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Beyond the fever: shotgun metagenomic sequencing of stool unveils pathogenic players in HIV-infected children with non-malarial febrile illness

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Abstract

Background Non-malarial febrile illnesses (NMFI) pose significant challenges in HIV-infected children, often leading to severe complications and increased morbidity. While traditional diagnostic approaches focus on specific pathogens, shotgun metagenomic sequencing offers a comprehensive tool to explore the microbial landscape underlying NMFI in this vulnerable population ensuring effective management.

Methods In this study, we employed shotgun metagenomics to analyse stool samples from HIV-infected children at the Baylor Children's Clinic Uganda presenting with non-malarial febrile illness. Samples were collected and subjected to DNA extraction at the Molecular and Genomics Laboratory, Makerere University followed by shotgun metagenomics sequencing at the Chan Zuckerberg Biohub San Francisco. Bioinformatics analysis was conducted to identify and characterise the microbial composition and potential pathogenic taxa associated with NMFI using the CZID pipeline.

Results Our findings reveal a diverse array of microbial taxa in the stool samples of HIV-infected children with NMFI. Importantly, shotgun metagenomics revealed potentially pathogenic players including *Trichomonas vaginalis*, *Candida albicans*, Giardia, and Bacteroides in stool from this patient population. This sheds light on the complexities of microbial interactions that potentially underpin non-malarial febrile illness in this group. Taxonomic profiling identified recognised pathogens and comorbidities and revealed possible new correlations with NMFI, shedding light on the pathophysiology of fever in HIV-infected children.

Conclusion Shotgun metagenomics is a valuable method for understanding the gut microbial landscape of NMFI in HIV-infected children, providing a comprehensive approach to pathogen identification and characterisation. By revealing potential pathogenic actors beyond the fever, this work demonstrates how metagenomic sequencing may

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improve our knowledge of infectious illnesses in vulnerable groups and inspire targeted therapies for better clinical care and outcomes.

Keywords Children, HIV-Infected, Non-malarial febrile illness, Shotgun metagenomic sequencing, Uganda

Introduction

In Uganda, where children living with HIV confront numerous health challenges, non-malarial febrile illnesses (NMFI) present a substantial health concern. Malaria, caused by *Plasmodium* species parasites transmitted through the bite of infected mosquitoes, remains the primary cause of febrile illnesses in the region, with 284 per 1000 of the population at risk of malaria between 2020 and 2021 [1]. It is usually diagnosed by microscopic inspection of thick and thin blood smears for the presence of Plasmodium parasites. At the same time, rapid diagnostic tests (RDTs) are now widely used due to their ease of use and quick turnaround time. Once malaria is ruled out, emphasis shifts to the vast array of infections that cause NMFI in this vulnerable group. Many pathogens cause NMFI and yet are not the subject of effective surveillance programs, and, the backbone of clinical treatment and monitoring in peripheral care settings, are inaccessible [2].

Comprehensive monitoring efforts that capture numerous pathogen types improve relevant public health actions. Our complete awareness of the whole landscape of prevalent causes of NMFI at the community level also helps. Untargeted sequencing, or metagenomic sequencing (mNGS), is a potent method for detecting microbial nucleic acids in clinical samples without prior knowledge of the disease [3, 4] and is increasingly used for surveillance in regions at high risk of emerging and re-emerging diseases [5–7]. Because the mNGS method sequences all nucleic acid in a sample, these techniques are usually less sensitive for identifying any one pathogen than focused procedures (e.g., Polymerase Chain Reaction) due to the quantity of the host relative to pathogen nucleic acids [3, 4].

A broad range of pathogens can cause NMFI. Surveillance studies often focus on one or a few pathogens and are even limited to hospitalised patients, and, therefore, are limited in detecting all disease causes. Surveillance studies of severely ill hospitalised patients have identified bacterial pathogens, including vaccine-preventable illnesses such as *Streptococcus pneumoniae* and *Neisseria meningitidis* [8, 9]. Arboviruses such as Dengue and Chikungunya have been detected in time-limited outbreaks in Senegal [10, 11]. Community and clinic-based studies of patients with fever have revealed bacterial zoonoses are a common cause of ambulatory febrile illness in Senegal, including tick-borne relapsing fever, Rickettsioses, Q fever, and Bartonella [12, 13].

Parasitic pathogens also warrant consideration in the pathogen spectrum of NMFI in HIV-infected children in Uganda. While malaria may be excluded as the cause of fever in these children, other parasitic infections, such as schistosomiasis and intestinal parasites, remain prevalent in the region. These parasitic infections exacerbate the clinical course of HIV and contribute to the overall morbidity burden in affected children [14]. Therefore, having these pathogens undetected due to the narrow spectrum diagnostic tools or the absence of an all-pathogen detection tool undervalues the routine management of vulnerable individuals. The dynamic epidemiology of pathogens causing NMFI in HIV-infected children underscores the importance of comprehensive diagnostic approaches and targeted management strategies. Integrated healthcare programs emphasising early detection, prompt treatment, and ongoing monitoring are crucial for optimizing outcomes in this vulnerable population. This study aimed to show the importance of metagenomics as a complementary tool in the clinical management of HIV-infected patients with NMFIs.

