American Journal of Medicine and Medical Sciences 2014, 4(6): 206-211
DOI: 10.5923/j.ajmms.20140406.02

Scaling up Molecular Epidemiology of Lassa Fever Virus among High Risk Groups in Abuja F.C.T. "Are These Challenges Summontable?"

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Abstract Haemoharrgic fever virus disease caused by Lassa virus that belong to the Arena virus family, is now a big threat to human existence in Africa if not the whole world. The painful aspect in the management of this disease in Nigeria and other parts of Africa is the lack of the state of arts equipment required for diagnosis and the relatively poor attitude of stakeholders in giving it the attention it deserves!. This work critically examined the scaling up processes necessary in terms of the Molecular Epidemiology of the Lassa Fever Virus among high risk groups in Abuja F.C.T., challenges ahead, and strategies that must be put in place in nipping the task on the bud. As no drug has been approved yet by FAD i.e. Food and Drug for combating the Lassa fever virus disease; developing a preventative vaccine for researchers, health care providers and their patients therefore becomes imperative. Although, this work is a proposal, implementation of ideas raised here could be a giant step forward in curtailing the threat of haemoharrgic fever virus diseases, not only limited to *Lassa* virus disease, but others such as *Marburg*, *Ebola*, *Dengue*, Yellow fever, e.t.c.

Keywords Haemoharrgic fever virus, FAD, Food and Drugs, *Lassa* fever virus, *Ebola* virus, *Dengue* virus, Molecular

1. Background

Among the viruses causing severe hemorrhagic fever in Africa, Lassa fever is a significant public health problem in Nigeria, Liberia, Sierra Leone, and the Republic of Guinea. It is estimated that Lassa virus infects over 200,000 individuals per year across this region, causing over 3,000 deaths [1]. The case fatality rate for Lassa fever is around 15% in hospitalized patients and has been greater than 50% in several outbreaks [2, 3]. Human infection is associated with contact with a widely distributed and highly commensal rodent, *Mastomys natalensis*, or by contact with infected patients. Recent importation of Lassa fever into Germany, the Netherlands, the United Kingdom, and the United States by travelers on commercial airlines [4-8] illustrates the potential for the spread of this highly dangerous and contagious pathogen. In addition, Lassa virus has gained notoriety because it is classified as a Category A bioweapon agent [9].

Lassa virus is an enveloped, bisegmented RNA virus belonging to the Old World group within the family Arenaviridae [10]. Arenavirus particles contain a genome consisting of two ambisense single-stranded RNA molecules, designated small (S) and large (L), of a length about 3.4 kb and 7.2 kb, respectively. The S segment contains two genes that encode three structural proteins, the nucleoprotein (NP) and the envelope glycoproteins GP1 and GP2. The L segment contains two genes that encode two proteins, the viral polymerase (L protein) and the Z protein. NP and L protein associate with the genomic RNA in a ribonucleoprotein complex or nucleocapsid structure. It is thought that the Z protein functions as a matrix protein and is responsible for the formation of virus particles [11]. GP1 and GP2 are initially synthesized as a precursor molecule, glycoprotein C (GPC), which is posttranslationally cleaved by the protease SKI-1/S1P [12]. GP1 is the portion of the surface glycoprotein spike that is thought to be the effector for receptor binding, while GP2 is structurally consistent with viral transmembrane fusion proteins of other enveloped viruses [13].

Currently, there are no vaccines or antiviral drugs approved for Lassa fever. Treatment with intravenous ribavirin was shown to reduce mortality from Lassa fever in high-risk patients and presumably decreases morbidity in all patients with Lassa fever [14]. However, the availability of

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Published online at http://journal.sapub.org/ajmms

ribavirin is very limited in endemic areas, and treatment is most effective if initiated within the first week of disease onset [14]. Preventing contact with the reservoir host in endemic areas is currently unachievable; therefore, a preventive vaccine is a critical public health need, especially to protect health care providers, who are often the most at risk. Indeed, the recent untimely death of a well-known doctor in Sierra Leone who treated thousands of cases of Lassa fever and ultimately contracted the disease as a result of these laudable efforts [5] underscores the need for a preventive vaccine.

