Hepatitis E virus infection in high-risk populations in Osun State, Nigeria

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\begin{abstract}
Hepatitis E virus (HEV) infection is an emerging infection that is of major public health concern, especially in some vulnerable groups like immunosuppressed individuals, pregnant women and HBV-coinfected individuals. HEV is transmitted faecal/oral or zoonotically depending on the HEV-genotype. This study aimed at investigating HEV infections among different at-risk populations in Osun State, Southwestern Nigeria. A total of 720 serum samples were collected from animal handlers, pregnant women, people living with HIV/AIDS, and Hepatitis B virus (HBV) infected individuals. Commercially available Enzyme-Linked Immunosorbent Assays (ELISA) were used for the detection of anti-HEV total and IgM antibodies. Polymerase chain reaction (PCR) was carried out in the HEV seropositive samples and all the samples from individuals infected with HBV. Descriptive analysis and chi-square test of association were performed.

The anti-HEV total antibody seroprevalence in HIV-positive individuals, animal handlers and pregnant women was 11.4\% (n = 47/411), 7.9\% (n = 7/89), and 6.3\% (n = 10/160), respectively. Markers of acute HEV infection (anti-HEV IgM) were detected in 2.2\% of HIV-positive individuals (n = 9/411) and 1.8\% of animal handlers (n = 2/89), respectively, and in 0.6\% of pregnant women (n = 1/160). However, all samples were HEV RNA negative. This study analysed the presence of markers of HEV infection among different at-risk populations without clinical symptoms of HEV infection. Our results showed that HEV is an underestimated threat to public health in Nigeria and underlines the need of an HEV surveillance system to understand the distribution and transmission of HEV infection in animals and/to humans.
\end{abstract}

1. Introduction

Hepatitis E virus (HEV) has emerged as a threat to public health, with an estimated number of 70,000 deaths from genotypes 1 and 2 globally \cite{1}. HEV is a quasi-enveloped, single-stranded, positive-sense RNA virus with a genome size between 6.4 and 7.2 kb \cite{2,3}. Classified into the family \textit{Hepeviridae} family \textit{Orthohepeviridae} \textit{A}, HEV has eight known genotypes of which four are pathogenic to humans (HEV-genotypes 1–4); however, the HEV-genotype 7 initially found in dromedary could be also detected in immunosuppressed individuals \cite{4,5}.

HEV-genotypes 1 and 2 (HEV-1 and -2) are transmitted via the faecal-oral route and exclusively infect humans. Infections with HEV-1 can be more severe in pregnant women, where case fatality rates up to 25\% have been reported \cite{6}. Also, certain population groups like immunosuppressed persons and individuals with pre-existing liver disease are more vulnerable to infections with HEV \cite{7}. HEV genotypes 3 and 4, on the other hand, have been found in both humans and animals and are transmitted zoonotically via the ingestion of raw or undercooked meat and close contact to infected animals \cite{8}. Furthermore, HEV-3 and -4 can lead to chronic infections, especially in...
immunocompromised individuals. In patients with chronic HBV infection, super-infection with HEV is a common cause of liver failure, accounting for 20% of cases in regions endemic for HEV [8,9]. Furthermore, in some studies [10] a higher HEV seroprevalence in individuals with HBV-related liver disease in comparison to healthy individuals has been observed.

In Nigeria, only a few HEV seroprevalence studies have been performed until today which detected a seroprevalence (IgG and total antibodies) between 7.0%–66.7% in different populations [11–13]. In one study, the prevalence of HBV/HEV coinfection was analysed in Nigerian healthcare workers, reporting a coinfection rate of 27.3% [14]. Furthermore, an anti-HEV IgM antibody seroprevalence between 0.4%–9.0% was reported [13,15,16]. Two outbreaks of HEV have been documented in Nigeria; one small outbreak in Port Harcourt South-South Nigeria involving ten people between November 1997–June 1998 [17] and a recent outbreak in 2017 with about 1815 individuals infected with HEV [18]. While during the first outbreak, only HEV-2 was reported, the second outbreak was caused both by genotypes 1 and 2 [19,20]. There is a dearth of information on HEV infection in Nigeria, especially in at-risk populations. Therefore, this study aimed to analyse the prevalence of serological markers of HEV infection and/or HEV RNA in HIV-positive individuals, HBV-positive individuals, animal handlers and pregnant women.

