COMPARATIVE ANALYSIS OF THE IMPACT OF TWO VARIATIONS OF QIAGEN EXTRACTION PROCEDURE ON PCR AMPLIFICATION IN LASSA FEVER DIAGNOSIS IN OWO

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Abstract

Lassa fever, also known as Lassa hemorrhagic fever, is an acute and often fatal viral hemorrhagic fever. It is caused by the Lassa virus, which is an enveloped RNA virus from the Arenavirus family of viruses. The purpose of this study was to compare two different methods of Qiagen extraction in respect to their IC values, GPC and L gene Ct Values for Lassa fever diagnosis. Seventy Three (73) samples which were previously analyzed positive for Lassa virus and stored under appropriate storage condition at the Molecular Laboratory, Federal Medical Center, Owo, Ondo state was used for the study. Data obtained were analyzed using Sample T-test and graphical correlation. Sample T-test was used to compare differences in mean and relationship between methods were represented graphically. The study showed that data for GPC-Gene Cycle Threshold between the two methods was not statistically significant (p=0.393). On the other hand, data obtained for IC-1

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for G gene, L- Gene Cycle Threshold and IC-2 for L gene was statistically signification (p=0.000). There was a positive correlation between values obtained using method 1 and 2 for the GPC gene (r, 0.686), IC for the GPC gene (r, 0.486) and IC2 for L-gene (r, 0.705) and negative, but weak correlation for the L-gene(r, -0.161). This study has shown that two variations of Qiagen Extraction procedure could be used for Lassa fever diagnosis because it gives comparable yield with PCR amplification

Keywords*: two variations of Qiagen extraction, PCR, Lassa fever, GPC gene*

INTRODUCTION

The acute viral haemorrhagic fever known as Lassa was initially identified in 1969 in the town of Lassa in the Nigerian state of Borno, which is situated in the Yedseram River Valley near the southern tip of Lake Chad. (Frame *et al.*, 2017). The disease is endemic to West African nations and results in 300,000–500,000 cases annually, with about 5000 fatalities (Ogbuet *et al.,* 2007). Nigeria, Liberia, Sierra Leone, Guinea, and the Central African Republic all experience outbreaks of the diseases (Ogbu *et al.,* 2007). Mali, Senegal, and the Democratic Republic of the Congo are thought to also have human infections (Atkins *et al.,* 2009). Travelers who were infected have also exported cases to other nations. The Lassa virus, an RNA virus belonging to the Arenaviridae family, is the cause. The African soft furred rat (Mastomys natalensis), which may be found throughout West Africa and coexists closely with people, is the natural reservoir for Lassa disease. Mastomys discharge the virus in the urine, and human food contamination is a possible mechanism of transmission. The virus may also spread from person to person by contact with bodily fluids of infected individuals in medical facilities, leading to nosocomial epidemics with case fatality rates (CFR) of up to 65 percent (Fisher-Hoch *et al.,*2012). The majority of Lassa virus cases, though, show just minor or absent symptoms. A serious illness will strike one in five individuals (Fisher-Hoch *et al.,* 2012). The National Institute of Allergy and Infectious Diseases' classification of the Lassa virus as a category **A** pathogen has restricted the availability of laboratory testing. For handling possibly infectious specimens, bio-safety level 4 (BSL-4) precautions are advised. 3 weeks after Lassa Virus infection, persons begin to feel sick (CDC, 2016). The earliest signs and symptoms of Lassa fever are vague and may include: Fever, achy, headache, sore throat, coughing, discomfort in the chest or abdomen, and vomiting (WHO, 2016). The majority of the time, symptoms are severe; nevertheless, about 20% of cases develop abnormal bleeding, generalized edema, respiratory distress, hypotension, proteinuria, transaminitis, deafness, encephalopathy, or hypotension (Frame, 2012). Despite the fact that Lassa has a low overall fatality rate (Richard, 2003). It is between 15 and 20 percent among hospitalized patients (McCormick *et al.,* 2012). In

outbreaks and among pregnant women, particularly in the third trimester of pregnancy, increased death rates have been documented (Price *et al.,* 2005).

