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Article

Evidence of Exposure to Multiple Zoonotic Pathogens in Humans in Lusaka, Zambia: Insights from Metagenomic Next-Generation Sequencing

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Simple Summary: Zoonotic diseases—those that spread from animals to humans—pose a major health risk in sub-Saharan Africa due to close human-animal contact and limited diagnostic tools. This study examined 47 patients in Zambia suspected of having COVID-19, collecting samples from November 2020 to February 2021 at two major hospitals in Lusaka. Using advanced genetic testing (mNGS), researchers detected a variety of bacteria, viruses, fungi, and parasites, with 57.4% of patients carrying zoonotic pathogens. Some key infections included anthrax, plague, brucellosis, leptospirosis, and rickettsial diseases. However, no strong links were found between infections and factors like age, gender, or underlying health conditions. The study underscores the need for more research to better understand and prevent zoonotic disease transmission in Zambia.

Abstract: Zoonotic diseases present a growing public health challenge, particularly in sub-Saharan Africa (SSA) due to close interactions between humans and animals and poor diagnostic capacity. This pilot study investigated human exposure to zoonotic pathogens in Zambia, among 47 COVID-19 suspected patients from whom nasopharyngeal samples were collected between November 2020 and February 2021 at two major COVID-19 referral centers in Lusaka. Using metagenomic next-generation sequencing (mNGS), the study identified a diverse range of pathogens, including bacterial, fungal, viral, and parasitic species. The prevalence zoonotic pathogens was 57.4%. Noteworthy zoonoses included *Bacillus anthracis*, *Sporothrix schenckii*, *Listeria monocytogenes*, *Yersinia pestis*, *Streptococcus suis*, *Vibrio parahaemolyticus*, *Brucella melitensis*, *Rickettsia prowazekii*, *Shewanella algae*, *Rickettsia japonica*, *Coxiella burnetii*, *Leptospira borgpetersenii*, *Erysipelothrix rhusiopathiae*, *Brucella abortus*, *Bartonella quintana*, *Banna virus*, *Vibrio alginolyticus*, *Bartonella clarridgeiae*, *Rickettsia canadensis*, *Leishmania braziliensis*, *Trypanosoma brucei*, *Pasteurella multocida*, and *Arcobacter butzleri*. Despite moderate diversity in the microbial community, no significant demographic or health-related factors, including age, gender, or comorbidities such as HIV, were found to be statistically associated with zoonotic pathogen infection. The findings provide valuable data on the presence of zoonotic pathogens in humans in Zambia and highlight the need for more comprehensive research into zoonotic diseases in both clinical and non-clinical settings.

Keywords: zoonoses; zoonotic pathogens; metagenomic next-generation sequencing; COVID-19 and zoonotic pathogens; Zambia; zoonotic pathogens in humans

1. Introduction

Zoonotic diseases, which are transmitted between animals and humans, represent a major and growing public health challenge, particularly in sub-Saharan Africa (SSA) [1]. The close interaction between humans, livestock, and wildlife in this region creates a perfect environment for pathogen spill-over [2–5]. A key concern in SSA is the potential for zoonotic coinfections, where the presence of multiple pathogens in an individual can complicate diagnosis and worsen disease outcomes. For instance, co-infections with malaria - a leading endemic disease in SSA - and zoonotic pathogens like leptospirosis or rickettsiosis can result in more severe clinical manifestations, making it difficult to pinpoint the underlying cause and delay timely treatment [1,6]. Similarly, co-infection between zoonotic agents and emerging diseases like Coronavirus Disease 2019 (COVID-19) and others can exacerbate disease severity and increase mortality rates [1,6–8].