2. Materials and methods

2.1. Sampling

A total of 660 serum samples were collected from consenting human participants that included apparently healthy animal handlers (n = 89; of which pig farmers n = 15 and cow butchers n = 74), pregnant women (n = 160) and HIV-infected individuals (n = 411) in Osun State, Southwestern Nigeria (Table 1). Convenience sampling technique [21] was used in recruiting the study participants, and demographic data were obtained. Samples were collected between February 2014 to May 2017.

2.2. Ethical approval

The Health Planning Research and Statistics Department of Osun State Ministry of Health gave the ethical clearance (OSHREC/PRS/569 T/52) for this study. Informed consent for participants under the age of 18 years was obtained from consenting parents or guardians. The molecular analysis also included 60 serum samples from HBV-infected individuals collected in 2014 from outpatients in three tertiary hospitals from an initial study [22].

2.3. Serology

To detect anti-HEV total antibodies, samples were analysed using the MP diagnostics HEV ELISA 4.0 (MP Biomedicals Asia Pacific, Singapore). Samples positive for anti-HEV total antibodies were further screened for acute infection using the MP diagnostics HEV IgM ELISA 3.0 (MP Biomedicals Asia Pacific, Singapore). For the samples of HBV-infected individuals only PCR was performed due to the low sample material.

2.4. RT-PCR and genotyping

RNA was extracted from a 140 μl mixture (135 μl serum and 5 μl MS2 phage-internal control) by using the QiAmp Viral RNA kit (Qiagen, Hilden, Germany) and the QiaCUBE robotic machine according to the manufacturer’s instruction. The extracted products were stored at −80 °C before use. HEV RNA detection and subsequent genotyping of samples positive for anti-HEV total antibodies and the samples from HBV-infected individuals was performed using two different PCR assays: a one-step RT-nested PCR and a one-step RT-semi-nested PCR assay with primers lying in the ORF 1 and ORF 2 region of the HEV genome, respectively (Table 2). Primers were designed based on conserved regions in multiple sequence alignments of the ORF 1 and ORF 2 region of HEV-1 - HEV-4 [23]. The primers were made available by the FG 15 group of the Robert Koch Institute Berlin, Germany.

The same amplification conditions were used for the ORF 1 and ORF 2 PCR assays as described previously [13]. Briefly, the first-round PCR was performed in a volume of 25 μl (5.8 μl water, 5 μl 5 RT/PCR buffer (2.5 mM Mg), 5 μl 5 Q-solution, 1 μl 10 mM dNTP’s, 1 μl of antisense primer, 1 μl of sense primer, 0.2 μl RNasin (400u/μl), 1 μl enzyme mix (RT/TaqPol) and 5 μl template) using the Qiagen One-Step RT-PCR Kit (Qiagen, Hilden, Germany) and a Biometra Trio thermocycler (Biometra, Jena, Germany). Cycling conditions for the first reaction were 50 °C for 30 min, 95 °C for 5 min followed by 10 cycles of 95 °C for 30 s, 60 °C (1-cycle) for 30 s, 72 °C for 45 s, and another 35 cycles at 95 °C for 30 s, 52 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 7 min.

Twenty-five microlitre volumes containing 10.5 μl of water, 0.5 μl of sense and antisense primer, 12.5 μl Hot start Master Mix (Qiagen, Hilden, Germany) and 1 μl template- product from the first-round PCR was used for the second-round PCR.

Cycling parameters for the nested and semi-nested PCR were as follows: 95 °C for 10 min, followed by 40 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 45 s, with a final extension at 72 °C for 5 min. A plasmid containing the HEV genotype 3 (HEV_RKI [GenBank accession no. FJ956757]) was served as positive control.

2.5. Statistical analysis

The data generated from this study were analysed anonymously using descriptive analysis and chi-square for the test of association with STATA package version 14.2 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). Significance was set at p < 0.05 and X² is the Pearson chi square value. The descriptive analyses are presented as simple percentages and interquartile range.