Lassa fever is difficult to identify from other febrile disorders based solely on clinical symptoms, so laboratory testing is necessary to make a diagnosis (Fukushi *et al.,* 2012). To enable proper case management, such as treatment, the adoption of isolation measures, or the tracking of contact parties, a suspect must be quickly isolated or verified. By enabling early diagnosis, which may need quick antiviral therapy and reduce morbidity and mortality, assisting in the tracing of community contacts, and providing the full picture of the epidemic, diagnostics play a crucial role in the control of an outbreak of Lassa fever (Hamblion *et al.,* 2018). Enzyme-linked immunosorbent serologic assays (ELISA), which can identify both IgM and IgG antibodies as well as Lassa antigen, are the most frequently used to diagnose Lassa fever. During the first few days of sickness, only a small percentage of patients have IgM and IgG antibodies detectable, and patients with deadly Lassa fever may never develop antibodies (Khan *et al.,* 2008). To quickly and accurately diagnose Lassa fever, RT-PCR is a useful tool. It is possible to detect diseases in their early stages using reverse transcription-polymerase chain reaction (RT-PCR). The virus itself can be cultivated in 7–10 days, however this method can only be carried out in a high containment lab using proper laboratory techniques. It is possible to achieve a post-mortem diagnosis using immunohistochemistry on formalin-fixed tissue samples. Although diagnostics are important for responding to outbreaks of Lassa fever, their availability is restricted for a number of reasons. Clinically, the majority of Lassa fever sufferers show no symptoms, and even those who do may not have any particular signs (Yun and Walker, 2012). Due to the intricacy of the Lassa virus sequence variety, there are also a lot of difficulties in creating an effective test (Raabe and Koehler, 2017). Additionally, Lassa fever specimens must be collected, stored, and handled under biosafety measures comparable to those used for the Ebola virus. Lassa fever assay development and validation studies may have been constrained due to the demand for high containment safety standards and the dearth of high containment facilities in many regions of the world (Raabe and Koehler, 2017). However, given that the World Health Organization (WHO) has called for early diagnostic tests for Lassa (WHO, 2016) and has identified the Lassa fever virus as a priority pathogen with epidemic potential in its research and development program (WHO, 2015), it is essential to thoroughly review the diagnostic tests that are currently available in order to identify any gaps in diagnostic research and development. Several modifications of the Qiagen extraction procedure are in use to detect Lassa fever virus in Nigeria. This study is designed to compare two variations of the Qiagen extraction method used in Nigeria in a bid to determine the most effective of both methods in obtaining better diagnosis. The result of this study will be useful to provide more information on which procedure is best to be used in Lassa fever diagnosis, being a highly infectious disease; false negative results due to low sensitive procedure for viral RNA extraction can result in high public health cost.

MATERIALS AND METHODS

STUDY AREA

The study was carried out at the Molecular Laboratory of Federal Medical Center, Owo, Ondo state, Nigeria. Owo is located on latitude 07°C11'N and longitude 05°55'E. It is at the southern edge of the Yoruba Hills, and at the intersection of roads from Akure, Kabba, and Benin City. Administratively, The Owo local government has a population of 222,262, based on 2006 population census. Owo is predominantly made up of people of the Yoruba tribe but urbanization and industrialization have brought in many other ethnic groups.

RESEARCH DESIGN

This study is an Experimental study conducted with a scientific approach using two sets of variables.

SAMPLING TECHNIQUE

Simple Random sampling technique was used in which an aliquot of a Lassa Positive Sample that has been stored were randomly picked and used.

