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Current research on serological analyses of infectious diseases

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Published in
Frontiers in Public Health
Frontiers in Medicine

ISSN 1664-8714
ISBN 978-2-83251-813-7
DOI 10.3389/978-2-83251-813-7

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Current research on serological analyses of infectious diseases

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Citation

Sepulveda, N., Giorgi, E., Tetteh, K. K. A., Rogier, E. W., eds. (2023). *Current research on serological analyses of infectious diseases*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-813-7

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frontiers | Frontiers in Medicine

TYPE Editorial
PUBLISHED 17 February 2023
DOI 10.3389/fmed.2023.1154584

OPEN ACCESS

EDITED AND REVIEWED BY
Shisan Bao,
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SPECIALTY SECTION
This article was submitted to Infectious Diseases: Pathogenesis and Therapy, a section of the journal Frontiers in Medicine

RECEIVED 30 January 2023
ACCEPTED 07 February 2023
PUBLISHED 17 February 2023

CITATION
Rogier EW, Giorgi E, Tetteh K and Sepúlveda N (2023) Editorial: Current research on serological analyses of infectious diseases. *Front. Med.* 10:1154584. doi: 10.3389/fmed.2023.1154584

Editorial: Current research on serological analyses of infectious diseases

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KEYWORDS

microarray, immunochromatography test, enzyme-linked immunosorbent assay (ELISA), multiplex bead array, neglected diseases, tropical diseases, SARS-CoV-2, disease diagnosis and analysis

Editorial on the Research Topic
Current research on serological analyses of infectious diseases

Introduction

Serology based on antibody detection or quantification is a key research tool in the analysis of human infectious diseases. In Public Health and Epidemiology, it allows the estimation of the disease burden beyond the classical measures based on the presence or frequency of active infections in the population (1, 2). It also allows the prediction of when individuals were previously infected for tailoring novel disease control strategies (3, 4). In Medicine, it can assist in diagnosis (5), in the inference of disease etiology and pathology (6–8), and in the stratification of patients for better disease management and treatment (9). All these research opportunities motivated a discussion about the creation of a World Serum bank for infectious diseases (10–12).

Until recently, the enzyme-linked immunoassay (ELISA) and other related tests were at the core of the research made in infectious diseases. These tests typically detect or quantify antibodies against a single antigen. Nowadays high-throughput serological technologies, such as microarrays and multiplex bead assays, are becoming competing rivals of these standard tests due to the possibility of measuring multiple antibodies in the same biological sample at a reasonable cost. As such, these new technologies are giving rise to multiple system serology analyses (13–16).

In this Research Topic, we took the pulse of current research of infectious diseases based on serology with a special focus on applications in Medicine, Epidemiology, and Public Health. Among more than 20 submissions received, we were able to collect 9 original Research Topics (Figure 1A) and one systematic review (Figure 1B). These papers featured diverse infectious diseases, including neglected, re-emerging, tropical, established and novel. We were pleased to have at least one study investigating populations from different continents. This suggests that the findings of this Research Topic are likely to benefit multiple human populations globally. Below we provide a brief context of the published studies.

Serological studies using standard antibody assays

Notwithstanding the technological advances in serology, standard antibody assays seem the only viable option for investigating many diseases or populations from low- and middle-income countries (LMIC). This idea is illustrated in three independent studies included in the Research Topic. In one study, Qin et al. used serum agglutination tests to estimate the seroprevalence of animal plague and brucellosis in the neglected populations of Qinghai-Tibet plateau; these two neglected diseases are re-emerging in the area and, therefore, the reported findings have the potential to support more effective disease control interventions in the affected populations. In another study, Fernández Villaslobo et al. estimated the seroprevalence of Hepatitis E virus in children and adolescents in Colombia's capital city of Bogotá using commercial ELISA. Several study limitations were identified by the authors (e.g., massive school closure due to COVID-19 pandemic), but the findings suggested a low seroprevalence of IgG antibodies (~1%) and a single case of active infection (detected by IgM reactivity) among the study participants. It would be interesting to know whether these findings hold true if antibodies against multiple antigens were measured in the study. In a third study, Maamor et al. summarized data of 25 reports on the seroprevalence of Hepatitis B virus among health workers. These reports were restricted to African and Asian LMIC (Figure 1B) and, therefore, it is no surprise that the respective data were based on ELISA or rapid antibody tests whose performance can be affected by transport and storage conditions among other factors.

In the emergency of containing a disease outbreak, it is desirable to conduct a large-scale epidemiological study in real time. In this scenario, classical serological assays are invaluable tools in the field due to their low cost, the use of relatively simple technology, and the easiness of protocol's standardization across participating labs. The valuable use of these assays was illustrated in the peak of the COVID-19 pandemic with the fast execution of studies using ELISA protocols or rapid immunochromatography tests (17–19). In this scenario, it is fundamental to know the performance of available assays or tests in the field, as done in the United Kingdom and Denmark (20, 21). With a similar purpose of these benchmark studies, Girl et al. evaluated the performance of 2 lateral flow assays and 2 surrogate ELISA tests to detect SARS-CoV-2-neutralizing antibodies using data from more than 300 German individuals. Notwithstanding the current dampening of the COVID-19 threat in many parts of the world, this and similar studies provide a solid basis of evidence for facing future resurgences of SARS-CoV-2 in the respective populations.

Serological studies using high-throughput antibody assays

High-throughput antibody technologies are becoming more popular among the scientific community. Our Research Topic captures somehow this increasing popularity given that more than half of the published studies featured the use of these avant-garde assays in different epidemiological contexts. Similar to a nationwide seroprevalence study from Spain (22), Willeit et al. illustrated the benefits of using a commercial high-throughput and automated immunoassay to screen rapidly antibodies against the spike protein of SARS-CoV-2 B1.351 variant in almost 2,500 individuals from an Austrian district. In Sepúlveda et al., more than 3,000 different antibody responses to the common Epstein-Barr virus were screened in German patients with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) and healthy controls using a seroarray. The rationale for conducting a massive antibody screening lies on the fact that this complex and neglected disease remains without a diagnostic biomarker. This study highlighted two candidate antibody targets (EBNA4_0529 and EBNA6_0070) that could not only help in diagnosing a large subset of ME/CFS patients, but also further support the autoimmune hypothesis for the pathogenesis of this disease (23). In the realm of tropical diseases, Rogier et al. conducted a detailed

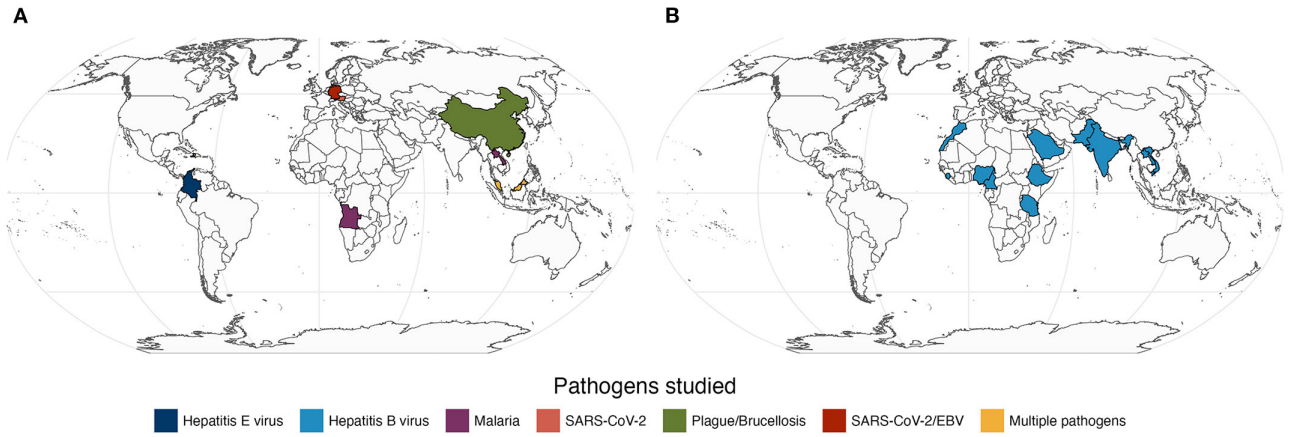

FIGURE 1
Map of the study populations and the respective pathogens featured in 9 original research papers **(A)** and in one systematic review **(B)** related to seroprevalence of the Hepatitis B virus among health workers (Maamor et al.). Note that Germany contributed with two independent papers (Girl et al. and Sepúlveda et al.) in plot A.

analysis of multiple antibody responses to the malaria-causing *Plasmodium falciparum* in Angolan children using a multiplex bead assay. The respective findings provide additional evidence for the current understanding of malaria immunity. These and other findings from the literature set the foundation to innovate on malaria vaccine development and immune-related treatments. In another study of Malaria, Byrne et al. reported data from more than 5,000 individuals from the Lao People's Democratic Republic using a multiplex bead assay. This study illustrates how multiplex data can be used to construct risk maps that could detect important foci of infection for close surveillance and future interventions.

The most impressive applications of multiplex serological assays in this Research Topic are related to two studies where data allowed to investigate multiple diseases at the same time; other examples of studies based on similar ideas can be found elsewhere (24–26). In Chan et al., the study was performed in the Malaysian province of Sabah and contemplated the sampling of more than 10,000 individuals. The data comprised antibodies against twelve antigens related to 6 neglected tropical diseases, including lymphatic filariasis, yaws, and trachoma. In another study, Chan et al. extended the number of measured antibodies to investigate the epidemiology of 11 pathogens in Haiti. This study integrates a series of research efforts to support malaria elimination in the country (27–31). This study showed the benefit of using multiplex data to screen secondary diseases in a single timepoint, thus, avoiding the negative effects (e.g., increasing cost and participation fatigue) of conducting multiple sampling in the same population.

Conclusion

This Research Topic provides an interesting contrast between studies using standard serological tools and those using more advanced technology. In our perspective, this contrast is particularly important to judge the pros and cons of using one or another technology across different research contexts. Above all, the availability of more advanced serological technology is a great opportunity to foster collaboration among researchers and to enhance capacity building (e.g., lab automation and data analysis skills) where it is needed the most.

In summary, this Research Topic shows the increasing popularity of high-throughput serological data and how these can be useful in the support of public health and epidemiological interventions (e.g., creating of multiple risk maps based on different antibodies to identify the key foci of infection). It also shows how the same type of data can expand our current knowledge on the pathogenesis and diagnosis not only of infectious disease but also of diseases with unknown etiology, as illustrated for ME/CFS.

Author contributions

NS drafted the editorial. All authors revised and approved the final version of the editorial.

Funding

NS received funding from the Fundação para a Ciência e Tecnologia, Portugal (ref. UIDB/00006/2020), and the Polish National Agency for Academic Exchange, Poland (ref. PPN/ULM/2020/1/00069/U/00001) during the editorial work related to this Research Topic.

Acknowledgments

We would like to thank all the authors, reviewers, and study participants who contributed one way or another to this Research Topic. We also thank João Malato for helping with the design of Figure 1.

Conflict of interest

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

Publisher's note

References

1. Wiegand RE, Deng Y, Deng X, Lee A, Meyer WA, Letovsky S, et al. Estimated SARS-CoV-2 antibody seroprevalence trends and relationship to reported case prevalence from a repeated, cross-sectional study in the 50 states and the District of Columbia, United States-October 25, 2020-February 26, 2022. *Lancet Reg Heal Am.* (2023) 18:104. doi: 10.1016/j.lana.2022.100403

2. Corran P, Coleman P, Riley E, Drakeley C. Serology: a robust indicator of malaria transmission intensity? *Trends Parasitol.* (2007) 23:575–82. doi: 10.1016/j.pt.2007.08.023

3. Longley RJ, White MT, Takashima E, Brewster J, Morita M, Harbers M, et al. Development and validation of serological markers for detecting recent Plasmodium vivax infection. *Nat Med.* (2020) 26:741–9. doi: 10.1038/s41591-020-0841-4

4. Helb DA, Tetteh KKA, Felgner PL, Skinner J, Hubbard A, Arinaitwe E, et al. Novel serologic biomarkers provide accurate estimates of recent Plasmodium falciparum exposure for individuals and communities. *Proc Natl Acad Sci U S A.* (2015) 112:E4438–47. doi: 10.1073/pnas.1501705112

5. Li Z, Yi Y, Luo X, Xiong N, Liu Y, Li S, et al. Development and clinical application of a rapid IgM-IgG combined antibody test for SARS-CoV-2 infection diagnosis. *J Med Virol.* (2020) 92:1518–24. doi: 10.1002/jmv.25727

6. Bjornevik K, Cortese M, Healy BC, Kuhle J, Mina MJ, Leng Y, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science.* (2022) 375:296–301. doi: 10.1126/science.abj8222

7. Loebel M, Eckey M, Sotzny F, Hahn E, Bauer S, Grabowski P, et al. Serological profiling of the EBV immune response in Chronic Fatigue Syndrome using a peptide microarray. *PLoS ONE.* (2017) 12:e0179124. doi: 10.1371/journal.pone.0179124

8. Ruprecht K, Wunderlich B, Gieß R, Meyer P, Loebel M, Lenz K, et al. Multiple sclerosis: the elevated antibody response to Epstein-Barr virus primarily targets, but is not confined to, the glycine-alanine repeat of Epstein-Barr nuclear antigen-1. *J Neuroimmunol.* (2014) 272:56–61. doi: 10.1016/j.jneuroim.2014.04.005

9. Domingues TD, Grabowska AD, Lee JS, Ameijeiras-Alonso J, Westermeier F, Scheibenbogen C, et al. Herpesviruses Serology Distinguishes Different Subgroups of Patients From the United Kingdom Myalgic Encephalomyelitis/Chronic Fatigue Syndrome Biobank. *Front Med.* (2021) 8:686736. doi: 10.3389/fmed.2021.686736

10. Metcalf CJE, Farrar J, Cutts FT, Basta NE, Graham AL, Lessler J, et al. Use of serological surveys to generate key insights into the changing global landscape of infectious disease. *Lancet.* (2016) 388:728–30. doi: 10.1016/S0140-6736(16)30164-7

11. de Lusignan S, Correa A. Opportunities and challenges of a World Serum Bank. *Lancet.* (2017) 389:250–1. doi: 10.1016/S0140-6736(17)30046-6

12. Coates KM. Opportunities and challenges of a World Serum Bank. *Lancet.* (2017) 389:251–2. doi: 10.1016/S0140-6736(17)30052-1

13. Wine Y, Horton AP, Ippolito GC, Georgiou G. Serology in the 21st Century: The Molecular-Level Analysis of the Serum Antibody Repertoire. *Curr Opin Immunol.* (2015) 35:89. doi: 10.1016/j.coi.2015.06.009

14. Pittala S, Morrison KS, Ackerman ME. Systems Serology for Decoding Infection and Vaccine-Induced Antibody Responses to HIV-1. *Curr Opin HIV AIDS.* (2019) 14:253. doi: 10.1097/COH.0000000000000558

15. Selva KJ, van de Sandt CE, Lemke MM, Lee CY, Shoffner SK, Chua BY, et al. Systems serology detects functionally distinct coronavirus antibody features in children and elderly. *Nat Commun.* (2021) 12:1–14. doi: 10.1038/s41467-021-22236-7

16. Wang EY, Mao T, Klein J, Dai Y, Huck JD, Jaycox JR, et al. Diverse functional autoantibodies in patients with COVID-19. *Nature.* (2021) 595:283–8. doi: 10.1101/2020.12.10.20247205

17. Gonçalves J, Sousa RL, Jacinto MJ, Silva DA, Paula F, Sousa R, et al. Evaluating SARS-CoV-2 Seroconversion Following Relieve of Confinement Measures. *Front Med.* (2020) 7:971. doi: 10.3389/fmed.2020.603996

18. Stringhini S, Wisniak A, Piumatti G, Azman AS, Lauer SA, Baysson H, et al. Seroprevalence of anti-SARS-CoV-2 IgG antibodies in Geneva, Switzerland (SEROCoV-POP): a population-based study. *Lancet.* (2020) 396:313–9. doi: 10.1016/S0140-6736(20)31304-0

19. Khalagi K, Gharibzadeh S, Khalili D, Mansournia MA, Mirab Samiee S, Aghamohamadi S, et al. Prevalence of COVID-19 in Iran: results of the first survey of the Iranian COVID-19 Serological Surveillance programme. *Clin Microbiol Infect.* (2021) 27:1666–71. doi: 10.1016/j.cmi.2021.06.002

20. Ainsworth M, Andersson M, Auckland K, Baillie JK, Barnes E, Beer S, et al. Performance characteristics of five immunoassays for SARS-CoV-2: a head-to-head benchmark comparison. *Lancet Infect Dis.* (2020) 20:1390–400. doi: 10.1016/S1473-3099(20)30634-4-4

21. Harritshøj LH, Gybel-Brask M, Afzal S, Kamstrup PR, Jørgensen CS, Thomsen MK, et al. Comparison of 16 serological SARS-CoV-2 immunoassays in 16 clinical laboratories. *J Clin Microbiol.* (2021) 59:2596–616. doi: 10.1128/JCM.02596-20

22. Pollán M, Pérez-Gómez B, Pastor-Barriuso R, Oteo J, Hernán MA, Pérez-Olmeda M, et al. Prevalence of SARS-CoV-2 in Spain (ENE-COVID): a nationwide, population-based seroepidemiological study. *Lancet.* (2020) 396:535–44. doi: 10.1016/S0140-6736(20)32266-2

23. Sotzny F, Blanco J, Capelli E, Castro-Marrero J, Steiner S, Murovska M, et al. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome – Evidence for an autoimmune disease. *Autoimmun Rev.* (2018) 17:601–9. doi: 10.1016/j.autrev.2018.01.009

24. O'Neal AJ, Glass KA, Emig CJ, Vitug AA, Henry SJ, Shungu DC, et al. Survey of Anti-Pathogen Antibody Levels in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. *Proteomes.* (2022) 10:21. doi: 10.3390/proteomes10020021

25. Brenner N, Mentzer AJ, Butt J, Braband KL, Michel A, Jeffery K, et al. Validation of Multiplex Serology for human hepatitis viruses B and C, human T-lymphotropic virus 1 and Toxoplasma gondii. *PLoS ONE.* (2019) 14:407. doi: 10.1371/journal.pone.0210407

26. Mentzer AJ, Brenner N, Allen N, Littlejohns TJ, Chong AY, Cortes A, et al. Identification of host-pathogen-disease relationships using a scalable multiplex serology platform in UK Biobank. *Nat Commun.* (2022) 13:1818. doi: 10.1038/s41467-022-29307-3

27. van den Hoogen LL, Présumé J, Romilus I, Mondélus G, Elismé T, Sepúlveda N, et al. Quality control of multiplex antibody detection in samples from large-scale surveys: the example of malaria in Haiti. *Sci Rep.* (2020) 10:1–10. doi: 10.1038/s41598-020-57876-0

28. Rogier E, Van Den Hoogen L, Herman C, Gurrala K, Joseph V, Stresman G, et al. High-throughput malaria serosurveillance using a one-step multiplex bead assay. *Malar J.* (2019) 18:3027. doi: 10.1186/s12936-019-3027-0

29. van den Hoogen LL, Stresman G, Présumé J, Romilus I, Mondélus G, Elismé T, et al. Selection of Antibody Responses Associated With Plasmodium falciparum Infections in the Context of Malaria Elimination. *Front Immunol.* (2020) 11:928. doi: 10.3389/fimmu.2020.00928

30. Druetz T, Stresman G, Ashton RA, Joseph V, Van Den Hoogen L, Worges M, et al. The Immediate Effects of a Combined Mass Drug Administration and Indoor Residual Spraying Campaign to Accelerate Progress Toward Malaria Elimination in Grande-Anse, Haiti. *J Infect Dis.* (2022) 225:1611–20. doi: 10.1093/infdis/jiab259

31. Jaramillo-Underwood A, Impoinvil D, Sutcliff A, Hamre KES, Joseph V, Hoogen L van den, et al. Factors Associated With Human IgG Antibody Response to Anopheles albimanus Salivary Gland Extract, Artibonite Department, Haiti, 2017. *J Infect Dis.* (2022) 226:1461–9. doi: 10.1093/infdis/jiac245

frontiers in Medicine

ORIGINAL RESEARCH
published: 04 February 2022
doi: 10.3389/fmed.2022.820151

Evaluation of Two Rapid Lateral Flow Tests and Two Surrogate ELISAs for the Detection of SARS-CoV-2 Specific Neutralizing Antibodies

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As vaccination against SARS-CoV-2 progresses rapidly around the world, reliable detection of SARS-CoV-2 specific neutralizing antibodies (NAb) has become an indispensable component of serological diagnostics. We evaluated the performance of four commercially available tests, i.e. two lateral flow assays (Coris BioConcept COVID-19 Sero NP/RBD and Concile InfectCheck COVID-19 NAb) and two surrogate ELISA (sELISA) tests (EUROIMMUN SARS-CoV-2 NeutraLISA and AdipoGen SARS-CoV-2 Neutralizing Antibodies Detection Kit) in comparison with an in-house SARS-CoV-2 micro neutralization test as reference. A total of 334 sera were tested, including 30 samples collected prior to the emergence of SARS-CoV-2, 128 sera from convalescent patients as well as 176 sera from partially or fully vaccinated individuals. The overall sensitivity of LFAs differed and was 71.6% for the Coris and 98.4% for the Concile. In contrast, overall sensitivity of the NeutraLISA was 86 and 98% for the AdipoGen. All test showed the highest sensitivity when testing samples from fully vaccinated individuals with both sELISA achieving 100% sensitivity. Overall specificity was 89.3% for the Coris and only 58.3% for the Concile. Similarly, significant differences were observed for both sELISA, with an overall specificity of 82.1% for the NeutraLISA and only 54.8% for the AdipoGen. All tests showed a 100% specificity when testing negative control samples while specificities were lowest when testing samples from only partially vaccinated individuals.

Keywords: COVID-19, SARS-CoV-2, neutralizing antibodies, ELISA, lateral flow assay

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *22 November 2021*
Accepted: *03 January 2022*
Published: *04 February 2022*

Citation:
Girl P, Zwirglmaier K, von Buttlar H, Wölfel R and Müller K (2022) Evaluation of Two Rapid Lateral Flow Tests and Two Surrogate ELISAs for the Detection of SARS-CoV-2 Specific Neutralizing Antibodies. Front. Med. 9:820151. doi: 10.3389/fmed.2022.820151

INTRODUCTION

Serological testing has become a useful tool in the fight against the SARS-CoV-2 pandemic. Among others, it can be used to estimate the prevalence and incidence in a given population (1, 2). Countless serological tests have been commercialized by now, including enzyme-linked immunosorbent assays (ELISAs) as well as point-of-care lateral flow assays (LFAs). These tests typically measure SARS-CoV-2 specific antibodies, often even specific immunoglobulin subclasses (i.e. IgG, IgM or IgA). In this context, neutralizing antibodies (NAbs) have emerged as a strong correlate of protection and are therefore being used to evaluate vaccine efficacy or select appropriate convalescent plasma for therapeutic use (3). However, they represent only a small subset of the total polyclonal immune response.

The main target of NAbs is the spike (S) protein, more precisely the Receptor Binding Domain (RBD) located at the outer end of the S1 subunit. This is responsible for the binding of SARS-CoV-2 to the Angiotensin-Converting Enzyme-2 (ACE2) receptor on the surface of the cell (4). Thus, it is not surprising that a recent study found that at least 90% of SARS-CoV-2 NAbs are RBD specific. However, not all RBD-specific antibodies are able to neutralize the virus (5). Thus, to efficiently assess the level of protection from (re-)infection rather than just demonstrate a prior encounter with SARS-CoV-2, serological tests need to be able to differentiate between neutralizing and non-neutralizing antibodies.

Plaque-reduction (PRNT) and neutralization (NT) tests are the current gold standards for the detection and quantification of NAbs (6). However, these assays are labor-intensive and require appropriate biocontainment laboratories (BSL-3) because they depend on working with infectious virus. The number of laboratories that can perform these tests is therefore limited (7). Previously described pseudo virus-based techniques can be performed under BSL-2 conditions, but also require the cultivation of reporter virus and therefore do not provide a significant time advantage (8, 9). A potential alternative to classic neutralization assays comes in the form of surrogate ELISA (sELISA). They rely on the ability of NAbs to bind RBD and prevent its interaction with ACE2. sELISAs use recombinant proteins and colorimetric analysis to determine this competitive inhibition and several versions of this type of assay have been commercialized (10). In addition, LFAs have been developed and are particularly appealing as point-of-care (POC) tests due to their rapid turnaround times and simplicity of use (11). In contrast to sELISAs, they are designed as sandwich assays to detect RBD-specific antibodies with neutralizing capability.

In this study, we investigated four commercially available tests for the detection of SARS-CoV-2 specific NAbs: two surrogate sELISAs (Euroimmun SARS-CoV-2 NeutraLISA and AdipoGen SARS-CoV-2 Neutralizing Antibodies Detection Kit) and two LFAs (Coris BioConcept COVID-19 Sero NP/RBD and Concile InfectCheck COVID-19 NAb). According to the manufacturers, all four tests are suitable for the detection of antibodies with neutralizing capabilities. However, the conceptual design differs between both types of tests. Both sELISAs are very similar in design and imitate the interaction of RBD and ACE2 to determine the inhibitory effect of neutralizing antibodies capable of interrupting this interaction. Based on the information provided by the manufacturers both LFAs simply detect RBD-specific antibodies without assessing functionality.

We tested all four assays side-by-side and in direct comparison with our in-house NT to determine both sensitivity and specificity and to test their potential as an alternative to conventional NT. While several studies have attempted to compare SARS-CoV-2 immunoassays in the past (12–14), our study focuses on immunoassays designed to detect antibodies with neutralizing function rather than antibodies in general. Furthermore, our study population differs from previous studies in that we specifically included samples from vaccinated individuals in addition to convalescent individuals in order to also investigate the potential use of such assays in the context of vaccine evaluation as well as the evaluation of the humoral immune response post vaccination.

MATERIALS AND METHODS

Serum Samples

We tested a total of 334 human serum samples. Samples were selected by non-probability sampling (convenience/purposive sampling). Of these samples, 304 had previously tested positive for SARS-CoV-2 antibodies in a commercial IgG ELISA (Euroimmun, Lübeck, Germany), which was used as a pre-screening assay (**Supplementary Table 1**). Of these, 128 samples came from PCR-confirmed convalescent patients of which 78 were registered convalescent plasma donors; all samples were taken six weeks after full recovery according to the official guidelines provided by the German Federal Institute for Vaccines and Biomedicine (15). All samples were from patients, which were hospitalized for their SARS-CoV-2 infection, but did not require mechanical ventilation. Another 177 samples came from vaccinated individuals and were taken between four and six weeks after vaccination. Of these, 40 individuals had received their primary vaccination with Vaxzevria (AstraZeneca) while the other 136 individuals were fully vaccinated either with Comirnaty (Pfizer/BioNTech), Spikevax (Moderna) or a heterologous vaccination with Vaxzevria (AstraZeneca) as prime and Spikevax as boost. The remaining 30 serum samples were collected before the occurrence of SARS-CoV-2 (mid to late 2018) and served as negative control samples. An overview of all samples is given in **Figure 1**.

All samples evaluated were residual diagnostic material. Therefore, no specific information (e.g. demographic characteristics, individual disease progression etc.) could be assigned to individual samples or patients.

Ethical Statement

The study was carried out in-line with "The Code of Ethics of the World Medical Association (Declaration of Helsinki)". The use of serum samples complied with the guidelines of the Central Ethics Committee of the German Medical Association [Dtsch Arztebl 2003; 100(23): A-1632]. In accordance with these guidelines, the anonymized use of residual material from the samples sent to our laboratory for diagnostic purposes is permissible, provided that the patients have not decided against this procedure. Samples from patients who had decided against this procedure were excluded from the analyses.

Neutralization Test (NT)

SARS-CoV-2 NAb titers were determined as previously described (16). Briefly, serum samples (duplicates) were serially diluted in 96-well tissue culture plates starting at 5 to a maximum of 640 along with positive and negative control samples. SARS-CoV-2 stocks (50 TCID/50 µl, MUC-IMB-1 [EPI_ISL_406862]) were prepared on Vero E6 cells and aliquots were stored at −80°C until further use. Virus was pre-incubated (1 h, 37°C) with diluted serum samples before Vero E6 cells (1 x 10e4 cells/50 µl) were added to each well. After 72 h (37°C), supernatants were discarded and wells were fixed (13% formalin/PBS) and

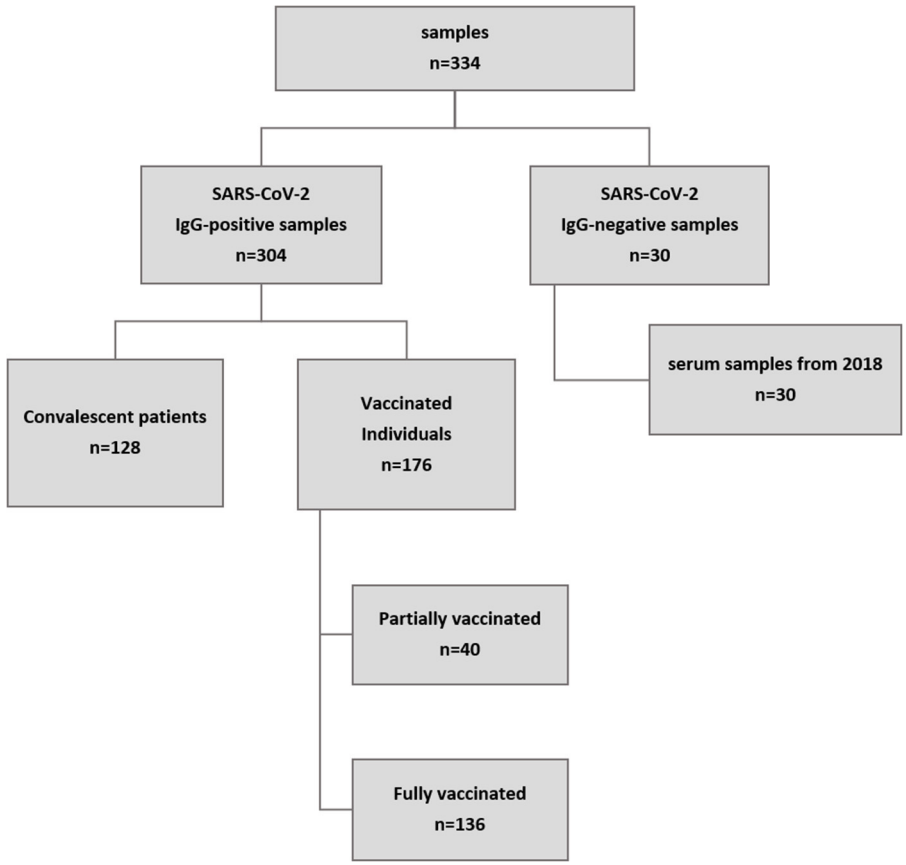

FIGURE 1 | Overview of sample origin. A total of 334 samples were tested. A commercial IgG ELISA (Euroimmun, Lübeck, Germany) was used for pre-screening. All but 30 samples were positive for SARS-CoV-2 IgG antibodies, of which 128 came from patients who had recently recovered from confirmed COVID-19 and 177 were from vaccinated individuals (collected at least three weeks after vaccination). In addition, 30 samples collected in mid to late 2018 served as negative control samples.

stained with crystal violet (0.1%). The NAb titer corresponded to the reciprocal of the highest sample dilution showing complete inhibition of cytopathic effects. A virus re-titration was performed in triplicates on every plate and exact titers were determined by retrograde calculation.

Surrogate ELISA (sELISA)

Two commercially available sELISA kits were tested in this study: The SARS-CoV-2 NeutraLISA (Euroimmun, Lübeck, Germany) and the SARS-CoV-2 Neutralizing Antibodies Detection Kit (AdipoGen Life Sciences, Liestal, Switzerland). Both tests function very similarly and are based on the competitive inhibition of the RBD-ACE2 interaction by NAbs. AdipoGen provides RBD-coated plates and uses HRP-conjugated ACE2 while Euroimmun uses the total S1 subunit of the S protein for coating and biotin-conjugated ACE2 (**Figures 2A,B**). Both tests were carried out strictly according to the manufacturer's instructions.

The NeutraLISA is the only test studied here that classifies results not only as positive or negative, but also as "indeterminate." We followed a conservative approach for the evaluation of the test results and evaluated indeterminate results as negative.

The sensitivity and specificity of the SARS-CoV-2 NeutraLISA were specified by the manufacturer as follows: Sensitivity = 95.9%; Specificity = 99.7%.

For the SARS-CoV-2 Neutralizing Antibodies Detection Kit (AdipoGen) no data on sensitivity and specificity was provided by the manufacturer.

Lateral Flow Assay (LFA)

Two commercially available LFAs were evaluated in this study: COVID-19 Sero NP/RBD test (Coris BioConcept, Belgium) and the InfectCheck COVID-19 NAb test (Concile GmbH Germany/Affimedix Inc. USA). Both are sandwich immuno assays. The Concile LFA uses colloidal gold particles that are conjugated with the RBD to bind to the NAb and mouse-anti-human IgG immobilized on the test line of the membrane to capture the NAb bound to the gold (**Figure 2C**). The Coris BioConcept detects not only RBD antibodies, but also antibodies against the nucleocapsid protein. Since all vaccines currently in use elicit an immune response against the spike protein, while an infection elicits an immune response against all components of SARS-CoV-2, the LFA can differentiate between immune response acquired through vaccination or infection. The test uses N protein and RBD immobilized on separate test lines to

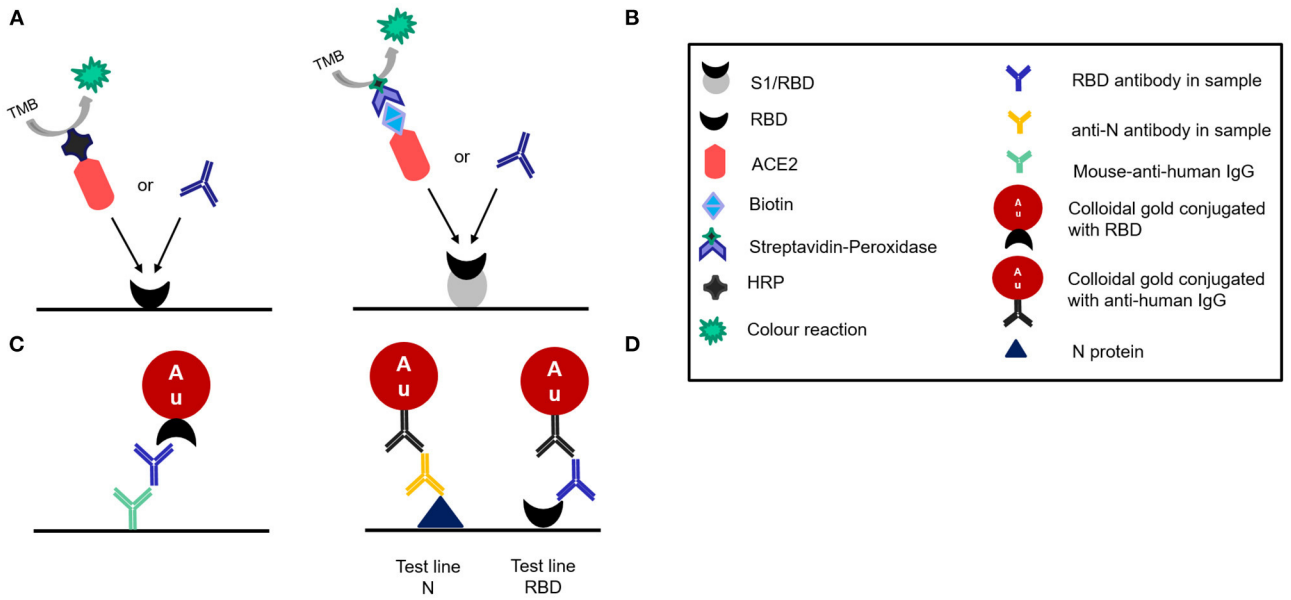

FIGURE 2 | Schematic illustration of the different designs of the four tests evaluated as disclosed by the manufacturers. **(A)** Adipogen:Competitive ELISA **(B)** Euroimmun. NeutraLISA:competitive ELISA **(C)** Affimedix TestNow:Lateral flow rapid test **(D)** Coris Bioconcept:Lateral flow rapid test.

capture the respective antibodies (**Figure 2D**). The other half of the immune sandwich consists of gold particles that are presumably (not detailed in the instructions for use) conjugated with anti-human IgG.

Both tests were carried out according to manufacturer's instructions. Briefly, for the COVID-19 Sero NP/RBD test 30 µl serum were applied to the test cassette followed by 4 drops of buffer 10 s later. Results were read out after 15 min and photographically documented within 15–20 min. Line intensities were evaluated visually by comparison to a visual analog color scale (**Supplementary Figure 1**). Intensities on the scale range from 0–9 and were recorded separately for test line and control line. Values of 2 or greater were scored as positive. A value of 1 corresponds to a very faint line that could not be reliably identified by different test operators and was therefore scored as negative. For the InfectCheck COVID-19 Nab test 5 µl serum were mixed with 5 drops of buffer and applied to the test cassette. Results were read out visually after 15 min as described above and photographically documented within 15–20 min. In addition to that, results were also measured with the concile α1 reader.

The sensitivity and specificity of the COVID-19 Sero NP/RBD (Coris) were specified by the manufacturer as follows: Sensitivity NP = 95.2%, Sensitivity RBD = 91.9%; Specificity NP= 98.5% Specificity RBD = 100.0%.

The sensitivity and specificity of the InfectCheck COVID-19 NAb test (Concile) were specified by the manufacturer as follows: Sensitivity = 100.0%; Specificity = 98.67%.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8 for Windows 64-bit [Version 8.4.3 (686)]. Sensitivity was defined as the ability of a test to identify the presence of SARS-CoV-2 specific NAb as determined by NT. Specificity was defined as the ability of a test to identify the absence of SARS-CoV-2 specific NAb as determined by NT.

RESULTS

Detection of SARS-CoV-2 Specific Neutralizing Antibodies by NT

Initially, all 334 samples were examined in our in-house NT. Serum samples with a titer ≥5 were considered positive as previously determined and described (17). In total, 84 samples tested negative for neutralizing antibodies while the remaining 250 tested positive. Among the samples that tested negative were all 30 negative control samples, 14 samples from convalescent patients and all serum sample from individuals who had only received their primary vaccination. Positive samples consisted of 114 sera from convalescent patients and 136 sera from fully vaccinated individuals. In summary, 100% of sera from fully vaccinated individuals and 89% of convalescent sera tested positive for NAbs while 100% of both negative control samples and samples from individuals that received their primary vaccination tested negative. An overview of the distribution of NT results by sample origin is given in **Table 1**.

Influence of Natural Infection and Immunization on Neutralizing Antibody Titers

The titer values determined by NT significantly differ between convalescent patients and fully vaccinated individuals. While the median titer of sera from convalescent patients was 37, it was 3.5 times higher (129) for sera from vaccinated individuals. **Figure 3**

TABLE 1 | Distribution of the results (positive/negative) from the in-house NT in relation to sample origin.

Sample origin	Number of samples	NT positive	NT negative
convalescent patients	128	114	14
partially vaccinated individuals	40	0	40
fully vaccinated individuals	136	136	0
negative control samples	30	0	30
Total	334	250	84

All (100%) of the NT positive samples came from fully vaccinated individuals and convalescent patients while the majority (83.3%) of NT negative samples came from only partially vaccinated individuals and negative control samples.

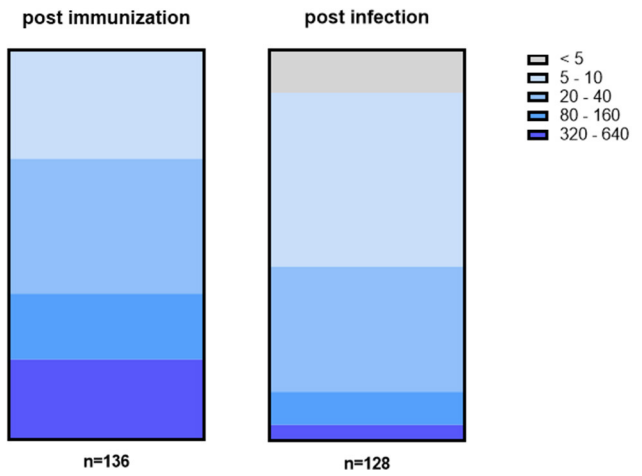

FIGURE 3 | Titer values among fully vaccinated (left) differ from titer values of recovered patients (right). In general, titers were higher among fully vaccinated individuals with 38% having a titer of at least 80 or higher including 21% with a titer of ≥320. In contrast, 45% of convalescent patients had a titer of 5 or 10 while in 11% no NAbs could be detected at all.

shows the distribution of titer values among fully vaccinated individuals (left) and recovered patients (right).

Among serum samples from vaccinated individuals, one-third (28%) have low titers of 5 to 10. At the same time, 34% show a slightly higher titer of either 20 or 40 while the remaining samples (38%) have a titer ≥80. In more detail, 17% of vaccinated individuals have a titer between 80 and 160 while another 21% reached the highest titer values of 320 or more. In contrast, only 4% of convalescent patients have titer values that high while another 8% have a titer between 80 and 160. Instead, the majority (45%) of convalescent patients have a titer of either 5 or 10 and another one third (32%) have a titer of 20 to 40. In addition, no NAbs at all could be detected in 11% of sera.

Sensitivity and Specificity of Commercial Immunoassays

In addition, all 334 samples were subsequently also analyzed using two sELISAs (AdipoGen and NeutraLISA) as well as two LFAs (Coris and Concile). Sensitivity and specificity were determined in direct comparison to the results of the NT. A detailed overview of the results of the individual immunoassays, including subgroup specific results, is shown in **Table 2**. In addition, a detailed overview of the distribution of inhibition values of both sELISAs within NT positive and NT negative samples as well as a two dimensional distribution of inhibition values is given in **Supplementary Figure 2**.

With regard to the entirety of samples examined all but five NT-positive samples were identified by the AdipoGen sELISA, resulting in the overall highest sensitivity of 98%. In comparison, of the 250 NT-positive samples, 215 samples tested positive in the NeutraLISA giving an overall sensitivity of 86%. However, of the 84 NT-negative samples, 38 tested positive in the AdipoGen whereas the NeutraLISA detected NAbs in 15 of them, giving an overall specificity of 54.8% (AdipoGen) and 82% (NeutraLISA) respectively.

Among vaccinated individuals both sELISAs detected NAbs in all 136 NT-positive samples resulting in a sensitivity and specificity of 100%. At the same time, of the 114 NT-positive samples from convalescent patients, the AdipoGen identified 109 samples as positive (95.6% sensitivity) while the NeutraLISA detected 79 positive samples (69.3% sensitivity). In addition, of the 14 NT-negative samples from convalescent patients the AdipoGen identified more than half ($n = 8$) as positive (42.9% specificity) while NeutraLISA detected no NAbs in all 14 samples (100% specificity). Of the 30 negative control samples, all were identified as negative by both sELISAs resulting in 100% specificity each.

With regard to both LFAs tested, sensitivity and specificity also varied between tests. The Concile identified all but four NT positive samples resulting in the highest overall sensitivity (98.4%) of all four tests evaluated. At the same time, the Coris identified significantly less NT positive samples, resulting in an overall sensitivity of 71.6%. Similar results were obtained among samples from convalescent and fully vaccinated individuals with the Concile showing continuously high sensitivities (97.4–99.3%). In contrast, the Coris showed the lowest sensitivities among all four tests (65.8–76.5%). Overall, all tests achieved their highest sensitivity with samples from fully vaccinated individuals while sensitivities were lowest when testing samples from convalescent patients. In terms of specificity, the Concile identified 35 of 84 negative samples as positive resulting in an overall specificity of 58.3%. Similar results were obtained among samples from convalescent (42.9%) and partially vaccinated (32.5%) individuals. In contrast, a specificity of 100% was achieved when testing negative control samples. At the same time, of the 84 negative samples the Coris identified all but nine samples as negative, giving an overall specificity of 89.3%. Similar results were obtained for samples from convalescent patients (85.7%), partially vaccinated individuals (82.5%) and control samples (100%).

The percentage of positive results of all four tests in relation to the results obtained by NT is shown in **Figure 4**. While both the Coris (light green) and the NeutraLISA (light blue) have moderately low levels of false positive test results for NT-negative samples (i.e. 10.7 and 17.8%), the Concile and AdipoGen both have similarly high numbers of false positive among NT-negative samples (i.e. 41.7 and 45.2%). However,

TABLE 2 | Detailed overview of overall sensitivity and specificity of the four evaluated commercial immunoassays in comparison to NT results.

			sELISA		LFA	
NT			**AdipoGen**	**NeutraLISA**	**Coris**	**Concile**
Sensitivity: positive samples (titer ≥ 5)	all samples	*n = 250*	98% (245/250)	86% (215/250)	71.6% (179/250)	98.4% (246/250)
	convalescent	*n = 114*	95.6% (109/114)	69.3% (79/114)	65.8% (75/114)	97.4% (111/114)
	fully vaccinated	*n = 136*	100% (136/136)	100% (136/136)	76.5% (104/136)	99.3% (135/136)
Specificity: negative samples (titer < 5)	all samples	*n = 84*	54.8% (46/84)	82.1% (69/84)	89.3% (75/84)	58.3% (49/84)
	convalescent	*n = 14*	42.9% (6/14)	100% (14/14)	85.7% (12/14)	42.9% (6/14)
	partially vaccinated	*n = 40*	25% (10/40)	62.5% (25/40)	82.5% (33/40)	32.5% (13/40)
	control samples	*n = 30*	100% (30/30)	100% (30/30)	100% (30/30)	100% (30/30)

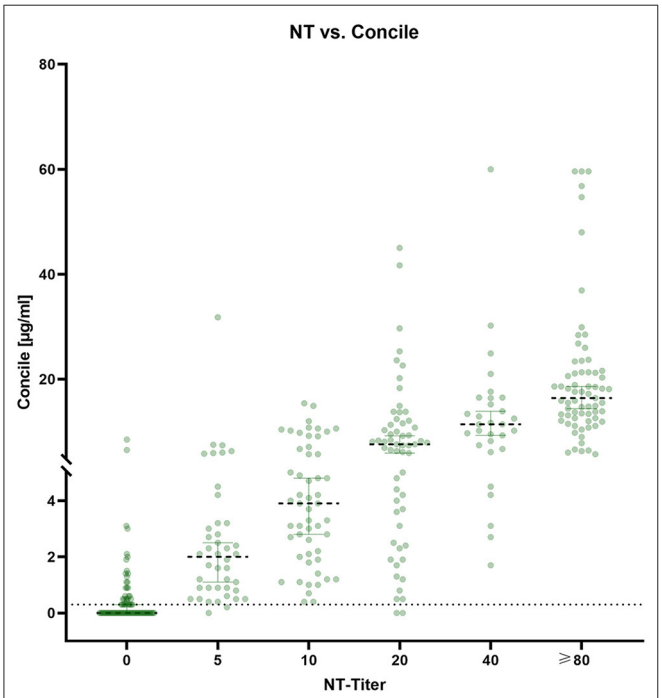

FIGURE 4 | Plot of the percentage of positive results of all four tests in comparison to the respective NT titer.

TABLE 3 | Correlation between all immunoassays and with NT.

	NT	AdipoGen	NeutraLISA	Coris (NP)	Coris	Concile
NT	1,00	0,84	0,85	0,13	0,80	0,84
AdipoGen		1,00	0,96	−0,17	0,67	0,83
NeutraLISA			1,00	−0,12	0,73	0,90
Coris (NP)				1,00	0,19	0,13
Coris					1,00	0,76
Concile						1,00

All coefficients are significant ($p < 0.0001$) with the only exception of the Coris NP assay; correlations are marked in color; strong correlations are marked in green shades while weaker correlations are marked in yellow, orange and red.

when looking at NT-positive samples, the Concile and AdipoGen have consistently good detection rates of ≥ 94% among all titer levels. Detection rates of the NeutraLISA improve with rising NT titers and reach 100% for samples with a titer ≥40 along with the Concile and the AdipoGen. Detection rates of the Coris also gradually improve with rising titers but remain overall low compared to the other three tests (the only exception being samples with a titer of ≥80).

The correlation between all four immunoassays as well as the correlation of each assay with the NT are shown in **Table 3**. Correlations were mostly medium to strong with the only exception of the Coris NP assay. The closest correlation (0.96) was observed between both sELISAs followed by the NeutraLISA and the Concile (0.9). A medium correlation (0.8–0.85) was demonstrated between all assays and the NT with the NeutraLISA exhibiting the closest correlation to the NT. In general, the Coris exhibited the weakest correlations with all other assays including the weakest correlation (0.67) that was observed between the Coris and the AdipoGen. As expected, no correlations at all were observed between the Coris specifically targeted at NP-specific antibodies and all other (spike protein specific) assays.

As the Coris not only detects RBD specific antibodies, but also antibodies against the nucleocapsid protein (NP), we examined all samples from convalescent patients ($n = 128$) with a previous PCR-confirmed infection specifically for antibodies against NP. NP specific antibodies were detected in a little over half of the samples (83/128) resulting in a NP specific sensitivity of 65%. At the same time, we also tested the 30 negative control samples, of which all were NP negative, giving a specificity of 100%. We did not include the samples from partially/fully vaccinated individuals, as our information is not sufficient to exclude a past SARS-CoV-2 infection with absolute certainty.

Antibody Quantities Determined by the Concile (InfectCheck COVID-19 Nab)

In addition to qualitative results, the Concile LFA also offers quantification of antibodies (µg/ml). An overview of quantitative results is given in **Figure 5**. Despite a noticeable wide distribution of results within each NT titer level, a low grade linear trend could still be observed (R^2 0.3, $p < 0.0001$). The highest precision was observed for samples with NT titer levels ≥80.

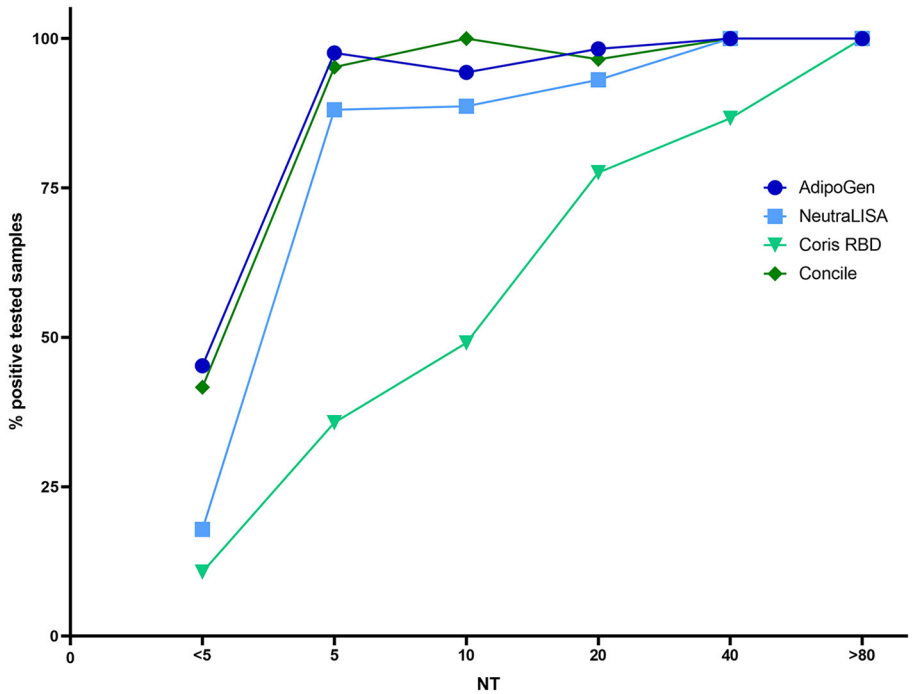

FIGURE 5 | Distribution of antibody quantities (μg/ml) determined by the Concile InfectCheck COVID-19 Nab in relation to the results of the NT. In accordance with the manufacturer's instructions, cut-off was set to 0.3 μg/ml. Despite a wide distribution of results (R^2 0.3, $p < 0.0001$), a low-grade linear trend could be observed with the highest precision observed for samples with high NT-titers (≥80).

DISCUSSION

Infection with SARS-CoV-2 elicits a humoral immune response that results in antibodies against specific viral proteins, including neutralizing antibodies (NAb). In this study, we investigated four immunassays, i.e. two surrogate ELISAs (sELISA) and two lateral flow assays (LFA), specifically designed for the detection of SARS-CoV-2 NAbs and evaluated their sensitivity and specificity in direct comparison to a gold standard virus based method (NT).

Of the 334 serum samples evaluated in this study, the majority came from convalescent patients (38%) and fully vaccinated individuals (41%). Interestingly, mean NT titers differed markedly between convalescent and vaccinated individuals, with titers nearly four times higher in the latter. In addition, we were able to detect NAb in all samples from vaccinated individuals while 11% of samples from convalescent patients were negative for NAb. This is also in line with a recent study by Cavanaugh et al. who found that a full vaccination provides significantly better protection against reinfection compared to natural infection (18). Remarkably, no NAb at all could be detected in partially vaccinated individuals. This observation was somewhat surprising because it contrasts with other studies in which at least low levels of neutralizing antibodies were detected after primary vaccination (19, 20). Since the samples were collected at similar time points after vaccination, this discrepancy is likely due to differences in testing methods. Our NT was essentially designed as a PRNT100 to maximize specificity and detect actual protective neutralizing antibodies with the highest possible confidence. However, this approach implies a reduction in test sensitivity and may explain the differences with studies using other methods such as PRNT50 or surrogate ELISA tests. This explanation is also supported by our finding that, although the NT results were negative, both sELISAs and both LFAs tested in this study detected NAb in many samples from partially vaccinated individuals.

The highest overall sensitivity (98.4%) was observed for the Concile despite it being a LFA, closely followed by the AdipoGen sELISA (98%). In contrast, sensitivity was lower for the NeutraLISA sELISA (86%) while the Coris exhibited the least sensitivity (71.6%). However, while the NeutraLISA demonstrated a similar overall specificity of 82.1%, the AdipoGen and Concile both exhibited a markedly lower overall specificity (54.8 and 58.3% respectively). In contrast, the Coris was the only test with a specificity (89.3%) higher than the sensitivity. Sensitivity of all four tests was highest when testing samples of fully vaccinated individuals. The fact that the Coris LFA offers the simultaneous detection of NP specific antibodies is potentially interesting. However, the NP specific sensitivity of only 65% is not sufficient to reliably confirm or exclude a previous infection. The low specificity of the Concile and the AdipoGen is due to a large number of apparent false positive results within the groups of partially vaccinated and convalescent individuals. In this context, we observed strikingly different specificities when testing different subgroups. Most interestingly, all four tests exhibited a specificity of 100% in the group of negative control samples, i.e. samples taken prior to the appearance of SARS-CoV-2. This

is in stark contrast to the specificities determined based on testing samples from partially vaccinated individuals, which were as low as 25%. Similar results were observed for NT-negative samples from convalescent individuals, raising the question if these samples are truly negative. As already mentioned above, this discrepancy could be due to the fact that our NT is very conservatively designed to be as specific as possible. This limitation might lead to an underestimation of specificity of the evaluated test. In this context, the observation that samples after prime immunization have detectable but generally low antibody levels (20–22) is consistent. At the same time, another possible explanation for this discrepancy could be the presence of SARS-CoV-2 specific antibodies without neutralizing activity. Such non-neutralizing antibodies may cross-react with both sELISA and LFA and lead to false positive results due to their artificial nature. In addition, we observed only very little correlation (R^2 0.26, $p < 0.0001$) between the entirety of IgG antibodies and NAb, including high levels of antibodies but no neutralizing activity which could favor cross-reactivity. The wide distribution of inhibition values that was observed for both sELISAs visualizes the overall low specificity yet high sensitivity. At the same time, the two dimensional distribution shows a wide spread of inhibition values, especially within negative to low NT titers, and underlines the non-quantitative character of both tests. Thus, no conclusion about titer levels can be drawn from inhibition values determined by either sELISA, which is a clear disadvantage compared to NT. The possibility of antibody quantification by the Concile LFA is potentially appealing. However, due to the wide distribution of results within each titer level together with the low overall specificity, the clinical use and interpretation of quantitative results remains questionable.

Another aspect to consider when assessing and interpreting these results is the different conceptual design of the four assays. As described and illustrated in **Figure 2**, both competitive sELISAs are similar in design and mimic the interaction of RBD and the ACE2 receptor to determine the inhibitory effect of neutralizing antibodies that are capable of interrupting this interaction. Therefore, they can at least artificially test the biological function of RBD-specific NAb. However, NAb are directed against different viral structures (23), which in principle gives virus neutralization assays a clear advantage in terms of biological sensitivity because they can detect all NAb regardless of the target structure. The fact that both sELISAs show high sensitivities despite this methodological disadvantage is likely due to the fact, that the vast majority of NAb was shown to be directed against the RBD (5). Therefore, their imperfect sensitivity is likely due to a combination of the disadvantage in biological sensitivity and the performance of the test itself (i.e. technical sensitivity). While the same study has also shown that not all RBD-specific antibodies are neutralizing, this could still explain the high sensitivity of the Concile LFA. Although it only detects RBD-specific antibodies without assessing functionality. While this should have a detrimental effect on (biological) sensitivity, it is again likely that at least a large proportion of these RBD-specific antibodies are in fact able to neutralize. Admittedly, this is in contrast to the significantly lower sensitivity of the Coris LFA, which also detects RBD-specific antibodies. However, this might be a methodological issue as the Concile uses anti-human-IgG for capture and gold conjugated RBD for detection while the Coris works the other way around and uses RBD for capture and gold conjugated anti-human-IgG for detection, which seems to lead to a lower technical sensitivity and should be explored in more detail in further studies.

Currently, there is a global effort to promote SARS-CoV-2 immunization. However, many countries have recently reported stagnating numbers of vaccinations. At the same time, countries are trying to return to normality and gradually ease restrictions, mostly for fully vaccinated people, while at the same time reports have begun to emerge about suboptimal immune responses after vaccination, especially among risk groups (24–26). Albeit still missing data on correlate of protection (27), this has led to an increased demand for NAbs testing to assess vaccination success. In addition, recent publications have indicated a beneficial effect of booster vaccinations to mitigate the reported decline in NAb (28–31), which has further increased the demand for NAb testing to select eligible groups.

Based on the results of this study, both sELISAs are suitable to be used as alternative testing methods in determining NAb levels post immunization (and with minor compromises also post infection) when NT testing is not available in standard laboratories. It must be noted, however, that all tests seem unsuitable to test patient cohorts with an expected low immune responses (e.g. patients under B cell depletion therapy, oncology patients and overall immunocompromised patients etc.) as there is probably a high risk of false positive results due to the previously described potential cross-reactivity with non-neutralizing antibodies.

A huge advantage of both sELISAs is, of course, the significantly reduces turn-around time of only a few hours compared to several days. The same can be said about the Concile LFA, which can be used by medical practices and other non-laboratory institutions due to its quick and easy workflow and turn-around of only 20 min.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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 for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

PG and KZ: conceptualization, methodology, visualization, writing – review and editing. HV and RW: data curation,

writing – review and editing. KM: conceptualization, methodology, writing – original draft, and visualization. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Medical Biodefense Research Program of the Bundeswehr Medical Service.

ACKNOWLEDGMENTS

The content of the manuscript has been uploaded to SSRN as a preprint: http://dx.doi.org/10.2139/ssrn.3963559 (32).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.820151/full#supplementary-material

REFERENCES

1. Gudina EK, Ali S, Girma E, Gize A, Tegene B, Hundie GB, et al. Seroepidemiology and model-based prediction of SARS-CoV-2 in Ethiopia: longitudinal cohort study among front-line hospital workers and communities. *Lancet Global Health.* (2021) 9:e1517–27. doi: 10.1016/S2214-109X(21)00386-7
2. Aziz NA, Corman VM, Echterhoff AKC, Müller MA, Richter A, Schmandke A, et al. Seroprevalence and correlates of SARS-CoV-2 neutralizing antibodies from a population-based study in Bonn, Germany. *Nat Commun.* (2021) 12:2117. doi: 10.1038/s41467-021-22351-5
3. Khoury DS, Cromer D, Reynaldi A, Schlub TE, Wheatley AK, Juno JA, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nat Med.* (2021) 27:1205–11. doi: 10.1038/s41591-021-01377-8
4. Barnes CO, Jette CA, Abernathy ME, Dam K-MA, Esswein SR, Gristick HB, et al. SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies. *Nature.* (2020) 588:682–7. doi: 10.1038/s41586-020-2852-1
5. Piccoli L, Park Y-J, Tortorici MA, Czudnochowski N, Walls AC, Beltramello M, et al. Mapping neutralizing and immunodominant sites on the SARS-CoV-2 spike receptor-binding domain by structure-guided high-resolution serology. *Cell.* (2020) 183:1024–42.e21. doi: 10.1016/j.cell.2020.09.037
6. Khoury DS, Wheatley AK, Ramuta MD, Reynaldi A, Cromer D, Subbarao K, et al. Measuring immunity to SARS-CoV-2 infection: comparing assays and animal models. *Nat Rev Immunol.* (2020) 20:727–38. doi: 10.1038/s41577-020-00471-1
7. GeurtsvanKessel CH, Okba NMA, Igloi Z, Embregts CWE, Laksono BM, Leijten L, et al. Towards the next phase: evaluation of serological assays for diagnostics and exposure assessment. *medRxiv [Preprints].* (2020). doi: 10.1101/2020.04.23.20077156
8. Nie J, Li Q, Wu J, Zhao C, Hao H, Liu H, et al. Establishment and validation of a pseudovirus neutralization assay for SARS-CoV-2. *Emerg Microbes Infect.* (2020) 9:680–6. doi: 10.1080/22221751.2020.1743767
9. Yang R, Huang B, Ruhan A, Li W, Wang W, Deng Y, et al. Development and effectiveness of Pseudotyped SARS-CoV-2 system as determined by neutralizing efficiency and entry inhibition test *in vitro*. *Biosaf Health.* (2020) 2:226–31. doi: 10.1016/j.bsheal.2020.08.004
10. Kohmer N, Rühl C, Ciesek S, Rabenau HF. Utility of different Surrogate Enzyme-Linked Immunosorbent Assays (sELISAs) for Detection of SARS-CoV-2 Neutralizing Antibodies. *J Clin Med.* (2021) 10:2128. doi: 10.3390/jcm10102128
11. Valdivia A, Torres I, Latorre V, Francés-Gómez C, Ferrer J, Forqué L, et al. Suitability of two rapid lateral flow immunochromatographic assays for predicting SARS-CoV-2 neutralizing activity of sera. *J Med Virol.* (2020) 93:2301–6. doi: 10.1002/jmv.26697
12. National SARS-CoV-2 Serology Assay Evaluation Group. Performance characteristics of five immunoassays for SARS-CoV-2: a head-to-head benchmark comparison. *Lancet Infect Dis.* (2020) 20:1390–400. doi: 10.1016/S1473-3099(20)30634-4
13. Olbrich L, Castelletti N, Schälte Y, Garí M, Pütz P, Bakuli A, et al. Head-to-head evaluation of seven different seroassays including direct viral neutralisation in a representative cohort for SARS-CoV-2. *J Gen Virol.* (2021) 102:001653. doi: 10.1099/jgv.0.001653
14. Lassaunière R, Frische A, Harboe ZB, Nielsen ACY, Fomsgaard A, Krogfelt KA, et al. Evaluation of nine commercial SARS-CoV-2 immunoassays. *medRxiv [Preprints].* (2020). doi: 10.1101/2020.04.09.20056325
15. Paul-Ehrlich-Institut – News – Recommendation of the Paul-Ehrlich-Institut for the Collection and Manufacture of COVID-19 Convalescent Plasma. (2021). Available online at: https://www.pei.de/EN/newsroom/hp-news/2020/200407-recommendation-pei-covid-19-convalescent-plasma.html;jsessionid=75FE2CC97FB1AC74E8529A023B965614.intranet241 (accessed April 4, 2020).
16. Haselmann V, Özçürümez MK, Klawonn F, Ast V, Gerhards C, Eichner R, et al. Results of the first pilot external quality assessment (EQA) scheme for anti-SARS-CoV2-antibody testing. *Clin Chem Lab Med.* (2020) 58:2121–30. doi: 10.1515/cclm-2020-1183
17. Müller K, Girl P, von Buttlar H, Dobler G, Wölfel R. Comparison of two commercial surrogate ELISAs to detect a neutralising antibody response to SARS-CoV-2. *J Virol Methods.* (2021) 292:114122. doi: 10.1016/j.jviromet.2021.114122
18. Cavanaugh AM, Spicer KB, Thoroughman D, Glick C, Winter K. Reduced risk of reinfection with SARS-CoV-2 after COVID-19 vaccination – Kentucky, May-June 2021. *MMWR Morb Mortal Wkly Rep.* (2021) 70:1081–3. doi: 10.15585/mmwr.mm7032e1
19. Wall EC, Wu M, Harvey R, Kelly G, Warchal S, Sawyer C. et al. Neutralising antibody activity against SARS-CoV-2 VOCs B16172 and B1351 by BNT162b2 vaccination. *Lancet.* (2021) 397:2331–3. doi: 10.1016/S0140-6736(21)01290-3
20. Bradley T, Grundberg E, Selvarangan R, LeMaster C, Fraley E, Banerjee D, et al. Antibody responses after a single dose of SARS-CoV-2 mRNA vaccine. *N Engl J Med.* (2021) 384:1959–61. doi: 10.1056/NEJMc2102051
21. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, et al. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. *N Engl J Med.* (2020) 383:2603–15. doi: 10.1056/NEJMoa2034577
22. Jeewandara C, Kamaladasa A, Pushpakumara PD, Jayathilaka D, Aberathna IS, Danasekara DRSR, et al. Immune responses to a single dose of the AZD1222/Covishield vaccine in health care workers. *Nat Commun.* (2021) 12:4617. doi: 10.1038/s41467-021-24579-7
23. Barnes CO, West AP, Huey-Tubman KE, Hoffmann MAG, Sharaf NG, Hoffman PR, et al. Structures of human antibodies bound to SARS-CoV-2 spike reveal common epitopes and recurrent features of antibodies. *Cell.* (2020) 182:828–42.e16. doi: 10.1016/j.cell.2020.06.025
24. Agha M, Blake M, Chilleo C, Wells A, Haidar G. Suboptimal response to COVID-19 mRNA vaccines in hematologic malignancies patients. *medRxiv [Preprints].* (2021). doi: 10.1101/2021.04.06.21254949
25. Shroff RT, Chalasani P, Wei R, Pennington D, Quirk G, Schoenle MV, et al. Immune responses to two and three doses of the BNT162b2 mRNA vaccine in adults with solid tumors. *Nat Med.* (2021) 27:2002–11. doi: 10.1038/s41591-021-01542-z
26. Lippi G, Henry BM, Plebani M. Anti-SARS-CoV-2 antibodies testing in recipients of COVID-19 vaccination: why, when, and how? *Diagnostics.* (2021) 11:941. doi: 10.3390/diagnostics11060941
27. Krammer F. A correlate of protection for SARS-CoV-2 vaccines is urgently needed. *Nat Med.* (2021) 27:1147–8. doi: 10.1038/s41591-021-01432-4

28. Bar-On YM, Goldberg Y, Mandel M, Bodenheimer O, Freedman L, Kalkstein N, et al. Protection of BNT162b2 vaccine booster against Covid-19 in Israel. *N Engl J Med.* (2021) 385:1393–400. doi: 10.1056/NEJMoa 2114255
29. Krause PR, Fleming TR, Peto R, Longini IM, Figueroa JP, Sterne JAC, et al. Considerations in boosting COVID-19 vaccine immune responses. *Lancet.* (2021) 398:1377–80. doi: 10.1016/S0140-6736(21)02046-8
30. Bar-On YM, Goldberg Y, Mandel M, Bodenheimer O, Freedman L, Kalkstein N, et al. BNT162b2 vaccine booster dose protection: a nationwide study from Israel. *medRxiv [Preprints].* (2021). doi: 10.1101/2021.08.27.21262679
31. Yue L, Xie T, Yang T, Zhou J, Chen H, Zhu H, et al. A third booster dose may be necessary to mitigate neutralizing antibody fading after inoculation with two doses of an inactivated SARS-CoV-2 vaccine. *J Med Virol.* (2021) 94:35–8. doi: 10.1002/jmv.27334
32. Girl P, Zwirglmaier K, von Buttlar H, Wölfel R, Müller K. Evaluation of two rapid lateral flow tests and two surrogate ELISAs for the detection of SARS-CoV-2 specific neutralizing antibodies. *SSRN J.* (2021). doi: 10.2139/ssrn.3963559. [Epub ahead of print].

