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Molecular Detection and Characterisation of Lassa Fever Virus among Patients Attending Tertiary Hospitals in Jos, Central Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author SCC did the sample collection, laboratory assays and wrote the manuscript, author DZE supervised the work and proof-read the manuscript, author PL supervised the research and proof-read the manuscript, author SO assisted with project design while author AA designed the project. All authors read the manuscript

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ABSTRACT

Background: Lassa fever is a highly contagious viral haemorrhagic fever that was first identified in 1969 following lethal infection of a missionary in Lassa, North eastern Nigeria.
Aim: The aim of the study was to carry out molecular detection and characterisation of Lassa viruses in Jos.
Study Design: Cross sectional study.
Materials and Methods: We targeted patients presenting with Lassa fever-like symptoms, including people who had lived in close association with them in Jos, central Nigeria. A total of 156 blood samples were collected from consented participants.
Results: Lassa IgG and IgM was detected using ELISA technique while S- RNA gene of Lassa

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virus was detected using RT-PCR. Partial sequencing and phylogenetic analysis of the S-RNA gene was done using appropriate tools. A Lassa virus seroprevalence of 24% was established in the study area. RT-PCR detected Lassa virus S-gene in 8 (5.1%) samples, while gene sequencing and phylogenetic analysis showed Lassa virus strains aligning with lineages II and III of Lassa viruses in the GenBank. Statistically, a positive correlation (r=0.057) among suspected cases and exposed subjects was established. Also, a negative correlation (r= -0.005) was seen among outcome of serology and molecular detection among suspected cases while a positive correlation (r= 0.086) was observed in the sub-population of suspected cases with regards to outcome of serology and molecular detection.

Conclusion: The discovery of Lassa strains aligning with lineage II Lassa viruses in GenBank is novel and remains the first report on the strains in central Nigeria. Similar work is recommended in other regions of the country.

Keywords: Lassa virus IgM; IgG; lineage; Jos; Central Nigeria.

1. INTRODUCTION

Lassa virus (LASV) is an Old World complex of the Arenavirus family. It causes haemorrhagic fever in humans with the natural host as the African rodent called *Mastomys natalensis*, which is a multimamate, peridomestic rat. Lassa fever has been reported to be responsible for over 300-5000 mortalities annually in West Africa [1]. It was first identified in Lassa town, north east Nigeria in 1969. Today, the disease is endemic in Nigeria with increasing annual outbreaks [2]. The virus is also known to circulate in Sierra Leone, Guinea and Liberia. Imported cases have however been reported in Europe, America and Asia, with traces of their ancestry pointing to strains from Africa [3]. The known traditional LASV epidemic-prone regions in Africa have also been modified with findings indicating that the virus is circulating in Ivory Coast and Mali and recently, Ghana [4,5,6,7].

Like other arenaviruses, LASV is an enveloped virus with a bisegmented single-stranded RNA genome encoding 4 proteins [8]. The small segment contains genes for the glycoprotein precursor (GPC) and nucleoprotein (NP), which serves as the main viral capsid protein. The large segment encodes the small zinc-binding protein (Z), which contains a RING motif, and another gene (L) containing the RNA-dependent RNA polymerase domain.

Complete and partial genome sequences for several LASV strains with origins from man and rodent host have been done and archived [9,10,11]. Leski et al. [12] reported that sequence analyses of some LASV showed the existence of high sequence diversity of up to 27% nucleotide of LASV. Bowen et al. [13] documented that sequence analysis enabled for categorisation of Lassa viruses into 4 major lineages which correlates with geographic location. Lineages I, II, and III are found in Nigeria, whereas lineage IV strains of the virus are found in Guinea, Sierra Leone, and Liberia. Also, recently published sequences of LASV from rodents in Mali [9] pointed to the existence of an additional clade (proposed as lineage V) [13]. Phylogenetic analysis has previously showed that LASV sequences of isolates from humans and rodents are interspersed, affirming the notion that human cases typically result from transmission from rodents [13].

The high degree of sequence divergence of LASV genomes is a major problem affecting the development of molecular and immune-based diagnostic technologies, vaccines, and possibly antiviral drugs [14,15,16].