This challenge is compounded by the limitations of current diagnostic strategies, particularly the reliance on syndromic diagnosis. This often fails to account for the wide diversity of pathogens that could be causing illness, leading to misdiagnosis or under-diagnosis [9]. Lack of comprehensive surveillance and pathogen-specific diagnostics may hinder timely interventions [10,11]. In SSA, including Zambia, the full range of zoonotic pathogens contribution to human illness outside large outbreaks remains poorly understood. A few studies have reported a 63% increase in zoonotic disease outbreaks between 2012 and 2022 [3]. Additionally, between 2000 and 2022, some countries in SSA (Cameroon, Central African Republic, Cote d'Ivoire, Democratic Republic of Congo, Gabon, Ghana, Kenya, Liberia, Madagascar, Mauritania, Mozambique, Namibia, Nigeria, Senegal, Sierra Leone, South Africa, South Sudan, Sudan, Tanzania, Uganda, Zambia, and Zimbabwe) reported a combined 28,934 human cases of zoonotic diseases, with 1,182 associated deaths (a fatality rate of 345.4 per 1000 population) [3]. Pathogens such as *Rickettsia* sp (770.4 per 1000), *Toxoplasma* sp (678.11 per 1000), Influenza A (523.81 per 1000), *Coxiella burnetii* (489.04 per 1000), and *Brucella* sp (484.00 per 1000) had some of the highest attack rates in the region, while filoviruses (>600 per 1000) and *Leptospira* sp (500 per 1000) were most frequently associated with fatalities [3].

Despite some reports of zoonotic diseases in humans in SSA, much of the research has focused on their presence in domestic and wild animals, with less attention paid to their impact on human health [12–15]. In Zambia, for instance, zoonoses are often neglected in diagnostic protocols at public health facilities, contributing to a lack of awareness and poor recognition of their impact on human morbidity and mortality [13]. This gap in knowledge is particularly concerning given Zambia's vulnerability to emerging infectious diseases and the increasing risks of pathogen spill-over. A more comprehensive understanding of zoonotic diseases in humans is crucial not only to observe trends but also to actively intervene and mitigate these threats. By identifying the most common pathogens, determining infection hotspots, and pinpointing vulnerable populations, we can use this data to design pre-emptive, effective public health and clinical interventions and prevention strategies.

To address these knowledge gaps, this study aimed to investigate human exposure to zoonotic pathogens in Zambia, using samples from suspected COVID-19 patients in Lusaka. The findings are intended to raise awareness of zoonotic pathogens among public health decision-makers and clinicians, and lay the groundwork for larger, collaborative investigations into the broader landscape of clinically relevant zoonotic diseases in human populations across Zambia.

2. Materials and Methods

2.1. Study Population and Participant Enrolment

This pilot study was carried out at two major COVID-19 referral centers in Lusaka, Zambia: the University Teaching Hospital (UTH) and Levy Mwanawasa University Teaching Hospital (LMUTH), between November 2020 and February 2021 as previously reported [16]. Individuals aged 18 years and older, presenting to the emergency department with suspected SARS-CoV-2 infection, were eligible for participation. Following informed consent, participants were enrolled and completed a

detailed questionnaire capturing their demographic information, clinical symptoms, pre-existing health conditions, and comorbidities (HIV, diabetes, hypertension, cancer, chronic respiratory disease).

2.2. Specimen Collection and Laboratory Testing

Each participant provided a nasopharyngeal swab. The nasopharyngeal swab was placed into 3 mL of viral transport media for both rapid antigen and reverse transcriptase polymerase chain reaction (RT-PCR) testing. The RealStar SARS-CoV-2 RT-PCR kit (Altona Diagnostics GmbH, Germany) was used for RT-PCR testing. The positivity of samples and presence of zoonotic pathogens was also evaluated using metagenomic next-generation sequencing (mNGS) through the NovaSeq 6000 (Illumina, San Diego, CA, USA) PCR machine as previously reported [14,17,18].

2.3. Metagenomic Next-Generation Sequencing

RNA extracted from each of the nasopharyngeal swabs from 47 patients was subjected to mNGS for microbiome analysis. The library preparation was performed using the NEBNext Ultra II RNA protocol (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's guidelines (New England Biolabs, 2024). In brief, RNA extracts were fragmented and spiked with the External RNA Controls 103 Consortium (ERCC) collection (ThermoFisher, Waltham, MA, USA), followed by complementary DNA (cDNA) synthesis. The cDNA was then ligated with adaptors, which were subsequently digested. The DNA was barcoded and purified using a magnetic rack. Library size and concentration were assessed using the 4150 TapeStation system (Agilent, MA, USA). The flow cell was washed to remove salts, and the library was denatured with sodium hydroxide (NaOH). PhiX was included as a calibration control. Sequencing was performed on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA).

