCR positive.*

***Among those with valid serology (68.1% or 30/44).*

TABLE 4 | Features of enrolled Associates in Thailand and Ecuador.

	KPP	Machala
Number	1,246	384
Median number of Associates per Initiate	3 (1–17)	8 (4–17)
Median age in years (range)	30 (0–96)	34 (0–87)
% female	57.4%	65.9%
History of flavivirus vaccine*	18.5%	11.7%
Total % with acute or recent infection	24.3%	25.0%
Within Initiate's house	22.0%	29.1%
Neighboring house**	26.8%	23.9%
RT-PCR positive (*n*)	44.2%	43.2%
Among RT-PCR positive Associates, infecting DENV serotype		
DENV-1	24.6%	15.8%
DENV-2	50.0%	57.9%
DENV-3	20.9%	26.3%
DENV-4	4.5%	0%
% concordant with Initiate serotype	94.0%	66.7%
% primary	11.8%	39.6%
% asymptomatic	25.1%	33.3%
Mean # adult *Aedes* females/house	1.67 (3.41)	0.95 (1.62)
Homes with infections	1.90 (3.83)	1.00 (1.76)
Homes without infections	0.99 (1.75)	0.80 (1.16)

**Reported history of JEV vaccine (Thailand) or YFV vaccine (Ecuador).*

***Note that for Thailand, Associate houses were enrolled out to a radius of 200 m if anyone in the home reported a history of recent fever; for Ecuador, Associate houses were enrolled in the four cardinal directions and 94% were within 100 m of the Initiate house (15).*

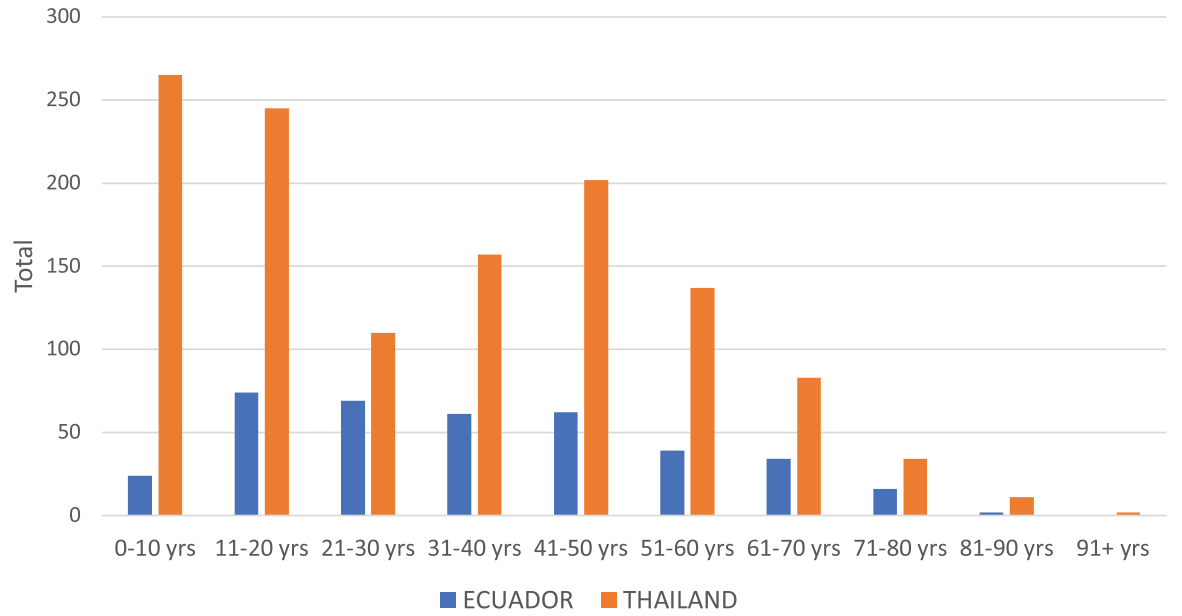

FIGURE 2 | Histogram of ages of enrolled Associates in Thailand (orange) and Ecuador (blue).

of JEV (in Thailand) or YFV vaccination (in Ecuador); however, 96.1% of Thai children (aged <18 years) reported a history of JEV vaccination and 19.6% of Ecuadorian children reported a history of YFV vaccination. Three hundred three Associates in Thailand (24.3%) and 96 in Ecuador (25.0%) were confirmed to have acute or recent DENV infection. Eliminating the results from the convalescent blood draw from the Thailand data (e.g., forcing a mirroring of study methods for the two sites by considering only the diagnostics testing results from the enrollment specimen), the number of infected Associates detected in the Thai study decreased to 202 (data not shown). Thus, extending the surveillance period by 15 days and incorporating a convalescent blood draw increased the detection of DENV infections in Associates by 33% (from 202 to 303 infections).

In Thailand, the DENV serotype detected in Associates matched the Initiate's serotype 94.0% of the time. In contrast, concordance was only 66.7% in Ecuador (i.e., one in three Associates were infected with a different serotype than the Initiate for that cluster). The clear majority of DENV infections in Associates in Thailand and Ecuador were symptomatic (defined as the report of any symptom within 7 days of enrollment through the 15-day follow-up for Thailand and within the past 7 days from enrollment for Ecuador). The mean number of *Aedes* females per home was higher in Thailand than in Ecuador ($p = 0.034$ by Mann Whitney *U*-test); for both sites, albeit non-significantly, the number of mosquitoes captured was higher in Associate homes with identified DENV infections than in those without DENV infections ($p > 0.05$ for both comparisons). In Thailand, the largest number of Associates was enrolled in the age group comprising children aged 0–10 years (**Figure 2**). In Ecuador, the largest age group enrolled comprised individuals aged 11–20 years.

Characteristics of DENV Infections in Associates

The highest infection rates among Associates for both Thailand and Ecuador were observed in the age group 11–20 years (**Figure 3**). The rates were roughly similar but generally higher for Ecuador than for Thailand. Incidence rate decreased with age at both sites.

The proportion of DENV infections that were primary by serology (EIA) was much higher in Ecuador than in Thailand overall (**Figure 4**). In the age group 0–10 years, 80.0% of DENV infections were primary in Ecuador, vs. 17.1% in Thailand. In Ecuador, the proportion of infections that were primary generally decreased with age, while in Thailand the proportion remained relatively level between 0 and 20%.

Children were more likely to experience symptomatic infection in Thailand as compared to Ecuador (**Figure 5**). The proportion of DENV infections that were asymptomatic increased steadily with age in Thailand, while in Ecuador the proportion asymptomatic remained relatively level between 15 and 40%.

Symptoms of DENV Infection in Associates

In Thailand, children were more likely than adults to report fever, headache, upper respiratory symptoms (rhinorrhea, cough), and abdominal symptoms (pain, nausea/vomiting) (**Table 5**). Children were also more likely to be hospitalized. Individuals experiencing a secondary DENV infection were more likely to be hospitalized and to demonstrate all symptoms solicited (significant for headache, anorexia, nausea/vomiting, drowsiness, muscle/joint pain, and abdominal pain). Children experiencing secondary infection reported the highest frequency of symptoms, notably with 82.9% reporting fever and 25.6% becoming hospitalized.

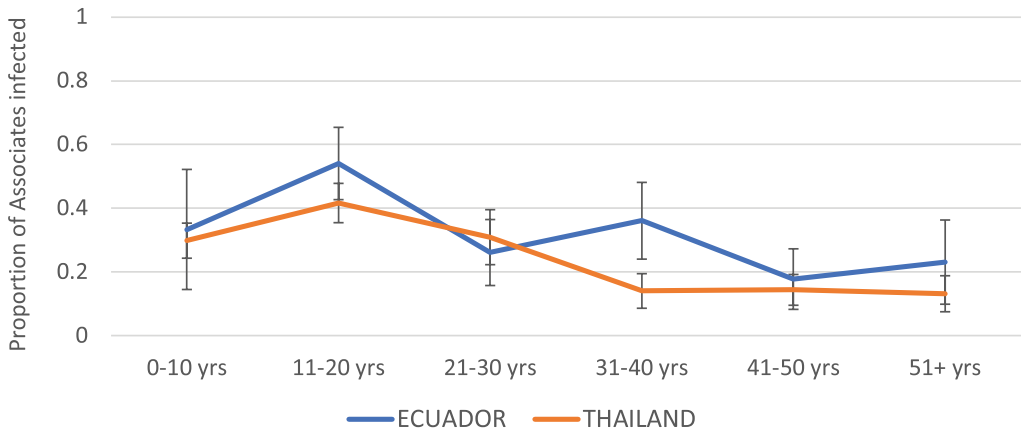

FIGURE 3 | Proportion of Associates confirmed to have DENV infection, by age and study site. Thailand is shown in orange and Ecuador in blue. Error bars reflect the 95% confidence intervals for the proportions.

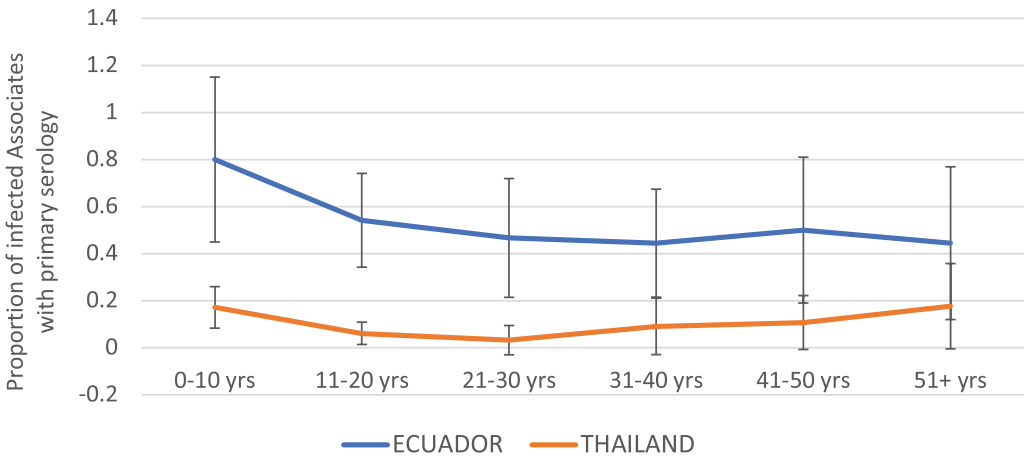

FIGURE 4 | Proportion of DENV-infected Associates found to have primary DENV infection (by ELISA), by age and study site. Thailand is shown in orange and Ecuador in blue. Error bars reflect the 95% confidence intervals for the proportions.

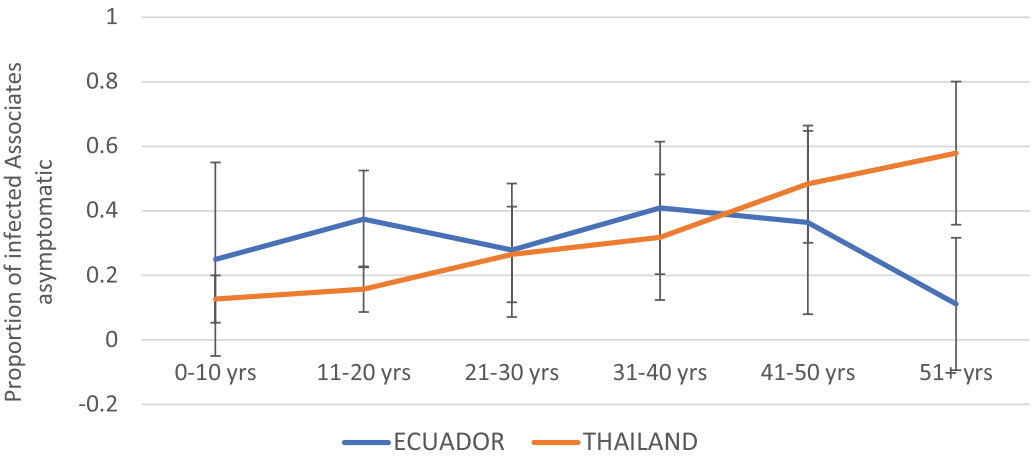

FIGURE 5 | Proportion of DENV-infected Associates with asymptomatic infection (i.e., denying any of the solicited symptoms) by age and study site. Thailand is shown in orange and Ecuador in blue. Error bars reflect the 95% confidence intervals for the proportions.

TABLE 5 | Symptoms reported by enrolled Associates by age (adult = age ≥ 18 years, child = age ≤ 18 years), among those with symptomatic infections (defined as the presence of any solicited symptom) in Thailand.

	Thailand											
	Total—by age			Total—by serology*			Primary			Secondary		
	Child	Adult	*p*-value	1°	2°	*p*-value	Child	Adult	*p*-value	Child	Adult	*p*-value
# Dengue illnesses**	163	140	NA	32	238	NA	17	15	NA	129	109	NA
Asymptomatic	13.5%	38.6%	<0.001	37.5%	21.4%	0.044	17.6%	60.0%	0.027	10.9%	33.9%	<0.001
Hospitalized	21.5%	7.9%	<0.001	3.1%	18.5%	0.024	5.9%	0.0%	1.000	25.6%	10.1%	0.002
Fever	80.4%	51.4%	<0.001	59.4%	69.3%	0.312	76.5%	40.0%	0.070	82.9%	53.2%	<0.001
Headache	56.4%	42.1%	0.016	15.6%	56.7%	<0.001	11.8%	20.0%	0.645	65.9%	45.9%	0.002
Rhinorrhea	30.1%	6.4%	<0.001	15.6%	20.6%	0.641	23.5%	6.7%	0.338	31.8%	7.3%	<0.001
Cough	35.6%	15.0%	<0.001	15.6%	28.6%	0.141	11.8%	20.0%	0.645	39.5%	15.6%	<0.001
Anorexia	46.6%	26.4%	<0.001	18.8%	39.9%	0.020	23.5%	13.3%	0.659	51.2%	26.6%	<0.001
Nausea/vomiting	42.3%	19.3%	<0.001	6.3%	33.6%	0.001	11.8%	0.0%	0.486	45.0%	20.2%	<0.001
Drowsiness	30.7%	21.4%	0.089	6.3%	29.4%	0.005	53.1%	46.9%	0.212	34.9%	22.9%	0.047
Muscle/joint pain	38.7%	45.7%	0.243	15.6%	47.5%	0.001	5.9%	26.7%	0.161	47.3%	47.7%	1.000
Abdominal pain	27.0%	11.4%	0.001	6.3%	23.1%	0.035	0.0%	13.3%	0.212	32.6%	11.9%	<0.001
Rash	12.3%	7.9%	0.255	9.4%	11.3%	1.000	17.6%	0.0%	0.229	13.2%	9.2%	0.413
Diarrhea	19.6%	11.4%	0.059	9.4%	16.4%	0.437	11.8%	6.6%	1.000	20.2%	11.9%	0.113
Retroorbital pain	17.8%	24.3%	0.201	9.4%	21.8%	0.158	5.9%	13.3%	0.589	20.9%	22.9%	0.754
Bleeding	6.7%	2.9%	0.183	0.0%	5.9%	0.386	0.0%	0.0%	NA	8.5%	2.8%	0.094

**Of those with serologically-confirmed DENV infection (i.e., either primary or secondary DENV infection).*

***Among all those with confirmed DENV infection (i.e., whether symptomatic or asymptomatic).*

P-values < 0.05 indicate statistically significant comparisons, applying Mantel-Haenszel chi-squared testing.

NA indicates not applicable.

In Ecuador, children experiencing DENV infection were more likely than adults to report rash, and adults were more likely than children to report muscle and joint pain (**Table 6**). Symptoms were more common in Associates experiencing secondary DENV infections. In Ecuador, most symptoms were more common in Associates experiencing secondary DENV infections although there was limited power to detect significant associations given low numbers. Rash was more common in children experiencing primary DENV infection.

DISCUSSION

DENV pose a significant and increasing threat to human health across broad regions of the globe. Counter measures to prevent human exposure to infected *Aedes* mosquitoes and to prevent illness, once exposed, are urgently needed. With promising interventions on the horizon, including multiple vaccine candidates under development (23) and a renewed and innovative focus on the vector, *Aedes aegypti* (12, 13), there persist significant gaps in our understanding of the similarities and differences in DENV epidemiology across regions of potential implementation and evaluation. In this manuscript, we highlight and compare findings from two analogous cluster-based studies for DENV transmission and pathogenesis conducted in Thailand and Ecuador to identify key features and questions for further pursuit.