In Nigeria, it has been established that LASV strains also have geographical peculiarities. Strains from northern Nigeria have been documented to align with lineage I. Those in central Nigeria align with lineage III while those in southern Nigeria aligned with lineage II [17]. However, whether the geographical colonisation of the strains and lineages of LASV in Nigeria has been altered due to viral circulation is the main intention of this study.

The purpose of this study, therefore, is to use molecular techniques to detect and characterise Lassa viruses in Jos, Central Nigeria in view of the upsurge in the epidemic of Lassa fever in the study area in the recent past. Also, since new strains of the LASV are being identified in new locations globally, the need to examine the situation in Jos, a major wetland for LASV becomes imperative.

2. MATERIALS AND METHODS

2.1 Study Area

Plateau State is located in Nigeria's middle belt. With an area of 26,899 square kilometres, the State has an estimated population of about three million people. It is located between latitude 08°24'N and longitude 008°32' and 010°38' east. The state is named after the picturesque Jos Plateau, a mountainous area in the north of the state with captivating rock formations [18].

2.2 Study Design

We implemented a cross sectional study design between 2013 and 2016 by surveying tertiary hospitals in Jos, Plateau State for patients presenting with symptoms suggestive of Lassa fever. Two categories of enrollees were targeted: those with Lassa-like symptoms (cases) and those who have lived in close association the cases (exposed). Ethical clearance was obtained from the hospitals (Health Research & Ethics Committee of Bingham University Teaching Hospital, Jos Ref: BHUTH/HREC/RE/SND/00067 and Ethical Committee of Plateau State Specialist Hospital, Jos Ref: NHREC/05/01/2010b).

While consent forms were administered to prospective study participants. Upon consent by a participant, 5ml blood sample was taken in vacutaner and questionnaire was administered to retrieve clinical and demographic data. In total, 156 participants were recruited for the study and had their blood samples taken. Sample size was determined scientifically according to the method of Krejcie and Morgan [19]. Details of the characteristics of the study participants is as presented in Table 1.

2.3 Serology

Lassa IgG and IgM were screened for using Enzyme Linked Immunosorbent Assay (ELISA) kit manufactured and validated by Bernhard Nocht Institute ofHamburg (Germany) and has been used in previous studies [20]. The solid phase of the test kit is impregnated with IgG Ag: NP 18.6.14 and IgM Ag: NP 24.1.12. The test was done and results read and computed as cut off point of optical density as provided for in the manufacturer's manual.

2.4 RNA Extraction

Viral RNA was extracted from serum with a QIAamp viral RNA minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA was eluted in 60µl AVE buffer (Qiagen) and stored at -70°C.

2.5 Lassa Virus GPC RT-PCRs

We used the new GPC RT-PCR (called GPC RT-PCR/2007) OneStep RT-PCR kit reagents (Qiagen) which targeted the 300bp S-gene of LASV according to the protocol of Olschlager et al. [16]. The 25µl assay mixture contained 5 µl RNA, 0.6 µM primer 36E2 (ACC GGG GAT CCT AGG CAT TT), 0.6 µM primer LVS-339-rev (GTT CTT TGT GCA GGA MAG GGG CAT KGT CAT), 0.4 mM dNTP, 1µlRT-PCR buffer, 1µl Q solution, and 1µl enzyme mixture. The cycling conditions were 50°C for 30 min and 95°C for 15 min, followed by 45 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. The assay was set up on ice and placed into a Primus25advanced thermocycler (PeqLab), after the heating block had reached 50°C. Amplified products were subjected to electrophoresis on 1% agarose gel at 100volts for 45mins. Migration of protein bands was visualised through a transilluminator and image was captured using an inbuilt camera. Migrated protein bands of 300bp were recorded as positive for LASV S-gene as shown in Plate 1.