SAMPLE SIZE DETERMINATION

The sample size was calculated using the formula from Daniel and Joseph (2010).
Applying the formula, a sample size of 73 was calculated for this study using the prevalence rate of 5% Lassa fever in Ondo state

$$N = \frac{t^2 \times p(1 - p)}{m^2}$$ (Daniel and Joseph, 2010)

P = prevalence rate 5% = 0.05
m = marginal of error =0.05
T = level confidence 95% =1.96

$$N = \frac{(1.96)^2 \times 0.05(1-0.05)}{(0.05)^2}$$

$$N= \frac{3.8416 \times 0.05(0.95)}{0.0025}$$

= 72.9
= **73**

ETHICAL CONSIDERATION

A protocol of this work was submitted to the ethical committee of Federal Medical Center, Owo for ethical clearance. After a successful review and consideration, ethical clearance was given by ethical review board with identification number FMC/OW/380/VOL.CLII/167.

SAMPLE COLLECTION

An aliquot of a Lassa Positive Sample that has been stored were randomly picked and used. The different samples were subjected to different extraction method to observe the differences in the Cycle Threshold Values (CT values) of G and L gene of the positive samples.

SAMPLE PREPARATION

The Principle of Qiagen: The QIAamp Viral RNA Mini Kits provide the fastest and easiest way to purify viral RNA for reliable use in amplification technologies. Plasma (treated with anticoagulants other than heparin), serum, and other cell-free bodily fluids can all be used to purify viral RNA. Samples may be frozen or fresh, however they must not be thawed more than once if they are frozen. Plasma samples should not be repeatedly frozen and thawed as this will diminish virus titers and reduce sensitivity. When samples are subjected to numerous freeze-thaw cycles, cryoprecipitates build up. When employing the vacuum procedure, this could result in the QIAamp membrane becoming clogged. QIAamp Viral RNA Mini Kits are for general use and can be used for isolation of viral RNA from a wide variety of viruses, but performance cannot be guaranteed for every virus.

INACTIVATION PROCEDURE

The carrier RNA was added to the AVL. 260 µl of the above mixture was pipetted into the elution column. 6 µl of internal control was added to all the elution column and this will be placed in the safety cabinet. 35 µl of Lassa sample plasma was pipetted into the the spin column. The mixture was vortexed. The mixture was incubated at room temperature for 10 minutes. 260 µl of Ethanol was added to the tube and will be vortexed

EXTRACTION PROCEDURE

The inactivated samples were transferred from the tubes to corresponding labeled fresh spin columns. The collection was centrifuged at 8000rpm for 1 minute and the collection tubes will be emptied and changed. Washing was done by adding 500 µl of AW1 on the spin column The above was centrifuged at 8000rpm for 1 minute 500 µl of AW2 was put into the spin column The above was centrifuged at 14000rpm for 3 minutes and collection tubes was changed 500 µl of AW2 was put into the spin column. The above was centrifuged at 14000rpm for 3 minutes and collection tubes was changed. The above was centrifuged at 14000rpm for 10 minutes to dry the RNA. The spin column was placed on the elution tube. Elution was done by adding 60ul of AVE into the elution tube. The above was incubated for 1 minute. Step (xii) was centrifuged at 8000rpm for 1 minute. The spin column was discarded and the trapped RNA will be transported to the master mix room in the elution tube.

MASTER MIXING

The RealStar® Lassa Virus RT-PCR Kit 2.0 contains 2 different RT-PCR assays with 48 reactions each. It contains two different Positive Controls: one for the GPC gene specific amplification and detection system and one for the L gene specific amplification and detection system. 20 µlof the Master G reagent was pipetted into the G labeled tubes (0.1ml tube). 20 µl of the Master L reagent was pipetted into the L labeled tubes (0.1ml tube).1 µl of Internal control was added into the tubes. 10 µl of the sample (extracted RNA) from the elution tube was added into the 0.1ml tube10 µl of positive control was added to the positive tubes of both the G tube and L tube. Nuclease Free Water was added to the negative control tubes, this served as negative control for the diagnosis. The above was mixed and transported to the PCR room and the thermocycler was used.