The incidence of DENV infection among Associates was remarkably similar across age groups in both countries. Applying the same definition to the Thai Associates as to the Ecuadorian Associates (i.e., based upon the enrollment specimen only), the incidence rate in Ecuador was at least 33% higher. This is somewhat surprising, given prior estimates suggesting a higher transmission intensity in Asia than in the Americas (8, 9). Multiple possible explanations exist for this. First, the incidence of DENV has been shown to vary significantly in time and space (24). The studies were conducted at different time points and for relatively short intervals and thus infection rates by country may be confounded by year. Further, the clinical severity and transmissibility of DENV has been demonstrated to vary by serotype (25–27). Second, the underlying susceptibility of the Associate populations is not known, by nature of the study design (i.e., cluster investigations based upon the identification of a DENV infection in a neighbor). If the Thai Associates had a higher level of pre-existing DENV immunity, a similar or even lower incidence may still reflect a high force of infection (see hypothetical illustration in **Figure 6**). This distinction is important, because the force of infection directly translates to the risk experienced by DENV-naïve subjects visiting or born into an area as well as the level of coverage needed by interventions to decrease transmission.

Interestingly, intra-cluster concordance of DENV serotypes between Initiates and Associates was only 67% in Ecuador, as compared to 94% in Thailand. This focal, concurrent micro-circulation of multiple serotypes has not, to our knowledge, been documented previously. Potential explanations for this finding may include: greater population-level immunological

TABLE 6 | Symptoms reported by enrolled Associates by age (adult = age ≥ 18 years, child = age ≤ 18 years), among those with symptomatic infections (defined as the presence of any solicited symptom) in Ecuador.

Ecuador	Total—by age			Total—by serology*			Primary			Secondary		
	Child	Adult	*p*-value	1°	2°	*p*-value	Child	Adult	*p*-value	Child	Adult	*p*-value
# Dengue illnesses**	27	67	NA	42	45	NA	13	29		8	37	
Asymptomatic	34.2%	32.9%	0.889	42.9%	22.2%	0.040	53.8%	37.9%	0.501	25.0%	21.6%	1.000
Hospitalized	0%	0%	NA				0%	0%	NA	0%	0%	NA
Fever	22.9%	14.1%	0.282	9.8%	20.0%	0.235	8.3%	10.3%	1.000	25.0%	18.9%	0.651
Headache	33.3%	27.8%	0.660	19.5%	37.8%	0.095	16.7%	20.7%	1.000	25.0%	40.5%	0.690
Rhinorrhea	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cough	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nausea/vomiting	13.2%	7.6%	0.333	4.8%	8.9%	0.677	0.0%	6.9%	1.000	0.0%	10.8%	1.000
Drowsiness	16.7%	21.5%	0.623	14.6%	26.7%	0.195	8.3%	17.2%	0.651	12.5%	29.7%	0.419
Muscle/joint pain	11.1%	34.2%	0.012	31.7%	33.3%	1.000	25.0%	34.5%	0.719	12.5%	37.8%	0.236
Abdominal pain	13.9%	22.8%	0.323	17.1%	26.7%	0.311	8.3%	20.7%	0.651	25.0%	27.0%	1.000
Rash	22.2%	7.6%	0.034	14.6%	13.3%	1.000	33.3%	6.9%	0.050	25.0%	10.8%	0.286
Diarrhea	2.8%	11.4%	0.168	4.9%	15.6%	0.161	0.0%	6.9%	1.000	0.0%	18.9%	0.321
Retroorbital pain	13.9%	26.6%	0.155	17.1%	35.6%	0.087	16.7%	17.2%	1.000	25.0%	37.8%	0.691
Bleeding	0.0%	1.3%	1.000	2.4%	0.0%	0.477	0.0%	3.4%	1.000	0.0%	0.0%	NA

Of those with valid serological results (i.e., either primary or secondary DENV infection).

**Among all those with confirmed DENV infection (i.e., whether symptomatic or asymptomatic).*

P-values < 0.05 indicate statistically significant comparisons, applying Mantel-Haenszel chi-squared testing.

NA indicates not applicable.

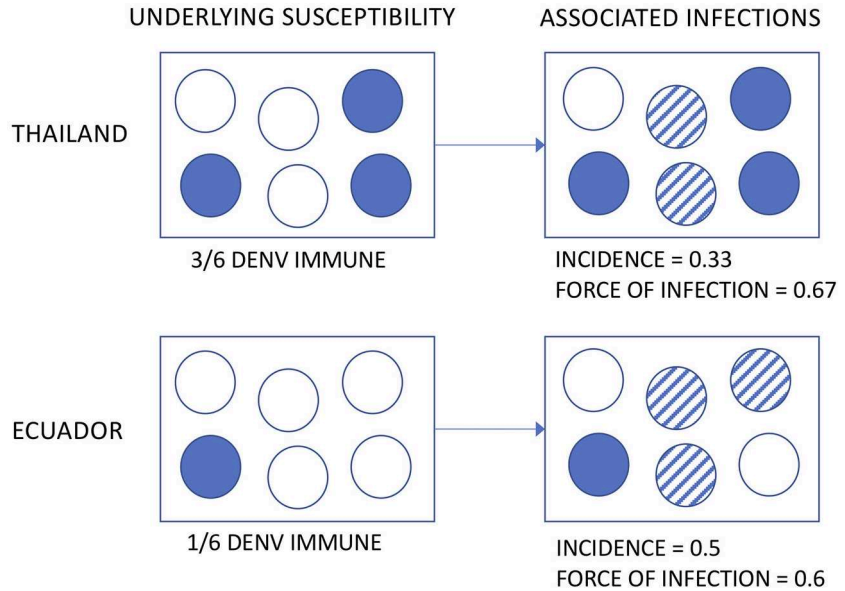

FIGURE 6 | Hypothetical illustration of the possible relationships between underlying susceptibility to DENV, observed incidence, and force of infection.

bottle necks for serotype co-circulation in Thailand, resulting from many decades of hyperendemic transmission, differences in human movement patterns, and/or differences in the spatial scale or hot-spots for transmission between the two sites. Long-term, parallel prospective cohort studies conducted across regions would contribute significantly to our understanding of DENV epidemiology, permitting characterization of baseline immune status (susceptibility), shifts in DENV serotypes and genotypes over time, and diverse risk factors for DENV infection and dengue illness.

The incidence of symptomatic DENV infection among Associates for both studies was much higher than has been reported in previous prospective cohort studies, at 25 and 33% for Thailand and Ecuador, respectively (7, 28) This may reflect recall bias, wherein individuals enrolled in cluster studies are more likely to notice and report even minor symptoms given that a neighbor has recently been diagnosed with a DENV infection. It is also possible that the enrollment of ill Initiates at their point of entry into the healthcare system imposes a sampling bias, selecting for more severe DENV serotypes, genotypes, and/or strains with an increased ability to infect and cause disease in Associates. Finally, it should be noted that the case definition for "symptomatic infection" applied in this analysis is more sensitive than the definition applied in some other studies. For example, prior analyses from KPP have required the presence of fever to define symptomatic illness, a symptom reported by 89% of DENV-infected Thai Associates with any clinical symptoms and only 24% of symptomatic, DENV-infected Ecuadorian Associates in the current analyses. This suggests that using fever as the sole criterion for "symptomatic infection" in field studies for DENV may result in the misclassification of potentially large numbers of ill subjects as "asymptomatic." Interestingly, the incidence of asymptomatic DENV infection increased with age in Thailand but remained relatively flat in Ecuador; this may reflect a higher force of infection for DENV in Thailand, with accumulated cross-protective immunity through multiple DENV exposures over time.

The clinical severity and manifestations of DENV infection in Associates differed between Thailand and Ecuador. 21.5% of children and 7.9% of adults enrolled as Associates in Thailand were hospitalized with dengue illnesses, as compared to 0% in Ecuador. This may reflect the greater occurrence of secondary DENV infection in Thai children, for whom rates of hospitalization were 25.6% and for whom most clinical symptoms were also more common (fever, headache, abdominal symptoms, etc.). Other possibilities for the greater clinical severity in Thailand include differences in the virulence of circulating DENV between regions and/or differences in study design, given that Thai Associate households were enrolled on the basis of reported fever and Ecuadorian households simply on the basis of their location relative to the Initiate house. Region-specific differences in patterns of care-seeking and criteria for hospitalization likely exist and may bias our comparisons; for example, individuals in Thailand may have been less likely to seek care for milder dengue illnesses as compared to individuals in Ecuador, and/or more likely to be hospitalized for a given clinical presentation. It is likely that human immunogenetic differences influence the clinical outcome to DENV infection and will differ across populations (29). Finally, undetected parasitic co-infections may play a role in modulating the immune response and thus the clinical outcome of DENV infection (30); for example, it is possible (but currently untested) that helminthic infections are more common in Ecuador than in Thailand and/or other parasitic co-infections such as *Trypanosoma cruzi* in the Americas may shape the clinical outcome of DENV infection.

The proportion of DENV infections identified as primary by DENV serology was much lower among similarly aged children in Thailand compared to Ecuador. This is presumably a

reflection of the high rates of coverage for JEV vaccination in Thai children, manifesting as an anamnestic, secondary-type response to primary DENV infection. YFV and JEV are both well-known to cross-react with DENV in serological assays. Prior analyses from KPP suggest that prior JEV immunity may predispose toward symptomatic DENV infection (31); the potential for YFV to shape the clinical outcome of DENV infection is unknown. Potential differences in the force of infection for ZIKV between the Americas and Asia remain poorly understood, though there is increasing evidence that ZIKV has been endemic in Thailand, possibly at low levels, for decades (19). Serological cross-reactivity between DENV and ZIKV currently complicates the reliability of serologically-confirming infections due to either virus (32), however, assays promising improved specificity are in development and under validation (33, 34). Future studies should seek to further clarify the potential for exposure (natural or vaccine-derived) to a range of non-DENV flaviviruses to modulate the clinical and immunological outcomes of DENV infection; this knowledge will have particular relevance when evaluating the immunogenicity and efficacy of DENV vaccines across regions.

In addition to addressing the questions above, long-term, parallel prospective cohort studies would allow valuable characterization of larger patterns in DENV transmission across regions. The average age of DENV infection has been increasing in Thailand and other parts of Asia, indicating a decreased force of infection possibly due to a demographic transition toward an older population (35, 36). While Thailand has had all four DENV serotype in circulation for many decades, DENV is re-emerging in the Americas, and Ecuador became hyperendemic for all four serotypes as recently as 2000. It is, therefore, conceivable that Thailand may represent the future for Ecuador with regards to long-standing hyperendemic DENV transmission, and Ecuador, in turn, the future for areas that are currently DENV-naïve or low-endemicity but at risk for DENV introduction or expansion with climate change.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author and with completion of appropriate regulatory requirements.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by for the Ecuador study, the protocol was reviewed and approval by Institutional Review Boards (IRBs) at SUNY Upstate Medical University, the Human Research Protection Office (HRPO) of the U.S. Department of Defense, the Luis Vernaza Hospital in Guayaquil, Ecuador, and the Ecuadorean Ministry of Health. For the Thai study, the protocol was approved by the IRBs of the Thai Ministry of Public Health (MOPH), Walter Reed Army Institute of Research (WRAIR), and SUNY Upstate Medical University. The IRBs of the University of California, Davis (UCD), University of Rhode Island (URI), and University at Buffalo established relying agreements with WRAIR IRB. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KA and AS-I performed the comparative analyses and drafted the manuscript. AS-I, EB, SR, and TE participated in the design, conduct, and analysis of the field study in Ecuador. DB, ST, RJ, and TE participated in the design and conduct of the field study in Thailand. TE was the PI for the NIH-funded R01 that provided support for the Thai study. SI provided support and guidance from the Thai Ministry of Public Health. SF provided support for the current analysis of the Thai data as current head of the department of virology, AFRIMS. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. *Nature.* (2013) 496:504–7. doi: 10.1038/nature12060
2. Alava A, Mosquera C, Vargas W, Real J. Dengue en el Ecuador 1989-2002. *Rev Ecuat Hig Med Trop.* (2005) 42:11–34.
3. Gutierrez E, Real J, Alava A, Mosquera C. Epidemia de Dengue Hemorragico en el Ecuador, 2003. *Rev. Ecuat. Hig. Med. Trop.* (2005) 42:35–49.
4. Endy TP, Chunsuttiwat S, Nisalak A, Libraty DH, Green S, Rothman AL, et al. Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. *Am J Epidemiol.* (2002) 156:40–51. doi: 10.1093/aje/kwf005
5. Rocha C, Morrison AC, Forshey BM, Blair PJ, Olson JG, Stancil JD, et al. Comparison of two active surveillance programs for the detection of clinical dengue cases in Iquitos, Peru. *Am J Trop Med Hyg.* (2009) 80:656–60. doi: 10.4269/ajtmh.2009.80.656
6. Alera MT, Srikiatkhachorn A, Velasco JM, Tac-An IA, Lago CB, Clapham HE, et al. Incidence of dengue virus infection in adults and children in a prospective longitudinal cohort in the Philippines. *PLoS Negl Trop Dis.* (2016) 10:e0004337. doi: 10.1371/journal.pntd.0004337
7. Gordon A, Kuan G, Mercado JC, Gresh L, Aviles W, Balmaseda A, et al. The Nicaraguan pediatric dengue cohort study: incidence of inapparent and symptomatic dengue virus infections, 2004-2010. *PLoS Negl Trop Dis.* (2013) 7:e2462. doi: 10.1371/journal.pntd.0002462
8. Imai N, Dorigatti I, Cauchemez S, Ferguson NM. Estimating dengue transmission intensity from sero-prevalence surveys in multiple countries. *PLoS Negl Trop Dis.* (2015) 9:e0003719. doi: 10.1371/journal.pntd.0003719
9. Imai N, Dorigatti I, Cauchemez S, Ferguson NM. Estimating dengue transmission intensity from case-notification data from multiple countries.