Materials and methods

Study design and settings

This was a cross-sectional study. It was conducted at Baylor College of Medicine Children's Foundation, Uganda (Baylor - Uganda). Baylor - Uganda is located at Mulago National Referral Hospital in Kampala, Uganda. Baylor - Uganda is a Non-Governmental Organization (NGO) involved in the prevention, care, and treatment of children and adolescents living with HIV and their families in Uganda. The Foundation is donor-funded and runs a clinic that offers free clinical care to all its patients. The clinic is an outpatient treatment centre that operates five days a week with an average daily attendance of 170 patients. More than 90% of the patients at the clinic are children between six weeks and 18 years old. Infants enrolled for care at the clinic are referred from the nearby Mulago National Referal Hospital wards and other centres involved in the Prevention of mother-to-child HIV Transmission (PMTCT). Some infants are referred from HIV treatment centres within the country, and others are self-referrals.

Sample collection

From November 2022 to October 2023, 147 stool samples were collected from children living with HIV at Baylor College of Medicine Children's Foundation, Uganda. Children under 14 who presented with fevers at Baylor

Children's Clinic were screened for malaria using the Olympus microscope. Those found negative for malaria were requested to consent and assent from their guardians to participate in the study. Upon consent, stool and blood samples were collected for shotgun metagenomics sequencing and a complete blood count analysis was performed at the clinic respectively. The stool samples were stored at -80ºC until DNA extraction was done.

DNA extraction

The procedure was conducted at the Makerere University Biomedical Research Centre Limited (MakBRC) using DNA-free equipment to prevent sample cross-contamination. A 200-µL slurry was lysed with lysis buffer and vortexed for 10 min using bead bashing tubes. Ready-to-use tubes containing beads were utilised in the process. Subsequently, DNA extraction was performed using the DNeasy PowerSoil Pro Kit (QIAGEN). Finally, the DNA was eluted in 100 µL of elution buffer. All the above procedures were performed as per the DNeasy PowerSoil Pro Kit's instructions Post-extraction, DNA quality was assessed using a NanoDrop spectrophotometer (Thermo-Scientific). At the same time, concentrations were measured using the Qubit 3.0 fluorometer (Invitrogen, Life technologies) with the Qubit dsDNA HS kit (Thermo Fisher Scientific). DNA samples were preserved at -80 °C until shipment for sequencing at the Chan Zuckerberg Biohub San Francisco.

Library preparation and sequencing

Library preparation for metagenomic sequencing was completed using the extracted DNA according to the Chan Zuckerberg Biohub SF standard protocols. Sequencing libraries were prepared on a non-normalized, equal volume of DNA (or negative water control) per sample using the NEB Next Ultra II FS DNA Library prep kit (New England Biolabs) and unique barcode primers (Integrated DNA Technologies) for amplification and sample multiplexing. The 192 generated libraries (inclusive of 7 negative and 39 water controls) were sequenced on an Illumina NextSeq2000 P3 flow cell, resulting in high-quality sequencing data representing the genetic material of the microbial community found in the stool samples.

Bioinformatic analysis

Raw fastq sequencing reads obtained from the Illumina platform were uploaded into the CZID web-based platform for metagenomics analysis (https://czid.org/) [15]. The reads were subjected to quality control and preprocessing steps to remove adapters, low-quality bases, and reads aligning to the human genome. The cleaned reads were then aligned to the NCBI database (excluding deuterostomes) for taxonomic profiling using the CZID metagenomic analysis pipeline [15]. Taxonomic profiling was performed to identify and quantify the composition of microbial taxa present in the samples at various taxonomic levels (e.g., species, genus) with controls as the background dataset. The results from this analysis were visualised using personalised *R* scripts with the collected metadata (https://github.com/Kanyerezi30/phicams_cz). The customised scripts aimed at determining the relationship of antiretroviral therapy (ART) regimen on the pathogens and how the different white blood cells were affected by the present pathogens segregated into increase (above average), decrease (below average), and no change (standard) cases (Fig. 1).

Results

The study enrolled 73 male and 71 female HIV-infected children. Among them, 29 were under 2 years old, 30 were between 2 and 5 years, and 85 were over 5 years. The mean ages for males and females were 7.1 years and 6.9 years, respectively. One hundred forty-seven stool samples were collected, but one was eliminated due to poor sample collection; therefore, 147 samples were collected and 144 were included in the study after 3 were removed for quality purposes. From DNA extraction, DNA was quantified, with all samples displaying concentrations ranging from − 0.316 ng/µl to 382.643 ng/µl (supplementary file 1: sheet1) (https://docs.google.com/spreadsheets/d/1sPxRZtVPUsJGMwlMwZ6AfchoRPHYb3Ibb1aR6OhpWnw/edit?gid=0#gid=0).