Table 1

| Samples (n) | Source |
|------------|--------|
| Animal handlers (89) | Abattoirs around the State |
| Pig handlers (15) | Farms and Association meeting |
| Pregnant women (160) | Antenatal clinics around the State Capital (5 clinics) |
| HIV-infected Individuals (411) | HIV Clinic (Institute of Human Virology, Nigeria Clinics LAUTECH Osogbo) |
| HBV-infected individuals (60) | Outpatients [24] |

Table 2

| Primer sequences | Sequence (5’-3’) | Location | Polarity |
|-----------------|-----------------|----------|----------|
| ORF 1 | GAGGCGATGGTGGAGAARG | 4084–4102 | + |
| HEV-39 | GCCATGTCAGACGACGTTC | 4622–4641 | – |
| HEV-37 | GTCTGCGTGTGTAAGAARG | 4277–4297 | + |
| HEV-27 | TCRCCAGAGTGYTTCTTCC | 4583–4566 | – |
| ORF 2 | CCGACGAATTGTAGTGC | 6296–6317 | + |
| HEV-31 | GTCTGCACTTGCTG | 6750–6733 | – |
| HEV-32 | GTCTGACAAATTGGGAGC | 6350–6372 | + |

*Based on HEV-1 GenBank accession number M73218
3. Results

3.1. Characteristics of the study cohort

In total, 720 serum samples from HIV-positive individuals, animal handlers, HBV-infected individuals and pregnant women were analysed for markers of HEV infection. The total study participants were mainly female (70.7%; n = 509/720) and had a median age of 34.5 years (IQR: 28–42). The median age in the HIV-infected individuals, animal handlers, pregnant women and HBV-positive individuals was 39 (IQR: 33–47), 35 (IQR: 27–45), 27 (IQR: 24–31.5) and 32 (IQR: 24.5–39) respectively (Table 3). Of the 160 pregnant women, 27.5% (n = 44/160) were in the first trimester, while 48.8% (n = 78/160) and 23.8% (n = 38/160) were in the second and third trimester, respectively. Of the sixty individuals with HBV, 48.3% (n = 29/60) were female and 51.7% (n = 31/60) were male. The treatment status of the HBV- and HIV-infected individuals is not known.

3.2. Anti-HEV seroprevalence

The seroprevalence of anti-HEV total antibodies was highest in HIV-positive individuals 11.4% (n = 47/411), followed by animal handlers 7.9% (n = 7/89) and pregnant women 6.3% (n = 10/160) (Table 4). For anti-HEV IgM, a seroprevalence of 2.2% was detected in both, HIV-positive individuals and animal handlers (n = 9/411 and n = 2/89, respectively), while in pregnant women 0.6% of individuals (n = 1/160) were anti-HEV IgM-positive. In the HIV-positive individuals, the highest seroprevalence of both anti-HEV total and IgM antibodies was detected in the age group of >60 year olds (22.7%; n = 5/22 and 9.1%; n = 2/22) (Table 4). In contrast, in the animal handlers only individuals <40 years of age tested positive for anti-HEV total and IgM antibodies. In the subgroup of pregnant women, the highest anti-HEV total antibody seroprevalence was detected in the age group of 10–19 year olds.

In the HIV-positive individuals, the seroprevalence of anti-HEV total and IgM antibodies was higher in men (total antibodies: 13.9%; IgM: 6.5%) than in women (total antibodies: 10.6%; IgM: 0.7%). None of the women from the animal handler’s cohort was positive for anti-HEV antibodies (total and IgM), while in men a prevalence of anti-HEV total antibodies and IgM was detected in 9.7% (n = 7/72) and 2.8% (n = 2/72), respectively. In the animal handlers anti-HEV antibodies were exclusively detected in cow butchers (total antibodies; 9.5%; IgM: 2.7%).

A statistical significance was only found for the anti-HEV IgM seroprevalence in HIV-infected individuals, which was significantly higher in men than in women (p < 0.01) (Table 5). In the different stages of pregnancy, the highest seroprevalence of anti-HEV total antibodies was detected in women in the second trimester with 7.7% (n = 6/78) followed by first trimester 6.8% (n = 3/44), and least in the third trimester, 2.6% (n = 1/38) (Table 6). Anti-HEV IgM antibodies were detected in only one woman (1.3%) in the second trimester.