2.3.1. Analysis of mNGS Reads

The mNGS data was processed and analysed in the CZ ID sequencing pipeline (Chan Zuckerberg Biohub, Inc, San Francisco, USA), a cloud-based bioinformatics tool (Kalantar et al., 2020; Munjita et al., 2022). At first, the raw read sequences were filtered to exclude host sequences to ensure data quality. The process involved data validation using the CZ ID software, adapter trimming and removal of low quality reads as well as removal of host and human data as well as duplicate reads. This was followed by read alignment against taxonomic categories in NCBI NT and NR databases by Minimap2 and Diamond (Buchfink et al., 2015; Li, 2018). The contigs were assembled by SPAdes (Bankevich et al., 2012) followed by contig alignment against custom nucleotide and protein NCBI databases. The final step was reporting and visualisation of results. Heatmaps for the identified species of microbes were generated after application of a background model and setting threshold limits.

2.3.2. Quality Control Metrics

The background correction model was applied to eliminate taxa that may have been prevalent in water controls and passed through filtration. This model was generated using sequence data from negative water controls. The water used for RNA extraction served as the control. In accordance with the model's application, the Z-score metric was set at 100 to account for potential background contamination from the water controls, ensuring that only microbes present in the samples were included in the analysis. The threshold for nucleotide reads per million (NT_rPM) was set to a minimum of 5 to comprehensively assess the mosquito microbiome. NT_rPM is a scaled metric that reflects the abundance of taxa, with smaller values indicating a lower likelihood of a particular organism's presence in the sample. To maintain specificity, the NT_rPM threshold was set to include only reads specific to particular taxa.

2.3.3. Bioinformatic Analysis

The relative abundance of microbes was assessed using the NT_rPM metric within the CZ ID pipeline. Heatmaps were generated to present the relative abundance of zoonotic microbial species only, as the dataset was too large to display all heatmaps. Microbial diversity across all microbes and within specific categories (bacteria, fungi, parasites, and viruses) was measured using the Shannon diversity index, calculated with the AL Young Biodiversity calculator and manual data manipulation in Excel. Shannon index values typically range from 0 (no diversity) to 4.5 (high diversity). The dominance of microbial species (proportion of most abundant species in a community) was evaluated using the Berger-Parker Index. The Berger-Parker Index is represented by 0 (perfect evenness, where all species are equally represented) and 1 (one species dominates entirely).

2.4. Statistical Analysis

Descriptive data were analyzed in Excel and results were presented in tables and graphs. Differences in species diversity and abundance among bacteria, fungi, parasites, and viral microbes were assessed using the Kruskal-Wallis test in SPSS Ver. 21 (IBM Corp, Armonk, NY, USA). A p-value of less than 0.05 was considered statistically significant. To examine associations between demographic and health-related factors (age, gender, HIV status, hypertension status, diabetes status, cancer status, and CRD status) and positivity for zoonotic pathogens, the Pearson chi-square test was applied. The association was considered significant if $p < 0.05$. A multivariable logistic regression analysis was then conducted to identify independent factors associated with zoonotic pathogen positivity. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to assess the strength of these associations. Independent variables were considered significant risk factors when $p < 0.05$.

2.5. Ethical Considerations

Ethical approval for this study was granted by the Excellence in Research Ethics and Science (ERES) Converge Ethics Committee IRB (Ref. No. 2020-JUL-07). Additionally, consent was obtained from all participants and/or their guardians. All participants were informed about the study procedures through an information sheet and asked to sign or provide a thumbprint on an informed consent form before sample collection. Participant information was kept anonymous using codes.

3. Results

3.1. Demographic Characteristics

A total of 47 study participants were enrolled, consisting of 42.6% females (20/47) and 57.4% males (27/47). Among the participants, 17.0% (8/47) were living with HIV while 19.1% (9/47) had various other comorbidity conditions. In terms of zoonotic pathogen positivity, 57.4% (27/47) of participants tested positive for at least one zoonotic pathogen. Among those with comorbidities (17/47), 52.9% (9/17) tested positive for at least one zoonotic pathogen. The majority of these individuals were HIV positive, with 66.7% (6/9) of the zoonotic pathogen-positive participants being HIV-infected.