Pathobiome analysis

To describe the pathobiome, we considered all potential known pathogens by the Chan Zuckerberg pipeline that have (supplementary file 1: 36 pathogen) were found to be non-normal flora in humans but rather pathogenic with *Streptococcus suis* being the most abundant while the rest doubled as normal flora and pathogenic under special circumstances. From the distribution of exclusively pathogenic microorganisms among females and males, most of the pathogens were evenly distributed between the two genders. However, ten of these were uniquely found in each of the genders; *Brachyspira hyodysentriae*, *Aeromonas caviae*, *Streptobacillus moniliformis*, *Schistosoma mansoni*, and *Orf virus* in females and *Providencia rettgeri*, *Providencia alcalifaciens*, *Human polyomavirus* 4, *Haemophilus ducreyi*, and *Entamoeba histolytica* in males. (Fig. 2)

We also sought to determine the differential distribution of the exclusively pathogenic microorganisms between 2 age groups; below the mean age (BMA) and equal to or above the mean age (EAMA) of the cohort. Similar to the findings from the gender investigation, the distribution here also showed that most of the pathogens were evenly distributed between the 2 age groups.

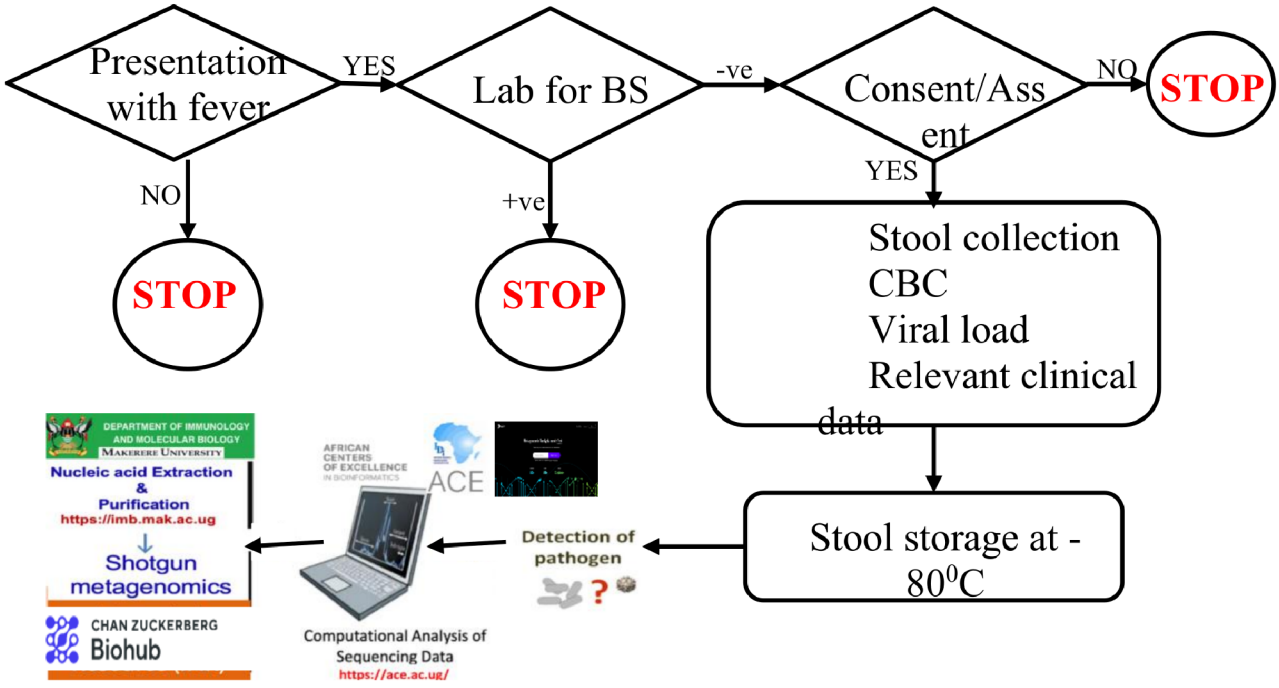

Fig. 1 The study workflow

Table Stating the clinical characteristics of the cohort

Clinical attribute	Total number
Female	71
Male	73
Mean Age for female	6.9
Mean Age for male	7.1
DTG based drugs	108
Non-DTG-based drugs	36

P. rettgeri, *S. moniliformis*, *S. mansoni*, *P. alcalifaciens*, *O. virus*, *H. ducreyi*, and *E. histolytica* were exclusively found in the BMA group and only *A. caviae* plus *H. polyomavirus 4* were exclusively found in the EAMA group. (Fig. 3)

Antiretroviral Therapy (ART)

To understand the effect of HIV drug combinations on the patient's pathogen microbiome composition, we went on to see which pathogens were present or absent while taking particular drug combinations. 10 pathogens (*Clostridioides difficile*, *Clostridium botulinum*, *Clostridium perfringens*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Prevotella intermedia*, *Salmonella enterica*, *Streptococcus pyogenes*, *S. suis*) were common to all drug combinations. 10, 1, 4, 1, 7, 1, and 7 pathogens were uniquely in abc-3tc-dtg, abc-3tc-lpv, azt-3tc-dtg, azt-3tc-lpv, newly diagnosed, sero-exposed, and tdf-3tc-dtg respective drug combinations. Dtg-3tc-1 and dtg-etv-drv-ri drug combinations had no unique pathogens. (Fig. 4). We analyzed the unique pathogens associated with DTG-based and non-DTG-based regimens. Our findings revealed a higher number of unique pathogens in individuals on DTG-based regimens compared to those on non-DTG regimens (Fig. 5). We performed a Chi-square test to see whether there was an association between the pathogens and ART drug regimen. However, the statistical result showed no significant association (*P*-value of 1.0) between the pathogens and the ART drug regimen.