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

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SYSTEMATIC REVIEW
published: 28 April 2022
doi: 10.3389/fpubh.2022.859350

Seroprevalence of Hepatitis B Among Healthcare Workers in Asia and Africa and Its Association With Their Knowledge and Awareness: A Systematic Review and Meta-Analysis

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Edited by:
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Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: 21 January 2022
Accepted: 04 April 2022
Published: 28 April 2022

Citation:
Maamor NH, Muhamad NA, Mohd Dali NS, Abdul Mutalip MH, Leman FN, Aris T, Lai NM and Abu Hassan MR (2022) Seroprevalence of Hepatitis B Among Healthcare Workers in Asia and Africa and Its Association With Their Knowledge and Awareness: A Systematic Review and Meta-Analysis. Front. Public Health 10:859350. doi: 10.3389/fpubh.2022.859350

Introduction: The hepatitis B virus (HBV) is a blood-borne virus that can be transmitted by percutaneous and mucocutaneous contact with infected bodily fluid. Healthcare workers (HCWs) are more exposed to HBV infection. They must have a thorough understanding of HBV infection since they can contract and spread the virus. In this study, we systematically reviewed all published evidence on the seroprevalence of Hepatitis B virus (HBV) infection among HCWs. and synthesize evidence on the association between knowledge and awareness with HBV infection.

Methods: We searched PubMed, EMBASE, Cochrane Library and Scopus for studies reporting on HBV seroprevalence from January 1997 to September 2021 among healthcare workers. We used random-effects meta-analyses to estimate the pool prevalence of HBV infection.

Results: We identified 25 studies that met our inclusion criteria, with data on 10,043 adults from 11 countries and two regions: Africa and Asia. The overall seroprevalence of HBV was 5.0% (95% confidence interval [CI] 3.6%), with Africa reporting higher estimates (5.0%, 95% CI 3.7%) than Asia population (4.0%, 95% CI 1.9%). The highest pooled prevalence estimate in African countries came from studies published in the Cameroon region (8.0%, 95% CI 5–10%) while the lowest came from Ethiopia (4.0%, 95% CI 2.6%). The overall seroprevalence estimates in the African population were significantly higher than those in the Asian group. Studies in Africa found that the average knowledge and seroprevalence were 1.4% and 11.0%, respectively where, eight studies (53.3%) reported good knowledge and seven studies (46.7%) reported average knowledge. In Asia, two studies (40.0%) reported good knowledge, one study (20.0%) reporting average knowledge, and two studies (40.0%) reporting poor knowledge. African studies

demonstrated good knowledge despite the fact that their HBV infection rate was higher than 6.7%.

Conclusion: Africa and Asia have the highest seroprevalence of HBV infection. Improving the comparability of epidemiological and clinical studies constitutes an important step forward. More high-quality data is needed to improve the precision of burden estimates.

Systematic Review Registration: PROSPERO CRD42021279905.

Keywords: hepatitis B virus, seroprevalence, prevalence, infection, healthcare workers, knowledge, awareness, epidemiology

INTRODUCTION

The Hepatitis B virus (HBV) is a bloodborne virus that has become a major global public health concern. HBV, which belongs to the *Hepadnaviridae* family, has only one known natural host: humans. The virus enters the liver through the bloodstream and replicates in the tissue of the liver (1). Acute hepatitis B infection causes inflammation and jaundice in the liver, while chronic hepatitis B infection can lead to potentially fatal diseases such as liver cirrhosis and hepatocellular carcinoma (2). Globally, HBV infected over 2 billion individuals with 250 million of them suffering from chronic HBV infection (3). According to the World Health Organization (WHO), 325 million people are infected with HBV, with the African and Western Pacific regions having the highest rates of HBV infection at 68% (4), and approximately 900,000 people dying from HBV each year (5). Hepatitis B is most common in Sub-Saharan Africa and Southeast Asia (8.0–10.0%). This is followed by Eastern and Southern Europe, the Middle East, and Japan (2.0-7.0%), and the United States and Northern Europe (0.5–2.0%) (6–8). Furthermore, it is estimated that 40% of the healthcare workers (HCWs) are infected with HBV infections in the developing countries (9).

Healthcare workers are four times more likely to be infected with HBV compared to the general population (10). This may be due to a lack of compliance with infection control recommendations from established guidelines such as the Center for Disease Control and Prevention (CDC) (11). Handwashing, glove use, and correct disposal of sharp instruments are all part of the CDC's recommended precaution, which is aimed to prevent the spread of blood-borne infections like HBV (12). In the case of HBV infection, knowledge includes information gathering, experience, skill, and disease prevention strategies (13, 14). A lack of understanding among HCWs in both low- and middle-income countries leads to low adherence to safety measures, aggravating the HBV situation (15). A better understanding of HBV infection is essential to reduce the rate of infection among HCWs in the healthcare context (16). Knowledge is usually assessed to investigate how far the community know the concepts of disease including causes and symptoms of disease. Attitude is defined as a product of a complex interaction on values, feelings and beliefs (17). Practice is defined as an action of the habitual community to prevent the disease (18). Awareness is the knowledgeable person being conscious and behavior under the receiving in taxonomy of affective domain (19).

Although HCWs are more aware of Hepatitis B, several countries lack a comprehensive grasp of the disease biology, transmission methods, risk of transmission, clinical characteristics, and vaccination availability (20). Hepatitis B virus seroprevalence among HCWs was reported in a prior study by Mahamat et al. (21), but there was no relationship study between seroprevalence and knowledge or awareness (21). Hepatitis B awareness is lower among HCWs in developing countries, which is linked to poorer preventive attitudes, including lower Hepatitis B vaccine coverage (22). On the other hand, the prevalence of HBV infection fluctuates and is influenced by a variety of factors including geographical region, host factor, and environmental or behavioral factors. The low prevalence of HBV in Europe, for example, may be attributed to the high standard of living there (10). As a result, Hepatitis B prevalence can also predict the risk factors for HBV transmission, such as injections, occupational injuries, body tattoos, and a history of not being been vaccinated, among others (23). Therefore, this study aimed to determine the pooled prevalence of hepatitis B infection among HCWs and to compare the pooled prevalence of HBV infection across different regions. We also compiled data to determine the association between seroprevalence and level of knowledge or awareness on HBV infection.

MATERIALS AND METHODS

We conducted a systematic review by following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (24) and Guidelines for Accurate and Transparent Health Estimates Reporting (GATHER) statement (25). The PROSPERO registration number is CRD42021279905. Our primary outcome was the seroprevalence of HBV infection among HCWs. Our secondary outcome was the estimation of knowledge and awareness attributable to HBV infection.

Search Strategy

A comprehensive systematic literature search was conducted in electronic databases (PubMed, EMBASE, Cochrane Library, and Scopus) to identify relevant studies from inception to September 2021. A search strategy was developed for PubMed (**Supplementary Table 1**) and adapted for use in the other

databases. Other electronic search was performed in the WHO International Clinical Trials Registry Platform (ICTRP). In the search, key words and equivalent Medical Subject Heading (MeSH) phrases were combined when applicable, with no language or publication year restrictions. Specific search terms are as follows: "hepatitis B" OR "HBV" OR "hepatitis B virus" AND "etiology" OR "etiology" OR "prevalence" OR "epidemiology" OR "infection" AND "healthcare workers" OR "healthcare" OR "doctor" OR "doctors" OR "nurse" OR "nurses" OR "medical" OR "medical staff" OR "medical assistant" OR "health personnel" OR "health care personnel" OR "healthcare personnel" OR "health care worker" OR "health care workers" OR "healthcare worker" OR "healthcare workers" OR "health worker" OR "health workers" OR "healthcare professionals" OR "medical care personnel" AND "knowledge" AND "awareness" (**Supplementary Table 1**) for the MEDLINE search. We also scanned through cross-references of identified primary studies and review articles for eligible studies.

Eligibility and Exclusion Criteria

We included studies according to the following criteria; Population/Intervention/Comparator/Outcome/Study Design (PICOS) approach. For this review, we included HCWs (P) defined as individuals such as doctors, dentists, nurses, midwives, medical personnel, medical assistant or laboratory scientists who are in direct contact with the following: (i) patient bodily fluids or biological samples such as blood, saliva, sperm, sweat and stool, (ii) new-born delivery process in which mother-to-child transmission was possible via a transplacental route, and (iii) people who were exposed sexually, and (iv) sharp or needle-stick injuries. Instead of an intervention (I), we included observational studies that report the seroprevalence of HBV with either one or both of the components of knowledge or awareness in relation to HBV seroprevalence. There was no comparator (C), and lastly, the determined outcome (O) was seroprevalence of hepatitis B infection. We considered all articles published in English language. When numerous studies with the same cohort were conducted, the research with the most detailed information on the participants or the largest number of participants was chosen.

We excluded abstracts, letters to the editor, reviews, commentaries, editorials, and studies without either primary data or described study methods. We excluded systematic reviews, non-empirical studies, conference, abstracts, editorials, commentaries, book reviews, and abstracts not accompanied by a full text.

Study Selection

Two review authors (NHM and NAM) independently screened all titles and abstracts to look for potential studies found through the search and coded them as "retrieve" (eligible or potentially eligible/unclear) or "do not retrieve". Two more review authors (NSMD and MHAM) independently retrieved full-text study reports/publications to identify studies for inclusion, and to identify and record reasons for studies' exclusion. When there were disagreements, a consensus was obtained through discussion with a third reviewer (MRAH).

Data Extraction Process and Data Items

Two review authors (FNL and TA) independently evaluated each included study 's methodological quality and extracted data using a piloted form; discrepancies were resolved through discussion with a third review author (NML). Data on the year of publication, country of origin, study design, sample size, sampling procedure (if available), study period, and setting (country/continent/region) were extracted using a standardized data collection form.

Risk of Bias in Included Studies

To assess the risk of bias for each study, a domain-based questionnaire developed from the Newcastle-Ottawa Scale (NOS) was used to assess methodological quality (26) (**Supplementary Table 2**). In the following domains: participant selection (selection bias), sample size justification (selection bias), outcome measurement (detection bias), and confounding adjustment, we evaluated the risk of bias as low, moderate, high, or uncertain. We assigned a score of 7 and above as good quality, and below 6 as having some concerns to determine the overall quality.

Data Synthesis and Analysis

We used Stata software version 16 for all statistical analysis (27). The pooled prevalence rates, as well as their 95% confidence intervals, were calculated using a random-effects model (28). The heterogeneity of the studies was assessed using I^2 statistics and Cochran's Q Test (29). The I^2 statistics were used to assess the explained variance attributable to study heterogeneity, with I^2 score of 25.0, 50.0 and 75.0% denoting low, moderate and high, respectively (28, 30).

RESULTS

Identification of Studies

We identified a total of 4,329 studies published between January 1997 and September 2021. We could not find any studies that evaluated seroprevalence of Hepatitis B among HCW and its association with knowledge, awareness and attitude of HBV in the Americas, Europe, Eurasia, Australia, or Antarctica regions. We excluded duplicates and collated multiple reports of the same study so that each study, rather than individual report, is the focus of our analysis. We meticulously documented the selection process to complete a PRISMA flow diagram (**Figure 1**). We shortlisted 25 studies from a pool of 28 for an in-depth full-text examination of their suitability.

Characteristics of Included Studies

Among the 25 included studies, nineteen were from Africa (10, 14, 20, 31–46) and six from Asia (47–52). The studies were conducted across eleven countries with a total of 10,043 participants. The characteristics of the included studies are depicted in **Table 1**.

Quality Assessment of Included Studies

Two reviewers (FNL and MHAM) independently assessed the quality of the included studies using an adapted version of the

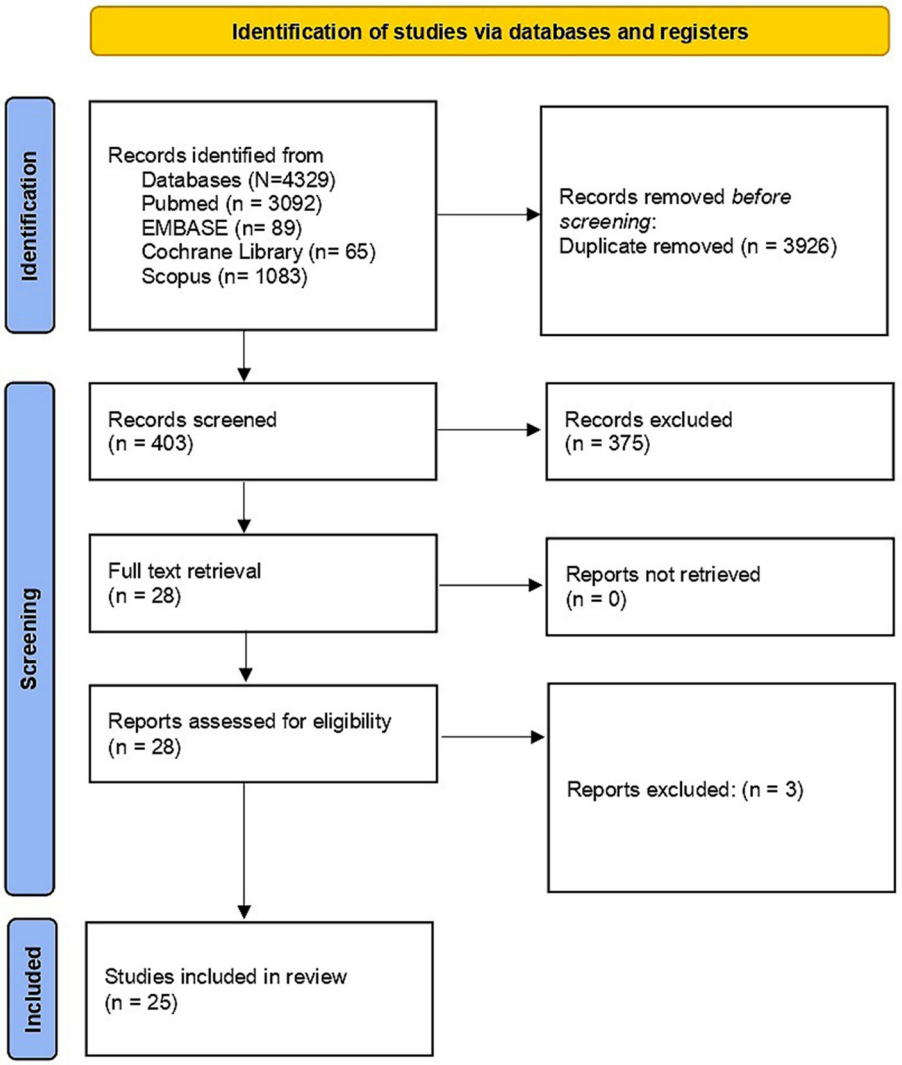

FIGURE 1 | PRISMA flow diagram on selection process. From: Page et al. (24). For more information, visit: http://www.prisma-statement.org/.

NOS for prevalence studies. The quality of evidence was rated as low to begin with, due to the non-randomized nature of the study. The quality of evidence in the outcomes were based on the NOS criteria, where a maximum score of 4 stars for selection, 2 stars for comparability, and 3 stars for exposure and outcome assessment. Studies with fewer than 2 stars were considered low quality; 2 to 6 stars, moderate quality; and 7 stars and more, high quality. Twenty-one studies were observed to be of good quality (NOS score 7 and above), and four studies were identified as fair quality (NOS score between 2 and 6 inclusive) (**Supplementary Table 2**).

The Estimates of Pool Seroprevalence of Hepatitis B

As shown in **Figure 2**, the overall estimate for pooled seroprevalence of Hepatitis B was 5.0% (95% CI: 0.03–0.06), with a high-level of heterogeneity between studies ($I^2 = 94.6\%$, $P = 0.001$). The study was divided into two parts: Africa and Asia. In Asia, the overall pooled seroprevalence of Hepatitis B was 4.0% (95% CI: 0.01–0.07) with a high-level of heterogeneity between studies ($I^2 = 95.7\%$, $P = 0.001$) (**Figure 3**), whereas in Africa, the overall pooled seroprevalence of Hepatitis B in Africa was 5.0% (95% CI: 0.03–0.07) with a high-level of heterogeneity between studies ($I^2 = 92.2\%$, $P = 0.001$) (**Figure 4**). The subgroup analysis was performed in Africa since there were nineteen publications categorized under the African region. We pooled the seroprevalence of Hepatitis B in African countries with two or more publications for this study. This subgroup analysis was performed in Nigeria, Ethiopia, and Cameroon (**Supplementary Figures 1–3**). In Nigeria, the overall pooled seroprevalence of hepatitis B was 4.0% (95% CI: 0.01–0.06) with high-level heterogeneity ($I^2 = 94.4\%$, $P = 0.001$) (**Supplementary Figure 1**). Ethiopia had a pooled seroprevalence of 4.0% (95% CI: 0.02–0.06), but the level of heterogeneity was

TABLE 1 | List of included studies.

No	References	Study population (country)	Continent/region	Sample size	Seroprevalence (%) /HBV marker	Knowledge/ Awareness findings
1	Djeriri et al. (31)	Morocco	Africa	276	1.0 (HBsAg)	Awareness: Overall good awareness. 95% aware the complications of chronic Hepatitis B, 68% aware Hepatitis could be fatal, 100% aware HBV can be transmitted by blood transfusion, 85% aware HBV transmitted by sexually transmitted disease and 97% by vertical transmission. 93% aware condom as an effective for prevention, 87% aware washing hands prevent HBV transmission and 96% aware the use of disposal gloves to prevent Hepatitis B
2	Shao et al. (32)	Tanzania	Africa	442	5.7 (HBsAg)	Knowledge: Overall average knowledge. Quarter (25.4%) had good knowledge and about half (49.6%) had fair knowledge about HBV infection. Most of the participants (85.9%) correctly identified that HBV is more contagious than HIV, while (91.3%) knew that there is effective and safe hepatitis B vaccine. Knowledge questions: HBV transmit via sexual intercourse and partner, known as blood-borne pathogen via accidental exposure to blood and its product, needle stick, broken skin, mucous membrane, infected blood, oral-fecal route, mom to fetus, immunoglobulin, and vaccine. Awareness: Overall poor awareness. 17.9% of participants were aware, unprotected sex with multiple partners was the risks for HBV infection.
3	Hebo et al. (33)	Ethiopia	Africa	240	4.4 (HBsAg)	Knowledge: Overall good knowledge on the virus (73.9%) including the transmission and the treatment. 26.1% had average knowledge
4	Desalegn and Selsassie (34)	Ethiopia	Africa	254	2.4 (HBsAg)	Knowledge: Overall good knowledge of universal precautions (UPs). 52.4% consistent use of gloves was reported by of the respondents, 61.0% concerning needle stick injury (NSI) and from other sharp injury and 50.0% had a history of NSI and sharp injury. 80.7% and 42.5% of HCWs knew about universal precaution guideline and were trained on infection prevention, respectively.
5	Anagaw et al. (35)	Ethiopia	Africa	100	6.0 (HBsAg)	Awareness: Overall good awareness. Aware on the viral hepatitis transmission via sexual contact, sharing special tools (i.e., eye goggle, thick gloves, protective gown, tooth brushes, shaving razor, etc.) and intravenous drug abuse.
6	Abiola et al. (10)	Nigeria	Africa	134	1.5 (HBsAg)	Knowledge: Overall good knowledge (56.7%) and 43.3% with average knowledge
7	Ngekeng et al. (36)	Nigeria	Africa	188	5.0 (HBsAg)	Knowledge: Overall average knowledge. 58.72% had good knowledge and 41.28% had poor knowledge. Average knowledge on the HBV transmission (66.9%) and good knowledge (80.0%) know HBV cause liver disease
8	Osagiede et al. (20)	Nigeria	Africa	280	1.4 (HBsAg)	Knowledge: Overall average knowledge. 32.5% had poor, 20% had average and 47.5% have good knowledge. Awareness: Overall good awareness with 86.4% aware about HBV.

(Continued)

TABLE 1 | Continued

No	References	Study population (country)	Continent/region	Sample size	Seroprevalence (%) /HBV marker	Knowledge/ Awareness findings
9	Ijoma et al. (37)	Nigeria	Africa	3,123	2.3 (HBsAg)	Knowledge: Overall good knowledge on HBV infection (97.0%) and 68.1% correctly identify risk factors and transmission. Poor knowledge on sexual intercourse and sharp objects
10	Ogundele et al. (14)	Nigeria	Africa	209	6.7 (HBsAg)	Knowledge: Overall adequate knowledge with 61.7% had adequate knowledge while 38.3% had poor knowledge range. The knowledge score was only significantly associated with work duration ($p = 0.018$). 89% of the participants ever heard of HBV prior to the study. Awareness: Overall good awareness with 83.7% were aware that HBV is contagious, only 125(59.8%) described it as a lethal disease
11	Oladokun et al. (38)	Nigeria	Africa	140	5 (HBsAg)	Knowledge: Overall good knowledge of the infection though some have had needle stick injury (12.14%) Awareness: Overall good awareness. Aware of the infection (92.86%) and its modes of transmission (72.86%).
12	Muhammad et al. (39)	Nigeria	Africa	283	6.0 (HBsAg)	Knowledge: Overall adequate knowledge with 58.3% had knowledge on HBV and 41.7% with poor knowledge Awareness: Overall good awareness with high awareness level observed in individuals wearing hand gloves. However, 70.1% do not recap needles after the injection
13	Amiwero et al. (40)	Nigeria	Africa	248	1.3 (HBsAg)	Awareness: Overall good awareness with 70.6% aware of various types of hepatitis and suggested that awareness increased with the increased of education level.
14	Mbaawuaga et al. (41)	Nigeria	Africa	255	10.6 (HBsAg)	Awareness: Overall good awareness with 79.6% had awareness about HBV infection.
15	Akazong et al. (42)	Cameroon	Africa	395	10.6 (HBsAg)	Knowledge: Overall average knowledge. 32.4% had poor knowledge while 67.6% had average knowledge
16	Rodrigue et al. (43)	Cameroon	Africa	171	7.0 (HBsAg)	Knowledge: Overall, good knowledge. 94.7% had good knowledge and 5.3% had poor knowledge. Good knowledge with 93% know it's come from virus. Most of HCWs believed HBV cause by sexual intercourse (96.5%), scarifications (34.5%) and blood exposure (19.3%)
17	Tatsilong et al. (44)	Cameroon	Africa	100	11.0 (HBsAg)	Knowledge: Overall had average knowledge. 47% had good knowledge in HBV mostly in men (3.2 times than women). Higher education, knowledge on the present of HB vaccine, needle injury, knowing the mode of HBV transmitted are named as a contribution factor to higher knowledge of HBV.
18	Qin et al. (45)	Sierra Leone	Africa	211	10.0 (HBsAg)	Knowledge: Overall average knowledge with 29.0% had poor knowledge on transmission, preventive HBV measure (44.1%). Longer working experience is associated with greater knowledge & medical doctor. Awareness: Overall poor awareness with 77.3% not aware about HBV clinical outcome.
19	Massaquoi et al. (46)	Sierra Leone	Africa	446	8.7 (HBsAg)	Knowledge: Overall good knowledge with 90.4% of participants were aware that hepatitis B could cause liver cancer. About 96.9% healthcare workers were concerned about their risk of hepatitis B at work

(Continued)

TABLE 1 | Continued

No	References	Study population (country)	Continent/region	Sample size	Seroprevalence (%) /HBV marker	Knowledge/ Awareness findings
20	Mangkara et al. (47)	Laos	Asia	317	5.0 (HBsAg)	Knowledge: Overall poor knowledge with 20% of dentists and 45% of assistants were unaware that HBV can be transmitted by blood. 8.2% of the dentists and 18.1% of assistants were not familiar or did not recognize serology as a way to test for Hepatitis B infection.
21	Nguyen et al. (48)	Vietnam	Asia	203	9.8 (HBsAg)	Knowledge: Overall good knowledge on the mode of HBV transmission. Majority believed that asymptomatic people can have chronic HBV or HCV infection (89%) and that HBV-HCV are lifelong infections which can cause liver cancer (95%) and can be lethal (86%). Physicians exhibit better knowledge than nurses or midwives and other HCWs.
22	Ptil et al. (49)	India	Asia	555	0.2 (HBsAg)	Awareness: Overall good awareness, with 98% aware of health consequences of HBV accidental exposure (needle prick and post exposure prophylaxis) and concerned about follow up
23	Aziz et al. (52)	India	Asia	250	2.4 (HBsAg)	Knowledge: Overall good knowledge with 90% know HBV can be transmission in hospital, needle stick (62%), sexual (59%), vertical (71%), hand washing (13%), precaution to avoid needle stick injury (23%), wear gloves (30%), proper vaccine (14%), regular screen (10%), no knowledge (1%). About quarter of them had needle stick injury during hospital job but few were tested against it. Less than half of them were previously vaccinated for HBV but majority of them knew about the risk of transmission of HBV, HCV and I-IIV during hospital job.
24	Memon et al. (51)	Pakistan	Asia	923	4.7 (HBsAg)	Knowledge: Overall poor knowledge regarding the importance of HBV prevention, 20%
25	Alqahtani et al. (50)	Saudi Arabia	Asia	300	8.7 (HBsAg)	Knowledge: Overall average knowledge observed among HCWs regarding occupationally transmitted blood-borne diseases, safe injection practices, and standard precautions to prevent occupationally transmitted blood-borne infections. Awareness: Overall good awareness with 99.0% of HCWs were aware of all blood-borne diseases, 53.0% felt all safe injection practices that may protect them and 72.6% said all standard isolation precautions to prevent occupationally transmitted blood-borne infections.

moderate ($I^2 = 42.6\%$, $P = 0.19$) (**Supplementary Figure 2**). Cameroon had the highest overall pooled seroprevalence of Hepatitis B in Africa, at 8.0% (95% CI: 0.05–0.11), with a moderate level of heterogeneity between studies ($I^2 = 63.7\%$, $P = 0.03$) (**Supplementary Figure 3**).

Knowledge and Awareness on Hepatitis B Infection Among Healthcare Workers

There were twenty studies that reported findings on seroprevalence and knowledge, with fifteen from Africa and five from Asia. Some of the studies mentioned the knowledge level (good, average, or poor knowledge) in the publication. In Africa, the majority of the studies found that participants have a strong knowledge of hepatitis B infection, with eight studies (53.3%) reporting good knowledge and seven studies (46.7%) reporting average knowledge. In Asia, five studies reported seroprevalence and knowledge findings, with two studies (40.0%) reporting good knowledge, one study (20.0%) reporting average knowledge, and two studies (40.0%) reporting poor knowledge. Seven studies in Africa found that the average knowledge and seroprevalence were 1.4 and 11.0%, respectively. Surprisingly, few African studies demonstrated good knowledge despite the fact that their HBV infection rate was higher than 6.7% (14, 42, 45). The average

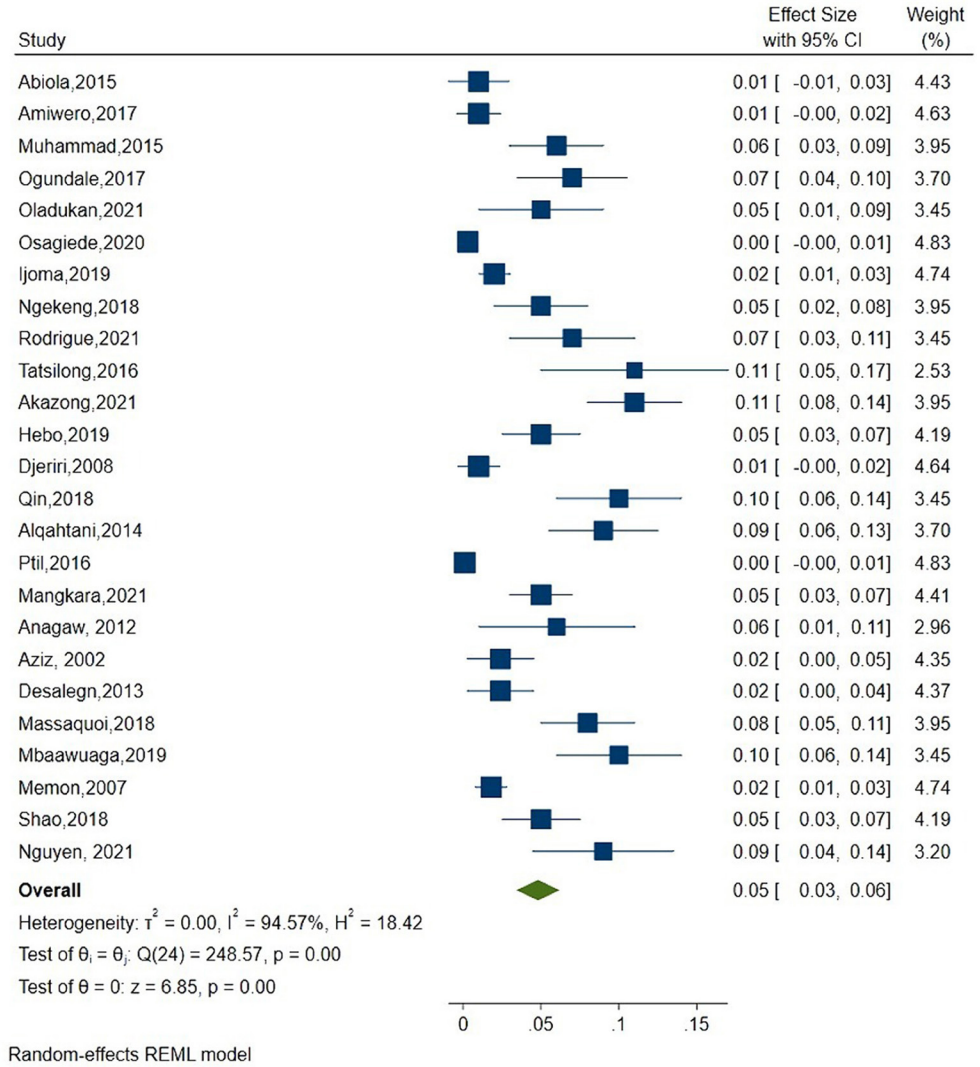

FIGURE 2 | Forest plot of overall seroprevalence estimate of the Hepatitis B infection among healthcare workers.

knowledge was reported in the cohort with a seroprevalence of 8.7% in the Asia region (49) Memon et al. (51) and Mangkara et al. (47) found poor knowledge in their participants with 4.7% and 5.0% HBV seroprevalence, respectively. However, Nguyen et al. (48), who found the highest seroprevalence (9.8%), claimed that participants had a good knowledge of HBV infection (48). The assessment of knowledge among HCWs were presented in the **Supplementary Table 3**.

There were eleven studies that found an association between seroprevalence and participants' level of awareness, including nine (81.8%) from Africa and two (18.2%) from Asia. Some of the studies mentioned the participants' level of awareness (good, average, or poor awareness). The scale for awareness level was adopted from Vaishali et al. (53) for those studies that did not mention it. Nine studies (81.8%) were reported to have good awareness, whereas two (18.2%) in the Africa region were found to have poor awareness. Seroprevalence was poor in Tanzania (32) and Sierra Leone (45), with 5.7 and 10.0%, respectively. Even though three publications had higher seroprevalence than Shao et al. (32) and Qin et al. (45), the publications showed good awareness: 6.0% Anagaw et al. (35), 6.7% Ogundele et al. (14), and 10.6% Mbaawuaga et al. (41). **Supplementary Table 4** summarizes the awareness evaluation, the instrument employed, and the conclusion.

DISCUSSION

Healthcare workers have a higher risk of contracting HBV infection than the general population. Simultaneously, they play a vital role in preventing and controlling HBV infection by disseminating and transmitting HBV knowledge to the public, as well as assisting in behavior changes that may aid in infectious diseases prevention (54). After utilizing

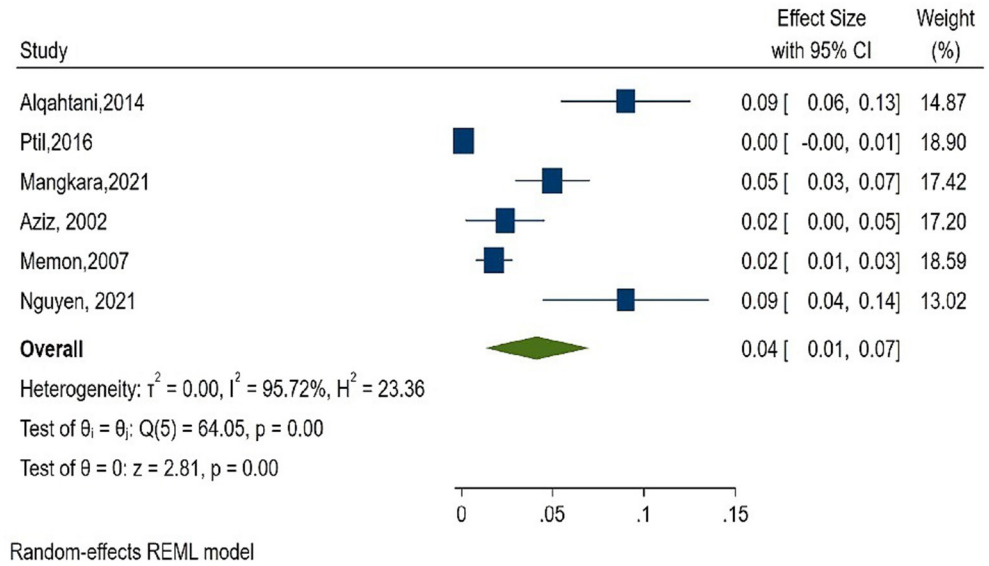

FIGURE 3 | Forest plot of seroprevalence estimate of the Hepatitis B infection among healthcare workers in Asia.

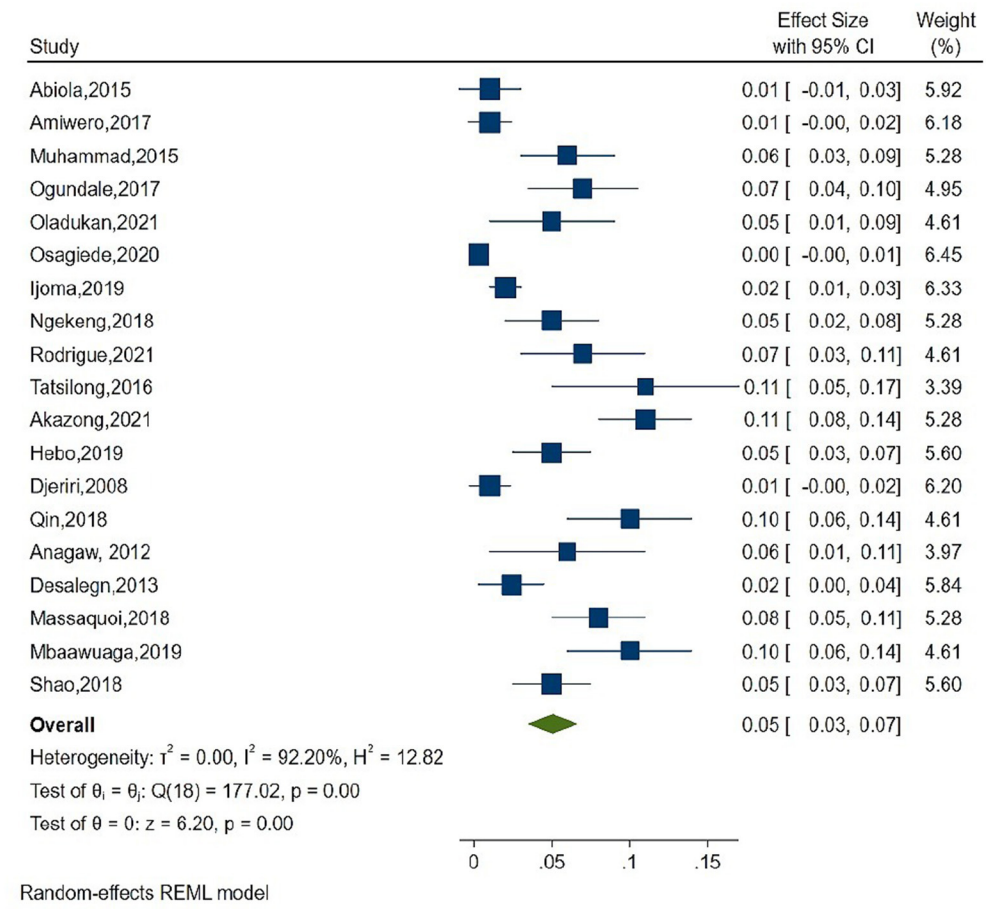

FIGURE 4 | Forest plot of seroprevalence estimate of the Hepatitis B infection among healthcare in Africa region.

NOS to assess methodological quality, only 21 out of the 25 studies included in this review showed a good risk of bias, while another four studies exhibited a fair risk of bias. Healthcare workers must consequently have a goof level of knowledge and awareness of HBV to limit their own and the public's risk of infection. According to Rayate et al. (55), the majority of HCWs are unaware that the virus can survive outside the body for seven days (55). The same study also reported that only 27.78% of HCWs are aware that the virus can survive in dried blood type form (55). Several factors influence the likelihood of getting hepatitis B, including the prevalence of the virus in the environment or in people's behavior, the frequency of blood and body fluid exposure to HCWs, HBV infectivity (14), geographical location, and host factors (10).

In comparison to other regions, the current study found that African countries have a high seroprevalence of HBV infection (**Figures 3**, **4**). Medical doctors, dentists, nurses, and laboratory workers made up the majority of HCWs infected with HBV. There were also cases of HBV infection among technicians, nurse assistant, cleaning operators, and housekeeping staff (17, 41, 48, 49). Accidental exposure to blood and blood products, occupational injuries such as needle-sticks and other injuries from sharp objects, lack of experience or practice with HBV infection, and not having been vaccinated were all risk factors for high seroprevalence of HBV infection in certain places (32–34, 36, 47, 51). Mahamat et al. (21) published a study on global seroprevalence among HCWs that was similar to ours. Our study, on the other hand, shows a link between HBV seroprevalence and HCWs knowledge or awareness of HBV infection, which was not addressed in the previous study (21).

Despite the high seroprevalence observed in a few studies, other publications claimed good knowledge or awareness of HBV infection (14, 35, 41, 43, 46). High HBV seroprevalence has been attributed to a lack of knowledge about HBV transmission routes in one study in Cameroon (42). Surprisingly, despite the lower seroprevalence, some studies reported average or poor knowledge or awareness of HBV infection (32, 39). This finding showed an inconsistency between the level of seroprevalence and the level of knowledge or awareness among HCWs. Notwithstanding the inconsistency, it is critical to increase HBV knowledge and awareness among HCWs (50).

A few approaches to increase an awareness of HBV infection among HCWs include strengthening immunization program, regularly screen the HBsAb and HBsAg of HCWs, media involvement, continuous medical educations, and provide trainings to the HCWs (14, 32, 33, 46, 50). This will encourage safer work practices and a higher degree of compliance with hospital policies. By assessing the level of knowledge among HCWs, not only is the general public indirectly examined, but also preventative implementation is improved. Separately, the long years of hospital service have contributed to raising increasing hepatitis B infection awareness. Furthermore, HCWs' lack of knowledge regarding Hepatitis B could have a significant impact on safety behaviors, such as vaccination. As a result, HCW awareness of Hepatitis B is vital, as knowledge plays a key role in changing prevention-related behavior (22). Lack of training or seminars for HCWs, insufficient information, or a poor awareness of HBV infection could all contribute to the lack of knowledge among HCWs. Moreover, insufficient health education programs and obtaining unreliable Hepatitis B information from friends, relatives, and co-workers may increase the likelihood of acquiring incorrect information (42). To increase HBV knowledge among HCWs, improvement in clinical practice, 53 training, and practical skills are required (56, 57).

The present study had limitations. First, we were unable to locate reports on the HBV seroprevalence and levels of knowledge or awareness in the developing countries. Thus, we were unable to compare the findings worldwide. Second, in some research, differences in score and inability to score on the level of knowledge or awareness may result in inconsistent conclusions that are either good, average, or poor. As a result, we were unable to determine some research' scores and compare them to seroprevalence.

In conclusion, hepatitis B virus was shown to be present in 4.0–5.0 % of the population tested, with an apparent higher prevalence in African countries than in Asian countries. Some HCWs were still infected with HBV despite having strong knowledge and awareness of HBV infection. Improved epidemiological data collection can help determine and identify key risk factors for a more effective public health response. Thus, if enough people are exposed to Hepatitis B virus knowledge, awareness, attitude, and practice, the goal of eliminating viral hepatitis by 2030 may be achieved.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

NHM and NAM carried out the study design, study selection, data extraction, and statistical analysis and drafted the manuscript. NSMD and MHAM participated in the study selection and data extraction and drafted the manuscript. FNL and TA evaluated the quality of included studies. MRAH and NML participated in the discussion for any discrepancies and supervised the study. All authors read and approved the final manuscript.

FUNDING

The open-access publication fee was supported by the National Institutes of Health, Ministry of Health, Malaysia.

ACKNOWLEDGMENTS

We would like to thank the Director General of Health, Ministry of Health, Malaysia for the permission to publish this article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.859350/full#supplementary-material

REFERENCES

1. Yizengaw E, Getahun T, Geta M, Mulu W, Ashagrie M, Hailu D, et al. Seroprevalence of hepatitis B virus infection and associated factors among health care workers and medical waste handlers in primary hospitals of North-West Ethiopia. *BMC Res Notes.* (2018) 11:437. doi: 10.1186/s13104-018-3538-8
2. Tang LSY, Covert E, Wilson E, Kottilil S. Chronic hepatitis B infection: a review. *JAMA.* (2018) 319:1802–13. doi: 10.1001/jama.2018.3795
3. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. *Lancet.* (2015) 386:1546–55. doi: 10.1016/S0140-6736(15)61412-X
4. World Health Organization. *Global hepatitis report 2017*. World Health Organization (2017).
5. World Health Organization. *World Hepatitis Day 2020*. World Health Organization (2020). Available online at: https://www.who.int/campaigns/world-hepatitis-day/2020 (accessed October 10, 2021).
6. Biset Ayalew M, Adugna B, Getachew N, Amare S, Getnet A. Knowledge and attitude of health care professionals regarding hepatitis B virus infection and its vaccination, university of gondar hospital, Ethiopia. *HMER.* (2016) 8:135–42. doi: 10.2147/HMER.S120477
7. Fatusi A, Esimai A, Onayade A, Ojo O. Aceptance of hepatitis B vaccine by workers in a Nigerian teaching hospital. *E Af Med Jrnl.* (2009) 77:608–12. doi: 10.4314/eamj.v77i11.46734
8. Hou J, Liu Z, Gu F. Epidemiology and prevention of hepatitis B virus infection. *Int J Med Sci.* (2005) 50–57. doi: 10.7150/ijms.2.50
9. Prüss-Üstün A, Rapiti E, Hutin YJF. *Sharps Injuries: Global Burden of Disease from Sharps Injuries to Health-Care Workers*. World Health Organization (2003). Available online at: https://apps.who.int/iris/handle/10665/42743
10. Abiola A-HO, Agunbiade AB, Badmos KB, Lesi AO, Lawal AO, Alli QO. Prevalence of HBsAg, knowledge, and vaccination practice against viral hepatitis B infection among doctors and nurses in a secondary health care facility in Lagos state, South-western Nigeria. *Pan Afr Med J.* (2016) 23:160. doi: 10.11604/pamj.2016.23.160.8710
11. Sadoh WE, Fawole AO, Sadoh AE, Oladimeji AO, Sotiloye OS. Practice of universal precautions among healthcare workers. *J Natl Med Assoc.* (2006) 98:722.
12. Garner JS. Hospital infection control practices advisory committee. guideline for isolation precautions in hospitals. *Infect Control Hosp Epidemiol.* (1996) 17:54–80. doi: 10.1017/S0195941700006123
13. Nasim A, Shah Y. ul Haq N, Tahir M, Mohammad F, Saood M, Riaz S. Knowledge, attitude and practice regarding hepatitis B in nurses working in different hospitals of Quetta city, Pakistan. *Int J Nurs Crit Care.* (2020) 6:24–34. Available online at: https://nursing.journalspub.info/index.php?journal=IJNCC&page=article&op=view&path%5B%5D=1427
14. Ogundele OA, Olorunsola A, Bakare B, Adegoke IA, Ogundele T, Fehintola F, et al. Seroprevalence and knowledge of hepatitis B and C among health care workers in a specialist hospital in Nigeria. *EJPM.* (2017) 5:7–12. doi: 10.11648/j.ejpm.s.2017050101.12
15. Elsheikh T, Balla SA, Abdalla AA, Elgasim M, Swareldahab Z, Bashir AA. Knowledge, attitude and practice of heath care workers regarding transmission and prevention of hepatitis B virus infection, White Nile state, Sudan, 2013. *Am J Health Res.* (2016) 4:18–22. doi: 10.11648/j.ajhr.20160402.11
16. Askarian M, McLaws M-L, Meylan M. Knowledge, attitude, and practices related to standard precautions of surgeons and physicians in university-affiliated hospitals of Shiraz, Iran. *Int J Infect Dis.* (2007) 11:213–9. doi: 10.1016/j.ijid.2006.01.006
17. Haq NU, Hassali MA, Shafie AA, Saleem F, Farooqui M, Aljadhey H, et al. cross sectional assessment of knowledge, attitude and practice towards Hepatiis B amng healthy population of Quetta, Pakistan. *BMC Public Health.* (2012) 12:692. doi: 10.1186/1471-2458-12-692
18. Kassahun CW, Mekonen AG. Knowledge, attitude, practice and their associated factors towards diabetes mellitus among non-diabetes community members of Bale zone administrative town, South East Ethiopia. a cross-sectional study. *PLoS ONE.* (2017) 12:e0170040. doi: 10.1371/journal.pone.0170040
19. Gafoor KA. Considerations in the Measurement of Awareness. *National Seminar on Emerging trends in education* (2012). Available online at: https://files.eric.ed.gov/fulltext/ED545374.pdf (accessed October 15, 2021).
20. Osagiede EF, Obekpa SA, Am IA, Tracy EO, Ehikioya JO, Johnbull J. Assessment of knowledge and seroprevalence of hepatitis B and C viral infection among health care personnel in a rural teaching hospital in South-South Nigeria. *J Environ Occupational Health.* (2020) 10:55–72. Available online at: https://www.jenvoh.com/abstract/assessment-of-knowledge-and-seroprevalence-of-hepatitis-b-and-c-viral-infection-among-health-care-personnel-in-a-rural-t-52567.html
21. Mahamat G, Kenmoe S, Akazong EW, Ebogo-Belobo JT, Mbaga DS, Bowo-Ngandji A, et al. Global prevalence of hepatitis B virus serological markers among healthcare workers: a systematic review and meta-analysis. *WJH.* (2021) 13:1190–202. doi: 10.4254/wjh.v13.i9.1190
22. Tepavčević DK, Kanazir M, Marić G, Zarić M, Lončarević G, Gazibara T. Hepatitis B-related awareness among health care workers in Belgrade, Serbia. *Vojnosanitetski Pregled.* (2020) 77:463–469. doi: 10.2298/VSP180227090K
23. Gyang MD, Madaki AJ, Dankyau M, Toma BO, Danjuma SA, Gyang BA. Prevalence and correlates of hepatitis B and C seropositivity among health care workers in a semi urban setting in north central Nigeria. *Highland Med Res J.* (2016) 16:75–9. Available online at: https://www.ajol.info/index.php/hmrj/article/view/148792
24. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ.* (2021) 372:n71. doi: 10.1136/bmj.n71
25. Stevens GA, Alkema L, Black RE, Boerma JT, Collins GS, Ezzati M, et al. Guidelines for accurate and transparent health estimates reporting: the GATHER statement. *PLoS Med.* (2016) 13:e1002056. doi: 10.1371/journal.pmed.1002056
26. Wells GA, Shea B, O'Connell D, Peterson J, Welch V, Losos M, et al. *The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyse* (2000)
27. StataCorp. *Stata Statistical Software: Release 16.* StataCorp LLC (2019).
28. Riley RD, Higgins JP, Deeks JJ. Interpretation of random effects meta-analyses. *BMJ.* (2011) 342:d549. doi: 10.1136/bmj.d549
29. Borenstein M, Hedges LV, Higgins JP, Rothstein HR. A basic introduction to fixed-effect and random-effects models for meta-analysis. *Res Synth Methods.* (2010) 1:97–111. doi: 10.1002/jrsm.12
30. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ.* (2003) 327:557–60. doi: 10.1136/bmj.327.7414.557
31. Djeriri K, Laurichesse H, Merle JL, Charof R, Abouyoub A, Fontana L, et al. Hepatitis B in moroccan health care workers. *Occup Med.* (2008) 58:419–24. doi: 10.1093/occmed/kqn071
32. Shao ER, Mboya IB, Gunda DW, Ruhangisa FG, Temu EM, Nkwama ML, et al. Seroprevalence of hepatitis B virus infection and associated factors among healthcare workers in northern Tanzania. *BMC Infect Dis.* (2018) 18:474. doi: 10.1186/s12879-018-3376-2
33. Hebo HJ, Gemeda DH, Abdusemed KA. Hepatitis B and C viral infection: prevalence, knowledge, attitude, practice, and occupational exposure among

healthcare workers of Jimma university medical center, Southwest Ethiopia. *Scientific World Journal.* (2019) 2019:1–11. doi: 10.1155/2019/9482607
34. Desalegn Z, Selsassie SG. Prevalence of hepatitis B surface antigen (HBsAg) among health professionals in public hospitals in Addis Ababa, Ethiopia. *Ethiopian J Health Dev.* (2013) 27:72–79. Available online at: https://www.ejhd.org/index.php/ejhd/article/view/314
35. Anagaw B, Shiferaw Y, Anagaw B, Belyhun Y, Erku W, Biadgelegn F, et al. Seroprevalence of hepatitis B and C viruses among medical waste handlers at Gondar town health institutions, Northwest Ethiopia. *BMC Res Notes.* (2012) 5:55. doi: 10.1186/1756-0500-5-55
36. Ngekeng S, Chichom-Mefire A, Nde P, Nsagha D, Nkuigue A, Tiogouo K, et al. Hepatitis B prevalence, knowledge and occupational factors among health care workers in Fako division, South West region Cameroon. *MRJI.* (2018) 23:1–9. doi: 10.9734/MRJI/2018/40445
37. Ijoma U, Meka I, Omotowo B, Nwagha T, Obienu O, Onodugo O, et al. Sero-prevalence of hepatitis B virus infection: a cross-sectional study of a large population of health care workers in Nigeria. *Niger J Clin Pract*. (2021) 24:38. Available online at: link.gale.com/apps/doc/A649545545/HRCA?u=anon~118ca5b4&sid=googleScholar&xid=2b45065f
38. Oladokun AO, Agidigbi EF, Oke MA, Otebolaku-Olajide TM, Adigun GA, Alao MA. Seroprevalence and knowledge of hepatitis B among healthcare workers in Saki, Southwest, Nigeria. *IOSR J Nurs Health Sci.* (2021) 10:9–13. doi: 10.9790/1959-1002050913
39. Muhammad AA, Ibrahim BC, Ramadan AM. Knowledge, attitude and practice regarding hepatitis B infection among nurses in public hospitals of Niger state, Nigeria. *J Obstet Gynaecol.* (2016) 12:1–9. doi: 10.9734/IJTDH/2016/18663
40. Awimero CE, Nelson EA, Yusuf M, Olaosebikan OF, Adeboye MAN, Adamu UG, et al. *Knowledge, awareness and prevalence of viral hepatitis among health care workers (HCWs) of the federal medical centre Bida, Nigeria* (2017).
41. Mbaawuaga EM, Hembah-Hilekaan SK, Iroegbu CU, Chibuogwu Ike A. Hepatitis B virus and human immunodeficiency virus infections among health care workers in some health care centers in Benue state, Nigeria. *Open J Med Micro.* (2019) 9:48–62. doi: 10.4236/ojmm.2019.92007
42. Akazong WE, Tume C, Njouom R, Ayong L, Fondoh V, Kuiate J-R. Knowledge, attitude and prevalence of hepatitis B virus among healthcare workers: a cross-sectional, hospital-based study in bamenda health district, NWR, Cameroon. *BMJ Open.* (2020) 10:e031075. doi: 10.1136/bmjopen-2019-031075
43. Rodrigue G, Ernest D, Marcel NN, Jeanne N. Determination of the prevalence of the HBs antigen and evaluation of the vaccination status against Hepatitis B among the staff of the Dschang district hospital in west Cameroon. G*SC Biol and Pharm Sci*. (2021) 15:128–139. doi: 10.30574/gscbps.2021.15.2.0127
44. Tatsilong HOP, Noubiap JJN, Nansseu JRN, Aminde LN, Bigna JJR, Ndze VN, et al. Hepatitis B infection awareness, vaccine perceptions and uptake, and serological profile of a group of health care workers in Yaoundé, Cameroon. *BMC Public Health.* (2016) 16:706. doi: 10.1186/s12889-016-3388-z
45. Qin Y-L, Li B, Zhou Y-S, Zhang X, Li L, Song B, et al. Prevalence and associated knowledge of hepatitis B infection among healthcare workers in Freetown, Sierra Leone. *BMC Infect Dis.* (2018) 18:315. doi: 10.1186/s12879-018-3235-1
46. Massaquoi TA, Burke RM, Yang G, Lakoh S, Sevalie S, Li B, et al. Cross sectional study of chronic hepatitis B prevalence among healthcare workers in an urban setting, Sierra Leone. *PLoS ONE.* (2018) 13:e0201820. doi: 10.1371/journal.pone.0201820
47. Mangkara B, Xaydalasouk K, Chanthavilay P, Kounnavong S, Sayasone S, Muller CP, et al. Hepatitis B virus in lao dentists: a cross-sectional serological study. *Ann Hepatol.* (2021) 22:100282. doi: 10.1016/j.aohep.2020.10.010
48. Nguyen T, Pham T, Tang HK, Phan L, Mize G, Lee WM, et al. Unmet needs in occupational health: prevention and management of viral hepatitis in healthcare workers in Ho Chi Minh city, Vietnam: a mixed-methods study. *BMJ Open.* (2021) 11:e052668. doi: 10.1136/bmjopen-2021-052668
49. Ptil S, Rao RS, Agarwal A. Awareness and risk perception of hepatitis B infection among auxiliary healthcare workers. *J Int Soc Prevent Communit Dent*. (2013) 3:67. doi: 10.4103/2231-0762.122434
50. Alqahtani JM, Abu-Eshy SA, Mahfouz AA, El-Mekki AA, Asaad AM. Seroprevalence of hepatitis B and C virus infections among health students and health care workers in the Najran region, southwestern Saudi Arabia: the need for national guidelines for health students. *BMC Public Health.* (2014) 14:577. doi: 10.1186/1471-2458-14-577
51. Memon MS, Ansari S, Rashid N, Khatri NK, Mirza MA, Jafri W. Hepatitis B vaccination status in health care workers of two universiry hospitals. *JLUMHS.* (2007) 48–51. doi: 10.22442/jlumhs.07610115
52. Aziz S, Tily HI, Rasheed K, Memon A, Jehangir K, Quraishy MS. Prevalence of H1V, hepatitis B and C amongst health workers of civil hospital karachi. *JPMA*. (2022) 52. Available online at: https://www.jpma.org.pk/PdfDownload/2142.pdf
53. Vaishali K, Zulfeequer C, Aanad R, Thakrar R, Alaparthi G, Kumar SK. Awareness in patients with COPD about the disease and pulmonary rehabilitation: a survey. *Lung India.* (2014) 31:134. doi: 10.4103/0970-2113.129837
54. Roien R, Mehterkhail S, Faizi MA, Haidari MH, Haidari E, Alimi B, et al. Knowledge, attitude and vaccination status of health care workers against hepatitis B virus infection in kabul. *Kateb Quarterly (Sci - Res).* (2019) 6: 119–32. Available online at: https://research.kateb.edu.af/dari/wp-content/uploads/sites/2/2019/12/9.pdf
55. Rayate AS, Barhate NR, Bhalge UU, Gavkare AM, Nagoba BS. Awareness about hepatitis B virus infection and vaccination among health care personnel at higher risk - a cross-sectional study. The European Journal of Innovative, Integrative and Translational Medicine. *Eur J Integr Trans Med.* (2021) 5. Available online at: https://www.resclinmed.eu/public/data_files/articles/136/article_136.pdf
56. Heinrich J. Occupational safety: selected cost and benefit implications of needlestick prevention devices for hospitals. *United States General Accounting Office* (2000).
57. Mahfouz M, Nguyen H, Tu J, Diaz CR, Anjan S, Brown S, et al. Knowledge and Perceptions of hepatitis B and hepatocellular carcinoma screening guidelines among trainees: a tale of three centers. *Dig Dis Sci.* (2020) 65:2551–61. doi: 10.1007/s10620-019-05980-1

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frontiers | Frontiers in Public Health

ORIGINAL RESEARCH
published: 09 June 2022
doi: 10.3389/fpubh.2022.897013

Multiplex Serology for Measurement of IgG Antibodies Against Eleven Infectious Diseases in a National Serosurvey: Haiti 2014–2015

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OPEN ACCESS

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Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: 15 March 2022
Accepted: 18 May 2022
Published: 09 June 2022

Citation:
Chan Y, Martin D, Mace KE, Jean SE, Stresman G, Drakeley C, Chang MA, Lemoine JF, Udhayakumar V, Lammie PJ, Priest JW and Rogier EW (2022) Multiplex Serology for Measurement of IgG Antibodies Against Eleven Infectious Diseases in a National Serosurvey: Haiti 2014–2015. Front. Public Health 10:897013. doi: 10.3389/fpubh.2022.897013

Background: Integrated surveillance for multiple diseases can be an efficient use of resources and advantageous for national public health programs. Detection of IgG antibodies typically indicates previous exposure to a pathogen but can potentially also serve to assess active infection status. Serological multiplex bead assays have recently been developed to simultaneously evaluate exposure to multiple antigenic targets. Haiti is an island nation in the Caribbean region with multiple endemic infectious diseases, many of which have a paucity of data for population-level prevalence or exposure.

Methods: A nationwide serosurvey occurred in Haiti from December 2014 to February 2015. Filter paper blood samples (n = 4,438) were collected from participants in 117 locations and assayed for IgG antibodies on a multiplex bead assay containing 15 different antigens from 11 pathogens: *Plasmodium falciparum*, *Toxoplasma gondii*, lymphatic filariasis roundworms, *Strongyloides stercoralis*, chikungunya virus, dengue virus, *Chlamydia trachomatis*, *Treponema pallidum*, enterotoxigenic *Escherichia coli*, *Entamoeba histolytica*, and *Cryptosporidium parvum*.

Results: Different proportions of the Haiti study population were IgG seropositive to the different targets, with antigens from *T. gondii*, *C. parvum*, dengue virus, chikungunya virus, and *C. trachomatis* showing the highest rates of seroprevalence. Antibody responses to *T. pallidum* and lymphatic filariasis were the lowest, with <5% of all samples IgG seropositive to antigens from these pathogens. Clear trends of increasing seropositivity and IgG levels with age were seen for all antigens except those from chikungunya virus and *E. histolytica*. Parametric models were able to estimate the rate of seroconversion and IgG acquisition per year for residents of Haiti.

Conclusions: Multiplex serological assays can provide a wealth of information about population exposure to different infectious diseases. This current Haitian study included IgG targets for arboviral, parasitic, and bacterial infectious diseases representing multiple

different modes of host transmission. Some of these infectious diseases had a paucity or complete absence of published serological studies in Haiti. Clear trends of disease burden with respect to age and location in Haiti can be used by national programs and partners for follow-up studies, resource allocation, and intervention planning.

Keywords: multiplex assay, IgG detection, Haiti, integrated serosurveillance, infectious disease, seroprevalence

INTRODUCTION

Tropical and other infectious diseases cause high morbidity and mortality worldwide, and many are co-endemic due to socioeconomic, environmental, climatological, and other factors (1). Epidemiology, control, and potential elimination of these diseases benefits from continued surveillance and monitoring for acute infection or past exposure. As symptomatic surveillance alone may not be a reliable indicator of infection for many tropical diseases, serological confirmation provides an effective way of estimating pathogen exposure within a population (2–6). Additionally, as infectious disease transmission is reduced in an area, standard diagnostic methods for many pathogens tend to provide less accurate estimates of true prevalence (7, 8). Serological assays that detect antibodies against pathogen-specific antigens are used for a variety of purposes such as providing history of infection of diseases within a population (9, 10), understanding transmission patterns (5), strategizing control and elimination efforts (11, 12), and assessing host immune status (13).

Conventionally, single-analyte detection methods such as Western blotting, lateral flow assays (LFAs), or enzyme-linked immunosorbent assays (ELISAs) have been used to detect human antibodies against infectious disease antigens. The bead-based multiplex platform for detecting and quantitating antibodies against multiple antigens is efficient for the concurrent analysis of an individual's serological profile to numerous infectious diseases (2, 9, 13, 14). Additional benefits include the time and reduced costs of multiplexing targets for several pathogens compared to traditional single-plex assays while remaining relatively easy to operate in a laboratory setting (15, 16). Thus, multiplex assays can offer a practical and more comprehensive understanding of epidemiologic patterns and co-endemic burdens of infectious diseases in an area (13, 17).

In this current study, a multiplex bead assay (MBA) was utilized to assess IgG antibody levels for 4,438 blood samples collected during a Haitian national community-based household survey that took place from December 2014 to February 2015. The MBA panel included 15 antigenic targets to evaluate exposure to 11 infectious diseases in the nation of Haiti. Data are displayed to estimate department-level and national-level seroprevalence estimates and trends by age categories.

MATERIALS AND METHODS

Ethical Approvals and Sample Collection

The study protocol was approved by the Haitian Ministry of Health. Participant consent (and parental assent if under 15 years) was verbal. The Haitian population was sampled from December 2014 to February 2015 as part of the Global Fund grant against malaria (Round 8) implemented by Population Services International (PSI) Haiti as Principal Recipient. Enumeration areas throughout the country (*sections d'énumération*, SDE) were chosen on a proportional sampling by predicted malaria risk strata as determined by predictive modeling (18). A target of 20 households were randomly selected by field teams within each SDE, and all members of the household were offered the opportunity to participate. Blood was collected by fingerprick on Whatman 903 Protein Saver cards (GE Healthcare, Chicago, IL), dried overnight, and individually stored in plastic bags with desiccant at −20° C until shipment to the Centers for Disease Control and Prevention in Atlanta, GA, USA. Samples were assigned unique identification numbers that were not traceable to the individual. A total of 4,535 persons were enrolled in the survey, of which 4,438 (97.9%) provided DBS for serological assays. Participants in the survey were aged 1–99 years, with a median number of 30 persons sampled per SDE and 117 total SDEs sampled throughout the country. For the Haiti tracking results continuously (TRaC) survey, the study protocol was approved by the Haitian Ministry of Health and approved as a non-research activity by the Center for Global Health Human Research Protection Office (HRPO), US Centers for Disease Control and Prevention (CDC; Center for Global Health determination #2015-04).

Samples from U.S. resident blood donors were used to represent a population of persons putatively seronegative to tropical diseases not endemic to the U.S. All blood samples were from consenting adults who had screened negative for HIV and hepatitis B viruses and had no reported history of international travel in the last 6 months, and use was approved by CDC's Center for Global Health Institutional Review Board under non-engagement in human subjects research status.

Antigens Used for Multiplex Bead Assay (MBA)

The 19-kDa fragment of the *P. falciparum* merozoite surface protein 1 (PfMSP1-19) was cloned from *P. falciparum* isolate 3D7 and expressed as previously described (17, 19, 20). The SAG2A antigen from *T. gondii* was cloned from the RH strain and produced recombinantly as described previously (21–23). The production of *Brugia malayi* roundworm recombinant antigens Bm33 and Bm14 have been described previously (24–27). *Wuchereria bancrofti* antigen Wb123 was a kind gift from T. Nutman (National Institutes of Health, Bethesda, MD) (28). The *Strongyloides stercoralis* NIE antigen (Ss-NIE-1) produced by L3 parasites was recombinantly produced as described previously

TABLE 1 | Infectious Diseases Represented and Antigens used for Multiplex Serology.

Pathogen	Disease	Antigen
Plasmodium falciparum	Malaria	PfMSP1-19
Toxoplasma gondii	Toxoplasmosis	Sag2A
Wuchereria bancrofti	Lymphatic filariasis	Wb123
Brugia malayi	Lymphatic filariasis	Bm14
Brugia malayi	Lymphatic filariasis	Bm33
Strongyloides stercoalis	Strongyloidiasis	NIE
Chikungunya virus (CHIKV)	Chikungunya	Chik E1
Dengue Virus Type 2 (DENV2)	Dengue	Dengue 2 VLP
Chlamydia spp.	Trachoma / Chlamydia	Pgp3
Chlalamydia trachomatis	Trachoma / Chlamydia	CT694
Treponema spp.	Yaws / Syphilis	rp17
Treponema pallidum	Yaws / Syphilis	TmpA
Enterotoxic *E. coli*	Diarrhea	ETEC-LTB
Entamoeba histolytica	Amoebiasis	LecA
Cryptosporidium parvum	Cryptosporidiosis	Cp23

(29, 30). The chikungunya virus envelope glycoprotein E1 was purchased through CTK Biotech (Porway, CA). The dengue virus serotype 2 virus-like particle was grown and isolated from transfected eukaryotic cell culture as described previously (31). The *Chlamydia trachomatis* antigens Pgp3 and CT694 were recombinantly expressed and purified as described previously (32). The recombinant *Treponema pallidum* antigen rp17 was purchased by Chembio Diagnostic Systems (Medford, NY) and recombinant TmpA through ViroGen (Boston, MA) and dialyzed overnight before bead coupling as described previously (2). Recombinant enterotoxigenic *E. coli* heat-labile enterotoxin B subunit (ETEC LTB) produced in *Pichia pastoris* was purchased from Sigma Aldrich (St. Louis, MO) (33). The *Entamoeba histolytica* LecA recombinant antigen was kindly provided by William Petri, Jr. (University of Virginia, Charlottesville, VA) and Joel Herbein (TechLab, Blacksburg, VA) (34, 35). The recombinant 27-kDa antigen from *Cryptosporidium parvum* (Cp23) has been previously described (36, 37). The antigen MBA panel is outlined in **Table 1** and **Supplementary Table 1**.