PLoS Negl Trop Dis. (2016) 10:e0004833. doi: 10.1371/journal.pntd.0004833
10. Sridhar S, Luedtke A, Langevin E, Zhu M, Bonaparte M, Machabert T, et al. Effect of dengue serostatus on dengue vaccine safety and efficacy. *N Engl J Med.* (2018) 379:327–40. doi: 10.1056/NEJMoa1800820
11. Ooi EE, Goh KT, Gubler DJ. Dengue prevention and 35 years of vector control in Singapore. *Emerg Infect Dis.* (2006) 12:887–93. doi: 10.3201/eid1206.051210
12. O'Neill SL, Ryan PA, Turley AP, Wilson G, Retzki K, Iturbe-Ormaetxe I, et al. Scaled deployment of Wolbachia to protect the community from dengue and other Aedes transmitted arboviruses. *Gates Open Res.* (2018) 2:36. doi: 10.12688/gatesopenres.12844.1
13. Carvalho DO, McKemey AR, Garziera L, Lacroix R, Donnelly CA, Alphey L, et al. Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Negl Trop Dis.* (2015) 9:e0003864. doi: 10.1371/journal.pntd.0003864
14. Thomas SJ, Aldstadt J, Jarman RG, Buddhari D, Yoon IK, Richardson JH, et al. Improving dengue virus capture rates in humans and vectors in Kamphaeng Phet Province, Thailand, using an enhanced spatiotemporal surveillance strategy. *Am J Trop Med Hyg.* (2015) 93:24–32. doi: 10.4269/ajtmh.14-0242
15. Stewart-Ibarra AM, Ryan SJ, Kenneson A, King CA, Abbott M, Barbachano-Guerrero A, et al. The burden of dengue fever and chikungunya in southern coastal ecuador: epidemiology, clinical presentation, and phylogenetics from the first two years of a prospective study. *Am J Trop Med Hyg.* (2018) 98:1444–59. doi: 10.4269/ajtmh.17-0762
16. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol.* (1992) 30:545–51. doi: 10.1128/JCM.30.3.545-551.1992
17. Santiago GA, Vergne E, Quiles Y, Cosme J, Vazquez J, Medina JF, et al. Analytical and clinical performance of the CDC real time RT-PCR assay for detection and typing of dengue virus. *PLoS Negl Trop Dis.* (2013) 7:e2311. doi: 10.1371/annotation/ae27d48b-025f-47ce-8427-4af59f821ad7
18. Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, Suntayakorn S, et al. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg.* (1989) 40:418–27. doi: 10.4269/ajtmh.1989.40.418
19. Ruchusatsawat K, Wongjaroen P, Posanacharoen A, Rodriguez-Barraquer I, Sangkitporn S, Cummings DAT, et al. Long-term circulation of Zika virus in Thailand: an observational study. *Lancet Infect Dis.* (2019) 19:439–46. doi: 10.1016/S1473-3099(18)30718-7
20. World Health Organization. *WHO Vaccine-Preventable Diseases: Monitoring System*. 2019 Global Summary (2019).
21. World Health Organization. *Ecuador: WHO and UNICEF Estimates of Immunization Coverage: 2018 Revision* (2018).
22. Sippy R, Herrera D, Gaus D, Gangnon RE, Patz JA, Osorio JE. Seasonal patterns of dengue fever in rural Ecuador: 2009-2016. *PLoS Negl Trop Dis.* (2019) 13:e0007360. doi: 10.1371/journal.pntd.0007360
23. Halstead SB, Dans LF. Dengue infection and advances in dengue vaccines for children. *Lancet Child Adolesc Health.* (2019). doi: 10.1016/S2352-4642(19)30205-6
24. Endy TP, Nisalak A, Chunsuttiwat S, Libraty DH, Green S, Rothman AL, et al. Spatial and temporal circulation of dengue virus serotypes: a prospective study of primary school children in Kamphaeng Phet, Thailand. *Am J Epidemiol.* (2002) 156:52–9. doi: 10.1093/aje/kwf006
25. Fried JR, Gibbons RV, Kalayanarooj S, Thomas SJ, Srikiatkhachorn A, Yoon IK, et al. Serotype-specific differences in the risk of dengue hemorrhagic fever: an analysis of data collected in Bangkok, Thailand from 1994 to 2006. *PLoS Negl Trop Dis.* (2010) 4:e617. doi: 10.1371/journal.pntd.0000617
26. Yung CF, Lee KS, Thein TL, Tan LK, Gan VC, Wong JG, et al. Dengue serotype-specific differences in clinical manifestation,laboratory parameters and risk of severe disease in adults, singapore. *Am J Trop Med Hyg.* (2015) 92:999–1005. doi: 10.4269/ajtmh.14-0628
27. Reiner RC Jr, Stoddard ST, Forshey BM, King AA, Ellis AM, Lloyd AL, et al. Time-varying, serotype-specific force of infection of dengue virus. *Proc Natl Acad Sci USA.* (2014) 111:E2694–702. doi: 10.1073/pnas.1314933111
28. Endy TP, Anderson KB, Nisalak A, Yoon IK, Green S, Rothman AL, et al. Determinants of inapparent and symptomatic dengue infection in a prospective study of primary school children in Kamphaeng Phet, Thailand. *PLoS Negl Trop Dis.* (2011) 5:e975. doi: 10.1371/journal.pntd.0000975
29. Xavier-Carvalho C, Cardoso CC, de Souza Kehdy F, Pacheco AG, Moraes MO. Host genetics and dengue fever. *Infect Genet Evol.* (2017) 56:99–110. doi: 10.1016/j.meegid.2017.11.009
30. Kamal SM, El Sayed Khalifa K. Immune modulation by helminthic infections: worms and viral infections. *Parasite Immunol.* (2006) 28:483–96. doi: 10.1111/j.1365-3024.2006.00909.x
31. Anderson KB, Gibbons RV, Thomas SJ, Rothman AL, Nisalak A, Berkelman RL, et al. Preexisting Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a school-based cohort in Thailand. *PLoS Negl Trop Dis.* (2011) 5:e1311. doi: 10.1371/journal.pntd.0001311
32. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis.* (2008) 14:1232–9. doi: 10.3201/eid1408.080287
33. Balmaseda A, Stettler K, Medialdea-Carrera R, Collado D, Jin X, Zambrana JV, et al. Antibody-based assay discriminates Zika virus infection from other flaviviruses. *Proc Natl Acad Sci USA.* (2017) 114:8384–9. doi: 10.1073/pnas.1704984114
34. Tsai WY, Youn HH, Tyson J, Brites C, Tsai JJ, Pedroso C, et al. Use of urea wash ELISA to distinguish Zika and dengue virus infections. *Emerg Infect Dis.* (2018) 24:1355–9. doi: 10.3201/eid2407.171170
35. Cummings DA, Iamsirithaworn S, Lessler JT, McDermott A, Prasanthong R, Nisalak A, et al. The impact of the demographic transition on dengue in Thailand: insights from a statistical analysis and mathematical modeling. *PLoS Med.* (2009) 6:e1000139. doi: 10.1371/journal.pmed.1000139
36. Egger JR, Ooi EE, Kelly DW, Woolhouse ME, Davies CR, Coleman PG. Reconstructing historical changes in the force of infection of dengue fever in Singapore: implications for surveillance and control. *Bull World Health Org.* (2008) 86:187–96. doi: 10.2471/BLT.07.040170

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Contextual, Social and Epidemiological Characteristics of the Ebola Virus Disease Outbreak in Likati Health Zone, Democratic Republic of the Congo, 2017

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While the clinical, laboratory and epidemiological investigation results of the Ebola outbreak in Likati Health Zone, Democratic Republic of the Congo (DRC) in May 2017 have been previously reported, we provide novel commentary on the contextual, social, and epidemiological characteristics of the epidemic. As first responders with the outbreak Surveillance Team, we explain the procedures that led to a successful epidemiological investigation and ultimately a rapid end to the epidemic. We discuss the role that several factors played in the trajectory of the epidemic, including traditional healers, insufficient knowledge of epidemiological case definitions, a lack of community-based surveillance systems and tools, and remote geography. We also demonstrate how a collaborative Rapid Response Team and implementation of community-based surveillance methods helped counter contextual challenges during the Likati epidemic and aid in identifying and reporting suspected cases and contacts in remote and rural settings. Understanding these factors can hinder or help in the rapid detection, notification, and response to future epidemics in the DRC.

Keywords: The Democratic Republic of the Congo, Ebola Virus Disease, outbreak investigation, contact tracing, surveillance, zoonotic disease

INTRODUCTION

In April 2017 (1, 2) the Likati Health Zone office in the northern province of Bas Uélé in the Democratic Republic of the Congo (DRC) identified a cluster of illnesses and deaths with Ebola-like symptoms. Following investigation by local health authorities on May 5, 2017 (3), the Provincial Health Office alerted the Ministry of Health (MOH) in Kinshasa of a potential Ebola Virus Disease (EVD) outbreak, which was subsequently reported to the World Health Organization (WHO) per International Health Regulation requirements (4). The MOH officially declared the EVD outbreak in the Likati Health Zone on May 11, 2017 (1) after a blood sample collected from one of five suspected cases tested positive by reverse transcription-polymerase chain reaction (RT-PCR) (5) for Ebola virus subtype Zaire at the national reference laboratory in Kinshasa (6).

The DRC is the second largest and fourth most populated country in Africa and has an environment favorable to zoonotic disease outbreaks such as yellow fever, monkeypox, EVD, and other viral hemorrhagic fevers (7, 8). Tropical forests rich in animal diversity and growing in population density, like those in the DRC, have been shown to increase the risk of emerging infectious diseases (9). These ecological factors, regional sociopolitical insecurity and instability, shared borders with nine other countries, and a mobile population, make DRC highly vulnerable to disease outbreaks (9, 10). While the DRC is experienced in outbreak response, having responded to more EVD outbreaks than any other country, the current (2018–2019) EVD outbreak in Ituri and North Kivu provinces—the longest-lasting in DRC's history—has demonstrated that when certain factors converge, outbreaks can still be a challenge to contain (11).

The Bas Uélé province—which houses the Likati Health Zone where the May-June 2017 EVD outbreak occurred—is situated in the northern part of the country on the border with the Central African Republic. Likati is a heavily forested, rural area (population of 74,648) (12) ∼140 km away from the provincial capital Buta. Likati is isolated and lacks infrastructure, has limited communication networks, and no paved roads; travel routes (dirt paths and rivers) become impassable during the rainy season April through December. These factors result in reduced access to healthcare and delays in detecting, reporting and responding to potential cases of epidemic-prone diseases (13). The poverty rate is high, and the economy is based on agriculture, fishing, and hunting; many rely on the bushmeat industry as a food staple and source of income, increasing risk of zoonotic diseases exposure and transmission (14). The DRC's vulnerability to emerging and re-emerging infectious diseases along with the challenging environmental, geographic and sociopolitical factors renders timely detection and reporting of epidemic-prone diseases difficult, and underscores the importance of continued investment in a strong epidemiological surveillance system to detect and monitor disease outbreaks.

In the 2017 Likati EVD outbreak, the MOH's National Coordination Committee was responsible for managing outbreak response activities, coordinating with national and international partners to develop the outbreak response plan and assembling a multi-sectoral Rapid Response Team (**Table 1**), which was deployed to the outbreak epicenter on May 13, 2017. As part of the rapid response, the MOH's Directorate of Disease Control was assigned primary responsibility to coordinate the outbreak surveillance and case investigation activities. The Rapid Response Surveillance Team was comprised of field epidemiologists and surveillance experts who were responsible for conducting active case investigations, identifying and monitoring case contacts, tracking case alerts (i.e., symptomatic individuals or unexplained deaths) at health centers and within the community, managing case and contact data, and producing daily Situation Reports. Using the epidemiological, clinical, and laboratory data collected during surveillance activities, the Surveillance Team conducted an epidemiological investigation to identify the chain of transmission, determine the origin of the outbreak, and understand the dynamics of this EVD outbreak. As members of the Rapid Response Team, we describe the methods of our epidemiological investigation and expand upon previously published results (6) by describing the contextual, social and epidemiological factors that contributed to the Likati outbreak, and the potential implications these findings have on future EVD outbreaks in the DRC.

Abbreviations: DLM, Direction de la Lutte contre la Maladie/Directorate of Disease Control; ELISA, Enzyme-linked immunosorbent assay; EVD, Ebola Virus Disease; IDSR, Integrated Disease Surveillance and Response; INRB, Institut National de Recherche Biomédicale/National Reference Laboratory; RT-PCR, Reverse transcription polymerase chain reaction; VHF, Viral Hemorrhagic Fever.

METHODS

Case Investigation and Contact Tracing

We visited remote villages throughout the Likati Health Zone to interview case contacts, health workers, traditional healers, community and family members who transported patients, and local authorities to determine how and when the outbreak began. We reviewed health records, investigated unexplained deaths and illnesses in humans and animals, and investigated evidence of animal-to-human transmission of EVD. A standard case investigation form was used to record demographic characteristics; determine methods of exposure; document illness onset and signs and symptoms; and identify potentially exposed contacts of suspect, confirmed and probable cases.

To improve the early detection of suspected cases, we established a community alert system and trained community health workers to rapidly report and effectively manage community alert cases. Based on the outbreak-specific case definitions (**Table 2**), all alerts in the community were investigated and those meeting the criteria as a suspected case were transported to a health facility for clinical assessment, confirmatory laboratory testing, and appropriate treatment per Integrated Disease Surveillance and Response guidelines (15, 16). Contact information for suspected cases was obtained, and individuals who came in contact with a suspected case in the previous 21 days were defined as case contacts (**Table 2**). These contacts were monitored by community health workers for 21 days and contacts that began exhibiting symptoms were classified and treated as a suspected case.

All information on suspected case contacts was aggregated into a contacts list register. Patient information such as identity, method of notification, history of symptoms and treatment seeking behavior, symptoms, laboratory testing, and final classification was aggregated in the case line listing register in Excel. Both registers were uploaded into the Epi Info™ Viral Hemorrhagic Fever (VHF) application, version 0.9.60 (17).

We documented the chain of transmission by analyzing the case investigation forms, the case line listing register, the contacts list register, and transcripts of interviews with EVD

TABLE 1 | Overview of Ebola Rapid Response Teams, Likati Health Zone, DRC, May–June 2017*.

Response pillar	Description	Lead partner	Other key partners
Surveillance team	Organized and implemented active case investigation, contact tracing, and monitoring activities in health center and community. Conducted epidemiological surveillance in the community to trace chain of transmission	MOH-DLM	WHO, RTI
Medical management team	Established Ebola Treatment Centers (ETCs) at Likati general reference hospital and Nambwa Health Center, provided palliative care to suspected cases, educated caregivers and family members on infection prevention	MSF	MOH, WHO, ALIMA
Water and hygiene team	Distributed protective equipment, provided community sensitization on safe burial, implemented infection control activities, installed WASH kits at health structures, public places, and several households	IFRC	UNICEF, WHO
Laboratory and research team	Conducted confirmatory testing in Kinshasa, established mobile laboratories in Likati and Buta, developed testing algorithm, implemented standardized procedures to collect samples from suspected cases at admission, collected second sample as control for negative results, responsible for animal testing	INRB	JICA, WHO
Psychosocial support team	Provided support to suspected cases at ETCs, survivors after they were released, and family members of deceased cases	MSF	ALIMA
Logistics team	Ensured efficient resource management and coordination of staff and materials arriving and departing from Likati and Buta	MONUSCO and WFP	UNICEF, WHO, DFID, USAID
Communication and social mobilization team	Organized awareness-raising activities in villages, schools, markets, and churches. These activities were carried out by CHWs, who used a variety of strategies based on target population (e.g., door-to-door, films, radio, megaphones)	UNICEF	MSF

**ALIMA, Alliance for International Medical Action; DFID, Department for International Development; DLM, Directorate of Disease Control and Prevention; ETC, Ebola Treatment Center; IFRC, International Federation of the Red Cross; INRB, Institut National de Recherche Biomédicale; JICA, Japanese International Cooperation Agency; KSPH, Kinshasa School of Public Health; MoH, Ministry of Health; MONUSCO, United Nations Organization Stabilization Mission in the Democratic Republic of the Congo; MSF, Médecins sans Frontières; RTI, RTI International; SitRep, Situational Report; UMIR/FARDC, Unité Médicale d'Intervention Rapide/Forces Armées de la République Démocratique du Congo; UNIKIN, University of Kinshasa; WFP, World Food Program; WHO, World Health Organization.*

cases, survivors, and relatives of deceased cases, extended family, contacts, and community members.

Classification of Cases

Standard case definitions from the 2011 Integrated Disease Surveillance and Response (IDSR) Technical Guide (16) were adapted for health workers and outbreak response teams to improve assessment and classification of probable, confirmed, or non-cases (**Table 2**). IDSR alert case definitions were broadened to include any unexplained death **or** anyone with a high fever **or** anyone with bloody diarrhea; previously an alert case was defined as anyone with a high fever **and** bloody diarrhea. Case definitions were posted on health facility walls, and community and facility-based health workers were trained on these definitions to ensure proper classifications in applying case definitions. Community health workers were trained to use the definition for an alert case and notified either the Surveillance Team or health center closest in proximity if an alert case (alive or dead) was identified. Surveillance Team members traveling in the remote health areas and health center personnel were trained to report based on the definition of a suspected case, and would then notify the Rapid Response Team to either transport the patient to receive appropriate medical care or to collect and safely despose of the human remains. Notifications of both alert and suspected cases prompted Surveillance Team investigation; based on the investigation results, alert and suspected cases received a final classification as a probable, confirmed, or non-case according to the specified definitions (**Table 2**).

RESULTS

Case Investigation and Contact Tracing

As previously reported, the outbreak resulted in eight cases, five of which were laboratory confirmed [two by RT-PCR and three by enzyme-linked immunosorbent assay (ELISA)] (5, 18), and three of which were classified as probable. There were four deaths (three men and one woman). Five of the eight confirmed or probable cases came from the Nambwa Health Area (in the Likati Health Zone), which was identified as the outbreak epicenter.

All contacts completed the 21-day monitoring period by June 2, 2017, with no additional cases identified. The WHO officially declared the EVD outbreak over on July 2, 2017, 42 days after the last confirmed case tested Ebola virus-negative the second time; this period, which is twice the maximum incubation period for Ebola virus, is used to confirm the end of human-to-human transmission (19).

Chain of Transmission and Outbreak Origin

Data suggest that all confirmed and probable cases originated from a single EVD case with bushmeat exposure, and all subsequent cases resulted from human-to-human transmission (6). The epidemiological investigation suggested that the origin of the outbreak began with the index case's contact with bushmeat on March 15, 2017. The index case's brother-in-law, a hunter, brought back a monkey and a wild boar, partially eaten by other wild animals. The investigation uncovered the death of

TABLE 2 | Definitions of alert, suspected, probable, confirmed, non-cases, and case contacts used in the Likati 2018 outbreak (15).

Classification	Definition
Alert case	Anyone with a sudden onset of high fever OR: bloody urine/diarrhea OR: sudden death
Suspected case	Anyone, alive or dead, presenting or having had a high fever with a sudden onset, and who has been in contact with a suspected, probable or confirmed case of Ebola AND/OR a dead or sick animal OR: Anyone with a high fever with a sudden onset and at least three of the following symptoms: - Headache - Vomiting - Anorexia/loss of appetite - Diarrhea - Intense tiredness - Abdominal pain - Muscle or joint pain - Difficulty swallowing - Difficulty breathing - Hiccups - Skin rash OR: Anyone with unexplained bleeding OR: Anyone dying suddenly and whose death is unexplained OR: Spontaneous abortion
Probable case	Suspected case evaluated by a clinician OR: Deceased case with epidemiological link with a confirmed case OR: Any suspect case that is unable to be confirmed with laboratory testing, but the surveillance team classifies as probable after a case classification meeting there is evidence of an epidemiological link to a confirmed case
Confirmed case	Any suspected case with a positive lab result for viral RNA or antibodies for Ebola (RT-PCR or ELISA)
Non-case	Any suspect case with a negative laboratory result. Non-cases do not have antibodies, RNA, or detectable antigens
Case contact	Anyone who has had contact with a confirmed case or a sick/deceased animal. Contact with a human case is classified as any person who has been in contact with a confirmed case in one or more of the following ways: - Stayed in the same household as the confirmed case in the month preceding symptom onset - Had direct physical contact with the confirmed case (living or dead) during his/her illness - Shared the same means of transport (e.g., plane, boat, vehicle, bike, motorcycle, canoe) - Touched bodily fluids of confirmed case during his/her illness - Handled confirmed case's clothes or linen - Was breastfed by the confirmed case Contact with dead or sick animal is classified as anyone who has been in contact with an animal found dead or sick in at least one of the following ways: - Touched - Handled - Prepared - Touched the blood of an animal - Ate bushmeat

84 pigs in three villages of the Nambwa health area between March 9, 2017 and May 22, 2017, however testing of the dead pigs by RT-PCR indicated they were not the origin of the outbreak.