PCR (Polymerase Chain Reaction)
The thermocycler is used to amplify the RNA samples by PCR. The device has a thermal block with holes where the tubes with the PCR reaction mixtures can be inserted.
The cycler rises and lowers the temperature of the block in discrete, pre-programmed steps.

Qiagen Extraction Methods to be compared

METHOD ONE (1):

The AVL tubes was quick spun after addition of Viral RNase**.** 35 µl of Plasma samples were used**.** The AW1 and AW2 aliquots were examined**.** The columns and elutions were lablled correctly**.** The micropipette tips was changed in between tips for every step**.** All the lysat AVL was added to the collection tube which had the spin column**.** The collection was centrifuged at 8000rpm for 1 minute and the collection tubes was emptied. Washing was done by adding 500 µl of AW1 on the spin column**.** The above was centrifuged at 8000rpm for 1 minute**.** 500 µlof AW2 was put into the spin column**.** The above was centrifuged at 14000rpm for 3 minutes and collection tubes were changed**.** 500 µl of AW2 was put into the spin column**.** The above was centrifuged at 14000rpm for 3 minutes and collection tubes were changed**.** The above was centrifuged at 14000rpm for 10 minutes to dry the RNA**.** The spin column was placed on the elution tube**.** Elution was done by adding 60 µl of AVE into the elution tube**.** This was incubated for 1 minute**.** This was centrifuged at 8000rpm for 1 minute**.** This was the taken into the master mix room where the Lassa Altona 2.0 kit was added.

METHOD 2:

The samples was inspected visually to ensure it is thawed and the sample volume is 650 µl**.** The cryotube lid was removed if BME was not added to AVL for sample inactivation. 5 µl of BME was added and vortexed for 10 seconds and was placed in a water bath at 56°c and incubated for 15 minutes. 560 µl of ethanol was added to the tube and was vortexed for 10 seconds and incubated at room temperature for 5 minutes. 140 µl of Plasma samples were used**.** The mixture was centrifuged briefly. 650 µl of lysat was added into the QiaAmp mini spin columns without wetting the rim. The mixture was centrifuged at 10,000rpm for 1 minute. The column was placed into new collection tubes. 500 µl of AW1 was added to the column to wash and centrifuged at 10,000rpm for 1 minute. The old collection tubes were discarded and changed after (ix) above. 500 µl of AW2 was added to the column to wash and centrifuged at 10,000rpm for 1 minute. The old collection tubes were discarded and changed after (xi) above. 500 µl of 100% EtOH was added to wash and was centrifuged at 10,000rpm for 1 minutes. The old collection tubes were discarded and changed after (xiii) above. The above was centrifuged for 3 minutes at maximum speed to dry the column of any residual ethanol. The old collection tubes were discarded and was placed into an RNase-free 1.5mL microcentrifuge tube. 60 µl of AVE was added to the center of the silica gel matrix. The AVE was allowed to incubate on the matrix for 5 minutes. The RNA was eluted by centrifuging for 1 minute at 8000rpm. The eluent was carefully removed and reapplied to the

column and was allowed to incubate at room temperature for 5 minutes. The First elution was done at 8,000 rpm and final elution was centrifuged at 12,000rpm for 1 minute. The extracted sample was then taken into the master mix room where the Lassa Altona 2.0 kit will be added.

Variable	Method 1 Mean ±SD	Method 2 Mean ±SD	T-Value	P-value	Remark
GPC-Gene Cycle Threshold	30.98 ± 3.63	30.40 ± 5.12	0.866	0.393	Not significant
IC-1 for G gene	30.75 ± 1.74	27.89 ± 1.90	8.610	0.000*	Significant
L- Gene Cycle Threshold	33.10 ± 5.82	27.51 ± 4.29	7.536	0.000*	Significant
IC-2 for L gene	31.81 ± 3.28	26.90 ±0.98	7.642	0.000*	Significant

DATA ANALYSIS

Data collected were analyzed using Sample t-test. Categorical data was compared using graphical correlation.