Antigen Binding to Beads

Antigens were covalently bound to polystyrene BioPlex® COOH beads (BioRad, Hercules, CA; 1715060XX) or Luminex® SeroMap beads (Luminex Corp, Austin, TX, L100-S0XX) by the commonly used EDC/Sulfo-NHS intermediate reaction. Previous comparisons between magnetic and non-magnetic beads have found comparable serological results between the two bead types (38–40). Reactive esters were formed on the carboxylated beads in the presence of 5 mg/mL EDAC (1-Ethyl-3-(3ℓ-dimethylaminopropyl)carbodiimide) (EMD Millipore; 341,006) and 5 mg/mL Sulfo-NHS (N-hydroxysulfosuccinimide, ThermoScientific; 24,510) under light agitation for 20 min. Carboxyl to primary amine crosslinking took place in buffer at pH 5 (0.85% NaCl and 0.05 M 2-(N-morpholino)ethanesulfonic acid, MES) or at pH 7.2 (phosphate buffered saline, PBS, 10 mM PO_4 and 0.85% NaCl) under light agitation for 2 h. Nonspecific protein binding was blocked by BSA incubation (PBS pH 7.2, + 1% bovine serum albumin, BSA) for 30 min, and beads were resuspended in blocking buffer with the addition of 0.02% NaN_3 and protease inhibitors as described previously (25). Each antigen had been previously optimized to the appropriate coupling concentration and pH: CHIK-E1 (pH 5, 17 μg/mL); Dengue 2 VLP (pH 7.2, 30 μg/mL); *Brugia malayi* Bm14 (pH 7.2, 120 μg /mL); *Wuchereria bancrofti* Wb123 (pH 7.2, 120 μg/mL); Bm33 (pH 6.0 with 2M urea, 20 μg/mL); Enterotoxigenic *E. coli* (ETEC) heat-labile enterotoxin beta subunit (pH 5, 30 μg/mL); *Chlamydia trachomatis* Pgp3 pCT03 (pH 7.2, 120 μg/mL); *C. trachomatis* CT694 (pH 7.2, 30 μg/mL); *Treponema pallidum* TmpA (pH 5, 15 μg/mL); *T. pallidum* rp17 (pH 5, 15 μg /mL); *Toxoplasma gondii* SAG2A (pH 5, 12.5 μg/mL); *Plasmodium falciparum* MSP1 (pH 5, 30 μg/mL); *Strongyloides stercoralis* NIE (pH 7.2 with 2M urea, 20 μg/mL); *Cryptosporidium parvum* Cp23 (pH 5, 12.5 μg/mL); *Entamoeba histolytica* LecA (pH 5.0, 30 ug/mL). As an internal control to test for non-specific binding or any serum IgG against *Schistosoma japonicum* glutathione-*S*-transferase (GST) fused to recombinant antigens (41), a bead was included in the panel that was coupled to GST (coupling concentration of 15 μg/mL at pH5).

Blood Spot Elution and MBA

A 6 mm circular punch corresponding to approximately 10 μL whole blood was taken from the center of each blood spot for elution. Samples were shaken in 100 μL protein elution buffer overnight at room temperature (PBS pH 7.2, 0.05% Tween-20, 0.05% NaN3) and stored at 4°C until further processing. Elution from blood spots provided an initial 1:10 dilution. Samples were further diluted 1:40 in Luminex sample diluent [PBS, 0.5% Polyvinyl alcohol (Sigma), 0.8% Polyvinylpyrrolidone (Sigma), 0.1% casein (ThermoFisher, Waltham, MA), 0.5% BSA (Millipore, Burlington, MA), 0.3% Tween-20, 0.02% NaN3, and 3 μg/mL *E. coli* extract to prevent non-specific binding] for a final whole blood dilution of 1:400, corresponding to a serum dilution of approximately 1:800 with the assumption of 50% hematocrit in whole blood. This serum dilution in the range of serum dilution previously utilized by our group and found to be able to provide accurate seroestimates for all infectious disease antigens on our multiplex panel.

For the MBA, a mix was prepared for all bead regions in 5 mL reagent diluent (PBS, 0.05% Tween20, 0.5% BSA, 0.02% NaN_3). Filter bottom plates (Multiscreen 1.2 μm, Millipore) were pre-wetted with PBS-T, 50 μL bead mix (approximately 1,500 beads/analyte) added to wells and wells washed twice, and beads incubated with the sample in duplicate for 1.5 h under gentle shaking. Secondary antibodies tagged with biotin (1:500 monoclonal mouse anti-human total IgG (Southern Biotech); 1:625 monoclonal mouse anti-human IgG_4 (Southern Biotech) were incubated with the beads for 45 min, and subsequent incubation with streptavidin-phycoerythrin (1:200, Invitrogen) for 30 min. Plates had a final wash incubation with reagent diluent for 30 min and were read on a Bio-Plex 200 machine to generate the median fluorescence intensity (MFI) signal for 50 beads/analyte. Background (bg) MFI was generated from blank wells containing only sample diluent, and this value was

FIGURE 1 | Sampling Locations in Haiti from the 2014/2015 Nationwide Survey. Each of the 117 sampling locations are indicated by a black dot. Boundaries of the ten Haitian departments are also shown.

subtracted from each antigen's raw MFI to give an MFI-bg. The mean of MFI-bg values from duplicate wells was used for analysis, though previous studies from our group and others have also shown high reproducibility for MBAs when only singlet assay wells are run (42). Due to limited volumes of antigen-coupled beads, not all samples had data collected for IgG against all antigens. Total number of persons with IgG antibody data collected for each antigen is summarized in **Supplementary Table 1**.

Determining Seropositivity Thresholds

Determining the MFI-bg assay signal threshold above which an individual was determined to be IgG positive (seropositive) for each of the antigens in the study was accomplished through different approaches. The MFI-bg signal thresholds are all shown in **Supplementary Table 1**. No cutoff estimate was available for Enterotoxigenic *E. coli* LT B subunit antigen as a negative population was not available for comparison (43).

Non-exposed U.S. Residents Approach

For all infectious diseases assayed in this study that were endemic only to tropical areas, the antigen panel for those diseases was assayed with blood samples from 92 U.S. residents who were unlikely to have been exposed to these infectious diseases. From this population of U.S. residents, the lognormal mean MFI signal plus three standard deviations were exponentiated to derive the seropositivity signal threshold (in MFI-bg units). This approach was used for the malaria (PfMSP1-19), lymphatic filariasis (Bm14, Bm33, Wb123), stronglyloides (NIE), chikungunya virus (E1), dengue virus (VLP), and *E. histolytica* (LecA) antigens. Histograms for MFI-bg signal distribution for all antigens included in this study for the Haitian study population vs. the US resident sample set are shown in **Supplemental Figure 1**.

Mixture Model Approach

Some pathogens included in this analysis are endemic in the US, and individuals cannot be assumed to be seronegative. To determine seropositive and seronegative subpopulations in a dataset, a 2-component mixture model strategy was used (**Supplementary Figure 2**). From the first distribution (component) of log-transformed data, which is assumed to be the distribution of the signal of the putative seronegative population, the mean plus three standard deviations were exponentiated to derive the seropositivity signal threshold (in MFI-bg units). This approach was used for the *T. gondii* (SAG2A), *C. trachomatis* (Pgp3, CT694), and *T. pallidum* (rp17, TmpA) antigens.

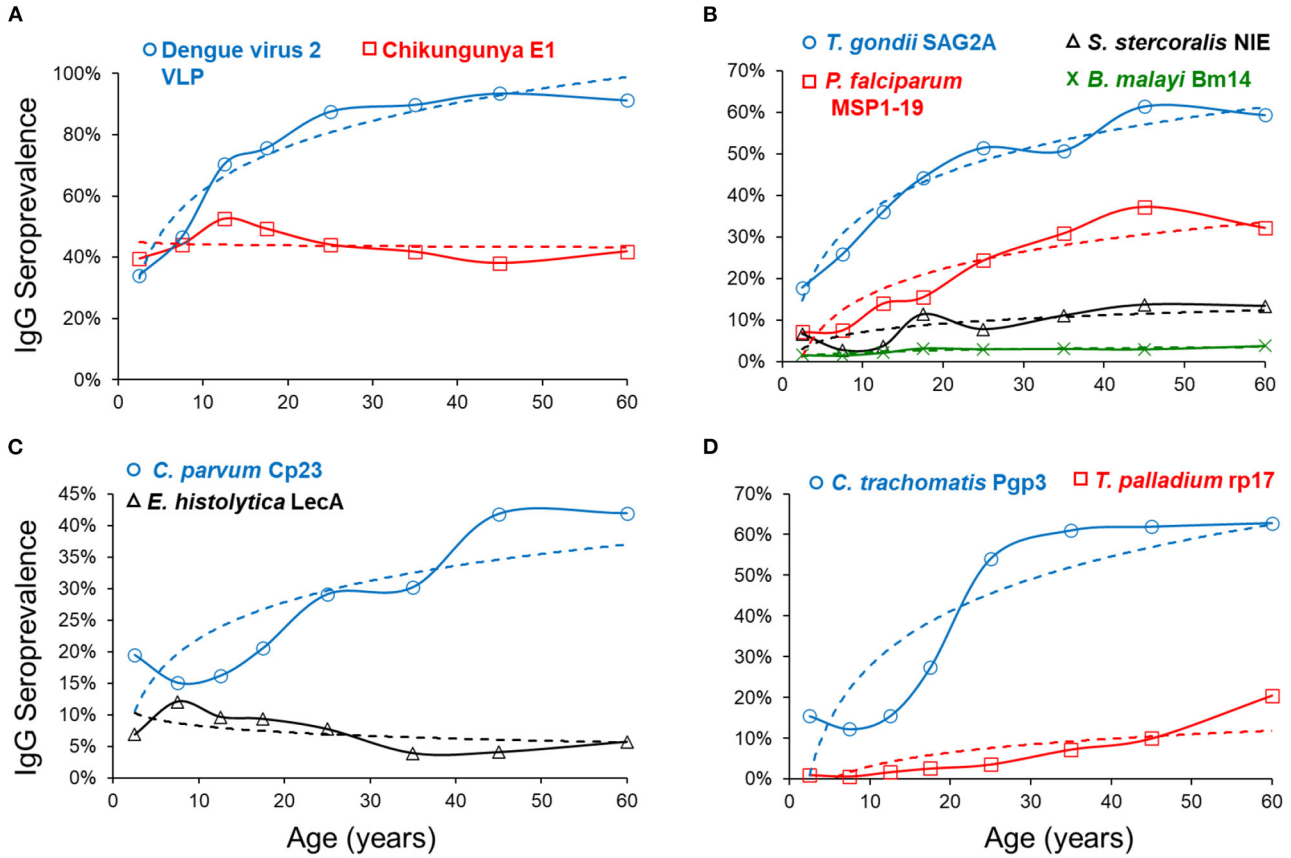

FIGURE 2 | Seroprevalence by Age and Regression Fitting for Selected Antigens. By age category, mean seroprevalence (as plotted by percent IgG positive) was plotted on y-axis and age on x-axis. Dashed regression lines were fitted to a logarithmic equation for positivity by age and grouped into similar categories of arboviruses **(A)**, non-waterborne parasites **(B)**, waterborne pathogens **(C)**, and other bacterial pathogens **(D)**.

Receiver Operator Characteristic Curve Analysis

For responses to the *C. parvum* Cp23 antigen, the typical approach is to use a panel of Western blot positives and negatives to establish a cutoff by Receiver operator characteristic curve analysis (44). The beads used in this study were previously determined to have a cutoff of 1,870 MFI-bg by this method (45). However, since this study used only 50% of the serum concentration in each assay as the previous work, the cutoff was adjusted to 935 MFI-bg to account for the difference.

Statistical Analysis

Statistical procedures were performed in SAS® 9.4 software (SAS Institute, Cary, NC), at the 5% significance level (alpha: 0.05), applying both Anderson-Darling and Cramér-von Mises null hypotheses. Descriptive statistics and histograms in SAS software were summarized with corresponding 95% confidence interval, using the PROC FREQ, PROC UNIVARIATE, and PROC MEANS statements. Ages were categorized into eight mutually exclusive groups (0–4 years, 5–10 years, 11–15 years, 16–20 years, 21–30 years, 31–40 years, 41–50 years, and >50 years) due to observed differences between antibody concentrations of younger and older populations. unweighted, two-component finite mixture models (FMM) of log-transformed data were compared using the FMM procedure in SAS. Logistic and linear regressions were created using PROC REG and PROC GLM. Analysis of potential correlation between antigens was produced through PROC LOGIT, and PROC CORR statements. Seroprevalence estimates were not generated for the enterotoxigenic *E. coli* LT B subunit antigen as exposure in the population is ubiquitous (43).

RESULTS

As part of the 2014/2015 TRaC survey, 117 communities were sampled throughout Haiti, as shown in **Figure 1**. Though the sampling design was powered to present nationwide malaria estimates to gauge the relative disparities in seroprevalence among three different malaria risk strata, IgG seropositivity to different antigens is displayed by Haitian departments in **Supplementary Table 2**. For the Ouest department, estimates for the city of Port-au-Prince were displayed separately from the more rural areas, as this is a broad urban area that is densely populated. A large percentage of the population in all departments was seropositive to dengue virus serotype 2

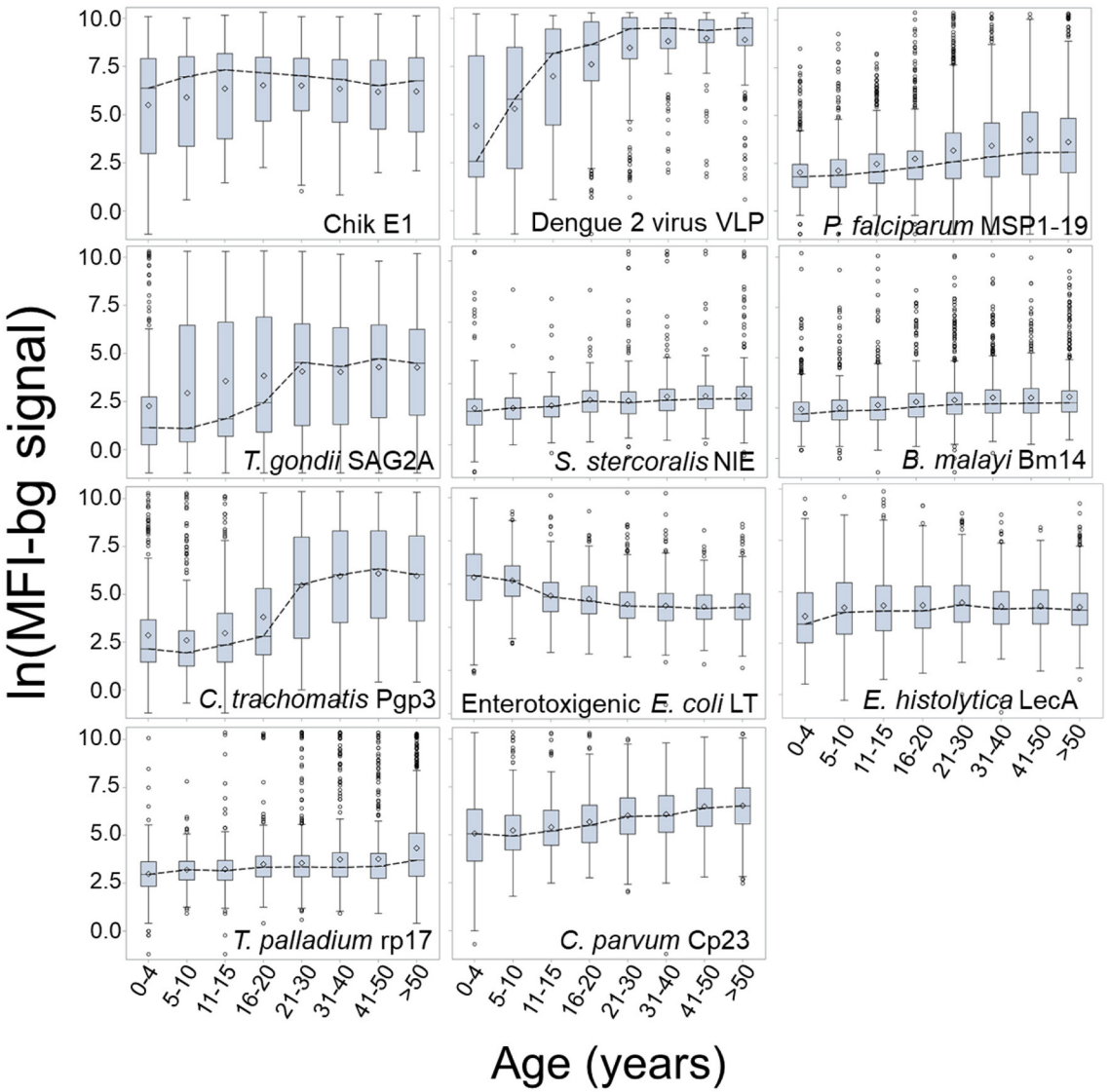

FIGURE 3 | Dynamics in MFI-bg IgG Signal by Age for Selected Antigens. Data shown as boxplots for log-transformed median fluorescence intensity minus background (MFI-bg) assay signal for selected antigens by age category. Boxplots are displayed as interquartile rage (IQR) with whiskers extending 1.5x above and below IQR and circles for observations beyond this. A dashed line connects boxplot medians.

(dengue 2 VLP) and chikungunya (Chik E1), ranging from 65.0–91.9% and 20.8–59.3%, respectively. Seropositivity to antigens for the parasitic pathogens *C. parvum* (Cp23) and *T. gondii* (SAG2A) was found to be high as well, with 26.1 and 45.0% of all persons seropositive, respectively. Among the lymphatic filariasis antigens, Wb123 and Bm14 never exceeded 3.6% seroprevalence for all persons within a department, whereas Bm33 ranged from 5.0–9.5% seroprevalence. Seropositivity to *P. falciparum* for the whole study population was 21.8% and ranged from 12.0% in urban Port-au-Prince to 37.1% in the rural Center department. The two antigens for *C. trachomatis* (Pgp3 and CT694) provided similar seropositivity estimates for the whole study population (41.7% vs. 35.2% respectively), as well as the two antigens for *T. pallidum* (rp17: 6.6% vs. TmpA: 5.0%).

Figure 2 depicts mean seroprevalence to a subset of antigens by age, grouped into categories of disease or pathogen similarities. In **Figure 2**, single antigens were included to represent *W. bancrofti*, *C. trachomatis,* and *T. pallidum*. Seropositivity data were fitted to a logarithmic equation with intercept and slope estimates for all antigens (**Supplementary Table 3**). Positive slope estimates were highest for the dengue virus, *T. gondii*, and the *C. trachomatis* antigens. Only two antigens provided negative slopes (chik E1 and *E. histolytica* LecA), both of which were non-significant. When modeling for seropositivity by age, logarithmic regression

provided strong goodness of fit (R^2 >0.75) for PfMSP1-19, SAG2A, Wb123, Bm14, dengue 2 VLP, pgp3, and Ct694 (**Supplementary Table 3**).

Figure 3 presents the log-transformed MFI-bg by age category for the same selected antigens in **Figure 2** with the addition of ETEC-LTB, and regression estimates for the effect of age on IgG titer are shown in **Supplementary Table 4**. When modeling for acquisition or loss of MFI-bg assay signal by age, most estimates for the age parameter were found to be statistically significant within the regression model (**Supplementary Table 4**), with only the Wb123, Bm33, and chik E1 antigens not significant. Though the *C. parvum* Cp23 showed clear increases in seropositivity with age, the other antigen from a waterborne pathogen (*E. histolytica* LecA) showed a consistent negative slope when modeling for seropositivity (**Supplementary Table 3**) or MFI-bg signal (**Supplementary Table 4**) by age. All other antigens had consistent positive slopes for seropositivity and IgG acquisition with age except SAG2A (positive slope for seropositivity by age, negative slope for MFI-bg IgG response by age) and chik E1 (negative slope for seropositivity with age, positive slope for MFI-bg IgG intensity by age). Slopes for seropositivity and antibody acquisition by age were positively correlated (**Supplementary Figure 3**). For the ETEC-LT and dengue 2 VLP antigens, regression estimates were also generated for only young children to show IgG response's rapid loss (or gain) throughout their first years of life (**Supplementary Table 4**). The correlation of MFI-bg signal among all antigens is shown in **Supplementary Table 5**. Specifically, antigens from the same pathogens showed the highest correlation values: Pgp3 and Ct694 (rho = 0.83), rp17 and TmpA (rho = 0.64), Bm14, and Wb123 (rho = 0.60).

DISCUSSION

In this report, we show the capacity of the MBA to investigate exposure to multiple diseases of interest from samples gathered from December 2014 to February 2015 during a nationwide malaria survey in Haiti. Analyses took into account participants' ages and areas of residence, but future studies with demographic and spatial data could allow for more informative epidemiological outputs (46, 47). Haitian estimates for population-level exposure to each of the pathogens will be described below as grouped by infectious disease category and include examples of how serological data generated by MBA can be applied.

Arboviruses

This serosurvey represented two arboviruses: chikungunya and dengue virus serotype 2. Our study found the transmission dynamics for these two arboviruses to be quite different, with the dengue virus antigen providing a population seroprevalence curve indicating increased likelihood of lifetime exposure as persons aged. Beginning immediately after birth, seroprevalence and IgG levels rise rapidly in the first 15 years of life. By age 30, Haitians had a >80% chance of being exposed to the dengue virus and typically displayed very high IgG titers. In contrast, the seroprevalence curve by age categories for chikungunya was mostly flat, likely indicative of the recent introduction of the disease into the country in 2014 and the rapid spread of this arbovirus among all age groups (48).

Waterborne Pathogens

Antigens for two waterborne pathogens are included in this survey: *C. parvum* and Enterotoxigenic *Escherichia coli* (ETEC), both of which are important causes of childhood diarrhea (49). We observed small but consistent increases in antibody levels to the Cp23 antigen with age, similar to what was previously seen in Haiti (35). We found the IgG levels against the ETEC-LT antigen to be high in the youngest ages; levels decrease during the first 15 years of life and remain low among older age groups. This finding may suggest immune tolerance to this *E. coli* antigen, as noted for another *E. coli* antigen, lipopolysaccharide (LPS) (50). In this study population, seropositivity to the *E. histolytica* LecA antigen peaked around 10 years of age, though IgG levels also slowly increased with persons' age. The use of this antigen to assess *E. histolytica* serology has been very limited but has shown clear increases in seroconversion during the first years of life (35), which mirrors exposure dynamics to this parasite in children (51, 52).

Other Parasites

Plasmodium falciparum is transmitted through *Anopheles* spp. mosquitos and is the primary causative agent of malaria in Haiti (53, 54). Our current study showed a consistent increase in seropositivity, and the population antibody levels with age. Modeled PfMSP1-19 seropositivity estimated that a person aged 45 years would be 3.7-fold more likely to be seropositive than someone aged 5 years. Malaria serology data can be applied to understanding areas of ongoing transmission (17). The percentages of population seropositive to PfMSP1-19 were lowest in the Port au Prince metropolitan area (12.0%) and highest in the Center department (37.1%), consistent with known lower *P. falciparum* exposure in urban areas due to poor mosquito vector habitat (55).

Toxoplasmosis is a zoonotic infection caused by a single celled parasitic protozoan, *Toxoplasma gondii.* Transmission occurs when eating undercooked, contaminated meat or by oral ingestion of the oocyst stage when humans come into contact with infected cat feces (56). As *T. gondii* infection typically goes into latency in the human host (57), seropositivity indicates lifetime infection (58). Our study found reliable increases in IgG prevalence to the SAG2A antigen with age, indicating past and current stable transmission of this parasite. Our data estimated that by the time a Haitian reaches 25 years of age, the risk of exposure to *T. gondii* is approximately 50%. A previous cohort study in Leogane, Haiti, had estimated seroprevalence to *T. gondii* of 25% among children 0–12 years old, similar to the estimates of 22% presented in this study (21).

Adult lymphatic filariasis (LF) worms live in the lymph system, and microfilariae circulate in the blood, and this disease is found throughout the tropical and subtropical areas of the world (33, 59). In Haiti, LF is caused by the roundworm *Wucheria bancrofti*, and current targets for elimination will benefit from continual serosurveillance efforts as the endemic range is reduced

(60). In this study, we employed three filarial antigens to identify seropositive persons in this low-transmission setting. A low proportion of the population was seropositive to the worm antigens, with low (but positive) slope estimates indicating an increase in seroprevalence and IgG titer with age, as has been seen in other low transmission settings (10).

The roundworm *Strongyloides stercoralis* is the causative agent of strongyloidiasis. This soil-transmitted helminth is transmitted when the skin comes into contact with free-living larvae in contaminated soil, and most people infected are asymptomatic (32, 61, 62). *Strongyloides* seroprevalence estimates have previously been proposed as a pragmatic tool for population exposure (41, 63), and our study found an overall low Haitian seroprevalence (<15% in any department) to the *S. stercoralis* NIE antigen with increasing seroprevalence by age. The lowest seroprevalence was found in Artibonite (4.6%), the highest in Grand'Anse (14.9%), and a nationwide seroprevalence of 9.2%. Surveillance for roundworms through serological data could be utilized for directing mass drug administration campaigns in areas where active infection and parasite prevalence are difficult to estimate.

C. trachomatis and *T. pallidum* are sexually transmitted infections (STIs, causing chlamydia, and syphilis, respectively) that also cause non-venereal infections in children. Ocular *C. trachomatis* infection can lead to trachoma, the world's leading infectious cause of blindness (33). Yaws, a skin infection that can lead to bone and soft tissue damage, is caused by *T. pallidum* spp *pertenue*. The childhood infection and STI are serologically indistinct for each of these pathogens. Our study found consistent increases in antibodies against *C. trachomatis* antigens Pgp3 and CT694 with age, with the most pronounced increases during the ages of sexual debut, likely indicative of STI exposure. Haiti is not thought to be endemic for trachoma, and these data do not suggest high trachoma transmission intensity (11, 64, 65).

Antibodies to the treponemal antigen rp17 represent historical exposure, whereas TmpA-specific antibodies represent recent infection (66). Only 0.52% of under 15–year-olds were antibody-positive to both antigens. No data on yaws are available from Haiti, while the sampling frame may not be granular enough to detect pockets of yaws transmission, these data are suggestive of little or no yaws transmission in the surveyed areas. An interesting contrast in seroprevalence of *C. trachomatis* and *T. pallidum* was seen in the adult population, with approximately 60% of the >20–year-olds seropositive to chlamydial antigens and <10% seropositive to antigens indicative of syphilis.

Limitations to this study include that laboratory-based serological assays depend on the sensitivity and specificity of the assays used for IgG detection. Some diseases do not have well-defined antigens that are known to elicit strong IgG responses or have antigens with known IgG cross-reactivity issues with responses from other pathogens. Additionally, some infectious diseases (such as those infecting the respiratory or gastrointestinal tract) are known to elicit strong IgA responses, so the measurement of only IgG in this study may have reduced the sensitivity of seropositivity measurement. As this study only used one serum dilution, for IgG quantification purposes against each individual antigen, different serum dilutions for each target would be most optimal. Seropositivity cut-off values need to be evaluated by each group employing these antigen targets, especially in elimination programs where populations have decreased exposure and finding active infections is difficult (10, 17). Increasing survey sample sizes can help overcome statistically biased estimates and increase precision. Defining seroconversion, boosting effects after re-exposure, antibody decay, and immunocompetency of the host are all primary concerns for some infectious diseases. Continued investigation is required to correctly interpret serology data for different diseases. The seropositivity cutoff for Cp23 was determined 1:800 serum dilution, whereas this was previously at done at a 1:400 dilution (35). This may have overestimated the true cutoff value and resulted in lower-than-expected seroprevalence values. Among the factors listed above, another limitation to this study is that the sampling design was powered for the modeled malaria active infection prevalence in Haiti (18), but the cluster design, enrollment in households, and attempt at nationwide representativeness can still provide insight into population exposure to other infectious diseases. Additionally, the survey was cross-sectional, and regression estimates of data representing trends over time assume consistent dynamics of endemicities and transmission intensity. Future studies in Haiti should investigate if similar findings would be observed.

This nationwide Haiti survey for malaria provided an opportunity to employ a 15-antigen MBA panel measuring IgG presence and titer to 11 infectious diseases. As some pathogens are cleared from the host within a few days or weeks, assaying for antibodies greatly augments the window of time in which to survey for exposure in a population. In addition, accurate surveillance for recurrent-type infectious diseases can be confounded by asymptomatic infections, poor access to healthcare or healthcare reporting, or poor diagnostics. Understanding the co-endemic disease burden on a national level allows for collaborative strategies of multiple stakeholders focused on combined interventions at the community level. Multiple programs, especially those targeting multiple diseases, can be monitored simultaneously through one well-designed, population-representative integrated survey (41, 60, 67). A follow-up cross-sectional survey throughout Haiti with the same sampling design could prove valuable for monitoring changes in seroestimates in the Haitian population since this 2014–2015 survey.

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 Haitian Ministry of Health; Center for Global Health Human Research Protection Office (HRPO), US Centers for Disease Control and Prevention. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SJ and JL facilitated field studies. MC, VU, PL, JP, and ER conceptualized the experiments. DM, PL, and JP provided laboratory supplies. ER collected data. YC and ER performed data analyses and drafted the manuscript. DM, KM, GS, CD, MC, and JP provided technical expertise. All authors reviewed and approved the manuscript for submission.

FUNDING

Global Fund to Fight AIDS, Tuberculosis and Malaria funded the Haiti nationwide survey. Funds for publication are from the Centers for Disease Control and Prevention.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Haitian survey teams and participants for their involvement in this study. The authors thank T. Nutman (NIH), W. Petri, Jr. (University of VA), and J. Herbein (TechLab) for reagent contribution, Gretchen Cooley (CDC) for assistance with bead couplings, and Delynn Moss (CDC) for assistance in running the multiplex immunoassay.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.897013/full#supplementary-material

REFERENCES

1. WHO. *Accelerating Work to Overcome the Global Impact of Neglected Tropical Diseases—A Roadmap for Implementation*. WHO (2012).
2. Cooley GM, Mitja O, Goodhew B, Pillay A, Lammie PJ, Castro A, et al. Evaluation of multiplex-based antibody testing for use in large-scale surveillance for yaws: a comparative study. *J Clin Microbiol.* (2016) 54:1321–5. doi: 10.1128/JCM.02572-15
3. Kurkjian KM, Vaz LE, Haque R, Cetre-Sossah C, Akhter S, Roy S, et al. Application of an improved method for the recombinant k 39 enzyme-linked immunosorbent assay to detect visceral leishmaniasis disease and infection in Bangladesh. *Clin Diagn Lab Immunol.* (2005) 12:1410–5. doi: 10.1128/CDLI.12.12.1410-1415.2005
4. Won KY, Kanyi HM, Mwende FM, Wiegand RE, Goodhew EB, Priest JW, et al. Multiplex serologic assessment of schistosomiasis in Western Kenya: antibody responses in preschool aged children as a measure of reduced transmission. *Am J Trop Med Hyg.* (2017) 96:1460–7. doi: 10.4269/ajtmh.16-0665
5. Rogier EW, Moss DM, Mace KE, Chang M, Jean SE, Bullard SM, et al. Use of bead-based serologic assay to evaluate chikungunya virus epidemic, Haiti. *Emerg Infect Dis.* (2018) 24:995–1001. doi: 10.3201/eid2406.171447
6. van Hooij A, Tjon Kon Fat EM, Batista da. Silva M, Carvalho Bouth R, Cunha Messias AC, Gobbo AR, et al. Evaluation of immunodiagnostic tests for leprosy in Brazil, China and Ethiopia. *Sci Rep.* (2018) 8:17920. doi: 10.1038/s41598-018-36323-1
7. Smith JL, Auala J, Tambo M, Haindongo E, Katokele S, Uusiku P, et al. Spatial clustering of patent and sub-patent malaria infections in northern Namibia: Implications for surveillance and response strategies for elimination. *PLoS ONE.* (2017) 12:e0180845. doi: 10.1371/journal.pone.0180845
8. Arnold BF, van der Laan MJ, Hubbard AE, Steel C, Kubofcik J, Hamlin KL, et al. Measuring changes in transmission of neglected tropical diseases, malaria, and enteric pathogens from quantitative antibody levels. *PLoS Negl Trop Dis.* (2017) 11:e0005616. doi: 10.1371/journal.pntd.0005616
9. Rogier E, Moss DM, Chard AN, Trinies V, Doumbia S, Freeman MC, et al. Evaluation of immunoglobulin g responses to plasmodium falciparum and plasmodium vivax in malian school children using multiplex bead assay. *Am J Trop Med Hyg.* (2017) 96:312–8. doi: 10.4269/ajtmh.16-0476
10. Won KY, Robinson K, Hamlin KL, Tufa J, Seespesara M, Wiegand RE, et al. Comparison of antigen and antibody responses in repeat lymphatic filariasis transmission assessment surveys in American Samoa. *PLoS Negl Trop Dis.* (2018) 12:e0006347. doi: 10.1371/journal.pntd.0006347
11. Pinsent A, Solomon AW, Bailey RL, Bid R, Cama A, Dean D, et al. The utility of serology for elimination surveillance of trachoma. *Nat Commun.* (2018) 9:5444. doi: 10.1038/s41467-018-07852-0
12. Won KY, Sambou S, Barry A, Robinson K, Jaye M, Sanneh B, et al. Use of antibody tools to provide serologic evidence of elimination of lymphatic filariasis in the Gambia. *Am J Trop Med Hyg.* (2018) 98:15–20. doi: 10.4269/ajtmh.17-0371
13. Fujii Y, Kaneko S, Nzou SM, Mwau M, Njenga SM, Tanigawa C, et al. Serological surveillance development for tropical infectious diseases using simultaneous microsphere-based multiplex assays and finite mixture models. *PLoS Negl Trop Dis.* (2014) 8:e3040. doi: 10.1371/journal.pntd.0003040
14. Lammie PJ, Moss DM, Brook Goodhew E, Hamlin K, Krolewiecki A, West SK, et al. Development of a new platform for neglected tropical disease surveillance. *Int J Parasitol.* (2012) 42:797–800. doi: 10.1016/j.ijpara.2012.07.002
15. Mohanty I, Dash M, Sahu S, Narasimham MV, Panda P, Padhi S. Seroprevalence of chikungunya in southern odisha. *J Family Med Prim Care.* (2013) 2:33–6. doi: 10.4103/2249-4863.109939
16. Elshal MF, McCoy JP. Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. *Methods.* (2006) 38:317–23. doi: 10.1016/j.ymeth.2005.11.010
17. Rogier E, Wiegand R, Moss D, Priest J, Angov E, Dutta S, et al. Multiple comparisons analysis of serological data from an area of low plasmodium falciparum transmission. *Malar J.* (2015) 14:436. doi: 10.1186/s12936-015-0955-1
18. Sutherland LJ, Cash AA, Huang YJ, Sang RC, Malhotra I, Moormann AM, et al. Serologic evidence of arboviral infections among humans in Kenya. *Am J Trop Med Hyg.* (2011) 85:158–61. doi: 10.4269/ajtmh.2011.10-0203
19. Blackman MJ, Ling IT, Nicholls SC, Holder AA. Proteolytic processing of the plasmodium falciparum merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor-like domains. *Mol Biochem Parasitol.* (1991) 49:29–33. doi: 10.1016/0166-6851(91)90127-R
20. Egan A, Waterfall M, Pinder M, Holder A, Riley E. Characterization of human T- and B-cell epitopes in the C terminus of plasmodium falciparum merozoite surface protein 1: evidence for poor T-cell recognition of polypeptides with numerous disulfide bonds. *Infect Immun.* (1997) 65:3024–31. doi: 10.1128/iai.65.8.3024-3031.1997
21. Priest JW, Moss DM, Arnold BF, Hamlin K, Jones CC, Lammie PJ. Seroepidemiology of toxoplasma in a coastal region of Haiti: multiplex bead assay detection of immunoglobulin G antibodies that recognize the SAG2A antigen. *Epidemiol Infect.* (2015) 143:618–30. doi: 10.1017/S0950268814001216
22. Prince JB, Auer KL, Huskinson J, Parmley SF, Araujo FG, Remington JS. Cloning, expression, and cDNA sequence of surface antigen P22 from toxoplasma gondii. *Mol Biochem Parasitol.* (1990) 43:97–106. doi: 10.1016/0166-6851(90)90134-8
23. Parmley SF, Sgarlato GD, Mark J, Prince JB, Remington JS. Expression, characterization, and serologic reactivity of recombinant surface antigen P22 of Toxoplasma gondii. *J Clin Microbiol.* (1992) 30:1127–33. doi: 10.1128/jcm.30.5.1127-1133.1992
24. Hamlin KL, Moss DM, Priest JW, Roberts J, Kubofcik J, Gass K, et al. Longitudinal monitoring of the development of antifilarial antibodies and acquisition of Wuchereria bancrofti in a highly endemic area of Haiti. *PLoS Negl Trop Dis.* (2012) 6:e1941. doi: 10.1371/journal.pntd.0001941

25. Moss DM, Priest JW, Boyd A, Weinkopff T, Kucerova Z, Beach MJ, et al. Multiplex bead assay for serum samples from children in Haiti enrolled in a drug study for the treatment of lymphatic filariasis. *Am J Trop Med Hyg.* (2011) 85:229–37. doi: 10.4269/ajtmh.2011.11-0029
26. Chandrashekar R, Curtis KC, Li BW, Weil GJ. Molecular characterization of a Brugia malayi intermediate filament protein which is an excretory-secretory product of adult worms. *Mol Biochem Parasitol.* (1995) 73:231–9. doi: 10.1016/0166-6851(95)00122-H
27. Dissanayake S, Xu M, Nkenfou C, Piessens WF. Molecular cloning and serological characterization of a Brugia malayi pepsin inhibitor homolog. *Mol Biochem Parasitol.* (1993) 62:143–6. doi: 10.1016/0166-6851(93)90191-Y
28. Kubofcik J, Fink DL, Nutman TB. Identification of Wb123 as an early and specific marker of Wuchereria bancrofti infection. *PLoS Negl Trop Dis.* (2012) 6:e1930. doi: 10.1371/journal.pntd.0001930
29. Rascoe LN, Price C, Shin SH, McAuliffe I, Priest JW, Handali S. Development of Ss-NIE-1 recombinant antigen based assays for immunodiagnosis of strongyloidiasis. *PLoS Negl Trop Dis.* (2015) 9:e0003694. doi: 10.1371/journal.pntd.0003694
30. Ravi V, Ramachandran S, Thompson RW, Andersen JF, Neva FA. Characterization of a recombinant immunodiagnostic antigen (NIE) from Strongyloides stercoralis L3-stage larvae. *Mol Biochem Parasitol.* (2002) 125:73–81. doi: 10.1016/S0166-6851(02)00214-1
31. Chang GJ, Hunt AR, Holmes DA, Springfield T, Chiueh TS, Roehrig JT, et al. Enhancing biosynthesis and secretion of premembrane and envelope proteins by the chimeric plasmid of dengue virus type 2 and Japanese encephalitis virus. *Virology.* (2003) 306:170–80. doi: 10.1016/S0042-6822(02)00028-4
32. Goodhew EB, Priest JW, Moss DM, Zhong G, Munoz B, Mkocha H, et al. CT694 and pgp3 as serological tools for monitoring trachoma programs. *PLoS Negl Trop Dis.* (2012) 6:e1873. doi: 10.1371/journal.pntd.0001873
33. Rezaee MA, Rezaee A, Moazzeni SM, Salmanian AH, Yasuda Y, Tochikubo K, et al. Expression of Escherichia coli heat-labile enterotoxin B subunit (LTB) in saccharomyces cerevisiae. *J Microbiol.* (2005) 43:354–60.
34. Houpt E, Barroso L, Lockhart L, Wright R, Cramer C, Lyerly D, et al. Prevention of intestinal amebiasis by vaccination with the entamoeba histolytica gal/galnac lectin. *Vaccine.* (2004) 22:611–7. doi: 10.1016/j.vaccine.2003.09.003
35. Moss DM, Priest JW, Hamlin K, Derado G, Herbein J, Petri WA Jr, et al. Longitudinal evaluation of enteric protozoa in Haitian children by stool exam and multiplex serologic assay. *Am J Trop Med Hyg.* (2014) 90:653–60. doi: 10.4269/ajtmh.13-0545
36. Perryman LE, Jasmer DP, Riggs MW, Bohnet SG, McGuire TC, Arrowood MJ, et al. Cloned gene of cryptosporidium parvum encodes neutralization-sensitive epitopes. *Mol Biochem Parasitol.* (1996) 80:137–47. doi: 10.1016/0166-6851(96)02681-3
37. Priest JW, Kwon JP, Moss DM, Roberts JM, Arrowood MJ, Dworkin MS, et al. Detection by enzyme immunoassay of serum immunoglobulin G antibodies that recognize specific Cryptosporidium parvum antigens. *J Clin Microbiol.* (1999) 37:1385–92. doi: 10.1128/JCM.37.5.1385-1392.1999
38. Mazhari R, Brewster J, Fong R, Bourke C, Liu ZSJ, Takashima E, et al. A comparison of non-magnetic and magnetic beads for measuring IgG antibodies against Plasmodium vivax antigens in a multiplexed bead-based assay using Luminex technology (Bio-Plex 200 or MAGPIX). *PLoS ONE.* (2020) 15:e0238010. doi: 10.1371/journal.pone.0238010
39. Coughlin MM, Matson Z, Sowers SB, Priest JW, Smits GP, van der Klis FRM, et al. Development of a measles and rubella multiplex bead serological assay for assessing population immunity. *J Clin Microbiol.* (2021) 59:e02716–20. doi: 10.1128/JCM.02716-20
40. Ondigo BN, Park GS, Ayieko C, Nyangahu DD, Wasswa R, John CC. Comparison of non-magnetic and magnetic beads multiplex assay for assessment of plasmodium falciparum antibodies. *PeerJ.* (2019) 7:e6120. doi: 10.7717/peerj.6120
41. Priest JW, Jenks MH, Moss DM, Mao B, Buth S, Wannemuehler K, et al. Integration of multiplex bead assays for parasitic diseases into a national, population-based serosurvey of women 15–39 years of age in Cambodia. *PLoS Negl Trop Dis.* (2016) 10:e0004699. doi: 10.1371/journal.pntd.0004699
42. Ubillos I, Aguilar R, Sanz H, Jimenez A, Vidal M, Valmaseda A, et al. Analysis of factors affecting the variability of a quantitative suspension bead array assay measuring IgG to multiple Plasmodium antigens. *PLoS ONE.* (2018) 13:e0199278. doi: 10.1371/journal.pone.0199278
43. Arnold BF, Martin DL, Juma J, Mkocha H, Ochieng JB, Cooley GM, et al. Enteropathogen antibody dynamics and force of infection among children in low-resource settings. *Elife.* (2019) 8:e45594. doi: 10.7554/eLife.45594
44. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem.* (1993) 39:561–77. doi: 10.1093/clinchem/39.4.561
45. Mosites E, Miernyk K, Priest JW, Bruden D, Hurlburt D, Parkinson A, et al. Giardia and cryptosporidium antibody prevalence and correlates of exposure among Alaska residents, 2007–2008. *Epidemiol Infect.* (2018) 146:888–94. doi: 10.1017/S095026881800078X
46. Biggs J, Raman J, Cook J, Hlongwana K, Drakeley C, Morris N, et al. Serology reveals heterogeneity of plasmodium falciparum transmission in northeastern South Africa: implications for malaria elimination. *Malar J.* (2017) 16:48. doi: 10.1186/s12936-017-1701-7
47. Kerkhof K, Sluydts V, Heng S, Kim S, Pareyn M, Willen L, et al. Geographical patterns of malaria transmission based on serological markers for falciparum and vivax malaria in Ratanakiri, Cambodia. *Malar J.* (2016) 15:510. doi: 10.1186/s12936-016-1558-1
48. Poirier MJ, Moss DM, Feeser KR, Streit TG, Chang GJ, Whitney M, et al. Measuring Haitian children's exposure to chikungunya, dengue and malaria. *Bull World Health Organ.* (2016) 94:817–25A. doi: 10.2471/BLT.16.173252
49. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet.* (2013) 382:209–22. doi: 10.1016/S0140-6736(13)60844-2
50. Seeley JJ, Ghosh S. Molecular mechanisms of innate memory and tolerance to LPS. *J Leukoc Biol.* (2017) 101:107–19. doi: 10.1189/jlb.3MR0316-118RR
51. Calegar DA, Monteiro KJL, Bacelar PAA, Evangelista BBC, Almeida MM, Dos Santos JP, et al. Epidemiology, species composition and genetic diversity of tetra- and octonucleated Entamoeba spp in different Brazilian biomes. *Parasit Vectors.* (2021) 14:160. doi: 10.1186/s13071-021-04672-y
52. Hailu GG, Ayele ET. Assessment of the prevalence of intestinal parasitic infections and associated habit and culture-related risk factors among primary schoolchildren in Debre Berhan town, Northeast Ethiopia. *BMC Public Health.* (2021) 21:112. doi: 10.1186/s12889-020-10148-y
53. Frederick J, Saint Jean Y, Lemoine JF, Dotson EM, Mace KE, Chang M, et al. Malaria vector research and control in Haiti: a systematic review. *Malar J.* (2016) 15:376. doi: 10.1186/s12936-016-1436-x
54. Lemoine JF, Boncy J, Filler S, Kachur SP, Fitter D, Chang MA. Haiti's commitment to malaria elimination: progress in the face of challenges, 2010–2016. *Am J Trop Med Hyg.* (2017) 97:43–8. doi: 10.4269/ajtmh.16-0902
55. Doumbe-Belisse P, Kopya E, Ngadjeu CS, Sonhafouo-Chiana N, Talipouo A, Djamouko-Djonkam L, et al. Urban malaria in sub-Saharan Africa: dynamic of the vectorial system and the entomological inoculation rate. *Malar J.* (2021) 20:364. doi: 10.1186/s12936-021-03891-z
56. Hill D, Dubey JP. Toxoplasma gondii: transmission, diagnosis and prevention. *Clin Microbiol Infect.* (2002) 8:634–40. doi: 10.1046/j.1469-0691.2002.00485.x
57. Zainodini N, Zare-Bidaki M, Abdollahi SH, Afrooz M, Ziaali N, Ebrahimian M, et al. Molecular and serological detection of acute and latent toxoplasmosis using real-time PCR and ELISA techniques in blood donors of rafsanjan city, iran, 2013. *Iran J Parasitol.* (2014) 9:336–41.
58. Bela SR, Oliveira Silva DA, Cunha-Junior JP, Pirovani CP, Chaves-Borges FA, Reis de. Carvalho F, et al. Use of SAG2A recombinant toxoplasma gondii surface antigen as a diagnostic marker for human acute toxoplasmosis: analysis of titers and avidity of IgG and IgG1 antibodies. *Diagn Microbiol Infect Dis.* (2008) 62:245–54. doi: 10.1016/j.diagmicrobio.2008.05.017
59. Joseph H, Maiava F, Naseri T, Silva U, Lammie P, Melrose W. Epidemiological assessment of continuing transmission of lymphatic filariasis in Samoa. *Ann Trop Med Parasitol.* (2011) 105:567–78. doi: 10.1179/2047773211Y.0000000008
60. Knipes AK, Lemoine JF, Monestime F, Fayette CR, Direny AN, Desir L, et al. Partnering for impact: integrated transmission assessment surveys for lymphatic filariasis, soil transmitted helminths and malaria in Haiti. *PLoS Negl Trop Dis.* (2017) 11:e0005387. doi: 10.1371/journal.pntd.0005387

61. Paradies P, Iarussi F, Sasanelli M, Capogna A, Lia RP, Zucca D, et al. Occurrence of strongyloidiasis in privately owned and sheltered dogs: clinical presentation and treatment outcome. *Parasit Vectors.* (2017) 10:345. doi: 10.1186/s13071-017-2275-5
62. Greaves D, Coggle S, Pollard C, Aliyu SH, Moore EM. Strongyloides stercoralis infection. *BMJ.* (2013) 347:f4610. doi: 10.1136/bmj.f4610
63. Krolewiecki AJ, Lammie P, Jacobson J, Gabrielli AF, Levecke B, Socias E, et al. A public health response against strongyloides stercoralis: time to look at soil-transmitted helminthiasis in full. *PLoS Negl Trop Dis.* (2013) 7:e2165. doi: 10.1371/journal.pntd.0002165
64. Kim JS, Oldenburg CE, Cooley G, Amza A, Kadri B, Nassirou B, et al. Community-level chlamydial serology for assessing trachoma elimination in trachoma-endemic Niger. *PLoS Negl Trop Dis.* (2019) 13:e0007127. doi: 10.1371/journal.pntd.0007127
65. Woodhall SC, Gorwitz RJ, Migchelsen SJ, Gottlieb SL, Horner PJ, Geisler WM, et al. Advancing the public health applications of chlamydia trachomatis serology. *Lancet Infect Dis.* (2018) 18:e399–407. doi: 10.1016/S1473-3099(18)30159-2
66. Ijsselmuiden OE, Schouls LM, Stolz E, Aelbers GN, Agterberg CM, Top J, et al. Sensitivity and specificity of an enzyme-linked immunosorbent assay using the recombinant DNA-derived treponema pallidum protein TmpA for serodiagnosis of syphilis and the potential use of TmpA for assessing the effect of antibiotic therapy. *J Clin Microbiol.* (1989) 27:152–7. doi: 10.1128/jcm.27.1.152-157.1989
67. Solomon AW, Engels D, Bailey RL, Blake IM, Brooker S, Chen JX, et al. A diagnostics platform for the integrated mapping, monitoring, and surveillance of neglected tropical diseases: rationale and target product profiles. *PLoS Negl Trop Dis.* (2012) 6:e1746. doi: 10.1371/journal.pntd.0001746

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ORIGINAL RESEARCH
published: 24 June 2022
doi: 10.3389/fmed.2022.921101

Revisiting IgG Antibody Reactivity to Epstein-Barr Virus in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and Its Potential Application to Disease Diagnosis

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Infections by the Epstein-Barr virus (EBV) are often at the disease onset of patients suffering from Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS). However, serological analyses of these infections remain inconclusive when comparing patients with healthy controls (HCs). In particular, it is unclear if certain EBV-derived antigens eliciting antibody responses have a biomarker potential for disease diagnosis. With this purpose, we re-analyzed a previously published microarray data on the IgG antibody responses against 3,054 EBV-related antigens in 92 patients with ME/CFS and 50 HCs. This re-analysis consisted of constructing different regression models for binary outcomes with the ability to classify patients and HCs. In these models, we tested for a possible interaction of different antibodies with age and gender. When analyzing the whole data set, there were no antibody responses that could distinguish patients from healthy controls. A similar finding was obtained when comparing patients with non-infectious or unknown disease trigger with healthy controls. However, when data analysis was restricted to the comparison between HCs and patients with a putative infection at their disease onset, we could identify stronger antibody responses against two candidate antigens (EBNA4_0529 and EBNA6_0070). Using antibody responses to these two antigens together with age and gender, the final classification model had an estimated sensitivity and specificity of 0.833 and 0.720, respectively. This reliable case-control discrimination suggested the use of the antibody levels related to these

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Edited by:
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Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 15 April 2022
Accepted: 26 May 2022
Published: 24 June 2022

Citation:
Sepúlveda N, Malato J, Sotzny F, Grabowska AD, Fonseca A, Cordeiro C, Graça L, Biecek P, Behrends U, Mautner J, Westermeier F, Lacerda EM and Scheibenbogen C (2022) Revisiting IgG Antibody Reactivity to Epstein-Barr Virus in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and Its Potential Application to Disease Diagnosis. Front. Med. 9:921101. doi: 10.3389/fmed.2022.921101

candidate viral epitopes as biomarkers for disease diagnosis in this subgroup of patients. To confirm this finding, a follow-up study will be conducted in a separate cohort of patients.

Keywords: Epstein-Barr virus, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, antigen mimicry, biomarker discovery, patient stratification

INTRODUCTION

Infections by the ubiquitous Epstein-Barr virus (EBV) are linked to multiple sclerosis, rheumatoid arthritis, systemic erythematosus lupus, lymphomas, among other known diseases (1–3). A less-known disease where EBV infections are also important is Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) (4–6). The hallmark symptom of this condition is an unexplained but persistent fatigue that cannot be alleviated by rest and that can increase upon minimal physical and emotional effort (7, 8). In ME/CFS, acute EBV infections are reported by a subset of patients at the onset of their symptoms (9, 10). Reactivation of latent EBV infections has also been described during the disease course (11). However, current evidence remains inconclusive on whether the prevalence of these reactivations is either higher or lower in patients than in healthy controls (12). This conflicting evidence notwithstanding, ME/CFS patients show deficient B- and T-cell responses against EBV and altered antibody profiles when compared with healthy controls (10, 13–15). Finally, CD4+ T cells recognizing self-peptides on HLA-DR15, the strongest genetic risk factor for multiple sclerosis, have been shown to cross-react with peptides derived from EBV (16). Multiple sclerosis patients share many symptoms with the ones suffering from ME/CFS (17–19). EBV antigens were also reported to share sequence homology with human peptides derived from the myelin basic protein (20–22), lactoperoxidase (23), and anoctamin-2 (24, 25). These observations suggest that molecular mimicry between human and EBV-derived antigens could play a role in the pathogenesis of ME/CFS. This suggestion is in line with our recent hypothesis that links the pathogenesis of ME/CFS to chronically activated immune responses (26). Our assumption raises the possibility that the immune system of some ME/CFS patients is oscillating between an activation state that attempts controlling latent herpesviruses infections and the suppression of deleterious autoimmune responses *via* the activation of regulatory T cells (26). Thus, considering the growing body of evidence that links EBV infection to the pathogenesis of ME/CFS, studies that aim at elucidating underlying mechanisms are needed.

A major problem in investigating ME/CFS is the inexistence of a robust biomarker that could ascertain the disease diagnosis. In the past, different discovery studies suggested certain cytokines, antibodies against self and non-self-antigens, microRNAs, and methylation markers as potential disease biomarkers (27). Antibodies against EBV antigens are of particular interest as disease biomarkers given the above evidence connecting this virus with the disease and routine application of serological assays in the clinical practice. However, EBV antigens included in commercial kits are mostly markers of exposure to the infection and are unable to distinguish between patients with ME/CFS and healthy controls (28). This distinction can only be made when comparing a subset of clinically diagnosed ME/CFS patients with an EBV infection trigger to healthy controls (10). A serological evaluation of antibodies against less-studied EBV antigens did not identify any that could be used as a specific disease biomarker (29). However, this antibody evaluation was done using a limited number of EBV-derived antigens and no subgroup analysis was performed. The lack of patient stratification in ME/CFS studies reduces the chance of reproducing the same findings in follow-up studies (27, 30). Therefore, it is still possible to identify alternative antigens whose antibody responses could be used as disease biomarkers for a subgroup of patients.

Recently, we analyzed antibody responses against more than 3,000 overlapping antigens derived from 14 EBV proteins (23). The aim of this study was to extract an antibody signature against EBV in ME/CFS patients when compared to healthy controls. In the present study, we extended the analysis of the obtained data with the specific objective of optimizing biomarker discovery. In particular, we compared patients with or without an infectious trigger at disease onset to healthy controls in order to discover EBV-derived antigens whose antibody responses could be used for ME/CFS diagnosis.

MATERIALS AND METHODS

Study Participants

Ninety-two ME/CFS patients were recruited between 2011 and 2015 at the Charité outpatient clinic for immunodeficiencies at the Institute of Medical Immunology in the Charité Universitatsmedizin Berlin, Germany. Additional fifty individuals were recruited from the employees of the same clinic, who self-reported to be healthy and to not suffer from fatigue. However, neither clinical nor laboratory assessment was performed to confirm the healthy status of those individuals. ME/CFS patients and healthy controls were matched for gender and age (**Table 1**) with 50% of women and an overall average of ∼43 years of age. Fifty-four out of 92 patients (58.7%) reported an acute infection at their disease onset, whilst the remaining 38 patients (41.3%) reported either a disease trigger other than an infection, did not know their disease onset or the information about the disease trigger was missing. These two subgroups were also matched for age and gender (**Table 1**).

TABLE 1 | Basic characteristics of ME/CFS patients and healthy controls, where *p*-values refer to the comparison between ME/CFS groups and healthy controls.

	Female			Age, years	
Group	***N***	**%**	***P*-value**	**Mean (age range)**	***P*-value**
Healthy controls	50	50.0	N/A	42.4 (25-61)	N/A
ME/CFS (all)	92	51.1	0.901	43.7 (25-66)	0.453
With infectious trigger	54	50.0	∼1.000	43.2 (17–66)	0.585
Unknown trigger or without infectious trigger	38	52.6	0.807	44.4 (24-66)	0.679

Peptide Array

Data under analyses refer to the signal intensities derived from IgG antibody responses to 3,054 EBV-associated peptides measured by a seroarray described in detail in the original study (23). These peptides consisted of partially overlapping 15 amino acids (15-mer) and covered the full length of the following proteins (**Supplementary Table 1**): BALF-2, BALF-5, BFRF-3, BLLF-1, BLLF-3, BLRF-2, BMRF-1, BZLF-1, EBNA-1, EBNA-3, EBNA-4, EBNA-6, LMP-1, and LMP-2. The 15-mer peptides overlapped in 11 amino acids. The amino-acid sequences of these peptides were representative of the following EBV strains: AG876 (West Africa, EBV type 2), B95-8 (USA, EBV type 1), GD1 (China, EBV type 1), Cao (China, EBV type 1), Raji (Nigeria, EBV type 1), and P3HR-1 (Nigeria, EBV type 2). These data are freely available in Supplementary File S1 of the original study (23).

Statistical Analysis

We used the Chi-square test to compare ME/CFS patients to healthy controls in terms of gender distribution. The non-parametric Mann-Whitney test was used to compare the medians of the respective age distributions. There was evidence for age- and gender-matched distributions if the *p*-values of these tests were greater than the significance level of 0.05.

We first performed a multivariate analysis using (i) the classical principal component analysis (PCA) and (ii) computing different correlation matrices using Spearman's correlation coefficient (which is invariant to monotonic changes in the scale of the data, is robust against the presence of outliers, and does not depend on the normality assumption). We then performed linear discriminant analyses (LDA) to determine the best linear combination of all the antibody responses that could distinguish ME/CFS patients and their subgroups from healthy individuals. A similar analysis was done to compare the two subgroups of ME/CFS patients.

The outcome of each LDA was the estimated classification probability for each individual. These estimated probabilities were then analyzed by the respective receiver operating characteristic (ROC) curve where 1-specificity and sensitivity are plotted against each other as a function of the cutoff of the underlying classification probability. After computing each ROC curve, we calculated the respective area under the curve (AUC) and its 95% confidence interval to determine the accuracy of the classification irrespective of the cut-off used. In general, an AUC = 0.50 is indicative of a complete random classification of the individuals, while AUC = 1.00 implies that the constructed classifier perfectly predicts the true class membership of each individual.

We performed further antibody-wide association analyses related to the following comparisons (or classification exercises): (i) healthy controls versus all the ME/CFS patients; (ii) healthy controls versus ME/CFS patients with an infectious trigger; (iii) healthy controls versus ME/CFS patients with a non-infectious or unknown trigger; and (iv) ME/CFS patients with an infectious trigger versus the remaining ME/CFS patients. In each association analysis, we first estimated three regression models: logistic model, probit model, and complementary log-log model. In these models, the disease status was the outcome variable, age and gender were the respective covariates. To determine the best link function for the outcome variable, we selected the model with the lowest Akaike's information criterion (AIC). For the best link function ("the null model"), we estimated the respective ROC and its AUC as described above.

We fitted five different logistic models, including the main effects and all the interaction terms related to age, gender, and the antibody response under analysis: (i) a model with main effects only and no interaction terms; (ii) a model with an interaction term between age and the antibody response; (iii) a model with an interaction term between gender and the antibody response; (iv) a model with two interaction terms between age and the antibody response and between gender and the antibody response; (v) a model with all two-way and three-way interaction terms related to age, gender, and the antibody response. We compared each of these models with the null one using Wilks's likelihood ratio test, where low *p*-values provide evidence for these models, including effects of an antibody response. We reported the minimum *p*-value obtained from these model comparisons. Finally, we adjusted the minimum *p*-values of each analysis. This adjustment was made using the Benjamini-Yekutieli procedure ensuring a global false discovery rate (FDR) of 5% under the assumption of dependent tests (31). In this analysis, adjusted p-values < 0.05 indicated statistically significant results.

To filter out redundant antibody responses, we pooled all the significant antibody responses in a single model. The effect and interaction terms of these antibody responses were defined according to the most significant model obtained in the previous stage of analysis. We performed a backward stepwise model selection. The resulting model was finally evaluated in terms of predictive performance using ROC analysis as described above.

The above analysis was primarily done for the whole data set irrespective of the ME/CFS subgroups. We repeated the same analysis to compare each subgroup of ME/CFS patients (with infectious and non-infectious or unknown disease trigger) with the healthy controls. Finally, we repeated the analysis to compare the two subgroups of ME/CFS patients.

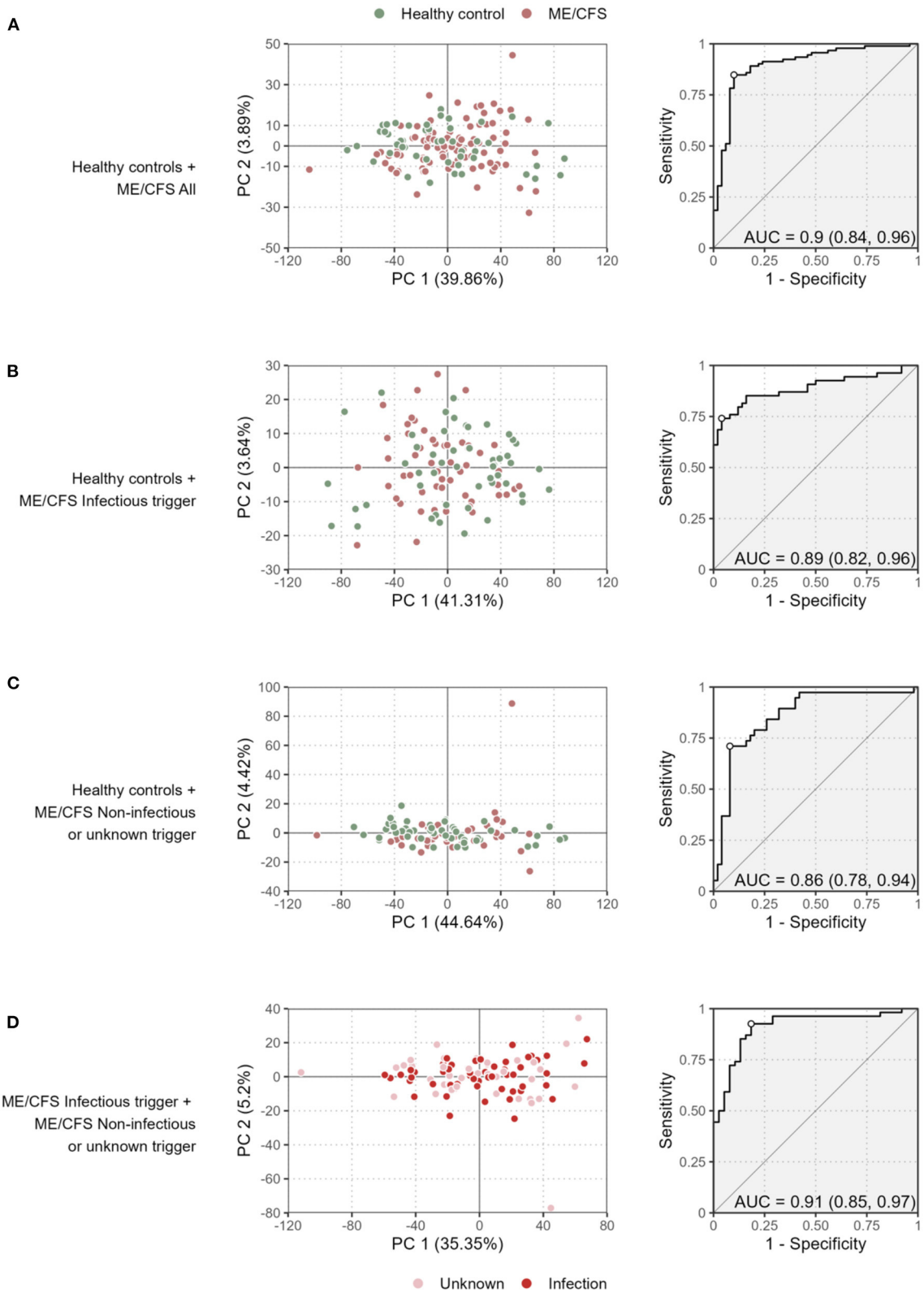

FIGURE 1 | Preliminary multivariate analysis of the data. Scatterplots of the first two principal components (left plots) and the ROC curve and its AUC of the respective LDA (right plots) when comparing all the ME/CFS patients to healthy controls **(A)**, ME/CFS patients with an infectious trigger to healthy controls **(B)**, ME/CFS patients with a non-infectious or unknown trigger to healthy controls **(C)**, and ME/CFS patients with an infectious trigger to the remaining patients **(D)**. The percentage of the variance explained by each principal component is shown in each axis within brackets.

Statistical Software

The statistical analysis was performed in the R software version 4.0.3 with core functions and the following packages: MASS v7.3-56 to perform stepwise model selection (32), pROC v1.18.0 to estimate the ROC curve and the respective AUC (33), OptimalCutpoints v1.1-5 to estimate the optimal cutoff and the associated sensitivity/specificity (34). The full reproducible code is freely available from NS or JMal upon request.

RESULTS

Principal Component and Linear Discriminant Analyses

We first performed a PCA to discriminate patients with ME/CFS and their subgroups from healthy controls (**Figures 1A–C**). A similar analysis was done for discriminating patients with an infectious trigger from the remaining patients (**Figure 1D**).

The proportion of variance explained by the first principal component varied from 35.4% (**Figure 1D**) to 44.6% (**Figure 1C**) referring to the comparisons between the two subgroups of ME/CFS patients, and between healthy controls and patients with non-infectious or unknown disease trigger, respectively. These high estimates suggested that different antibody levels were correlated with each other. This interpretation was confirmed by determining the distributions of Spearman's correlation coefficient between all possible pairs of antibodies using data from each study group (**Supplementary Figure 1**). In particular, the antibody levels were positively correlated with each other with median correlation estimates of 0.56, 0.56, 0.40, and 0.48 for healthy controls, all the ME/CFS patients, ME/CFS patients with an infectious disease trigger, and the remaining ME/CFS patients, respectively. Interestingly, the median correlation estimate was decreased in ME/CFS patients with an infectious trigger when compared to other study groups. This finding suggested that the production of the antibodies against the EBV-derived antigens could be reduced in these patients when compared to healthy controls or patients with non-infectious or unknown disease trigger.

The visualization of the first two components did not reveal a clear discrimination between healthy controls and ME/CFS patients (or their subgroups). To improve this analysis, we then performed different LDAs in search of a linear combination of the antibody measurements that could be used for disease diagnosis. The performance of the constructed classifiers ranged from 0.86 (**Figure 1C**) to 0.91 (**Figure 1D**) referring to the classification of healthy controls and ME/CFS patients with non-infectious or unknown disease trigger and the classification of the two subgroups of ME/CFS patients, respectively. Therefore, the results of this analysis indicate that the antibody data could discriminate different study groups.