The epidemiological investigation found that the index case became symptomatic (with fever, arthralgia and muscle pain, nausea, vomiting) on March 27, 2017, within the incubation period after exposure to bushmeat on March 15, 2017. The index case was treated at a private health facility for ∼6 days. The index case experienced hematemesis on April 2, 2017; believing it to be a sign of poisoning, the index case's family brought them to a traditional healer. Showing no signs of improvement after 2 days, the traditional healer referred the patient to a private health center, and upon arrival their temperature was 103.1 degrees Fahrenheit (39.5 degrees Celsius). Symptoms did not improve, and after 1 day of observation they were advised to transfer to the Likati General Reference Hospital. The index case died en route on April 5, 2017, 9 days after symptom onset. Transportation to the hospital was via motorcycle, with a driver and a person assisting with transport. The driver, who later died, was classified as a probable case. The person assisting with transport was classified a confirmed case (serology), and was initially believed to be the index case until the epidemiological investigation, instead, identified them as a contact.

Classification of Cases

Standardizing case definitions, establishing the community alert system, and training community health workers helped to detect, report, and effectively manage community alerts.

Coordination with the Communication and Social Mobilization Team (**Table 1**) was crucial to ensure alerts were investigated by the Surveillance Team and classified according to standard case definitions; the Communication and Social Mobilization Team organized community awareness campaigns through local radio, churches, market, schools, and other public places to remind the population to report suspected cases or deaths in the community. This collaboration resulted in identifying suspected cases in eight of 11 health areas, with 98 classified as non-cases following laboratory testing and epidemiological investigation. All suspected cases and 583 contacts were monitored for 21 days without any lost to follow-up.

The epidemiological investigation discovered a limited understanding of EVD among community health workers and healthcare facility staff in Likati. In response to this observed gap, we trained 98 community health workers in seven health areas of Likati Health Zone on the EVD community case definition to identify community alerts, case contact identification, data collection and follow-up procedures, and collection of body temperature.

DISCUSSION

In DRC, previous experience with EVD outbreaks has contributed to improved national preparedness to swiftly coordinate and manage an outbreak response. Decades of experience has led to successful containment strategies that involve both formal health workers, traditional healers, and village social and religious leaders, and substantial efforts have been made in the DRC for capacity-building in epidemiology, laboratory analysis, and patient care, resulting in readily available local expertise that can quickly respond to outbreaks (20). These preparatory efforts contributed to the Rapid Response Team's ability to continually assess and strategically adapt to the evolving situation during the Likati 2017 EVD outbreak. The Surveillance Team succeeded in identifying how and when the outbreak began and developing a detailed description of the chain of transmission, which resulted in effectively interrupting the transmission chain to contain the Likati outbreak in 51 days. The person originally thought to be the index case was determined to be a contact instead; thus, the epidemiological investigation found that the outbreak started on March 27, 2017, a month earlier than was originally reported. Using the adjusted timeline, the MOH outbreak declaration on May 11, 2017 was 45 days after the index case first developed symptoms and was shortly thereafter seen in a private health facilty. Additionally, the epidemiological investigation confirmed that the index case had contact with monkey and wild boar bushmeat. While monkeys are known animal reservoirs for EVD, a wild boar has not been a documented likely orgin of a previous EVD outbreak in the DRC (21).

Understanding the contextual factors that contribute to notification delays may allow for targeted improvement of the surveillance system in DRC in preparation for future EVD outbreaks. In the 2017 Likati EVD outbreak, first responders identified several factors that contributed to the delays in detection and reporting: the use of traditional healers as first-line healthcare and treatment, insufficient knowledge of EVD case definitions at the health center and among community health workers, lack of community-based surveillance systems and tools, and remote rural geographic characteristics.

Use of Traditional Healers

Interviews with key informants during the epidemiological investigation found that EVD cases—including the index case—received care from traditional healers, which can result in delayed detection of a potential epidemic and the coordinated response necessary to halt viral transmission (22). Traditional healers are often the first point-of-care in rural areas where access to the formal healthcare system may be limited, or when one believes an illness is spiritual and cannot be cured with a medical intervention (23). To improve healthcare linkages for populations in rural settings, the DRC MOH put a national program of traditional medicine in place in 2001 to help regulate care provision in rural areas; however, for various reasons including mistrust between traditional and modern practitioners, traditional healers were not integrated into the national healthcare system (24). Currently, due to the informal nature in which traditional healers operate, they can be difficult to identify for EVD control measures. Despite this challenge, it is critical that future EVD communication campaigns sensitize traditional healers (and private health facilities, where the index case first received treatment) to recognize symptoms and refer sustected cases. Of note, among the eight confirmed and probable EVD cases in the Likati outbreak, only one was determined to be exposed at a healthcare facility and the remaining seven were most likely exposed to the virus in the community. This is an important finding because exposure to EVD in healthcare facilities can lead to rapid amplification of an outbreak as was demonstrated in the 1995 Kikwit outbreak where 25% of cases were among health workers exposed in a healthcare facility (25). Proper infection prevention procedures by healthcare workers at the affected healthcare facility may have also contributed to more rapid containment of the Likati outbreak.

Community and Facility Health Worker Knowledge

Despite treatment at two local healthcare facilities and a traditional healer, the index case was not properly diagnosed with EVD, leading to a substantial delay in notification of the case. This points to the importance of health workers and communities' ability to recognize the signs and symptoms of EVD. The Surveillance Team observed a limited understanding of EVD among facility-based and community-based health workers in remote health areas of Likati Health Zone; as care seeking from traditional healers or religious leaders often replaces or precedes the formal healthcare system, community health workers should be routinely trained to detect unusual health events in their communities (including suspected EVD cases) and report these events to health authorities. The Surveillance Team's knowledge of this factor led to targeted training of community

health workers during the Likati epidemic, emphasizing the important role of community-surveillance systems in remote and rural settings.

Community-Based Surveillance Systems and Tools

The Likati epidemic demonstrated the important role of communities in contributing to EVD response efforts. Training and equipping community and facility-based health workers with the tools to collect, manage, and properly report community alerts and suspected cases in line with national and international surveillance rules and regulations is critical to containing epidemics. Tools such as low-literacy flip charts and posters with visual depictions of case definitions and data collection and reporting forms, should be standardized and available for use in both the informal and formal healthcare system to bridge the gap between event- and indicator-based surveillance systems (26). To be most effective, the community-based surveillance system in the DRC should incorporate notifications and reporting of Ebola-like symptoms and suspected deaths from traditional healers in the communities. The Likati community alert system developed by the Surveillance Team aimed to address this gap, especially in the more remote health areas in Likati that were far from formal healthcare structures.

Remote and Rural Geographic Characteristics

Likati's remote and rural geography presented challenges that impacted the ability to conduct outbreak investigation and response activities. Impassable roads and poor network coverage affected timely and accurate communication and reporting from remote health areas. Limited transportation infrastructure between Likati and the general reference laboratory in Kinshasa slowed the diagnosis of initial suspected cases until a mobile laboratory unit could be deployed. To address these challenges, Rapid Response Teams (**Table 1**) used canoes and motorbikes to traverse rivers and difficult terrain inaccessible by car, brought generators to address inconsistent power supply in the district health office, and used Very Small Aperture Terminal (VSAT) satellites and satellite telephones with solar chargers for connectivity in remote health areas. Further, the Surveillance Team placed satellite phones in communities deemed high-risk to ensure direct, real-time case reporting. Nevertheless, Likati's challenging geographic characteristics may have contributed to the confinement of the EVD outbreak and reduced the risk of transmission to more densely populated urban areas in neighboring health zones (6, 13). The low population density limits human contacts, and lack of infrastructure decreases chances of EVD rapidly spreading between large cities. This is in stark contrast to the 2014-2015 EVD epidemic in West Africa.

CONCLUSION

The context in which an EVD outbreak occurs can contribute delays in detection, notification, and rapid response. The 2017 Likati outbreak response was a success; despite delays in notification, the Rapid Response Team successfully worked together to contain the EVD outbreak. Case investigation and contact tracing efforts provided important information about how and when the outbreak began, confirmed the true index case, and developed a comprehensive chain of transmission. The investigation also highlighted epidemiological characteristics that can hinder rapid response efforts; understanding these factors that contribute to notification delays allows for targeted improvement of the DRC's surveillance system to best prepare for future EVD outbreaks. Ongoing efforts to identify gaps, and the motivation of the MOH and international community to implement sustainable solutions, may support improved response to and prevent morbidity and mortality from infectious disease epidemics in the DRC and the wider global community.

DATA AVAILABILITY STATEMENT

The data analyzed in this study was obtained from the Directorate of Disease Control, Ministry of Public Health, Kinshasa, and Democratic Republic of the Congo. The following licenses/restrictions apply: all data requested related to the Likati EVD outbreak investigation will be deidentified prior to sharing. Requests to access these datasets should be directed to Dr. Benoit Kebela Ilunga, kebelailunga@gmail.com.

AUTHOR CONTRIBUTIONS

BN, LL, MM, and AO were members of the Rapid Response Surveillance Team responsible for developing the epidemiological investigation plan, conducting the investigation, collecting and analyzing epidemiological data, and establishing the chain of transmission. BI provided technical oversight to the Rapid Response Ream and contributed to the design of the outbreak epidemiological surveillance strategy. KG, KS, JH-F, and PM provided technical assistance for surveillance activities during the outbreak and compiled the findings of the epidemiological investigation for the manuscript. KG wrote the first draft of the manuscript and steadily improved based on comments from KS, JH-F, and PM. All authors reviewed 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/fpubh.2020.00349/full#supplementary-material

REFERENCES

1. Ministere de la Santé Publique. *Communication speciale de Son Excellence Monsieur le Ministre de la Sante Publique en Rapport avec la situation epidemiologique du pays*. Republique Democratique du Congo (2017). Available online at: https://reliefweb.int/sites/reliefweb.int/files/resources/20170511---communication-de-sem-le-ministre-de-la-sante%CC%81-sur-situation-e%CC%81p..._0.pdf (accessed August 2, 2019).
2. World Health Organization. *Dr Oly Ilunga Kalenga, Minister of Public Health, Announces an Outbreak of Ebola Virus Disease in Likati district, Bas-Uélé Province (northern DRC) following confirmation by the National Biomedical Research Institute*. Geneva: World Health Organization. (2017). Available online at: https://www.afro.who.int/news/dr-oly-ilunga-kalenga-minister-public-health-announces-outbreak-ebola-virus-disease-likati
3. World Health Organization. *Ebola Virus Disease. Democratic Republic of the Congo. External Situation Report 1*. Switzerland: World Health Organization (2017). Available online at: https://apps.who.int/iris/bitstream/handle/10665/255419/EbolaDRC-1552017-eng.pdf?sequence=1 (accessed May 15, 2017).
4. World Health Organization. *International Health Regulations (IHR)*. Switzerland: World Health Organization (2019) Available online at: https://www.who.int/topics/international_health_regulations/en/ (accessed April 10, 2019).
5. Cherpillod P, Schibler M, Vieille G, Cordey S, Mamin A, Vetter P, et al. Ebola virus disease diagnosis by real-time RT-PCR: a comparative study of 11 different procedures. *J Clin Virol.* (2016) 77:9–14. doi: 10.1016/j.jcv.2016.01.017
6. Nsio J, Kapetshi J, Makiala S, Raymond F, Tshapenda G, Boucher N, et al. 2017 outbreak of ebola virus disease in northern Democratic Republic of Congo. *J Infect Dis*. (2019) 22701–6. doi: 10.1093/infdis/jiz107
7. Ministere de la Santé Publique Secrétariat General. *Plan de Développement Sanitaire de la Zone de Santé de Likati*. Kinshasa: Ministere de la Santé Publique Secrétariat General (2015).
8. Stolka KB, Ngoyi BF, Grimes KEL, Hemingway-Foday JJ, Lubula L, Nzanzu Magazani A, et al. Assessing the surveillance system for priority zoonotic diseases in the Democratic Republic of the Congo, 2017. *Health Secur.* (2018) 16:S44–53. doi: 10.1089/hs.2018.0060
9. Allen T, Murray KA, Zambrana-Torrelio C, Morse SS, Rondinini C, Di Marco M, et al. Global hotspots and correlates of emerging zoonotic diseases. *Nat Commun.* (2017) 8:1124. doi: 10.1038/s41467-017-00923-8
10. Moore M, Gelfeld B, Okunogbe A, Paul C. *Identifying Future Disease Hot Spots. Infectious Disease Vulnerability Index*. Santa Monica, CA: RAND Corporation (2016). doi: 10.7249/RR1605
11. Ilunga Kalenga O, Moeti M, Sparrow A, Nguyen VK, Lucey D, Ghebreyesus TA. The ongoing ebola epidemic in the Democratic Republic of Congo, 2018-2019. *N Engl J Med.* (2019) 381:373–83. doi: 10.1056/NEJMsr1904253
12. *Plan National de Riposte à l'épidémie de la maladie a virus Ébola dans la zone de sante de Likati en province du bas-Uélé MNCC*. Bas-Uele Province: National Ebola Response Plan in Likati Health Zone (2017).
13. World Health Organization. *Managing Epidemics: Key Facts About Major Deadly Diseases*. Version 1. Switzerland: World Health Organization; (2018). Available online at: https://www.who.int/emergencies/diseases/managing-epidemics-interactive.pdf?ua=1 (accessed April 10, 2019).
14. Wolfe ND, Daszak P, Kilpatrick AM, Burke DS. Bushmeat hunting, deforestation, and prediction of zoonotic disease emergence. *Emerg Infect Dis.* (2005) 11:6. doi: 10.3201/eid1112.040789
15. Democratic Republic of Congo Ministry of Health, Directorate of Disease Control. *Internal Situation Report 7. Definition de cas de Maladie a Virus Ebola a Partir du Mois de Mars. Situation de la réponse à l'épidémie de la MVE à Likati. May 21 ed*. Kinshasa: Democratic Republic of Congo Ministry of Health, Directorate of Disease Control (2017).
16. Organisation mondiale de la Santé, Centers for Disease Control and Prevention. *Guide technique pour la Surveillance Intégrée de la Maladie et la Riposte (SIMR) dans la Région Africaine*. Second edition. (2011). Available online at: https://www.afro.who.int/sites/default/files/2017-06/IDSR-Technical%20-Guidelines-2010_French%20_final.pdf (accessed April 10, 2019).
17. EpiData Software. *What is EpiData Software? Denmark: EpiData Association*. RRID: SCR_008485; 2001. Available online at: http://www.epidata.dk (accessed August 6, 2019).
18. Ksiazek TG, West CP, Rollin PE, Jahrling PB, Peters CJ. ELISA for the detection of antibodies to Ebola viruses. *J Infect Dis.* (1999) 179(Suppl 1):S192–8. doi: 10.1086/514313
19. World Health Organization. *Declaration of the end of Ebola Virus Disease outbreak in the Democratic Republic of the Congo [Internet]. Ebola Outbreak Situation Reports*. Switzerland: World Health Organization; (2017). Available online at: http://apps.who.int/iris/bitstream/handle/10665/255798/EbolaDRC-02072017.pdf;jsessionid=52B61BDE9691418191747ECA86EE1CD5?sequence=1 (accessed March 14, 2019)
20. Maganga GD, Kapetshi J, Berthet N, Kebela Ilunga B, Kabange F, Mbala Kingebeni P, et al. Ebola virus disease in the Democratic Republic of Congo. *N Engl J Med.* (2014) 371:2083–91. doi: 10.1056/NEJMoa1411099
21. Rosello A, Mossoko M, Flasche S, Van Hoek AJ, Mbala P, Camacho A, et al. Ebola virus disease in the Democratic Republic of the Congo, 1976-2014. *Elife.* (2015) 4:e09015. doi: 10.7554/eLife.09015
22. Manguvo A, Mafuvadze B. The impact of traditional and religious practices on the spread of Ebola in West Africa: time for a strategic shift. *Pan Afr Med J.* (2015) 22(Suppl 1):9. doi: 10.11694/pamj.supp.2015.22.1.6190
23. Kasereka, MC, Hawkes, MT. "The cat that kills people:" community beliefs about Ebola origins and implications for disease control in Eastern Democratic Republic of the Congo. *Pathog Glob Health*. (2019). 113:149–157. doi: 10.1080/20477724.2019.1650227
24. Democratic Republic of Congo Ministry of Health. *Plan National de Developpement Sanitaire 2016-2020: vers la Couverture Sanitaire Universelle [Internet]*. (2016). Available online at: http://www.nationalplanningcycles.org/sites/default/files/planning_cycle_repository/democratic_republic_of_congo/pnds_2016-2020_version_finale_29_avril_2016.pdf (accessed April 27, 2018).
25. Khan AS, Tshioko FK, Heymann DL, Le Guenno B, Nabeth P, Kerstiens B, et al. The reemergence of Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995. Commission de Lutte contre les Epidemies a Kikwit. *J Infect Dis.* (1999) 179(Suppl. 1):S76–86. doi: 10.1086/514306
26. Centers for Disease Control and Prevention. *Global Disease Detection Operations Center: Event-Based Surveillance*. Atlanta, GA: Centers for Disease Control and Prevention (2019). Available online at: https://www.cdc.gov/globalhealth/healthprotection/gddopscenter/how.html (accessed April 10, 2019).