White blood cell counts

We also investigated the correlation of blood cell counts with the patient's pathogen microbiome. We focused on the distribution of bacteria, Eukaryota, and viruses in different white blood cell counts (normal, above normal, below normal as defined by the CBC machine). Under the normal range, the distribution of the 3 domains was even in all the different white blood cells. In the below range, only neutrophils and lymphocytes had all the 3 domains with bacterial and Eukaryota abundance being higher in the neutrophils. In the same range, basophils, eosinophils, and monocytes had only the bacterial domain with basophils and eosinophils having an equal distribution. In the above range, lymphocytes, monocytes, and neutrophils had all the 3 domains present while basophils and eosinophils had only the Eukaryota and bacterial domains present (Fig. 6).

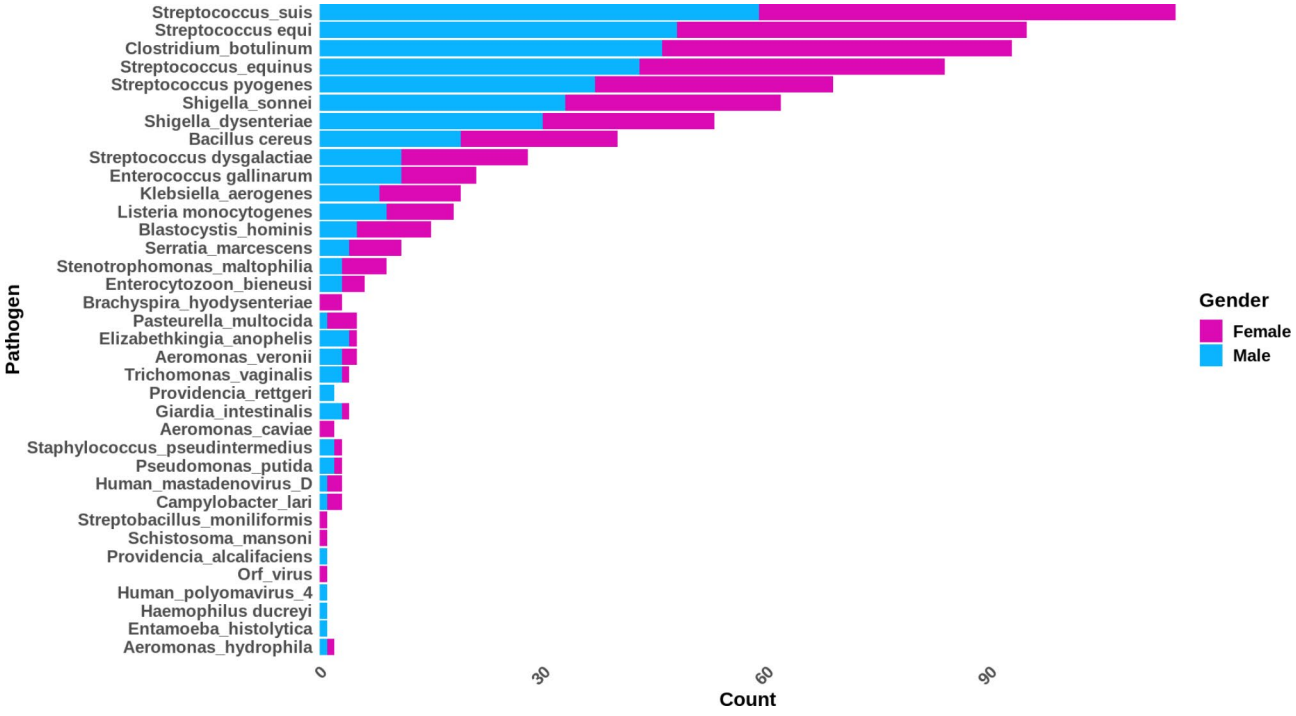

Fig. 2 Bar graph showing the distribution of the 36 known pathogens across gender