Antibody-Wide Association Analysis

The next step of the analysis was to identify specific antibody responses that could be used to discriminate the different study groups. With this purpose, we first determined the best "null" model among the logistic, probit, and complementary log-log models. All of them included age and gender and their interaction as covariates for each comparison between any two study groups (**Supplementary Table 2**). The best "null" models were the following: (i) complementary log-log - comparison between healthy controls and all the ME/CFS patients [AUC = 0.574; 95% CI = (0.475; 0.672)]; (ii) probit-comparison between healthy controls and ME/CFS patients with an infectious trigger [AUC = 0.606; 95% CI = (0.496; 0.715)]; (iii) complementary log-log - comparison between healthy controls and ME/CFS patients with a non-infectious or unknown trigger [AUC = 0.556; 95% CI = (0.429; 0.683)]; and (iv) logit - comparison between the two subgroups of ME/CFS groups [AUC = 0.596; 95% CI = (0.471; 0.720)]. The 95% confidence interval for the AUC of these null models included 0.50 and therefore, the respective predicted classification was consistent with a random guess. Such a result was in agreement with the age and gender matching between different study groups and healthy controls (**Table 1**).

TABLE 2 | Estimates of the final complementary log-log model to discriminate ME/CFS patients with an infectious disease trigger from healthy controls.

Model term	Coefficient estimate (SE)	*P*-value
Intercept	10.67 (10.33)	0.302
Age (in years)	−0.49 (0.26)	0.060
Gender (Woman)	−17.33 (6.85)	0.011
EBNA4_0529	2.25 (1.09)	0.039
EBNA6_0070	−5.62 (3.09)	0.069
Age × Gender	0.07 (0.04)	0.070
Gender × EBNA6_0070	4.05 (1.75)	0.021
Age × EBNA6_0070	0.15 (0.08)	0.062

We performed further antibody-wide association analyses controlling for a global FDR of 5%. The comparison between healthy controls and all the ME/CFS patients did not identify any significant antibody associations with the disease (**Figure 2A**). The top 5 antibodies, although not statistically significant, were EBNA6_0066, BLRF2_0005, EBNA4_0392, EBNA4_0497, and EBNA4_0529 (adjusted p-values = 0.181, 0.326, 0.326, 0.326, and 0.326, respectively).

When the comparison was limited to healthy controls and ME/CFS patients with an infectious trigger, we identified three significant antibodies related to the following antigens (**Figure 2B**): EBNA6_0066, EBNA6_0070, and EBNA4_0529 (adjusted p-values = 0.005, 0.005, 0.038, respectively). The first two antigens were shared between AG876, B95-8, and GD1 strains, while the third one was derived from the B95-8 strain. We compared ME/CFS patients with non-infectious or unknown disease trigger to healthy controls, and found no significant differences in the antibody responses (**Figure 2C**). The same finding was obtained when we compared the two subgroups of ME/CFS patients (**Figure 2D**). The top 5 antibodies related to these analyses can be found in **Supplementary Table 3**.

Analysis of Candidate Antigens for Classifying ME/CFS Patients With an Infectious Trigger

We then analyzed in detail the impact of the antibody levels against the three candidate antigens on the classification of ME/CFS patients with an infectious trigger. Antibody levels

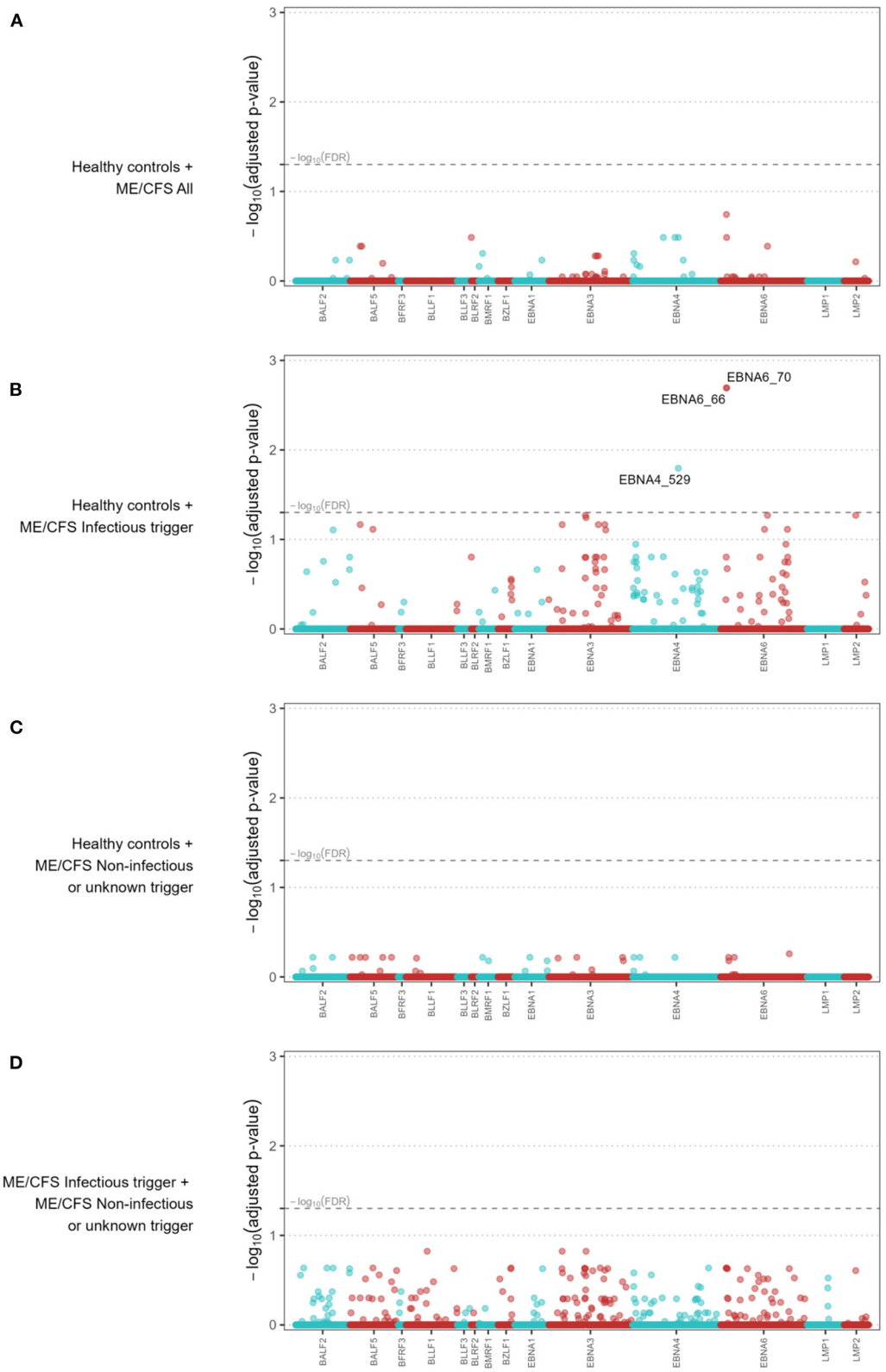

FIGURE 2 | Antibody-wide association analyses when comparing all the ME/CFS patients to healthy controls **(A)**, ME/CFS patients with an infectious trigger to healthy controls **(B)**, ME/CFS patients with a noninfectious or unknown trigger to healthy controls **(C)**, and ME/CFS patients with an infectious trigger to the remaining patients **(D)**. The *x*-axes comprise each antibody while the *y*-axes represent the $-\log_{10}$(adjusted *p*-value) of the respective association. In the *x*-axes, the antibodies

(Continued)

FIGURE 2 | were ordered alphabetically first by the protein name and then by the starting point of the antigen within the protein. Adjusted p-values were calculated according to the Benjamini-Yekutieli procedure for a global FDR of 5% under the assumption of dependent data. Dashed line represents the threshold for statistical significance (i.e., $-\log_{10}(\text{FDR} = 0.05)$) and $-\log_{10}(\text{adjusted } p\text{-values}) > 1.30$ were considered statistically significant.

were increased in this subgroup of ME/CFS patients when compared to healthy controls (**Figure 3A**). The same evidence could not be found when comparing all the ME/CFS patients to healthy controls (**Figure 3A**). Data related to EBNA4_0529, EBNA6_0066 and EBNA6_0070 were significantly correlated with each other (Spearman's correlation coefficients higher than 0.58; **Figure 3B**). The correlation between the levels of antibodies against EBNA6_0066 and EBNA6_0070 could be explained by the fact that these two peptides are 15-mers overlapping 11 amino acids with each other (23). In contrast, it was unclear why the levels of antibodies against EBNA4_0529 and EBNA6_0066 were highly correlated (Spearman's correlation coefficient = 0.79), considering that these antigens did not share a high sequence homology (**Figure 3C**).

Given the high correlation between antibody levels related to these antigens, a statistical redundancy was expected when using their data for patients' classification purpose. This redundancy was confirmed when the three candidate antibodies were included as covariates in the same model. A stepwise variable selection procedure led to the exclusion of the antibody levels related to EBNA6_0066 from the final classification model.

The final model included the main effects of antibodies to EBNA4_0529 and EBNA6_0070 and the two-way interaction of the latter with age and gender (**Table 2**). On the one hand, the $\log_{10}$-levels of antibodies related to EBNA4 increased the probability of being a patient (coefficient estimate = 2.25, Standard error = 1.09). In particular, the odds of being a patient were estimated to increase $\sim$9.5 ($e^{2.25}$) times per fold-change in the levels of these antibodies. On the other hand, the effects of antibody levels related to EBNA6_0070 on the probability of an individual being an ME/CFS patients were not so trivial to ascertain (**Figure 4A**). In particular, women with high EBNA6_0070 antibody levels showed an increasing estimated probability of being a patient with increasing age. In contrast, the probability profile of being patient was different in men. In that case, younger men with low EBNA6_0070 antibody levels or older men with high EBNA6 antibody levels had a higher probability of being a patient.

The AUC of the classification predicted by the final model was estimated at 0.835 with a 95% CI = (0.759; 0.911; **Figure 4B**). This estimate suggested that the combination of these two antibodies together with age and gender could be used for the diagnosis of patients with an infectious trigger. The optimal sensitivity and specificity were estimated at 0.833 and 0.720, respectively. Therefore, ME/CFS patients were better discriminated than healthy controls by this model.

When the same classification model was applied to the whole cohort of ME/CFS patients, the AUC decreased to 0.731 with a 95% CI = (0.648, 0.814). This could be explained by the cohort of patients with a non-infectious or unknown trigger in which the performance of the classification model was close to a random guess [AUC = 0.583; 95% CI = (0.461; 0.705)].

DISCUSSION

This study, based on previously published data, aimed to discover EBV-derived antigens that could elicit distinct antibody responses in ME/CFS patients when compared to healthy controls. The key finding was the identification of two candidate antigens inducing increased antibody responses in ME/CFS patients with an infectious trigger. The high sensitivity and specificity of our classification model including these antibodies suggest their potential for diagnosis of this subgroup of affected individuals. For ME/CFS patients without an infectious trigger, we could not find any antigens causing antibody responses that could be used for diagnostic purposes. This finding is in agreement with an extensive serological investigation of different herpesviruses in ME/CFS patients (29). This negative finding supports the hypothesis that EBV plays a role in the group of ME/CFS patients with an infectious trigger. In a subset of patients, infectious mononucleosis caused by primary EBV infection can be documented as a trigger (10). In many others, no infection with a specific pathogen could be associated with the disease onset (5). A tempting hypothesis from our finding is that EBV reactivation which can occur during other infections may play until now an underestimated role in triggering ME/CFS. In line with this concept, a recent study showed that EBV reactivation during COVID-19 is a risk factor for Post COVID Syndrome which also includes ME/CFS (35). Alternatively, the responses to the EBNA6 peptides are due to a cross-reactivity to other pathogens, as outlined below.

Other findings of this study pointed to three key challenges associated with the discovery of a biomarker. Firstly, it is difficult to identify a disease-specific biomarker for all the ME/CFS patients. Thus, given the heterogeneous nature of ME/CFS, it is pivotal to stratify patients adequately (30), based on age, gender, and disease trigger for biomarker discovery (27). In this regard, the identification of antibody patterns specific to ME/CFS patients with an infectious trigger was in agreement with other studies where significant results could be found for the same subgroup of patients (10, 36, 37). However, given the vast number of infectious agents associated with ME/CFS (5, 38), it is worth noting that this subgroup of patients could be further subdivided according to the nature of the causative infection. In this regard, the data about the infectious agents that could have initiated ME/CFS are either inconclusive or simply based on self-reported history in most patients, as demonstrated by the data from the United Kingdom ME/CFS Biobank, where only a minority of patients had their infection confirmed with the lab test (10). Secondly, the final classification model included non-trivial statistical interactions of antibodies against EBNA6_0070

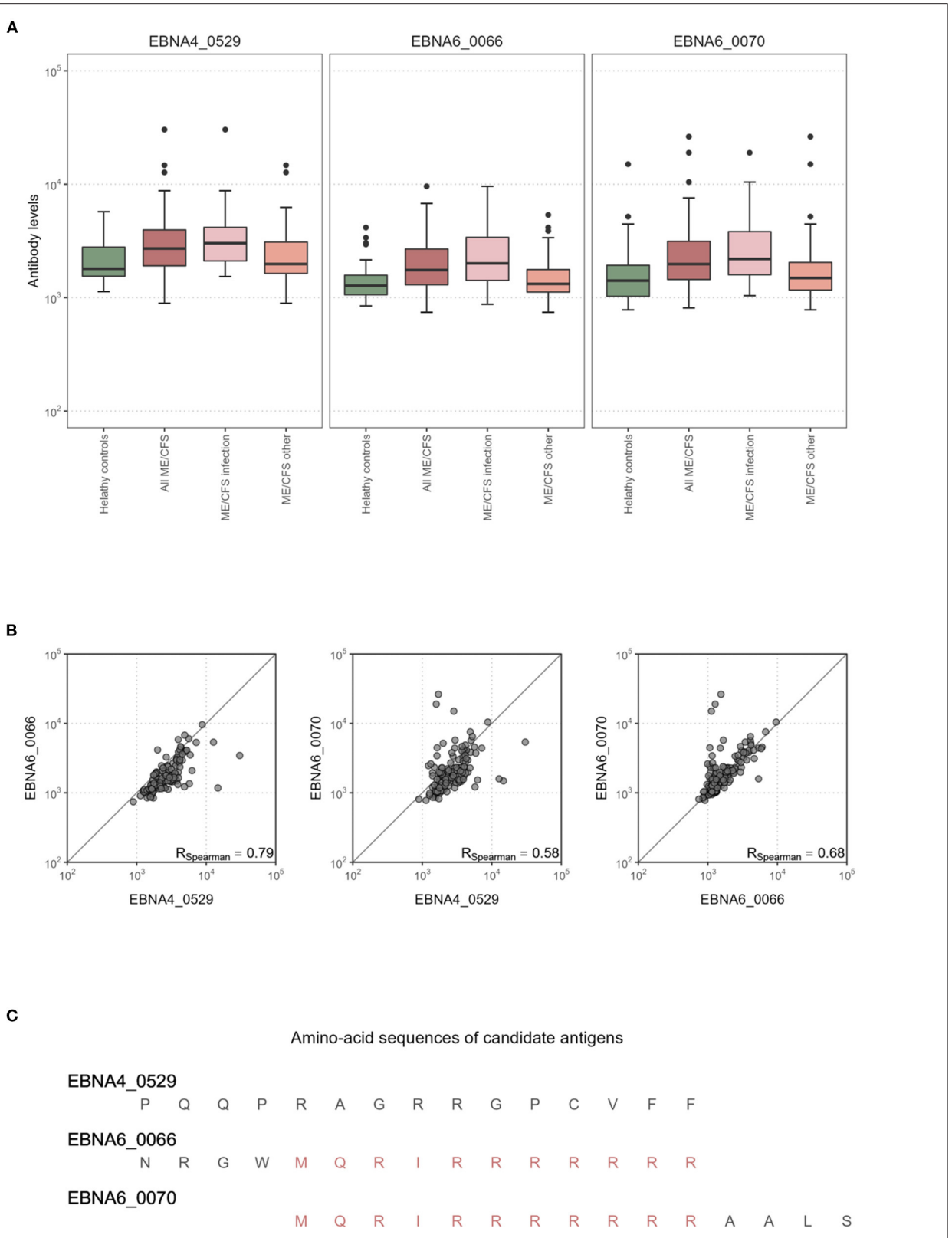

FIGURE 3 | Statistical analysis of the antibody levels related to EBNA4_0529, EBNA6_0066, and EBNA6_0070. **(A)** Boxplots of the data per study group. **(B)** Scatterplots and the respectively Spearman's correlation coefficients (R) in the whole dataset. **(C)** Amino acid sequences of EBNA4_0529, EBNA6_0066, and EBNA6_0070.

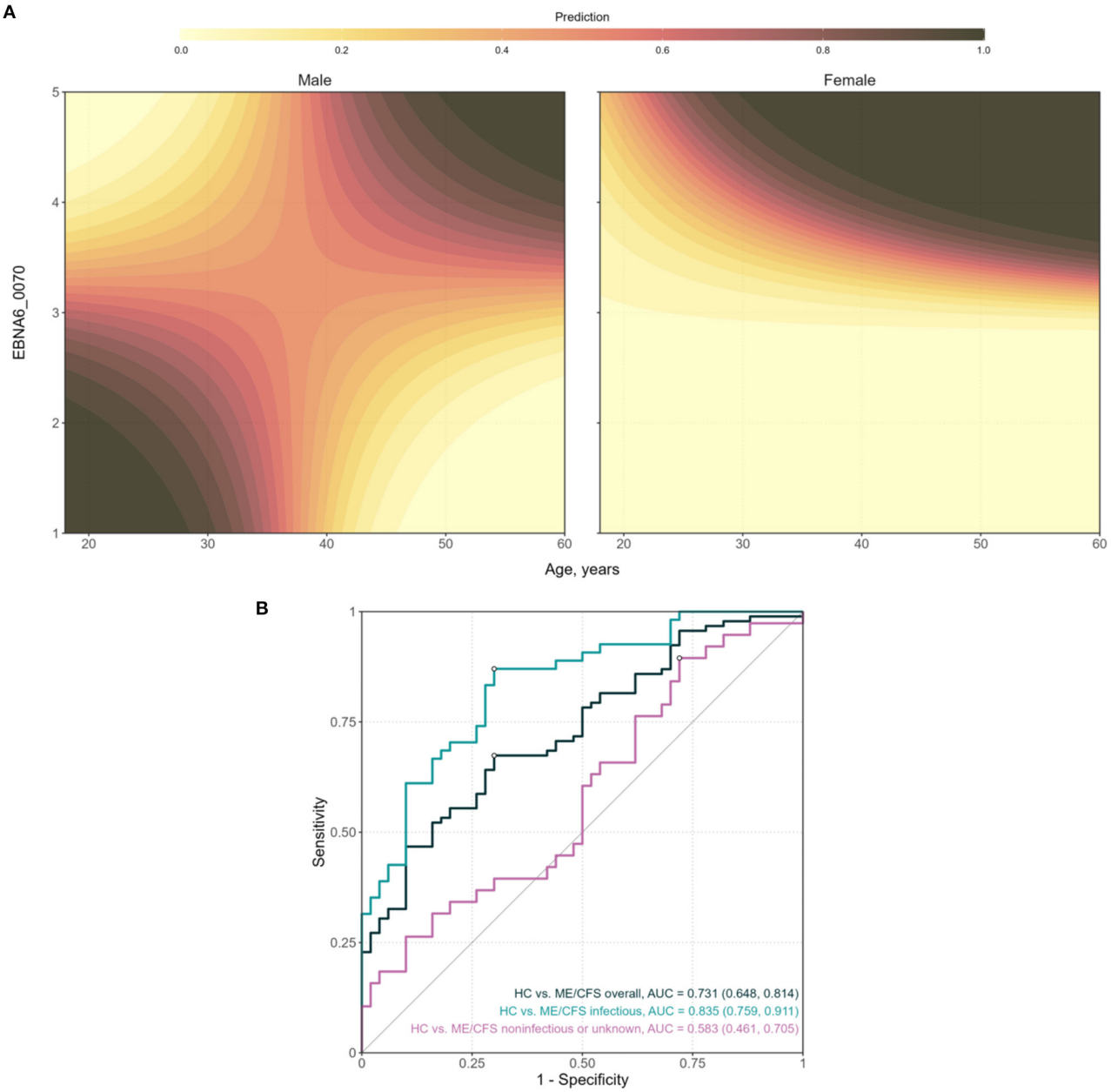

FIGURE 4 | Analysis of the final classification model for predicting ME/CFS patients with an infectious trigger when compared to healthy controls. **(A)** Contour plots of the probability of being a patient as a function of age and EBNA6_0070 antibody levels, for men and women, respectively. The prediction values were calculated by fixing $\log_{10}$(EBNA4_0529) at the respective mean value. **(B)** ROC curves and the respective AUC (95% confidence interval shown within brackets) when using the model to compare different groups of ME/CFS patients to healthy controls.

with both age and gender. This finding implies that significant interactions between candidate biomarkers and confounding factors might be overlooked by analysts or, even when tested, they are likely to be discarded due to the small sample sizes to detect them. The presence of these interactions might be yet another factor that contributes to the lack of reproducibility between biomarker studies on ME/CFS. A proposed strategy to overcome this limitation is to conduct more advanced statistical analyses including the application of machine learning techniques which intrinsically consider the complexity of a large set of clinical and biological data, as demonstrated in drug discovery (39). Thirdly, the interaction between the candidate antibodies against EBNA6_0070 and gender implied a remarkable distinct antibody signature between male and female patients. Again, this finding is in line with gender differences in immunity to viral infection (40). In particular, men have typically lower antibody responses

when vaccinated and are more susceptible to infections than women (41). In this regard, our study suggested that the higher probability of younger man being an ME/CFS patient is associated with lower levels of antibodies against the antigen EBNA6_0070. In contrast, female and male patients seemed to be at higher risk with higher antibodies at increasing age suggesting that at least a subset develop these antibody responses later in life. An implication of having a different antibody profiling between men and women is that analysis of each gender should be performed separately. At the same time, it is important to note that epidemiological data on ME/CFS suggested approximately a disease ratio of three women to one man (42–44). Therefore, if gender is an important stratification factor for biomarker discovery, studies should be designed toward a more balanced gender ratio. Similar sample sizes between male and female cohorts ensure comparable statistical power when analyzing data from each sex separately.

Both EBNA4_0529 and EBNA6_0070 antigens are derived from proteins whose genetic expression typically occurs during the EBV type III latency. Therefore, the acquisition of the respective antibodies might have occurred during initial B-cell transformation and immortalization. It could also be acquired slowly over time, given that the type III latency pattern can be detected sporadically in lymphoid follicles where EBV-infected B cells can proliferate and mimic a germinal center reaction program (45). We can hypothesize from our data that both male and female patients developing higher antibody responses against this antigen later in life are at an increased risk of developing ME/CFS suggesting that reactivation of EBV plays a role. In male patients a subgroup with lower EBNA6 antibodies early in live is at risk of developing ME/CFS, too. Using the recent analytical framework of ME/CFS natural progression (46), antibodies against these antigens are more likely to be biomarkers of patients suffering from ME/CFS more than 2 years of disease rather than the ones either in prodromal period or at early stages in line with our findings. Based on that assumption, these antibodies seemed more appropriate for diagnosing putative patients with delayed disease diagnosis rather than early suspected cases. However, it is known that the delay of ME/CFS diagnosis is a recurrent problem in the clinic (8, 47). As such, we anticipate a higher utility of these antibodies when redeployed to real-world screening. Another practical implication of using these antibodies as biomarkers is the possibility of developing routine ELISA kits that can be standardized across different laboratories and easily scalable for large population screenings. Notwithstanding these promising practical expectations, it is important to emphasize that past studies also suggested potential disease biomarkers (27) and, therefore, it is imperative to replicate the findings of this study with different cohorts of patients.

An interesting observation is that both EBNA6_0066 and EBNA6_0070 contain an arginine-repeat sequence. Such a sequence has homologies with putative epitopes from several human proteins (48). Such homologies suggest a potential molecular mimicry between the viral and human antigens. Molecular mimicry can trigger deleterious autoimmune responses as hypothesized for ME/CFS pathogenesis (38, 49). Molecular mimicry between human and microbial antigens has been also hypothesized for several autoimmune diseases (50), such as multiple sclerosis and rheumatoid arthritis, and Post COVID syndrome, whose patients share similar symptoms with ME/CFS ones (19, 51–53). Interestingly, T cell clones recognizing such arginine-repeat sequences were isolated from a patient with multiple sclerosis supporting our concept of epitope mimicry (48). Finally, arginine-repeat sequences are found in various other pathogens including enteroviruses and human papillomavirus which are also triggers of ME/CFS (5).

Further we can hypothesize that peptides highly enriched in arginine residues might be particularly susceptible to citrullination, in which arginine residues are post-translationally converted to citrulline. These post-translational modifications occur during cell death under normal physiological conditions. However, under chronic inflammation, the accumulation of citrullinated (auto)antigens in inflamed sites might lead to deleterious autoimmune responses, thus, promoting the onset of different autoimmune diseases (54). A potential cross-reactivity between microbial and citrullinated human antigens could also be a mechanism by which an autoimmune disease can be triggered. In rheumatoid arthritis, antibodies against EBNA-1 peptides were shown to cross-react with denatured collagen and keratin (55). However, in the present study, we could not find any antibodies against EBNA-1-derived peptides to be associated with ME/CFS. Interestingly, the serum levels of citrulline were reported to be elevated in ME/CFS patients when compared to healthy controls (56). However, another study could not confirm this finding, but instead provided evidence for increased plasma levels of arginine residues (57). Another source of antigen modification is the process of generating new and more immunogenic epitopes from ubiquitous molecules upon oxidative and nitrosative stress. In ME/CFS, IgM antibodies against several of these neoepitopes, including NO-Arginine, were increased in patients (58). In all of these possible scenarios, it is imperative to investigate the stability of this candidate biomarker antigen to post-translational modifications that could be occurred and eventually increased during the disease course.

In conclusion, this study identified two candidate antigens whose antibodies could be used to identify ME/CFS patients with an infectious trigger. To strengthen our findings, two other cohorts of patients are currently studied, including the well-characterized ME/CFS patients with different disease triggers and healthy controls from the United Kingdom ME/CFS biobank (10).

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: Loebel et al. (23).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Charité Universitatsmedizin Berlin in accordance with the 1964 World Medical Association Declaration of Helsinki and its later amendments (23). The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NS and CS conceived this research. NS, JMal, AG, and AF performed the data analysis. FS, UB, EML, and CS collected and provided the data. All authors interpreted and discussed the results. NS wrote the paper. All authors have read, revised, and approved the final version of the manuscript.

FUNDING

NS, FW, EML, and CS received funding from ME Research UK (SCIO Charity Number SC036942) for this project. NS acknowledges funding from the Fundação para a Ciência e Tecnologia, Portugal (ref. UIDB/00006/2020) and the Polish National Agency for Academic Exchange, Poland (ref. PPN/ULM/2020/1/00069/U/00001). JMal and AF were funded by the Fundação para a Ciência e Tecnologia, Portugal (ref. SFRH/BD/149758/2019 and SFRH/BD/147629/2019, respectively). CC work was partially financed by national funds through FCT – Fundação para a Ciência e a Tecnologia under the project UIDB/00006/2020. EML acknowledges funding from the National Institutes of Health (ref. NIH 2R01AI103629) and the ME Association for the maintenance of the UK ME/CFS Biobank (UKMEB – ME Association (Grant PF8947_ME Association). CS acknowledges funding from the Weidenhammer Zoebele and Lost Voices Foundation, Germany.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.921101/full#supplementary-material

REFERENCES

1. Houen G, Trier NH. Epstein-barr virus and systemic autoimmune diseases. *Front Immunol.* (2020) 11:587380. doi: 10.3389/fimmu.2020.587380
2. Shannon-Lowe C, Rickinson AB, Bell AI. Epstein–Barr virus-associated lymphomas. *Philos Trans R Soc B Biol Sci.* (2017) 372:20160271. doi: 10.1098/rstb.2016.0271
3. Bjornevik K, Cortese M, Healy BC, Kuhle J, Mina MJ, Leng Y, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science.* (2022) 375:296–301. doi: 10.1126/science.abj8222
4. Koo D. Chronic fatigue syndrome. A critical appraisal of the role of Epstein-Barr virus. *West J Med.* (1989) 150:590–6.
5. Rasa S, Nora-Krukle Z, Henning N, Eliassen E, Shikova E, Harrer T, et al. Chronic viral infections in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). *J Transl Med.* (2018) 16:268. doi: 10.1186/s12967-018-1644-y
6. Ruiz-Pablos M, Paiva B, Montero-Mateo R, Garcia N, Zabaleta A. Epstein-barr virus and the origin of myalgic encephalomyelitis or chronic fatigue syndrome. *Front Immunol.* (2021) 12:656797. doi: 10.3389/fimmu.2021.656797
7. Rivera MC, Mastronardi C, Silva-Aldana CT, Arcos-Burgos M, Lidbury BA. Myalgic encephalomyelitis/chronic fatigue syndrome: a comprehensive review. *Diagnostics.* (2019) 9:91. doi: 10.3390/diagnostics9030091
8. Bateman L, Bested AC, Bonilla HF, Chheda BV, Chu L, Curtin JM, et al. Myalgic encephalomyelitis/chronic fatigue syndrome: essentials of diagnosis and management. *Mayo Clin Proc.* (2021) 96:2861–78. doi: 10.1016/j.mayocp.2021.07.004
9. Hickie I, Davenport T, Wakefield D, Vollmer-Conna U, Cameron B, Vernon SD, et al. Post-infective and chronic fatigue syndromes precipitated by viral and non-viral pathogens: prospective cohort study. *BMJ.* (2006) 333:575. doi: 10.1136/bmj.38933.585764.AE
10. Domingues TD, Grabowska AD, Lee JS, Ameijeiras-Alonso J, Westermeier F, Scheibenbogen C, et al. Herpesviruses serology distinguishes different subgroups of patients from the United Kingdom myalgic encephalomyelitis/chronic fatigue syndrome biobank. *Front Med.* (2021) 8:686736. doi: 10.3389/fmed.2021.686736
11. Shikova E, Reshkova V. Kumanova A, Raleva S, Alexandrova D, Capo N, Murovska MB. Cytomegalovirus, Epstein-Barr virus, and human herpesvirus-6 infections in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *J Med Virol.* (2020) 92:3682. doi: 10.1002/jmv.25744
12. Lee JS, Lacerda EM, Nacul L, Kingdon CC, Norris J, O'Boyle S, et al. Salivary DNA loads for human herpesviruses 6 and 7 are correlated with disease phenotype in myalgic encephalomyelitis/chronic fatigue syndrome. *Front Med.* (2021) 8:1129. doi: 10.3389/fmed.2021.656692
13. Kerr JR. Epstein-Barr virus induced gene-2 upregulation identifies a particular subtype of chronic fatigue syndrome/myalgic encephalomyelitis. *Front Pediatr.* (2019) 7:59. doi: 10.3389/fped.2019.00059
14. Lerner AM, Ariza ME, Williams M, Jason L, Beqaj S, Fitzgerald JT, et al. Antibody to Epstein-Barr virus deoxyuridine triphosphate nucleotidohydrolase and deoxyribonucleotide polymerase in a chronic fatigue syndrome subset. *PLoS ONE.* (2012) 7:e47891. doi: 10.1371/journal.pone.0047891
15. Loebel M, Strohschein K, Giannini C, Koelsch U, Bauer S, Doebis C, et al. Deficient EBV-specific B- and T-cell response in patients with Chronic Fatigue Syndrome. *PLoS ONE.* (2014) 9:e85387. doi: 10.1371/journal.pone.0085387
16. Wang J, Jelcic I, Mühlenbruch L, Haunerdinger V, Toussaint NC, Zhao Y, et al. HLA-DR15 molecules jointly shape an autoreactive T cell repertoire in multiple sclerosis. *Cell.* (2020) 183:1264–81.e20. doi: 10.1016/j.cell.2020.09.054
17. Malato J, Graça L, Nacul L, Lacerda E, Sepúlveda N. Statistical challenges of investigating a disease with a complex diagnosis. *medRxiv.* (2021) 2021:2021.03.19.21253905. doi: 10.1101/2021.03.19.21253905
18. Morris G, Maes M. Myalgic encephalomyelitis/chronic fatigue syndrome and encephalomyelitis disseminata/multiple sclerosis show remarkable levels of similarity in phenomenology and neuroimmune characteristics. *BMC Med.* (2013) 11:205. doi: 10.1186/1741-7015-11-205
19. Gaber TAZK, Oo WW, Ringrose H. Multiple sclerosis/chronic fatigue syndrome overlap: when two common disorders collide. *NeuroRehabilitation.* (2014) 35:529–34. doi: 10.3233/NRE-141146
20. Wucherpfennig KW, Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell.* (1995) 80:695–705. doi: 10.1016/0092-8674(95)90348-8
21. Holmøy T, Kvale EØ, Vartdal F. Cerebrospinal fluid CD4+ T cells from a multiple sclerosis patient cross-recognize Epstein-Barr virus and myelin basic protein. *J NeuroVirology.* (2004) 105:278–83. doi: 10.1080/13550280490499524
22. Lünemann JD, Jelčić I, Roberts S, Lutterotti A, Tackenberg B, Martin R, et al. EBNA1-specific T cells from patients with multiple

sclerosis cross react with myelin antigens and co-produce IFN-γ and IL-2. *J Exp Med.* (2008) 205:1763–73. doi: 10.1084/jem.20072397

23. Loebel M, Eckey M, Sotzny F, Hahn E, Bauer S, Grabowski P, et al. Serological profiling of the EBV immune response in Chronic Fatigue Syndrome using a peptide microarray. *PLoS ONE.* (2017) 12:e0179124. doi: 10.1371/journal.pone.0179124
24. Tengvall K, Huang J, Hellström C, Kammer P, Biström M, Ayoglu B, et al. Molecular mimicry between Anoctamin 2 and Epstein-Barr virus nuclear antigen 1 associates with multiple sclerosis risk. *Proc Natl Acad Sci USA.* (2019) 116:16955–60. doi: 10.1073/pnas.1902623116
25. Sepúlveda N. Impact of genetic variation on the molecular mimicry between Anoctamin-2 and Epstein-Barr virus nuclear antigen 1 in Multiple Sclerosis. *Immunol Lett.* (2021) 238:29–31. doi: 10.1016/j.imlet.2021.07.007
26. Sepúlveda N, Carneiro J, Lacerda E, Nacul L. Myalgic encephalomyelitis/chronic fatigue syndrome as a hyper-regulated immune system driven by an interplay between regulatory T cells and chronic human herpesvirus infections. *Front Immunol.* (2019) 10:2684. doi: 10.3389/fimmu.2019.02684
27. Scheibenbogen C, Freitag H, Blanco J, Capelli E, Lacerda E, Authier J, et al. The European ME/CFS biomarker landscape project: an initiative of the European network EUROMENE. *J Transl Med.* (2017) 15:162. doi: 10.1186/s12967-017-1263-z
28. Cliff JM, King EC, Lee JS, Sepúlveda N, Wolf AS, Kingdon C, et al. Cellular immune function in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). *Front Immunol.* (2019) 10:796. doi: 10.3389/fimmu.2019.00796
29. Blomberg J, Rizwan M, Böhlin-Wiener A, Elfaitouri A, Julin P, Zachrisson O, et al. Antibodies to human herpesviruses in myalgic encephalomyelitis/chronic fatigue syndrome patients. *Front Immunol.* (2019) 10:1946. doi: 10.3389/fimmu.2019.01946
30. Jason LA, Corradi K, Torres-Harding S, Taylor RR, King C. Chronic fatigue syndrome: the need for subtypes. *Neuropsychol Rev.* (2005) 15:29–58. doi: 10.1007/s11065-005-3588-2
31. Benjamini Y, Yekutieli D. The control of the false discovery rate in multiple testing under dependency. *Ann Stat.* (2001) 29:1165–88. doi: 10.1214/aos/1013699998
32. Venables WN, Ripley BD. *Modern Applied Statistics with S. Fourth*. New York, NY: Springer (2002). Available online at: https://www.stats.ox.ac.uk/pub/MASS4/ (accessed May 28, 2021)
33. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: An open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics.* (2011) 12:1–8. doi: 10.1186/1471-2105-12-77
34. López-Ratón M, Rodríguez-Álvarez MX, Cadarso-Suárez C, Gude-Sampedro F. Optimalcutpoints: an R package for selecting optimal cutpoints in diagnostic tests. *J Stat Softw.* (2014) 61:1–36. doi: 10.18637/jss.v061.i08
35. Su Y, Yuan D, Chen DG, Ng RH, Wang K, Choi J, et al. Multiple early factors anticipate post-acute COVID-19 sequelae. *Cell.* (2022) 185:881–95.e20. doi: 10.1016/j.cell.2022.01.014
36. Steiner S, Becker SC, Hartwig J, Sotzny F, Lorenz S, Bauer S, et al. Autoimmunity-related risk variants in PTPN22 and CTLA4 are associated with ME/CFS with infectious onset. *Front Immunol.* (2020) 11:578. doi: 10.3389/fimmu.2020.00578
37. Szklarski M, Freitag H, Lorenz S, Becker SC, Sotzny F, Bauer S, et al. Delineating the association between soluble CD26 and autoantibodies against G-protein coupled receptors, immunological and cardiovascular parameters identifies distinct patterns in post-infectious vs. non-infection-triggered myalgic encephalomyelitis/chro. *Front Immunol.* (2021) 12:1077. doi: 10.3389/fimmu.2021.644548
38. Blomberg J, Gottfries CG, Elfaitouri A, Rizwan M, Rosén A. Infection elicited autoimmunity and Myalgic encephalomyelitis/chronic fatigue syndrome: an explanatory model. *Front Immunol.* (2018) 9:229. doi: 10.3389/fimmu.2018.00229
39. Gupta R, Srivastava D, Sahu M, Tiwari S, Ambasta RK, Kumar P. Artificial intelligence to deep learning: machine intelligence approach for drug discovery. *Mol Divers.* (2021) 25:1315–60. doi: 10.1007/s11030-021-10217-3
40. Jacobsen H, Klein SL. Sex differences in immunity to viral infections. *Front Immunol.* (2021) 12:3483. doi: 10.3389/fimmu.2021.720952
41. Aaby P, Benn CS, Flanagan KL, Klein SL, Kollmann TR, Lynn DJ, et al. The non-specific and sex-differential effects of vaccines. *Nat Rev Immunol.* (2020) 20:464–70. doi: 10.1038/s41577-020-0338-x
42. Chu L, Valencia IJ, Garvert DW, Montoya JG. Onset patterns and course of myalgic encephalomyelitis/chronic fatigue syndrome. *Front Pediatr.* (2019) 7:12. doi: 10.3389/fped.2019.00012
43. Johnston SC, Staines DR, Marshall-Gradisnik SM. Epidemiological characteristics of chronic fatigue syndrome/myalgic encephalomyelitis in Australian patients. *Clin Epidemiol.* (2016) 8:97–107. doi: 10.2147/CLEP.S96797
44. Nacul LC, Lacerda EM, Pheby D, Campion P, Molokhia M, Fayyaz S, et al. Prevalence of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) in three regions of England: a repeated cross-sectional study in primary care. *BMC Med.* (2011) 9:91. doi: 10.1186/1741-7015-9-91
45. Thorley-Lawson DA. EBV persistence—introducing the virus. *Curr Top Microbiol Immunol.* (2015) 390:151. doi: 10.1007/978-3-319-22822-8_8
46. Nacul L, O'Boyle S, Palla L, Nacul FE, Mudie K, Kingdon CC, et al. How myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) progresses: the natural history of ME/CFS. *Front Neurol.* (2020) 11:826. doi: 10.3389/fneur.2020.00826
47. Nacul L, Authier FJ, Scheibenbogen C, Lorusso L, Helland IB, Martin JA, et al. European network on myalgic encephalomyelitis/chronic fatigue syndrome (EUROMENE): expert consensus on the diagnosis, service provision, and care of people with ME/CFS in Europe. *Medicina.* (2021) 57:510. doi: 10.3390/medicina57050510
48. Sospedra M, Zhao Y, Hausen H, Muraro PA, Hamashin C, De Villiers EM, et al. Recognition of conserved amino acid motifs of common viruses and its role in autoimmunity. *PLoS Pathog.* (2005) 1:0335–48. doi: 10.1371/journal.ppat.0010041
49. Phelan J, Grabowska AD, Sepúlveda N. A potential antigenic mimicry between viral and human proteins linking Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) with autoimmunity: the case of HPV immunization. *Autoimmun Rev.* (2020) 19:102487. doi: 10.1016/j.autrev.2020.102487
50. Rojas M, Restrepo-Jiménez P, Monsalve DM, Pacheco Y, Acosta-Ampudia Y, Ramírez-Santana C, et al. Molecular mimicry and autoimmunity. *J Autoimmun.* (2018) 95:100–23. doi: 10.1016/j.jaut.2018.10.012
51. Ali S, Matcham F, Irving K, Chalder T. Fatigue and psychosocial variables in autoimmune rheumatic disease and chronic fatigue syndrome: a cross-sectional comparison. *J Psychosom Res.* (2017) 92:1–8. doi: 10.1016/j.jpsychores.2016.11.002
52. Moss-Morris R, Chalder T. Illness perceptions and levels of disability in patients with chronic fatigue syndrome and rheumatoid arthritis. *J Psychosom Res.* (2003) 55:305–8. doi: 10.1016/S0022-3999(03)00013-8
53. Komaroff AL, Lipkin WI. Insights from myalgic encephalomyelitis/chronic fatigue syndrome may help unravel the pathogenesis of postacute COVID-19 syndrome. *Trends Mol Med.* (2021) 27:895–906. doi: 10.1016/j.molmed.2021.06.002
54. Alghamdi M, Alasmari D, Assiri A, Mattar E, Aljaddawi AA, Alattas SG, et al. An overview of the intrinsic role of citrullination in autoimmune disorders. *J Immunol Res.* (2019) 2019:7592851. doi: 10.1155/2019/7592851
55. Birkenfeld P, Haratz N, Klein G, Sulitzeanu D. Cross-reactivity between the EBNA-1 p107 peptide, collagen, and keratin: implications for the pathogenesis of rheumatoid arthritis. *Clin Immunol Immunopathol.* (1990) 54:14–25. doi: 10.1016/0090-1229(90)90002-8
56. Pall ML. Levels of nitric oxide synthase product citrulline are elevated in sera of chronic fatigue syndrome patients. *J Chronic Fatigue Syndr.* (2002) 10:37–41. doi: 10.1300/J092v10n03_04
57. Naviaux RK, Naviaux JC Li K, Bright AT, Alaynick WA, Wang L, et al. Metabolic features of chronic fatigue syndrome. *Proc Natl Acad Sci USA.* (2016) 113:E5472–80. doi: 10.1073/pnas.1607571113

58. Maes M, Mihaylova I, Leunis J. Chronic fatigue syndrome is accompanied by an IgM-related immune response directed against neoepitopes formed by oxidative or nitrosative damage to lipids. *Neuro Endocrinol Lett.* (2006) 27:615–21. doi: 10.1097/YCO.0b013e32831a4728

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 19 July 2022
DOI 10.3389/fmed.2022.869028

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

RECEIVED 03 February 2022
ACCEPTED 29 June 2022
PUBLISHED 19 July 2022

CITATION
Rogier E, Nace D, Dimbu PR, Wakeman B, Beeson JG, Drakeley C, Tetteh K and Plucinski M (2022) Antibody dynamics in children with first or repeat *Plasmodium falciparum* infections.
Front. Med. 9:869028.
doi: 10.3389/fmed.2022.869028

Antibody dynamics in children with first or repeat *Plasmodium falciparum* infections

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Immunoglobulin (Ig) production during and after infection with *Plasmodium* parasites is one of the greatest adaptive immune defenses the human host has against this parasite. Infection with *P. falciparum* has been shown to induce different B cell maturation responses dependent upon the age of the patient, number of previous exposures, and severity of the disease. Described here are dynamics of Ig responses to a panel of 32 *P. falciparum* antigens by patients followed for 42 days and classified individuals as showing characteristics of an apparent first *P. falciparum* infection (naïve) or a repeat exposure (non-naïve). Six parameters were modeled to characterize the dynamics of IgM, IgG_1, IgG_3, and IgA for these two exposure groups with differences assessed among Ig isotypes/subclasses and unique antigens. Naïve patients had significantly longer periods of time to reach peak Ig titer (range 4–7 days longer) and lower maximum Ig titers when compared with non-naïve patients. Modeled time to seronegativity was significantly higher in non-naïve patients for IgM and IgA, but not for the two IgG subclasses. IgG_1 responses to Rh2030, HSP40, and PfAMA1 were at the highest levels for non-naïve participants and may be used to predict previous or nascent exposure by themselves. The analyses presented here demonstrate the differences in the development of the Ig response to *P. falciparum* if the infection represents a boosting response or a primary exposure. Consistency in Ig isotype/subclasses estimates and specific data for *P. falciparum* antigens can better guide interpretation of seroepidemiological data among symptomatic persons.

KEYWORDS

malaria, antibodies, isotypes, boosting, exposure

Introduction

The human host mounts a vigorous adaptive immune response to the *Plasmodium falciparum* parasite, and B cell responses through antibody-mediated immunity have been shown to be protective against malaria (1), and even passive transfer of serum antibodies from persons living in endemic settings reduces *P. falciparum* parasite burden of symptomatic children (2). As malaria infection is a bloodborne infectious disease, anti-*P. falciparum* immunoglobulin (Ig) titers are generally highest for IgG in humans upon natural exposure, though both IgM and IgA antibodies are observed in substantial quantities as well (3–5). The IgG response to *P. falciparum* can be further subdivided by the four subclasses of this isotype in humans, with highest serum levels of IgG_1 followed by IgG_3, IgG_4, and IgG_2 (6–9); however, the relative abundance of IgG subclass response varies for different malaria antigens. IgG1 and IgG3 are generally the predominant response and effectively mediate interactions with complement and Fcγ-receptors expressed on immune cells, which play roles in immunity (10, 11). Understanding the induction, function, and dynamics of the Ig response against *P. falciparum* antigens has greatly enhanced vaccine development (12–14) and interpretation of seroepidemiological studies (15, 16).

In *P. falciparum*-endemic areas throughout the world, infants or children may become exposed to this parasite at a very early age. Passive placental transfer of IgG to the fetus provides a degree of clinical protection early in life (17), though these antibodies are generally lost by 6 months of age (18, 19). Formation and maturation of the host anti-*P. falciparum* B cell response is an area of active research for several decades, though human studies have obvious limitations due to the inherent need for immediate treatment when any infection is diagnosed. The response in humans appears to follow many of the classical assumptions regarding class switching, affinity maturation, and clonal selection, though recent work has emphasized the contribution of atypical B cell populations early in development which appear be able to respond faster to antigen challenge, but are less efficient at establishing protective and long-term antibody production (20–22). Evidence has been presented showing the early B activation in response to *P. falciparum* in naïve humans to be dominated by short-lived and metabolically-active plasmablasts which have the capacity for prolific antibody secretion, but may inhibit the formation of durable immunity (23). Additionally, as with B cell maturation to many other immunogenic agents, the importance of CD4 + T follicular helper cells has been documented for the development of the *P. falciparum* antibody response with T helper (Th) cell Th1 and Th2 subsets likely playing distinct roles (24, 25). Recent studies have also highlighted the prominence of IgM responses to malaria infection, including repeat infections and the persistence of IgM responses over time (26, 27).

Individual *P. falciparum* antigens have been identified for their specific abilities to induce B cell responses and antibody production in exposed endemic populations (16, 28–31), as well as controlled human malaria infections (CHMIs) (24, 26, 32, 33). The study presented here investigates the short-term immunoglobulin response to natural *P. falciparum* infection by categorizing a study population of children into first or repeat infection and comparing Ig responses for 42 days following antimalarial treatment. This study aims to understand the Ig dynamics arising from a B cell response in the nascent host vs. a host with previous immunological memory. Ig responses were broadly investigated in a population of children against a panel of 32 *P. falciparum* antigens encompassing all life stages in the human host was investigated for the ability to bind IgM, IgG_1, IgG_3, and IgA in blood samples in order to obtain detail on the specificity and nature of immune responses. These data are used to estimate the nascent B cell response to *P. falciparum* exposure through the dynamics of Ig production to multiple parasite antigens, and how this differs from individuals experiencing a repeat *P. falciparum* infection.

Materials and methods

Study design

Dried blood spots were collected during a therapeutic efficacy monitoring study (TES) in Angola in 2017 (34). Samples from all three sentinel TES sites were included: high-transmission M'Banza Congo, Zaire Province and Saurimo, Lunda Sul Province; and low\mid-transmission Benguela, Benguela Province. Due to different transmission levels, the parasite density criteria for enrollment were lower in Benguela province (1,000–100,000 p/μL blood) vs. Lunda Sul and Zaire (2,000–200,000 p/μL blood). Children aged 6 months to 11 years old with microscopically confirmed acute *P. falciparum* infection were treated with one of three artemisinin-based combination therapies (ACT) and followed weekly for 28 (participants treated with artemether-lumefantrine or artesunate-amodiaquine) or 42 (participants treated with dihydroartemisinin-piperaquine) days. Patients with severe or complicated malaria infections were excluded from enrollment. Participant samples were collected on Days 0 (enrollment), 2, 3, 7, 14, 21, 28, 35, and 42 after initiation of ACT.

Ethics approval

Study participants consented to collection of malaria data from provided blood samples. The study received human subjects approval from the Angolan Ministry of Health. Secondary analysis of anonymized samples was approved by

the office of the Associate Director of Science in the Center for Global Health at the CDC (Project ID: 0900f3eb8193aa9d).

Laboratory analysis

Samples were assayed for antibody responses to a panel of *P. falciparum* antigens using a multiple bead-based assay as described previously (5). Assay signal was provided as mean fluorescent intensity (MFI) minus the signal from blank wells on each plate to provide a final signal of MFI-bg for analysis.

Statistical analysis

To classify study participants into *P. falciparum* naïve (first lifetime infection) and non-naïve (repeat infection) categories, the assay signals for baseline (Day 0) samples for IgG_1 response to PfMSP1 and PfAMA1 were compared. Natural exposure to PfMSP1 and PfAMA1 is highly immunogenic in humans, and IgG_1 responses to these two antigens are long-lived and generally considered to be indicative of any prior exposure to *P. falciparum* (5, 35–37). MFI-bg seropositivity thresholds for PfMSP1 and PfAMA1 were 115 and 113, respectively, and generated as described previously (5). Children that had IgG_1 responses to both the PfMSP1 and PfAMA1 antigens below the seropositivity threshold at Day 0 were considered "naive" and it was assumed that their presenting *P. falciparum* infection was their first-ever *P. falciparum* infection. This classification scheme is also supported by the three TES enrollment sites being located in meso- to high-endemic *P. falciparum* settings in Angola (34), and the relatively young ages of participants, meaning it is unlikely they would have had time for IgG_1 seroreversion from a previous *P. falciparum* exposure. In order to have higher confidence in the classification scheme, children seropositive for PfMSP1 and/or PfAMA1 IgG_1 but having assay signal less than one $\log_{10}$ fold greater than the seropositivity threshold (MFI-bg value of 1,150 and 1,130, respectively) were classified as indeterminate and excluded from further analysis. All other children with high IgG_1 assay signals to these *P. falciparum* antigens were considered "non-naive" with strong evidence for previous *P. falciparum* blood-stage infection.

For all antigens included in the panel, individual decay curves were characterized with six key parameters: C_{max}, the maximum antibody signal; Δ_C, the difference between C_{max} and the antibody signal at Day 0; C_{end}, the antibody signal at last day of follow up; t_{max}, the time in days to maximum antibody signal; $t_{1/2}$, the post-peak half-life; and t_{neg}, the expected time to seronegativity (5). The distribution of the estimates for these parameters across all antigens was compared between children classified as naive and non-naive. The analysis was separately done for IgG_1, IgG_3, IgM, and IgA. Though IgG2, IgG4, IgE, and IgD responses were also measured in these same samples, these isotypes/subclasses were either undetectable (at 1:100 serum concentration) or too few children displayed responses for these Igs to allow for parameter estimates (5).

Differences in the empirical distributions were assessed using the Kolmogorov–Smirnov non-parametric test. Heatmaps were generated to simultaneously assess clustering patterns of antibody responses by antigen and participant. All analysis was done in R version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Classification of study population into *Plasmodium falciparum* naïve and non-naïve

Of 104 enrolled participants, 89 (85.6%) provided samples for the entire 42-day follow-up period (Days 0, 2, 3, 7, 14, 21, 28, 35, and 42 after enrollment), while 2 (1.9%) provided samples only up to 35 days, 1 (1.0%) to 28 days, 6 (5.8%) to 21 days, and 6 (5.8%) to 14 days. As described and listed previously, a total of 32 *P. falciparum* antigens were utilized for the multiplex antibody detection assay (5). The distribution of IgG_1 responses to PfMSP1 and PfAMA1 for the entire study population were bimodal at Day 0, and by the end of follow-up sampling, nearly all participants registered high responses to both of these antigens (**Figure 1**). A similar pattern was observed for the IgG_3 response to both antigens, but the distributions were not as distinct for IgM and IgA antibodies (**Supplementary Figure 1**). As described in "Materials and methods," the bivariate correlation for participant IgG_1 responses to PfMSP1 and PfAMA1 at day of enrollment (Day 0) was considered in order to classify participants as previously exposed to *P. falciparum* (participant was non-naïve), or if the current infection represented potential first *P. falciparum* exposure (participant was naïve). Using the PfMSP1 and PfAMA1 assay signal thresholds for IgG_1, of the 104 participants, 26 (25%) were classified as naïve (seronegative to both antigens), 66 (63%) as non-naïve (high levels of IgG_1 to either), and 12 as indeterminate (seropositive to either, but of lower assay signal) (**Figure 2**). Of the 66 persons classified as non-naive, 64 (97.0%) were seropositive to both of these antigens. For the remaining two individuals, one was IgG_1 seropositive to PfMSP1 only, and one was seropositive to PfAMA1 only. Participants classified as exposure naïve were on average 1.2 years younger than non-naive participants (mean age 2.3 years vs. 3.5, Student's *t*-test *p*-value 0.001). Parasite density at Day 0 and sex were not statistically different between the two classification groups (**Table 1**).

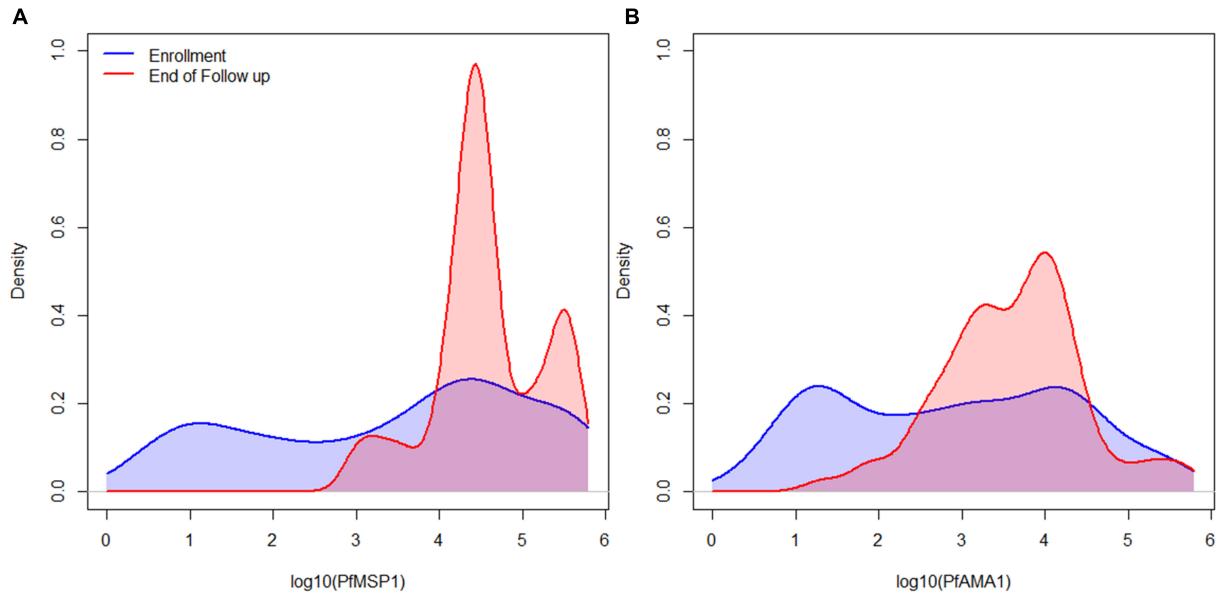

FIGURE 1
IgG_1 antibody levels for all participants for the immunogenic *P. falciparum* PfMSP1 and PfAMA1 antigens. Smoothed distribution of IgG_1 antibody responses to PfMSP1 **(A)** and PfAMA1 **(B)** at enrollment (baseline) in blue, and last day of follow-up in red for children treated for *P. falciparum* infection.

Differences in parameter estimates by Ig isotype and subclass

When stratifying by Ig isotype and subclass for IgG_1, IgG_3, IgM, and IgA, considerable differences were observed in many of the six parameters used to describe Ig dynamics between the naïve and non-naïve groups for the aggregate responses to the 32 *P. falciparum* antigens (Figure 3). For IgG_1, five of the six parameters showed statistically significant differences between the naïve and non-naïve groups (Table 2). Of particular note was the difference in IgG_1 C_{max} (MFI-bg of 407 for non-naïve vs. 117 for naïve) and in t_{max}, which was 7 days later for the naïve IgG_1 response. Aggregate estimates for three of the five parameters for dynamics in IgG_3 response were significantly different with C_{max} again higher for non-naives, and t_{max} again 1 week later for naives. For IgM, five of the six parameters showed significant differences in aggregate estimates, and for IgA, all six parameters were significantly different between the two groups. When considering the six parameter estimates for all malaria antigens and Ig isotypes together, naïve participants had significantly lower maximum antibody responses (C_{max}), higher absolute changes in antibody response (Δ_C), took longer to reach maximum antibody response (t_{max}), and were seropositive for a shorter period of time (t_{neg}) compared to non-naive participants (p-values < 0.001) (Supplementary Figure 2).

Differences in specific *Plasmodium falciparum* antigen Ig responses by participant classification

When assessing differences in Ig dynamics parameter estimates for individual *P. falciparum* antigens, some general trends were observed, but with variability within each isotype/subclass. Certain antigens were particularly discriminatory between naive and non-naive participants. For example, HSP40, Rh2030 and PfAMA1 tended to have a substantially higher C_{max} and C_{end} in non-naive than naive participants for the IgG_1 response (Figure 4 and Supplementary Figure 3). All three of these antigens also stimulated significantly higher IgA responses on the last day of follow-up in non-naive vs. naive participants. Interestingly, C_{max} for the anti-PfMSP1 IgM response was substantially higher in naïve participants, which translated to significantly higher Δ_C and C_{end} estimates in naïve persons. Estimates for t_{max}, $t_{1/2}$, and t_{neg} yielded some striking findings for individual antigens: t_{max} 21 days longer for MSP2_Dd2 for IgG_1 in naïve persons, $t_{1/2}$ 30 days longer for Rh_2030 for IgG_1 in naïve persons, $t_{1/2}$ 15 days longer for Etramp5 Ag1 for IgG_3 in naïve persons, and $t_{1/2}$ significantly longer for IgM (25 days) and IgA (39 days) against PfMSP1 in non-naïve persons. In time to apex antibody levels (t_{max}), all isotypes showed the predominance of naïve children taking longer to reach this apex with statistically

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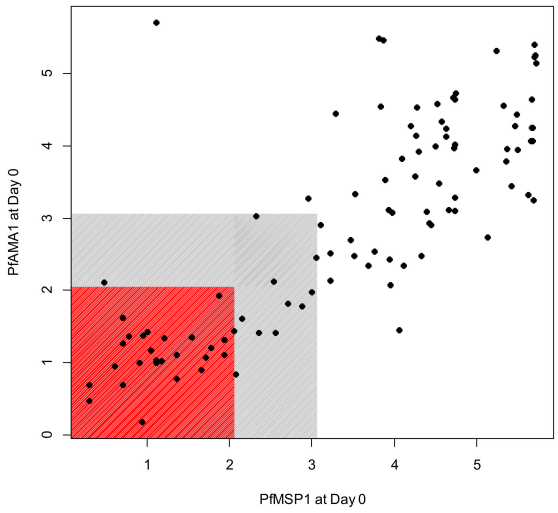

FIGURE 2
Relationship of the IgG_1 antibody responses to PfMSP1 and PfAMA1 at the first day of follow-up (Day 0) in children treated for *P. falciparum* infection. Axes display assay log_{10}-transformed assay signal to the two respective *P. falciparum* antigens. Children IgG_1 seronegative for both antigens are shown by markers in the red box, and children nominally IgG_1 seropositive to either (or both) antigens shown in gray box. Children seronegative to both targets are classified as *P. falciparum* naïve, and children with assay signals greater than the gray shading classified as non-naïve.

TABLE 1 Characteristics of children with *P. falciparum* infection stratified into exposure naïve and exposure non-naïve.

	Non-naïve ($n = 66$)	Naïve ($n = 26$)	*P*-value*
Age, mean (standard deviation)	3.5 (± 1.9)	2.3 (± 1.3)	0.001
Parasite density at Day 0 (pg/μL), median (range)	23704 (3916–184465)	15682 (4113–184243)	0.500
Female, %	48%	50%	1.000

**Difference in age was assessed using a t-test, difference in parasitemia using a t-test after log-transformation, and difference in sex using a chi-square test.*

significant differences for 13/32 (40.6%) of antigens for IgG_1 detection, 7/32 (21.9%) of antigens for IgG_3, 11/32 (34.4%) for IgM, and 8/32 antigens (25.0%) for IgA. Antibodies against the PfMSP1, PfAMA1, GLURP R_o, Etramp4Ag2, and Etramp5Ag1 antigens were all significantly delayed in reaching apex levels in naïve children for all four Ig isotypes/sub-classes.

Hierarchical clustering based on Ig response parameters

Estimates among the panel of *P. falciparum* antigens for Δ_C for IgG_1 and IgM were sufficiently consistent to allow clustering based on naïve or non-naïve classification (**Figure 5**). Clustering based on IgG_1 Δ_C was largely driven by higher estimates in the naïves for PfMSP1, PfAMA1, and Etramp5Ag1, and lower estimates in Rh_2030, HSP40, HRP2, Etramp4Ag2, and the three EBA antigens. Clustering based on IgM Δ_C was largely driven by the higher estimates in naïves for PfMSP1, GLURP R_o, Etramp5Ag1, and PfAMA1. For children classified as indeterminate (neither naïve or non-naïve), the IgG_1 Δ_C estimates among antigens did not cluster with either naïve/non-naïve categories, though the IgM Δ_C estimates were more similar with the children classified as naïve.

Discussion

Here is described the quantitative comparison of six parameters describing Ig dynamics upon successful treatment of *P. falciparum* infection when study participants were classified as previously exposed to *P. falciparum* or if the current *P. falciparum* infection was their first exposure. Infection with *P. falciparum* parasites is known to induce a robust IgG response in humans with IgG_1 levels showing the highest titers followed by IgG_3, IgG_4, and IgG_2 (6–9). Multiple individual *P. falciparum* antigens have been identified as immunogens in the human host and utilized in candidate malaria vaccine development, association with clinical disease, or seroepidemiological studies (15, 16, 26, 38, 39). Previous work has estimated that upon natural exposure to *P. falciparum* and generation of IgG_1 antibodies, children would remain seropositive to this subclass for an estimated 408 days for PfMSP1 and 153 days for PfAMA1 (5). Studies by others evaluating all age ranges (likely from persons with multiple past infections) have also confirmed this longevity of IgG to these two antigens with estimates for IgG half-life in the host of years to decades (40, 41). Ultimately, quantitative empirical estimates for an individual's retention of IgG antibodies against any *P. falciparum* antigen would be a factor of numerous immunological and parasitological factors, so broad assumptions could not be made for a human population. Interestingly, though known to both be among the longer-lasting anti-*Plasmodium* antibodies, IgG levels to the PfMSP1 and PfAMA1 antigens show low correlation in individuals (42), and it has been hypothesized that different factors control antibody responses to these antigens (43). Based on this information from previous studies, binary classification for this study population to estimate *P. falciparum* exposure appeared to be appropriate based on IgG_1 responses to both of these antigens. To reduce classification error, an additional margin was added to the binary categorization, so only those children with much higher IgG_1 levels (well beyond the seropositivity threshold) were considered as non-naïve for *P. falciparum*. This classification scheme is also supported by the three TES enrollment sites being located in meso- to high-endemic *P. falciparum* settings in Angola (34), and the relatively young ages of participants (6 months to 11 years old), meaning it is unlikely they would have had enough years of life for

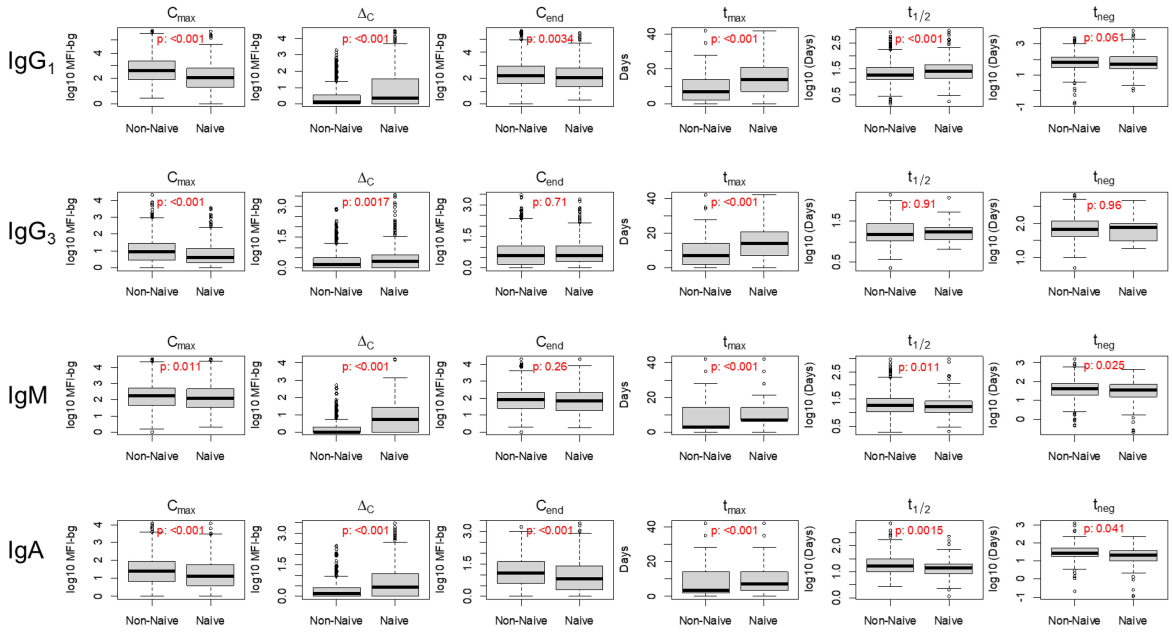

FIGURE 3
Distribution of six key post-treatment clearance parameters in non-naïve and naïve Angolan children treated for malaria, stratifying by immunoglobulin (Ig) class/subclass. *P*-value for Kolmogorov–Smirnov test for difference in empiric distribution between non-naïve and naïve participants. Mean values between non-naïve and naïve categories are displayed in Table 2.