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frontiers in Medicine

CASE REPORT
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Case Report: Congenital Arthrogryposis and Unilateral Absences of Distal Arm in Congenital Zika Syndrome

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Zika virus was recognized as a teratogen in 2015, when prenatal Zika infection was associated with neonatal microcephaly. The transmission, virulence, tropism, and consequences of Zika virus infection during pregnancy are currently studied. Decreased neural progenitor cells, arrest in neuronal migration and/or disruption of the maturation process of the fetus central nervous system have been associated. Congenital Zika Syndrome produces a fetal brain disruption sequence resulting in structural brain abnormalities, microcephaly, intracranial calcifications, fetal akinesia and arthrogryposis. Vascular abnormalities like unique umbilical artery and decreased cerebral vascular flow have been described in some patients. This article reports a Zika positive patient with sequence of fetal brain disruption, arthrogryposis and absence of distal third of the right forearm. This report expands the clinical observations of congenital Zika syndrome that may be related to disruptive vascular events.

Keywords: Zika virus, birth defects, congenital infection, arthrogryposis, microcephaly, sequence, disruption

INTRODUCTION

Since the identification of the Zika virus (ZIKV) in a rhesus monkey in 1947 and its isolation in humans in 1954, this virus has caused outbreaks in different populations from 2007 to 2013, and recently in 2015 in Brazil, with different public health impacts (1–4). ZIKV infection in humans is related to blood dyscrasias such as thrombocytopenia, Guillain Barre Syndrome and structural morphological abnormalities in fetus of infected pregnant mothers (5–8). But, up to 80% of those infected will course asymptomatic. ZIKV is transmitted by the bite of infected *Aedes aegypti* mosquito, sexual or vertical transmission during pregnancy, through blood transfusions, among others (3, 9–12). ZIKV is a flavivirus with two identified, Asian and African, lineages. Its RNA genome (10.8 kb) encodes for a 3,419-amino acid polyprotein which forms a capsid (C), a membrane precursor (prM), a wrap (E), and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B y NS5) (13). Revers transcription polymerase chain reaction (RT-PCR) and reverse transcription quantitative polymerase chain reaction (RT-qPCR) are reliable tests for detection of viral ZIKV RNA in serum or urine of infected patients but they are limited because of fast

decline of virus presence in these tissues (1 to 2 weeks post-infection). Tests for ZIKV-specific IgM antibodies in serum are also used as a diagnostic evidence for ZIKV infection expanding the diagnostic opportunity for several months, but cross-reactivity with other flaviviruses like dengue, must be taken into account, especially in endemic areas (14, 15).

The pathogenicity of ZIKV is related to cellular events like apoptosis, vascular damage, restriction in the cell maturity, and the signal cascade activation, but its virulence and cellular pathology is not totally elucidated (16–18). ZIKV interferes with the neural development through decreased neural progenitor cells, arrest in neuronal migration and/or disruption of the maturation process of the fetus central nervous system (CNS). This is clinically translated into microcephaly, lissencephaly, and others brain abnormalities (19–22). The objective of this article is to report one patient with brain sequence disruption, arthrogryposis and absence of the distal segment of the right arm with ZIKV RNA detected in the cerebrospinal fluid.

CASE REPORT

During the 2016 ZIKV outbreak in Merida, México, a 27-year-old woman in the third trimester of pregnancy was referred to medical geneticist because multiple malformations detected in the fetus. Informed consent was obtained for sampling, clinical evaluations, and for the publication report. Exploring the medical records, she reported unquantified fever, preauricular nodes, pruritus and rash in the shoulder girdle and thorax in the first trimester when the pregnancy was unnoticed. No serological tests were performed for ZIKV at that time. Ultrasound was performed at 16.4 weeks of gestation with report of fetal growth within normal ranges; but at 23 weeks of gestation, the fetal hands were not identified. At 27.4 weeks of gestation, fetus was reported with microcephaly (DBP 58 mm); nuchal thickening, ventriculomegaly, hemisphere hypoplasia and cerebellar vermis were detected in the brain, and micrognathia, right radial aplasia, and arthrogryposis were also reported at that time.

A stillbirth with generalized subcutaneous edema was obtained via cesarean section at 35 weeks of gestation. At physical exploration showed craniofacial disproportion, microcephaly, irregular anterior and lower posterior hairline. Posterior sloping of the forehead and hypertelorism were observed. The nasal bridge, the nostrils and the filtrum were normal. Retrognathia and normal oral cavity were found. The ears were cupped with low implantation and thickened helix. The shoulders were short, with internal rotation and presented limitation to abduction. The left upper limb presented an extended elbow with limitation to the reduction, pronation arm, flexed wrist, non-reducible hand with cyanotic coloration. The upper right limb was conformed only to the proximal third of the arm. At this level, soft tissue defect was found with the presence of an irregular cutaneous line, exposure of subcutaneous tissue and the humeral condyle, no tissue bleeding was detected (**Figure 1**). The lower extremities presented limitation to hip abduction, knee extension and flexion of both feet. The genitalia anatomy showed 1 cm penis and a complete rough scrotum without testes inside.

On the skull x-ray, everted sutures and partial collapse of the cranial bones with a hypoplastic occipital was observed. The radiograph of the upper extremities shows a right humerus shorter than the left, with preserved of the distal region of the humerus (**Figure 2**). Computational axial tomography reported subcortical calcifications, lissencephaly, ventriculomegaly, and generalized cortical degeneration. The karyotype was normal, 46, XY. The serological test for toxoplasma, rubella, cytomegalovirus and herpes virus were negative in the mother and the patient. The RT-PCR for ZIKV/Dengue/Chikungunya in the patient's cerebrospinal fluid detected the presence of Zika viral RNA (23). Autopsy was not authorized.

DISCUSSION

The Zika virus outbreak in Brazil in 2015 became emergent due to catastrophic consequences in infected newborns during the prenatal period (7, 8). Current research investigates the virulence and pathogenicity of the African and Asian ZIKV lineages to understand why this teratogenic effect was not observed in earlier outbreaks (24, 25). Epidemiology during the outbreak in Brazil allowed to observe that: (1) Pregnant women infected with Zika were asymptomatic or symptomatic as well as the general population; (6) (2) Not all pregnant women with ZIKV infection had perinatal complications or their products had congenital abnormalities, it was estimated that up to 5–10% of these women had children with morphological abnormalities (8), (3) Structural abnormalities found in fetus and newborns were related to brain tissue disruption sequence and growth restriction (7, 21, 22). (4) Establishing a conclusive diagnosis of Congenital Zika Syndrome (CZS) is a challenge due to the prolonged time between acute (symptomatic or asymptomatic) maternal infection and the time when fetal abnormalities are detected (8), and finally, (5) Although the presence of viral RNA has been demonstrated for prolonged periods in serum, urine, semen and other tissues of infected patients, to stablish ZIKV diagnosis is still a challenged because is related to optimal RNA recovery methods. These methods are under investigations actually (14, 15).

In CZS, a fetal brain disruption sequence (FBDS) was described, thus numerous events would produce variable findings in brain imaging tests. The sequence of disruption is a congenital, static morphological abnormality, caused by the developmental failure of a body structure that had the normal (genetic) developmental potential. The embryological or fetal moment at which the tissue is interrupted or the development determines subsequent destruction; may therefore, be heterogeneous (26). Until now, different etiologies of FBDS are described, being the infections and vascular injuries more frequent (22). ZIKV interferes with neural development through the decrease of neural progenitor cells, the arrest in neuronal migration and/or disruption of the CNS maturation process (19, 20, 27). The involvement of neuronal stem cells in human fetuses through non-structural proteins (NS4B and NS4A) has been associated with the inhibition of Akt-mTor signaling that participates in

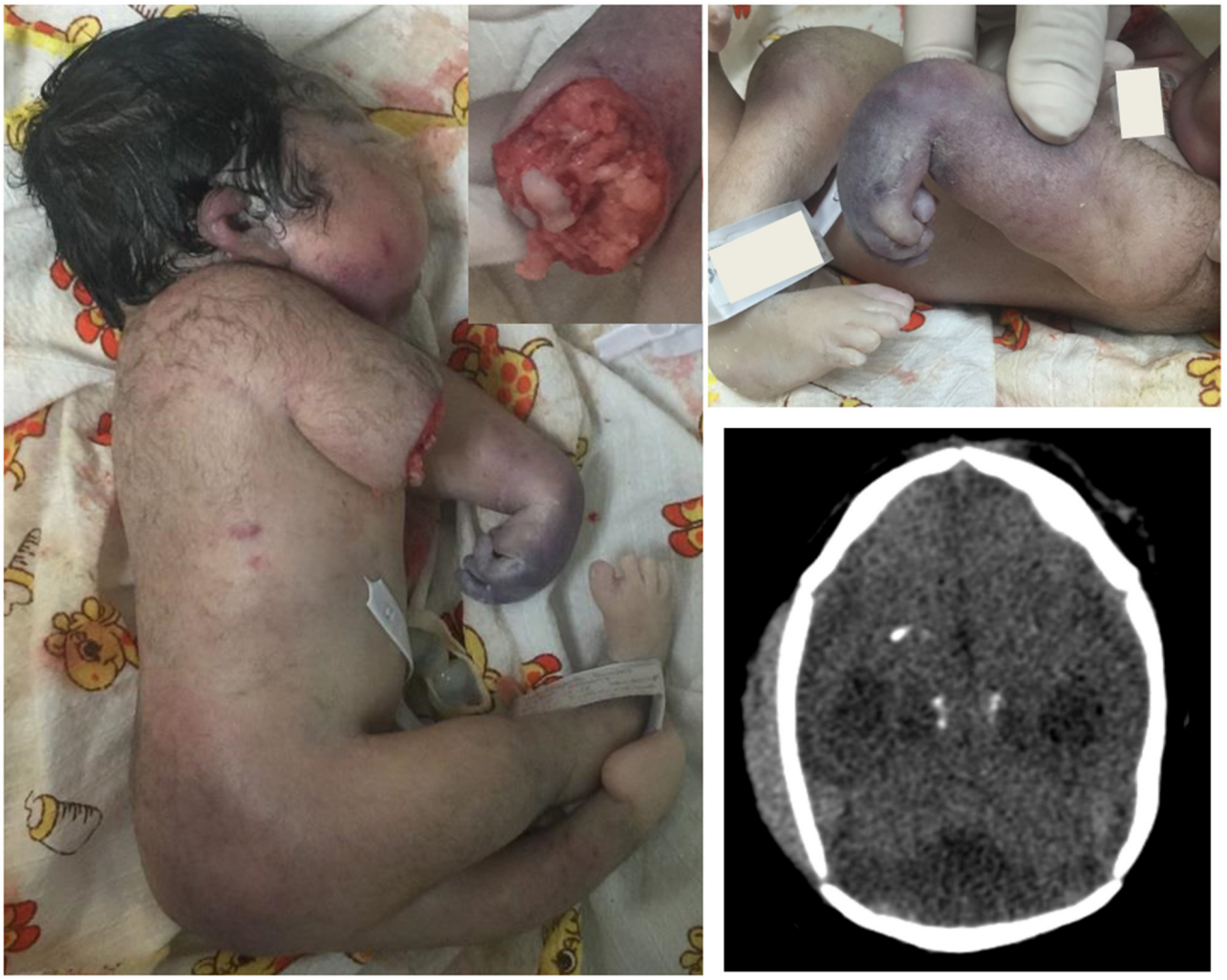

FIGURE 1 | (A) In this picture it is appreciate the patient phenotype with arthrogryposis and the absence of the distal part of the right arm The frontal view of the arm injury is inserted in the upper part of the photo. **(B)** Observe cyanotic coloration in the distal left arm and hand in comparison of the foot. **(C)** Axial-cut cranial tomography showing subcortical calcifications.

brain development (28). Depending on the damage to brain tissue, microcephaly can be observed and subsequent fetal skull collapse would result (22). Microcephaly, intracranial calcifications and brain disruption were the most frequent abnormalities shown in SCZ (21).

As a consequence of brain or peripheral nerves damage, can occur decrease in fetal movements and joint contractures (arthrogryposis) in consequence. It is known while limitation of fetal movement is earlier in gestational age and lower range of joint movement happens, the greater joint involvement and contractures will be observed at birth (29). This was one of the proposed mechanisms of the most severely affected patients with CZS (30). Arthrogryposis can occur as an isolated manifestation or as part of other genetic syndromes. Its etiology is not well-known; however, abnormalities in connective, nervous, muscular and vascular tissue have been related as possible pathological mechanisms (29).

In the patient here described, the contractures observed in the left arm were internal shoulder rotation, extended elbow and flexed wrist corresponding to amyoplasia, which is the most frequent arthrogryposis (31). However, the left arm was cyanotic from the middle part of the forearm to the acroterminal region and the wrist was hyperflexed with overlapping fingers with no reducible position. The isquemic pattern in the left hand could suggest vascular disruption. No constrictor rings were detected (32). The transverse terminal deficiency of the right limb is suspected of being lost during the last 8 weeks of pregnancy, because an obstetric ultrasound reported radial aplasia at 27 weeks gestation. In the distal stump, the skin was irregular and no bleeding was observed. Also, granulation tissue was found in the stump and fetal remains were not founded inside the uterus so that, this lost limb was not considered a traumatic event. The right arm injury presented in this patient was found different from those reported

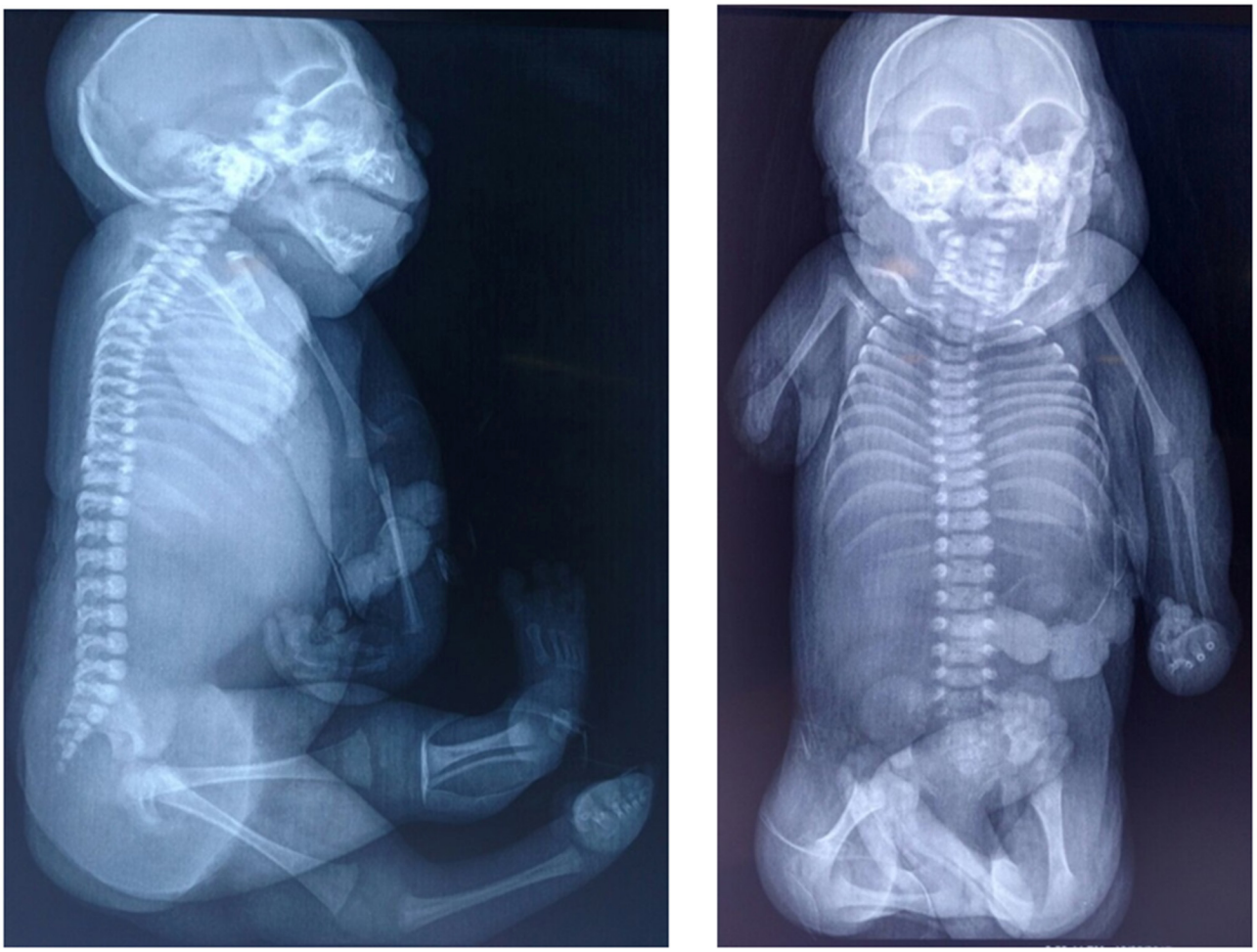

FIGURE 2 | X-ray imaging. In both image it appreciate skull with everted sutures and partial collapse of the cranial bones. Asymmetry is observed in the length of the right and left humerus.