### Table 3

| Age (years) | Total | Male | Female | Gender | 0-9 | 10-19 | 20-29 | 30-39 | 40-49 | 50-59 | >60 | Median age | IQR | N (%) |
|-------------|-------|------|--------|--------|-----|-------|-------|-------|-------|-------|----|----------|-----|-------|
| Total       | 411   | 108  | 303    | Gender | 108 (26.0) | 303 (73.7) | 7 (1.7) | 6 (1.5) | 44 (10.7) | 156 (38.0) | 123 (30.0) | 53 (13.0) | 22 (5.4) | 39 | 33-47 |
| 0-9         | 7     | 7    | 0      |        | 4 (4.5) | 20 (22.5) | 29 (32.6) | 17 (19.1) | 13 (8.3) | 9 (10.1) | 3 (8.3) | 2 (3.3) | 7 (8.3) | 2 (2.5) | 7 (8.3) |
| 10-19       | 6     | 6    | 0      |        | 4 (4.5) | 20 (22.5) | 29 (32.6) | 17 (19.1) | 13 (8.3) | 9 (10.1) | 3 (8.3) | 2 (3.3) | 7 (8.3) | 2 (2.5) | 7 (8.3) |
| 20-29       | 44    | 44   | 0      |        | 4 (4.5) | 20 (22.5) | 29 (32.6) | 17 (19.1) | 13 (8.3) | 9 (10.1) | 3 (8.3) | 2 (3.3) | 7 (8.3) | 2 (2.5) | 7 (8.3) |
| 30-39       | 156   | 64   | 92     |        | 4 (4.5) | 20 (22.5) | 29 (32.6) | 17 (19.1) | 13 (8.3) | 9 (10.1) | 3 (8.3) | 2 (3.3) | 7 (8.3) | 2 (2.5) | 7 (8.3) |
| 40-49       | 123   | 77   | 46     |        | 4 (4.5) | 20 (22.5) | 29 (32.6) | 17 (19.1) | 13 (8.3) | 9 (10.1) | 3 (8.3) | 2 (3.3) | 7 (8.3) | 2 (2.5) | 7 (8.3) |
| 50-59       | 53    | 34   | 19     |        | 4 (4.5) | 20 (22.5) | 29 (32.6) | 17 (19.1) | 13 (8.3) | 9 (10.1) | 3 (8.3) | 2 (3.3) | 7 (8.3) | 2 (2.5) | 7 (8.3) |
| >60         | 22    | 15   | 7      |        | 4 (4.5) | 20 (22.5) | 29 (32.6) | 17 (19.1) | 13 (8.3) | 9 (10.1) | 3 (8.3) | 2 (3.3) | 7 (8.3) | 2 (2.5) | 7 (8.3) |

3.3. PCR detection of HEV

All samples, which tested positive for anti-HEV total antibodies (n = 64) and individuals infected with HBV (n = 60) were subjected to PCR analyses for the detection of HEV RNA. However, all samples were tested negative for HEV RNA.

4. Discussion

In this study, the anti-HEV seroprevalence was analysed in populations at risk for HEV infection, namely HIV-positive individuals, animal handlers and pregnant women. The highest seroprevalence (total antibodies) was detected in HIV-positive individuals (11.4%), followed by animal handlers (7.9%) and pregnant women (6.3%). None of the samples was tested positive for HEV RNA.

In this study, 11.4% of HIV-infected individuals tested positive for anti-HEV total antibodies indicating that this group is at higher risk of acquiring HEV infection compared to the other groups analysed here. Moreover, acute HEV infection is correlated with a higher mortality rate in individuals with underlying liver disease, which is often present in HIV-infected individuals due to co-infection with hepatitis B or C virus [24]. This calls for close monitoring of people living with HIV for better care and management. While the seroprevalence observed in this study is lower than seroprevalences reported from HIV-positive individuals from other African countries where HEV is endemic [25,26], it is in line with the results reported from studies in Nigeria, where anti-HEV seroprevalences of 5.5% in Ogbomoso (South-West) [27], 11.1% in Oyo state (South-West) [28] and 31.1% in Plateau state (North-Central) [12] were detected. The total anti-HEV IgM seroprevalence detected in this study (1.8%, n = 12/660) is similar to studies observed in different regions of Nigeria. The anti-HEV IgM seroprevalence observed among people living with HIV in this study (2.2%) is comparable with the seroprevalences observed among HIV-positive individuals in Ibadan and Plateau State, Nigeria, where a seroprevalence of 1.7% and 1.3%, was reported, respectively [12,28].

Although there was no statistically significant difference in the seroprevalence of anti-HEV total antibodies in male and female HIV-positive study participants (13.9% in men vs 10.6% in women), the seroprevalence of anti-HEV IgM was significantly higher in men (6.5%) than in women (0.7%). Also, all the HEV total antibodies and HEV IgM was only found in male among animal handlers. A possible factor might be the intake of alcohol, as in Nigeria, men are more involved in alcoholism [29] which has been described as a cofounding risk factor for HEV infection [13]. Alcohol may destroy the liver integrity increasing the susceptibility to viral infections [7,30]. It could also mean that females engage in better personal hygiene practices than males [31].