RESULTS

Table 1: Comparison of Mean, Standard Deviation of Variables between Method 1 and Method 2

***p<0.05 (i.e. Significant).**

There is no significant difference between Glycoprotein complex (GPC)-gene cycle threshold value in Method 1 and G-gene cycle threshold value in Method 2 (p>0.05); and this means that the GPC-gene cycle threshold value in Method 1 and GPC-gene cycle threshold value in Method 2 are statistically the same. There is a significant difference between Internal control (IC)value for GPC gene in Method 1 and Internal control (IC) value in Method 2 (P<0.05) ; and this means that the IC value in Method 1 and IC value in Method 2 are statistically not the same. The IC value is lower and better in Method 2 than in Method 1 (27.89 Vs 30.75).

Similarly, there is a significant difference between L-gene Cycle Threshold value in Method 1 and L-gene Cycle Threshold value in Method 2 (P<0.05); and this means that the L-gene Cycle

Threshold value in Methods 1 and Method 2 are statistically not the same. The L-gene Cycle Threshold value is higher in Method 1 than in Method 2 (33.10 Vs 27.51).

Also, there is a significant difference between IC value for L-gene in Method 1 and IC value in method 2 (P<0.05); and this means that the IC value in Methods 1 and Method 2 are statistically not the same. The IC value is lower and better in Method 2 than in Method 1 (26.90 Vs 31.81).