for gangrene, ischemia and necrosis, as well as in the cases described of compartmental neonatal syndrome. Various reviews of sequences of amniotic bands show constriction rings, with hypoplasia of the post-ring region; even so, post-ring lesion usually has normal skin (31, 33). Teratogens associated with disruptive events in limbs have been described for a long time. Even so, limb amputations in uterus are infrequently reported (29–34). In ZIKV infection, cell cycle arrest and apoptosis happen in neuronal cell, but abnormalities in the density and vascular diameter of the brain had been reported (21). In addition, patients with abnormalities of cerebral flow and umbilical cord with single artery also were reported, so the vascular damage in others ZIKV affected tissues should still be clarified.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

SC-C, NP-R, and PM-S coordinated all work and did most of the writing. JP-B was responsible for the evaluation of medical records and ultrasonographic data. HB-P was responsible for macroscopy pathology data. DP-E and NV-G were responsible for biochemical and genetics data. All authors reviewed and commented on drafts and approved the final manuscript and the decision to submit for publication.

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REFERENCES

1. Dick GWA, Kitchen SF, Haddow AH. Zika virus (I). Isolations and serological specificity. *Transac R Soc Trop Med Hyg.* (1952) 46:509–20. doi: 10.1016/0035-9203(52)90042-4
2. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS. Zika virus outbreak on Yap Island, Federated States of Micronesia. *N Engl J Med.* (2009) 360:2536–43. doi: 10.1056/NEJMoa0805715
3. Besnard M, Lastére S, Teissier A, Cao-Lourmeau VM, Musso D. Evidence of perinatal transmission of Zika virus, French Polynesia, December 2013 and February 2014. *Euro Surveill.* (2014) 19:1–4. doi: 10.2807/1560-7917.ES2014.19.13.20751
4. Musso D, Gubler DJ. Zika Virus. *Clin Microbiol Rev.* (2016) 28:487–524. doi: 10.1128/CMR.00072-15
5. Parra B, Lizarzo J, Jiménez-Arango JA, Zea-Vera A, González-Manrique G, Vargas J, et al. Guillain-barré Syndrome associated with Zika virus infection in Colombia. *N Engl J Med.* (2016) 375:1513–23. doi: 10.1056/NEJMoa1605564
6. Brasil P, Pereira JP, Moreira ME, Ribeiro Nogueira RM, Damasceno L, Wakimoto M. Zika virus infection in pregnant women in Rio de Janeiro. *N Engl J Med.* (2016) 375:2321–34. doi: 10.1056/NEJMoa1602412
7. Mattar S, Ojeda C, Arboleda J, Arrieta G, Bosch I, Boha I, et al. Case report: microcephaly associated with ZIKV infection, Colombia. *BMC Infect Dis.* (2017) 17:425. doi: 10.1186/s12879-017-2522-6
8. Reynolds MR, Jones AM, Petersen Em Lee EH, Rice ME, Bingham A, Ellingtos SR, et al. Vital signs: update on Zika virus-associated birth defects and evaluation of all U.S. infants with congenital Zika virus exposure- U.S. Zika Pregnancy. *MMWR.* (2017) 66:1–9. doi: 10.15585/mmwr.mm6613e1
9. Haddow AD, Schuh AJ, Yasuda CY, Kasper MR, Heang V, Huy R, et al. Genetic characterization of Zika virus strains: geographic expansion for the Asian lineage. *PLoS Negl Trop Dis.* (2012) 6:e1477. doi: 10.1371/journal.pntd.0001477
10. Musso D, Nhan T, Robin E, Roche C, Bierlaire D, Zisou K, et al. Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. *Euro Surveill.* (2014) 19:1–3. doi: 10.2807/1560-7917.ES2014.19.14.20761
11. Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau V-M. Potential sexual transmission of Zika virus. *Emerg Infect Dis.* (2015) 21:359–61. doi: 10.3201/eid2102.141363
12. Donald CL, Brennan B, Cumberworth SL, Rezelj VV, Clark JJ, Cordeiro MT, et al. Full genome sewuence and sfRNA interferon antagonist activity of Zika virus from recife Brazil. *PLoS Negl Trop Dis.* (2016) 10:e0005048. doi: 10.1371/journal.pntd.0005048
13. Fleming AM, Ding Y, Alenko A, Burrows CJ. Zika virus genomic RNA possesses conserved G-Quadruplexes characteristics of the Flaviviridae family. *ACS Infect Dis.* (2016) 2:674–81. doi: 10.1021/acsinfecdis.6b00109
14. Gorchakov R, Berry RM, Patel SM, El Sahly HM, Ronca SE, Murray KO. Optimizing PCR detection of Zika virus from various body fluids. *Am J Trop Med Hyg.* (2019) 100:427–33. doi: 10.4269/ajtmh.18-0755
15. Del Pilar Martinez Viedma M, Puri V, Oldfield LM, Shabman RS, Tan GS, Pickett BE. Optimization of qRT-PCR assay for Zika virus detection in human serum and urine. *Virus Res.* (2019) 263:173–8. doi: 10.1016/j.virusres.2019.01.013
16. Beys-da-Silva WO, Rosa RL, Santi L, Berger M, Kyu PS, Campos AR. Zika virus infection of human mesenchymal stem cells promotes differential expression of proteins linked to several neurological diseases. *Mol Neurobiol.* (2018) 56:4708–17. doi: 10.1007/s12035-018-1417-x
17. Annamalai AS, Pattnaik A, Sahoo BR, Muhukrishnan E, Kumar NS, Steffen D, et al. Zika virus encoding nonglycosylated envelope protein is attenuated and defective in neuroinvasion. *Virol.* (2019) 91:e01348–17. doi: 10.1128/JVI.01348-17
18. Rinkenberger N, Schoggins JW. Comparative analysis of viral entry for Asian and African lineages of Zika virus. *Virology.* (2019) 533:59–67. doi: 10.1016/j.virol.2019.04.008
19. Shao Q, Herrlinger S, Yang SL, Lai F, Moore JM, Brindley MA, et al. Zika virus infection disrupts neurovascular development and results in postnatal microcephaly with brain damage. *Development.* (2016) 143:4127–36. doi: 10.1242/dev.143768
20. Acosta-Reyes J, Navarro E, Herrera MJ, Goenaga E, Ospina ML, Parra E, et al. Severe neurologic disorders in 2 fetuses with Zika virus infection, Colombia. *Emerg Infect Dis.* (2017) 23:982–4. doi: 10.3201/eid2306.161702
21. Del Campo M, Feitosa IM, Ribeiro EM, Horovitz DD, Pessoa AL, França GV, et al. The phenotypic spectrum of congenital Zika syndrome. *Am J Med Genet.* (2017) 173:841–57. doi: 10.1002/ajmg.a.38170
22. Moore CA, Stables JE, Dobyns WB, Pessoa A, Ventura CV, Borges da Fonseca E, et al. Characterizing the pattern of anomalies in Congenital Zika Syndrome for pediatric clinicians. *JAMA Pediatr.* (2017) 171:288–995. doi: 10.1001/jamapediatrics.2016.3982
23. *Trioplex Real-time RT-PCR Assay*. CDC (2016). Available online at: https://www.cdc.gov/zika/pdfs/trioplex-real-time-rt-pcr-assay-instructions-for-use.pdf
24. Yuan L, Huang X-Y, Liu Z-Y, Zhang F, Zhu X-L, Yu J-Y, et al. A single mutation in the prM protein of Zika Virus contributes to fetal microcephaly. *Science.* (2017) 358:933–6. doi: 10.1126/science.aam7120
25. Xavier-Neto J, Carvalho M, Pascoalino BDS, Cardoso AC, Costa AMS, Pereira AHM, et al. Hidrocephalus and arthrogryposis in an immunocompetent Mous model of Zika teratogeny: a developmental study. *PLoS Negl Trop Dis.* (2017) 11:e0005363. doi: 10.1371/journal.pntd.0005363
26. Hennekam RC, Biesecker LG, Allanson JE, Hall JG, Opitz JM, Temple IK, et al. Elements of morphology: general terms for congenital anomalies. *Am J Med Genet Part A.* (2013) 161A:2726–33. doi: 10.1002/ajmg.a.36249
27. Tang H, Hammack C, Ogden SC, Wen Z, Quian X, Li Y, et al. Zika virus infects human cortical neural progenitors and attenuates their growth. *Cell Stem Cell.* (2016) 18:1–4. doi: 10.1016/j.stem.2016.02.016
28. Liang Q, Luo Z, Zeng J, Chen W, Foo SS, Lee SA, et al. Zika virus NS4A and NS4B proteins deregulate Akt-mTOR signaling in human fetal neural stem cells to inhibit neurgenesis and induce autophagy. *Cell Stem Cell.* (2016) 195:663–71. doi: 10.1016/j.stem.2016.07.019
29. Hall JG. Arthrogryposis (multiple congenital contractures): diagnostic approach to etiology, classification, genetics, and general principles. *Eur J Med Genet.* (2014) 57:464e472. doi: 10.1016/j.ejmg.2014.03.008
30. van der Linden V, Filho EL, Lins OG, van der Linden A, de Aragão MF, Brainer-Lima AM, et al. Congenital Zika syndrome with arthrogryposis: retrospective case series study. *BMJ.* (2016) 354:i3899. doi: 10.1136/bmj.i3899
31. Hall JG. Amyoplasia involving only the upper limbs or only involving the lower limbs with review of the relevant differential diagnoses. *Am J Med Genet Part A.* (2014) 164A:859–73. doi: 10.1002/ajmg.a.36397
32. Naidich TP, Griffiths PD, Rosenbloom L. Central nervous system injury in utero: selected entities. *Pediatr Radiol.* (2015) 45:S454–62. doi: 10.1007/s00247-015-3344-6
33. Gold NB, Westgate M-N, Holmes LB. Anatomic and etiological classification of congenital limb deficiencies. *Am J Med Genet Part A.* (2011) 155:1225–35. doi: 10.1002/ajmg.a.33999
34. Nichols T. *Natural history study of arthrogryposis multiplex congenita, amyoplasia type* [UT GSBS dissertations and theses] (2011). Available

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A Flow Cytometry-Based Serological Assay to Detect Visceral Leishmaniasis in HIV-Infected Patients

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da Silva ED, de Oliveira BC, Pereira AMdS, Guedes DL, de Melo Neto OP, Costa CHN, de Medeiros ZM and Pereira VRA (2021) A Flow Cytometry-Based Serological Assay to Detect Visceral Leishmaniasis in HIV-Infected Patients. Front. Med. 8:553280. doi: 10.3389/fmed.2021.553280

Visceral Leishmaniasis (VL) is a severe parasitic disease that has emerged as an important opportunistic condition in HIV-infected patients and whose control is impaired by inaccurate identification. This is mainly due to the serological tests used for VL having a reduced performance in cases of VL-HIV coinfection due to a low humoral response. In this situation, however, a positive test has even greater diagnostic value when combined with the clinical status. This study aimed to evaluate the application and performance of flow cytometry to detect anti-*Leishmania infantum* antibodies in HIV-infected patients. Sera from VL/HIV coinfected patients, characterized using "gold standard" techniques, were compared with sera from healthy controls plus sera from HIV-infected individuals. The flow cytometry results were expressed as levels of IgG reactivity, based on the percentage of positive fluorescent parasites (PPFP). A ROC curve analysis of a serum titration indicated a PPFP of 1.26% as being the cutoff point to segregate positive and negative results. At the 1:2,048 dilution, with 89% sensitivity and 83% specificity, flow cytometry showed greater sensitivity in relation to the serological tests evaluated. Futhermore, flow cytometry was the only assay that positively identified all VL-HIV patients with quantified HIV load. Together, these findings suggest that flow cytometry may be used as an alternative serological approach for VL identification and as a tool to characterize the humoral response against *Leishmania infantum* in HIV-infected patients.

Keywords: visceral leishmaniasis, HIV infection, aids, diagnosis, flow cytometry

INTRODUCTION

Visceral Leishmaniasis (VL) is a potentially fatal disease that has emerged as an important opportunistic condition in HIV infected patients, resulting in a substantial number of VL–HIV coinfection cases which have been reported from 35 countries. The coinfection generates an impact in the immunopathogenesis, clinical manifestation, therapeutic response and diagnosis of both diseases (1). Case definition of HIV-VL requires confirmation of HIV infection by serological tests and positive results for VL diagnosis based on parasitological (bone marrow aspirate),

serological or molecular methods, in addition to clinical symptoms. The microscopic examination or isolation of the parasite, the protozoan *Leishmania infantum*, is considered the gold standard for laboratorial confirmation of VL. Although this technique has high specificity, its use in clinical laboratories has some limitations, mainly due to the low sensitivity levels. The procedures involved are also invasive, time-consuming and require experienced personnel (2). Furthermore, due to the immunodepressed status of HIV-infected individuals, the parasites may not be found in the bone marrow, but rather in less common sites such as the oral mucosa, skin, stomach, colon and lungs (3–5). Serological approaches which detect specific antibodies against *L. infantum* constitute a valuable alternative as an early, rapid, and user-friendly diagnostic test. In the VL-HIV coinfection, however, the conventional VL serological assays, which includes indirect immunofluorescence test and the rK39 rapid test, are not considered accurate due to the low antibody production in these individuals (6–8).

The development of an effective VL diagnosis for the VL-HIV coinfections represents still a relevant challenge since it needs to be precise in order to reduce the lethality and mortality of afflicted individuals. Considering the limitations of the available diagnostic techniques, alternative methodologies have been employed (9, 10). One of them is flow cytometry, a technique that has been seen to be useful for a diversity of diagnostic applications, such as immunodeficiency disorders and cancer (11, 12). In addition, it can also be applied to parasitic diseases, such as Chagas Disease and leishmaniasis (13, 14). This technique has several advantages for immunoassays, such as high throughput capacity, possibility of analyte quantification, reduced sample volume, high reproducibility and sensitivity (14, 15). More importantly, it allows the development of multiplex studies using recombinant antigens, and it can be used as a monitoring tool for cured patients, allowing a more sensitive detection of anti-*Leishmania* antibodies (16–18). Therefore, the aim of this study was to evaluate the performance and to verify the possible application of an alternative diagnostic method using flow cytometry to detect anti-*L. infantum* antibodies in HIV-infected patients.

METHODS

Serum Samples and Study Population

The study population was defined by the convenience of the sample size from two states from Northeastern Brazil (Pernambuco and Piaui). The sera used were from 18 VL-HIV coinfected (diagnosed by *Leishmania* positive bone marrow aspirate and rapid HIV test) and 18 VL negative-HIV positive patients as well as 18 healthy control individuals, with VL negative sera confirmed using conventional serological tests (rK39 rapid test and DAT). For the VL-HIV coinfected group, eight patients (five from Pernambuco and three from Piaui) had been more thoroughly investigated prior to this study during their clinical evaluation, with more detailed immunological records available (CD4 T cell count and viral load). All serum samples were collected in vacutainer tubes (BD Biosciences), processed by centrifugation (1,000 g, 10 min, room temperature), inactivated by heating (30 min at 56°C) and centrifuged at 4°C, 1,000 g for 5 min. After centrifugation, the supernatants were aliquoted and kept at −20°C until further use.