The seroprevalence of anti-HEV total antibodies in animal handlers in this study (7.9%) is lower than the seroprevalence reported from animal handlers (66.3%) in Plateau State, Nigeria [12]. Also, anti-HEV IgM seroprevalence reported from animal handlers in this study

N = number; IQR = interquartile range.
positive for HEV infection, but antibodies have been found in goats, routes of HEV transmission. infected animals, and infected animal faecal samples, among other Nigeria may be due to zoonotic transfer from direct contact with [38x185]— only a few handled pigs. However, it is worthy of note that none of the risk of HEV which may explain the discrepancy with the results found [38x216]— and Junaid et al., [12] animal handlers and especially pig handlers were [38x237]— of the pig handlers (n = 15). Further, a higher seroprevalence of HEV has been found in pigs in the North-central and North-eastern Nigeria as compared with the South-western and South-southern regions [33]. As such, an increased sero-prevalence in the animal handlers from Plateau State, North-central Nigeria may be due to zoonotic transfer from direct contact with infected animals, and infected animal faecal samples, among other routes of HEV transmission.

To the best of our knowledge, cows in Nigeria have not been found positive for HEV infection, but antibodies have been found in goats, sheep and pigs [34]. This might be a pointer that the route of transmission of HEV in animal handlers in this study could be from other routes like contaminated water, not zoonotic transfer. Nevertheless, markers of HEV infection have been detected in cows [35] and HEV has also been isolated from cow milk [36,37], so that a zoonotic transmission cannot be excluded.

All the HEV-seropositive samples were observed in male animal handlers. Animal handling is more of a male job than a female role in the Nigeria setting, and the number of female animal handlers was rather low.

In this study, an anti-HEV total antibody seroprevalence of 6.3% in pregnant women has been observed which in comparison is lower than the seroprevalence reported from pregnant women in Plateau State, Nigeria (42.6%) [12] and Ghana (12.31%) [38] but closer to the anti-HEV IgG seroprevalence (5.7%) recorded in Mexico [39]. Most of the pregnant women recruited in the study of Junaid et al., [12] were from the rural community of Plateau State while most of the women in this study were from the urban region. The anti-HEV IgM seroprevalence observed among pregnant women in this study (0.6%) is comparably similar to the results from the studies of Obiri-Yeboah et al., [38] in Ghana and Ifeorah et al., [15] in pregnant women in Ibadan, Nigeria (anti-HEV IgM seroprevalence: 0.2% and 0.4% respectively).

The only anti-HEV IgM positive pregnant woman was in the age range of 30–39 years and in the second trimester of pregnancy. HEV infection in the third trimester of pregnancy has been associated with a high fetomaternal mortality rate [40]. HEV has been found to take a more aggressive course in pregnant women in comparison to non-pregnant women than any other hepatotropic virus as reported by several authors [40,41]. The high maternal-child mortality rate in Nigeria [42] may be partly due to HEV infection as Krain et al. [43] suggested that HEV might be the underlying cause of more than 3000 stillbirths occurring annually in developing countries. These claims are substantiated with the HEV outbreak that occurred in Borno State, Northeast of Nigeria, where a case fatality of 0.6% was recorded; infected pregnant women inclusive [44]. It is therefore expedient to enlighten this group of people on the need for better hygiene and the employment of HEV vaccine to prevent HEV infection in women of reproductive age.

A limitation of our study may be that the sample size is somewhat small; however, in our opinion 660 samples divided in three subgroups will generate valid comparison and statistics. The disparities in the seroprevalences observed in this study compared to other studies may be attributed to the prevailing conditions that operate in rural areas of a low-income country such as inadequate potable water, poor living conditions, poor nutritional status, and ill-equipped health facilities [45] which are known to be associated with a higher risk of HEV infection. Various factors fuelling the transmission of HEV in different localities, sample size and differences in sensitivities of the assays used may account for the differences in anti-HEV seroprevalences in individuals within Nigeria and across Africa. Furthermore, foodborne transmission could be a factor for the different anti-HEV seroprevalences