3.2. Diversity and Dominance of Microbes

RNAs from a total of 3171 microbes, of which 87 were pathogenic, were detected in all the 47 samples representing 50.9% (1615/3171) bacterial species, 47.9% (1519/3171) fungi, 0.79% (25/3171) viruses, and 0.38% (12/3171) parasites. A total of 723,813 microbial sequencing reads were obtained, with bacteria comprising 67.95% (491,807.1) of the reads, followed by fungi at 28.36% (20,294.11), viruses at 3.52% (25,453.25), and parasites at 0.17% (1,259.52). In terms of overall species diversity across all microbial categories, the Shannon index (H') of 2.393 indicated moderate biodiversity within the microbial community. There was a significant difference in the diversity of microbes across

different microbial groups ($p = 0.00002$). Bacterial microbes exhibited the highest diversity ($H' = 3.221$, $d = 0.080$), followed by fungi ($H' = 3.421$, $d = 0.071$). In contrast, viruses and parasites displayed low species richness and uneven distribution, with H' values of 1.029 ($d = 0.470$) and 1.900 ($d = 0.274$), respectively.

3.3. Prevalence and Abundance of Zoonotic Pathogens

Pathogenic microbes made up a small proportion of the total microbes, at 2.7% (87/3,171), with 0.8% (24/3,171) being zoonotic pathogens. Of the 87 pathogenic microbes, 27.6% (24/87) were of zoonotic origin (Figure 1). The overall prevalence of zoonotic pathogens in the study population was 57.4% (27/47). The prevalence among males was 55.6% (15/27) compared to 60% (12/20) among females. The most prevalent zoonotic pathogens were *Staphylococcus pseudintermedius* (21.3%), *Bacillus anthracis* (8.5%), *Sporothrix schenckii* (8.5%), *Arcobacter butzleri* (6.4%), *Listeria monocytogenes* (4.3%), *Yersinia pestis* (4.3%), and *Coxiella burnetii* (4.3%) (Figure 1). Meanwhile, *B. anthracis* and *Pasteurella multocida* were the most abundant organisms. The abundance and distribution of zoonotic pathogens is shown in the heatmap in Figure 2.