This study was approved by the Ethics Committees from the Federal University of Piauí (0116/2005) and from the Aggeu Magalhães Institute, Oswaldo Cruz Foundation (CAEE 51603115.7.0000.5190).

Conventional Tests for VL Diagnosis

Bone marrow (1 mL) aspirates were obtained for *Leishmania* detection and used to prepare smears by slide apposition. The slides were stained with a panoptic staining kit (Ranylab, Barbacena, Brazil) and were evaluated under a light microscope (100× objective). At least three bone marrow smears were evaluated for each patient and the process was performed according to Da Silva et al. (19). Rapid tests based on rK39 (IT LEISH) were purchased from Bio Rad Laboratories (Marnes-la-Coquette, France) and performed according to the manufacturer's instructions. The DAT was carried out according to the manufacturer's instructions (Royal Tropical Institute, Amsterdam, NL), with sera having dilution titers of 1:6,400 considered positive, as defined by El Harith et al. (20).

In-house Immunofluorescence Antibody Test

The IFAT test was performed with an in-house protocol, where 20 μl of a *L. infantum* promastigote antigenic suspension were applied to the delimited region of IFAT slides (PERFECTLAB, São Paulo, Brazil) and kept for 2 h at 37°C. The slides were then coated with 10 μl of the patients' serum, in titers ranging from 1:20 to 1:320, diluted in PBS, pH 7.2. Two control sera (positive and negative) were incubated in a humid chamber for 30 min at 37°C. After incubation, the slides were washed three times through immersion in PBS, in intervals of 10 min. Anti-human IgG conjugated to fluorescein isothiocyanate-FITC (Sigma Chemical Corp., St. Louis, MO) prepared in Evans blue (40 mg) in PBS (previously diluted at 1:10 ratio in the same buffer) was added to the slides in a 1:50 dilution, and incubated under the same conditions as mentioned before. The slides were then washed three times for 10 min in PBS and left at room temperature. The assembly was made with buffered glycerin pH 8.5 and the slides then observed under a fluorescence microscope, with a 100× objective. Sera were considered positive from the dilution 1:40.

ELISA

The ELISA test was performed as described by Oliveira et al. (21), using 600 ng per well of crude *L. infantum* antigen assayed with the various sera diluted 1:900, followed by incubation with the peroxidase-conjugated anti-IgG (Calbiochem, EMD Millipore, Billerica, MA) diluted 1:2,000. After enzymatic detection with o-phenylenediamine (OPD) and H_2O_2, the reaction was quenched by adding 2M H_2SO_4 (50 μl/well) and the plates read at 490 nm (Spectra Max 190, Molecular Devices, Sunnyvale, USA or MRX II, Dynex Technologies, Chantilly, USA). Positive and negative controls were added to each 96-well plate to standardize the readings and variations. The cutoff point between non-reagent

and reagent samples was calculated as the mean of the negative controls plus two standard deviations.

Flow Cytometry

The flow cytometry assay was performed as originally described by Rocha et al. (22). Cultured *L. infantum* promastigotes (strain MHOM/BR/70/BH46) were harvested and washed three times in ice-cold PBS supplemented with 10% fetal bovine serum (FBS), prior to resuspension in 1% paraformaldehyde and incubation overnight. Following by a new wash and resuspension in PBS+ 10% FBS, the parasite suspension was incubated in 96-well, U-bottom plates (2.5×10^5/well) at 37°C for 30 min in the presence of different serum dilutions (1:64–1:8,192), followed by two washes with PBS-10% FBS. The parasites were then incubated at 37°C for 30 min protected from the light and in the presence of anti-human IgG conjugated to fluorescein isothiocyanate-FITC (Sigma Chemical Corp., St. Louis, MO) diluted 1:200 in PBS–10% FBS. After yet another wash, FITC labeled parasites were fixed with 200 μL of 1% paraformaldehyde and kept away from direct light for 30 min at 4°C until data acquisition on the flow cytometer (FACScalibur, Becton Dickinson), using the software "Cell Quest Pro," with 20.000 events per sample. Promastigotes were identified based on their specific frontal (FSC) and side (SSC) light scattering properties. After FSC and SSC gain adjustments, the parasites assumed a characteristic distribution with these parameters. The relative FITC fluorescence intensity of each event was analyzed with a single histogram representation. A delimitation was set on the FITC-conjugated internal control histogram and it was applied to all data analyses reported here in order to determine the percentage of positive fluorescent parasites (PPFP) for each sample (**Supplementary Figure 1**). The optimal serum dilution and PPFP cutoff point were then selected to gather the IgG reactivity data with the best performance indexes. The values obtained were plotted as the mean of the PPFP related to the inverse dilution of the evaluated sera. For each assay, in addition to the FITC-conjugated internal control, unlabeled controls in quadruplicates and negative (a pool of negative sera) and positive (a pool of positive sera) controls were included to validate the assay.

Statistics

For each test, the sensitivity was determined as the fraction of the confirmed VL-HIV coinfected sera that were reagent, and the specificity was calculated as the fraction of non-reagent sera (Healthy controls and HIV mono–infected groups) that were identified to be truly test negative. Statistical analyses were performed using a two-by-two contingency table with exact binomial 95% CIs using the OpenEpi Software (Version 2.3.1, Centers for Disease Control, Atlanta, GA, USA). The degree of agreement was determined by the kappa index, using the Landis and Koch interpretation criteria. A kappa-value of 0.60–0.80 represents a substantial agreement beyond chance and a kappa-value of >0.80 represents almost perfect agreement beyond chance (23). The graphs were generated by the GraphPad Prism version 7.0 (GraphPad Prism Inc., San Diego, CA).

RESULTS

Defining the Flow Cytometry Parameters for the Diagnosis of VL-HIV Coinfected Cases

To evaluate the use of flow cytometry serology to clearly differentiate between positive and negative VL samples from HIV co-infected individuals, a serum dilution curve was used to assess the IgG reactivity data from sera from VL-HIV coinfected patients in comparison with a VL-negative control group. The VL-HIV coinfected samples all consisted of true positive cases identified by a positive parasitological test for VL and positive HIV serology. The control group consisted of sera from healthy individuals, with no obvious signs and symptoms of any disease and living in the non-endemic regions for VL, as well as HIV mono-infected individuals, with positive serology for HIV and negative serology for VL. This negative VL serology was confirmed through three independent assays: an in-house Immunofluorescence Antibody Test (IFAT), a commercial Direct Agglutination Test (DAT) and ELISA using a crude *L. infantum* antigen preparation. **Figure 1** shows the mean values of the percentage of positive fluorescent parasites (PPFP) from VL-HIV coinfection and control groups vs. a sera dilution curve ranging from 1:64 to 1:8,192. The difference between the reactivity of positive and negative samples (Δ) showed that the best performance in segregating these groups was at the dilution of 1:2,048. Thus, we used this dilution to better define the optimal PPFP value for VL diagnosis.

Defining a Cutoff Point for the Diagnosis of VL-HIV Coinfected Cases

Next, we sought to define an ideal cutoff point for the flow-cytometry, which would be able to differentiate the IgG reactivity data with the best performance indexes. This was evaluated through a Receiver Operating Characteristic (ROC) curve, generated by plotting sensitivity on the y-axis and the complement of specificity (100—specificity) on the x-axis and thus able to discriminate negative from low positive and high positive PPFP results. The data analysis of the ROC curve demonstrated that the PPFP value of 1.26 was the most appropriated cutoff to distinguish negative (PPFP $\leq$ 1.26%) from positive (PPFP > 1.26%) results (**Figure 2**). The tests' global accuracy determined by the area under the ROC curve (AUC), which was calculated at 0.93 [95%, with a confidence interval (CI) between 0.85 and 1.0]. Using this approach, flow cytometry displayed 89% of sensitivity (CI 95% = 65–99%) and 83% of specificity (CI 95% = 67–94%). The mean PPFP values was 36% (CI 95% = 22–50%) for the VL-HIV coinfection group, 1.4% (CI 95% = 0.9–1.8%) for the healthy controls and 0.2% (CI 95% = 0.1–0.35%) for the mono-infected HIV group.

Comparative Analysis of Flow Cytometry and Conventional Serological Tests for VL-HIV Diagnosis

Aiming to evaluate the global performance of flow cytometry, we used the same serum panel with serological tests conventionally

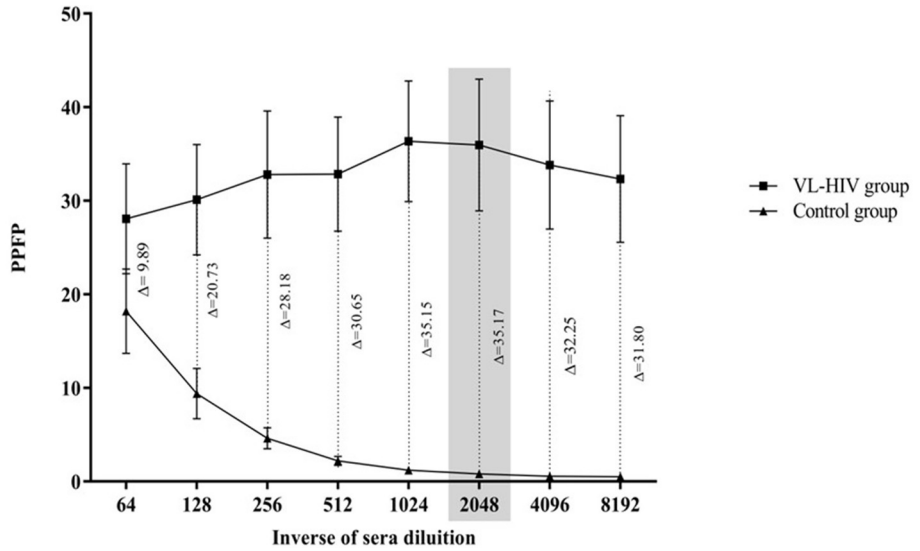

FIGURE 1 | IgG antibody titration curve of anti-fixed *Leishmania infantum* promastigotes defected by flow cytometry with sera from the two stratified groups assayed here. (|) VL-HIV group, $n = 18$, and (▲) control group, $n = 36$. The gray rectangle corresponds to the titration of 1:2,048 which was the region of greatest separation between groups. PPFP, Percentage of Positive Fluorescent Parasites. Δ = difference seen for the reacitivity between groups.

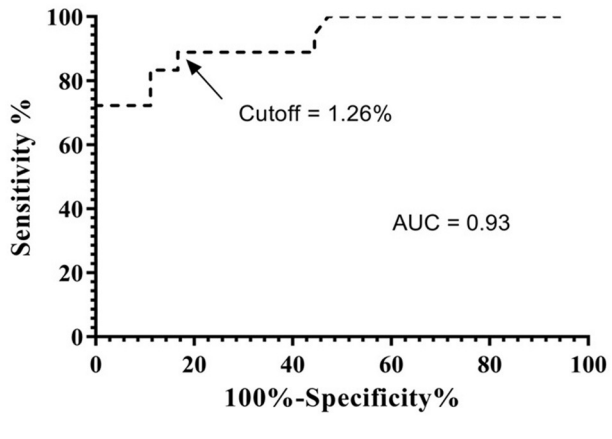

FIGURE 2 | ROC curve analysis of the performance indexes, sensitivity, and specificity of flow cytometry. The ROC curve was applied to confirm the best cutoff point which was able to discriminate PPFP values from positive and negetive samples and to indicate the area under the curve (AUC = global accuracy). ROC curve of the samples at dilution 1:2,048 indicating a cutoff point of 1.26.

used for the diagnosis of VL (DAT, rK39 rapid test, ELISA, and IFAT). Flow cytometry had the best values in terms of sensitivity and negative predictive value (89 and 94%, respectively), however, the other tests were more specific (100%) when compared to flow cytometry, which had a specificity of 83% (**Table 1**). When the different tests were individually compared to flow cytometry, we could identify a substantial agreement between DAT and ELISA tests ($\kappa > 0.6$; agreement $> 80\%$) and a moderate agreement between rK39 rapid test and IFAT ($\kappa < 0.6$; agreement < 80).

Performance of Flow Cytometry and Standard Serological Tests in Relation to the Immunological Status of Patients Co-Infected With VL-HIV

Eight of the VL-HIV coinfected sera were derived from patients whose immune statuses had been evaluated and the HIV viral load quantified (**Table 2**). We observed that flow cytometry was able to positively identify all of these sera, including those with a more severe immunossupression, with CD4+ T cell counts below 200 cells/mm^3, and even taking into account the very large variations in HIV viral load. Although the various conventional tests evaluated also gave positive results even in the patients with the most severe immunosuppressions, all the other tests had at least one negative result for the series of cases analyzed, with IFAT having the worst performance (three false negative results). However, no clear correlation between immunosuppression, viral load and positivity in the assays was observed for the any of these tests.

DISCUSSION

To our knowledge, this is the first study using the detection of antibodies anti-*L. infantum* by flow cytometry for VL diagnosis in HIV-infected individuals. Previous reports started by using this technique to evaluate IgG binding to live promastigotes to assay individuals with VL (24, 25) and with American tegumentary leishmaniasis (26). The used of fixed cells as an alternative was also investigated with both Chagas disease and tegumentary leishmaniasis (27, 28). In a previous study, we also

TABLE 1 | Values of sensitivity, specificity, positive and negative predictive values, and accuracy of the serological tests used for the diagnosis of VL-HIV coinfection ($N = 54$)*.

	Flow cytometry	DAT	rK39 rapid test	ELISA	IFAT
Sensitivity (95%CI)[a]	89% (67–97%)	83% (61–94%)	72% (49–87.5%)	72% (49–87.5%)	61% (39–80%)
Specificity (95%CI)	83% (68–92%)	100% (90–100%)	100% (90–100%)	100% (90–100%)	100% (90–100%)
PPV[b] (95%CI)	73% (52–87%)	100% (80–100%)	100% (80–100%)	100% (80–100%)	100% (74–100%)
NPV[c] (95%CI)	94% (80–98%)	92% (80–97%)	88% (74–95%)	88% (74–95%)	84% (70–92%)
Accuracy (95%CI)	85% (73–92%)	94% (85–98%)	91% (80–96%)	91% (80–96%)	87% (76–94%)

The samples included 18 VL-HIV coinfected patients, 18 VL negative-HIV positive patients and 18 healthy individuals (negative control).

[a] *CI, Confidence Interval.*

[b] *PPV, Positive Predictive Value.*

[c] *NPV, Negative Predictive Value.*

TABLE 2 | Laboratorial findings of eight cases of the VL-HIV/AIDS coinfected group.

Patient	Flow cytometry (%PPFP)	DAT (Titer)	rK39 rapid test	ELISA (Absorbance-490 nm)	IFAT (Titer)	T CD4+ (cells/mm^3)	Viral load (copies/mL)
1	Positive (1.37)	Positive (1:51,200)	Positive	Positive (0.64)	Negative	399	3,722
2	Positive (23.47)	Positive (1:24,600)	Positive	Positive (0.85)	Positive (1:160)	56	50,000
3	Positive (1.96)	Negative	Negative	Negative (0.02)	Negative	392	<50
4	Positive (37.91)	Positive (1:102,400)	Positive	Positive (3.5)	Positive (1:160)	2	<50
5	Positive (21.56)	Positive (1:51,200)	Positive	Positive (0.85)	Positive (1:160)	<50	54
6	Positive (59.76)	Positive (1:51,200)	Positive	Positive (0.65)	Positive (1:40)	157	45,795
7	Positive (4.89)	Positive (1:6,400)	Negative	Negative (0.04)	Negative	345	39,529
8	Positive (88.32)	Positive (1:51,200)	Positive	Positive (3.1)	Positive (1:320)	92	1,027

directly investigated the use of fixed promastigotes to assess IgG binding by flow cytometry for the diagnosis of individuals with VL, reaching a sensitivity of 92–96% in these individuals (15). In the present study, we found a good, but not ideal, sensitivity, although in relation to the conventional tests used for comparison, the sensitivity of flow cytometry was greater. As described here, the technique is even more relevant for the diagnosis of VL in cases of VL-HIV co-infections.

The rk39 rapid test and IFAT are the serological tests most used for the diagnosis of VL, but they show the lowest sensitivity (<60%) in VL-HIV coinfected individuals (6). Therefore, particularly for this group of patients, the VL diagnosis is a great challenge. Our results showed higher sensitivity levels for these tests than previously reported, but with a performance still inferior to flow cytometry and DAT.