2.6 Partial Sequencing and Phylogenetic Analysis of S RNA

The protocol of Olschlager et al. [16] was adopted with minor modifications. Briefly, the 5´portion of S RNA (positions 10 to 300), which covers the target region of the GPC RT-PCR, was amplified with primers and a Qiagen OneStep RT-PCR kit (Qiagen). The 20µl assay mixture contained 2 µl RNA, 0.5 µM primer 36E2 (ACC GGG GAT CCT AGG CAT TT), 0.6 µM primer LVS-339-rev (GTT CTT TGT GCA GGA MAG GGG CAT KGT CAT), 0.4 mM deoxynucleoside triphosphate (dNTP), 1x RT-PCR buffer, and 0.8µl enzyme mixture. The reaction was performed in a Primus25advanced thermocycler (PeqLab, Erlangen, Germany) using the following temperature profile: 50°C for 30 min and 95°C for 15 min, followed by 45 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 1 min. Both strands of the amplified fragments were sequenced.

Sequence analysis was performed with a Bayesian approach through BEAST software, with a GTR + gamma 6 categories + invariant sites substitution model. The clock was strict and the viral population was considered as constant.

Table 1. Characteristics of recruited study participants

	Gender			Location					Age Bracket (yrs)						
	Male	Female	Total	PN	PC	PS	OP	Total	11-20	21-30	31-40	41-50	51-60	61-70	71-100
Case	19	23	42	17	9	10	6	42	1	9	18	8	5	1	-
Exposed	62	52	114	53	21	25	15	114	7	52	29	16	6	3	1
Total	81	75	156	70	30	35	21	156	8	61	47	24	11	4	1

Key: PN-Plateau North; PC-Plateau Centre; PS-Plateau South; OP-Outside Plateau

Plate 1. Picture of LASV S-gene (300bp) on gel electrophoresis

The tree present the sequences of glycoprotein (GP) truncated at 256 nucleotides. The numbers at the node represent the posterior values and give the confidence of the analysis. Values between 0.7 and 1 are good and imply that the node is well supported below these values, the node is unstable and the tree can change (Fig. 1).

2.7 Statistical Analysis

Correlation statistical tool was deployed to compare outcomes of clinical parameters at 95% confidence limit.

3. RESULTS

One hundred and fifty six (156) study participants were recruited for the study. The breakdown showed that 81 (51.9%) were male participants while 75 (48.1%) were female participants. Forty two (26.9%) were suspected cases while 114 (73.1%) were of exposed subjects (Table 1).

More people (44.9%) were recruited from Plateau north senatorial zone than from central (19.2%) and southern zones (22.4%). A further 13.5% was recruited from locations bordering Plateau State. In terms of age distribution, ages 21-30 and 31-40 has more people recruited into the study as the number declines with increased age (Table 1).

Thirty eight 38(24%) were positive for Lassa serology. Seventeen (11%) were positive for Lassa IgM and 21(13.5%) were positive for Lassa IgG. Eight 8(5.1%) had both IgG and IgM, while 102 (65.3%) had neither Lassa IgG nor IgM (Table 2).

RT-PCR detected Lassa 300bp S-gene in 8 (5.1%) samples. Seven (4.5%) were from suspected cases while 1(0.6%) was from exposed subjects. Sequences obtained from the partial analysis 300bp of S-gene and their phylogenetic showed all but one of the Lassa virus strains aligning with the lineage II of the Lassa viruses in the Genebank. Only one strain aligned with lineage III while none aligned with lineages I and IV.

Statistical analysis showed a positive correlation (r=0.057) between Lassa suspected cases and Lassa exposed subjects. Also, a negative correlation (r= -0.005) was seen among outcomes of serology and molecular detection