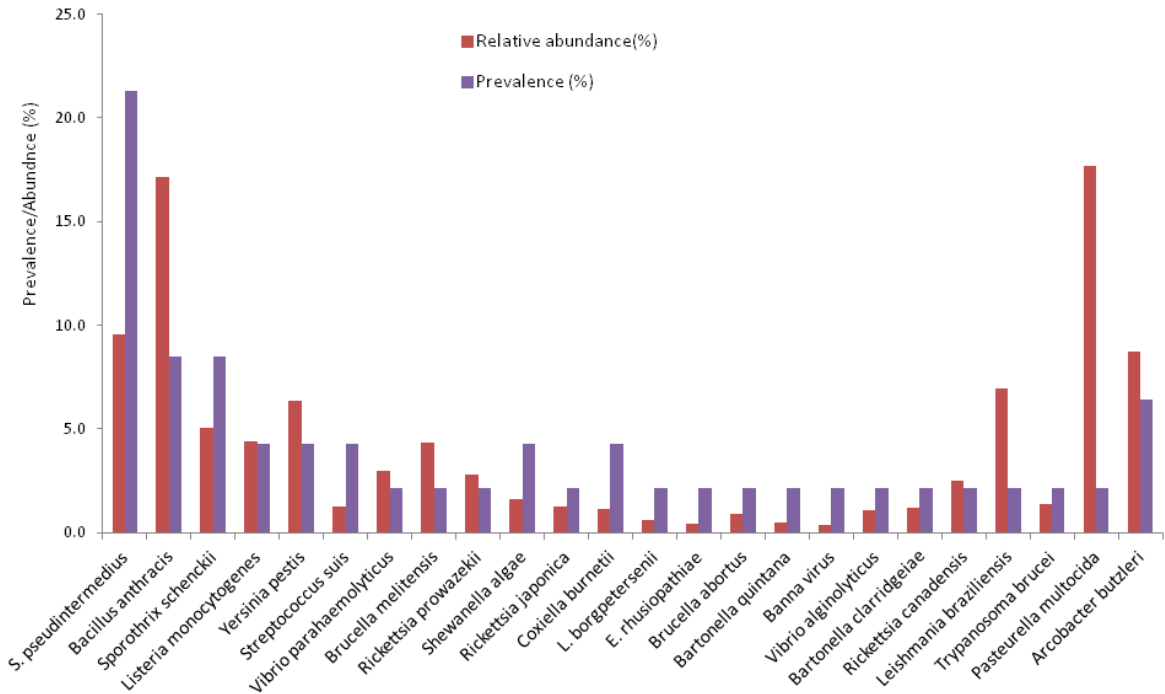


Figure 1. Prevalence and abundance of all 24 zoonotic pathogens detected in 27 out of 47 patients.



Figure 2. Heatmap of zoonotic pathogens detected in 27 human samples. Microbes which are not present in the sample are represented by white and gray cells. White cells mean that the pathogens were completely absent while gray cells mean the pathogens were filtered out after application of the background model and other metrics as defined in the methods. For the other colours, a denser shade indicates a higher likelihood of high pathogen abundance.

3.4. Risk Factors for Infection with Zoonotic Pathogens

Although the sample size was low, the chi-square (χ^2) test and multivariate logistic regression analysis were used to assess the potential influence of demographic and health-related factors on infection with zoonotic pathogens. There was no association between the factors and infection with zoonotic pathogens and none of the variables (gender, age, comorbidities, or HIV status) were found to be statistically significant predictors of zoonotic pathogen infection ($p > 0.05$) (Table 1).

Table 1. Likelihood estimates for occurrence of Zoonoses in humans.

Variable	chi-square (χ^2) test	Multivariate logistic regression			
	<i>p-value</i>	Level	Odds ratio	95% CI	<i>p-value</i>
Gender	0.502	Male	0.951	0.280 - 3.227	0.936
		Female	Ref		
Age	0.306	Adult	1.191	0.300 - 4.723	0.804
		Youth	Ref		
Comorbidities	0.434	Present	2.667	0.441- 16.147	0.286
		Absent	Ref		
HIV	0.242	Positive	0.179	0.020 - 1.593	0.179
		Negative	Ref		

* CI = Confidence interval; Ref = Reference category.

3. Discussion

This pilot study explored the presence and diversity of zoonotic pathogens in patients presenting with suspected SARS-CoV-2 infection at two major COVID-19 referral hospitals in Lusaka, Zambia. By utilizing metagenomic next-generation sequencing (mNGS), 24 zoonotic pathogens were identified in nasopharyngeal samples from 47 patients. Despite the high prevalence of zoonotic pathogens, no significant associations were found between demographic factors (such as gender, age) or health-related conditions (including HIV status and comorbidities) and the likelihood of infection with zoonotic pathogens.

This is the first comprehensive insight into the diversity of zoonotic pathogens in humans in Zambia. Prior to this, most studies largely focused on domestic and wildlife reservoirs, with limited understanding of the role such pathogens play in human illness [12,13,15,17–22]. Therefore, the study

fills a crucial gap in the current literature. The high prevalence of zoonotic pathogens could be indicative of under-recognized clinically relevant zoonotic risks in Zambia and may call for changes in public health guidelines concerning diagnosis and treatment [23].

A range of zoonotic pathogens were identified. Among these, *Coxiella burnetii*, known to cause Q fever, was detected for the first time in humans in the country and prior to this in rodents [22]. *C. burnetii* is associated with severe respiratory illness and is a known zoonotic threat [5,24]. Other notable first-time reports included *S. schenckii* and *Bartonella* species recently reported in rodents by Munjita et al, further highlighting the diverse range of zoonotic pathogens circulating in the human and animal population [14]. *S. schenckii*, is a dimorphic fungus commonly associated with zoonotic transmission through contact with infected dogs and cats [25]. It is known for causing sporotrichosis, a condition that can manifest as skin lesions and occasionally spread to the lymphatic system, bones, and other organs [25].