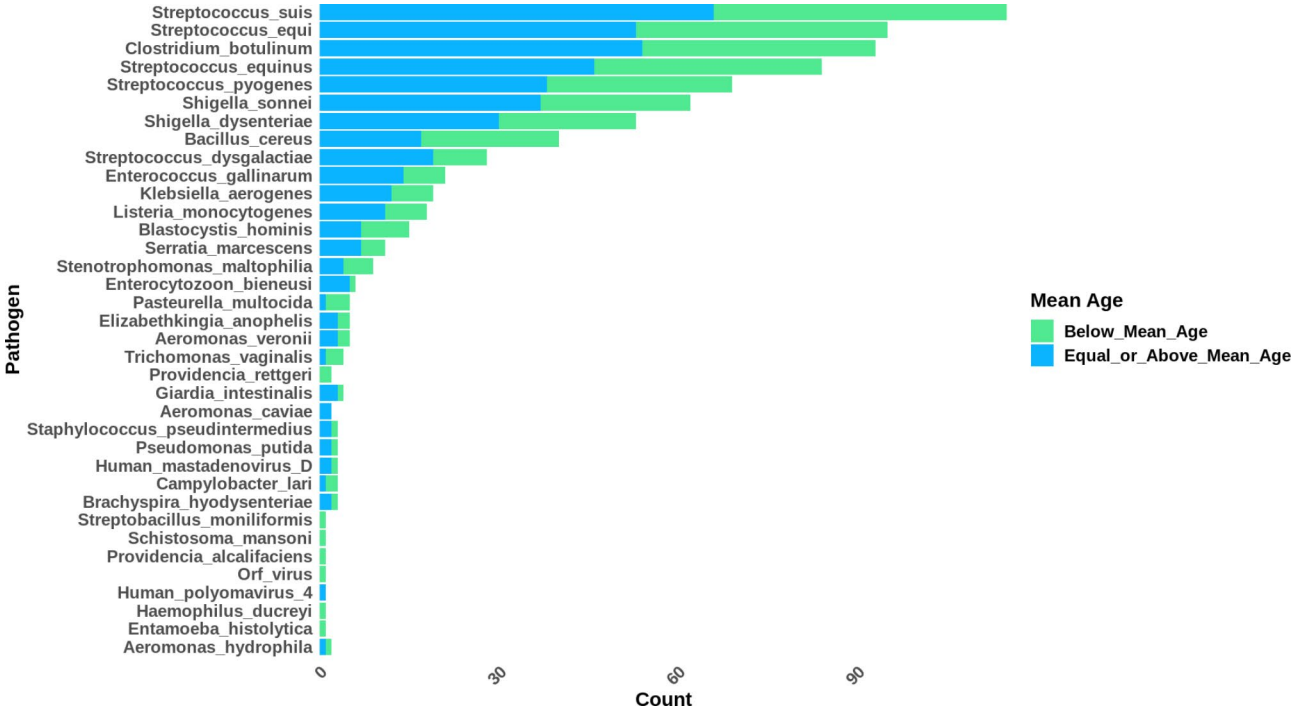

Fig. 3 Bar graph showing the distribution of the 36 known pathogens across age groups; above the mean age and below the mean age

Discussion

Pathobiome analysis

This study explored the utility of shotgun metagenomics of stool in pathogen discovery among HIV-infected children with NMFI in Uganda. We identified a wide range of infectious microorganisms, emphasizing the complexities of NMFI pathogenesis in this susceptible group. A total of 199 pathogens were found in the samples, 36 of which were solely pathogenic in which *S. suis* emerged as the most common pathogen. Among these, some cause

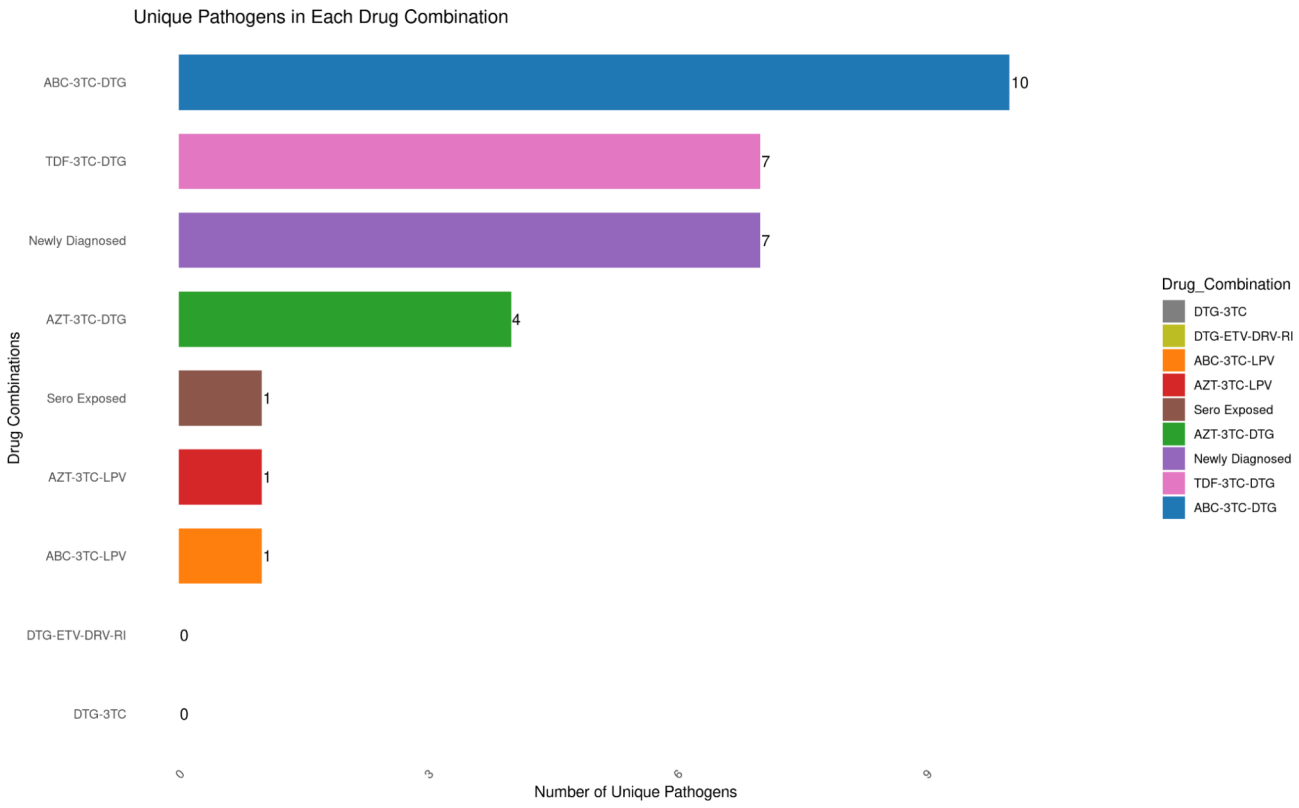

Fig. 4 A bar graph showing unique pathogens in each of the drug regimes taken by the study group