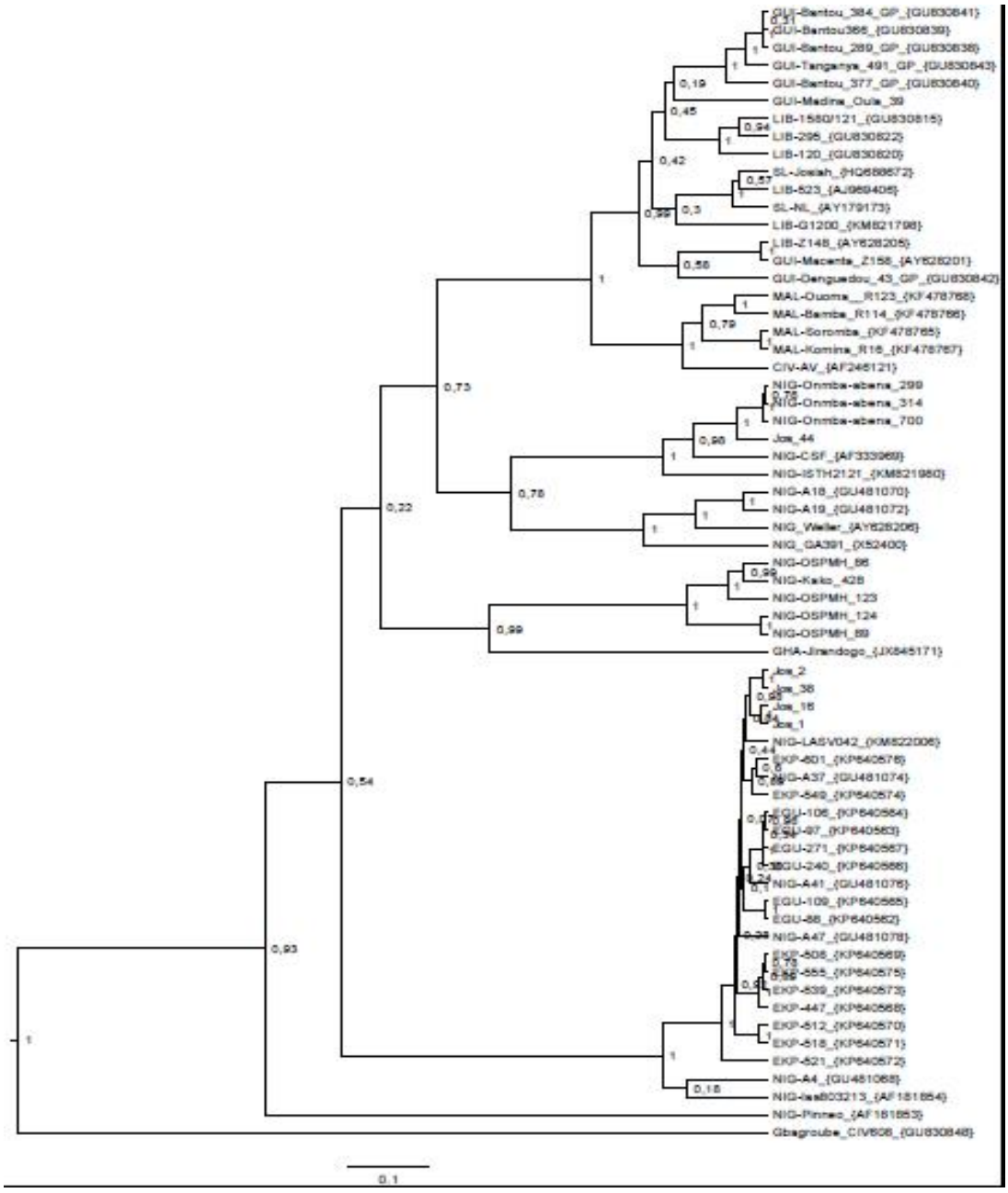

Fig. 1. Phylogenetic analysis of Lassa virus strains

among Lassa exposed subjects while a positive correlation (r= 0.086) was observed among sub-population of Lassa suspected cases with regards to outcome of serology and molecular detection (Table 2).

4. DISCUSSION

Lassa fever is endemic in West Africa where it accounts for several thousand mortalities and morbidities in the region. Due to absence of approved vaccines and drugs for intervention purposes, the disease has remained unabated in the region with improved prevalence, emerging epidemiology, genotypes and pathology in recent times.

In the present research, a Lassa sero-prevalence rate of 24% has been established in the study area. This is, however, lower than Lassa sero-

prevalence rate of 58.2% reported by Tobin et al. [21] amongst hospitalised patients in Irrua, Edo State, Southern Nigeria. Historically, however, it is slightly higher than 21% sero-prevalence earlier reported in Nigeria [22] and significantly higher than the about 6% sero-prevalence reported from sera collected in Lassa village, Nigeria, in August 1970 following the index case report in 1969 [23]. Interestingly, it is in tandem with average national prevalence of Lassa fever in Nigeria which earlier reports put at above 20% [24]. The sero-prevalence of Lassa fever obtained confirms the persistence of the virus in the study location following first reports in the 80s as documented earlier [17, 2].