Early attempts to develop a vaccine against Lassa fever focused on classical approaches such as killed vaccines. A whole-virion vaccine inactivated by gamma irradiation provided a good humoral response to Lassa viral proteins, NP, and GP, but failed to protect nonhuman primates from a lethal Lassa challenge [16]. As these animals were unprotected despite a strong humoral response, it was suggested that protection would depend on a robust cellular response. Subsequent efforts to develop an efficacious vaccine against Lassa fever focused on genetically engineered vaccines. Specifically, studies focused on recombinant vaccinia viruses expressing the NP, the full-length GPC, and combinations of GP1 and GP2. In a series of studies by Fisher-Hoch and colleagues, approximately 90% of nonhuman primates that received a vaccine containing both GP1 and GP2 survived a lethal Lassa challenge [1, 17, 18]. Protection did not correlate with humoral immunity, as none of these animals had demonstrable Lassa virus-specific antibody before challenge. Consequently, cell-mediated immunity was implicated in protecting these animals, although T-cell responses were not measured in these studies. In comparison, vaccination of macaques with NP alone resulted in the development of relatively high antibody titers before Lassa challenge, but only 20% survival.

While the recombinant vaccinia vaccines expressing the full-length Lassa GPC protected nearly 90% of nonhuman primates from a lethal challenge, it was noted that the vaccinia platform is not suitable for human use because of potential side effects, which is a particular concern in areas where HIV prevalence is high [1]. However, it appears from all previous studies that a strong cellular response may be required for protection. Vaccination with a live virus characteristically elicits strong cellular immune responses, and live vaccines are particularly attractive because they can confer protection in a single injection. Therefore, the ideal Lassa vaccine would be based on a replication-competent format engineered for enhanced safety and with the ability to overcome technical obstacles such as prior immunity to the vector. Recently, we described the generation of a live attenuated, recombinant vesicular stomatitis virus (rVSV) expressing the GPC of Lassa virus, strain Josiah [19]. Vaccines based on live attenuated rVSV have been highly effective in animal models and are particularly attractive because they can be administered by the mucosal route [20-23]. Furthermore, vesicular stomatitis virus (VSV) infections in humans occur fairly rarely worldwide, mainly in the enzootic regions of the Americas, and consequently, global pre-existing immunity is negligible [24]. Because the guinea pig model of Lassa fever does not faithfully mimic human disease [25] and is not generally predictive for efficacy of vaccines and antivirals [1], evaluation of the protective efficacy of the replicating rVSV vector in nonhuman primates was carried out.

2. Statement of Problem

Preventing contact with the reservoir host in endemic areas is currently unachievable; therefore, a preventive vaccine is a critical public health need, especially to protect health care providers, who are often the most at risk. Indeed, the recent untimely death of a well-known doctor in Sierra Leone who treated thousands of cases of Lassa fever and ultimately contracted the disease as a result of these laudable efforts [15] underscores the need for a preventive vaccine. And most recently, the death of a patient from Lassa fever in south Africa who had hitherto passed through a tertiary center of health excellence in Nigeria un detected ! was a big cause for worry in areas of diagnosis and vaccine development in this part of the Globe !!.

JUSTIFICATION OF STUDY; Epidemiology of genes (such as *LARGE AND INT21*) had only been paucitly documented in Nigeria. Some studies had documented these genes to be positively selected in the course of evolution over the years; The molecular epidemiology proposed intends to know the frequencies of these genes in Lassa fever virus infection- which confer resistance in appreciable proportion of the target population. Sound knowledge of this would go a long way in developing future effective split vaccine to combat the menace of the Lassa fever disease in Nigeria as a whole. Hence this proposed work is justified.

— *Function altering mutations (millions of years)*. When a protein is under strong selection, the number of non-synonymous (D_N) to synonymous (D_S) changes in its open reading frame may change dramatically. An excess of D_N suggests that positive selection has worked on the protein, whereas an excess of D_S suggests negative or purifying selection. Similarly, tests have been developed to identify an excess of potential function altering mutations in non-coding regions [8].

— *Reduction in genetic diversity around selected allele* (*less than 250 000 years*). As a variant under positive selection rises in frequency, 'hitch-hiking' nearby alleles increase in frequency as well. Such a 'selective sweep' leads to an overall decrease in diversity in the selected region with a simultaneous increase in the number of rare alleles as new SNPs are 'born' near the positively selected allele [8].

— *Increase in the frequency of derived alleles* (*less than 80 000 years*). When new alleles arise, they have a lower frequency than already present (ancestral) alleles. However, during a selective sweep, the selected allele

as well as nearby neutral derived alleles will rapidly rise in frequency. A region with a high proportion of many derived alleles is therefore good evidence for positive selection having occurred in that part of the genome [8].