TABLE 2 Mean values for each parameter by immunoglobulin isotype/subclass as aggregate for all *P. falciparum* antigen responses estimated for the naïve and non-naïve children.

		C_{max} (log_{10} MFI-bg)	Δ_C (log_{10} MFI-bg)	C_{end} (log_{10} MFI-bg)	t_{max} (days)	$t_{1/2}$ (days)	t_{neg} (days)
IgG_1	Non-Naïve	**2.61**	**0.12**	**2.23**	**7.0**	**19.5**	66.1
	Naïve	**2.07**	**0.34**	**2.04**	**14.0**	**24.5**	53.7
	p-value	**<0.001**	**<0.001**	**0.003**	**<0.001**	**<0.001**	0.06
IgG_3	Non-Naïve	**0.95**	**0.19**	0.60	**7.0**	14.8	64.6
	Naïve	**0.60**	**0.30**	0.60	**14.0**	17.0	75.9
	p-value	**<0.001**	**0.002**	0.71	**<0.001**	0.91	0.96
IgM	Non-Naïve	**2.23**	**0.00**	1.89	**3.0**	**18.6**	**39.8**
	Naïve	**2.09**	**0.74**	1.83	**7.0**	**16.6**	**33.9**
	p-value	**0.011**	**<0.001**	0.26	**<0.001**	**0.011**	**0.025**
IgA	Non-Naïve	**1.38**	**0.11**	**1.04**	**3.0**	**17.0**	**28.8**
	Naïve	**1.11**	**0.40**	**0.81**	**7.0**	**13.5**	**21.4**
	p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.002**	**0.041**

Statistically significant mean differences are displayed in bold.

IgG_1 seroreversion from a previous *P. falciparum* exposure. A previous report has also shown rapid acquisition of total IgG against *P. falciparum* antigens with time spent in Angola (44), indicating the high endemic nature of *P. falciparum* in this setting.

Between these two classification groups, no significant difference was noted between the peripheral parasite densities at presentation to the health facility. It may be expected that previous malaria exposure would suppress parasite burden (1, 32), but all children enrolled in this study were symptomatic, so the distributions presented here are not inclusive of lower-density asymptomatic infections in the general population (45, 46). Classification of the study population into naïve and non-naïve categories found many differences that highlighted classical assumptions of human adaptive humoral immunity. Additionally, as only children older than 6 months old were included in this study, any maternal antibodies would have likely been eliminated by this time (18, 19), and this Ig

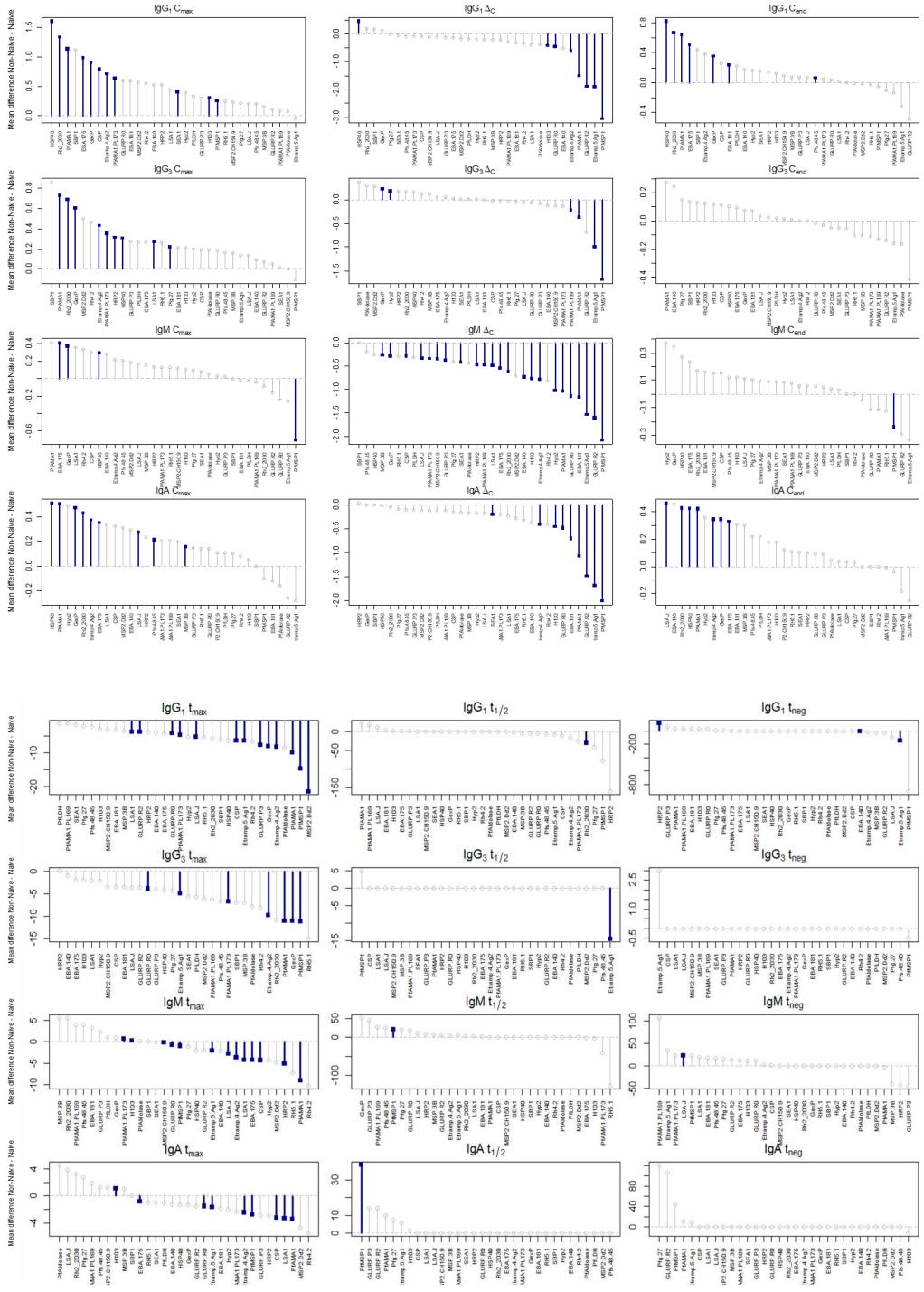

FIGURE 4
Mean differences for each parameter by Ig response for non-naïve and naïve children followed post-treatment. Positive values represent higher values in non-naïves, and lines with blue squares denote statistically significant differences.

data most certainly represents true host response. The current *P. falciparum* infection for non-naïve individuals appeared to have served as an antibody boosting event for IgG_1, IgG_3, IgM, and IgA antibodies when compared to naïve persons, with maximum antibody levels (C_{max}) significantly higher for all four of these Igs, though IgM boosting appears to be the most subdued when compared to the other isotypes/subclasses. Similar to IgG boosting seen in humans after successive

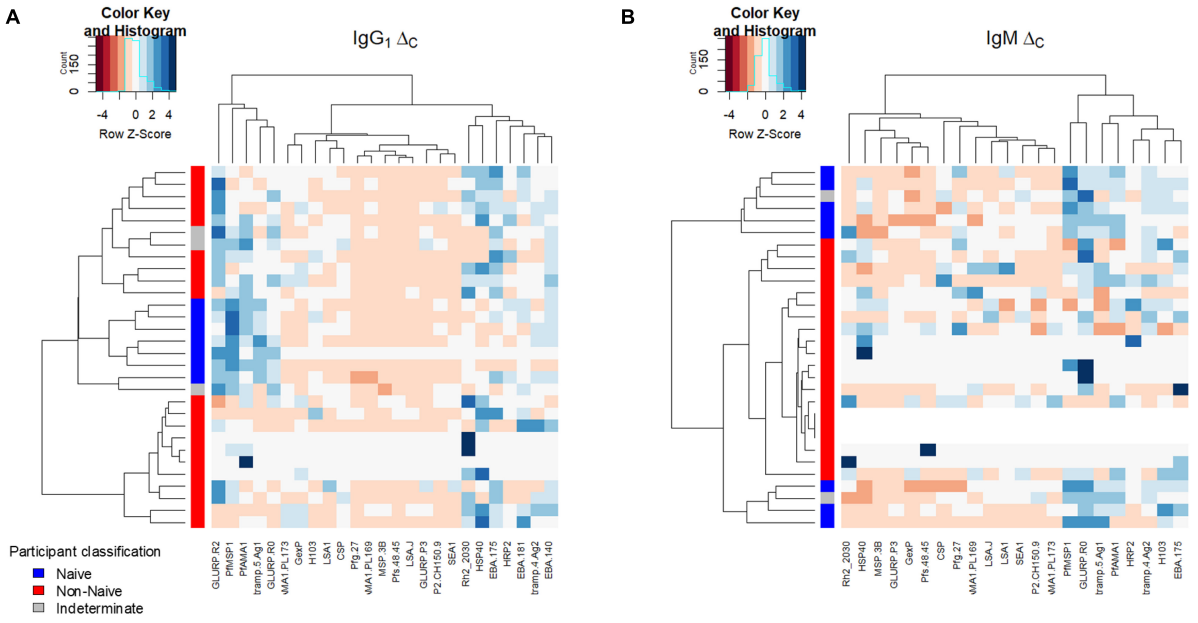

FIGURE 5
Hierarchical relationship among study participants shown for the Δ_C parameter. Clustering of Δ_C by *P. falciparum* antigen response (columns) and participants (rows) for IgG_1 **(A)** and IgM **(B)**. Intensity of color shading within the plot indicated by the magnitude of the standard score (Z score).

P. falciparum infections (37, 47), IgG_1 antibodies showed the most antigen targets with C_{max} values significantly higher (11/32, 34.4% of all antigens) for non-naïve vs. naïve children, though IgG_3 and IgA also showed multiple antigen targets (9 each) exhibiting boosting characteristics. Specifically, the IgG_1 responses to Rh2030, HSP40, and PfAMA1 were the highest boosted levels for non-naïve participants, and may (collectively or individually) be used to predict previous or nascent exposure. A prior study showed that in previously exposed individuals, the Rh2030 and PfAMA1 responses were highly correlated with each other and predictive of the *P. falciparum* pre-patent period (32). Recently, IgG sero-responses to all three of these antigens were significantly correlated to asymptomatic infection, whereas responses to the Etramp5Ag1 and PfMSP1 antigen were correlated with clinical disease (48), giving evidence that the non-naïve children in this current study had some form of previous *P. falciparum* exposure and were boosted during the current infection.

Recent work has expanded on contribution of the IgM response to *P. falciparum* infection with findings of IgM-positive memory B cell subsets being predominant in children, IgM inhibiting parasite invasion in a complement-dependent manner, and persistence of IgM response to merozoite surface antigen over time (26, 27). Among all four Ig isotypes/subclasses tested here, estimates for change in day of enrollment to peak Ig levels (Δ_C) were universally higher for IgM response in the naïve individuals, with 23/32 (71.9%) *P. falciparum* antigen Δ_C responses reaching statistical significance. These data suggest the presence of IgM + memory B cell response in non-naïve children (49), as the IgM response in naïve children is practically non-existent at the day of enrollment and 11/32 (34.4%) of IgM t_{max} estimates higher in naïves. Induction of IgA during natural (3, 50) and malaria vaccine (51) exposure has been well documented, though it's unclear if there's a specific immunological role this isotype plays in response to *P. falciparum* infection. Previous work to assess the value of IgA in protecting the host against malaria pathogenesis found no significant benefit from anti-PfMSP1 IgA when infecting mice with transgenic *P. berghei* expressing the PfMSP1 antigen (52). In the same manner as IgM, Δ_C estimates for IgA were nearly all higher in naïve children, but more similar to IgG_1 and IgG_3, C_{max} of nearly all antigens were higher in non-naïves. This data shows evidence for IgA boosting upon re-exposure similar to IgG subclasses with PfAMA1, Rh_2030, and HSP40 boosting as providing some of the strongest markers for previous exposure.

A limitation of this study was that presence and magnitude of IgG_1 response against two *P. falciparum* antigens were used as the only proxy for classification of any previous exposure. However, although previous studies from endemic settings have followed up persons and assessed Ig dynamics over long periods of time (8), previous malaria history (if assessed) comes from clinical episodes, and would miss asymptomatic infections. Additionally, infection events from longitudinal studies are typically noted by sparse intervals or clinical episodes, so true *P. falciparum* exposure could be missed by the sampling design. This current study only measures the absolute level of antibodies

binding to specific *P. falciparum* antigens, and experiments were not performed to measure binding strength among different antigens or the naïve/non-naïve groups. Proportions of B cell subsets were not able to be evaluated, nor was antibody functional activity (inhibition of parasite invasion, complement activation, etc.) assessed. The results presented here are data only from symptomatic Angolan children infected with *P. falciparum* from Angola, so it is possible that persons of older ages, persons living in different transmission settings, or different host and parasite genotypes would provide different outputs than the ones observed here. More robust statistical methods for looking across data from multiple Ig classes and antigens are also needed to gain a comprehensive understanding of the human B cell response to *P. falciparum* infection.

Classification of children into *P. falciparum* naïve and non-naïve categories and assessment of antibody dynamics weeks after resolved infection showed stark differences in Ig levels and temporal trends between these two groups. This data helps to elucidate Ig dynamics in a human population naturally exposed to *P. falciparum* malaria and provides generalizable results which can better assist in translating findings from seroepidemiological studies. Presentation here of Ig results by individual *P. falciparum* antigens will also aid future research studies utilizing these specific targets to put serological data into context

Data availability statement

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

Author contributions

PD and MP designed and coordinated the field study. ER and MP designed the laboratory study, conceptualized the experiments, drafted the manuscript, and performed statistical analyses. ER and DN performed laboratory assays. BW, JB, CD, and KT provided antigens and scientific expertise. All authors reviewed and approved the final version of the manuscript.

Funding

MP was supported by the U.S. President's Malaria Initiative. JB was supported by a Senior Research Fellowship of the National Health and Medical Research Council of Australia.

Acknowledgments

We thank Christine Langer and Linda Reiling (Burnet Institute) for their assistance with recombinant protein expression and purification. We thank Jan Pohl at CDC for the assistance with antigen production. We also thank Jing Jin and Simon Draper (University of Oxford, United Kingdom) for the provision of RH5.1 protein.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.869028/full#supplementary-material

References

1. Doolan DL, Dobano C, Baird JK. Acquired immunity to malaria. *Clin Microbiol Rev.* (2009) 22:13–36.

2. Cohen S, Mc GI, Carrington S. Gamma-globulin and acquired immunity to human malaria. *Nature.* (1961) 192:733–7.

3. Leoratti FM, Durlacher RR, Lacerda MV, Alecrim MG, Ferreira AW, Sanchez MC, et al. Pattern of humoral immune response to Plasmodium falciparum blood stages in individuals presenting different clinical expressions of malaria. *Malar J.* (2008) 7:186. doi: 10.1186/1475-2875-7-186

4. Ubillos I, Jimenez A, Vidal M, Bowyer PW, Gaur D, Dutta S, et al. Optimization of incubation conditions of Plasmodium falciparum antibody multiplex assays to measure IgG, IgG1-4, IgM and IgE using standard and customized reference pools for sero-epidemiological and vaccine studies. *Malar J.* (2018) 17:219. doi: 10.1186/s12936-018-2369-3

5. Rogier E, Nace D, Dimbu PR, Wakeman B, Pohl J, Beeson JG, et al. Framework for characterizing longitudinal antibody response in children after plasmodium falciparum infection. *Front Immunol.* (2021) 12:617951. doi: 10.3389/fimmu.2021.617951

6. Braga EM, Barros RM, Reis TA, Fontes CJ, Morais CG, Martins MS, et al. Association of the IgG response to Plasmodium falciparum merozoite protein (C-terminal 19 kD) with clinical immunity to malaria in the Brazilian Amazon region. *Am J Trop Med Hyg.* (2002) 66:461–6. doi: 10.4269/ajtmh.2002.66.461

7. Rouhani M, Zakeri S, Mehrizi AA, Djadid ND. Comparative analysis of the profiles of IgG subclass-specific responses to Plasmodium falciparum apical membrane antigen-1 and merozoite surface protein-1 in naturally exposed individuals living in malaria hypoendemic settings, Iran. *Malar J.* (2015) 14:58. doi: 10.1186/s12936-015-0547-0

8. Ssewanyana I, Rek J, Rodriguez I, Wu L, Arinaitwe E, Nankabirwa JI, et al. Impact of a rapid decline in malaria transmission on antimalarial IgG subclasses and avidity. *Front Immunol.* (2020) 11:576663. doi: 10.3389/fimmu.2020.576663

9. Dobano C, Quelhas D, Quinto L, Puyol L, Serra-Casas E, Mayor A, et al. Age-dependent IgG subclass responses to Plasmodium falciparum EBA-175 are differentially associated with incidence of malaria in Mozambican children. *Clin Vaccine Immunol.* (2012) 19:157–66. doi: 10.1128/CVI.05523-11

10. Boyle MJ, Reiling L, Feng G, Langer C, Osier FH, Aspeling-Jones H, et al. Human antibodies fix complement to inhibit Plasmodium falciparum invasion of erythrocytes and are associated with protection against malaria. *Immunity.* (2015) 42:580–90. doi: 10.1016/j.immuni.2015.02.012

11. Osier FH, Feng G, Boyle MJ, Langer C, Zhou J, Richards JS, et al. Opsonic phagocytosis of Plasmodium falciparum merozoites: mechanism in human immunity and a correlate of protection against malaria. *BMC Med.* (2014) 12:108. doi: 10.1186/1741-7015-12-108

12. Pholcharee T, Oyen D, Flores-Garcia Y, Gonzalez-Paez G, Han Z, Williams KL, et al. Structural and biophysical correlation of anti-NANP antibodies with in vivo protection against P. falciparum. *Nat Commun.* (2021) 12:1063. doi: 10.1038/s41467-021-21221-4

13. Livingstone MC, Bitzer AA, Giri A, Luo K, Sankhala RS, Choe M, et al. In vitro and in vivo inhibition of malaria parasite infection by monoclonal antibodies against Plasmodium falciparum circumsporozoite protein (CSP). *Sci Rep.* (2021) 11:5318.

14. Murugan R, Buchauer L, Triller G, Kreschel C, Costa G, Pidelaserra Marti G, et al. Clonal selection drives protective memory B cell responses in controlled human malaria infection. *Sci Immunol.* (2018) 3:eaa8029. doi: 10.1126/sciimmunol.aap8029

15. van den Hoogen LL, Stresman G, Presume J, Romilus I, Mondelus G, Elisme T, et al. Selection of antibody responses associated with plasmodium falciparum infections in the context of malaria elimination. *Front Immunol.* (2020) 11:928. doi: 10.3389/fimmu.2020.00928

16. Helb DA, Tetteh KK, Felgner PL, Skinner J, Hubbard A, Arinaitwe E, et al. Novel serologic biomarkers provide accurate estimates of recent Plasmodium falciparum exposure for individuals and communities. *Proc Natl Acad Sci USA.* (2015) 112:E4438–47. doi: 10.1073/pnas.1501705112

17. Dobbs KR, Dent AE. Plasmodium malaria and antimalarial antibodies in the first year of life. *Parasitology.* (2016) 143:129–38.

18. Dent AE, Malhotra I, Wang X, Babineau D, Yeo KT, Anderson T, et al. Contrasting patterns of serologic and functional antibody dynamics to plasmodium falciparum antigens in a Kenyan birth cohort. *Clin Vaccine Immunol.* (2016) 23:104–16. doi: 10.1128/CVI.00452-15

19. Moussiliou A, Turner L, Cottrell G, Doritchamou J, Gbedande K, Fievet N, et al. Dynamics of PfEMP1 antibody profile from birth to 12 months of age in beninese infants. *J Infect Dis.* (2020) 221:2010–7. doi: 10.1093/infdis/jiaa043

20. Portugal S, Tipton CM, Sohn H, Kone Y, Wang J, Li S, et al. Malaria-associated atypical memory B cells exhibit markedly reduced B cell receptor signaling and effector function. *Elife.* (2015) 4:e07218. doi: 10.7554/eLife.07218

21. Sutton HJ, Aye R, Idris AH, Vistein R, Nduati E, Kai O, et al. Atypical B cells are part of an alternative lineage of B cells that participates in responses to vaccination and infection in humans. *Cell Rep.* (2021) 34:108684. doi: 10.1016/j.celrep.2020.108684

22. Pérez-Mazliah D, Gardner PJ, Schweighoffer E, McLaughlin S, Hosking C, Tumwine I, et al. Plasmodium-specific atypical memory B cells are short-lived activated B cells. *Elife.* (2018) 7:e39800. doi: 10.7554/eLife.39800

23. Vijay R, Guthmiller JJ, Sturtz AJ, Surette FA, Rogers KJ, Sompallae RR, et al. Infection-induced plasmablasts are a nutrient sink that impairs humoral immunity to malaria. *Nat Immunol.* (2020) 21:790–801. doi: 10.1038/s41590-020-0678-5

24. Chan JA, Loughland JR, de Labastida Rivera F, SheelaNair A, Andrew DW, Dooley NL, et al. Th2-like T follicular helper cells promote functional antibody production during plasmodium falciparum infection. *Cell Rep Med.* (2020) 1:100157. doi: 10.1016/j.xcrm.2020.100157

25. Obeng-Adjei N, Portugal S, Tran TM, Yazew TB, Skinner J, Li S, et al. Circulating Th1-Cell-type Tfh Cells that exhibit impaired B cell help are preferentially activated during acute malaria in children. *Cell Rep.* (2015) 13:425–39. doi: 10.1016/j.celrep.2015.09.004

26. Boyle MJ, Chan JA, Handayuni I, Reiling L, Feng G, Hilton A, et al. IgM in human immunity to Plasmodium falciparum malaria. *Sci Adv.* (2019) 5:eaax4489.

27. Hopp CS, Sekar P, Diouf A, Miura K, Boswell K, Skinner J, et al. Plasmodium falciparum-specific IgM B cells dominate in children, expand with malaria, and produce functional IgM. *J Exp Med.* (2021) 218:e20200901. doi: 10.1084/jem.20200901

28. Fonseca AM, Quinto L, Jiménez A, González R, Bardají A, Maculuve S, et al. Multiplexing detection of IgG against Plasmodium falciparum pregnancy-specific antigens. *PLoS One.* (2017) 12:e0181150. doi: 10.1371/journal.pone.0181150

29. Kerkhof K, Canier L, Kim S, Heng S, Sochantha T, Sovannaroth S, et al. Implementation and application of a multiplex assay to detect malaria-specific antibodies: a promising tool for assessing malaria transmission in Southeast Asian pre-elimination areas. *Malar J.* (2015) 14:338. doi: 10.1186/s12936-015-0868-z

30. Richards JS, Arumugam TU, Reiling L, Healer J, Hodder AN, Fowkes FJ, et al. Identification and prioritization of merozoite antigens as targets of protective human immunity to Plasmodium falciparum malaria for vaccine and biomarker development. *J Immunol.* (2013) 191:795–809. doi: 10.4049/jimmunol.1300778

31. Crompton PD, Kayala MA, Traore B, Kayentao K, Ongoiba A, Weiss GE, et al. A prospective analysis of the Ab response to Plasmodium falciparum before and after a malaria season by protein microarray. *Proc Natl Acad Sci USA.* (2010) 107:6958–63. doi: 10.1073/pnas.1001323107

32. Achan J, Reuling IJ, Yap XZ, Dabira E, Ahmad A, Cox M, et al. Serologic markers of previous malaria exposure and functional antibodies inhibiting parasite growth are associated with parasite kinetics following a plasmodium falciparum controlled human infection. *Clin Infect Dis.* (2020) 70:2544–52. doi: 10.1093/cid/ciz740

33. van den Hoogen LL, Walk J, Oulton T, Reuling IJ, Reiling L, Beeson JG, et al. Antibody responses to antigenic targets of recent exposure are associated with low-density parasitemia in controlled human plasmodium falciparum infections. *Front Microbiol.* (2018) 9:3300. doi: 10.3389/fmicb.2018.03300

34. Davlantes E, Dimbu PR, Ferreira CM, Florinda Joao M, Pode D, Felix J, et al. Efficacy and safety of artemether-lumefantrine, artesunate-amodiaquine, and dihydroartemisinin-piperaquine for the treatment of uncomplicated Plasmodium falciparum malaria in three provinces in Angola, 2017. *Malar J.* (2018) 17:144. doi: 10.1186/s12936-018-2290-9

35. Egan AF, Chappel JA, Burghaus PA, Morris JS, McBride JS, Holder AA, et al. Serum antibodies from malaria-exposed people recognize conserved epitopes formed by the two epidermal growth factor motifs of MSP1(19), the carboxy-terminal fragment of the major merozoite surface protein of Plasmodium falciparum. *Infect Immun.* (1995) 63:456–66. doi: 10.1128/iai.63.2.456-466.1995

36. Stanisic DI, Richards JS, McCallum FJ, Michon P, King CL, Schoepflin S, et al. Immunoglobulin G subclass-specific responses against Plasmodium falciparum merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. *Infect Immun.* (2009) 77:1165–74. doi: 10.1128/IAI.01129-08

37. White MT, Griffin JT, Akpogheneta O, Conway DJ, Koram KA, Riley EM, et al. Dynamics of the antibody response to Plasmodium falciparum infection in African children. *J Infect Dis.* (2014) 210:1115–22.

38. Taghavian O, Jain A, Joyner CJ, Ketchum S, Nakajima R, Jasinskas A, et al. Antibody profiling by proteome microarray with multiplex isotype detection reveals overlap between human and aotus nancymaae controlled malaria infections. *Proteomics.* (2018) 18:201700277.

39. Fowkes FJ, Richards JS, Simpson JA, Beeson JG. The relationship between anti-merozoite antibodies and incidence of Plasmodium falciparum malaria: a systematic review and meta-analysis. *PLoS Med.* (2010) 7:e1000218. doi: 10.1371/journal.pmed.1000218

40. Drakeley CJ, Corran PH, Coleman PG, Tongren JE, McDonald SL, Carneiro I, et al. Estimating medium- and long-term trends in malaria transmission by using serological markers of malaria exposure. *Proc Natl Acad Sci USA.* (2005) 102:5108–13. doi: 10.1073/pnas.0408725102

41. Ondigo BN, Hodges JS, Ireland KF, Magak NG, Lanar DE, Dutta S, et al. Estimation of recent and long-term malaria transmission in a population by antibody testing to multiple Plasmodium falciparum antigens. *J Infect Dis.* (2014) 210:1123–32. doi: 10.1093/infdis/jiu225

42. Rogier E, Moss DM, Chard AN, Trinies V, Doumbia S, Freeman MC, et al. Evaluation of immunoglobulin G responses to plasmodium falciparum and Plasmodium vivax in Malian school children using multiplex bead assay. *Am J Trop Med Hyg.* (2017) 96:312–8. doi: 10.4269/ajtmh.16-0476

43. Johnson AH, Leke RG, Mendell NR, Shon D, Suh YJ, Bomba-Nkolo D, et al. Human leukocyte antigen class II alleles influence levels of antibodies to the Plasmodium falciparum asexual-stage apical membrane antigen 1 but not to merozoite surface antigen 2 and merozoite surface protein 1. *Infect Immun.* (2004) 72:2762–71. doi: 10.1128/IAI.72.5.2762-2771.2004

44. Martins JF, Marques C, Nieto-Andrade B, Kelley J, Patel D, Nace D, et al. Malaria risk and prevention in Asian migrants to Angola. *Am J Trop Med Hyg.* (2020) 103:1918–26. doi: 10.4269/ajtmh.20-0706

45. Galatas B, Bassat Q, Mayor A. Malaria parasites in the asymptomatic: looking for the hay in the haystack. *Trends Parasitol.* (2016) 32:296–308. doi: 10.1016/j.pt.2015.11.015

46. Wu L, van den Hoogen LL, Slater H, Walker PG, Ghani AC, Drakeley CJ, et al. Comparison of diagnostics for the detection of asymptomatic Plasmodium falciparum infections to inform control and elimination strategies. *Nature.* (2015) 528:S86–93.

47. Fowkes FJ, McGready R, Cross NJ, Hommel M, Simpson JA, Elliott SR, et al. New insights into acquisition, boosting, and longevity of immunity to malaria in pregnant women. *J Infect Dis.* (2012) 206:1612–21. doi: 10.1093/infdis/jis566

48. Wu L, Mwesigwa J, Affara M, Bah M, Correa S, Hall T, et al. Antibody responses to a suite of novel serological markers for malaria surveillance demonstrate strong correlation with clinical and parasitological infection across seasons and transmission settings in The Gambia. *BMC Med.* (2020) 18:304. doi: 10.1186/s12916-020-01724-5

49. Ly A, Hansen DS. Development of B cell memory in malaria. *Front Immunol.* (2019) 10:559. doi: 10.3389/fimmu.2019.00559

50. Doritchamou J, Teo A, Morrison R, Arora G, Kwan J, Manzella-Lapeira J, et al. Functional antibodies against placental malaria parasites are variant dependent and differ by geographic region. *Infect Immun.* (2019) 87:e865–818.

51. Suau R, Vidal M, Aguilar R, Ruiz-Olalla G, Vazquez-Santiago M, Jairoce C, et al. RTS,S/AS01E malaria vaccine induces IgA responses against CSP and vaccine-unrelated antigens in African children in the phase 3 trial. *Vaccine.* (2021) 39:687–98. doi: 10.1016/j.vaccine.2020.12.038

52. Shi J, McIntosh RS, Adame-Gallegos J, Dehal PK, van Egmond M, van de Winkel J, et al. The generation and evaluation of recombinant human IgA specific for Plasmodium falciparum merozoite surface protein 1-19 (PfMSP1 19). *BMC Biotechnol.* (2011) 11:77. doi: 10.1186/1472-6750-11-77

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 18 August 2022
DOI 10.3389/fmed.2022.929366

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

RECEIVED 26 April 2022
ACCEPTED 22 July 2022
PUBLISHED 18 August 2022

CITATION
Byrne I, Cramer E, Nelli L, Rerolle F, Wu L, Patterson C, Rosado J, Dumont E, Tetteh KKA, Dantzer E, Hongvanthong B, Fornace KM, Stresman G, Lover A, Bennett A and Drakeley C (2022) Characterizing the spatial distribution of multiple malaria diagnostic endpoints in a low-transmission setting in Lao PDR. *Front. Med.* 9:929366. doi: 10.3389/fmed.2022.929366

Characterizing the spatial distribution of multiple malaria diagnostic endpoints in a low-transmission setting in Lao PDR

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The epidemiology of malaria changes as prevalence falls in low-transmission settings, with remaining infections becoming more difficult to detect and diagnose. At this stage active surveillance is critical to detect residual hotspots of transmission. However, diagnostic tools used in active surveillance generally only detect concurrent infections, and surveys may benefit from sensitive tools such as serological assays. Serology can be used to interrogate and characterize individuals' previous exposure to malaria over longer durations, providing information essential to the detection of remaining foci of infection. We ran blood samples collected from a 2016 population-based survey in the low-transmission setting of northern Lao PDR on a multiplexed bead assay to characterize historic and recent exposures to *Plasmodium falciparum* and *vivax*. Using geostatistical methods and remote-sensing data we assessed the environmental and spatial associations with exposure, and created predictive maps of exposure within the study sites. We additionally linked the active surveillance PCR and serology data with passively collected surveillance data from health facility records. We aimed to highlight the added information which can be gained from serology as a tool in active surveillance surveys in low-transmission settings, and to identify priority areas for national surveillance programmes where malaria risk is higher. We also discuss

the issues faced when linking malaria data from multiple sources using multiple diagnostic endpoints.

KEYWORDS

malaria, serology, active surveillance, passive surveillance, geostatistics, elimination

Introduction

Through an intensification of their programmatic activities and an increased coverage of interventions (1), the Lao People's Democratic Republic (PDR) has seen substantial declines in malaria cases, with a fall in case incidence by 80% from 2016 to 2020, and no reported malaria deaths since 2018 (2). Transmission is very low in northern Lao PDR, with *P. vivax* cases making up the majority of malaria burden (1, 3). The nation aims to eliminate *Plasmodium vivax* and *Plasmodium falciparum* from northern areas by 2025, and all species nationwide by 2030 (2, 4).

As countries near elimination and transmission declines, they experience characteristic shifts in malaria epidemiology. Substantial areas become malaria-free, and malaria risk becomes increasingly heterogenous and geographically or demographically clustered (5, 6), with cases becoming more difficult to detect and diagnose. Here, passive surveillance systems become inadequate as the sole method of data collection to inform population-level burden estimates (5–7). Estimates may be biased by different treatment-seeking behavior in high-risk populations, and the quality of record keeping may vary between health facilities or administrative regions (8). Passive surveillance also fails to detect asymptomatic individuals, which act as parasite reservoirs and contribute to continued transmission, even in low-transmission settings (9–11). At this stage it is important to find remaining clusters of transmission where infection remains high in order to target resources effectively (6, 7). Finding these residual foci of transmission involves actively seeking out infections, often through screening or surveying populations irrespective of malaria symptoms. This active surveillance can complement passive surveillance, and can play a role in interrupting transmission as countries near elimination (11). Active surveillance surveys for malaria are typically cross-sectional and involve sampling communities using RDT diagnostics, often collecting valuable added information on cases and specific populations who are at higher risk of infection (1, 5). As prevalence drops in elimination settings, passive and active surveillance surveys face the challenge of detecting sufficient concurrent infections to obtain a full picture of the epidemiology within a population, even when robust sampling-strategies are applied (1). In these situations, more sensitive diagnostic tools are needed to improve burden estimates and understand whether transmission is ongoing (12).

Serological assays are useful in such low-transmission settings. Rather than solely capturing concurrent infections, serology measures specific antibody responses which reflect previous exposure to pathogens. Different malaria antigens elicit different antibody responses, each of which last for different durations in the immune system (13, 14). Longitudinal research into antibody kinetics has resulted in a highly informative and diverse set of biomarkers being identified for *P. vivax* and *P. falciparum* infections. These characterize an individual's exposure history, and when sampled en masse, can provide information on the short-, medium- and long-term trends in malaria transmission in a population, highlighting changes in transmission over longer durations than PCR-based surveys (13, 15–18). Serological methods have been shown to be a useful complementary tool where traditional parasitological tools are not sensitive enough to estimate recent and active exposure and transmission intensity in low-transmission settings (11, 19). Serological multiplex bead assays (MBA) make serological surveys operationally feasible and can be added as a supplemental aspect of population surveys, as they can measure a broad range of immune responses from a single blood spot (14, 20). Measuring population-level serological responses using MBA can be useful in showing spatial heterogeneity of malaria exposure, finding clustering or hotspots of transmission and to predict receptive areas at risk of outbreaks (12, 21, 22).

Geostatistical methods are increasingly being applied in disease research to relate infection metrics with environmental, spatial and temporal covariates (14). In malaria research there are numerous recent analyses projects involving geostatistical mapping of malaria incidence, prevalence and other metrics (23, 24). In low-resource and/or low-transmission settings where infection data is sparse and transmission becomes more spatially heterogeneous, geostatistical mapping can identify and highlight areas where risk is more concentrated and may require targeted interventions from programme implementers (23, 25). Alongside the useful predictions of disease burden, geostatistical analyses can also identify areas of uncertainty in predictions, which can be used to prioritize future data collection (23).

Integrating geostatistical methods with serology data collected during active surveillance surveys provides an opportunity to characterize the spatial distribution of recent

and historic exposure to different malaria antigens in a low-transmission setting. We ran blood samples from a 2016 active surveillance population survey (1) in northern Lao PDR using a serology MBA to gain an understanding of current and historic exposures to *P. vivax* and *P. falciparum*. We additionally used passive surveillance (case incidence) data collected from health facilities in the same districts (3) to compare serology and PCR-derived prevalences with burden estimates from active surveillance at the health facility catchment- level. We fit geostatistical models to predict historic and recent exposures to *P. vivax* and *P. falciparum*. We aimed to highlight the additional information which can be leveraged from serology as a complementary tool to passive and active surveillance in low-transmission settings, and to identify areas with elevated risks of malaria transmission requiring prioritization for national surveillance programmes.

Methods

Study site

The active surveillance survey was conducted in four districts (Et, Paktha, Nambak and Koua) of northern Lao PDR (Figure 1), which are situated in four northern provinces (Bokeo, Huaphanh, Phongsaly and Luang-Prabang). The survey was conducted following the rainy season, between September and October 2016. The districts were chosen to focus on areas of malaria hotspots, and to ensure representation by surveying from diverse epidemiological settings (1). At the time of the study *P. vivax* was endemic and *P. falciparum* had reached historical lows in these provinces (3). It is a mountainous region characterized by a diverse climate, with low population density and limited access to roads (1, 3). The region shares borders with China, Myanmar, Thailand and Vietnam.

Study design

The passive surveillance data were gathered according to Rerolle et al. (3). Briefly, a retrospective review of malaria registries between 2013 and 2016 was performed at health-facilities in the active survey districts. The data gathered from the registries included testing for malaria by RDT or microscopy, date, species-specific test results, village location and demographic variables including age and gender of those tested. For the present study, the dataset was sub-set to include records from 2016.

The survey data were collected according to Lover et al. (1). Briefly, a stratified two-stage cluster-sampling design was used. In each district catchment 25 survey clusters of 50 individuals were chosen for sampling, providing 1,250 participants per district and 5,000 overall. The district malaria office catchments were determined from local-level health office lists and did not always conform to official administrative boundaries. As a result, in the eastern district of Et a portion of the households fall outside of the official administrative boundaries (Figure 1). All residents and visitors who were over 18 months old and had spent the previous night in the household were invited to participate. Written or thumbprint consent was obtained from all participants. Upon informed consent, eligible individuals were tested with CareStart Ag Pf/Pv (SD Bioline, Cat #05FK80) rapid diagnostic tests and treated according to national guidelines if found positive. Four blood spots were collected on Whatman 903 "Protein Saver" sample cards (GE Healthcare; Cardiff UK). These were dried and cooled in refrigerators until subsequent analysis. Geographic coordinates were collected for all participating households.

Laboratory procedures

The chemical coupling of 17 *P.falciparum* and *P.vivax* antigens (Supplementary Table 1) to MagPlex© beads (Luminex Corporation, TX, USA) were previously optimized *via* titration as described by Wu et al. (26). 3 mm punches of one blood spot from each Whatman 903 Protein Saver card were eluted 1:100 in buffer B [1xPBS, 0.05% Tween, 0.5% BSA, 0.02% sodium azide, 0.1% casein, 0.5% polyvinyl alcohol (PVA), 0.5% polyvinyl pyrrolidone (PVP)] containing 15.25 ug/mL *E.coli lysate* to prevent non-specific binding to antigens expressed in *E.coli*. 50 uL of each 1:100 blood spot elution was co-incubated with 1,000 coupled beads per antigen and specific IgG antibody binding was detected using 50 uL of R-PE conjugated secondary antibody [Jackson Immunoresearch 109-116-098: Goat anti-human Fcy-fragment specific IgG conjugated to R-Phycoerythrin (R-PE)] diluted 1:200 as described previously (26). Background-adjusted median fluorescent intensity (MFI) of wells achieving at least a 35-bead count per antigen were measured using a Luminex MAGPIX© bioanalyzer and xPONENT software (version 4.2). *P. falciparum* positive control (NIBSC, 10/198), *P. vivax* positive control (72/96, NIBSC) and a curve of pooled hyperimmune *P.falciparum* sera (CP3, LSHTM) were included in singlicate on each test plate to assess interplate variability. 96 malaria-naïve sera from the UK (Public Health England 2016) were assayed at 1:100 using the same method.

PCR testing was performed on dried blood spots as described by Lover et al. (1), using previously described methods (27). Four blood spots were lysed on 96-well plates overnight at 4°C with 150 μl per well of HBS 1X/Saponin 0.5%. Samples were washed twice with HBS 1X and Instagene® Matric resin (Bio-Rad, Singapore) was used according to manufacturer's instructions to extract DNA. In order to limit the presence of inhibitors an additional centrifugation step (4,000 rpm, 20 min) was added, and a final volume of 50 μl of the supernatant was transferred into a new 96-well plate. Extracted DNA samples

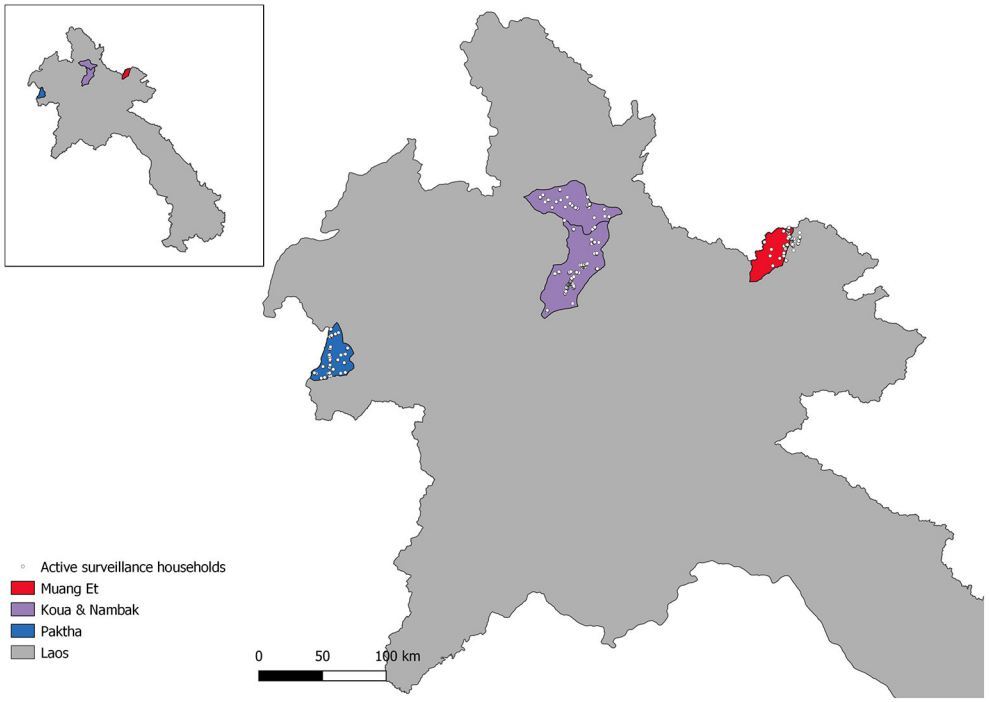

FIGURE 1
Map of Northern Lao PDR with active surveillance study households and districts of the study.

were screened for the presence of *Plasmodium* DNA using a qualitative real-time PCR assay which targeted *Plasmodium cytochrome b* gene (27). Positive samples were analyzed for *Plasmodium* species *P. falciparum, P. vivax, P. ovale* and *P. malariae,* using four real-time PCR assays (27).

Statistical analysis

Individuals were classified as seropositive or seronegative for historical and recent exposures to *P. vivax* and *P. falciparum* based on their responses to the antigens in Table 1. The antigens in Table 1 were chosen based on their known longevity in the immune system. Pf/PvMSP119 and Pf/PvAMA1 are known to persist in the blood for many years and can be used as a proxy for any previous exposure in an individual's lifetime (14). Etramp5.Ag1 and PvEBPII are known to be shorter-lived and are used here to represent exposure within 6–9 months (17, 27).

We used unsupervised machine learning K-means clustering algorithms on each antigen separately to group samples into positives or negatives based on their MFI values. The optimal numbers of clusters for each antigen was determined using within-cluster sum of squares and average silhouette testing (28). Historic exposure to *P. vivax* was calculated as a combined exposure to PvMSP119 and PvAMA1. If an individual was classified as seropositive to one or both of these antigens, they were classified as being seropositive to historical exposure to *P. vivax.* Recent exposure to *P. vivax* was determined by seropositivity to PvEBPII. If an individual was positive for PvEBPII, they were classified as recently exposed to *P. vivax.* Historic exposures to *P. falciparum* were calculated as a combined exposure to PfMSP119 and PfAMA1. If an individual was classified as seropositive to one or both of these antigens, they were classified as being seropositive to historical exposure to *P. falciparum.* Recent exposure to *P. falciparum* was defined by seropositivity to Etramp5Ag1 antigens. If an individual was positive for Etramp5Ag1, they were classified as recently exposed to *P. falciparum.* Age-stratified seroprevalence was estimated for proportional age groups for each species and each exposure.

Spatial analysis

The spatial distribution of exposure risks were assessed using geostatistical methods. Satellite-derived potential spatial and environmental covariates were assembled to assess associations with exposure risk. Covariates included topographic measures, distance to land cover types, forest cover and forest loss, population density, accessibility, and climactic variables (Supplementary Table 1). Pearson correlation coefficients were calculated, and highly correlated variables (correlation

TABLE 1 Table of malaria antigens used to define P. falciparum and P. vivax exposures, broken down by Plasmodium species. Including Plasmodb ID and reference source.

Species	Antigen	Description	Exposure period	Plasmodb ID	Reference
P.falciparum	PfAMA1	Apical membrane antigen 1	Historic	PF3D7_1133400	(44)
P.falciparum	PfMSP1_19	Merozoite surface protein 1-19	Historic	PF3D7_0930300	(45)
P.falciparum	Etramp 5 Ag 1	Early transcribed membrane protein 5 antigen (exon) 1	Recent	PF3D7_0532100	(46), Tetteh K unpublished
P.vivax	PvMSP119	Merozoite surface protein 1-19	Historic	PVX_099980	(47, 48)
P.vivax	PvAMA1	Apical membrane antigen 1	Historic	PVX_092275	(49)
P.vivax	PvEBPII	*P. vivax* erythrocyte binding protein	Recent	PVX_110810	(50–52)

coefficient > 0.8) were excluded from the final dataset. All covariates were resampled to 250 m for predictions.

Geostatistical models of household seroprevalence for each species exposure were fit separately for the 1,402 households in the active surveillance. The models were fit within a Bayesian framework where $p(x_i)$ denotes seroprevalence at locations x_i, $i = 1, \ldots, n$, the number of positive households *Yi* out of *Ni* people sampled follows a binomial distribution:

$$Yi|P(x_i) \sim Binomial(N_i, P(x_i)),$$
$$logit(P(x_i)) = \beta_0 + d(x_i)' \beta + w_i$$

Where β_0 denotes the intercept, $\boldsymbol{d(x_i)'\beta}$ denotes a vector of location specific covariate effects (within active survey district boundaries) and w_i represents the spatial effect. The spatial effects were modeled as a Matérn covariance function using the stochastic partial differential equation (SPDE) approach in Integrated Nested Laplace Approximation (R-INLA) (29). The intercepts and fixed effect coefficients were fitted with weakly informative priors of Normal (0,100). Deviance information criteria (DIC) were used to assess the final models. A continuous surface of prevalence predictions for the active survey districts were extracted as the mean of posteriors of the predictions for each model. For the eastern district of Et, we extended the predictions to include the neighboring district of Xienghor, as a portion of the survey households fell close to or over the official district border. Exceedance probabilities for a 20% seroprevalence threshold for *P. vivax* and a 5% seroprevalence threshold for *P. falciparum* exposures were also extracted. These metrics represent the probability of the seroprevalence in each location exceeding its given threshold, where probabilities around 0.5 represent high uncertainty around the threshold (30). The upper and lower limits of the 95% credible intervals were also extracted to visualize uncertainty. Prevalence predictions, exceedance probabilities and upper and lower limits of the 95% credible intervals were converted to raster files and visualized in QGIS.

To evaluate health facility catchment-level seroprevalences and PCR prevalences, we estimated catchment areas for all health facilities from an official Lao PDR Ministry of Health list (31) of 190 health facilities in the country. A friction surface map of motorized travel time in Laos (32) was used to create 190 rasters of travel time to each health facility. The travel time rasters were then combined into a final raster of minimum travel time to each of the health facilities and converted into a polygon shapefile of catchment boundaries based on lowest-travel time to health facility. The catchment areas were estimated using script adapted from Weiss et al. (32) in RStudio version 1.4. Household seroprevalence estimates were linked to catchment boundaries using QGIS, and catchment-level seroprevalences and PCR prevalences were calculated for each species exposure. Positive RDT and microscopy levels per capita for 2016 were calculated from the estimated population size for each catchment.

Ethics

Approvals for the field surveys (PI: Adam Bennett) were obtained from UCSF (approval 16-19649; 7-20-2016) and the Lao National Ethics Committee for Health Research, Lao Ministry of Health (approval 2016-014; 8-22-2016). Both approvals included provisions for future analysis of serological markers of malaria exposures.

Results

The results from the retrospective survey of 2016 passive surveillance records from the study regions are presented in Table 2. In total 343 *P. vivax* cases and 36 *P. falciparum* cases were confirmed by RDT. 23 *P. vivax* cases and 12 *P. falciparum* cases were confirmed by microscopy.

Table 3 provides descriptive statistics on the participants involved in the active survey. 5,084 individuals were samples from 1,402 households, with an average of 3.6 samples per household.

The mean MFI values for the antigens used to define historic and recent exposure to *P. falciparum* and *P. vivax* are as follows: PfMSP119 541.26 (±1334.88); PfAMA1 723.33 (±1785.43); Etramp5.Ag1 99.31 (±183.71); PvMSP119 169.272 (±181.789); PvAMA1 483.1 (±1772.88); PvEBPII 371.64 (±860.05). The range of individuals' MFI values by positivity and age are presented in Supplementary Figures 1A,B.

TABLE 2 Numbers of positive cases by species confirmed by RDT and microscopy from passive surveillance (health center) 2016 records.

District	Health Center	RDT Pf +ve	RDT Pv +ve	Microscopy Pf +ve	Microscopy Pv +ve
Khua	Buamaphan	5	60	1	2
Khua	Lardsang	1	1	0	0
Khua	Nayang	3	37	0	2
Khua	Vikocmueng	0	0	0	0
Nambak	Khunolum	0	0	0	0
Nambak	Makpouk	18	100	0	0
Nambak	Muengteng	0	0	0	0
Nambak	Numnga	1	1	0	0
Nambak	Numthuan	0	2	0	0
Et	Naphieng	0	118	0	0
Et	Xiengkhoun	3	13	11	19
Paktha	Hardsa	0	1	0	0
Paktha	Houisat	0	0	0	0
Paktha	Jiengtong	0	3	0	0
Paktha	Kengphak	0	0	0	0
Paktha	Kiewlom	0	2	0	0
Paktha	Konteum	5	5	0	0

TABLE 3 Age range and gender breakdown of participants in active survey.

Characteristic	*n*	% total (95% CI)
Sex		
Male	2,380	46.8 (45.7–48.0%)
Female	2,702	53.2 (52.0–54.3%)
Age Group		
<5	273	5.4 (4.6–6.3%)
5–15	1,198	23.6 (21.9–25.3%)
> 15	3,611	71.0 (73.0–76.1%)

Table 4 presents the results of the PCR and serological exposures from the active survey including the number of individuals and households sampled per district and the number positive for PCR and serological exposure to *P. vivax* and *P. falciparum*.

At the survey-level seroprevalences for exposures to *P. vivax* were higher (0.22 for historic and 0.07 for recent) than *P. falciparum* (0.03 for historic and 0.01 for recent). The age-stratified seroprevalences for each species exposure are shown in Figure 2. Exposure was positively associated with increasing age for historic exposure to *P. falciparum* and recent exposure to *P. vivax*. These increases began at around 25 years of age for both exposures. Recent exposure to *P. falciparum* was very low across all age groups.

The PCR survey also found higher case numbers of *P. vivax* ($n = 23$) than *P. falciparum* ($n = 8$). Of the 23 positive *P. vivax* PCR cases in the active survey, seven were classified as positive for recent exposure to *P. vivax* (30%). Of the eight positive *P. falciparum* PCR cases in the active survey, one was classified as positive for recent exposure to *P. falciparum* (12.5%). The central districts of Khua and Nambak recorded the highest number of positive PCR *P. vivax* cases. *P. falciparum* PCR case numbers were similar across the three study areas.

Figure 3 presents the results of the geostatistical modeling for household seroprevalences to *P. vivax* and *P. falciparum* exposure. The environmental and spatial covariates included in the final geostatistical models are listed in Supplementary Table 3.

The estimation of catchment size resulted in 190 catchments across Lao PDR (Supplementary Figure 1). Due to the imperfect alignment of the passive surveillance data (3) and the active surveillance survey (1), 286 of the cross-sectional survey households (1,008 samples) did not fall within the catchment boundaries. This resulted in a significant reduction in the sample size for estimating PCR and seroprevalences at the catchment-level. RDT and microscopy cases per capita were calculated using the estimated population size for each health facility for 2016 (3). The catchment-level seroprevalences for recent exposure to *P. vivax* and *P. falciparum* and RDT and microscopy cases per capita are presented in Figure 4.

Discussion

This study has demonstrated the use of integrating serology into active surveillance projects to provide additional

TABLE 4 Numbers of individuals and households tested in active surveillance survey per district, with number positive for P. vivax and P. falciparum by PCR and serology (with prevalence in brackets).

Districts	Individuals sampled	Households sampled	Serology *P. vivax* historic +ve	Serology *P. vivax* recent +ve	Serology *P. falciparum* historic +ve	Serology *P. falciparum* recent +ve	PCR *P. vivax* +ve	PCR *P. falciparum* +ve	RDT *P. falciparum* +ve	RDT *P. vivax* +ve
Paktha	983	357	308 (0.31)	88 (0.09)	52 (0.05)	21 (0.02)	3 (0.003)	2 (0.002)	0 (0.000)	0 (0.000)
Khua and Nambak	2,393	683	470 (0.2)	46 (0.02)	46 (0.02)	24 (0.01)	17 (0.007)	3 (0.001)	0 (0.000)	0 (0.000)
Et	1,418	362	289 (0.2)	52 (0.04)	19 (0.01)	10 (0.00)	3 (0.002)	3 (0.002)	0 (0.000)	0 (0.000)

+ve = positive.

information on historic and recent exposures to malaria. We have shown how geostatistical modeling with remote sensing-derived environmental variables can be used to predict and characterize the distribution of malaria exposures, and how these can be used to highlight priority areas for added data collection or targeted interventions. We found that historical exposures to *P. vivax* and *P. falciparum* were more widespread in northern Lao PDR, with recent exposures being more focally distributed, as is expected in an elimination setting. Additionally, we showed how retrospectively collected passive surveillance data can be linked to active surveillance data which were not collected in alignment.

The active surveillance survey involved rigorous population sampling which was powered to estimate PCR-based prevalences of malaria in northern Lao PDR as the country prepared for elimination (1). They found very low numbers of asymptomatic *Plasmodium* infections, with higher numbers of *P. vivax* (28 total, 0.005 prevalence) and lower numbers of *P. falciparum* (eight total, <0.000 prevalence). The seroprevalence rates estimated in this study for recent exposure to *P. vivax* and *P. falciparum* were higher than these PCR rates but followed similar trends. The higher numbers of *P. vivax* cases compared to *P. falciparum* detected by Lover et al. (1) and recorded by the health facilities (Figure 4) are consistent with our seroprevalence estimations. The almost complete absence of *P. falciparum* PCR-cases and very low numbers recorded in 2016 by health facilities are also aligned with our very low estimation of recent exposure to *P. falciparum*, suggesting that northern Lao PDR was close to eliminating this species during the year of study. We classified recent exposure to *P. vivax* and *P. falciparum* in 30 and 12.5% of PCR-confirmed cases. This shows that our classifications to recent exposure by serology detect some, but not all concurrent infections. Additionally, the findings show that antigens used for these classifications may be useful indicators of current as well as recent exposure. However, the very low sample size of PCR-confirmed cases for both species mean these findings cannot be confirmed in this study. Our findings provide evidence that at a broad level, our serological estimations reflect what is seen in both PCR-based and routine clinical case management. They also highlight the added information which can be extracted from active surveillance samples with the operationally feasible addition of MBA technology.

The age-stratified seroprevalence curves of recent exposure for *P. vivax* and historic exposure for *P. falciparum* are consistent with our expectations for a low-transmission setting in the Greater Mekong Region (GMR). Here, and in Lao PDR, malaria transmission is heterogenous, and transmission is more intense in forested areas (33, 34). Malaria exposure is largely an occupational hazard for forest workers, with transmission higher in remote forested areas, logging camps and plantations where conventional malaria vector control tools are inefficient (33). In these populations, exposure typically begins at around 20 years old when forest work begins

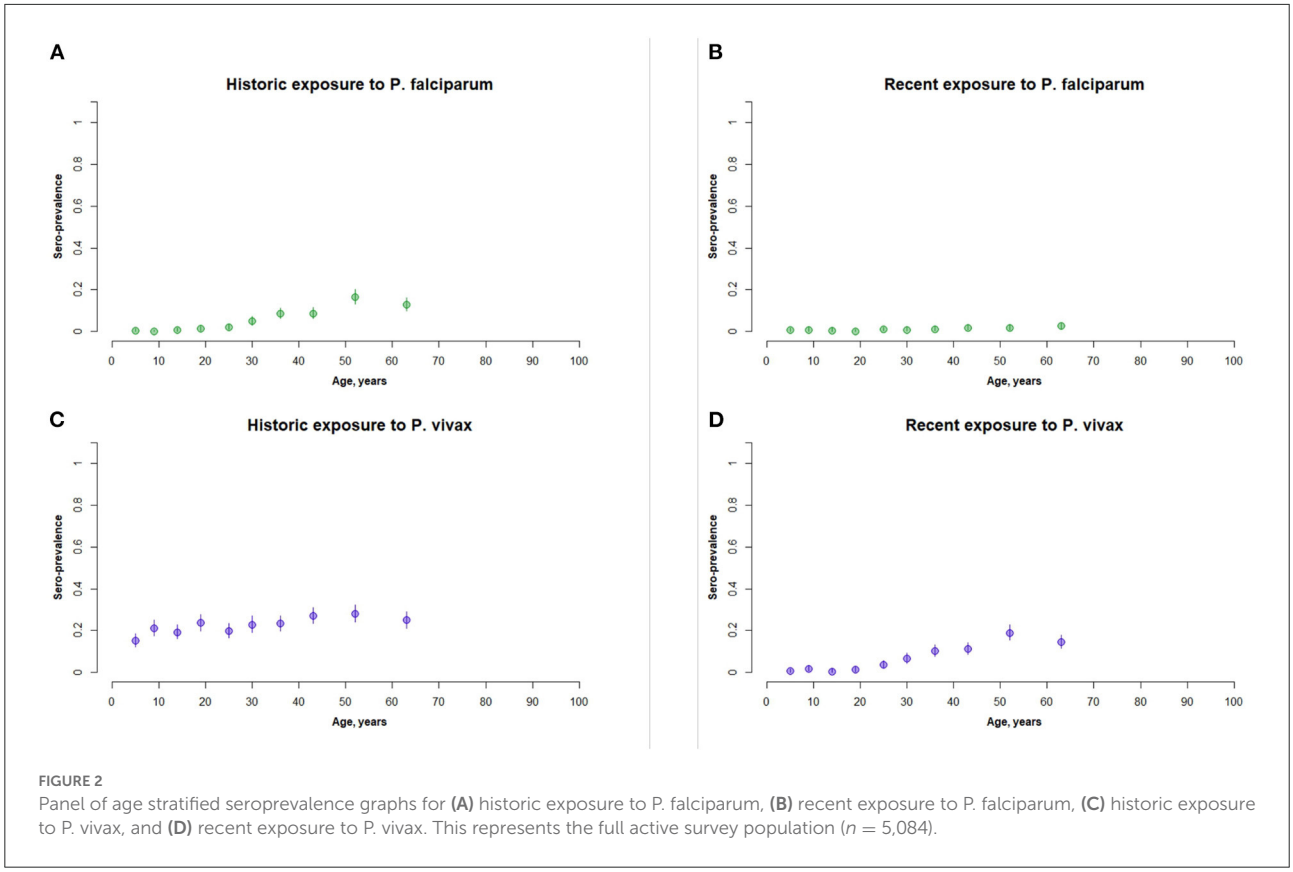

FIGURE 2
Panel of age stratified seroprevalence graphs for **(A)** historic exposure to P. falciparum, **(B)** recent exposure to P. falciparum, **(C)** historic exposure to P. vivax, and **(D)** recent exposure to P. vivax. This represents the full active survey population ($n = 5{,}084$).

(35–39). Historical exposures for *P. falciparum* increase with age, as is expected as transmission was higher during the lifetimes of older populations. We are therefore confident in our methodology using k-means algorithms for classifying seropositive and seronegative individuals, in the absence of international standards for estimating malaria seropositivity in a population. This characteristic age-stratified curve was not seen for historic exposure to *P. vivax*. One explanation for this is that it may be artifact of sampling bias, where the true highest-exposed groups were less likely to be captured at home and more likely to be working outside of the home (8, 34, 40).

The geostatistical mapping of serology data in this study allowed for the characterization of the spatial heterogeneity of remaining foci of *P. vivax* and *P. falciparum* infection. This mapping enables the estimation of seroprevalence at health-decision making units (probabilities of being over a given threshold), alongside measures of uncertainty (23). If taken up by National Malaria Control Programmes, this could allow prioritization of elimination efforts to the areas which they would be most impactful. In this study we arbitrarily chose thresholds of 20% exposure to *P. vivax* and 5% exposure to *P. falciparum* due to the local epidemiology of these species at the time of data collection. In future exercises where geostatistical maps of serology data are used to inform elimination programmes closer to the time of data collection, these thresholds could be set by the programmes according to their own criteria. The prediction maps and maps of exceedance probabilities follow the trend seen in the population-level classifications of seropositivity. We predicted higher and more widespread exposures to historical antigens and lower, more focalised exposures to recent antigens. In addition, predictions for recent and historic *P. vivax* burdens were higher than those for *P. falciparum*.

Despite the value of the geostatistical mapping, we faced a number of limitations. The survey households were clustered within three distinct areas in northern Lao PDR (Figure 1). Geostatistical mapping works by exploiting correlation between nearby data points and utilizing environmental and spatial covariates to produce estimates on a continuous surface (41, 42). Predicting at too far of a spatial range from sampling points results in higher uncertainty around predictions, which is less useful for public health programmes. Therefore, we limited the geostatistical mapping predictions to the districts the samples were collected from and did not produce larger province-level prediction maps. The spatial distribution of sampling points is an important consideration to take into account for projects planning to produce geostatistical maps, where a wider distribution of points can allow for predictions

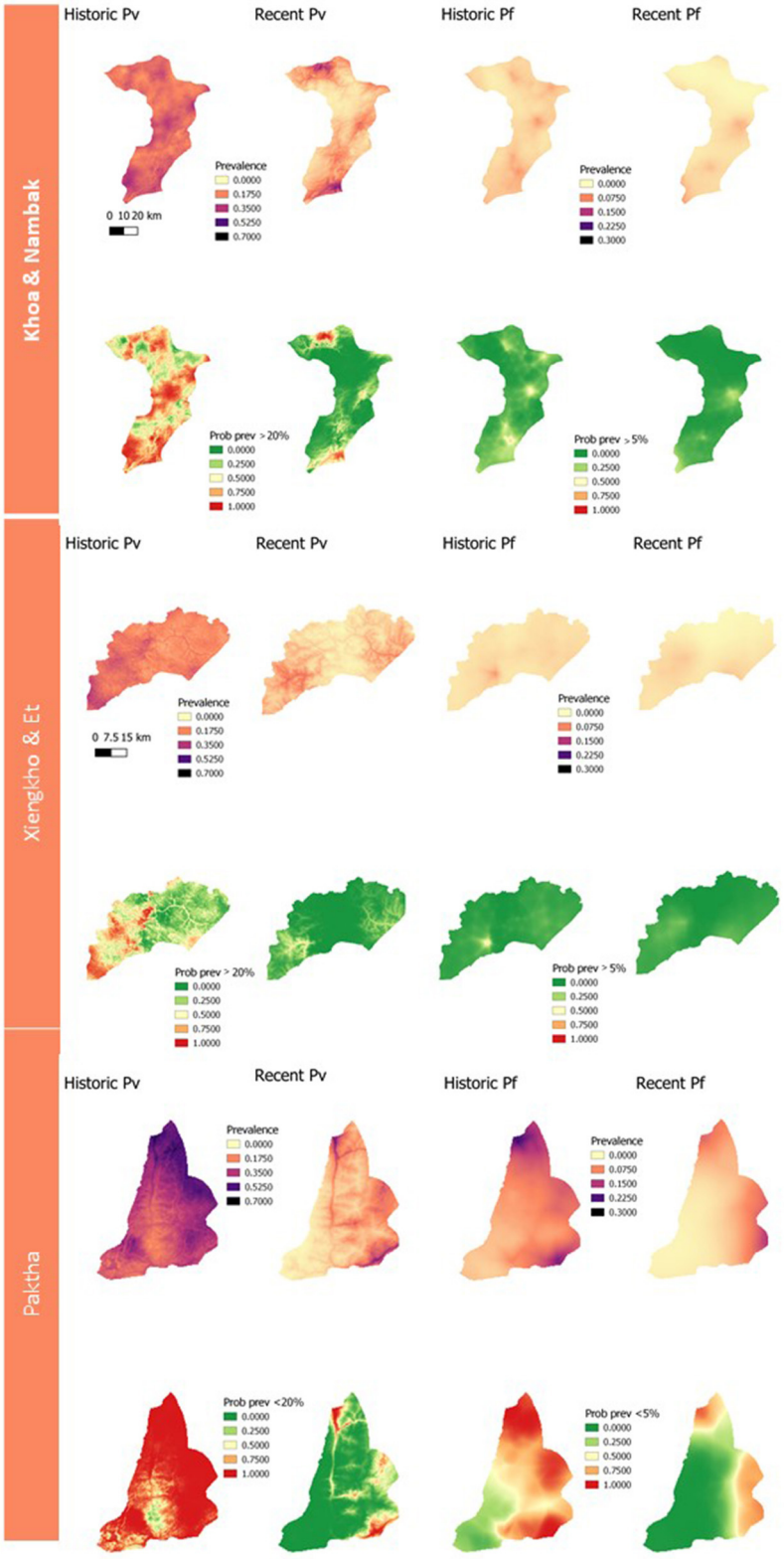

FIGURE 3
Panel of geostatistical maps of predicted seroprevalences and exceedance probabilities to historic and recent P. vivax and P. falciparum exposure for each district. Exceedance probabilities are on the bottom row of each district panel and refer to the probability of a location having >20% predicted prevalence for P. vivax exposures, and >5% for P. falciparum exposures.