Table 2. Outcome of serology and PCR of suspected Lassa cases and Lassa exposed subjects

	SLC (PCR)	LES (PCR)	Total
+ve IgM	11(4)	6(1)	17(5)
+ve IgG	7(0)	14(0)	21(0)
+ve IgM/IgG	6(3)	2(0)	8(3)
-ve IgM/IgG	18(0)	92(0)	110(0)
Total	42(7)	114(1)	156(8)

Coefficient of Correlation (r) between antibody and gene detection for LE is -0.005 while for SLC is 0.086
Key: SLC: Suspected Lassa Case; LES: Lassa Exposed Subject; PCR: Polymerase Chain Reaction

Interestingly, S-gene of Lassa virus was detected in only 8 (5.1%) of the study population using RT-PCR. Seven of the RT-PCR positive cases were from LSC with high titres of IgM. Statistically, positive RT-PCR outcomes correlated positively with presence of IgM amongst LSC (Table 2). This finding agrees with that of Shaffer et al. [25] who earlier reported that people with anti-Lassa IgM had higher chances of Lassa viraemia which is a marker of current infection with higher chances of laboratory detection by RT-PCR. Also, Branco et al. [26] had previously documented that Lassa viraemia correlated well with Lassa fever and high mortality.

On Lassa population studied, a positive correlation (r = 0.057) was seen to exist between LSC and LE subjects in all age groups. A positive correlation is a direct relationship where the outcome of one variable depends on the outcome of another. From our study, 42 LSC gave rise to 114 LES subjects with the data showing rise in number of exposed subject with rising figures of suspected cases. Archival data corroborates this as it has always shown through contact tracing that one suspected case could be responsible for as much as 5-10 exposed people depending on the disease and mobility of the suspect in question [27]. For example, it was reported by World Health Organisation in 2012 that 1,723 contacts were traced from 112 confirmed cases that died of Lassa fever in 23 states of Nigeria. The same report added that 12 people were on the contact tracing list from 5 deaths due to Lassa in Abuja, Nigeria [27]. The positive r value of 0.057 obtained in this study is therefore justifiable.

Also, a negative correlation (r = -0.005) was established between outcome of serology and molecular detection among LES. This is manifest in the decreased rate of virus detection by PCR among people with positive IgG serology. Since IgG appears much later in the course of infection, the presence of it has often been attributed to regressed infections with diminished or undetectable viraemia from laboratory tests [28].

A novel finding from this study is the clustering of all but one Lassa strains obtained from this study with lineage II Lassa viruses in the GenBank. Previous reports had it that only lineage III strains of Lassa viruses were circulating in Jos, Central Nigeria while lineages I and II were circulating in Northern and Southern Nigeria [17]. These findings show that there is now an ecological mix in the lineages of Lassa viruses in Jos, Central Nigeria and serves as the first report on the circulation of Lassa virus lineage II in Jos, Central Nigeria.

This ecological redefinition of the Lassa virus lineages is coming in the wake of an observed Lassa fever outbreak pattern that has seen increased in the consistency and gravity of the outbreaks both in the study area and in Nigeria as a whole. While urbanisation and increased movement of grains across the country may be fingered, previous reports on the existence of defined geographical Lassa wetlands now appear outdated. This is largely due to increased interactions between the virus vector-host, the vector-vector and host-host ecosystems.

5. CONCLUSION

The findings of this research indicate that Lassa fever is still a challenge in Jos, central Nigeria, fifty years after the detection of the index case. Also, contrary to previous assertions that only lineage III Lassa fever viruses circulate in central Nigeria, our findings have also revealed the presence of lineage II viruses in the study area.

CONSENT

Consent forms were administered to prospective study participants.

ETHICAL APPROVAL

Ethical clearance was obtained from the hospitals (Health Research & Ethics Committee of Bingham University Teaching Hospital, Jos Ref: BHUTH/HREC/RE/SND/00067 and Ethical Committee of Plateau State Specialist Hospital, Jos Ref: NHREC/05/01/2010b).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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