— *Increase in population differentiation* (*less than 75 000 years*) A particular allele may be beneficial in one population but not in another. In such a case, there will be a large difference between the frequency of the allele in one population versus the other [8].

— *Long-range haplotypes* (*less than 30 000 years*). Recombination during meiosis continuously breaks down associations between alleles on the same chromosomes. During a selective sweep, however, the selected variant rises quickly in frequency, leaving links with nearby alleles on the ancestral chromosome intact. This increase in 'linkage disequilibrium' leads to chromosomal regions where the haplotype is unusually long. This signature of positive selection can be measured using various haplotype-based tests, such as LRH [4], iHS [9] and XP-EHH [10].

— *Composite of multiple signals* (*CMS*) (*??? years*). While most of these tests have been successful in identifying regions under selection, in many cases, the individual genes or variants under selection remain obscured due to a lack of spatial resolution. Because strongly selected variants should contain many of the above five mentioned signatures, 'CMS' method to improve spatial resolution up to 100-fold [11] had recently been developed - allowing the identification and localization of specific variants under positive selection.

The Hypothesis that; - Natural selection of Lassa fever virus genes "*LARGE AND INT21 "targeted* regulatory regions controlling the expression of both.

1 H_0; Genes implicated in Lassa fever are not under positive selection in Nigeria, West Africa

H_a; Genes implicated in Lassa fever are under positive selection in Nigeria West Africa

2 H_0; Positive selection within *LARGE* does not localize to the first two introns

H_a; Positive selection within *LARGE* localizes to the first two introns

3 H_0 Selection around *IL21does not* localize to a region containing three different genes

Ha Selection around *IL21* localizes to a region containing three different genes

4 H_0 Natural selection of Lassa Fever virus genes "*LARGE AND INT21 "*does not target regulatory regions controlling the expression of both using qPCR technique.

H_a Natural selection of Lassa Fever virus genes "*LARGE AND INT21 "*targets regulatory regions controlling the expression of both using qPCR technique.

5 H_0 *There are* no different ancient patterns of evolution in *Large* and *IL21 genes*

H_a *Large* and *IL21* genes show different ancient patterns of evolution

SPECIFIC AIMS; As a matter of fact and urgency, we are strongly determined as a group of concerned medical scientists in F.C.T. Abuja Nigeria , to follow our stepping /scaling up strategies in alignment with the hypotheses stated above and also the specific aims defined below;

1. To test whether Genes implicated in Lassa fever are under positive selection in Nigeria, West Africa.
2. To identify the position of localization of *LARGE Genes* within the introns
3. To know the localization of genes following Selection around *IL21*
4. To establish that Natural selection of Lassa fever virus genes "*LARGE AND INT21 "targets* regulatory regions controlling the expression of both using qPCR technique.

PRELIMINARY OF SIMILAR STUDIES EARLIER CONDUCTED AND RESULTS GENERATED

***PRELIMINARY STUDIES;* Genes implicated in Lassa fever are under positive selection in West Africa**

Given the potential impact of a disease as severe as LF on human genome evolution, we decided to investigate genes involved in LF pathogenicity that show evidence of recent positive selection. Using the HapMap II dataset, the human genome was scanned using the long-range haplotype method iHS [9] and found evidence for positive selection at the *LARGE* locus on chromosome and *IL21* locus on chromosome in YRI. The *LARGE* gene has previously been identified using other long-range haplotype methods [10] and we found that the signal is consistently found as one of the top-scoring genes using a variety of selection methods and datasets (data not shown).

PRELIMINARY RESULTS; signal was consistently found as one of the top-scoring genes using a variety of selection methods and datasets (data not shown). Both *LARGE* and *IL21* have a strong biological association with LASV infection and disease. *LARGE* is a glycosyltransferase that modifies the mucin domain of the LASV entry receptor α-DG. It also interacts with the N-terminal domain of α-DG and is required for the ability of LASV to infect cells [12]. Therefore, it is possible that polymorphisms in *LARGE* found in the West African population may confer direct protection against LASV infection by decreasing the ability of the virus to enter the cell.

IL21 is a member of the common γ-chain family of cytokines, which also includes IL2 and IL15 [24]. It is crucial for effective clearance of chronic infection with another member of the arenavirus family—lymphocytic choriomeningitis virus (LCMV) [25-27]. This virus shares α-DG receptor binding and much of its biology with LASV and is a very commonly used pathogen in immunological

research [28]. Our data suggest that polymorphisms in the region surrounding *IL21* might have been positively selected in West Africa, potentially making individuals better able to cope with LASV infection.