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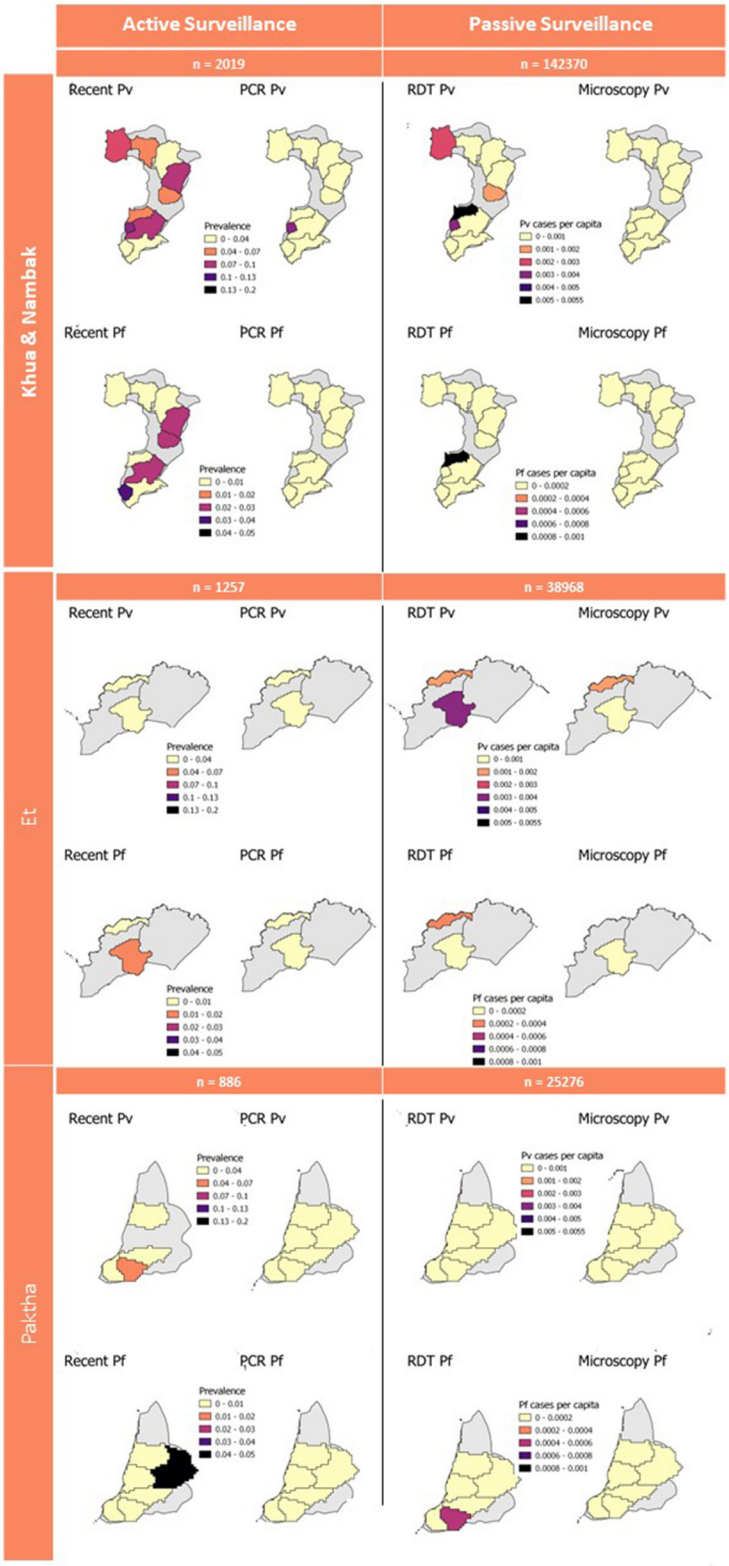

FIGURE 4
Active and passive survey diagnostic results aggregated to catchment level. Gray background represents full district area used for geostatistical predictions in Figure 3. Sample numbers for active surveillance are number of samples from active survey. Sample numbers for passive surveillance are the 2016 population within health facility catchments which was used to calculate cases per capita.

over larger spatial scales. The potential for relapses of *P. vivax* due to the reactivation of hypnozoite stage parasites should also be considered when interpreting the *P. vivax* maps and seroprevalence estimates. While our seroprevalence estimates and prediction maps followed the expected trend of declining in size and becoming more focal as exposure transitioned from historic to recent, the possibility of recurrent infections inflating exposure estimates and predictions should still be acknowledged. Differentiating between new and relapse cases remains challenge in *P. vivax* research and should be taken into account in mapping projects especially, as antibody production may occur in a different location to the original site of exposure (43).

While we have demonstrated a methodology for linking passively collected health-facility data to active surveillance data in the absence of health-facility catchment area boundaries, this aspect of the study had several important limitations. Firstly, the summary statistics presented for these areas are likely subject to modifiable areal unit problems. The list of health facilities used to create the catchment areas (31) (Supplementary Figure 1) was more expansive than that used by the retrospective review of malaria registries (3). This may be explained by the regular updating of the official online roster of health facilities, resulting in more locations listed in 2021 than during the year of the survey. As a result, some areas within the active surveillance districts were broken into catchments for health facilities which we did not have records for. 286 households comprising 1,008 samples were located in these areas and were thus lost from the catchment-level estimations of seroprevalences and PCR-based prevalences. As a result, the sample sizes of survey households per catchment were highly varied between health facilities, with one facility's records being excluded from this study as zero survey households were located within the estimated catchment area. Secondly, seroprevalences and PCR-prevalences were calculated using different denominators to the RDT and microscopy case metrics, therefore are not directly comparable. The active surveillance survey metrics were calculated using samples per area, and the passive surveillance metrics were calculated per capita from the estimated population size per catchment (3). Some households may also utilize near-by health facilities that are not the administratively assigned ones for their households, resulting in discrepancies in the catchment-area population estimations. While it is useful to visualize these various diagnostic endpoints on the same map (Figure 4), as they can help to pick up broad patterns in recent and current exposure, these limitations should be considered when interpreting these results.

Despite these limitations, we have shown that adding serology into passive surveillance projects can provide additional information on current and historic trends in exposure. The higher numbers of *P. vivax* compared to *P. falciparum* cases detected in the passive and active surveillance were reflected in the seroprevalence estimates for northern Lao PDR from this study, indicating that at a broad level, our serological estimations reflect the epidemiology of malaria in the area. We also demonstrated the use of health facility data to contextualize findings from serological burden estimations. The addition of serology in this study allowed for the characterization of the spatial distributions of exposures to *P. vivax* and *P. falciparum*, demonstrating how these methods can provide valuable information for control and elimination programmes which need to identify and target remaining foci of infection in low transmission settings. Additionally, we showed how active surveillance data can be linked to passive surveillance data, and the challenges which come with this. Future work should prioritize spatial and temporal alignment of sampling wherever possible, with design and implementation of user-friendly platforms to move these analyses into more routine public health use.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by University of California San Francisco. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

IB: investigation, formal analysis, and writing—original draft. EC, LN, and ED: support for analysis. EC, LN, FR, and ED: data interpretation. LN, FR, and BH: writing—review. LN, GS, AB, CD, and LW: conceptualization. FR and LW: data curation. CP and JR: conducting serological analyses. CP: completing quality control and normalization. KT: supporting laboratory analysis of serological samples. ED, GS, AL, AB, and CD: writing—review and editing. KF, GS, AL, and CD: supervision. All authors contributed to the article and approved the submitted version.

Funding

Funding for the collection of data used in study was provided to the MEI at the UCSF by the Bill and Melinda Gates

Foundation (OPP# 1116450). This study was funded by the Bill and Melinda Gates Foundation (OPP# 1177272).

Acknowledgments

We would like to thank Andrew Seaton at the University of Glasgow for his expertise and help with the geostatistical modeling. Thank you also to the reviewers of this manuscript for their insightful and constructive comments.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.929366/full#supplementary-material

References

1. Lover AA, Dantzer E, Hongvanthong B, Chindavongsa K, Welty S, Reza T, et al. Prevalence and risk factors for asymptomatic malaria and genotyping of glucose 6-phosphate (G6PD) deficiencies in a vivax-predominant setting, Lao PDR: implications for sub-national elimination goals. *Malar J.* (2018) 17:218. doi: 10.1186/s12936-018-2367-5

2. WHO. *Word Malaria Report.* (2021). Word Malaria report Geneva: World Health Organization. (2021). p. 2013–2015.

3. Rerolle F, Dantzer E, Lover A, Marshall JM, Hongvanthong B, Sturrock HJW, et al. Spatio-temporal associations between deforestation and malaria incidence in Lao Pdr. *Elife.* (2021) 1:10. doi: 10.7554/eLife.56974

4. World Health Organization. *Regional Office for the Western Pacific. Strategy for malaria elimination in the Greater Mekong Subregion : 2015-2030.* (2015).

5. Cotter C, Sturrock HJW, Hsiang MS, Liu J, Phillips AA, Hwang J, et al. The changing epidemiology of malaria elimination: new strategies for new challenges. *Lancet.* (2013) 382:900–11. doi: 10.1016/S0140-6736(13)60310-4

6. Hay SI, Smith DL, Snow RW. Measuring malaria endemicity from intense to interrupted transmission. *Lancet Infect Dis.* (2008) 8:369–78. doi: 10.1016/S1473-3099(08)70069-0

7. Reiner RC, Le Menach A, Kunene S, Ntshalintshali N, Hsiang MS, Perkins TA, et al. Mapping residual transmission for malaria elimination. *Elife.* (2015) 4. doi: 10.7554/eLife.09520

8. Wangdi K, Pasaribu AP, Clements ACA. Addressing hard-to-reach populations for achieving malaria elimination in the Asia Pacific Malaria Elimination Network countries. *Asia Pacific Policy Stud.* (2021) 8:176–88. doi: 10.1002/app5.315

9. Imwong M, Nguyen TN, Tripura R, Peto TJ, Lee SJ, Lwin KM, et al. The epidemiology of subclinical malaria infections in South-East Asia: findings from cross-sectional surveys in Thailand-Myanmar border areas, Cambodia, and Vietnam. *Malar J.* (2015) 14:1–13. doi: 10.1186/s12936-015-0906-x

10. Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. *Nat Rev Microbiol.* (2014) 12:833–40. doi: 10.1038/nrmicro3364

11. Wickremasinghe R, Fernando SD, Thillekaratne J, Wijeyaratne PM, Wickremasinghe AR. Importance of active case detection in a malaria elimination programme. *Malar J.* 13:186. doi: 10.1186/1475-2875-13-186

12. Surendra H, Wijayanti MA, Murhandarwati EH, Irnawati, Yuniarti T, Mardiati, et al. Analysis of serological data to investigate heterogeneity of malaria transmission: a community-based cross-sectional study in an area conducting elimination in Indonesia. *Malar J.* (2019) 18:1–12. doi: 10.1186/s12936-019-2866-z

13. Greenhouse B, Daily J, Guinovart C, Goncalves B, Beeson J, Bell D, et al. Priority use cases for antibody-detecting assays of recent malaria exposure as tools to achieve and sustain malaria elimination. *Gates Open Res.* (2019) 3:131. doi: 10.12688/gatesopenres.12897.1

14. Fornace Id KM, Id LS, Martin DL, Gwyn S, Schmidt E, Agyemangid D, et al. Characterising spatial patterns of neglected tropical disease transmission using integrated sero-surveillance in Northern Ghana. Lau EH, editor. *PLoS Negl Trop Dis.* (2022) 16:e0010227. doi: 10.1371/journal.pntd.0010227

15. Drakeley CJ, Corran PH, Coleman PG, Tongren JE, McDonald SLR, Carneiro I, et al. Estimating medium- and long-term trends in malaria transmission by using serological markers of malaria exposure. Proc Natl Acad Sci U S A. (2005) 102:5108–13. doi: 10.1073/pnas.0408725102

16. Helb DA, Tetteh KKA, Felgner PL, Skinner J, Hubbard A, Arinaitwe E, et al. Novel serologic biomarkers provide accurate estimates of recent Plasmodium falciparum exposure for individuals and communities. *Proc Natl Acad Sci U S A.* (2015) 112:E4438–47. doi: 10.1073/pnas.1501705112

17. Wu L, Mwesigwa J, Affara M, Bah M, Correa S, Hall T, et al. Antibody responses to a suite of novel serological markers for malaria surveillance demonstrate strong correlation with clinical and parasitological infection across seasons and transmission settings in The Gambia. *BMC Med.* (2020) 18:304. doi: 10.1186/s12916-020-01724-5

18. Longley RJ, White MT, Takashima E, Brewster J, Morita M, Harbers M, et al. Development and validation of serological markers for detecting recent Plasmodium vivax infection. Nat Med (2020) 26:741–9. doi: 10.1038/s41591-020-0841-4

19. Corran P, Coleman P, Riley E, Drakeley C. Serology: a robust indicator of malaria transmission intensity? *Trends Parasitol.* (2007) 23:575–82. doi: 10.1016/j.pt.2007.08.023

20. ThermoFisher Scientific. *Luminex 2018.* (2018). Available online at: https://www.thermofisher.com/uk/en/home/life-science/antibodies/immunoassays/procartaplex-assays-luminex.html (accessed November, 2021).

21. Cook J, Kleinschmidt I, Schwabe C, Nseng G, Bousema T, Corran PH, et al. Serological markers suggest heterogeneity of effectiveness of malaria control interventions on bioko island, equatorial Guinea. *PLoS ONE.* (2011) 6:e25137. doi: 10.1371/journal.pone.0025137

22. Surendra H. Supargiyono, Ahmad RA, Kusumasari RA, Rahayujati TB, Damayanti SY, et al. Using health facility-based serological surveillance to predict receptive areas at risk of malaria outbreaks in elimination areas. *BMC Med.* (2020) 18:1–14. doi: 10.1186/s12916-019-1482-7

23. Yankson R, Anto EA, Chipeta MG. Geostatistical analysis and mapping of malaria risk in children under 5 using point-referenced prevalence data in Ghana. *Malar J.* (2019) 18:1–12. doi: 10.1186/s12936-019-2709-y

24. Malaria Atlas Project. *Research Projects Archive - MAP.* Available online at: https://malariaatlas.org/research-project/ (accessed September, 2021).

25. Ashton RA, Joseph V, van den Hoogen LL, Tetteh KKA, Stresman G, Worges M, et al. Risk factors for malaria infection and seropositivity in the elimination Area of Grand'Anse, Haiti: a case–control study among febrile individuals seeking

treatment at public health facilities. *Am J Trop Med Hyg*. (2020) 103:767–77. doi: 10.4269/ajtmh.20-0097

26. Tetteh KKA, Wu L, Hall T, Ssewanyana I, Oulton T, Patterson C, et al. Optimisation and standardisation of a multiplex immunoassay of diverse *Plasmodium falciparum* antigens to assess changes in malaria transmission using sero-epidemiology. *Wellcome Open Res.* (2020) 4:26. doi: 10.12688/wellcomeopenres.14950.2

27. Canier L, Khim N, Kim S, Sluydts V, Heng S, Dourng D, et al. An innovative tool for moving malaria PCR detection of parasite reservoir into the field. Malar J. (2013). Nov 9 [cited (2022). Mar 24];12:1–12. Available from: https://link.springer.com/articles/10.1186/1475-2875-12-405 doi: 10.1186/1475-2875-12-405

28. Kaufman L, Rousseeuw PJ. Finding Groups in Data. Kaufman L, Rousseeuw PJ, editors. Hoboken, NJ, USA: John Wiley & Sons, Inc. (1990). Available online at: https://onlinelibrary.wiley.com/doi/book/10.1002/9780470316801

29. Lindgren F, Rue H. Bayesian spatial modelling with R-INLA. *J Stat Softw*. (2015) 63:1–25. doi: 10.18637/jss.v063.i19

30. Moraga P. *Geospatial Health Data: Modeling and Visualization with R-INLA and Shiny*. Chapman & Hall/CRC Biostatistics Series. Boca Raton: CRC press (2019). doi: 10.1201/9780429341823

31. Health Facility Master List Online (Lao PDR). Available online at: https://hfml.la/

32. Weiss DJ, Nelson A, Gibson HS, Temperley W, Peedell S, Lieber A, et al. A global map of travel time to cities to assess inequalities in accessibility in 2015. *Nature*. (2018) 553:333–6. doi: 10.1038/nature25181

33. Marcombe S, Maithaviphet S, Bobichon J, Phommavan N, Nambanya S, Corbel V, et al. New insights into malaria vector bionomics in Lao PDR: a nationwide entomology survey. *Malar J*. (2020) 19:1–17. doi: 10.1186/s12936-020-03453-9

34. Kounnavong S, Gopinath D, Hongvanthong B, Khamkong C, Sichanthongthip O. Malaria elimination in Lao PDR: the challenges associated with population mobility. *Infect Dis Poverty*. (2017) 6:81. doi: 10.1186/s40249-017-0283-5

35. Chaveepojnkamjorn W, Pichainarong N. Malaria infection among the migrant population along the Thai-Myanmar border area. *Southeast Asian J Trop Med Public Health*. (2004) 35:48–52.

36. Das NG, Talukdar PK, Das SC. Epidemiological and entomological aspects of malaria in forest-fringed villages of Sonitpur district, Assam. *J Vector Borne Dis*. (2004) 41: 5–9.

37. Lansang MAD, Belizario VY, Bustos MDG, Saul A, Aguirre A. Risk factors for infection with malaria in a low endemic community in Bataan, the Philippines. *Acta Trop*. (1997) 63:257–65. doi: 10.1016/S0001-706X(96)00625-0

38. Erhart A, Thang ND, Van Ky P, Tinh TT, Van Overmeir C, Speybroeck N, et al. Epidemiology of forest malaria in central Vietnam: a large scale cross-sectional survey. *Malar J*. (2005) 4:58. doi: 10.1186/1475-2875-4-58

39. Trung HD, Van Bortel W, Sochantha T, Keokenchanh K, Quang NT, Cong LD, et al. Malaria transmission and major malaria vectors in different geographical areas of Southeast Asia. *Trop Med Int Health*. (2004) 9:230–7. doi: 10.1046/j.1365-3156.2003.01179.x

40. Regional Office for South-East Asia WHO, Regional Office for South-East Asia WHO (2016). A for mobile and migrant populations in the context of malaria multi-drug resistance and malaria elimination in the GMS. Approaches for mobile and migrant populations in the context of malaria multi-drug resistance and malaria elimination in the Greater Mekong Subregion. (2016).

41. Moraga P, Cramb SM, Mengersen KL, Pagano M. A geostatistical model for combined analysis of point-level and area-level data using INLA and SPDE. *Spat Stat*. (2017) 21:27–41. doi: 10.1016/j.spasta.2017.04.006

42. Diggle PJ, Moraga P, Rowlingson B, Taylor BM. *Spatial and Spatio-Temporal Log-Gaussian Cox Processes: Extending the Geostatistical Paradigm*.

43. Ashton RA, Kefyalew T, Rand A, Sime H, Assefa A, Mekasha A, et al. Geostatistical modeling of malaria endemicity using serological indicators of exposure collected through school surveys. *Am J Trop Med Hyg*. (2015) 93:168–77. doi: 10.4269/ajtmh.14-0620

44. Collins CR, Withers-Martinez C, Bentley GA, Batchelor AH, Thomas AW, Blackman MJ. Fine mapping of an epitope recognized by an invasion-inhibitory monoclonal antibody on the malaria vaccine candidate apical membrane antigen 1. *J Biol Chem*. (2007) 282:7431–41. doi: 10.1074/jbc.M610562200

45. Burghaus PA, Holder AA. Expression of the 19-kilodalton carboxy-terminal fragment of the Plasmodium falciparum merozoite surface protein-1 in Escherichia coli as a correctly folded protein. *Mol Biochem Parasitol*. (1994) 64:165–9. doi: 10.1016/0166-6851(94)90144-9

46. Spielmann T, Fergusen DJP, Beck HP. etramps, a new Plasmodium falciparum gene family coding for developmentally regulated and highly charged membrane proteins located at the parasite-host cell interface. *Mol Biol Cell*. (2003) 14:1529–44. doi: 10.1091/mbc.e02-04-0240

47. Cunha MG, Rodrigues MM, Soares IS. Comparison of the immunogenic properties of recombinant proteins representing the Plasmodium vivax vaccine candidate MSP1(19) expressed in distinct bacterial vectors. *Vaccine*. (2001) 20:385–96. doi: 10.1016/S0264-410X(01)00359-0

48. Soares IS, Barnwell JW, Ferreira MU, Da Cunha MG, Laurino JP, Castilho BA, et al. A Plasmodium vivax vaccine candidate displays limited allele polymorphism, which does not restrict recognition by antibodies. *Mol Med*. (1999) 5:459. doi: 10.1007/BF03403539

49. Kocken CHM, Dubbeld MA, Van Der Wel A, Pronk JT, Waters AP, Langermans JAM, et al. High-level expression of Plasmodium vivax apical membrane antigen 1 (AMA-1) in Pichia pastoris: strong immunogenicity in Macaca mulatta immunized with P. vivax AMA-1 and adjuvant SBAS2. *Infect Immun*. (1999) 67:43–9. doi: 10.1128/IAI.67.1.43-49.1999

50. Hester J, Chan ER, Menard D, Mercereau-Puijalon O, Barnwell J, Zimmerman PA, et al. De Novo assembly of a field isolate genome reveals novel plasmodium vivax erythrocyte invasion genes. *PLoS Negl Trop Dis*. (2013) 7:e2569. doi: 10.1371/journal.pntd.0002569

51. Menard D, Chan ER, Benedet C, Ratsimbasoa A, Kim S, Chim P, et al. Whole genome sequencing of field isolates reveals a common duplication of the duffy binding protein gene in malagasy plasmodium vivax strains. *PLoS Negl Trop Dis*. (2013) 7:e2489. doi: 10.1371/journal.pntd.0002489

52. Roesch C, Popovici J, Bin S, Run V, Kim S, Ramboarina S, et al. Genetic diversity in two Plasmodium vivax protein ligands for reticulocyte invasion. *PLoS Negl Trop Dis*. (2018) 12:e0006555. doi: 10.1371/journal.pntd.0006555

frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 09 September 2022
DOI 10.3389/fpubh.2022.989337

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Infectious Diseases–Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

RECEIVED 08 July 2022
ACCEPTED 17 August 2022
PUBLISHED 09 September 2022

CITATION
Willeit P, Kimpel J, Winner H, Harthaller T, Schäfer H, Bante D, Falkensammer B, Rössler A, Riepler L, Ower C, Sacher M, von Laer D and Borena W (2022) Seroprevalence of SARS-CoV-2 infection in the Tyrolean district of Schwaz at the time of the rapid mass vaccination in March 2021 following B.1.351-variant outbreak. *Front. Public Health* 10:989337. doi: 10.3389/fpubh.2022.989337

Seroprevalence of SARS-CoV-2 infection in the Tyrolean district of Schwaz at the time of the rapid mass vaccination in March 2021 following B.1.351-variant outbreak

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In order to curb the rapid dissemination of the B.1.351 variant of SARS-CoV-2 in the district of Schwaz and beyond, the EU allocated additional vaccine doses at the beginning of March 2021 to implement a rapid mass vaccination of the population (16+). The aim of our study was to determine the seroprevalence of SARS-CoV-2 among the adult population in the district of Schwaz at the time of the implementation. Data on previous history of infections, symptoms and immunization status were collected using a structured questionnaire. Blood samples were used to determine SARS-CoV-2 specific anti-spike, anti-nucleocapsid and neutralizing antibodies. We recruited 2,474 individuals with a median age (IQR) of 42 (31–54) years. Using the official data on distribution of age and sex, we found a standardized prevalence of undocumented infections at 15.0% (95% CI: 13.2–16.7). Taken together with the officially documented infections, we estimated that 24.0% (95% CI: 22.5–25.6) of the adult population had prior SARS-CoV-2 infection. Hence, the proportion of undocumented infections identified by our study was 55.8% (95% CI: 52.7–58.5). With a vaccination coverage of 10% among the adults population at that time, we imply that a minimum of two-thirds of the target popuation was susceptible to the circulating threat when this unique campaign started.

KEYWORDS

seroprevalence, SARS-CoV-2, vaccination, Comirnaty, undocumented infection, seropositivity, anti-N, beta

Introduction

As global efforts are in progress to cope with the uncontrolled transmission of SARS-CoV-2 infection, new variants surface as obstacles against the process of containment. The variant B.1.351, detected for the first time in South Africa in October 2020 (1), was found to have impaired neutralization by convalescent plasma from the wild type infection. A potential dominance of such immune escape variants may pose a serious threat to real world vaccine effectiveness.

Almost simultaneously with the introduction of the first vaccines at the beginning of the year 2021, Europe notified increased circulation of the variant B.1.351 (2).

Having reported over 300 cases of infections with this variant of concern (VOC) at that time, Austria came into focus as a hotspot (3, 4). Almost all reported cases originated from the district of Schwaz in the western part of the country. In order to curb the spread of this variant, the European Union chose the district to serve as a model region and provided Austria with 100,000 doses of the BNT162b2 (Comirnaty) vaccine by BioNTech/Pfizer for the immediate immunization of 50,000 adults living in this district. According to official reports, approximately 41,700 (61% of the adult population) received both doses of the vaccine as part of this immunization programme (4, 5).

Vaccine donors, regional or national health authorities, vaccine policy makers, other relevant institutions or even the general public may legitimately wonder what proportion of the community had already been exposed to the virus or what proportion was completely immuno-naïve as this unique immunization campaign started. The results may be used as baseline information in evaluating the performance of the mass immunization in achieving the goal it was aimed for (6). Although for this purpose data from the official registry of SARS-CoV-2 infections may be utilized, it is highly likely that a non-negligible proportion of the population might have gone through an infection that has remained undetected and unreported (7–10). Anti-nucleocapsid antibodies may help identify, irrespective of vaccination status, subjects with prior infections which were not detected by the conventional confirmatory tests.

With this study, we aimed to determine the prevalence SARS-CoV-2 infection status through the use of serological assays in the district of Schwaz at the time of the mass vaccination.

Materials and methods

Study population

The study was conducted in March 2021. All adult residents of the district Schwaz in Tyrol, aged 18 years and above (n = 68,896), were invited through the local county office and local media. Consenting participants were asked to fill out a short questionnaire on sociodemographic aspects as well as their history of SARS-CoV-2 infection, history of hospitalization and the status of vaccination. Blood samples (EDTA) collected at the study site were used for the determination of SARS-CoV-2-specific antibodies targeting the spike (S) and nucleocapsid (N) proteins.

SARS-CoV-2 antibody test

All samples were tested using the Chemiluminescent-based immunoassay—SARS-CoV-2 IgG II Quant (Abbott, Illinois, USA). The assay detects antibodies directed against S protein and was performed on the ARCHITECT i2000SR platform. Using standards of various concentrations, results were provided in a quantitative manner as binding antibody units per milliliter (BAU/ml). According to the manufacturer, the cutoff for positivity was defined to be >7.1 BAU/ml. All samples were further analyzed using a second serological assay—the Elecsys Anti-SARS-CoV-2 (Roche Diagnostics, Indianapolis, USA)—which detected antibodies against the N protein of SARS-CoV-2. Detection of anti-N immunoglobulin (anti-N Ig) has the additional value of differentiating between post-infection and post-vaccination antibody positivity. Individuals with positive anti-N Ig were further tested for the presence of neutralizing antibodies using an in-house pseudovirus-based assay (pVNT) as described previously (11, 12). In short, replication defective vesicular stomatitis virus (VSVΔG-GFP) pseudotyped with Wuhan-1 spike protein was used to infect susceptible cells (293T-ACE2) after pre-incubation with participant's plasma in serial four-fold dilutions. Cells infected with the pseudovirus expressed GFP. This signal was quantified approximately 16 hours after infection using a spot reader (ImmunoSpot® S5 analyzer). Continuous titers that resulted in 50% reduction of GFP expression (50% inhibition titer) as compared to virus-only wells were determined using a non-linear regression method as described before (13). Titers ≥1:16 (continuous titer of ≥16) were considered positive.

Data analysis

Our study population showed a slight predominance of women and subjects between 25 to 55 years of age as compared to the general (source) population (Supplementary Figure 1). In order to account for these discrepancies, we estimated the overall prevalence through age and sex standardization. To counteract a potential selection-bias of the study (i.e., more subjects with a history or suspicion of previous SARS-CoV-2 infection preferentially willing to participate), we estimated

the serology-derived proportion of unreported infections and interpreted the result in combination with data from officially reported cases. For this purpose, we excluded subjects who gave a history of prior infection in the questionnaire and considered anti-N positivity among the rest of the study participants to represent the proportion of undocumented infections. Through direct age standardization using the official census data of the district (14), we estimated the seroprevalence in the adult population of Schwaz (source population). We defined the true cumulative incidence at the time of data collection to be the sum of the estimated undocumented infections and the officially reported daily numbers stratified by age and sex kindly provided by the Austrian Agency for Health and Food Safety (Dr. Daniela Schmid, AGES).

We used student's t-test or ANOVA to test for a difference in quantitative variables across groups. We applied non-parametric tests (Mann-Whitney U test and Kruskal-Wallis) for variables not fulfilling the criteria of normality. For variables of categorical nature, we used χ^2-test (Fisher's exact where appropriate). For the main analysis of seroprevalence of unreported infections using Roche anti-N Ig we also provided a Rogan-Gladen correction for an imperfect diagnostic test. 95% CIs were calculated using the Clopper-Pearson exact method (15). The level of significance was set at 5% using two-sided tests where applicable.

Ethical clearance

The study was approved by Ethics Committee of the Medical University of Innsbruck (EK Nr:1093/2021).

Results

Questionnaire data

As shown in Table 1, the total number of participants added up to 2,474 adults (n = 1,028 males and n = 1,446 females) between 18 and 89 years of age. While 593 participants (24%, 95% CI: 22.4–25.6) reported to have had a PCR (n = 591) or antigen (n = 2) confirmed SARS-CoV-2 infection, 94 (3.8%, 95% CI: 3.1–4.5) reported a previous infection based on a routine antibody test. Only 15 participants (2.1%) reported to have been hospitalized due to SARS-CoV-2 infection. Around 15% of subjects with a previous infection reported to have had no symptoms. Among participants with a history of infection and reporting symptoms (n = 578), the majority (52.8%) had only mild symptoms without being bedridden. Although symptom reporting was more common among females than males [OR, (95% CI) = 2.03 (1.34–3.14)], significantly more males reported to have been hospitalized [OR, (95% CI) = 3.04 (1.03–8.99)]. A total of 1,948 participants (79.2%) had received at least one dose of an mRNA or a vector vaccine approved in Europe at the time of the study (a single individual reported to have received BBIBP-CorV, Sinopharm). The majority (92%) of the vaccinated subjects had received Comirnaty—as part of the mass vaccination with a median (IQR) of 9 (7–10) days prior to the study—followed by Vaxzevria (ChAdOx1, AstraZeneca) (7.6%). No significant difference was observed in the vaccination status across sexes.

Seropositivity and previous infection

Independent of sex, the proportion of subjects positive for anti-S IgG antibody was 51.4% and positive for anti-N antibody 34.3% (p = 0.28 and 0.29, respectively) (Table 1). The proportion of anti-S positives was higher since there were participants who had been vaccinated prior to or as part of the mass immunization programme.

In order to assess the seroprevalence of SARS-CoV-2 antibodies before the mass vaccination, we characterized (as shown in Table 2) subjects with positive anti-N Ig antibodies (n = 848). We observed a significant association between anti-N Ig levels and age but no difference across sexes. The median concentration was three times higher among participants older than 60 years as compared to those below 40 years of age. Only 548 (64.6%) anti-N Ig positive subjects also reported a history of previous PCR- or antigen-confirmed SARS-CoV-2 infection. About one third of the remaining 296 subjects with no history of PCR- or antigen-confirmed SARS-CoV-2 infections reported to have found out about a previous infection based on an antibody test prior to the study, taking 205 participants (8.3% of the total study participants) by surprise. Participants reporting severe symptoms or a history of hospitalization had significantly higher anti-N Ig concentrations than participants without (p = 0.008 and 0.018, respectively). The mere presence or absence of symptoms showed no significant difference on the level of antibodies directed against N protein.

The majority of anti-N Ig positive subjects, 757 (89.3%), were also positive for neutralizing antibodies. However, a significant proportion had already received at least one dose of SARS-CoV-2 vaccines prior to our study impeding the interpretation of infection-induced neutralization activity. Compared to 98.2% of previously infected plus vaccinated individuals only 69.0% of the participants with previous infection without any history of vaccination were positive for neutralizing antibodies. The median 50% inhibition titer was 46 fold higher in the vaccinated plus infected group as compared to the non-vaccinated convalescent group (p < 0.0001). Concentrating on the group with a history of infection but no vaccination (n = 248), we observed a

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TABLE 1 Baseline characteristics of study participants (n = 2,474).

Participants	Total ($n = 2,474$)	Male ($n = 1,028$)	female ($n = 1446$)	p-value*	Missing (n)
Age, years					0
Mean (SD)	43.2 (14.3)	44.3 (14.3)	42,4 (14.2)	**0.001**	
Median IQR	42 (31-54)	44 (33-55)	42 (31-53.3)		
Range	18-86	18-82	18-86		
History of previous infection (reported) (n,%)				0.49	10
PCR-based	591 (23.9)	236 (23.3)	355 (24.6)		
Antibody-based	94 (3.8)	41 (4.0)	53 (3.7)		
Antigen-based	2 (0.1)	1 (0.1)	1 (0.1)		
None	1,777 (71.8)	746 (72.6)	1,031 (71.3)		
Symptoms (if yes to infection, n= 687 (n_{male} =278, n_{female} =409)) (n, %)**					
any symptom				**0.001**	2
Yes	578 (84.1)	218 (78,4)	360 (88.2)		
No	107 (15.6)	59 (21.1)	48 (11.7)		
bedridden				0.88	37
Yes	268 (39)	105 (37.8)	163 (41.5)		
No	382 (55.6)	152 (54.7)	230 (56.2)		
hospitalized	15 (2.1)	10 (3.6)	5 (1.2)	**0.035**	7
Vaccination status (n,%)					13
not vaccinated	513 (20.7)	195 (19,0)	318 (22.0)	0.097	
vaccinated with one dose	1,812 (73.2)	765 (74.7)	1,047 (72.9)		
vaccinated with two doses	136 (5.5)	64 (6.3)	72 (5.0)		
Vaccine type among vaccinated, (n=1,948) n_{male} =829, n_{female} =1,119) (n, %)				0.14	8
Comirnaty (BioNTech/Pfizer)	1,785 (91.6)	766 (92.4)	1,019 (91.1)		
Vaxzevria (AstraZeneca)	147 (7.5)	56 (6.8)	91 (8.1)		
Spikevax (Moderna Biotech)	7 (0.4)	1 (0.1)	6 (0.5)		
Others	1 (0.1)	1 (0.1)	0		
Antibody status (n,)					0
anti-S IgG positive	1,271 (51.4)	515 (50.1)	756 (52.3)	0.28	
Anti-N Ig positive	848 (34.3)	340 (31.3)	508 (35.1)	0.29	1
pVNT (50% inhibition titer)[§],					0
Overall (n=848) (n_{male} =340, n_{female} =508)					
pVNT positive (≥16), n (%)	757 (89.3)	299 (87.9)	458 (90.2)	0.31	
Median (GMT)	695.9 (265)	674.9 (260.2)	708.5 (265.4)	0.19	
By vaccination status					5
Non-vaccinated, (n=248) (n_{male} =89, n_{female} =159)					
pVNT positive (≥16), n (%)	171 (69.0)	56 (62.9)	115 (72.3)	0.13	
Median (GMT)	24.5 (17.7)	24.4 (16.1)	24.5 (18.6)	0.57	
Vaccinated, (n=595) (n_{male} =251, n_{female} =344)					
pVNT positive (≥16), n (%)	584 (98.2)	243 (96.8)	341 (99.1)	**0.04**	
Median (GMT)	1,129.0 (820,4)	1,075.9 (697.7)	1,163.2 (923.4)	0.05	

*Significance was calculated using chi-square test for the categorical variables and Mann-Whitney U test for the continuous variables,
**687 = 591 (PCR-positive) + 94 (antibody-positive) + 2 (antigen-positive),
[§]Includes only subjects positive for anti-N Ig.
SD, standard deviation; IQR, interquartile range; S, spike protein; N, nucleocapsid protein; pVNT, pseudovirus based virus neutralization test; GMT, geometric mean titer.

TABLE 2 Geometric mean (median) anti-N antibody concentrations across participant characteristics (n=848).

	n	Geometric mean (median), COI	*p*-value**	Missing (n)
Participants			0.25	0
male	340	35.1 (42.5)		
female	508	30.7 (37.4)		
Age (years)			**<0.0001**	0
<40	365	23.5 (24.8)		
40-<60	363	38.2 (44.5)		
>60	120	52.2 (68.3)		
History of infection			**0.034**	4
Yes (PCR, AG, AB)	639	35.1 (42.4)		
No	205	27.5 (35.3)		
History of infection			0.32	4
Yes (PCR, AG)	548	34.1 (41.5)		
No*	296	29.8 (37.4)		
Symptoms ($n_{historyofinfection}$ =639)			0.52	2
Yes	550	36.1 (42.9)		
No	87	30.1 (36.2)		
Non-mild symptoms (bed-bound), ($n_{historyofinfection}$ =639)			**0.008**	27
Yes	258	42.3 (46.0)		
No	354	31.5 (40.5)		
Hospitalized ($n_{historyofinfection}$ =639)			**0.018**	5
Yes	15	88.8 (119.8)		
No	619	34.3 (41.6)		
pVNT (50% inhibition titer)[§], (n=248)positive (≥ 16)	171	53.4 (40.6)	**<0.0001**	0
Negative (<16)	77	24.4 (19.4)		

*Including participants with a history of antibody positive, **P-values were calculated based Mann-Whitney test, [§]Non-vaccinated convalescent individuals.
AB, antibody-based; AG, antigen-based; COI, coefficient of index. significant findings (values <0.05) are in bold.

significant association between anti-N Ig level and positivity for neutralizing antibodies.

Undocumented SARS-CoV-2 infections and standardized seroprevalence for the general adult population of Schwaz

Undocumented infection was defined as being anti-N Ig positive despite reporting to have had no known SARS-CoV-2 infection in the past. After excluding 548 persons who explicitely reported to have had prior PCR- or antigen-based infection, we found the crude seroprevalence of undocumented infections among the study population reporting no history of infection to be 15.8% (95% CI: 14.2–17.6). Supplementary Figure 2 shows the quantitative distribution of anti-N Ig values across age.

Since the age and sex distribution of the study participants showed obvious deviation from the official distribution of the total population in Schwaz (Supplementary Figure 1), we estimated the age and sex standardized prevalence (95% CI) of undocumented infections as shown in Table 3. We initially estimated the number of individuals in the general population with undocumented infections by projecting the age-specific crude seroprevalence from the study population to the total population. We then determined the overall prevalence of undocumented infections across age categories and sex. The sum of the prevalences across these age strata (15.0%, 95% CI: 13.3–16.7) was the overall age standardized prevalence among adults in Schwaz. This translated into 9,228 (95% CI: 8,150–10,296) undocumented infections out of 61,576 adults officially never having a previous SARS-CoV-2 infection (Table 4). Taking 7,320 subjects officially reported to have had confirmed SARS-CoV-2 at the time of data collection (information obtained from AGES), the true total number of infections in the adult population of the district of Schwaz by March 2021 was estimated at 7,320 + 9,228 = 16,548 (95% CI: 15,494 – 17,616) which translates into an overall pre-mass vaccination SARS-CoV-2 prevalence of 24% (95% CI: 22.5–25.6) and a proportion of undocumented infections of 55.8% (95% CI: 52.7–58.5) in adults at that time.

In a sensitivity analysis that employed the Rogan-Gladen correction for an imperfect diagnostic test (Supplementary Table 1), the results were similar to the principal analysis, as expected given the sensitivity and

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TABLE 3 Characterizing study population and source population without a known history of officially reported SARS-CoV-2 infection.

Age group	Sex	Participants reporting no history of infection	Anti N positive participants	Age–specific *crude* prevalence, % (95% CI)*	No. of people in reference population with no report of previous infection**	(Expected number (n) of unreported cases in the reference population)§	Expected proportion (%, 95% CI) of unreported cases in the reference population]§§	% Reference population out of total
<40	Female	480	78	16.3 (13.1-19.9)	10,347	1,681	2.73 (2.60-2-86)	16.8%
<40	Male	316	50	15.8 (12.0-20.3)	10,708	1,694	2.75 (2.62-2.88)	17.4%
40-<60	Female	469	80	17.1 (13.8-20.8)	10,980	1,873	3.04 (2.91-3.18)	17.8%
40-<60	Male	345	58	16.8 (13.2-21.2)	11,045	1,857	3.02 (2.88-3.15)	17.9%
60+	Female	135	15	11.1 (6.35-17.7)	9,940	1,104	1.79 (1.69-1.90)	16.1%
60+	Male	126	15	11.9 (6.82-18.9)	8,556	1,019	1.65 (1.56-1.76)	13.9%
Total		**1,871**	**296**	**15.8 (14.2-17.6)**	**61,576**	**9,228**	**15.0%**	

*Proportion of anti-N positives across age and sex strata,

**Based on daily reports of SARS-CoV-2 infection in Schwaz since the beginning of the pandemic (data obtained from AGES),

§Calculated as: $\frac{age\text{-}specific\ crude\ prevalence\ X\ number\ of\ people\ in\ the\ reference\ population\ with\ no\ report\ of\ previous\ infection}{100}$,

§§Calculated as: $\left(\frac{Expected\ number\ (n)\ of\ unreported\ cases\ in\ the\ reference\ population}{61576}\right) X\ 100\%$. all 95% confidence intervals (CI) calculated based on Clopper-Pearson exact method.

TABLE 4 Projected undocumented and overall point prevalence of SARS-SoV-2 infection in the district of Schwaz just before the mass immunization campaign.

	Point estimate	95% confidence interval	
		lower bound	upper bound
Estimated no. of cases in Schwaz			
Expected number of unreported cases in the reference population	9,228	8,159	10,296
Previously documented*	7,320		
Documented and undocumented cases	16,548	15,479	17,616
% previously undocumented	55.87%	52.7%	58.5%
% of reference population including previously documented cases**	24.0%	22.5%	25.6%

*Data from AGES, **Calculated as: $\left(\frac{16548}{68896}\right) X$ 100%, all 95% confidence intervals (CI) calculated based on Clopper-Pearson exact method.

specificity of the anti-N Ig assay close to 100%. In specific, in participants without a known history of SARS-CoV-2 infection, the age- and sex-standardized seroprevalence was 14.9% (95% CI: 13.2–16.6%) and the expected number of unreported cases in the reference population was 9,169 (95% CI: 8,103–10,233) after correction.

Discussion

At the time of our data collection, the mass vaccination had been going on for about one week. In order to avoid a potential bias caused by anti-S IgG seroconversion following vaccination, we chose to use anti-N Ig in estimating the seroprevalence. This helps to doubtlessly exclude the effect of vaccination following SARS-CoV-2 infection, since earliest seroconversion has been described to occur within days post vaccination (16–20). The demonstrably good performance of the Roche anti-N immunoglobulin assay underscores the validity of our approach (21–23). Moreover, the assay proved a persistently high sensitivity even months after a confirmed infection (23).

With 7,320 officially documented cases above the age of 18 at the time of the study, the official SARS-CoV-2 prevalence would be estimated to 10.6% (95% CI: 10.4–10.9) at that time. The fact that 24% of the study participants reported to have had PCR- or antigen-confirmed infection led to the reasonable suspicion that our convenience sampling may have resulted in a selection bias. A seroprevalence of 34.3% based on anti-N positivity is thus likely to be an overestimation. Consequently we opted to include, in the main analysis, only subjects who reported no known history of infection (n = 1,871) and to estimate age standardized cumulative incidence of unreported infections in the general adult population of the district of Schwaz (n = 61,576). Using data obtained

from official statistics in Austria (14), we accounted for disproportional age and sex distribution of the study population by conducting direct age and sex standardization. Our finding of standardized seroprevalence of 15.0% translates into previously undocumented 9,228 cases (95% CI: 8,159–10,296) in the general adult population of Schwaz. Adding this to 7,320 (10.6%) subjects officially reported to have had SARS-CoV-2 at the time of data collection (data from AGES), the overall prevalence of SARS-CoV-2 infection at the start of the mass vaccination among adults added up to 24% (95% CI: 22.5–25.6). With a vaccination coverage of 10% among the adult population of Schwaz prior to the mass immunization campaign (6), our result implies that a maximum of one third of the adult popuation had at least one SARS-CoV-2 specific immunological event.

Previous studies

Comprehensive meta-analysis studies indicate that a plethora of seroprevalence studies has been conducted across the globe since early on in the pandemic. A wide range of cumulative incidence has been reported depending on the population studied, the sample size used, the serological method applied, the time of the study since the start of the pandemic or whether or not vaccination status was considered (24, 25), making direct comparison of estimates very challenging.

Based on a review on population-based studies in Europe until September 2020, for example, the seroprevalence ranged from as low as 0.42% in some studies to as high as 23.3% in a highly affected region of Lombardy, Italy following the first wave of infection (24). Another study conducted in Ischgl, a once-a-corona-hotspot ski resort in western Austria in April 2020, found an even higher seroprevalence of 42% (26). A nation-wide study in Austria, conducted in Autumn of 2020 using a representative sample of the Austrian population, found a seroprevalence of 4.7% (95% CI: 3.8–5.6) and reported that the estimate for western Austria was higher (5.7%, 95% CI: 4.1–7.4) (27).

Our study was conducted at the end of the second infection wave, hence the higher seroprevalence highlighting the temporal continuum of the rising infection numbers. A follow-up nationwide survey conducted between October 2020 and January 2021 and global epidemiological data on seroprevalence over time also corroborate this temporal trend (25, 28).

Regarding antibody concentration and age, our findings are in accord with several previous works showing higher antibody titres in older adult populations, presumably owing to the fact that age is a known risk factor for severe disease (29–32). Since the very beginning of the pandemic, even before vaccines were available, several studies reported higher levels of both binding and neutralizing antibodies correlating with disease severity, which in turn has been shown to correlate with age among other factors (33–37).

Similarly, a superiority of the concentration and quality of antibodies generated by vaccinees with a history of infection compared to immuno-naïve vaccinees has also been reported previously, even following a single dose (18, 38–45). The robustness of this finding is strengthened by the observation that not only humoral response but also cellular immunity was generally superior in the non-naïve group (40, 43, 45).

Seroprevalence post second infection wave

Since seroprevalence surveys conducted after 2021 paralleled the vaccination rollout, interpretation of data has been a challenge unless non-vaccine-induced antibodies like the nucleocapsid protein antibodies are targeted. Similar to ours, several other studies investigated seroprevalence based on anti-N Ig in the post-vaccine era. Studies targeting health care workers or patients or residents of nursing homes found a much higher seroprevalence than ours owing to the high-risk target population (46–48). For instance, a prospective study from England, which examined the prevalence of SARS-CoV-2 among both residents and staff in long-term care facilities over a period of March through the beginning of May 2021 found a prevalence of anti-N Ig positivity of 34.6% among the residents and 26.1% among staff (47).

Blood donors, on the other hand, make up a comparable group to our study population. Contemporaneous to ours, another survey assessed seroprevalence among Tyrolean blood donors targeting anti-N antibodies. Siller et al. found seropositivity (95% CI) of 14.0% (13.0–15.1) among donor samples collected in March 2021 (49), a finding notably lower than ours (24%) in the district of Schwaz. Several factors may be accountable for this: First, the two studies used different serological platforms—chemiluminescent microparticle immunoassay from Abbott vs. Electrochemiluminescence immunoassay from Roche. These two assays were shown to have a significant gap in sensitivity of detecting anti-N antibodies particularly later in the post-infection period in favor of the Roche assay (40): Six months post confirmed SARS-CoV-2 infection, the Roche assay had a sensitivity of 94,3% (95% CI: 84.3–98.8) in detecting anti-N antibodies whereas for the Abbott system it was only at 45.3 % (95% CI: 31.6–59.6%) (39). Second, reports from official data indicated that the district of Schwaz had higher incidence rates (49, 50) in the second infection wave as compared to the rest of the Tyrolean districts, further accounting for the discrepancy.

Common to the majority of previously published seroprevalence data, as underpinned by a large global meta-analysis, is that the estimates based on serological approach

are clearly higher than the officially reported ones with values ranging from as low as 1.5 times to as high as 10 times the reported cumulative incidence (51). With 55.8% of seropositive subjects having never been registered officially, our study underpins this notion.

Strengths and limitations

Beyond the large sample size, the availability of data on daily infections in the study district across age and sex since the start of the pandemic, as well as the official census data that enabled a direct age standardization and projection of estimates to the general population, is one strength of the study. The main serological assay we used to determine seroprevalence has a high sensitivity and specificity (22, 23).

A major limitation of this study is the convenience-sampling approach in which individuals who were willing to participate may be significantly different from those who were not attracted by this approach. This might have resulted in selection bias toward higher prevalence. We aimed to balance this effect by concentrating on subjects reporting no known history of previous infection and by coupling the unreported infections to the officially registered cases. A further limitation is that our data concentrated only on the adult population as we lacked ethical clearance to include minors.

Conclusion

Our conclusions are twofold. First, by accounting for the undocumented cases through serological approach, our study confirmed once again that officially reported data on infection status markedly underestimate the true prevalence. Second, adding our finding to the vaccine coverage of 10% among the adults population shortly before the mass immunization campaign (6), our result implies that at least two-thirds of the adult population was immuno-naïve and susceptible to the circulating threat as this unique campaign started.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical University of Innsbruck (EK Nr:1093/2021). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: DL and WB. Data and sample acquisition: HS, DB, BF, TH, CO, MS, and WB. Laboratory work: JK, WB, AR, and LR. Data cleaning: TH and WB. Formal data analysis: PW, WB, and HW. Manuscript drafting: WB and PW. All authors have read, critically reviewed, and agreed to this version of the manuscript.

Acknowledgments

We thank Maria Huber, Albert Falch, Evelyn Peer, Lisa-Maria Raschbichler, and the whole diagnostic and research team at the Institute of Virology for excellent technical support. We are also grateful to Bianca Neurauter, Eva Hochmuth, and Luiza Hoch and the whole organization team for excellent coordination of the study both at the laboratory and at the study site.

Conflict of interest

DB declares to hold stocks of Pfizer.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.989337/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Age structure of the study population as compared to the official age structure in Schwaz (source population) among men **(A)** and women **(B)** [Source population data from Statisik Austria (14)].

SUPPLEMENTARY FIGURE 2
Spearman's correlation coefficient (r) and 95% CI between anti N Ig and age of study participants with no reports of known prior infection (n = 1871) **(A)** and the whole study participants (n = 2472) **(B)**. Dotted horizontal lines represent the cutoff values as recommended by the manufacturer. COI, coefficient of index; N, nucleocapsid; Ig, Immunoglobulin.

SUPPLEMENTARY TABLE 1
Seroprevalence of unreported SARS-CoV-2 infection after Rogan-Gladen correction.

References

1. Tegally H, Wilkinson E, Giovanetti M, Iranzadeh A, Fonseca V, Giandhari J, et al. Detection of a SARS-CoV-2 variant of concern in South Africa. *Nature*. (2021) 592:438–43. doi: 10.1038/s41586-021-03402-9

2. European Center For Disease Prevention And Control. *Rapid Risk Assessment: SARS-CoV-2 – increased circulation of variants of concern and vaccine rollout in the EU/EEA, 14th update*. (2021). Available online at: https://www.ecdc.europa.eu/sites/default/files/documents/RRA-covid-19-14th-update-15-feb-2021.pdf (accessed November, 2021).

3. AGES, Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH. *SARS-CoV-2-Varianten in Österreich*. (2022). Available online at: https://www.ages.at/en/topics/pathogenic-organism/coronavirus/SARS-CoV-2-varianten-inoesterreich/"onavirus/SARS-CoV-2-varianten-in-oesterreich/ (accessed May, 2021).

4. AGES. *Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH*. SARS-CoV-2-Varianten in Österreich. (2022). Available online at: https://www.ages.at/themen/krankheitserreger/coronavirus/SARS-CoV-2-varianten-in-oesterreich/ (accessed May, 2021).

5. Gesundheitsministerium Österreich. *Impflage*. (2022). Available online at: https://info.gesundheitsministerium.at/impflage (accessed June, 2022).

6. Paetzold J, Kimpel J, Bates K, Hummer M, Krammer F, von Laer D, Winner H. Impacts of rapid mass vaccination against SARS-CoV2 in an early variant of concern hotspot. *Nat Commun*. (2022) 13:612. doi: 10.1038/s41467-022-28233-8

7. Li R, Pei S, Chen B, Song Y, Zhang T, Yang W, Shaman J. Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV-2). *Science*. (2020) 368:489–493. doi: 10.1126/science.abb3221

8. Grant R, Dub T, Andrianou X, Nohynek H, Wilder-Smith A, Pezzotti P, Fontanet A. SARS-CoV-2 population-based seroprevalence studies in Europe: a scoping review. *BMJ Open*. (2021) 11:e045425. doi: 10.1136/bmjopen-2020-045425

9. Sah P, Fitzpatrick MC, Zimmer CF, Abdollahi E, Juden-Kelly L, Moghadas SM, et al. Asymptomatic SARS-CoV-2 infection: a systematic review and meta-analysis. *Proc Natl Acad Sci U S A*. (2021) 118:e2109229118. doi: 10.1073/pnas.2109229118

10. Moghadas SM, Fitzpatrick MC, Sah P, Pandey A, Shoukat A, Singer BH, et al. The implications of silent transmission for the control of COVID-19 outbreaks. *Proc Natl Acad Sci U S A*. (2020) 117:17513–5. doi: 10.1073/pnas.2008373117

11. Riepler L, Rössler A, Falch A, Volland A, Borena W, von Laer D, et al. Comparison of four SARS-CoV-2 neutralization assays. *Vaccines (Basel)*. (2020) 9:13. doi: 10.3390/vaccines9010013

12. Borena W, Bánki Z, Bates K, Winner H, Riepler L, Rössler A, et al. Persistence of immunity to SARS-CoV-2 over time in the ski resort Ischgl. *EBioMedicine*. (2021) 70:103534. doi: 10.1016/j.ebiom.2021.103534

13. Ferrara F, Temperton N. Pseudotype neutralization assays: from laboratory bench to data analysis. *Methods Protoc*. (2018) 1:8. doi: 10.3390/mps1010008

14. Statistik Austria. *Bevölkerung nach Alter und Geschlecht*. (2021). Available online at: https://www.statistik.at/web_de/statistiken/menschen_und_gesellschaft/bevoelkerung/bevoelkerungsstruktur/bevoelkerung_nach_alter_geschlecht/index.html (accessed November, 2021).

15. Clopper CJ, Pearson ES. The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika*. (1934) 26:404–13. doi: 10.1093/biomet/26.4.404

16. Gonçalves J, Juliano AM, Charepe N, Alenquer M, Athayde D, Ferreira F, et al. Secretory IgA and T cells targeting SARS-CoV-2 spike protein are transferred to the breastmilk upon mRNA vaccination. *Cell Rep Med*. (2021) 2:100468. doi: 10.1016/j.xcrm.2021.100468

17. Lustig Y, Sapir E, Regev-Yochay G, Cohen C, Fluss R, Olmer L, et al. BNT162b2 COVID-19 vaccine and correlates of humoral immune responses and dynamics: a prospective, single-centre, longitudinal cohort study in health-care workers. *Lancet Respir Med*. (2021) 9:999–1009. doi: 10.1016/S2213-2600(21)00220-4

18. Krammer F, Srivastava K, Alshammary H, Amoako AA, Awawda MH, Beach KF, et al. Antibody responses in seropositive persons after a single dose of SARS-CoV-2 mRNA vaccine. *N Engl J Med*. (2021) 384:1372–4. doi: 10.1056/NEJMc2101667

19. Walsh EE, Frenck RW Jr, Falsey AR, Kitchin N, Absalon J, Gurtman A, et al. Safety and immunogenicity of two RNA-based covid-19 vaccine candidates. *N Engl J Med*. (2020) 383:2439–50. doi: 10.1056/NEJMoa2027906

20. Shachor-Meyouhas Y, Hussein K, Szwarcwort-Cohen M, Weissman A, Mekel M, Dabaja-Younis H, et al. Single BNT162b2 vaccine dose produces seroconversion in under 60 s cohort. *Vaccine*. (2021) 39:6902–6. doi: 10.1016/j.vaccine.2021.10.016

21. Olbrich L, Castelletti N, Schälte Y, Garí M, Pütz P, Bakuli A, et al. Head-to-head evaluation of seven different seroassays including direct viral neutralisation in a representative cohort for SARS-CoV-2. *J Gen Virol*. (2021) 102:001653. doi: 10.1099/jgv.0.001653

22. Poljak M, Oštrbenk Valenčak A, Štamol T, Seme K. Head-to-head comparison of two rapid high-throughput automated electrochemiluminescence immunoassays targeting total antibodies to the SARS-CoV-2 nucleoprotein and spike protein receptor binding domain. *J Clin Virol*. (2021) 137:104784. doi: 10.1016/j.jcv.2021.104784

23. Kannenberg J, Schnurra C, Reiners N, Henschler R, Buhmann R, Kaiser T, et al. Sensitivity of SARS-CoV-2 antibody tests with late convalescent sera. *J Clin Virol Plus*. (2021) 1:100038. doi: 10.1016/j.jcvp.2021.100038

24. Vaselli NM, Hungerford D, Shenton B, Khashkhusha A, Cunliffe NA, French N. The seroprevalence of SARS-CoV-2 during the first wave in Europe 2020: a systematic review. *PLoS ONE*. (2021) 16:e0250541. doi: 10.1371/journal.pone.0250541

25. Bergeri I, Whelan M, Ware H, Subissi L, Nardone A, Lewis HC, et al. Global epidemiology of SARS-CoV-2 infection: a systematic review and meta-analysis of standardized population-based seroprevalence studies, Jan 2020-Oct 2021. *medRxiv [preprint]*. (2021). doi: 10.1101/2021.12.14.21267791

26. Knabl L, Mitra T, Kimpel J, Rössler A, Volland A, Walser A, et al. High SARS-CoV-2 seroprevalence in children and adults in the Austrian ski resort of Ischgl. *Commun Med (London)*. (2021) 1:4. doi: 10.1038/s43856-021-00007-1

27. Medizinische Universität Wien. *Antikörperprevalenz*. (2020). Available online at: https://www.meduniwien.ac.at/web/en/ueber-uns/news/detailseite/2020/news-im-dezember-2020/47-der-oesterreichischen-bevoelkerung-hatten-mitte/ende-oktober-2020-antikoerper-gegen-SARS-CoV-2/ (accessed November, 2021).

28. Kerbl R, Strenger V, Bernar B, Zurl C, Simma B. "Corona Task Force" der Österreichischen Gesellschaft für Kinder- und Jugendheilkunde (ÖGKJ). SARS-CoV-2-Seroprävalenz in Österreich. Die Situation vor der Omikronwelle [SARS-CoV-2 seroprevalence in Austria. The situation before the omicron wave]. *Monatsschr Kinderheilkd*. (2022) 170:487–9. doi: 10.1007/s00112-022-01491-y

29. Zhang H, Wu Y, He Y, Liu X, Liu M, Tang Y, et al. Age-related risk factors and complications of patients with COVID-19: a population-based retrospective study. *Front Med (Lausanne)*. (2022) 8:757459. doi: 10.3389/fmed.2021.757459

30. Cordero-Franco HF, De La Garza-Salinas LH, Gomez-Garcia S, Moreno-Cuevas JE, Vargas-Villarreal J, González-Salazar F. Risk factors for SARS-CoV-2 infection, pneumonia, intubation, and death in northeast mexico. front public health. (2021) 9:645739. doi: 10.3389/fpubh.2021.645739

31. Herrera-Esposito D, de Los Campos G. Age-specific rate of severe and critical SARS-CoV-2 infections estimated with multi-country seroprevalence studies. *BMC Infect Dis*. (2022) 22:311. doi: 10.1186/s12879-022-07262-0

32. Poletti P, Tirani M, Cereda D, Trentini F, Guzzetta G, Marziano V, et al. Age-specific SARS-CoV-2 infection fatality ratio and associated risk factors, Italy, February to April (2020). *Euro Surveill*. (2020) 25:2001383. doi: 10.2807/1560-7917.ES.2020.25.31.2001383

33. Weisberg SP, Connors TJ, Zhu Y, Baldwin MR, Lin WH, Wontakal S, et al. Distinct antibody responses to SARS-CoV-2 in children and

adults across the COVID-19 clinical spectrum. *Nat Immunol.* (2021) 22:25–31. doi: 10.1038/s41590-020-00826-9

34. Schlickeiser S, Schwarz T, Steiner S, Wittke K, Al Besher N, Meyer O, et al. Disease severity, fever, age, and sex correlate with SARS-CoV-2 neutralizing antibody responses. *Front Immunol.* (2021) 11:628971. doi: 10.3389/fimmu.2020.628971

35. Klein SL, Pekosz A, Park HS, Ursin RL, Shapiro JR, Benner SE, et al. Sex, age, and hospitalization drive antibody responses in a COVID-19 convalescent plasma donor population. *J Clin Invest.* (2020) 130:6141–50. doi: 10.1172/JCI142004

36. Zhang B, Zhou X, Zhu C, Song Y, Feng F, Qiu Y, et al. Immune phenotyping based on the neutrophil-to-lymphocyte ratio and igg level predicts disease severity and outcome for patients with COVID-19. *Front Mol Biosci.* (2020) 7:157. doi: 10.3389/fmolb.2020.00157

37. Zhao J, Yuan Q, Wang H, Liu W, Liao X, Su Y, et al. Antibody responses to SARS-CoV-2 in patients with novel Coronavirus Disease (2019). *Clin Infect Dis.* (2020) 71:2027–34. doi: 10.1093/cid/ciaa344

38. Anichini G, Terrosi C, Gandolfo C, Gori Savellini G, Fabrizi S, Miceli GB, et al. SARS-CoV-2 antibody response in persons with past natural infection. *N Engl J Med.* (2021) 385:90–2. doi: 10.1056/NEJMc2103825

39. Manisty C, Otter AD, Treibel TA, McKnight Á, Altmann DM, Brooks T, et al. Antibody response to first BNT162b2 dose in previously SARS-CoV-2-infected individuals. *Lancet.* (2021) 397:1057–8. doi: 10.1016/S0140-6736(21)00501-8

40. Prendecki M, Clarke C, Brown J, Cox A, Gleeson S, Guckian M, et al. Effect of previous SARS-CoV-2 infection on humoral and T-cell responses to single-dose BNT162b2 vaccine. *Lancet.* (2021) 397:1178–81. doi: 10.1016/S0140-6736(21)00502-X

41. Stamatatos L, Czartoski J, Wan YH, Homad LJ, Rubin V, Glantz H, et al. mRNA vaccination boosts cross-variant neutralizing antibodies elicited by SARS-CoV-2 infection. *Science.* (2021) 372:1413–8. doi: 10.1126/science.abg9175

42. Ebinger JE, Fert-Bober J, Printsev I, Wu M, Sun N, Prostko JC, et al. Antibody responses to the BNT162b2 mRNA vaccine in individuals previously infected with SARS-CoV-2. *Nat Med.* (2021) 27:981–4. doi: 10.1038/s41591-021-01325-6

43. Goel RR, Apostolidis SA, Painter MM, Mathew D, Pattekar A, Kuthuru O, et al. Distinct antibody and memory B cell responses in SARS-CoV-2 naïve and recovered individuals following mRNA vaccination. *Sci Immunol.* (2021) 6:eabi6950. doi: 10.1126/sciimmunol.abi6950

44. Saadat S, Rikhtegaran Tehrani Z, Logue J, Newman M, Frieman MB, Harris AD, et al. Binding and neutralization antibody titers after a single vaccine dose in health care workers previously infected with SARS-CoV-2. *JAMA.* (2021) 325:1467–9. doi: 10.1001/jama.2021.3341

45. Reynolds CJ, Pade C, Gibbons JM, Butler DK, Otter AD, Menacho K, et al. Prior SARS-CoV-2 infection rescues B and T cell responses to variants after first vaccine dose. *Science.* (2021) 372:1418–23. doi: 10.1126/science.abh1282

46. Allen N, Brady M, Ni Riain U, Conlon N, Domegan L, Carrion Martin AI, et al. Prevalence of antibodies to SARS-CoV-2 following natural infection and vaccination in Irish hospital healthcare workers: changing epidemiology as the pandemic progresses. *Front Med (Lausanne).* (2022) 8:758118. doi: 10.3389/fmed.2021.758118

47. Krutikov M, Palmer T, Tut G, Fuller C, Azmi B, Giddings R, et al. Prevalence and duration of detectable SARS-CoV-2 nucleocapsid antibodies in staff and residents of long-term care facilities over the first year of the pandemic (VIVALDI study): prospective cohort study in England. *Lancet Healthy Longev.* (2022) 3:e13–21. doi: 10.1016/S2666-7568(21)00282-8

48. Gonçalves J, Sousa RL, Jacinto MJ, Silva DA, Paula F, Sousa R, et al. Evaluating SARS-CoV-2 seroconversion following relieve of confinement measures. *Front Med (Lausanne).* (2020) 7:603996. doi: 10.3389/fmed.2020.603996

49. Siller A, Seekircher L, Wachter GA, Astl M, Tschiderer L, Pfeifer B, et al. Seroprevalence, waning and correlates of anti-SARS-CoV-2 igg antibodies in tyrol, austria: large-scale study of 35,193 blood donors conducted between June 2020 and September 2021. Viruses. (2022) 14:568. doi: 10.3390/v14030568

50. AGES, Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH. *Dashboard COVID-19.* (2020). Available online at: https://covid19-dashboard.ages.at/. (accessed March, 2021).

51. Bobrovitz N, Arora RK, Cao C, Boucher E, Liu M, Donnici C, et al. Global seroprevalence of SARS-CoV-2 antibodies: a systematic review and meta-analysis. *PLoS ONE.* (2021) 16:e0252617. doi: 10.1371/journal.pone.0252617

frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 25 October 2022
DOI 10.3389/fpubh.2022.924316

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

RECEIVED 20 April 2022
ACCEPTED 07 October 2022
PUBLISHED 25 October 2022

CITATION
Chan YL, Patterson CL, Priest JW, Stresman G, William T, Chua TH, Tetteh K, Lammie P, Drakeley C and Fornace KM (2022) Assessing seroprevalence and associated risk factors for multiple infectious diseases in Sabah, Malaysia using serological multiplex bead assays. *Front. Public Health* 10:924316. doi: 10.3389/fpubh.2022.924316

Assessing seroprevalence and associated risk factors for multiple infectious diseases in Sabah, Malaysia using serological multiplex bead assays

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Background: Infectious diseases continue to burden populations in Malaysia, especially among rural communities where resources are limited and access to health care is difficult. Current epidemiological trends of several neglected tropical diseases in these populations are at present absent due to the lack of habitual and efficient surveillance. To date, various studies have explored the utility of serological multiplex beads to monitor numerous diseases simultaneously. We therefore applied this platform to assess population level exposure to six infectious diseases in Sabah, Malaysia. Furthermore, we concurrently investigated demographic and spatial risk factors that may be associated with exposure for each disease.

Methods: This study was conducted in four districts of Northern Sabah in Malaysian Borneo, using an environmentally stratified, population-based cross-sectional serological survey targeted to determine risk factors for malaria. Samples were collected between September to December 2015, from 919 villages totaling 10,100 persons. IgG responses to twelve antigens of six diseases (lymphatic filariasis- Bm33, Bm14, BmR1, Wb123; strongyloides- NIE; toxoplasmosis-SAG2A; yaws- Rp17 and TmpA; trachoma- Pgp3, Ct694; and giardiasis- VSP3, VSP5) were measured using serological multiplex bead assays. Eight demographic risk factors and twelve environmental covariates were included in this study to better understand transmission in this community.