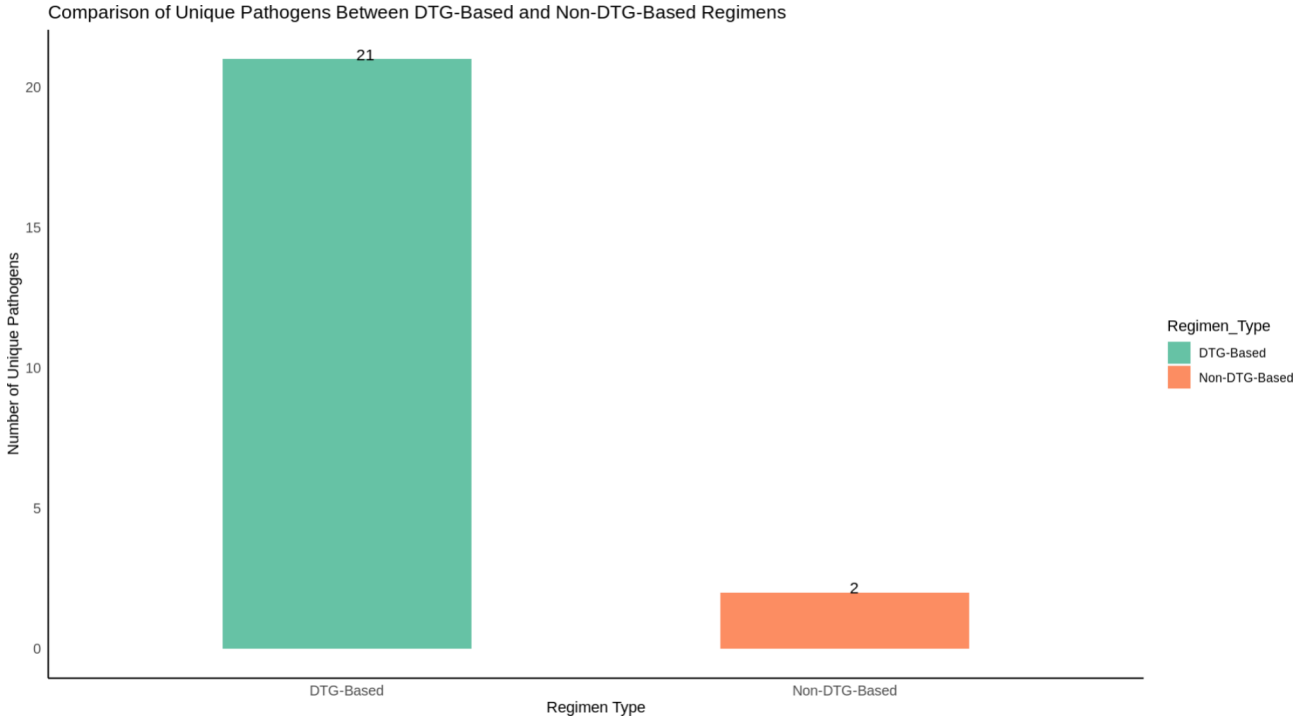

Fig. 5 A bar graph showing unique pathogens identified in DTG based and non DTG based drug regimens