Positive selection within *LARGE* localizes to the first two introns

PRELIMINARY STUDIES

Having identified a signal of selection at the *LARGE* locus, CMS was used [11] to further narrow down the signal. As true causal variants should display most signatures of positive selection (long haplotype, high-derived allele frequency and high population differentiation), it was expected that this test would give a much better spatial resolution [11]. Data from the HapMap II project (approx. three million SNPs) was used from the initial phase of the 1000 G (approx. 15 million SNPs) and compared with the results from both datasets.

PRELIMINARY RESULTS

It was found that using either dataset, the signal of positive selection localizes to the first two introns of *LARGE*. While the HapMap II analysis gave approximately 15 high-scoring SNPs, the 1000 G analysis narrowed that down to around five. Notably, all the five individual tests that form the basis for CMS [11] were able to pick up signals within *LARGE*, but with a much lower resolution. The fact that the selection signal is placed mainly within introns suggests that the selected allele of *LARGE* may be differentially regulated or alternatively spliced.

Selection around *IL21* localizes to a region containing three different genes

PRELIMINARY STUDIES

CMS was used to localize the signal of selection observed near the *IL21* locus. Using HapMap II and 1000 G analysis, it was found that the signal narrowed down to a 300 kb cluster on chromosome four containing the genes *ADAD1* and *IL2* in addition to *IL21*. Similar to that observed for *LARGE*, data from HapMap II or 1000 G gave comparable results, with 1000 G having fewer high-scoring SNPs. Again, CMS had much better power at localizing SNPs under selection than any of the five individual tests. As expected, the derived allele of *IL21* (59% frequency in YRI, 25% in CEU and 4% in CHB + JPT), unlike its ancestral counterpart, displayed long-range associations with nearby alleles when visualized in haplotype bifurcation diagrams.

PRELIMINARY RESULTS

The top-scoring SNPs by CMS fall outside any of the three genes' ORFs, suggesting that selection may have targeted variants that give rise to differential gene expression—either at individual genes, or over the whole cluster. Given the close spacing of the two common γ-chain cytokines *IL2* and *IL21*, it is interesting to speculate that selection may have targeted regulatory regions controlling the expression of both. Indeed, this potential appears to exist within these loci, as certain SNPs in this region have been implicated in an increased susceptibility to the autoimmune disease type I diabetes.

***Large* and *IL21* show different ancient patterns of evolution**

PRELIMINARY STUDIES

Having identified signatures of recent positive natural selection in the genomic regions containing *LARGE* and *IL21*, attention was focused on evidence of ancient natural selection within the ORFs of these two genes. CMS and haplotype-based methods allow for the detection of recent and ongoing selection in the human genome (within the last approx. 30 000 years) [3]. In contrast, methods to detect selection based on multiple species comparisons such as non-synonymous to synonymous (D_N/D_S) can elucidate evidence of natural selection as far back as the human split from other apes millions of years ago [3]. Codon-alignment of the ORFs of the genes from at least 10 mammals was done and a D_N/D_S analysis was performed across the entire coding sequence. At individual sites, an excess of D_N over D_S was suggestive of positive selection, whereas a larger number of D_S over D_N was suggestive of purifying selection. Using the random effects likelihood and fixed effects likelihood tests incorporated in the HYPHY package on the Data monkey website, the following results were generated;

PRELIMINARY RESULTS

The *LARGE* gene has been under very strong purifying selection in mammals and the gene is 100 per cent identical in humans and chimpanzees (data not shown). In contrast, *IL21* appears to have been under moderate positive selection in these species.

PRELIMINARY STUDIES

Next, McDonald–Kreitman tests were performed on the ORFs of *LARGE* and *IL21* to compare the level of genetic variation within human populations (polymorphisms) with that of genetic variation between species (divergence). For the purpose of minimizing the number of multiple mutations at individual sites, we limited our comparison to humans and macaques—two closely related species. However, we obtained very similar results when we compared humans with mice or rats (data not shown).