Bacillus anthracis, which causes anthrax, was detected during a period when Zambia was experiencing sporadic outbreaks of the disease culminating into a major outbreak in 2023 [26]. This temporal overlap suggests that the potential for zoonotic pathogens to cause public health crises remains high, particularly in areas with significant livestock populations. Additionally, pathogens commonly associated with seafood consumption, such as *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, were also identified, pointing to risks related to the consumption of marine products [27]. Furthermore, the study found *Banna virus* for the first time in human samples from Zambia, adding to the growing list of emerging viral zoonoses that have been identified in the region. *Banna virus* is an arbovirus that circulates in mosquitoes [28].

Rickettsial pathogens, which have been reported in humans in Zambia before [21], were also detected in a number of individuals in this study. The high presence of *rickettsiae* in this study mirrors similar findings from other African countries, which have increasingly recognized rickettsial diseases as important causes of febrile illness [3,29]. These glaring findings suggest the need to include rickettsial pathogens in diagnostic protocols in hospitals.

Meanwhile, detection of *Leptospira borgpetersenii* in this study also marked the first report of a *Leptospira* sp in humans in Zambia. Previous reports were in rodents and bats [30,31]. *Leptospira* sp cause leptospirosis in humans, a zoonotic disease that is transmitted through contact with water or soil contaminated by the urine of infected animals [4]. Leptospirosis is often associated with flooding and environmental contamination, and the detection of *L. borgpetersenii* in Zambia is particularly noteworthy, as the country shares ecological and epidemiological links with regions such as Tanzania, which experience frequent outbreaks of leptospirosis [32].

This study also provides the first report of *Leishmania braziliensis* and *A. butzleri* in human samples in Zambia. The most recent report about *Leishmania* sp was in dogs in the south of Zambia not long ago [33]. This study builds on that finding but this time in humans. *L. braziliensis*, a causative agent of cutaneous leishmaniasis, is a notable pathogen with significant public health implications, especially in tropical regions. The presence of this pathogen underscores the need for increased vigilance in monitoring neglected tropical diseases that may be emerging in Zambia.

A. butzleri, a bacterium associated with gastrointestinal infections, has been increasingly recognized as a zoonotic pathogen, particularly in the context of contaminated food and water [34]. Its identification in this study highlights the growing importance of foodborne pathogens in human health and emphasizes the need for comprehensive surveillance systems to detect and address these emerging threats. Additionally, *L. monocytogenes*, a pathogen typically associated with contaminated food sources was also detected in this study. While *L. monocytogenes* has primarily been reported in foods in Zambia in the absence of outbreaks, its identification in active human infections is rare [35,36]. The discovery of *L. monocytogenes* warrants further investigation into its role in human infections in the clinical and non-clinical settings, where asymptomatic or subclinical cases may go undiagnosed.

While the study's findings are illuminating, there are some limitations to consider. First, all participants were enrolled at hospitals due to suspected COVID-19 infection, and it is possible that

some individuals had self-treated with antibiotics prior to hospital visits. This may have reduced the abundance of bacterial pathogens detected by mNGS, as antibiotics could have suppressed microbial growth. Furthermore, the lack of data regarding the specific geographic locations of participants makes it difficult to conduct future follow-up studies on zoonotic risks in particular hotspots. The relatively small sample size of 47 participants, though revealing a high prevalence of zoonotic pathogens, may have been insufficient to fully understand the risk factors for zoonotic pathogen infection in the broader population. Additionally, blood samples were not used in this study meaning there could be under-reporting of most pathogens. Some of the pathogens may have come from the food residues that patients may have eaten and not really an infection in the patients, an indication of possible modes of exposure to zoonoses hence the title of this study. Despite these limitations, this study provides valuable data on the presence of zoonotic pathogens in humans in Zambia and highlights the need for more comprehensive research into zoonotic disease in clinical and non-clinical settings especially in the light of poor health systems in low income countries [37].

5. Conclusions

The detection of a diverse array of zoonotic pathogens, many of which are emerging or under-recognized, underscores the importance of strengthening surveillance in humans and diagnostic capacity. This is critical for mitigating risks associated with both known and unknown pathogens in this rapidly changing global health landscape.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Excellence in Research Ethics and Science (ERES) Converge Ethics Committee IRB of Lusaka, Zambia (Ref. No. 2020-JUL-07).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study is available from the corresponding author upon reasonable request.

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