Results: Seroprevalence of LF antigens included Bm33 (10.9%), Bm14+ BmR1 (3.5%), and Wb123 (1.7%). Seroprevalence of Strongyloides antigen NIE was 16.8%, for Toxoplasma antigen SAG2A was 29.9%, and Giardia antigens GVSP3

+ GVSP5 was 23.2%. Seroprevalence estimates for yaws Rp17 was 4.91%, for TmpA was 4.81%, and for combined seropositivity to both antigens was 1.2%. Seroprevalence estimates for trachoma Pgp3 + Ct694 were 4.5%. Age was a significant risk factors consistent among all antigens assessed, while other risk factors varied among the different antigens. Spatial heterogeneity of seroprevalence was observed more prominently in lymphatic filariasis and toxoplasmosis.

Conclusions: Multiplex bead assays can be used to assess serological responses to numerous pathogens simultaneously to support infectious disease surveillance in rural communities, especially where prevalences estimates are lacking for neglected tropical diseases. Demographic and spatial data collected alongside serosurveys can prove useful in identifying risk factors associated with exposure and geographic distribution of transmission.

KEYWORDS

neglected tropical disease (NTD), serology, multiplex bead assay analysis, epidemiology - analytic (risk factors), Malaysia

Introduction

Within the last decade, disease control efforts including mass drug administration, improved sanitation, and public health awareness have helped reduce the burden of neglected tropical (NTDs) and other infectious diseases in Malaysia. However, many of these diseases persist, especially among isolated, resource-constrained, and aboriginal communities in Sabah, resulting in sustained morbidity and chronic impact on quality of life (1). For example, helminth diseases in Malaysia include strongyloidiasis (2–4) and lymphatic filariasis (LF) (5) that can cause a range of illnesses leading to malnutrition and disability (6–8). Persistent protozoan diseases in Malaysia include giardiasis (9), toxoplasmosis (10), and malaria (11, 12). Giardiasis can result in malnutrition from chronic diarrhea (13, 14) while toxoplasmosis symptoms can vary from asymptomatic to severe clinical manifestations that occur typically in immunocompromised patients (15). In Malaysia, bacterial diseases include leptospirosis (16, 17), trachoma (18), and yaws (19) that can impact the skin, eyes, joints, and other parts of the body.

Current epidemiological trends are unknown for many of these infections due to the dearth of routine and reliable surveillance (18). Characterizing disease burden can be particularly difficult in low-transmission and post-elimination settings, especially if sub-clinical infections are common. Assessing cross-sectional population prevalence can help identify areas of transmission resurgence or introduction, but low transmission rates, mild morbidity, and limited resources may have reduced public health priority of systematic monitoring of these diseases. Since transmission of many of these pathogens geographically overlap and can result in co-infections, integrated, multi-disease monitoring would provide resource efficient alternatives compared with single disease surveillance (20). While diverse biological targets of tropical infections often require different laboratory methods to capture disease burden (e.g., stool microscopy, polymerase chain reaction, or antibody testing), a unified platform for monitoring exposure to diverse pathogens may help to overcome some of these logistical challenges toward concurrent NTD monitoring.

Integrated monitoring may be attainable using serological multiplex bead assays (MBA). MBAs can quantify immune responses to multiple pathogens from a single blood spot (21). Serology can effectively capture asymptomatic infections and reveal historical pathogen exposure by measuring pathogen-specific antibody responses (22). The use of serology in monitoring NTDs and vaccine preventable diseases (VPDs) has been applied in numerous settings (21–25). Furthermore, demographic and environmental data collected in population-based surveys provide key opportunities to assess potential and shared risk factors of the different diseases that may enhance controls strategies and community awareness. While certain socio-economic risk factors have been studied for several parasitic infections in Malaysia (4, 13, 26, 27), spatial and other risk factors are not well-characterized for many of these diseases.

To our knowledge, multiplex bead assays have yet to be applied to assessing NTD and parasitic disease seroprevalence and associated risk factors in Malaysia. In this study, we used MBA on samples collected during a 2015 cross-sectional survey in Northern Sabah, Malaysia to estimate population exposure to multiple pathogens. We aimed to (1) describe population level exposure to six infectious diseases, (2) assess pathogen-specific individual risk factors for exposure; and (3) determine spatial

and environmental risk factors and predict population-level exposure probabilities.

Methods

Study site and sampling

This study was conducted in four districts of Northern Sabah in Malaysian Borneo (Figure 1). This area is tropical with elevations ranging from sea-level to over 4,000 meters above sea level (MSL). The population is predominantly rural, and most occupations are associated with agricultural or plantation activities. This study was designed to determine the risk factors for malaria using an environmentally stratified, population-based cross-sectional survey that was conducted from September 17, 2015 to December 12, 2015, as described by Fornace et al. (28). Briefly, seroprevalence was estimated using a non-self-weighting two-stage sampling design of 919 villages stratified by forest cover, with a target sample size of 2,650 households and 36 households sampled per village (powered for *Plasmodium knowlesi* seroprevalence). All individuals residing in selected households were asked to participate (ages 3 months—105 years). Finger prick blood sampling was used to prepare blood spots of filter paper (3MM, Whatman, Maidstone, UK).

Ethics approval

The Medical Research Sub-Committee of the Malaysian Ministry of Health (NMRR-14-713-21117) and the Research Ethics Committee of the London School of Hygiene and Tropical Medicine (8340) approved the Malaysian study and written informed consent was obtained from all study participants. Because CDC authors did not interact with study participants and had no access to personal identifying information, they were determined to be "not engaged" in human subjects research.

Multiplex IgG detection assay

The IgG responses to 12 antigens from six pathogens were assayed (Table 1; Supplementary Table 1). Merozoite surface protein 1-19 (MSP1-19) and apical membrane antigen-1 (AMA-1) antigens from *Plasmodium falciparum* and *P. vivax* were also included with appropriate control sera as internal positive controls. Excluding the malaria proteins, all antigen-coupled microspheres were provided by the Centers for Disease Control and Prevention (Atlanta, GA, USA) and coupled according to standard Luminex protocols to minimize the signal-to-noise ratio (29). Malaria antigen coupling was optimized in-house as described previously (28, 30) (Luminex Corporation, Austin, TX, USA).

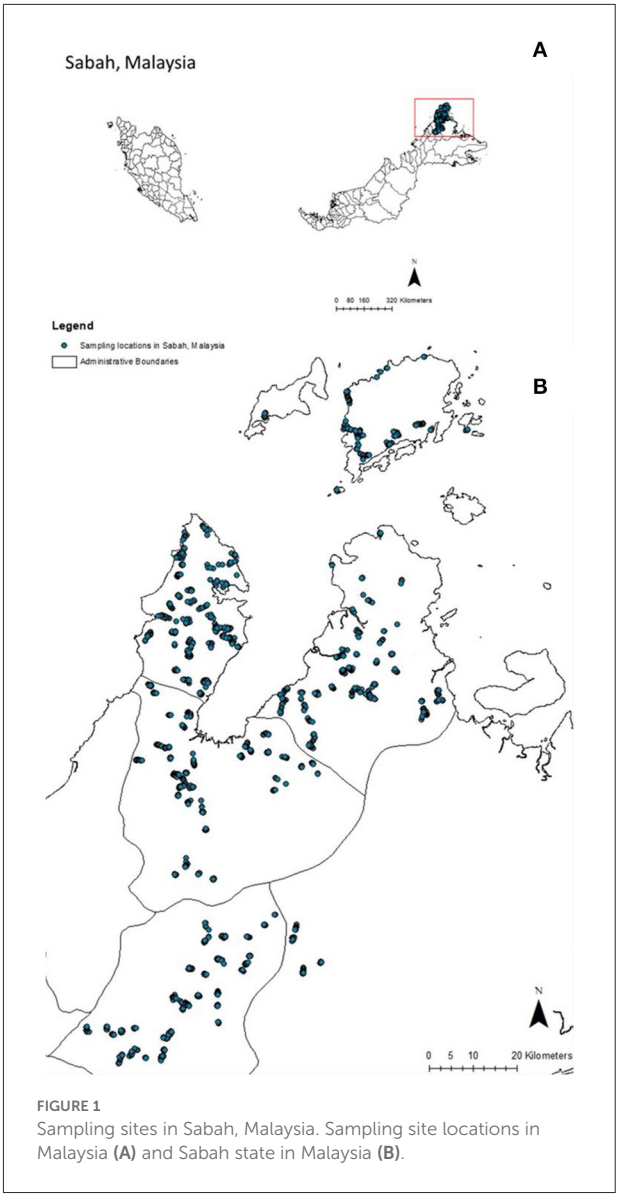

FIGURE 1
Sampling sites in Sabah, Malaysia. Sampling site locations in Malaysia **(A)** and Sabah state in Malaysia **(B)**.

Test samples were eluted from a 3-mm dried blood spot (DBS) punch, corresponding to 2.1 μl of whole blood, and shaken overnight at room temperature in 200 μl of elution buffer (1xPBS, 0.05% sodium azide and 0.05% Tween-20), resulting in a 1:200 pre-dilution, assuming 50% hematocrit. At least 1 day prior to testing, samples were diluted to a final 1:400 dilution using Luminex buffer B (1xPBS, 0.05% Tween, 0.5% BSA, 0.02% sodium azide, 0.1% casein, 0.5% polyvinyl alcohol (PVA), 0.5% polyvinyl pyrrolidone (PVP) and 15.25 μg/ml *E. coli* extract) to prevent non-specific binding. Negative and positive controls were also incubated in buffer B at least 1 day before testing, with negative controls prepared

TABLE 1 Cut-off method, seroprevalence and vaccine exposure in percentages, and number of individuals per antigen.

Antigen, pathogen	Disease	Percent seroprevalence with 95% CI	*n*
Gaussian mixture model (2 distributions)			
Bm33, *Brugia malayi*	Lymphatic Filariasis	10.9 (10.2, 11.6)	8129
Wb123, *Wucheria Bancrofti*	Lymphatic Filariasis	1.7 (1.5, 2.02)	8128
NIE, *Strongyloides stercoralis*	Strongyloidiasis	16.8 (16.7, 16.9)	8131
SAG2A, *Toxoplasma gondii*	Toxoplasmosis	29.9 (28.9, 30.1)	7430
Rp17, *Treponemal pallidum pertenue*	Yaws	4.9 (3.9, 6.1)	1529
TmpA, *Treponemal pallidum pertenue*	Yaws	4.8 (4.00, 5.8)	1660
Rp17 TmpA double positive	Yaws	1.2 (0.7, 1.8)	1638*
VSP3 + VSP5, *Giardia duodenalis*	Giardiasis	23.2 (22.3-24.2)	7682
***K*-means clustering ($k = 3$)**			
Bm14+BmR1, *Brugia malayi*	Lymphatic Filariasis	3.5 (3.1, 4.00)	6855
Pgp3+Ct694, *Chlamydia trachomatis*	Trachoma	4.5 (3.7, 5.5)	1970

TABLE 2 Study site characteristics.

Demographic variable	*n*
Study population	8,205
Males	3,389
Females	4,312
Mean age in years (range)	29 (0–105)
Occupation	***n***
Farmer	1,153
Student	3,745
Other occupation	997
No occupation	3,745
Ethnic groups in Malaysia	***n***
Bajau	752
Dusun	4,137
Other	1,135
Rungus	2,091
Environmental variable	**Mean (range)**
Population density (per km^2)	1.8 (0–183.4)
Elevation (meters above sea level)	166.4 (4.0–1,258.0)
NDVI	0.5 (-0.2 to 0.9)
Average temperature, 1970–2000 (°C)	26.7 (21.5–27.5)
Mean diurnal range, 1970–2000 (°C)	8.2 (7.00–10.3)
Maximum temperature of warmest month, 1970–2000 (°C)	31.9 (28.1–32.8)
Minimum temperature of coldest month, 1970–2000 (°C)	21.5 (14.8–22.9)
Precipitation of the wettest month. 1970–2000 (mm)	2,417 (2,167–2,754)
Precipitation seasonality, 1970–2000 (coefficient of variation)	44.1 (16.9–59.5)
Distance to intact forest (m)	3,647 (0–19,836)
Distance to irrigated farmland (m)	2,794 (0–23,716)
Distance to oil palm plantation (m)	1098 (0–20,940)

at 1:400, a pooled *P. falciparum* positive prepared at 1:400 and 1:4,000, and a pooled *P. vivax* positive control prepared in a 6-point 2-fold serial dilution (1:400–1:12,800). Fifty microliter of the samples were co-incubated with antigen-coupled beads in a 1-day multiplex serological assay described previously (30). Using a Luminex MAGPIX bioanalyzer and xPONENT software (version 4.2), the background-adjusted median fluorescent intensity (MFI) of wells achieving at least a 30-bead count per analyte were recorded. The *P. vivax* control curve was included on each plate to standardize data between plates (28).

Determination of seropositivity

Different cut-off approaches were used to determine seropositivity per antigen (Supplementary Table 2). To determine seropositivity, antigen-specific cut-off values from log transformed MFI with background subtracted (MFI-bg) were calculated in R using the mixtools package (31). Gaussian mixture models using the mean of the lower component plus three standard deviations were used to determine cut-off thresholds for eight antigens on this panel. To ensure sufficient negatives for estimating population level exposure, we included individuals of all ages in cut-off determination for LF (Bm33, LF Wb123) and toxoplasma (SAG2A) antigens (32). Data from individuals <3, 5, and 14 years of age were used to determine cut-offs for antigens of strongyloides (NIE), giardia (VSP3, VSP5), and yaws (Rp17, TmpA), respectively. For giardia and yaws, we examined double seropositivity as an indicator of more recent exposure.

As multiple antigens were measured for specific diseases, we also analyzed highly correlated antigens (Pearson's correlation co-efficient > 0.65) for the same pathogen together as representative of individuals exposed to the same pathogen (Supplementary Figure 1). This was done for lymphatic filariasis (Bm14 and BmR1) and trachoma (Pgp3 and Ct694) antigens using K-means clustering (three clusters, highest cluster of MFI responses to multiple antigens are considered seropositive) to classify seropositive and seronegative. We limited analysis of antigens for trachoma to children under 10 years old to exclude sexually acquired venereal chlamydia.

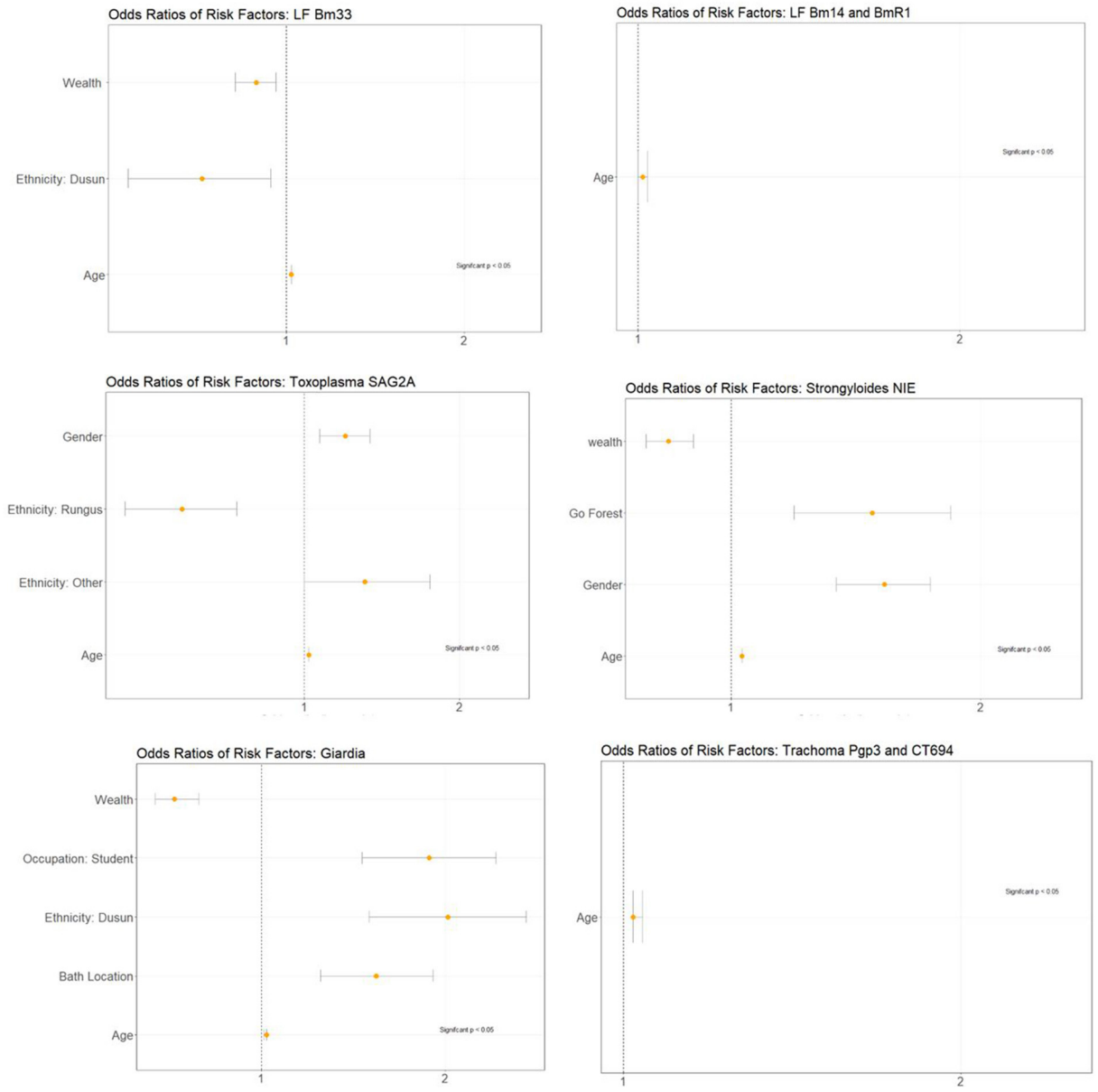

FIGURE 2
Adjusted odd ratio plots for associated disease risk factors.

Statistical analysis of risk factors

We assessed eight demographic, health, and socioeconomic risk factors (Supplementary Table 3). Logistic regression was used to evaluate risk factors association to seroprevalence for each antigen, with household included as a random effect to control for sampling design. Associations with a $p < 0.05$ were considered statistically significant using adjusted odds ratios (Supplementary Table 4). Variables were assessed using variation inflation factor < 5 to assess for potential collinearity, and final models were selected using backwards elimination ($p < 0.05$).

Spatial patterns of exposure risks

To assess the spatial distribution of exposure risks, we additionally assembled potential spatial environmental covariates, including topographic measures, distance to land

cover and forest types, population density, accessibility, and climatic variables (Table 2). Pearson correlation analysis was used to exclude highly correlated variables (correlation coefficient > 0.7) with the final dataset including 21 potential spatial and environmental predictors (Supplementary Tables 5, 6). As demographic data was not available for all locations within this region, we did not include additional questionnaire data. All covariates were resampled to 500 m resolution for predictions.

Using the seropositivity thresholds defined above, we fit geostatistical models of household seroprevalence for each disease separately. Models were fit in a Bayesian framework with $p(x_i)$ denoting the seroprevalence at locations x_i, $i = 1 \ldots n$, with m_i individuals sampled per household location. The full model was specified as:

$$Y_i \sim \text{Binomial}\left(\text{m}_\text{i},\ \text{p}(\text{x}_\text{i})\right)$$

With the linear predictor for the binomial model specified as:

$$\text{logit}\left(\text{p}(\text{x}_\text{i})\right) = \beta_0 + \mathbf{d}(\mathbf{x_i})'\beta + \text{w}_\text{i}$$

Where β_0 represents the intercept, $\mathbf{d}(\mathbf{x_i})'\beta$ represents a vector of location specific covariate effects and w_i represents the spatial effect. Residual spatial autocorrelation was assessed using Moran's I, with spatial effects modeled as a Matern covariance function using the stochastic partial differential equation approach implemented in Integrated Nested Laplace Approximation (R-INLA) (33). Weakly informative priors of Normal (0, 100) were used for intercepts and fixed effect coefficients and penalized complexity priors were used for the spatial effect (34). Final models were assessed using the deviance information criteria (DIC) and root mean squared error. Posterior probabilities were estimated using 1,000 posterior samples. Additionally, to visualize the uncertainty around these predictions, we calculated exceedance probabilities using a 10% seroprevalence threshold (35). These exceedance probabilities represent the probability a location exceeds this threshold; locations with exceedance probabilities around 50% represent areas where there is high uncertainty around this threshold. All analysis was conducted in R statistical software (36), with maps visualized in ArcGIS (ESRI, Redlands, USA).

Results

Seroprevalence

Cross-sectional serological survey data was available for 10,100 individuals, with varying number of individuals available for analysis based on sample and antigen availability. Seroprevalence estimates of the whole study site in northern Sabah are shown in Table 1. The seroprevalence of LF antigens were highest in Bm33 (10.9%), then Bm14+ BmR1 (3.5%), and lowest in Wb123 (1.7%). Seroprevalence of *Strongyloides* antigen NIE was 16.8%, for *Toxoplasma* antigen SAG2A was 29.9%, and *Giardia* antigens GVSP3 + GVSP5 was 23.2%. Seroprevalence estimates for yaws antigens in school children <10 years of age for Rp17 was 4.91% and for TmpA was 4.81%. As Rp17 may indicate historical exposure and TmpA may indicate more recent exposure, combined seropositivity to both antigens was 1.2%. Seroprevalence estimates for trachoma Pgp3 +Ct694 were 4.5%.

Study site characteristics that include demographic and environmental variables are listed in Table 2. Seroprevalence of demographic risk factors are listed in Supplementary Table 3. Seropositivity to all assessed antigens showed potential age effects, demonstrating differences in exposure by age category (Supplementary Figures 2–4).

Risk factor analysis

Multivariate analysis using logistic regression identified associations between seropositivity and risk factors that were considered significant at $p < 0.05$ (Figure 2; Supplementary Table 4). For LF Bm33 antigen, significant associations were observed for age, wealth, and Dusun ethnicity. Higher socio-economic status and Dusun ethnicity demonstrated decreased odds of risk of exposure. For LF Wb123 antigens, no significant associations were observed, potentially due to the low overall seroprevalence in the population. For LF Bm14 + BmR1, significant associations for risk factors were observed for age only. For toxoplasma SAG2A antigen, significant associations were observed with age, gender, ethnicity, and bath location. Increased odds of exposure were observed for males compared to females and for ethnicity within the "Other" category. Decreased odds of exposure were observed for Rungus ethnic group. For the *Strongyloides* antigen, significant associations were observed for age, wealth, going to the forest, and gender. Higher socio-economic status was associated with decreased odds of exposure, while going to the forest and being male demonstrated increased odds of exposure. For *Giardia* antigens, age, student occupation, Dusun ethnicity, and bath location (i.e., bathing in outdoor locations or with water pipes) were shown to increase odds of exposure, while decreased odds of exposure was observed with higher socio-economic status. For trachoma antigens, age was the only significant risk factor.

Environmental risk factors and spatial distribution of exposure

The spatial distribution of seroprevalence of antigens are presented in Figure 3 and Supplementary Figure 6. The study

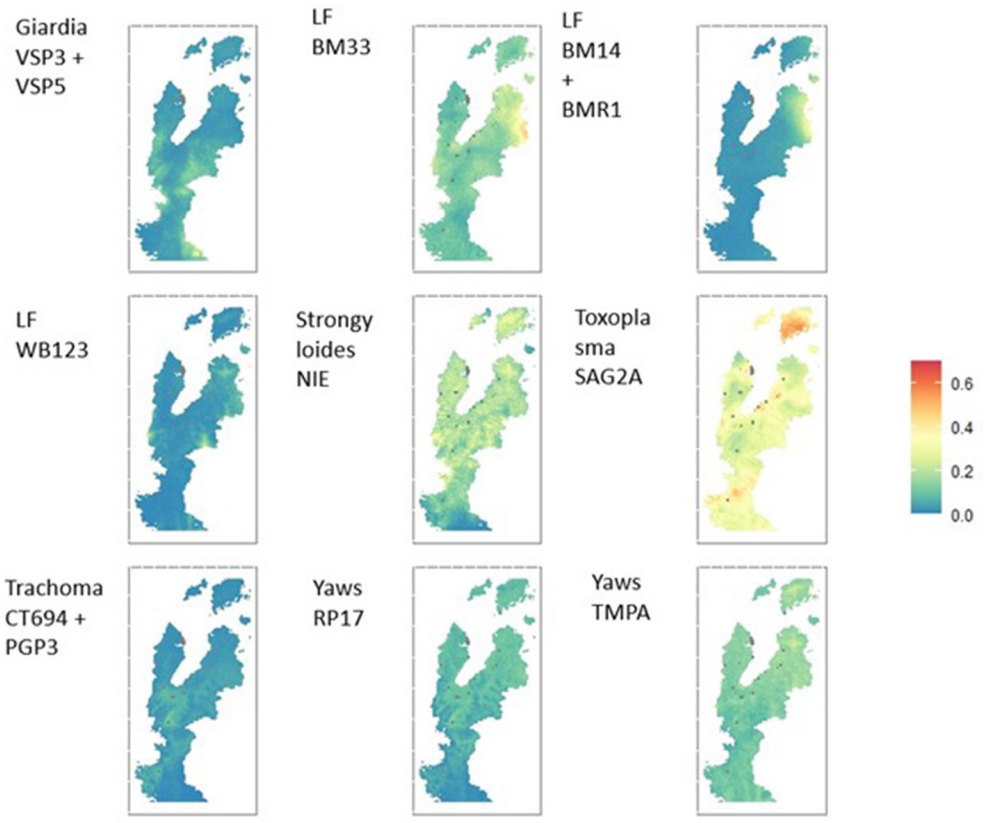

FIGURE 3
Geostatistical maps showing mean posterior estimated seroprevalence per antigen.

area represented a wide range of ecologies with varying land cover, topography, and population densities (Table 2; Supplementary Table 5). Using these data, we additionally identified predictive spatial and environmental factors for exposure to diseases (Supplementary Table 6). Geostatistical models identified marked differences in the spatial distribution of exposure to the different antigens, revealing areas of potential persistent exposure. Mean posterior estimates of seroprevalence for each pathogen are shown in Figure 3, with estimates of the probability of over 10% of the population being exposed to a particular pathogen shown in Supplementary Figure 5. For example, the spatial distribution of seroprevalence for strongyloides NIE and Yaws TmpA demonstrated broad homogeneity in community exposure, while the spatial distribution of toxoplasma SAG2A and LF BM antigens identified areas of higher seroprevalence compared to the rest of the community.

Discussion

Serological surveys provide a platform for integrated monitoring of numerous pathogens. In our study, we applied multiplex bead assays to assess seroprevalences and associated risk factors to six NTDs. The seroprevalence results provided evidence of exposure for all NTDs in Malaysia during 2015. Integrating this data within a geostatistical framework enables visualization of spatial dispersal of exposure, detecting priority areas for follow up, surveillance, and targeted public health initiatives.

Analysis of disease specific responses allowed identification of risk factors and spatial distribution of exposure for all diseases, showing broad agreement with other sources of epidemiological data. For example, persistent LF transmission and LF MDA was on-going during the year of the survey [World Health Organization (WHO) Global Health Observatory (GHO), accessed August 19, 2020]. Preventative chemotherapy treatments for strongyloidiasis (prevalence = 16.8%) and other STHs were also administered to parts of the country during the same year of this survey (according to WHO GHO, accessed August 19, 2020), and prevalence estimates were similar to previously reported estimates (31.5%, using ELISA) in Malaysia (Orang Asli) (2). Seroprevalence estimate for toxoplasmosis in this study was 29.9% (CI: 28.9–30.1%) and consistent with similar estimates of previous studies (10, 37). For giardiasis, the

seroprevalence estimate was 23.24% (CI: 22.31–24.19%), higher than prior estimates using molecular techniques, which varied between 0.2 and 20% (38, 39). Composite antigen responses for trachoma were detected among 4.52% (CI: 3.68–5.53%) of children 1–9 years of age. This is similar to what was previously observed in areas suspected not to be endemic for trachoma in the Pacific Island nations of the Solomon Islands, Fiji, and Vanuatu (40–42).

For LF, trachoma, yaws, and *Giardia*, multiple antigens were included in determining seropositivity. For LF prevalence, estimates varied using antigens of the same pathogen. This may be due to differing immunogenicity of antigens, antibody kinetics as markers of recent or historical exposure, or possible cross reactivity with antibodies elicited by infection with other pathogens (43, 44). For yaws antigens, seroprevalence was 4.91% (CI: 3.93–6.11%) for Rp17 and 4.81% (CI: 3.98–5.79%) for TmpA. We observed a lack of correlation between the two yaws antigen, which may be due to individual antigen function (Supplementary Figure 6). For example, Cooley et al. have found that Rp17 captures long-lived treponemal antibodies, while TmpA can be potentially used to differentiate exposure based on antibody titer concentrations (45). In our study, we presented double seropositivity for both antigens (1.16%, CI: 0.74–1.80). If further information was available on antibody decay rates, more accurate estimates of infection status and time since infection could be identified. For highly correlated antigens of LF and trachoma, we determined seropositivity by applying K-means clustering approach to classify seroprevalence. This approach to classifying antibody responses may potentially enhance seroprevalence approximations by examining multiple highly correlated antigens within the population, thereby maximizing the use of information from multiple antigens.

We examined several risk factors in this study to demonstrate the utility of multiplex bead assays in supporting integrated disease control efforts. Given the age effect on antibody acquisition, we hypothesized that this association would be present among differing concentrations of the antigen levels and age, within our study population. We found age to be associated with seroprevalence for all antigens, indicating increased likelihood for exposure over time. For giardiasis, however, consistent exposure and chronic infection among children and adults may dampen any age effects on seroprevalence. Differences in antibody concentrations in age may support targeted public health initiatives and further examination of historical exposure patterns among age groups.

Previous studies in Malaysia have found associations between low socioeconomic backgrounds and burden of disease, which is attributable to living standards, working conditions and access to health care (46, 47). We hypothesized that high wealth index would be an acceptable indicator of adequate nutrition, better living conditions, clean water, and easier access to health care, thus reducing seroprevalence in higher socio-economic classes for all NTDs and parasitic disease infections (14, 48, 49). We found associations of higher wealth index and decreased seroprevalence among antigens of LF, *Strongyloides*, and *Giardia*, but no associations were observed for *Toxoplasma* or trachoma.

Common socio-demographic risk factors such as gender, ethnicity, education, occupation, toilet usage, and contact with animals have also been previously studied for LF, toxoplasmosis, and giardiasis in Malaysia (5, 15, 37, 50, 51). We examined these potential risk factors for the diseases represented by our panel of antigens. In this study, significant risk of exposure for occupation was not observed for any disease. Previous studies have found limited data on human seroprevalence in relation to animal exposure for toxoplasmosis in Malaysia, including domestic and livestock animals (52), although Ngui et al. found significant associations with seropositivity for individuals coming in close contact with cats and other pets (10). In our study, we did not find any significant associations with owning animals and increased odds of exposure for any disease marker. We also included bath location in this risk factor analysis, as clean water is important in the prevention of diseases such as STHs and giardiasis, and we found significant associations in decreased prevalence with the use of bathrooms compared to outside bathing for giardiasis in this study. Variation in seroprevalence by ethnic groups may be attributable to cultural norms, occupations, genetics, and geographic dispersion that may warrant more detailed investigation of these differences to aid public health initiatives.

In addition to identifying risk factors, we demonstrate how serological data can be used to characterize the spatial distribution of exposure. Simple visualizations of cluster level mean antibody responses can be used to quickly identify clusters with high responses to multiple pathogens. By integrating serological data into geostatistical models, we identified areas with differential exposure of diseases such as filariasis or focalised transmission such as toxoplasmosis; this data can be used to supplement available infection reports to support elimination campaigns and targeted control. Conversely, we also identify diseases with widespread transmission, such as giardiasis. Characterizing these differences in spatial distribution allows development of appropriate control and surveillance strategies for diseases with vastly different transmission levels. Additionally, this provides further data on the immune status of different populations, with potential implications on susceptibility to disease.

Within this study there are several limitations. Serological standards to determine cut-offs have not been established for most pathogens on this panel, and choice of cut-off method may have impacted the accuracy of seroprevalence approximations. Another limitation within the survey is the lack of individual information about survey respondent's migratory status for coastal Sabah, thus it is unclear whether serological responses represent regional or imported cases. Lastly, we applied a non-conventional method to cluster seropositives using k-means algorithm for correlated antigens

of the same pathogens. While trachoma estimates were similar to what was found previously, the discrepancy in seroprevalence estimates among mixture models and k-means clustering for LF antigens implores further exploration of using this method paired with clinically confirmed data or gold standard approaches.

Despite these limitations, this study supports the utility of MBAs for simultaneous disease monitoring of diverse pathogens in low transmission settings. As integrated disease management is being adopted in the WHO NTD Roadmap of 2021 (53), MBAs with serological surveys can provide valuable information regarding population exposure and associated socio-demographic or environmental risk factors impacting transmission of numerous co-endemic pathogens.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Research Sub-Committee of the Malaysian Ministry of Health (NMRR-14-713-21117) and the Research Ethics Committee of the London School of Hygiene and Tropical Medicine (8340). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

CD, KF, and YC conceptualized research questions and analysis. TW and TC were involved in sample collections and survey design. CP and KT ran multiplex bead assays and provided support to analysis. YC and KF performed the analysis. YC wrote manuscript draft. CD, GS, JP, KF, and PL provided critical reviews and revisions of manuscript draft. All authors contributed to the article and approved the submitted version.

Funding

We acknowledge the UK Medical Research Council, Natural Environment Research Council, Economic and Social Research Council and Biotechnology and Biosciences Research Council for the funding received for this project through the Environmental and Social Ecology of Human Infectious Diseases Initiative (Grant Number G1100796). KF was supported by a Sir Henry Dale fellowship jointly funded by the Wellcome Trust and Royal Society (Grant Number 221963/Z/20/Z).

Acknowledgments

The authors thank T. Nutman (NIH, Bethesda, MD) for Wb123 protein, Diana L. Martin (CDC, Atlanta, GA) for providing TmpA, rp17, Pgp3, and CT694 antigens, and Prof. Rahmah Noordin (Institute for Research in Molecular Medicine and School of Medical Sciences, Universiti Sains Malaysia, Malaysia) for providing the BmR1 antigen.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.924316/full#supplementary-material

References

1. Hotez PJ. Aboriginal populations and their neglected tropical diseases. *PLoS Negl Trop Dis.* (2014) 8:e2286. doi: 10.1371/journal.pntd.0002286

2. Ahmad AF, Hadip F, Ngui R, Lim YAL, Mahmud R. Serological and molecular detection of Strongyloides stercoralis infection among an Orang Asli community in Malaysia. *Parasitol Res.* (2013) 112:2811–6. doi: 10.1007/s00436-013-3450-z

3. Lim YAL, Romano N, Colin N, Chow SC, Smith HV. Intestinal parasitic infections amongst Orang Asli (indigenous) in Malaysia: has socioeconomic development alleviated the problem? *Trop Biomed.* (2009) 26:110–22.

4. Lim-Leroy A, Chua TH. Prevalence and risk factors of geohelminthiasis among the rural village children in Kota Marudu, Sabah, Malaysia. *PLoS ONE.* (2020) 15:e0239680. doi: 10.1371/journal.pone.0239680

5. Al-Abd NM, Nor ZM, Ahmed A, Al-Adhroey AH, Mansor M, Kassim M. Lymphatic filariasis in Peninsular Malaysia: a cross-sectional survey of the knowledge, attitudes, and practices of residents. *Parasit Vect.* (2014) 7:545. doi: 10.1186/s13071-014-0545-z

6. Al-Mekhlafi HM, Azlin M, Aini UN, Shaik A, Sa'iah A, Fatmah MS, et al. Protein-energy malnutrition and soil-transmitted helminthiases among Orang Asli children in Selangor, Malaysia. *Asia Pac J Clin Nutr.* (2005) 14:188–94.

7. Dreyfuss ML, Stoltzfus RJ, Shrestha JB, Pradhan EK, LeClerq SC, Khatry SK, et al. Hookworms, malaria and vitamin A deficiency contribute to anemia and iron deficiency among pregnant women in the plains of Nepal. *J Nutr.* (2000) 130:2527–36. doi: 10.1093/jn/130.10.2527

8. Simonsen PE, Derua YA, Magesa SM, Pedersen EM, Stensgaard AS, Malecela MN, et al. Lymphatic filariasis control in Tanga Region, Tanzania: status after eight rounds of mass drug administration. *Parasit Vect.* (2014) 7:507. doi: 10.1186/s13071-014-0507-5

9. Sinniah B, Hassan A KR, Sabaridah I, Soe MM, Ibrahim Z, Ali O. Prevalence of intestinal parasitic infections among communities living in different habitats and its comparison with one hundred and one studies conducted over the past 42 years (1970 to 2013) in Malaysia. *Trop Biomed.* (2014) 31:190–206.

10. Ngui R, Lim YAL, Amir NFH, Nissapatorn V, Mahmud R. Seroprevalence and sources of toxoplasmosis among orang Asli (indigenous) communities in Peninsular Malaysia. *Am J Trop Med Hyg.* (2011) 85:660–6. doi: 10.4269/ajtmh.2011.11-0058

11. Yusof R, Lau YL, Mahmud R, Fong MY, Jelip J, Ngian HU, et al. High proportion of knowlesi malaria in recent malaria cases in Malaysia. *Malaria J.* (2014) 13:168. doi: 10.1186/1475-2875-13-168

12. Ramdzan AR, Ismail A, Mohd Zanib ZS. Prevalence of malaria and its risk factors in Sabah, Malaysia. *Int J Infect Dis.* (2020) 91:68–72. doi: 10.1016/j.ijid.2019.11.026

13. Choy SH, Al-Mekhlafi HM, Mahdy MAK, Nasr NN, Sulaiman M, Lim YAL, et al. Prevalence and associated risk factors of giardia infection among indigenous communities in rural Malaysia. *Sci Rep*. (2014) 4:6909. doi: 10.1038/srep06909

14. Al-Mekhlafi HM, Al-Maktari MT, Jani R, Ahmed A, Anuar TS, Moktar N, et al. Burden of Giardia duodenalis infection and its adverse effects on growth of schoolchildren in rural Malaysia. *PLoS Negl Trop Dis.* (2013) 7:e2516. doi: 10.1371/journal.pntd.0002516

15. Sahimin N, Lim YAL, Ariffin F, Behnke JM, Basanez MG, Walker M, et al. Socio-demographic determinants of Toxoplasma gondii seroprevalence in migrant workers of Peninsular Malaysia. *Parasit Vect*. (2017) 10:238. doi: 10.1186/s13071-017-2167-8

16. Thayaparan S, Robertson ID, Fairuz A, Suut L, Abdullah MT. Leptospirosis, an emerging zoonotic disease in Malaysia. *Malays J Pathol*. (2013) 35:123–32.

17. Benacer D, Thong KL, Min NC, Bin Verasahib K, Galloway RL, Hartskeerl RA, et al. Epidemiology of human leptospirosis in Malaysia, 2004-2012. *Acta Trop*. (2016) 157:162–8. doi: 10.1016/j.actatropica.2016.01.031

18. Hotez PJ, Bottazzi ME, Strych U, Chang LY, Lim YAL, Goodenow MM, et al. Neglected tropical diseases among the Association of Southeast Asian Nations (ASEAN): overview and update. *PLoS Negl Trop Dis*. (2015) 9:e0003575. doi: 10.1371/journal.pntd.0003575

19. Lo EKC. Yaws in Malaysia. *Rev Infect Dis.* (1985) 7:S251–S3. doi: 10.1093/clinids/7-Supplement_2.S251

20. Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, et al. Control of neglected tropical diseases. *N Engl J Med.* (2007) 357:1018–27. doi: 10.1056/NEJMra064142

21. Lammie PJ, Moss DM, Brook Goodhew E, Hamlin K, Krolewiecki A, West SK, et al. Development of a new platform for neglected tropical disease surveillance. *Int J Parasitol.* (2012) 42:797–800. doi: 10.1016/j.ijpara.2012.07.002

22. Arnold BF, van der Laan MJ, Hubbard AE, Steel C, Kubofcik J, Hamlin KL, et al. Measuring changes in transmission of neglected tropical diseases, malaria, and enteric pathogens from quantitative antibody levels. *PLoS Negl Trop Dis.* (2017) 11:e0005616. doi: 10.1371/journal.pntd.0005616

23. Njenga SM, Kanyi HM, Arnold BF, Matendechero SH, Onsongo JK, Won KY, et al. Integrated cross-sectional multiplex serosurveillance of IgG antibody responses to parasitic diseases and vaccines in coastal Kenya. *Am J Trop Med Hyg.* (2020) 102:164–76. doi: 10.4269/ajtmh.19-0365

24. Ondigo BN, Muok EMO, Oguso JK, Njenga SM, Kanyi HM, Ndombi EM, et al. Impact of mothers' schistosomiasis status during gestation on children's IgG antibody responses to routine vaccines 2 years later and anti-schistosome and anti-malarial responses by neonates in Western Kenya. *Front Immunol.* (2018) 9:1402. doi: 10.3389/fimmu.2018.01402

25. Priest JW, Jenks MH, Moss DM, Mao B, Buth S, Wannemuehler K, et al. Integration of multiplex bead assays for parasitic diseases into a national, population-based serosurvey of women 15-39 years of age in Cambodia. *PLoS Negl Trop Dis.* (2016) 10:e0004699. doi: 10.1371/journal.pntd.0004699

26. Nissapatorn V, Suwanrath C, Sawangjaroen N, Ling LY, Chandeying V. Toxoplasmosis-serological evidence and associated risk factors among pregnant women in southern Thailand. *Am J Trop Med Hyg.* (2011) 85:243–7. doi: 10.4269/ajtmh.2011.10-0633

27. Ngui R, Halim NA, Rajoo Y, Lim YA, Ambu S, Rajoo K, et al. Epidemiological characteristics of strongyloidiasis in inhabitants of indigenous communities in Borneo Island, Malaysia. *Korean J Parasitol.* (2016) 54:673–8. doi: 10.3347/kjp.2016.54.5.673

28. Fornace KM, Brock PM, Abidin TR, Grignard L, Herman LS, Chua TH, et al. Environmental risk factors and exposure to the zoonotic malaria parasite Plasmodium knowlesi across northern Sabah, Malaysia: a population-based cross-sectional survey. *Lancet Planet Health.* (2019) 3:e179–86. doi: 10.1016/S2542-5196(19)30045-2

29. Priest JW, Moss DM. Measuring cryptosporidium serologic responses by multiplex bead assay. *Methods Mol Biol.* (2020) 2052:61–85. doi: 10.1007/978-1-4939-9748-0_5

30. Wu L, Hall T, Ssewanyana I, Oulton T, Patterson C, Vasileva H, et al. Optimisation and standardisation of a multiplex immunoassay of diverse *Plasmodium falciparum* antigens to assess changes in malaria transmission using sero-epidemiology. *Wellcome Open Res.* (2019) 4:26. doi: 10.12688/wellcomeopenres.14950.1

31. Benaglia T CD, Hunter DR, Young D. Mixtools: an R package for analyzing finite mixture models. *J Stat Softw.* (2009) 32:1–29. doi: 10.18637/jss.v032.i06

32. Arnold BF, Martin DL, Juma J, Mkocha H, Ochieng JB, Cooley GM, et al. Enteropathogen antibody dynamics and force of infection among children in low-resource settings. *Elife*. (2019) 8:e45594. doi: 10.7554/eLife.45594.034

33. Lindgren F, Rue H. Bayesian spatial modelling with R-INLA. *J Stat Softw.* (2015) 63:10–13. doi: 10.18637/jss.v063.i19

34. Simpson DP, Illian JB, Lindren F, Sorbye SH, Rue H. Penalising model component complexity: a principled, practical approach to constructing priors. *Stat Sci.* (2017) 32:16–20. doi: 10.1214/16-STS576

35. Giorgi E, Diggle PJ, Snow RW, Noor AM. Geostatistical methods for disease mapping and visualisation using data from spatio-temporally referenced prevalence surveys. *Int Stat Rev.* (2018) 86:571–97. doi: 10.1111/insr.12268

36. R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing (2017).

37. Yahaya N. Review of toxoplasmosis in Malaysia. *Southeast Asian J Trop Med Public Health.* (1991) 22(Suppl.):102–6.

38. Anuar TS, Azreen SN, Salleh FM, Moktar N. Molecular epidemiology of giardiasis among Orang Asli in Malaysia: application of the triosephosphate isomerase gene. *BMC Infect Dis*. (2014) 14:78. doi: 10.1186/1471-2334-14-78

39. Norhayati M, Penggabean M, Oothuman P, Fatmah MS. Prevalence and some risk factors of Giardia duodenalis infection in a rural community in Malaysia. *Southeast Asian J Trop Med Public Health.* (1998) 29:735–8.

40. Butcher R, Sokana O, Jack K, Sui L, Russell C, Last A, et al. Clinical signs of trachoma are prevalent among Solomon Islanders who have no persistent markers of prior infection with Chlamydia trachomatis. *Wellcome Open Res.* (2018) 3:14. doi: 10.12688/wellcomeopenres.13423.2

41. Cocks N, Rainima-Qaniuci M, Yalen C, Macleod C, Nakolinivalu A, Migchelsen S, et al. Community seroprevalence survey for yaws and trachoma

in the Western Division of Fiji. *Trans R Soc Trop Med Hyg.* (2016) 110:582–7. doi: 10.1093/trstmh/trw069

42. Butcher R, Handley B, Garae M, Taoaba R, Pickering H, Bong A, et al. Ocular Chlamydia trachomatis infection, anti-Pgp3 antibodies and conjunctival scarring in Vanuatu and Tarawa, Kiribati before antibiotic treatment for trachoma. *J Infect.* (2020) 80:454–61. doi: 10.1016/j.jinf.2020.01.015

43. Kubofcik J, Fink DL, Nutman TB. Identification of Wb123 as an early and specific marker of Wuchereria bancrofti infection. *PLoS Negl Trop Dis.* (2012) 6:e1930. doi: 10.1371/journal.pntd.0001930

44. Hamlin KL, Moss DM, Priest JW, Roberts J, Kubofcik J, Gass K, et al. Longitudinal monitoring of the development of antifilarial antibodies and acquisition of Wuchereria bancrofti in a highly endemic area of Haiti. *PLoS Negl Trop Dis.* (2012) 6:e1941. doi: 10.1371/journal.pntd.0001941

45. Cooley GM, Mitja O, Goodhew B, Pillay A, Lammie PJ, Castro A, et al. Evaluation of multiplex-based antibody testing for use in large-scale surveillance for yaws: a comparative study. *J Clin Microbiol.* (2016) 54:1321–5. doi: 10.1128/JCM.02572-15

46. Houweling TA, Karim-Kos HE, Kulik MC, Stolk WA, Haagsma JA, Lenk EJ, et al. Socioeconomic inequalities in neglected tropical diseases: a systematic review. *PLoS Negl Trop Dis.* (2016) 10:e0004546. doi: 10.1371/journal.pntd.0004546

47. Ngui R, Lim YA, Chong Kin L, Sek Chuen C, Jaffar S. Association between anaemia, iron deficiency anaemia, neglected parasitic infections and socioeconomic factors in rural children of West Malaysia. *PLoS Negl Trop Dis.* (2012) 6:e1550. doi: 10.1371/journal.pntd.0001550

48. Bangert M, Molyneux DH, Lindsay SW, Fitzpatrick C, Engels D. The cross-cutting contribution of the end of neglected tropical diseases to the sustainable development goals. *Infect Dis Poverty.* (2017) 6:73. doi: 10.1186/s40249-017-0288-0

49. Addiss DG. Soil-transmitted helminthiasis: back to the original point. *Lancet Infect Dis.* (2015) 15:871–2. doi: 10.1016/S1473-3099(15)70095-2

50. Lim KC, Pillai R, Singh M. A study on the prevalence of antibodies to Toxoplasma gondii in Singapore. *Southeast Asian J Trop Med Public Health.* (1982) 13:547–50.

51. Brandon-Mong GJ, Che Mat Seri NA, Sharma RS, Andiappan H, Tan TC, Lim YA, et al. Seroepidemiology of toxoplasmosis among people having close contact with animals. *Front Immunol.* (2015) 6:143. doi: 10.3389/fimmu.2015.00143

52. Nasiru Wana M, Mohd Moklas MA, Watanabe M, Nordin N, Zasmy Unyah N, Alhassan Abdullahi S, et al. A review on the prevalence of toxoplasma gondii in humans and animals reported in Malaysia from 2008-2018. *Int J Environ Res Public Health.* (2020) 17:4809. doi: 10.3390/ijerph17134809

53. World Health Organization. *Ending the Neglect to Attain the Sustainable Development Goals: A Framework for Monitoring and Evaluating Progress of the Road Map for Neglected Tropical Diseases 2021–2030.* Geneva (2021).

frontiers | Frontiers in Public Health

TYPE Brief Research Report
PUBLISHED 18 November 2022
DOI 10.3389/fpubh.2022.990218

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Infectious Diseases–Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

RECEIVED 09 July 2022
ACCEPTED 01 November 2022
PUBLISHED 18 November 2022

CITATION
Qin S, Liang J, Tang D, Chen Y, Duan R, Lu X, Bukai A, Zheng X, Lv D, He Z, Wu W, Han H, Jing H and Wang X (2022) Serological investigation of plague and brucellosis infection in *Marmota himalayana* plague foci in the Altun Mountains on the Qinghai-Tibet Plateau.
Front. Public Health 10:990218.
doi: 10.3389/fpubh.2022.990218

Serological investigation of plague and brucellosis infection in *Marmota himalayana* plague foci in the Altun Mountains on the Qinghai-Tibet Plateau

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The Altun Mountains are among the most active regions of *Marmota himalayana* plague foci of the Qinghai-Tibet Plateau where animal plague is prevalent, whereas only three human cases have been found since 1960. Animal husbandry is the main income for the local economy; brucellosis appears sometimes in animals and less often in humans. In this study, a retrospective investigation of plague and brucellosis seroprevalence among humans and animals was conducted to improve prevention and control measures for the two diseases. Animal and human sera were collected for routine surveillance from 2018 to 2021 and screened for plague and brucellosis. *Yersinia pestis* F1 antibody was preliminarily screened by the colloidal gold method at the monitoring site to identify previous infections with positive serology. Previous plague infection was found in 3.2% (14/432) of the studied human population having close contact with livestock, which indicates evidence of exposure to the *Yersinia* antigen (dead or live pathogenic materials) in the Altun Mountains. Seroprevalence of brucellosis was higher in camels (6.2%) and sheepdogs (1.8%) than in other livestock such as cattle and sheep, suggesting a possible transmission route from secondary host animals to humans.

KEYWORDS

plague, brucellosis, seroprevalence, *Marmota himalayana* plague foci, previous infection

Introduction

Brucellosis and plague are both natural focus diseases that are separately recognized as neglected diseases and re-emerging diseases by the World Health Organization (1–3). With the economic globalization and rapid development of the transportation industry, the possibility of occurrence of imported cases in non-endemic foci is increasing (4–8). *Marmota himalayana* plague foci of the Qinghai-Tibet Plateau are the most active foci in China, whereas the Alutun Mountains are the most active region (9, 10). In the *M. himalayana* plague focus in China before the 1990s, most human cases occurred here. Since the 1990s, rat-associated plague epidemics have erupted in southern China, but beginning in 2004, the *M. himalayana* plague focus re-emerged as the main source of human cases. Outbreaks have occurred here every few years (11). Each year, *Y. pestis* is isolated in a number of marmots found dead in the environment (12). However, only three human cases have been found since 1960 (13). The reason for this paradox is not known. Brucellosis is also an important zoonosis in the Altun Mountains where animal husbandry is practiced (14). In 2020, a brucellosis outbreak occurred in camel herd. The local transmission of brucellosis was of concern. In this study, the findings of previous plague infection in humans and transmission of brucellosis from a secondary host can help improve the prevention and control of these two significant zoonoses (15).

Materials and methods

The Altun Mountains located on the north of the *M. himalayana* plague foci of the Qinghai-Tibet Plateau (Figure 1A) are mainly desert and semi-desert grasslands (Figure 1B). The area where the residents live is vast and sparsely populated (10,000 people in 31,000 km^2). Among the livestock raised, the number of sheep is the largest, which is about 120,000 per year. That of cattle, horses, camels, and other large livestock is about 6,000 per year. Free-ranging assisted by sheepdogs is the main husbandry pattern. *M. himalayana* are plentiful in number, widely distributed, have a high natural carrier rate of *Y. pestis,* and are the foci's main reservoir of *Y. pestis* (16).

This retrospective study was conducted by the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. To analyze the average seroprevalence levels of plague and brucellosis, at least two years of human and animal samples collected as part of routine surveillance were included in this study. Based on the screening test adopted by the laboratory at the monitoring site, the positive samples were further confirmed by the superior laboratory.

Blood samples were collected and sera were separated by centrifugation and frozen at −80°C. Serological monitoring of brucellosis in livestock (camel, cattle, sheep, and sheepdogs) and persons whose occupations were breeder, herder, veterinarian, and other occupations that were in close contact with livestock was carried out. The serum samples were collected during routine surveillance of plague and brucellosis from 2018 to 2021. The gender, age group (17), and occupation information was also collected. Human sera collected for brucellosis surveillance was also tested for the plague. The sera of marmots collected for plague surveillance was both tested for plague and brucellosis.

The colloidal gold method was used for screening for *Y. pestis* F1 antibodies (Beijing Jianaixi Biotechnology Co., Ltd., Beijing, China) and the indirect hemagglutination assay was performed for verification (Qinghai Province Endemic Disease Prevention and Control Institute, Xining, Qinghai Province, China). F1 antigen inhibition controls, negative controls, and positive controls were established. An antibody titer ≥ 1:16 was identified as positive.

The rose bengal plate test was used for screening for brucella antibodies (Idexx Laboratories, Westbrook, Maine, United States; Lanzhou Institute of Biological Products Co., Ltd., Lanzhou, Gansu Province, China). A total of 30 μL antigen and 30 μL serum samples were mixed on a flat plate. Results were read immediately after 4 min. Positive samples were further tested by Wright's serum agglutination tests (Idexx Laboratories, Westbrook, Maine, United States). Sera were diluted by 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160, and then equal-volume brucellosis antigen was added to each tube. Tubes were thoroughly mixed and incubated for 18–20 h at 37 ± 3°C. Turbidimetric tubes were used to compare the serum agglutination degree of samples. Samples ≥30 IU/ml were identified as positive. The antigen used in the study can detect *B. suis*, *B. melitensis,* and *B. abortus*.

Statistical analysis was conducted to compare seroprevalence among different groups (SPSS Version 26.0). According to specific theoretical frequency, Pearson's chi-square test ($T \geq 35$), Yates's continuity correction ($1 \leq T < 5$), or Fisher's exact test ($T < 1$) was applied to assess associations between variables of concern and the seroprevalence of brucellosis or plague.

Results

A total of 432 individuals between ages of 7 and 70 with certain occupations that were in close contact with livestock from January 2020 to July 2021 were tested for brucella and *Y. pestis*. The sample population was engaged in animal husbandry, including breeders (168), herders (167) veterinarians (59), and other occupations, including 38 individuals who purchase, process, or sale livestock products, such as fur, milk, meat, etc. A total of 5,799 livestock serum samples were tested for brucella. Samples included sera from cattle (987), camels (3,820), and sheep (882) collected from 2019 to 2020, and sheepdogs (110)

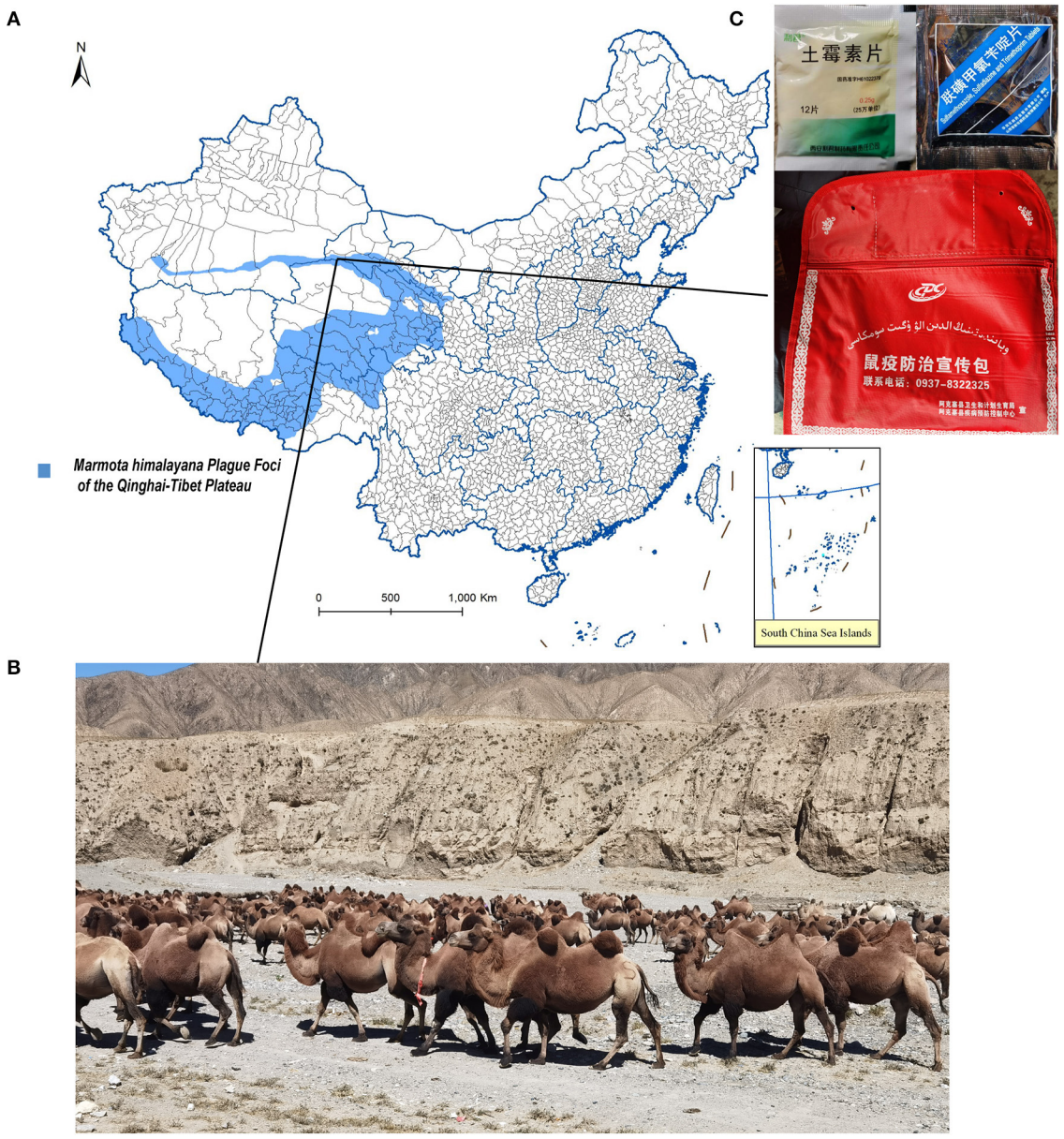

FIGURE 1
Ecology of the studied region and plague first-aid kit given to herdsmen. **(A)** Geographic region of this study. **(B)** Landscape of camel's living habitat. **(C) (Left)** Tablet oxytetracycline contained in the Plague first-aid kit. (Right) Sulfamethoxazole, sulfadiazine, and trimethoprim contained in the Plague first-aid kit. **(Bottom)** Plague first-aid kit for herdsmen.

collected from January 2020 to July 2021. To analyze the average seroprevalence level of brucellosis, human and dog samples collected in 2020 and 2021 were included because samples collected in 2019 were not available. No positive marmots were detected in the same period, so the range of detection years was expanded. A total of 360 marmot sera samples collected from January 2018 to July 2021 were tested for brucella and *Y. pestis* antibodies (because of insufficient sample volume, 73 marmot samples collected from 2018 to 2019 were tested only for brucella, while 287 marmot samples collected from January 2020 to July 2021 were tested for brucella and *Y. pestis*).

The seroprevalence for plague in marmots was 25.1% (72/287). Among 72 positive samples, the titers accounting for the top two largest proportions were 1:128 (25.0%, 18/72) and 1:2,048 (18.1%, 13/72), the highest titer was 1:16,384 (1.4%, 1/72), and the lowest titer was 1:16 (4.2%, 3/72) (Figure 2). The seroprevalence for human plague infection was 3.2% (14/432) (Table 1). All of the 14 seropositive individuals were identified

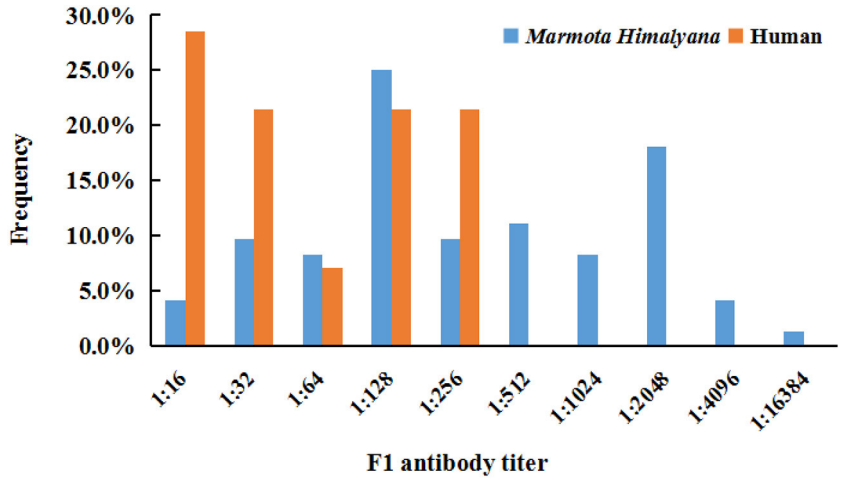

FIGURE 2
Frequency of *Yersinia pestis* F1 antibody titer in seropositive humans and *Marmota himalayana*.

TABLE 1 Seroprevalence of brucellosis and plague in studied human populations.

	Brucellosis, Seroprevalence, 95% CI (Positive /Total)	*P*-value	Plague, Seroprevalence, 95% CI (Positive /Total)	*P*-value
Gender		0.999		0.397
Male	1.1%, 0.2–3.1% (3/275)		2.6%, 0.7–4.3% (7/275)	
Female	1.3%, 0.1–4.5% (2/157)		4.5%, 1.2–7.4% (7/157)	
Age group		0.820		0.525
7∼19	0.0%, 0.0–33.6% (0/9)		0.0%, 0.0–3.6% (0/9)	
20∼44	1.7%, 0.3–4.7% (3/181)		2.8%, 0.4–5.0% (5/181)	
45∼59	1.0%, 0.1–3.5% (2/202)		4.5%, 1.5–7.0% (9/202)	
60∼70	0.0%, 0.0–8.8% (0/40)		0.0%,0.0–8.8% (0/40)	
Occupation		0.906		0.259
Breeder	1.8%, 0.4–5.0% (3/168)		1.8%,0.4–5.0% (3/168)	
Herder	1.2%, 0.1–4.2% (2/167)		3.6%,0.7–6.2% (6/167)	
Veterinarian	0.0%, 0.0–6.1% (0/59)		6.8%,1.8–15.5% (4/59)	
Other occupations	0.0%, 0.0–9.3% (0/38)		2.6%,0.1–13.5% (1/38)	
Total	1.2%, 0.2–2.1% (5/432)		3.2%,1.5–4.8% (14/432)	

as previous plague infection cases. The highest titer was 1:256, accounting for 21.4% (3/14), and the lowest titer was 1:16, accounting for 28.6% (4/14) (Figure 2). Occupations with the highest seroprevalence were veterinarians (6.8%, 4/59) and herders (3.6%, 6/167).

The seroprevalence for livestock brucellosis infection was 4.2% (243/5,799). It was higher in camels (6.2%, 236/3,820) and sheepdogs (1.8%, 2/110) than in cattle (0.4%, 4/987) and sheep (0.1%, 1/882); the seroprevalence for marmots was 0 (0%, 0/360).

In humans, the seroprevalence for brucellosis was 1.2% (5/432) (Table 2). The titers were 1:40 for two samples, and 1:20, 1:80, and 1:160 for the other three. Occupations with the highest seroprevalence were breeders (1.8%, 3/168) and herders (1.2%, 2/167). No statistically significant differences were found in seroprevalence between different groups in plague or brucellosis infection.

Discussion

The potential danger of animal plague prevalence should not be underestimated: one-fourth of the marmots were positive for F1-antibody, and seroprevalence for people having contact with livestock animals was 3.2%, which indicates evidence

10.3389/fpubh.2022.990218

TABLE 2 Brucellosis seroprevalence in different hosts.

Host	Collection period	No. specimens	No. positive specimens	Seroprevalence (%)
Cattle	2019–2020	987	4	0.4
Sheep	2019–2020	882	1	0.1
Camel	2019–2020	3,820	236	6.2
Sheepdog	2020–2021	110	2	1.8
Marmot	2018–2021	360	0	0.0
Human	2020–2021	432	5	1.2

of exposure to the *Yersinia* antigen (dead or live pathogenic materials). On the other hand, the findings of F1 antibody-positive unreported cases suggests that these previous plague infection cases had been ignored or misdiagnosed on routine clinical examination. Hence, routine surveillance of plague should be strengthened as some plage infection cases could be missed on routine clinical examination.

Several reasons might explain why previous plague infections have been missed and why severe plague cases are rare in the most active regions of the *M. himalayana* plague foci of the Qinghai-Tibet Plateau, Altun Mountains. First, the risk of human transmission is low because humans live in vast, sparsely populated areas. Second, the local Centers for Disease Control and Prevention distributed plague first-aid kits (Figure 1C) for herdsmen and breeders containing tablets of oxytetracycline, sulfamethoxazole, sulfadiazine, and trimethoprim, with a reminder to take the medicine and seek prompt medical advice if fever and other typical plague symptoms develop after contacting rodents such as marmots and hares. The majority of studied people were breeders and herders, living on vast land away from hospitals (Table 1). It is not known how many of them took the medicine, but the F1 antibody-positive cases had high chances. They may have taken medicine from a first-aid kit and recovered from the plague. Third, because of propaganda and customs, most local people will not eat dead animals that are found, reducing the risk of contracting pneumonic plague. The bubonic plague caused by fleabites was likely the plague type in plague cases, which has a long incubation period, no human-to-human transmission, and low mortality (11). Drugs in the incubation period can control infection progression in the early stages, avoid deterioration in the condition, and greatly reduce the case fatality rate (18). This indicates the importance of early prophylactic medication.

Brucellosis outbreaks occurred among camels in the region where brucella seroprevalence in camels was 6.2% but that of the studied human population was only 1.2% (19, 20). The rate was lower than that in Shanxi (2.91%) and Xinjiang (1.68%), which are also areas with high brucellosis incidence (21). The relatively low rate might be due to humans having less chance of contact with livestock in pastoral areas than that in captive breeding. In addition, most of the people living in the area are Kazak ethnicity, and they reduce risk of brucella infection by practicing good hygiene, including not eating raw meat and or found dead animals, washing their hands under running water before meals, and not optional touching food when visiting as a guest.