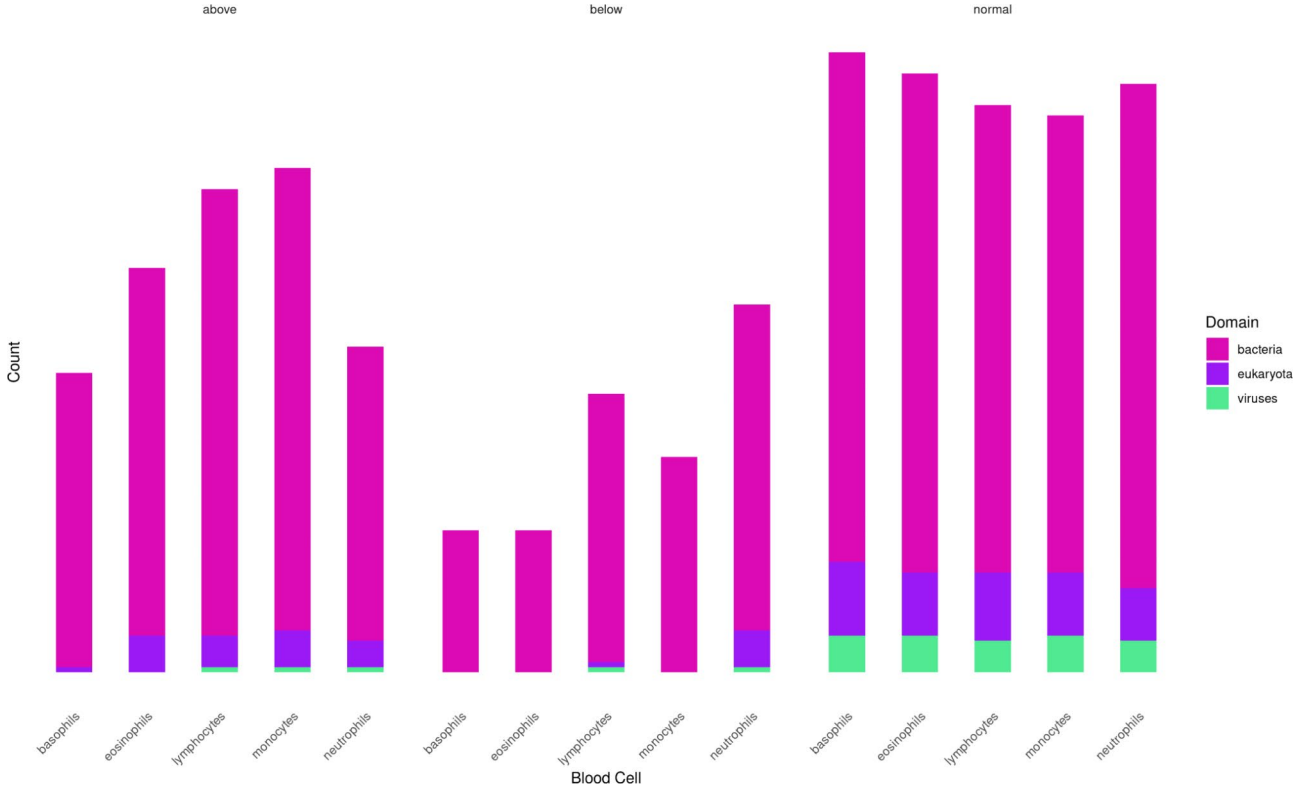

Fig. 6 A bar group showing the distribution of the 36 known pathogens across the different white blood cells under different ranges; Above the normal range, below the normal range and the normal range

sexually transmitted diseases, skin infections, viral, foodborne, zoonotic, and hospital-acquired diseases not forgetting parasitic infections. The variable prevalence of microbes across genders and age groups highlighted the importance of tailored diagnosis and treatment techniques. The data revealed that some microbes were unique to either gender or age group, indicating potential differences in susceptibility or exposure.

Gender and age

The discovery of gender-specific pathogens such as *S. mansoni* [16], *Brachyspira hyodysenteriae*, *A. caviae*, *O. virus and S. moniliformis* in females and *E. histolytica*, *P. alcalifaciens*, *H. polyomavirus 4* and *H. mastadenovirus D* in males suggests that gender-specific factors might influence pathogen exposure and infection. Similarly, age-specific pathogens like *P. rettgeri* in younger children point towards developmental or environmental factors playing a role in pathogen distribution. This emphasizes the need for targeted surveillance and interventions that consider these demographic differences.

Route of transmission and infections

Various pathogens detected spanned multiple transmission routes and caused a range of symptoms. These included sexually transmitted pathogens, zoonotic pathogens, foodborne pathogens, urinary tract pathogens, and parasites. Notable parasites such as *S. mansoni*, *T. vaginalis*, *B. hominis*, *G. intestinalis*, *E. histolytica*, and *E. bieneusi* have been identified in patients who complained of diarrhoea [17]. The detection of sexually transmitted diseases like *H. ducreyi* and *T. vaginalis* in children has raised concerns about potential sexual abuse or transmission during vaginal delivery and caregiver interaction [18, 19]. Foodborne pathogens such as *B. cereus*, *L. monocytogenes*, *C. botulinum*, and *Shigella* species posed significant threats due to their capacity to induce gastrointestinal problems and high infectivity [20, 21]. Zoonotic pathogens such as *Staphylococcus pseudintermedius*, *Streptococcus suis*, and *Pasteurella multocida* are frequently overlooked in routine clinical practices. Understanding and quantifying cross-species interactions and the potential risks posed by emerging zoonotic infectious diseases are crucial, underscoring the importance of considering animal contact as a factor in disease transmission [22]. Hospital-acquired infections added another layer of complexity like *Stenotrophomonas maltophilia*, *P. putida*, and *E. anophelis* complicating treatment and infection control. Furthermore, environmental and opportunistic bacteria such as *Aeromonas species*, *K. aerogenes*, and *S. marcescens* have substantial medication resistance, making infections difficult to

treat [23]. This diverse pathogen profile underscores the complexities and severity of managing febrile diseases in HIV-infected children, necessitating comprehensive and diversified treatment interventions.

Antiretroviral Therapy (ART) regimen

The study delved into the effects of various HIV drug combinations on the pathogen composition within patients' microbiomes. Despite identifying unique pathogen profiles linked to different drug regimens like abc-3tc-dtg, abc-3tc-lpv, and others, chi-square analysis showed no statistically significant association between specific pathogens and the drug regimens.

These findings emphasize the stability of the microbiome in the context of HIV drug therapy, carrying significant implications for treatment strategies and patient management. Importantly, the immunological impact of these regimens on the microbiome needs further exploration, especially regarding how these drug combinations affect immune responses and pathogen dynamics in HIV-infected individuals with non-malarial febrile illness [24–26]. This research highlights the potential for tailored HIV treatments to maintain microbiome stability while managing pathogen presence and patient health.