Indeed, among the serological tests conventionally used for the VL diagnosis in coinfected individuals, DAT stands out as having the highest sensitivities in multiple studies: 89% (29), 81% (6), 82.3–89.7% (30), 91.3% (31), 89.5% (32), and 90% (33). This performance was also corroborated by our study. Both DAT and flow cytometry use serial dilutions that allow the identification of antibodies in low serum concentrations, even in immunosuppressive conditions (CD4+ T cells <200 cells/mm^3). Nevertheless, flow cytometer uses photomultiplier detectors and its quantitative assessment excludes the operator subjectivity which exists in DAT. In this context, flow cytometry shows the potential to be an alternative serological method for VL detection in HIV-infected patients, since a positive test, even at low titers, has diagnostic value when combined with the clinical case definition.

All serological tests, except for flow cytometry, had 100% specificity. This may have been overestimated in our study, since we used the rK39 rapid test and DAT for the original screening for the group of VL negative-HIV positive samples. In previous studies, specificities varying from 83.3 to 90% for DAT and 97.4 to 100% for rapid tests have been observed in VL-HIV coinfections (6, 31, 32). Ideally, it would be best to evaluate different control groups, such as individuals from endemic regions and with other confirmed pathologies, to have a more reliable specificity value. Despite the good sensitivity of our flow cytometry data, further investigations are needed in order to reduce the high incidence of false positive results seen here among healthy controls from non-endemic regions (**Supplementary Figure 2**). It is particularly important to investigate VL-related diseases that are co-endemic, since cross-reactivity with other trypanosomatid infections still represents an important issue regarding the applicability of flow cytometry (24, 34).

So far, it has been a challenge to find a more practical and safer antigen preparation which would allow greater sensitivity and specificity levels with low cross-reactivity. Improvements which include the use of fixed parasites and solutions that are able to preserve their morphology, such as formaldehyde, were strategies developed to facilitate the use of these parasites and to enable the development of diagnostic kits (35, 36). It is also

noteworthy that the development of algorithms which allow the elimination of cross-reactivity are important for the differential diagnosis of trypanosomatids (14), but the use of molecularly defined antigens seems to be the best option capable of addressing this limitation (16).

With the emergence of monoclonal antibodies and flow cytometry, it was possible to clarify the role of CD4+ T cells in HIV-AIDS. Indeed, CD4 quantitation is currently one of the most widespread tests performed in diagnostic centers for the prognosis and evaluation of anti-retroviral treatments in HIV-infected individuals (37). In our study, the immunological data were collected retrospectively from medical records, limiting the complete evaluation of all patients. Nevertheless, for those with the data available, flow cytometry was able to detect anti-*L. infantum* antibodies even in cases with low CD4+ T cell counts. It can thus be an additional tool to improve the evaluation of individuals in endemic regions for VL with lower CD4+ T counts. **In this context,** it would be interesting to take advantage of the operational and technical settings that flow cytometers use to quantify CD4 and apply them also for the detection of anti-*Leishmania* antibodies in countries which are endemic for VL, such as Brazil. This would imply adding an algorithm for the VL diagnosis in people with HIV from endemic areas, enabling a more sensitive diagnosis in cases with a prior negative VL result based on techniques such as DAT and rapid tests. As observed in a study carried out in Ethiopia, the need for a different algorithm for this population is evident due to the substantial reduction in the sensitivity of conventional techniques in HIV-infected individuals (38).

In conclusion, although it is a preliminary assessment, our results emphasize that flow cytometry can contribute to the correct identification of cases, especially in cases of immunosuppression, being a useful tool to characterize the humoral response to *Leishmania* in HIV-infected patients. Therefore, we encourage the evaluation of this technique in a larger number of samples and in other regions, such as those affected by *Leishmania donovani*.

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 Aggeu Magalhães Institute, Oswaldo Cruz Foundation, Recife, Pernambuco, Brazil. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ES, ZM, and VP conceived and designed the study. ES, BO, and AP drafted the manuscript. ES and DG analyzed the data. ES, BO, AP, DG, OM, CC, ZM, and VP critically revised the manuscript. All 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.2021.553280/full#supplementary-material

REFERENCES

1. WHO technical. Control of the leishmaniases: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases. *World Health Organ Tech Rep Ser*. (2010) xii–xiii:1–186, back cover.
2. Sakkas H, Gartzonika C, Levidiotou S. Laboratory diagnosis of human visceral leishmaniasis laboratory diagnosis of human visceral leishmaniasis. *J Vector Borne Dis.* (2016) 53:8–16.
3. Diro E, van Griensven J, Mohammed R, Colebunders R, Asefa M, Hailu A, et al. Atypical manifestations of visceral leishmaniasis in patients with HIV in north Ethiopia: a gap in guidelines for the management of opportunistic infections in resource poor settings. *Lancet Infect Dis.* (2015) 15:122–9. doi: 10.1016/S1473-3099(14)70833-3
4. González-Beato MJ, Moyano B, Sánchez C, González-Beato MT, Pérez-Molina JA, Miralles P, et al. Kaposi's sarcoma-like lesions and other nodules as cutaneous involvement in AIDS-related visceral leishmaniasis. *Br J Dermatol.* (2000) 143:1316–8. doi: 10.1046/j.1365-2133.2000.03909.x
5. Santos-Oliveira JR, Da-Cruz AM, Pires LHS, Cupolillo E, Kuhls K, Giacoia-Gripp CBW, et al. Atypical lesions as a sign of cutaneous dissemination of visceral leishmaniasis in a human immunodeficiency virus-positive patient simultaneously infected by two viscerotropic Leishmania species. *Am J Trop Med Hyg.* (2011) 85:55–9. doi: 10.4269/ajtmh.2011.10-0398
6. Cota GF, de Sousa MR, Demarqui FN, Rabello A. The diagnostic accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV infected patients: meta-analysis. *PLoS Negl Trop Dis.* (2012) 6:e1665. doi: 10.1371/journal.pntd.0001665
7. Lindoso JAL, Valente CH, Cunha MA, Queiroz IT. Visceral leishmaniasis and HIV coinfection: current perspectives. *HIV/AIDS Res Palliat Care.* (2018) 10:193–201. doi: 10.2147/HIV.S143929
8. Silva MRB, Brandão NAA, Colovati M, Sousa MMP, Lima LC, Dorta ML, et al. Performance of two immunochromatographic tests for diagnosis of visceral leishmaniasis in patients coinfected with HIV. *Parasitol. Res.* (2017) 117:419–427. doi: 10.1007/s00436-017-5716-3

9. Abass E, Bollig N, Reinhard K, Camara B, Mansour D, Visekruna A, et al. rKLO8, a novel *Leishmania donovani*—derived recombinant immunodominant protein for sensitive detection of visceral leishmaniasis in Sudan. *PLoS Negl Trop Dis.* (2013) 7:2322. doi: 10.1371/journal.pntd.0002322
10. Bhattacharyya T, Bowes DE, El-Safi S, Sundar S, Falconar AK, Singh OP, et al. Significantly lower anti-leishmania IgG responses in Sudanese versus Indian visceral leishmaniasis. *PLoS Negl Trop Dis.* (2014) 8:2675. doi: 10.1371/journal.pntd.0002675
11. Cabral-Marques O, Schimke LF, de Oliveira EB, El Khawanky N, Ramos RN, Al-Ramadi BK, et al. Flow cytometry contributions for the diagnosis and immunopathological characterization of primary immunodeficiency diseases with immune dysregulation. *Front Immunol.* (2019) 10:2742. doi: 10.3389/fimmu.2019.02742
12. Del Principe MI, De Bellis E, Gurnari C, Buzzati E, Savi A, Consalvo MAI, et al. Applications and efficiency of flow cytometry for leukemia diagnostics. *Expert Rev Mol Diagn.* (2019) 19:1089–97. doi: 10.1080/14737159.2019.1691918
13. Martins-Filho OA, Pereira ME, Carvalho JF, Cançado JR, Brener Z. Flow cytometry, a new approach to detect anti-live trypomastigote antibodies and monitor the efficacy of specific treatment in human Chagas' disease. *Clin Diagn Lab Immunol.* (1995) 2:569–73.
14. Teixeira-Carvalho A, Campos FMF, Geiger SM, Rocha RDR, de Araújo FF, Vitelli-Avelar DM, et al. FC-TRIPLEX Chagas/Leish IgG1: a multiplexed flow cytometry method for differential serological diagnosis of chagas disease and leishmaniasis. *PLoS ONE.* (2015) 10:e0122938. doi: 10.1371/journal.pone.0122938
15. Silva ED, Oliveira BC, Oliveira AP, Santos WJT, Diniz GT, de Melo Neto OP, et al. Performance evaluation of anti-fixed Leishmania infantum promastigotes immunoglobulin G (IgG) detected by flow cytometry as a diagnostic tool for visceral Leishmaniasis. *J Immunol Methods.* (2019) 469:18–25. doi: 10.1016/j.jim.2019.02.009
16. Sousa S, Cardoso L, Reed S, Reis AB, Martins-filho OA, Silvestre R, et al. Development of a fluorescent based immunosensor for the serodiagnosis of canine leishmaniasis combining immunomagnetic separation and flow cytometry. *PLoS Negl Trop Dis.* (2013) 7:2371. doi: 10.1371/journal.pntd.0002371
17. Ker HG, Coura-Vital W, Valadares DG, Aguiar-Soares RDO, de Brito RCF, Veras PST, et al. Multiplex flow cytometry serology to diagnosis of canine visceral leishmaniasis. *Appl Microbiol Biotechnol.* (2019) 103:8179–90. doi: 10.1007/s00253-019-10068-x
18. Mendes APO, Oliveira BC, Pereira AMS, Castro MCAB, Souza MA, et al. American tegumentary leishmaniasis diagnosis using L. (V.) braziliensis fixed promastigotes: A comparative performance of serological tests and spontaneous cure identification. *BMC Infect Dis.* (2019) 19:1–11. doi: 10.1186/s12879-019-4642-7
19. Da Silva MRB, Stewart JM, Costa CHN. Sensitivity of bone marrow aspirates in the diagnosis of visceral leishmaniasis. *Am J Trop Med Hyg.* (2005) 72:811–4. doi: 10.4269/ajtmh.2005.72.811
20. El Harith A, Kolk AHJ, Leewenburg J, Muigai R, Huigen E, Jelsma T, et al. Improvement of a direct agglutination test for field studies of visceral leishmaniasis. *J Clin Microbiol.* (1988) 26:1321–5.
21. Oliveira GGS, Magalhães FB, Teixeira MCA, Pereira AM, Pinheiro CGM, Santos LR, et al. Characterization of novel Leishmania infantum recombinant proteins encoded by genes from five families with distinct capacities for serodiagnosis of canine and human visceral leishmaniasis. *Am J Trop Med Hyg.* (2011) 85:1025–34. doi: 10.4269/ajtmh.2011.11-0102
22. Rocha RDR, Gontijo CMF, Elói-Santos SM, Carvalho AT, Corrôa-Oliveira R, Marques MJ, et al. Anticorpos antipromastigotas vivas de Leishmania (Viannia) braziliensis, detectados pela citometria de fluxo, para identifição da infecção ativa na leishmaniose tegumentar americana. *Rev Soc Bras Med Trop.* (2002) 35:551–62. doi: 10.1590/S0037-86822002000600002
23. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics.* (1977) 33:159–74.
24. Garcia LM, Coelho-Dos-Reis JGA, Peruhype-Magalhães V, Teixeira-Carvalho A, Rocha RDR, Araújo MSS, et al. Anti-fixed *Leishmania chagasi* promastigotes IgG antibodies detected by flow cytometry (FC-AFPA-IgG) as a tool for serodiagnosis and for post-therapeutic cure assessment in American visceral leishmaniasis. *J Immunol Methods.* (2009) 350:36–45. doi: 10.1016/j.jim.2009.07.004
25. Lemos EM, Gomes IT, Carvalho SFG, Rocha RDR, Pissinate JF, Martins-Filho OA, et al. Detection of anti-leishmania (Leishmania) chagasi immunoglobulin G by flow cytometry for cure assessment following chemotherapeutic treatment of American visceral leishmaniasis. *Clin Vaccine Immunol.* (2007) 14:569–76. doi: 10.1128/CVI.00354-06
26. Pereira de Oliveira A, Accioly Brelaz de Castro MC, Ferreirade Almeida A, De Assis Souza M, Coutinho de Oliveira B, Campos Reis L, et al. Comparison of flow cytometry and indirect immunofluorescence assay in the diagnosis and cure criterion after therapy of American tegumentary leishmaniasis by anti-live Leishmania (Viannia) braziliensis immunoglobulin G. *J Imunol Methods.* (2013) 387:245–53. doi: 10.1016/j.jim.2012.11.002
27. Matos CS, Coelho-dos-Reis JGA, Rassi A, Luquetti AO, Dias JCP, Eloi-Santos SM, et al. Applicability of an optimized non-conventional flow cytometry method to detect anti-*Trypanosoma cruzi* immunoglobulin G for the serological diagnosis and cure assessment following chemotherapeutic treatment of Chagas disease. *J Immunol Methods.* (2011) 369:22–32. doi: 10.1016/j.jim.2011.03.007
28. Pereira VRA, Reis LDC, Souza MDA, de Oliveira AP, de Brito MEF, Lage PS, et al. Evaluation of anti-lived and anti-fixed Leishmania (Viannia) braziliensis promastigote IgG antibodies detected by flow cytometry for diagnosis and post-therapeutic cure assessment in localized cutaneous leishmaniasis. *Diagn Microbiol Infect Dis.* (2012) 74:292–8. doi: 10.1016/j.diagmicrobio.2012.06.025
29. ter Horst R, Tefera T, Assefa G, Ebrahim AZ, Davidson RN, Ritmeijer K. Field evaluation of rK39 test and direct agglutination test for diagnosis of visceral leishmaniasis in a population with high prevalence of human immunodeficiency virus in Ethiopia. *Am J Trop Med Hyg.* (2009) 80:929–34. doi: 10.4269/ajtmh.2009.80.929
30. Cota GF, de Sousa MR, Fereguetti TO, Rabello A. Efficacy of anti-leishmania therapy in visceral leishmaniasis among HIV infected patients: a systematic review with indirect comparison. *PLoS Negl Trop Dis.* (2013) 7:e2195. doi: 10.1371/journal.pntd.0002195
31. Bangert M, Flores-Chávez MD, Llanes-Acevedo IP, Arcones C, Chicharro C, García E, et al. Validation of rK39 immunochromatographic test and direct agglutination test for the diagnosis of Mediterranean visceral leishmaniasis in Spain. *PLoS Negl Trop Dis.* (2018) 12:6277. doi: 10.1371/journal.pntd.0006277
32. Freire ML, Assis M, De Oliveira E, Moreira D, Avelar D. Siqueira IC, et al. Performance of serological tests available in Brazil for the diagnosis of human visceral leishmaniasis. *PLoS Negl Trop Dis.* (2019) 13:e0007484. doi: 10.1371/journal.pntd.0007484
33. Sanchez MCA, Celeste BJ, Lindoso JAL, Fujimori M, De Almeida RP, Fortaleza, et al. Performance of rK39-based immunochromatographic rapid diagnostic test for serodiagnosis of visceral leishmaniasis using whole blood, serum and oral fluid. *PLoS ONE.* (2020) 15:e0230610. doi: 10.1371/journal.pone.0230610
34. Pissinate JF, Gomes IT, Peruhype-Magalhães V, Dietze R, Martins-Filho OA, Lemos EM. Upgrading the flow-cytometric analysis of anti-Leishmania immunoglobulins for the diagnosis of American tegumentary leishmaniasis. *J Immunol Methods.* (2008) 336:193–202. doi: 10.1016/j.jim.2008.04.018
35. Ker HG, Coura-Vital W, Aguiar-Soares, RDDO, Roatt BM, das Dores Moreira N, et al. Evaluation of a prototype flow cytometry test for serodiagnosis of canine visceral leishmaniasis. *Clin Vaccine Immunol.* (2013) 20:1792–8. doi: 10.1128/CVI.00575-13
36. Vitelli-Avelar DM, Sathler-Avelar R, Wendling APB, Rocha RDR, Teixeira-Carvalho A, Martins NÉ, et al. Non-conventional flow cytometry approaches to detect anti-*Trypanosoma cruzi* immunoglobulin G in the clinical laboratory. *J Immunol Methods.* (2007) 318:102–12. doi: 10.1016/j.jim.2006.10.009
37. Kagan JM, Sanchez AM, Landay A, Denny TN, Diseases I, Services H. A brief chronicle of CD4 as a biomarker for HIV/AIDS: a tribute to the memory of John L. Fahey Jonathan. *HHS Public Access.* (2015) 6:55–64. doi: 10.1615/ForumImmunDisTher.2016014169.A
38. Kassa M, Abdellati S, Cnops L, Bremer Hinckel BC, Yeshanew A, Hailemichael W, et al. Diagnostic accuracy of direct agglutination test, rk39 elisa

and six rapid diagnostic tests among visceral leishmaniasis patients with and without hiv coinfection in ethiopia. *PLoS Negl Trop Dis.* (2020) 14:8963. doi: 10.1371/journal.pntd.0008963

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