PRELIMINARY RESULTS

For *LARGE*, the ratio of non-synonymous to synonymous changes between species was significantly lower than the ratio of non-synonymous to synonymous polymorphisms 0.02 versus 0.58; neutrality index: 23.92; $p < 0.0001$). This is vastly different from the 1: 1 ratio (neutrality index: 1) expected under neutral conditions and is unlike that observed for the genome as a whole. The very low proportion of non-synonymous substitutions between species suggests that the gene has been under strong purifying selection within the mammalian lineage. On the other hand, it was identified that there was an unusually high proportion of polymorphisms in

the ORF of human *LARGE*. When comparison was made with other polymorphism datasets in the Single Nucleotide Polymorphism Database (dbSNP), it was found that the gene had substantially more polymorphisms in human populations than in rats, mice and chimpanzees—even when corrected for the larger number of described human SNPs. While there were significant number of both synonymous and non-synonymous SNPs in human *LARGE*, it was observed that that there were only synonymous SNPs in mice and no SNPs at all in chimpanzees and rats. This combined with sliding-window analysis the research group earlier carried out suggests that whereas *LARGE* has been under strong purifying selection in the mammalian lineage, it may be more recently under continuous diversifying selection in humans.

For *IL21*, the scenario was the opposite of that observed for *LARGE*. For this gene, the ratio of non-synonymous to synonymous changes between species was significantly greater than the ratio of non-synonymous to synonymous polymorphisms (7 versus 0; neutrality index: 0.00; *p*-value: 0.015). This was different from the ratio expected under neutral conditions and was also unlike the genome-wide observation. Rather, this suggests that *IL21* has undergone positive selection in the mammalian lineage, whereas its ORF appears fixed in the human population

WORRIES AND CHALLENGES

Potential problems likely to be encountered in accomplishing these lofty aims;

The above work was carried out in level four containment in America. The first problem envisaged is logistics of getting this level four containment in key tertiary centers that would be earmarked for the future work and also qPCR for the regulatory gene assay earlier mentioned. Most of these tertiary health institutions pay little or no attention to haemoharrgic fever viruses; they only tend to look for non-existing research centers for this sort of work. The questions that often agitate our minds are;

How many patients know about such centers?

If critically ill patients look onto these "centers of excellence for health!"

What should prevent these centers from having their own research centers where patient samples that poise challenges can be given adequate attention?

At best, and in the interim, why should there be barriers between these centers of excellence and conspicuous research centers in Nigeria; in other words why is there no inter-institutional collaborations with this regard??

ARE THESE WORRIES SUMMONTABLE?

YES they are; -the popular Nigerian adage says where there is **a will** there is **a way**; with full **commitment** on the part of the government and by **dint of hard** work from those of us proposing to undertake and face these challenges squarely we shall definitely succeed.

Experimental Design:

This would be a crossectional study employing a cluster sampling collection technique from endemic areas of Lassa fever infections within F.C.T. Abuja and its environs. Collected samples shall be processed in America where the preliminary results were done in order to acquire the expected skills and expertise.

STUDY LOCATION; our proposed study location shall be in the Federal Capital Territory Abuja Nigeria. However for skill acquisition and capacity development with this regard, we hope to carry out our sample analysis in Harvard University in the U.S.A. i.e. United States of America- where some group are already engaged in similar work. After which, God's willing we shall establish and replicate similar idea in our prominent tertiary health institutions in Abuja Nigeria and also other areas.

STUDY DURATION; A maximum of three years with quarterly feed back to home based institutions.

SOURCE OF RESEARCH SUPPORT/FUNDING

Seeks to get support from Nigerian government- as haemoharrgic fever virus disease is a major threat to human existence now in Africa if not the whole world.

CURRENT *STATI OF* STAKE HOLDERS; PhD Microbiology/Virology, MSc. Parasitology, Microbiology**, F.I.M.L.S.**, A.I.M.L.S. Medical Laboratory Sciences BSc. Hons.

CURRENT POSITIONS OF STAKE HOLDERS IN NATIONAL HOSPITAL ABUJA NIGERIA: Assistant Director**, Chief** Medical Laboratory Scientist, Assistant Chief Medical laboratory scientists.

3. Conclusions

With rigorous and intense pursuit of the molecular epidemiology of the *LARGE* **and** *IL21 Genes* in Lassa fever virus endemic in Nigeria- the pace of development of future lasting split gene preventative vaccine would be highly stepped up. As these genes suggest that they are most likely target of selection in differential gene regulation. This needs to be confirmed through extensive experimental studies, such as qPCR profiles for Lassa Fever patients and controls viz-avis reporter assays and biochemical tests *in vitro*. Combined, agnostic computational methods such as CMS and rigorous reductionist hypothesis testing in the laboratory should enable us to take the ever-increasing list of natural selection candidates to validated examples of evolution in the human species.

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