We observed a greater diversity of unique pathogens in individuals on dolutegravir (DTG)-based regimens compared to those on non-DTG-based regimens. This finding may indicate that antiretroviral therapy influences gut microbial composition. However, it is also plausible that these differences are linked to protease inhibitor-induced diarrhea, which can disrupt the gut microbiota [27]. The influence of pre-existing microbial communities and host factors on these trends requires further investigation. Gaining a deeper understanding of how different antiretroviral regimens affect pathogen dynamics is essential for evaluating their long-term impact on individuals living with HIV.

White blood cells

The analysis of immune cell responses reveals distinct patterns in microbiome composition, with variations linked to the type and severity of infection. Elevated levels of basophils and eosinophils, commonly associated with allergic reactions and parasitic infections, show a correlation with increased bacterial content. This may indicate inflammatory or hypersensitivity responses occurring alongside allergic conditions or parasitic co-infections such as *S. mansoni* [28–30]. Elevated counts of lymphocytes and monocytes are predominantly observed in bacterial infections, underscoring their critical roles in adaptive and innate immune responses essential for managing severe or chronic bacterial challenges such as *S. aureus* [31, 32].

Neutrophils, critical for bacterial defense, play a key role in initial pathogen clearance and the management of opportunistic infections in immunocompromised individuals. Reduced basophil levels in bacterial-dominated cases may reflect diminished inflammatory responses or immune suppression. Conversely, elevated eosinophil and lymphocyte counts, along with lower levels of eukaryotic and viral pathogens, may indicate shifts in immune response dynamics commonly seen in chronic infections or immunocompromised states such as HIV.

Monocytes and neutrophils show a strong correlation with bacterial presence and certain eukaryotic pathogens, emphasizing their roles in phagocytosis and pathogen clearance. Normal levels of eosinophils, lymphocytes, monocytes, and neutrophils are associated with high bacterial counts, moderate eukaryotic presence, and some viral presence, suggesting a balanced and effective immune response [33, 34].

These findings enhance our understanding of immune cell dynamics and highlight the significance of incorporating differential cell counts into tailored clinical strategies for diagnosing and managing diverse infections.

Conclusion

This study highlights the transformative potential of shotgun metagenomics in pathogen identification, providing a diagnostic tool far superior to traditional methods. By detecting a wide array of pathogens without prior knowledge, this approach is crucial for improving the management of NMFI in HIV-infected children, who are immunocompromised and vulnerable to numerous infections. The findings make a compelling case for incorporating metagenomic sequencing into routine diagnostics, particularly in resource-limited settings where NMFI is rampant, to enhance pathogen detection and improve patient outcomes significantly.

Limitations and recommendations

The absence of control groups in our study limited our ability to definitively differentiate between pathogenic, commensal, and opportunistic organisms. This limitation, combined with the inherent challenges of establishing causality—particularly in immunocompromised individuals who are more susceptible to opportunistic infections—represents a significant constraint of our work. Resource and logistical challenges further prevented the inclusion of traditional diagnostic tests, a limitation we have acknowledged in the manuscript. To address these challenges, future studies should incorporate a combination of metagenomics and traditional diagnostic methods to validate findings and enhance understanding of metagenomics' applicability in this context. Furthermore, the lack of detailed data on CD4 counts, viremia, and ART treatment duration restricts

the ability to fully explore immune responses and pathogen dynamics in this population.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-025-10517-1.

Supplementary Material 1

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Author contributions
P.N., S.K., I.S., G.K., and G.M. developed the conceptP.N., S.K., S.N, M.S., K.G., P.N.N., B.M., G.A., S.N., A.K., G.P., A.G., M.P., K.R.C., R.A., P.O., C.M.T., E.K., N.M.L., F.A.K., L.K., and A.K. did the data generation and curation.P.N., S.K, I.S., G.M., B.M., G.A., S.N., A.K., G.P., A.G., M.P., K.R.C., R.A., P.O., C.M.T., E.K., N.M.L., F.A.K., L.K., and A.K. did the methodologyP.N., S.K, I.S., G.M., and D.J did the validationP.N., S.K, I.S., G.K., G.M., and D.J did the visualizationP.N., S.K., S.N, M.S., K.G., P.N.N., B.M., G.A., S.N., A.K., G.P., A.G., M.P., K.R.C., R.A., P.O., C.M.T., G.M., and D.J wrote the original draftAll authors participated in reviewing and editing.

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Data availability
The raw sequences from this project have been deposited in NCBI/SRA under BioProject PRJNA1137832.

Declarations

Ethics approval and consent to participate
This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. All study procedures involving human participants and sample collection were approved by the Infectious Diseases. Institute Research. Ethics Committee. (IDI-REC-046/2022) and the Uganda National Council for Science and Technology (UNCST), ensuring compliance with ethical standards and regulations. Informed consent and assent were obtained from all participants and their legal guardians before sample collection and inclusion.

Consent for publication
All the participants consented to the publication of their identifying data while they were consenting to participate in the study.

Competing interests
The authors declare no competing interests.

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