### Table 4
Age distribution and subgroup seroprevalence of anti-HEV antibodies.

| Subgroup | Anti-HEV total antibodies | Anti-HEV IgM |
|----------|---------------------------|--------------|
|          | Positive n (%) | Positive n (%) |
| HIV-positive individuals (n = 411) | | |
| Male (n = 108) | 15 (13.9) | 7 (6.5) |
| Female (n = 303) | 32 (10.6) | 2 (0.7) |
| p value (X²) | 0.351 (0.871) | 0.000 (12.598) |
| Animal handlers-by type of animal | | |
| Cow handlers (n = 74) | 7 (9.5) | 2 (2.7) |
| Pig handlers (n = 15) | 0 (0) | 0 (0) |
| p value (X²) | 0.215 (1.540) | 0.520 (0.415) |

### Table 5
Distribution of anti-HEV antibodies according to HIV status, gender, and animal type.

| Subgroup | Anti-HEV total antibodies | Anti-HEV IgM |
|----------|---------------------------|--------------|
|          | Positive n (%) | Positive n (%) |
| HIV-positive individuals—by gender | | |
| Male (n = 72) | 0 | 0 |
| Female (n = 17) | 0 | 0 |
| p value (X²) | 0.180 (1.794) | 0.487 (0.483) |
| Animal handlers—by type of animal | | |
| Cow handlers (n = 74) | 7 (9.5) | 2 (2.7) |
| Pig handlers (n = 15) | 0 (0) | 0 (0) |
| p value (X²) | 0.215 (1.540) | 0.520 (0.415) |

### Table 6
Distribution of anti-HEV antibodies according to pregnancy stage.

| Subgroup | Anti-HEV total antibodies | Anti-HEV IgM |
|----------|---------------------------|--------------|
|          | Positive n (%) | Positive n (%) |
| First trimester (n = 44) | 3 (6.8) | 0 |
| Second trimester (n = 78) | 6 (7.7) | 1 (1.3) |
| Third trimester (n = 38) | 1 (2.6) | 0 |
| p value (X²) | 0.563 (1.150) | 0.589 (1.058) |
observed in different regions of Nigeria. Junaid et al. observed a significant association between improper washing of fruit and vegetables and anti-HEV IgG seroprevalence [12]. In comparison to Osun State, in Plateau State, ready to eat vegetables such as apples, pear, cabbages, and lettuce, among others are grown and more widely consumed [46]. Some of these vegetables and fruits may have been contaminated as farmers may have used both untreated animal and human faecal samples as manure in place of inorganic fertilisers that must be purchased. The assertion of faecal contamination of vegetables is supported by the findings of Pam et al., [47] in vegetables sold in different markets in south Jos. Vegetables and fruits have been implicated as a vehicle for transmission of HEV in some countries [48,49].

All the HBV infected serum sample were negative for HEV RNA. Due to the suboptimal quantity of the serum, the serological evidence of HEV transmission of HEV in some countries [48,49]. In comparison to Osun State, in south Jos. Vegetables and fruits have been implicated as a vehicle for transmission of HEV in some countries [48,49].

5. Conclusion

While HEV infection is still a neglected disease in Nigeria, the data from this study shows that HEV is of major health concern with a significant number of individuals being exposed to HEV. Therefore, the awareness of this disease should be increased by systematic screening, especially of at-risk populations having contact to zoonotically transmitted HEV.

Author contributions

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Funding resources

This study was supported by a grant from the German Federal Ministry of Health regarding a decision of the German Bundestag by the Federal Government (NiGeDe-Project grant No: ZMV1-2519GHP711, and CHERD-Project grant No: ZMV1-2511FHSB705). F.A.O. and O.O.O. are funded by a sub-project of the Global Health Protection Programme supported by the German Federal Ministry of Health based on a decision by the German Bundestag, the Partnership in Postgraduate Education (PPE), Robert Koch Institute, Berlin, Germany. O.A.A. is supported by funding of DAAD, Germany (German Academic Exchange Service; grant ID 57299293). B.W. is supported by the China Scholarship Council (CSC), Beijing, China. D.H. is supported by the Claussen-Simon-Stiftung (Claussen-Simon Foundation; CSF) “Dissertation Plus” program, Germany, and the Fazit-Stiftung, Germany, “Promotions Stipendium”. The funders BMG, CSC, DAAD, Fazit, CSF, and GHPP/PPE had no role in study design, data collection and interpretation, or the decision to submit the work for publication. The content is the responsibility only of the authors and does not represent the views of the funders.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest and personal relationship that could inappropriately influence the presented work.

Acknowledgments

We are grateful to Olamide Omopo (LAUTECH), Adebile Olumuremi (LAUTECH), Marcel Schulze (RKI), Steffen Zander (RKI), and Hannes Jarmuth (RKI) for their excellent technical assistance.

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