The way camels got infected is of concern, as they are usually raised separately with other livestock in the pastoral areas. Wild marmots have chances of contact with camels, but their negative results for brucellosis suggest that this is infrequent. Therefore, marmots and other livestock were unlikely the sources of infection. Sheepdogs had the second highest brucellosis seroprevalence after that of camels. Dogs are usually affected by *Brucella canis* but can also be infected with *Brucella melitensis* which camels are highly susceptible to. Camels can have close contact with sheepdogs when grazing. It is likely that diseased camels infected the dogs and the infection is circulating in the population. There is another possibility that the sheepdogs may be the source of infection in camels. Infected camels are difficult to detect because they are nearly asymptomatic (22), which increases the possibility of mutual infection among camels as the cause of outbreaks. Brucellosis in humans and animals caused by dogs has also been reported previously (23). Dog–camel transmission as a possible cause of brucellosis outbreaks indicates that host animals with low infection rates may also become secondary or transient sources of infection. Therefore, secondary hosts also need to be considered in zoonoses prevention and control, especially in natural foci associated with developed animal husbandry.

This serological investigation confirmed the existence of missed previous infection with plague in these foci and indicated the risk of the secondary host animal in the transmission of brucellosis infection. In the investigation of the transmission chain of brucellosis infection in camels, the serological investigation has shown that sheepdogs have a higher risk of transmission than other animals. However, because of the lack of a questionnaire survey on possible risk factors, the source of camel infection can only be speculated. More detailed epidemiological exposure history and etiological analysis will be helpful to determine the risk factors and infectious chains

of these two zoonotic diseases and should be confirmed in further studies.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Laboratory Animal Welfare & Ethics Committee of the National Institute for Communicable Disease Control and Prevention of the Chinese Center for Disease Control and Prevention. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by the Laboratory Animal Welfare & Ethics Committee of the National Institute for Communicable Disease Control and Prevention of the Chinese Center for Disease Control and Prevention. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

XW contributed to the conception, design of the work, and supervised the work. SQ, JL, DT, YC, DL, ZH, WW, and HH performed the experiments. SQ, DT, and RD performed the analysis and interpretation of the data. SQ, JL, YC, and HJ drafted the manuscript. RD, XL, AB, and XZ conceived the work and critically review the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Science and Technology Major Project (2018ZX10713-003-002 and 2018ZX10713-001-002).

Acknowledgments

We thank Charlesworth Author Services (paper no. 108748) for their critical editing and helpful comments regarding our manuscript.

Conflict of interest

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

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References

1. Ghanbari MK, Gorji HA, Behzadifar M, Sanee N, Mehedi N, Bragazzi NL. One health approach to tackle brucellosis: a systematic review. *Trop Med Health*. (2020) 48:86. doi: 10.1186/s41182-020-00272-1

2. *Operational Guidelines on Plague Surveillance, Diagnosis, Prevention and Control*. New Delhi: WHO Regional Office for South-East Asia (2009). Available online at: https://apps.who.int/iris/handle/10665/205593 (accessed October 31, 2019).

3. Johansen MV, Welburn SC, Dorny P, Brattig NW, Johansen MV, Welburn SC, et al. Control of neglected zoonotic diseases. *Acta Trop*. (2017) 165:1–2. doi: 10.1016/j.actatropica.2016.11.036

4. Peric L, Sabadi D, Rubil I, Bogdan M, Guzvinec M, Dakovic Rode O, et al. Imported brucellosis and Q-fever coinfection in Croatia: a case report. *J Infect Dev Countr*. (2018) 12:499–503. doi: 10.3855/jidc.10151

5. Norman FF, Monge-Maillo B, Chamorro-Tojeiro S, Pérez-Molina J, López-Vélez R. Imported brucellosis: a case series and literature review. *Travel Med Infect Di*. (2016) 14:182–99. doi: 10.1016/j.tmaid.2016.05.005

6. Zhou H, Guo S. Two cases of imported pneumonic plague in Beijing, China. *Medicine*. (2020) 99:e22932. doi: 10.1097/MD.0000000000022932

7. Melman SD, Ettestad PE, VinHatton ES, Ragsdale JM, Takacs N, Onischuk LM, et al. Human case of bubonic plague resulting from the bite of a wild Gunnison's prairie dog during translocation from a plague-endemic area. *Zoonoses Public Health*. (2018) 65:e254–8. doi: 10.1111/zph.12419

8. Danforth M, Novak M, Petersen J, Mead P, Kingry L, Weinburke M, et al. Investigation of and response to 2 plague cases, Yosemite National Park, California, USA, 2015. *Emerg Infect Dis*. (2016) 22:2045–53. doi: 10.3201/eid2212.160560

9. Wang X, Wei X, Song Z, Wang M, Xi J, Liang J, et al. Mechanism study on a plague outbreak driven by the construction of a large reservoir in southwest China (surveillance from 2000–2015). *PLoS Neglect Trop Dis*. (2017) 11:e5425. doi: 10.1371/journal.pntd.0005425

10. Qian Q, Zhao J, Fang L, Zhou H, Zhang W, Wei L, et al. Mapping risk of plague in Qinghai-Tibetan Plateau, China. *BMC Infect Dis*. (2014) 14:382. doi: 10.1186/1471-2334-14-382

11. He Z, Wei B, Zhang Y, Liu J, Xi J, Ciren D, et al. Distribution and characteristics of human plague cases and Yersinia pestis isolates from 4 marmota plague foci, China, 1950–2019. *Emerg Infect Dis*. (2021) 27:2544–53. doi: 10.3201/eid2710.202239

12. Xu D, Xi J, Wang D, Wang P, Wang S, Miao K, et al. Analysis of the plague epidemic characteristics in the natural foci of the Qilian Mountains-A -erh-chin Mountains Himalayan marmot plague in Gansu Province from 2011 to 2018. *Chin J Epidemiol*. (2021) 40:137–41. doi: 10.3760/cma.j.cn231583-20200116-00016

13. Ge P, Xi J, Ding J, Jin F, Zhang H, Guo L, et al. Primary case of human pneumonic plague occurring in a Himalayan marmot natural focus area Gansu Province, China. *Int J Infect Dis*. (2015) 33:67–70. doi: 10.1016/j.ijid.2014.12.044

14. Wei K, Zhang H, He J, Yu D, Yang X, Jiang Z, et al. Epidemiological and spatial-temporal distribution of several focus disease in Gansu Province, 2014–2018. *Chin J Epidemiol*. (2019) 40:947–52. doi: 10.3760/cma.j.issn.0254-6450.2019.08.014

15. Feldmann H, Czub M, Jones S, Dick D, Garbutt M, Grolla A, et al. Emerging and re-emerging infectious diseases. *Med Microbiol Immun*. (2002) 191:63–74. doi: 10.1007/s00430-002-0122-5

16. Yu D. *Plague Epizootiology*. (2009). Beijing: Science Press.

17. Zhao Q, Wang J, Nicholas S, Maitland E, Sun J, Jiao C, et al. Health-related quality of life and health service use among multimorbid middle-aged and older-aged adults in China: a cross-sectional study in Shandong Province. *Int J Env Res Pub Health*. (2020) 17:9261. doi: 10.3390/ijerph17249261

18. Yang R. Plague: recognition, treatment, and prevention. *J Clin Microbiol*. (2018) 56:e1517–9. doi: 10.1128/JCM.01519-17

19. Deqiu S, Donglou X, Jiming Y. Epidemiology and control of brucellosis in China. *Vet Microbiol*. (2002) 90: 65–82. doi: 10.1016/S0378-1135(02)00252-3

20. Wei K, Zhang H, He J, Gou F, Cheng Y, Liu X. Spatial distribution of Brucellosis in Gansu Province, 2013–2018. *Chin J Epidemiol*. (2019) 40:1099–105. doi: 10.3760/cma.j.issn.0254-6450.2019.09.015

21. Lin S, Wang Z, Liu X, Yu A, Muhtar H, Jiensi B, et al. Serological prevalence survey among the high-risk populations of brucellosis-endemic areas - China, 2019–2020. *CCDC Weekly*. (2021) 3:101–5. doi: 10.46234/ccdcw2021.027

22. Gwida M, El-Gohary A, Melzer F, Khan I, Rösler U, Neubauer H. Brucellosis in camels. *Res Vet Sci*. (2012) 92:351–5. doi: 10.1016/j.rvsc.2011.05.002

23. Hubbard K, Wang M, Smith DR. Seroprevalence of brucellosis in Mississippi shelter dogs. *Prev Vet Med*. (2018) 159:82–6. doi: 10.1016/j.prevetmed.2018.09.002

frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 07 February 2023
DOI 10.3389/fpubh.2023.981172

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Infectious Diseases–Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

RECEIVED 29 June 2022
ACCEPTED 06 January 2023
PUBLISHED 07 February 2023

CITATION
Fernández Villalobos NV, Kessel B, Torres Páez JC, Strömpl J, Kerrinnes T, de la Hoz Restrepo FP, Strengert M and Krause G (2023) Seroprevalence of Hepatitis E virus in children and adolescents living in urban Bogotá: An explorative cross-sectional study. *Front. Public Health* 11:981172. doi: 10.3389/fpubh.2023.981172

Seroprevalence of Hepatitis E virus in children and adolescents living in urban Bogotá: An explorative cross-sectional study

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The majority of Hepatitis E Virus (HEV)-related studies are carried out in adults whereas information about HEV seroprevalence, clinical disease manifestation, molecular epidemiology, and transmission patterns in children is limited. To estimate HEV seroprevalence among scholar children living in an urban setting and to analyze risk factors for an infection, we invited children aged 5–18 years from Bogotá (Colombia) for a cross-sectional survey. We collected self-reported data on demographics, social, clinical, and exposure variables in a structured interview. Venous blood samples were analyzed with two commercially available ELISAs for HEV-specific IgG antibodies. Among the 263 participants, we found three HEV IgG-reactive samples (1.1%) using both assays. We additionally characterized the samples for HEV IgM using a commercially available IgM ELISA and for HEV RNA. Here, we found one IgM-reactive sample, which was also reactive for IgG. In contrast, none of the IgM- and IgG-reactive sera samples showed detectable RNA levels indicating HEV exposure had not been recently. All participants reported access to drinking water and sanitary systems in their households and frequent hand washing routines (76–88%). Eighty percent of children reported no direct contact with pigs, but occasional pork consumption was common (90%). In contrast to the majority of studies performed in Colombian adults, we found a low unadjusted HEV seroprevalence of 1.1% (95% CI: 0.3–3.6%) for both HEV IgG ELISAs in our study population. While the majority of participants reported pork consumption, we speculate in the absence of viral RNA for genotyping in the affected individuals, that existing access to drinking water and sanitary systems within our study group contribute to the low HEV seroprevalence.

KEYWORDS

adolescents, children, Colombia, *Paslahepevirus balayani* (previously Hepatitis E virus), seroprevalence, risk factors, surveillance, transmission

1. Introduction

Paslahepevirus balayani (HEV), previously known as Hepatitis E virus, is responsible for a liver disease that affects ~ 20 million people worldwide, especially in low- and middle-income countries with poor socioeconomic conditions such as lack of sanitation, low-quality drinking water, or food supply (1, 2). While HEV liver disease is usually self-limiting with mild

symptoms, it can result in severe acute hepatitis, extrahepatic disorders, chronic hepatitis leading to fibrosis/cirrhosis, and fulminant hepatitis in some individuals (3). In particular, pregnant women face an increased risk of fulminant hepatitis with a mortality rate of 26.9% (4), whereas 66% of immunocompromised solid-organ transplant recipients develop a chronic course of infection (5).

HEV as a single-stranded RNA virus can be grouped in 8 genotypes (HEV-1-HEV-8) within the *Hepeviridae* family, *Orthohepevirinae* subfamily, *Paslahepevirus* genus, and *balayani* specie (6), but only genotype 1–4 and 7 infect humans (2, 7). HEV-1 and HEV-2 are dominant in low-middle-income countries within Africa and Asia where they cause both sporadic cases and larger outbreaks (8). While the fecal-oral HEV-1 and HEV-2 transmission route by contaminated water has been ascertained, HEV-3 and HEV-4 infection appear to be primarily associated with the consumption of contaminated or undercooked meat, or direct contact with an infected animal such as pigs, deer, or wild boar (8). Additionally, HEV-3 transmission after blood transfusions has also been documented (8–10). HEV-7 infection has been predominantly reported in camels (11), but one study has also found a patient to be infected by HEV-7 after consuming camel meat and milk (7). Other studies have also reported serological and molecular evidence of human infections with members of the *Rocahepevirus ratti* species, especially with the genotype C1 (12–17).

Global HEV seroprevalence estimates strongly vary and range from 0.25% to 74.76% (8). Those discrepancies are not only attributable to differences in hygienic standards, access to sanitation, or in zoonotic exposures, but also dependent on which serological assay is used to determine a previous HEV exposure (8, 18, 19). Although infections are thought to occur mainly in late childhood or young adulthood (20, 21), there are few studies that actually investigate HEV disease burden in children (22), and information about HEV seroprevalence, clinical manifestations, molecular epidemiology and transmission patterns in this population are equally less well examined (22, 23).

To address this gap, we performed a HEV seroprevalence study in children from Bogotá, Colombia. Colombia is an upper-middle-income country with clear social determinants of health inequalities especially between the rural and urban regions (24, 25). Bogotá, the country's capital has the lowest Unsatisfied Basic Needs indicator and the highest percentage coverage of public services in Colombia (26). To date, a limited number of studies has examined HEV disease burden in Colombian adults. Molecular and/or serological evidence of HEV infection has been detected in human sera or feces samples from acute hepatitis patients from different Colombian cities (27, 28), blood donors from Antioquia (29), swine farm workers from Medellin (30), and in slaughtered pigs or pig feces in Antioquia (31) or Medellin (32), respectively. HEV genome was also detected in both waste and drinking water in Antioquia (33). Concerning genotype distribution in Colombia, HEV-3 was first characterized in a 2008–2009 study (27, 34). HEV seroprevalence estimates were variable, while two studies in viral hepatitis patients showed anti-HEV proportions of 7.5% and 25.3% for IgG and of 1.74% and 5.6% for IgM (27, 33), the reported HEV IgG seroprevalence was 45.2% in blood donors (29).

We provide for the first time data on HEV seroprevalence estimates among scholar children living in the urban setting of Bogotá, and analyzed risk factors for infection by association with demographics, social, clinical, and exposure variables. Data about HEV seroprevalence in children is essential to understand the variable levels of seroprevalence not only observed in Colombian adults, but it also provides information for health authorities on the current extent of HEV infections to support the possible inclusion of HEV in the diagnosis and management plan of viral hepatitis in Colombia and to create control and prevention strategies for fecal-oral and zoonotic transmission.

2. Materials and methods

2.1. Study population

We designed a cross-sectional study, which was carried out in cooperation with the Universidad Nacional de Colombia (UNAL) from February 2020 until March 2021 in Bogotá, Colombia. Based on the division of Bogotá in 20 localities and 6 stratum areas (35, 36), we designed a two-stage cluster random sampling considering localities (37), and schools (38). The software Epidat version 4.2 was used to calculate a sample size of 280 (39) based on an estimate of 1.5 million individuals living in Bogotá aged between 5 and 18 years, an expected HEV seroprevalence of 3%, with a margin error of 2%, and confidence level of 95% (40).

Children and adolescents (further referred to as children) from 5 to 18 years old were invited to participate by advertising the study in different schools through electronic and paper-based documents. Prior to initiating the study, participating parents or legal guardians provided written informed consent. Inclusion criteria were to live in Bogotá, to study at the selected schools, and to have the authorization and company of a parent or legal guardian. We excluded children with any predisposition for bleeding, blood clots, cognitive deficits that prevent giving informed assent or consent, or suffering from primary or secondary immunodeficiency. Based on the socioeconomics characteristics of the respective population, we considered localities in Bogotá that had all strata represented (Suba, Usaquén, and Chapinero), and those with low socioeconomic conditions (San Cristóbal, Ciudad Bolívar, Usme, Bosa, and Santa Fe) for taking part in the study (41). Within the pre-identified localities, we randomly pre-selected two localities Ciudad Bolívar and Usaquén and different schools within those areas. However, due to the COVID-19 pandemic and the ensuing low numbers of participants, we invited other localities and schools to take part in the study (Supplementary Table 1). The COVID-19 pandemic also led to the exclusion of children and companions with risk factors or comorbidities such as diabetes, hypertension and others (Supplementary Table 2), or acute respiratory symptoms.

The study was conducted in line with the Declaration of Helsinki, and followed STROBE guidelines (42). It was approved by the Comité De Ética De Investigacion De La Facultad De Medicina, Universidad Nacional de Colombia, Bogotá, Colombia (N°.009-125-19) and by the Ethics Committee of Hannover Medical School, Hannover, Germany (Nr.9254_BO_K_2020).

2.2. Data collection and management

We collected self-reported data on demographic, social, clinical, and exposure variables through a structured questionnaire; using REDCap 7.3.6 electronic data capture tools hosted at Unidad

10.3389/fpubh.2023.981172

TABLE 1 General characteristics of the participants.

Characteristics of the participants		263 (100%) All participants *n* (%)	260 (98.9%) HEV IgG non-reactive participants *n* (%)	3 (1.1%) HEV IgG reactive participants *n* (%)
Social and demographic characteristics				
Age in years: Median (IQR)		9 (8-11)	9 (8-11)	8 (7-9)
Sex	Male	142 (54.0)	141 (54.2)	1 (33.0)
	Female	121 (46.0)	119 (45.8)	2 (67.0)
School type	Public	234 (89.0)	231 (88.8)	3 (100.0)
	Private	29 (11.0)	29 (11.2)	0 (0.0)
Social security affiliation	Subsidized	38 (14.5)	38 (14.6)	0 (0.0)
	Contributory	217 (82.5)	214 (82.3)	3 (100.0)
	Unaffiliated	8 (3.0)	8 (3.1)	0 (0.0)
Socioeconomical strata[†]	One	4 (1.5)	4 (1.5)	0 (0.0)
	Two	93 (35.4)	91 (35.0)	2 (67.0)
	Three	163 (61.9)	162 (62.3)	1 (33.0)
	Four	2 (0.8)	2 (0.8)	0 (0.0)
	Unknown	1 (0.4)	1 (0.4)	0 (0.0)
Income[‡]	Between one and two minimum wages	174 (66.2)	171 (65.9)	3 (100.0)
	2–6 minimum wages	69 (26.2)	69 (26.5)	0 (0.0)
	More than 6 minimum wages	5 (1.9)	5 (1.9)	0 (0.0)
	Do not want to inform	11 (4.2)	11 (4.2)	0 (0.0)
	Unknown	4 (1.5)	4 (1.5)	0 (0.0)
Country of birth	Colombia	251 (95.4)	248 (95.4)	3 (100.0)
	Venezuela	10 (3.8)	10 (3.8)	0 (0.0)
	Other	2 (0.8)	2 (0.8)	0 (0.0)
Behavioral characteristics				
Mother occupation	Occupation with animal/soil contact	3 (1.1)	3 (1.1)	0 (0.0)
	Other	259 (98.5)	256 (98.5)	3 (100.0)
	Unknown	1 (0.04)	1 (0.4)	0 (0.0)
Father occupation	Occupation with animal/soil contact	2 (0.8)	2 (0.8)	0 (0.0)
	Other	229 (87.1)	226 (86.9)	3 (100.0)
	Unknown	32 (12.1)	32 (12.3)	0 (0.0)
Contact with pigs	Yes	52 (19.8)	52 (20.0)	0 (0.0)
	No	209 (79.4)	206 (79.2)	3 (100.0)
	Unknown	2 (0.8)	2 (0.8)	0 (0.0)
Pork consumption	Never	12 (4.6)	11 (4.2)	1 (33.0)
	Occasionally	236 (89.7)	234 (90.0)	2 (67.0)
	Usually	14 (5.3)	14 (5.4)	0 (0.0)
	Always	1 (0.4)	1 (0.4)	0 (0.0)
Drinkable water	Bottled	42 (16.0)	41 (15.8)	1 (33.3)
	Filtered	30 (11.4)	29 (11.2)	1 (33.3)
	Boiled	71 (27.0)	70 (26.9)	1 (33.3)
	Tap water	120 (45.6)	120 (46.1)	0 (0.0)

(Continued)

TABLE 1 (Continued)

Characteristics of the participants		263 (100%) All participants *n* (%)	260 (98.9%) HEV IgG non-reactive participants *n* (%)	3 (1.1%) HEV IgG reactive participants *n* (%)
Hand washing after the toilet	Occasionally	27 (10.3)	27 (10.4)	0 (0.0)
	Usually	74 (28.1)	72 (27.7)	2 (67.0)
	Always	160 (60.8)	159 (61.1)	1 (33.0)
	Unknown	2 (0.8)	2 (0.8)	0 (0.0)
Hand washing before eating	Never	5 (1.9)	5 (1.9)	0 (0.0)
	Occasionally	55 (20.9)	53 (20.4)	2 (67.0)
	Usually	90 (34.2)	90 (34.6)	0 (0.0)
	Always	112 (42.6)	111 (42.7)	1 (33.0)
	Unknown	1 (0.4)	1 (0.4)	0 (0.0)
Recreational swimming in rivers or streams	Yes	155 (58.9)	154 (59.2)	1 (33.0)
	No	108 (41.1)	106 (40.8)	2 (67.0)
Health-related characteristics				
Blood transfusion	Yes	1 (0.4)	1 (0.4)	0 (0.0)
	No	262 (99.6)	259 (99.6)	3 (100.0)
Jaundice	Yes	2 (0.8)	2 (0.8)	0 (0.0)
	No	261 (99.2)	258 (99.2)	3 (100.0)
Viral hepatitis diagnosis	Yes	2 (0.8)	2 (0.8)	0 (0.0)
	No	261 (99.2)	258 (99.2)	3 (100.0)
Hepatitis symptoms	Yes	16 (6.1)	16 (6.2)	0 (0.0)
	No	247 (93.9)	244 (93.8)	3 (100.0)

†One represents the lowest strata and six the highest.
‡Colombian minimum wage ∼ 280 USD.

de Informática y Comunicaciones - Facultad de Medicina - Universidad Nacional de Colombia (43, 44). Details are listed in Table 1.

In addition, we took ∼ 5 ml of venous blood by venipuncture using an S-Monovette (cat no: 03.1397, Sarstedt, Sarstedt, Germany). Prior to freezing at <-20°C, samples were allowed to clot for 30 min at ambient temperature, and then centrifuged at 2,000 g for 10 min to obtain serum. In the absence of a gold standard for identification of anti-HEV IgG antibodies, two commercially available ELISAs were selected:

1. HEV IgG ELISA [cat no: 88 03 30, Axiom Diagnostic, Bürstadt, Germany; developed by Wantai, Beijing, China (19, 45)]: with a reported sensitivity of 93% (calculated with 90/91 patients with confirmed HEV infection) and a specificity of 99% (calculated with 414/418 blood donor samples) determined by Norder et al. (46).
2. recomWell HEV IgG [cat no: 5004, Mikrogen Diagnostik, Neuried, Germany]: with a reported sensitivity of 98.9% (calculated with 88/89 patients with acute HEV infections) and a specificity of 98.5% (calculated with 132/134 blood donor sera samples) defined by the manufacturer (47).

While the recomWell HEV IgG ELISA uses recombinant peptides of HEV ORF2/ORF3 genotypes 1 and 3 (46, 47) as antigens, the Axiom HEV IgG ELISA limits itself to the carboxy-terminal region of the ORF2 genotype 1 Burmese strain (46). Samples were measured once and classified as reactive for anti-HEV IgG based on an assay-specific signal to cut-off (S/CO) value of above 1.1 or 1.2 for the Axiom and the Mikrogen ELISA, respectively. HEV IgG-reactive samples after the first screening of all samples were re-measured in triplicates to confirm reactivity.

Because of the unavailability of the World Health Organization Reference Reagent for Hepatitis E Virus Antibody (NIBSC code: 95/584), we included two other available NIBSC quality control reagents QCRTHAVQC1 - Total Anti-Hepatitis A Virus Quality Control Reagent Sample 1 (NIBSC code: 17/B725) and QCRHEVQC1 - Anti-Hepatitis E Quality Control (NIBSC code: 17/B723) on every ELISA plate to monitor assay performance (Supplementary Table 3).

Next to an analysis for HEV IgG, all samples were also analyzed for HEV IgM using the recomWell HEV IgM [cat no: 5005, Mikrogen Diagnostik, Neuried, Germany, with a reported sensitivity of 98.9% (calculated with 87/89 patients with acute HEV infection) and a specificity of 98.5% (calculated with 354/359 of patients with a suspected non-HEV infection and blood donors) (47)], as IgM reactivity indicates a more recent infection (48). All IgM-reactive/-borderline samples in the first measurement were re-measured twice for an unequivocal IgM result. All IgG- and IgM-reactive samples were then further analyzed with the IVD-certified RealStar HEV

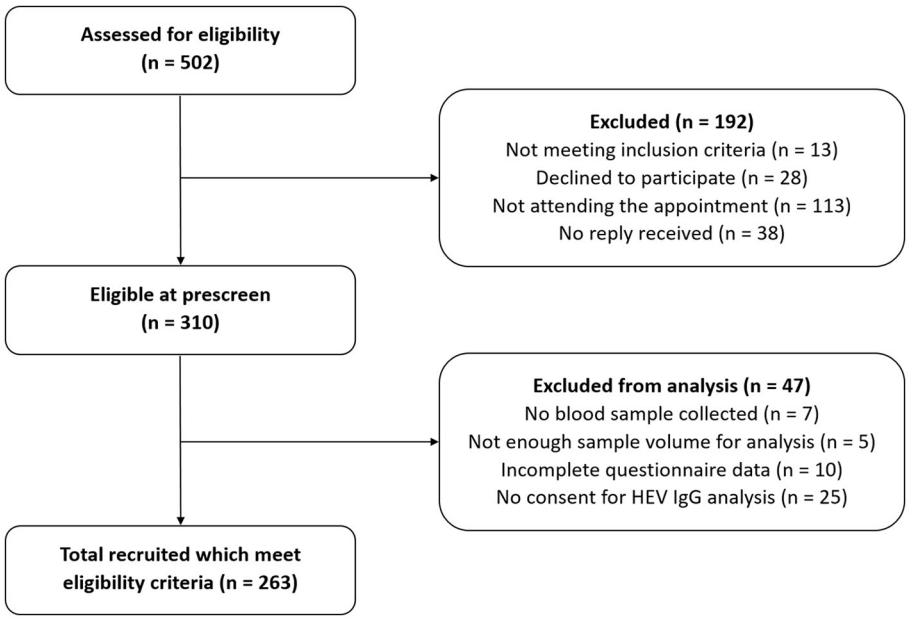

FIGURE 1
Flow chart of participants' selection.

RT-PCR Kit 2.0 (cat no: 272013, Altona Diagnostics, Hamburg, Germany) for the detection and, if applicable, the quantification of HEV-specific RNA. Prior to qRT-PCR analysis, nucleic acid from sera samples was isolated using the QIAamp MinElute Virus Spin Kit (cat no: 57704, Qiagen, Hildesheim, Germany). All laboratory analysis were performed according to the manufacturer's instruction. A more detailed description of the procedures can be found in Supplementary material.

2.3. Statistical analysis

Presence or absence of HEV IgG was defined as the main study outcome and considered as dependent variable. As exposures, we studied different socioeconomical and behavioral aspects, with special emphasis in fecal-oral and zoonotic transmission. We summarized categorical variables as counts and percentages, and continuous variables as medians and inter-quartile ranges (IQR). Due to only three reactive samples, we refrained from carrying out formal statistical tests assessing the association between HEV IgG reactivity and socioeconomical and behavioral variables.

We calculated the proportion of HEV IgG-reactive samples with their 95% confidence intervals (95% CI), score method with Yates' correction, function prop.test() in the R package "stats" (49) for each ELISA. After this, the crude seroprevalence was adjusted for the respective test's sensitivity and specificity as proposed by Lang and Reiczigel (50) using the R package "asht" (51). For calculation of those adjusted seroprevalence estimates, we used the sensitivity and specificity values determined by Norder et al. (46) for the Axiom assay and for the recomWell assay those provided by the manufacturer (47), as the latter reports information from an updated recomWell assay with altered performance characteristic that was also utilized in our study. We also assessed the inter-rater reliability between the two HEV IgG ELISAs by calculating Fleiss's *k* (52) with the R package "irrCAC" (53).

All statistical analysis were performed using RStudio (54) version 4.0.2, and the geographical representation was done using ArcGIS version 10.8.1 using the boundaries provided by Humanitarian Data Exchange (55) and data from Datos Abiertos Bogotá (56). HEV IgG/IgM S/CO calculation and quantification of HEV RNA were performed in Excel 2016 (Microsoft, Redmond, USA) or in GraphPad Prism 9.4.1 (GraphPadInc, SanDiego, USA), respectively.

3. Results

During the recruitment phase, 502 people showed interest to participate in the study. After the first contact with the team, 192 (38%) people were excluded because they did not meet the inclusion criteria, declined participation, did not attend the appointment, or did not reply further. Among the remaining 310 people (62%), 263 of them (85%) were included into the study, as they had a sufficiently complete data set suitable for analysis (Figure 1). The median age of those participants was 9 years (interquartile range 8–11 years), and 142 (54.0%) participants were male. Other characteristics of the participants are presented in Table 1.

We detected three HEV IgG-reactive samples with the recomWell HEV IgG ELISA and the Axiom HEV IgG ELISA in our final study population resulting in a crude seroprevalence of 1.1% (95% CI: 0.3–3.6%). When adjusting this crude estimate by each assay sensitivity and specificity, we found a seroprevalence of 0.0% (95% CI: 0.0–2.6%) for the recomWell assay and of 0.2% (95% CI: 0.0–2.6%) for the Axiom assay. When analyzing the concordance between serological test results (Table 2), we obtained a Fleiss's *k* agreement coefficient of 1, which demonstrates a perfect agreement between the assays.

All HEV IgG-reactive samples originated from participants born and raised in Colombia, who lived in areas of socioeconomic

TABLE 2 Concordance of test results between both HEV IgG ELISAs.

Axiom assay results	RecomWell assay results		
	Positive	Negative	Total (min–max S/CO)
Positive	3	0	3 (9.36–14.54)
Negative	0	260	260 (−0.01–0.89)
Total (min–max S/CO)	3 (4.51–8.55)	260 (0.01–0.87)	263

strata 2 and 3 (Table 1). Their parents received an income between one and two minimum wages (280–560 USD). All HEV IgG-reactive samples were from the locality Engativá but from different neighborhoods (Figure 2). None of the individuals with HEV IgG-reactive samples reported having an earlier blood transfusion, having suffered previously from jaundice or any other hepatitis-related symptoms, or having a diagnostic hepatitis panel done before our study took place.

All HEV IgG-reactive participants reported access to good-quality drinking water and sanitary systems in their homes making handwashing before eating food and after using the toilette possible. Pork consumption was reported by two people (67%), while recreational swimming in rivers or lakes was reported by one person (33%) (Table 3). Due to the low number of reactive cases, we refrained from further statistical evaluation to examine if age, gender, or other examined behavioral or environmental factors such as pork consumption, were associated with an earlier HEV infection.

After performing the IgM analysis, we found one reactive sample resulting in crude seroprevalence of 0.4% (95% CI: 0.0–2.4%) and an adjusted seroprevalence of 0% (95% CI: 0.0–1.3%). This reactive IgM sample was also reactive for IgG (Table 3). When analyzing those sera for HEV RNA by quantitative RT-PCR, no amplification traces were detectable in any of the samples.

4. Discussion

We explored the HEV seroprevalence in children aged 5–18 years living in an urban setting of Bogotá, Colombia together with social, clinical, and exposure variables to analyze risk factors for a HEV infection. We found an unadjusted HEV seroprevalence of 1.1% (95% CI: 0.3–3.6%) in our study population using two HEV IgG ELISAs. Our low HEV seroprevalence is in line with the few other studies performed in children in Latin America (57). While the first of two studies conducted in Argentina found a crude HEV IgG seroprevalence of 0.15% in participants from urban Buenos Aires with a mean age of 6.4 (58), the second one found a crude seroprevalence of 1.7% in participants from the rural Chaco Province with a median age of 14 years (59). HEV crude seroprevalence in children from urban Santa Cruz, Bolivia was 1.7% (95% CI: 1.5–1.8%) (60), in which all HEV IgG-reactive participants were exclusively from the poorest social class and between 13 and 18 years old. Other studies have shown higher seroprevalences. A study from Mato Grosso State, Brazil where a series number of hepatitis cases occurred in 1997 and 1998, reported a HEV IgG seroprevalence of 4.5% (95% CI 2.9–6.9%) in children aged 2–9 years (61), while a study from Mexico with representative regional and socioeconomic sampling found a HEV IgG seroprevalence of 4.4% in 5–14 year old children with increased Odds Ratios for age, type of community, and educational level (62). Even though our low seroprevalence is in line with similar studies in other Latin American countries, our study design did not allow us to identify an increase of seroprevalence with age, as shown by the study in Mexico (62). The latter study selected serum samples from a National Serologic Survey performed in 1987 and 1988, which included 3,549 participants aged 1 to 29 years old (62). Evidence suggests that increasing age is a risk factor for HEV infection (57). A systematic review by Belei et al. found that HEV seroprevalence estimates in individuals between 15 and 30 years can reach about 30% (63). Kmush et al. studied HEV seroprevalence in both children and adults and found an overall seroprevalence of HEV antibodies among adults of 9.52% (95% CI: 3.58–19.59%) in contrast to a seroprevalence of 0.7% (95% CI: 0.15–2.09%) in children (64).

As observed by Kmush et al. in New York, United States, our low HEV seroprevalence in children contrasts to increased levels of HEV seropositivity in the adult population of Colombia where study reported a seroprevalence of 1.74% for IgM antibodies and of 7.5% for IgG in 344 human sera samples from 16 Colombian departments (28). Another study that included 1,097 sera samples from 32 departments from patients with active viral hepatitis described a seroprevalence of 31.2% for IgG and of 11.5% for IgM (27). A further study performed in Medellin, Antioquia found nine (22.5%) cases of HEV infection in 40 fecal samples of patients with a clinical diagnosis of viral hepatitis using nested RT-PCR (65). In contrast to the previous studies, one study performed in 42 blood donors from the Municipality of Yarumal, Antioquia has identified 19 (45.2%) HEV IgG-reactive sera samples, but none for IgM (29). It is noteworthy that the majority of studies examining HEV epidemiology in Colombia not only in humans, but also in swine or in water samples originate from Antioquia. Interestingly, this region is the department with the highest pork production activity of 43.4% in Colombia (66). For environmental samples, one study in Antioquia detected HEV genome in 23.3% (7/30) of the samples from drinking water plants and in 16.7% (5/30) from sewage by RT-PCR (33). Several further studies provide evidence for HEV presence in pigs or pig products in Antioquia, one study reported that 41.3% of pig livers from slaughterhouses and 25% of livers from grocery stores in Medellin tested positive for HEV RNA by RT-PCR (67). Another study performed in fecal samples from 210 animals from 30 pig farms of Antioquia found that 100% of the samples were reactive for IgG antibodies, and 57% for IgM antibodies. Evidence of HEV genome was found in 26% of pig feces (31). A last study that included blood samples from pigs of Antioquia found 100% seropositivity for IgG antibodies and 82.06% for IgM antibodies using a commercial ELISA kit (32).

The most recent systematic reviews and meta-analysis' have identified risk factors for a HEV infection as consummation of raw meat, exposition to soil, having had a blood transfusion, travel to endemic areas, contact with dogs, living in rural areas and receiving lower level of education on a global level (8) and focused on the Americas as increasing age, contact with pigs and meat products, and low socioeconomic conditions (57). Our discrepant levels of HEV seropositivity in Colombian children and adults are reflected in the above identified risk factors where potential occupation and/or living in (rural) areas with pig farming and meat production are given. However, other factors such as improved hygiene standards, access to sanitation, or changes in behavioral conducts such as

10.3389/fpubh.2023.981172

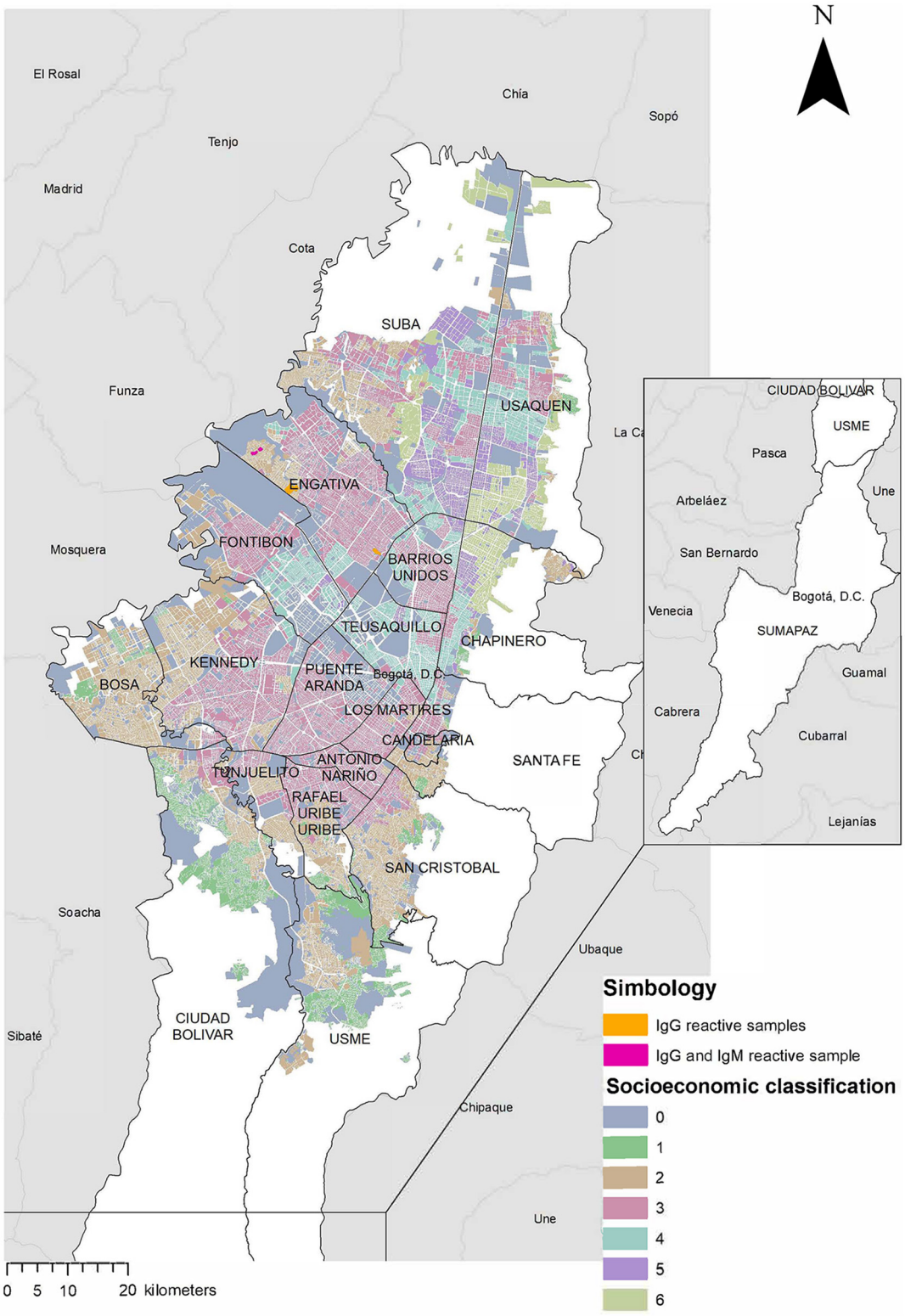

FIGURE 2
Neighborhoods in Bogotá with HEV antibody-reactive samples. The map shows the distribution of the localities and their socioeconomic classification. Neighborhoods with HEV IgG-reactive samples are highlighted in orange, the neighborhood with a HEV IgG- and IgM-reactive sample is highlighted in fuchsia.

increased awareness of risks from undercooked meat combined with avoiding consumption of raw meat in adults/parents might contribute also to the low seroprevalence in the current children population. Longitudinal follow-up exams in regular intervals in our study population until adulthood could contribute to further clarify those discrepant levels of HEV seroprevalence. As already shown

TABLE 3 Characteristics of the HEV IgM- and IgG-reactive samples within the study population.

Results	Sex	Age group	Contact with pigs	Pork consumption	Most frequent source of drinking water	Hand washing after the toilet	Hand washing before eating	Recreational swimming in rivers or streams	Mean IgG Axiom S/CO	Mean IgG Mikrogen S/CO	IgM Mikrogen S/CO
IgG^+	Female	5–8	No	Never	Bottled	Usually	Occasionally	No	9.70	4.51	0.38
IgG^+	Male	5–8	No	Occasioally	Boiled	Always	Always	No	9.36	5.35	0.25
IgG^+ & IgM^+	Female	9–11	No	Occasionally	Filtered	Usually	Occasionally	Yes	14.54	8.55	1.43

in other studies (68), none of HEV-IgG reactive participants of the current study reported any hepatitis-related symptoms indicating that HEV infection is mostly asymptomatic in children. Interestingly, the three participants with detectable HEV IgG in both assays showed high S/CO values pointing toward a robust immune response. Unfortunately, we were not able to convert our semi-quantitative antibody titers for further standardization using the World Health Organization Reference Reagent for Hepatitis E Virus Antibody due to its current unavailability (69) to substantiate our observation. Interestingly, we observed no differences in the number of HEV IgG-reactive samples between the two immunoassay used which differ in the peptide antigens for antibody capture. This is in contrast to an observation of Pezzoni et al. who found that 12% of tested swine sera were only reactive toward ORF3 protein (70). In addition, we observed slight discrepancies in adjusted seroprevalences in the two commercially developed anti-HEV IgG immunoassays. Those originated from different sensitivity and specificity, which can even be observed when the same sample sets were used for validation (19, 46). Those discrepancies in assay performance might have further implications in particular in a low-prevalence setting as ours and underline the need for standardization in HEV serology. Adjusting seroprevalence estimates for an assay's sensitivity and specificity, as done by us, does not only reflect better the underlying population seroprevalence, but it also makes results across studies directly comparable.

We found one HEV IgM-reactive sample, and no HEV-specific RNA was detectable in any of the IgM- or IgG-reactive sera samples. The only IgM-reactive sample was also reactive for IgG, which might represent an acute infection case, even in the absence of detectable RNA levels. While IgM antibodies decline more rapidly after an acute infection and can be detectable only for a few months after onset of symptoms, IgG antibodies can persists for at least 1 year (71, 72). RNA on the contrary declines even more rapidly, and it is not detectable in the serum by day 20 after onset of symptoms (73). Therefore, the other two HEV IgG-reactive samples might indicate an even earlier infection as no IgM were identified in them.

Our study has several limitations. Although our sample size is comparable to the majority of other studies in the region with a population screened between 99 and 1,848 children (22, 74), our recruitment and sampling processes were hampered by massive and long-lasting school closure due to the COVID-19 pandemic. We had to include more localities and schools than those previously selected by changing our random to a convenient sampling. Nevertheless, we were able to gather diverse samples in terms of sociodemographic characteristics such as different strata, broad age ranges, and localities of origin. Moreover, our study included self-reported medical and behavioral information, which may be inaccurate and threatened by self-reporting bias (75). We could not find any factor associated with HEV reactivity due to the few observations within groups in our study. Lastly, we only examine B-cell and not T-cell mediated immune responses, which also offer protection from reinfection (76). While the presence of antibodies is regarded as immune correlates of protection against HEV infection, we equally cannot provide insight into their persistence due to the lack of a longitudinal follow-up component in our study design. We were also not able to define the HEV genotype responsible for the infection to gain further insight into mode of transmission. Data on viral circulation in non-human samples were neither gathered nor available to correlate with findings in human bio-samples.

In conclusion, the unadjusted HEV IgG seroprevalence in the study population was 1.1% (95% CI: 0.3–3.6%) using both assays; the recomWell HEV IgG ELISA which detects antibodies reactive to ORF2 and ORF3 protein and the Axiom assay which only uses the ORF2 peptide as antigen. We can highlight that the participants living in an urban setting of Bogotá, Colombia, have good access to drinkable water and sanitary systems, have good hand-washing practices, rare contact with pigs, and moderate consumption of pork. As serological testing cannot define the viral genotype responsible for the previous infection, we speculate that those factors might explain the low HEV infection numbers found in our study.

Data availability statement

The raw data supporting the conclusions of this article have been provided by the authors in a public repository (https://zenodo.org/badge/latestdoi/559831870).

Ethics statement

The study involving human participants was reviewed and approved by the Comité De Ética De Investigacion De La Facultad De Medicina, Universidad Nacional de Colombia, Bogotá, Colombia (N°.009-125-19) and the Ethics Committee of Hannover Medical School, Hannover, Germany (Nr.9254_BO_K_2020). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

GK, FH, TK, NF, MS, and JT designed the study. NF and JT executed the study and collected data. GK, MS, and FH supervised the study. MS and JS performed the laboratory experiments. MS supervised and coordinated laboratory work and analyzed laboratory data. NF cleaned the database and prepared data for analysis. BK, MS, and NF analyzed and interpreted the data. NF and MS prepared the initial manuscript. MS and BK verified the underlying data and provided advice on data analysis. All authors have revised the manuscript, read, and approved the final version. All authors confirm full access to all the data in the study and accept responsibility to submit for publication.

Funding

The research was funded by intramural funds of the HZI and by the DZIF (German Center for Infection Research). NF receives a scholarship by Studienstiftung des deutschen Volkes.

Acknowledgments

We thank the laboratory biologists (Nicolás Lalinde Ruiz, Manuela Llano León, Laura Camila Martínez Enríquez), nurses (Lina María Beltrán Toca, Carlos David Zabala Vega, Oscar Stiven Alméziga Clavijo, Martha Yelitza Rodríguez Malagón), and medical personnel (Rosanna Camerano Ruiz, Karem Johanna Delgado Garcia, Andrés Felipe Mora Salamanca, Laura Johanna Piña Jaramillo) at UNAL for their assistance with data collection and processing. We further thank the UNAL administrative personal for their support in study planning and implementation. We also thank all members of the HZI team that supported administrative and laboratory processes.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.981172/full#supplementary-material

References

1. Hepatitis E. World Health Organization. (2021). Available online at: https://www.who.int/news-room/fact-sheets/detail/hepatitis-e (accessed February 8, 2021).

2. Nimgaonkar I, Ding Q, Schwartz RE, Ploss A. Hepatitis E virus: advances and challenges. *Nat Rev Gastroenterol Hepatol.* (2018) 15:96–110. doi: 10.1038/nrgastro.2017.150

3. Kamar N, Dalton HR, Abravanel F, Izopet J. Hepatitis E virus infection. *Clin Microbiol Rev.* (2014) 27:116–38. doi: 10.1128/CMR.00057-13

4. Kumar A, Beniwal M, Kar P, Sharma JB, Murthy NS. Hepatitis E in pregnancy. *Int J Gynecol Obstet.* (2004) 85:240–4. doi: 10.1016/j.ijgo.2003.11.018

5. Kamar N, Garrouste C, Haagsma EB, Garrigue V, Pischke S, Chauvet C, et al. Factors associated with chronic hepatitis in patients with hepatitis e virus infection who have received solid organ transplants. *Gastroenterology.* (2011) 140:1481–9. doi: 10.1053/j.gastro.2011.02.050

6. Purdy MA, Harrison TJ, Jameel S, Meng XJ, Okamoto H, Van der Poel WH, et al. ICTV virus taxonomy profile: hepeviridae. *J General Virol.* (2022) 98:2645. doi: 10.1099/jgv.0.000940

7. Lee G-H, Tan B-H, Chi-Yuan Teo E, Lim S-G, Dan Y-Y, Wee A, et al. Chronic infection with camelid hepatitis e virus in a liver transplant recipient who regularly consumes camel meat and milk. *Gastroenterology.* (2016) 150:355–357.e3. doi: 10.1053/j.gastro.2015.10.048

8. Li P, Liu J, Li Y, Su J, Ma Z, Bramer WM, et al. The global epidemiology of hepatitis E virus infection: A systematic review and meta-analysis. *Liver Int.* (2020) 40:1516–28. doi: 10.1111/liv.14468

9. Hewitt PE, Ijaz S, Brailsford SR, Brett R, Dicks S, Haywood B, et al. Hepatitis e virus in blood components: a prevalence and transmission study in southeast England. *Lancet.* (2014) 384:1766–73. doi: 10.1016/S0140-6736(14)61034-5

10. Bi H, Yang R, Wu C, Xia J. Hepatitis E virus and blood transfusion safety. *Epidemiol Infec*. (2020) 148:e158.

11. Woo PCY, Lau SKP, Teng JLL, Tsang AKL, Joseph M, Wong EYM, et al. New Hepatitis E virus genotype in camels, the middle east. *Emerg Infect Dis.* (2014) 20:1044–8. doi: 10.3201/eid2006.140140

12. Dremsek P, Wenzel JJ, Johne R, Ziller M, Hofmann J, Groschup MH, et al. Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs. *Med Microbiol Immunol.* (2012) 201:189–200. doi: 10.1007/s00430-011-0221-2

13. Shimizu K, Hamaguchi S, Ngo CC, Li T-C, Ando S, Yoshimatsu K, et al. Serological evidence of infection with rodent-borne hepatitis E virus HEV-C1 or antigenically related virus in humans. *J Vet Med Sci.* (2016) 78:1677–81. doi: 10.1292/jvms.16-0200

14. Sridhar S, Yip CCY, Wu S, Cai J, Zhang AJ-X, Leung K-H, et al. Rat hepatitis e virus as cause of persistent hepatitis after liver transplant. *Emerg Infect Dis.* (2018) 24:2241–50. doi: 10.3201/eid2412.180937

15. Andonov A, Robbins M, Borlang J, Cao J, Hatchette T, Stueck A, et al. Rat hepatitis e virus linked to severe acute hepatitis in an immunocompetent patient. *J Infect Dis.* (2019) 220:951–5. doi: 10.1093/infdis/jiz025

16. Sridhar S, Yip CC, Wu S, Chew NF, Leung K, Chan JF, et al. Transmission of rat hepatitis e virus infection to humans in hong kong: a clinical and epidemiological analysis. *Hepatology.* (2021) 73:10–22. doi: 10.1002/hep.31138

17. Rivero-Juarez A, Frias M, Perez AB, Pineda JA, Reina G, Fuentes-Lopez A, et al. Orthohepevirus C infection as an emerging cause of acute hepatitis in Spain: First report in Europe. *J Hepatol.* (2022) 77:326–31. doi: 10.1016/j.jhep.2022.01.028

18. Shrestha AC, Flower RLP, Seed CR, Stramer SL, Faddy HM, A. Comparative study of assay performance of commercial hepatitis e virus enzyme-linked immunosorbent assay kits in australian blood donor samples. *J Blood Transfus.* (2016) 2016:1–6. doi: 10.1155/2016/9647675

19. Wenzel JJ, Preiss J, Schemmerer M, Huber B, Jilg W. Test performance characteristics of anti-HEV IgG assays strongly influence hepatitis e seroprevalence estimates. *J Infect Dis.* (2013) 207:497–500. doi: 10.1093/infdis/jis688

20. Bawazir AA, Hart CA, Sallam TA, Parry CM, Beeching NJ, Cuevas LE. Seroepidemiology of hepatitis a and hepatitis e viruses in aden, yemen. *Trans R Soc Trop Med Hyg.* (2010) 104:801–5. doi: 10.1016/j.trstmh.2010.08.007

21. Fischler B, Baumann U, Dezsofi A, Hadzic N, Hierro L, Jahnel J, et al. Hepatitis E in children: A position paper by the ESPGHAN hepatology committee. *J Pediatr Gastroenterol Nutr*. (2016) 63:288–94. doi: 10.1097/MPG.0000000000001231

22. Verghese VP, Robinson JL. A systematic review of hepatitis e virus infection in children. *Clin Infect Dis.* (2014) 59:689–97. doi: 10.1093/cid/ciu371

23. Realpe-Quintero M, Mirazo S, Viera-Segura O, Copado-Villagrana ED, Panduro A, Roman S, et al. Hepatitis e virus genotype 1 and hepatitis a virus dual infection in pediatric patients with a low socioeconomic status from Mexico. *Intervirology.* (2018) 61:105–10. doi: 10.1159/000492425

24. Gómez Jiménez A. Colombia: el contexto de la desigualdad y la pobreza rural en los noventa - Colombia: the context for inequality and rural poverty in the 1990's. *Cuad Econ.* (2003) 22:198–238.

25. Aristizábal Giraldo S. La diversidad étnica y cultural de Colombia: un desafío para la educación. *Pedagog y Saberes.* (2000) 15:61–8. doi: 10.17227/01212494.15pys61.68

26. Ministerio de Salud y Protección Social. *Análisis de Situación de Salud (ASIS) Colombia, 2020—Health Situation Analysis (ASIS) Colombia, 2020*. Bogotá, DC (2020).

27. Peláez-Carvajal D, Martínez-Vargas D, Escalante-Mora M, Palacios-Vivero M. Contreras-gómez, lady. Coinfection of hepatitis E virus and other hepatitis virus in Colombia and its genotypic characterization. *Biomedica.* (2016) 36:69–78. doi: 10.7705/biomedica.v36i0.2957

28. Peláez D, Hoyos MC, Rendón JC, Mantilla C, Ospina MC, Cortés-Mancera F, et al. Infección por el virus de la hepatitis E en pacientes con diagnóstico clínico de hepatitis viral en Colombia - Hepatitis E virus infection in patients with clinical diagnosis of viral hepatitis in Colombia. *Biomédica.* (2014) 34:354–65. doi: 10.7705/biomedica.v34i3.2236

29. Duque Jaramillo A, Restrepo LF, Mantilla-Rojas C, Toro M, Olarte JC, Ríos Ocampo WA, et al. Frequency of antibodies to hepatitis e in blood donors in the municipality of yarumal, antioquia. *Rev Col Gastroenterol.* (2016) 31:228–33. doi: 10.22516/25007440.95

30. Betancur Jiménez CA, Mejía MV, Portillo S. Seroprevalencia de hepatitis e en trabajadores de fincas porcícolas del valle de aburrá 2011–2012—seroprevalence of hepatitis e in swine farms workers in Aburrá Valley 2011-2012. *Acta Médica Colomb.* (2013) 38:68–70. doi: 10.36104/amc.2013.54

31. Gutiérrez Vergara CC, Ospina Vélez DA, Forero Duarte JE, Rodríguez BD, Gutiérrez Builes LA, López Herrera A, et al. Detección serológica y molecular del virus de la Hepatitis E en cerdos de granjas antioqueñas—Serological and molecular detection of Hepatitis E virus in pig farms of Antioquia. *Ces Med Vet Zootec*. (2014) 9:158–68.

32. Forero D J, Gutiérrez V C, Parra S J, Correa L G, Rodríguez B, Gutiérrez B L, et al. Evidencia serológica de infección por el Virus de Hepatitis E en cerdos faenados en Antioquia, Colombia TT - Serological evidence of Hepatitis E Virus infection in Antioquia, Colombia slaughtered pigs. *Rev MVZ Córdoba.* (2015) 20:4602–13. doi: 10.21897/rmvz.63

33. Baez PA, Lopez MC, Duque-Jaramillo A, Pelaez D, Molina F, Navas M-C. First evidence of the Hepatitis E virus in environmental waters in Colombia. *PLoS ONE.* (2017) 12:e0177525. doi: 10.1371/journal.pone.0177525

34. Rendón JC, Navas MC, Hoyos MC, Cortés FM, Correa G, Sepúlveda ME, et al. MO-38 Evidencia serológica y molecular de la circulación del virus de la hepatitis E en Medellín - Serological and molecular evidence of hepatitis E virus circulation in Medellín. *Rev Infect*. (2010) 14:34.

35. Secretaría Distrital de Salud de Bogotá DC. *Plan Territorial de Salud Bogotá D.C. 2020–2024—Territorial Health Plan Bogotá D.C. 2020–2024*. Bogotá, DC (2020).

36. de Bogotá AM. *Secretaría Distrital de Salud. Documento de Análisis de Situación de Salud con el Modelo de los Determinantes Sociales de Salud para el Distrito Capital - Health Situation Analysis Document with the Social Determinants of Health Model for the Capital District Bogotá.* (2019). doi: 10.56085/20277970.8

37. Yunda JG. Densificación y estratificación social en bogotá: Distribución sesgada de la inversión privada—Densification and social stratification in Bogota: Biased distribution of private investment. *Eure.* (2019) 45:237–57. doi: 10.4067/S0250-71612019000100237

38. Ministerio de Educación Nacional. *Listado Colegios Bogotá | Datos Abiertos Colombia - List of Bogotá Schools | Colombia Open Data.* (2017).

39. Christiansen T, Lauritsen J. *EpiData—Comprehensive Data Management and Basic Statistical Analysis System*. Odense Denmark: EpiData Association (2010).

40. Departamento Administrativo Nacional de Estadísticas, Alcaldía de Medellín. *Estimaciones de población 1985–2005 y proyecciones de población 2005–2020 nacional, departamental y municipal por sexo, grupos quinquenales de edad - Population estimates 1985–2005 and population projections 2005–2020 national, departmental and municipal*. Bogotá (2011). Available online at: http://formularios.dane.gov.co/Anda_4_1/index.php/catalog/MICRODATOS

41. Alcalcía Mayor de Bogotá Secretaría & Distrital de Planeación. Bogotá Ciudad de Estadísticas Boletín No.18: población, viviendas y hogares a Junio 30 de 2010, en relación con la estratificación socioeconómica vigente en el 2010—Bogota City of Statistics Bulletin No.18: population, dwellings and households as of Jun. Bogotá; 2010. Available online at: http://www.sdp.gov.co/sites/default/files/dice101-cartillavihopeestrato-2010.pdf

42. Cuschieri S. The STROBE guidelines. *Saudi J Anaesth.* (2019) 13:31. doi: 10.4103/sja.SJA_543_18

43. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)-A metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform.* (2009) 42:377–81. doi: 10.1016/j.jbi.2008.08.010

44. Harris PA, Taylor R, Minor BL, Elliott V, Fernandez M, O'Neal L, et al. The REDCap consortium: Building an international community of software platform partners. *J Biomed Inform.* (2019) 95:103208. doi: 10.1016/j.jbi.2019.103208

45. Bohm K, Strömpl J, Krumbholz A, Zell R, Krause G, Sievers C. Establishment of a highly sensitive assay for detection of hepatitis e virus-specific immunoglobulins. *J Clin Microbiol*. (2020) 58:e01029–19. doi: 10.1128/JCM.01029-19

46. Norder H, Karlsson M, Mellgren Å, Konar J, Sandberg E, Lasson A, et al. Diagnostic performance of five assays for anti-hepatitis e virus igg and igm in a large cohort study. *J Clin Microbiol.* (2016) 54:549–55. doi: 10.1128/JCM.02343-15

47. Mikrogen Diagnostik. *Mikrogen - recomWell HEV IgG/IgM*. Available online at: https://www.mikrogen.de/english/deutschland/products/product-overview/testsystem/hev-igg.html (accessed February 09, 2022).

48. Wen G-P, Tang Z-M, Yang F, Zhang K, Ji W-F, Cai W, et al. A valuable antigen detection method for diagnosis of acute hepatitis E. *J Clin Microbiol.* (2015) 53:782–8. doi: 10.1128/JCM.01853-14

49. R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing (2021).

50. Lang Z, Reiczigel J. Confidence limits for prevalence of disease adjusted for estimated sensitivity and specificity. *Prev Vet Med.* (2014) 113:13–22. doi: 10.1016/j.prevetmed.2013.09.015

51. Fay MP. *Asht: Applied Statistical Hypothesis Tests*. (2022). Available online at: https://cran.r-project.org/package=asht

52. Allen M. *The SAGE Encyclopedia of Communication Research Methods.* 2455 Teller Road, Thousand Oaks California 91320: SAGE Publications, Inc. (2017). Available online at: https://sk.sagepub.com/reference/the-sage-encyclopedia-of-communication-research-methods/ (accessed May 03, 2022).

53. Gwet KL. *Computing Chance-Corrected Agreement Coefficients (CAC).* (2019).

54. Andy Bunn MK. *A language and environment for statistical computing. Vol. 10.* Vienna, Austria: R Foundation for Statistical Computing (2017). p. 11–8.

55. Humanitarian Data Exchange. *Colombia—Subnational Administrative Boundaries*. (2020). Available online at: https://data.humdata.org/dataset/cod-ab-col (accessed March 8, 2022).

56. Alcaldía mayor de Bogotá. *Datos Abiertos Bogotá - Open Data Bogotá*. Available online at: https://datosabiertos.bogota.gov.co/dataset (accessed Mar 8, 2022).

57. Fernández Villalobos NV, Kessel B, Rodiah I, Ott JJ, Lange B, Krause G. Seroprevalence of hepatitis E virus infection in the Americas: estimates from a systematic review and meta-analysis. *PLoS ONE.* (2022) 17:e0269253. doi: 10.1371/journal.pone.0269253

58. Rey JA, Findor JA, Daruich JR, Velazco CC, Igartua EB, Schmee E, et al. Prevalence of IgG anti-HEV in Buenos Aires, a nonendemic area for hepatitis E. *J Travel Med.* (1997) 4:100–1. doi: 10.1111/j.1708-8305.1997.tb00788.x

59. Martínez AP, Pereson MJ, Pérez PS, Baeck MI, Mandó P, López Saubidet I, et al. Prevalence of hepatitis E virus in children from Northeast of Argentina. *J Med Virol.* (2021) 93:4015–7. doi: 10.1002/jmv.26274

60. Gandolfo GM, Ferri GM, Conti L, Antenucci A, Marrone R, Frasca AM, et al. Prevalence of infections by hepatitis A, B, C, and E viruses in two different socioeconomic groups of children from Santa Cruz, Bolivia. *Med Clin (Barc).* (2003) 120:725–7. doi: 10.1016/S0025-7753(03)73826-3

61. Assis SB, Souto FJD, Fontes CJF, Gaspar AMC. Prevalence of hepatitis A and E virus infection in school children of an Amazonian municipality in Mato Grosso State. *Rev Soc Bras Med Trop.* (2002) 35:155–8. doi: 10.1590/s0037-86822002000200005

62. Alvarez-Muñoz MT, Torres J, Damasio L, Gómez A, Tapia-Conyer R, Muñoz O. Seroepidemiology of hepatitis E virus infection in Mexican subjects 1 to 29 years of age. *Arch Med Res.* (1999) 30:251–4. doi: 10.1016/S0188-0128(99)00019-6

63. Belei O, Ancusa O, Mara A, Olariu L, Amaricai E, Folescu R, et al. Current paradigm of hepatitis e virus among pediatric and adult patients. *Front Pediatr.* (2021) 9:721918. doi: 10.3389/fped.2021.721918

64. Kmush BL, Lu AM, Spillane T, Hruska B, Gump BB, Bendinskas KG. Seroprevalence of hepatitis E virus antibodies in adults and children from upstate New York: A cross-sectional study. *PLoS ONE.* (2021) 16:e0245850. doi: 10.1371/journal.pone.0245850

65. Rendon J, Hoyos MC, Di Filippo D, Cortes-Mancera F, Mantilla C, Velasquez MM, et al. Hepatitis E virus genotype 3 in Colombia: survey in patients with clinical diagnosis of viral hepatitis. *PLoS ONE.* (2016) 11:1–12. doi: 10.1371/journal.pone.0148417

66. Barrón JF, García R, Mora JS, López S, Pro A, García RC. El valor de la actividad Porcícola - The value of swine farming. *Rev Porkcolombia.* (2022) 263:369–377.

67. Gutiérrez-Vergara C, Quintero J, Duarte JF, Suescún JP, López-Herrera A. Detection of hepatitis E virus genome in pig livers in Antioquia, Colombia. *Genet Mol Res.* (2015) 14:2890–9. doi: 10.4238/2015.March.31.20

68. Horvatits T, Zur Wiesch JS, Lütgehetmann M, Lohse AW, Pischke S. The clinical perspective on hepatitis E. *Viruses.* (2019) 11:1–19. doi: 10.3390/v11070617

69. Ferguson M, Walker D, Mast E, Fields H. Report of a collaborative study to assess the suitability of a reference reagent for antibodies to hepatitis e virus. *Biologicals.* (2002) 30:43–8. doi: 10.1006/biol.2001.0315

70. Pezzoni G, Stercoli L, Pegoiani E, Brocchi E. Antigenic characterization of ORF2 and ORF3 proteins of hepatitis e virus (HEV). *Viruses.* (2021) 13:1385. doi: 10.3390/v13071385

71. Koshy A, Grover S, Hyams KC, Shabrawy MA, Pacsa A, Al-Nakib B, et al. Short-term IgM and IGG antibody responses to hepatitis e virus infection. *Scand J Infect Dis.* (1996) 28:439–41. doi: 10.3109/00365549609037935

72. Favorov MO, Fields HA, Purdy MA, Yashina TL, Aleksandrov AG, Alter MJ, et al. Serologic identification of hepatitis E virus infections in epidemic and endemic settings. *J Med Virol.* (1992) 36:246–50. doi: 10.1002/jmv.1890360403

73. Goel A, Padmaprakash K, Benjamin M, Katiyar H, Aggarwal R. Temporal profile of HEV RNA concentration in blood and stool from patients with acute uncomplicated hepatitis E in a region with genotype 1 predominance. *J Viral Hepat.* (2020) 27:631–7. doi: 10.1111/jvh.13266

74. Humberto Ibarra V, Stella Riedemann G, Claudio Toledo A. Seguimiento de anticuerpos contra hepatitis A y E en una cohorte de niños de bajo nivel socioeconómico - Hepatitis A and E virus antibodies in Chilean children of low socioeconomic status. *A one year follow-up study Rev Med Chil.* (2006) 134:139–44. doi: 10.4067/S0034-98872006000200001

75. Althubaiti A. Information bias in health research: definition, pitfalls, and adjustment methods. *J Multidiscip Healthc.* (2016) 9:211. doi: 10.2147/JMDH.S104807

76. Kulkarni SP, Sharma M, Tripathy AS. Antibody and memory b cell responses in hepatitis e recovered individuals, 1–30 years post-hepatitis e virus infection. *Sci Rep.* (2019) 9:4090. doi: 10.1038/s41598-019-40603-9

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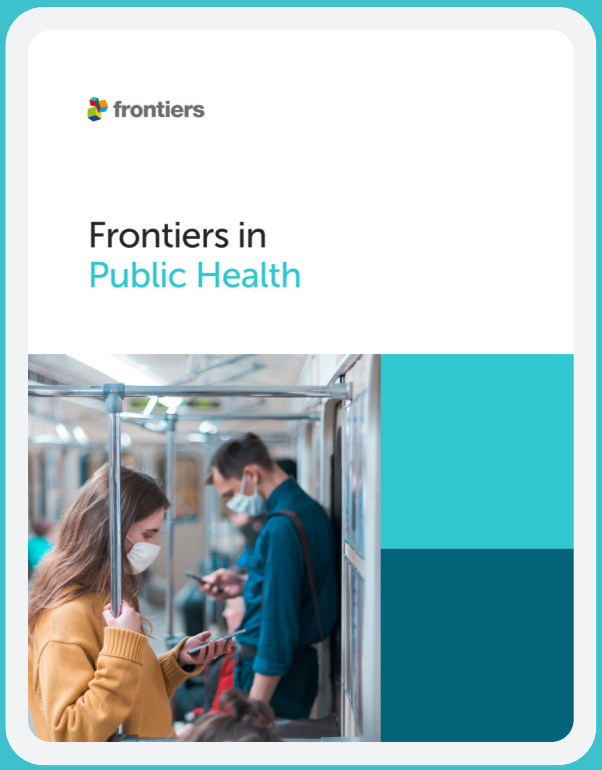

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