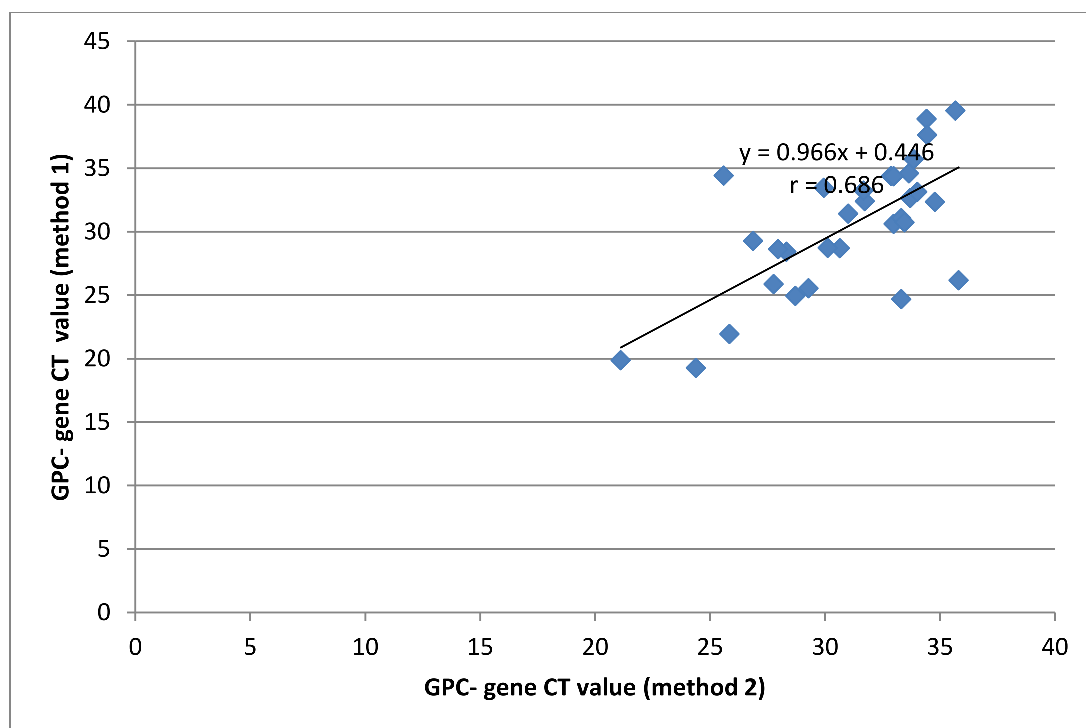

Fig 1: Scatter Diagram between GPC-gene CT Values of Method 1 and 2

The scatter diagram above showed that there is a very strong positive correlation between GPC-gene CT value in Method 1 and GPC-gene CT value in Method 2 (r = 0.686, p<0.05). Also, in every unit of G- gene Cycle Threshold in Method 2, there is a corresponding increase in G- gene Cycle Threshold in method 1 by 0.966.

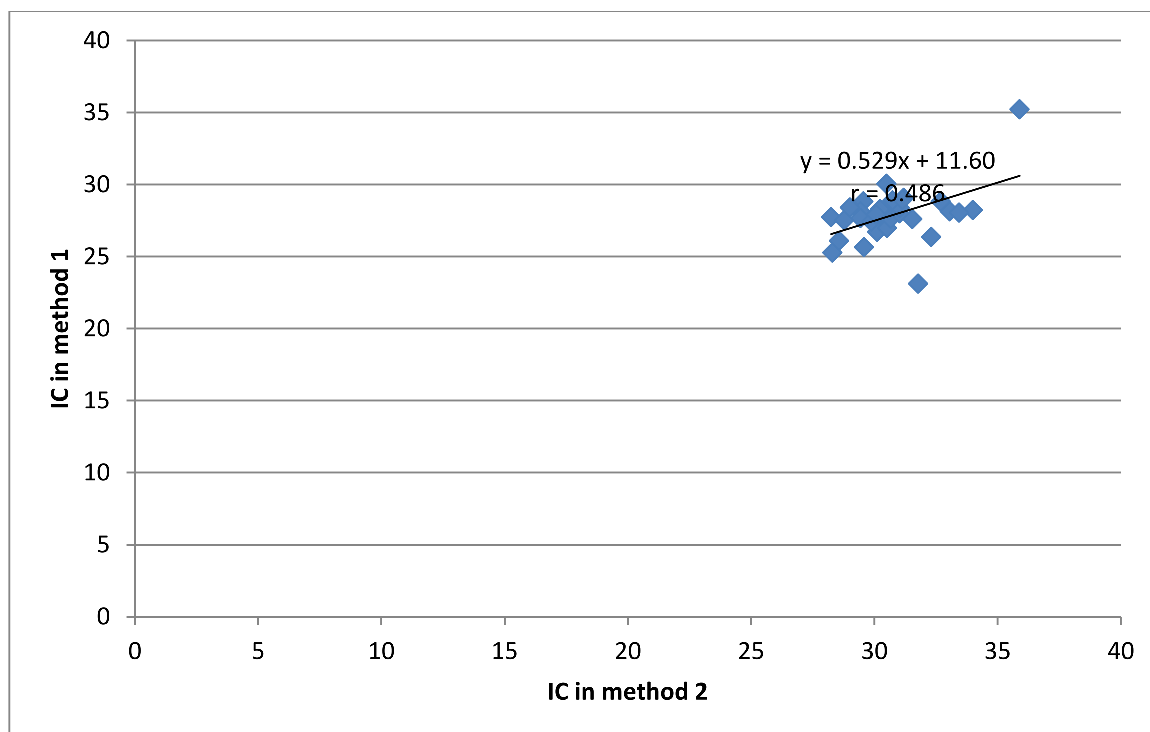

Fig 2 : Scatter Diagram between IC for GPC-gene Values of Method 1 and 2

The scatter diagram above showed that there is also a strong positive correlation between IC value in Method 1 and IC value in Method 2 (r=0.486, p<0.05). Also, in every unit of IC in Method 2, there is a corresponding increase in IC in Method 1 by 0.529

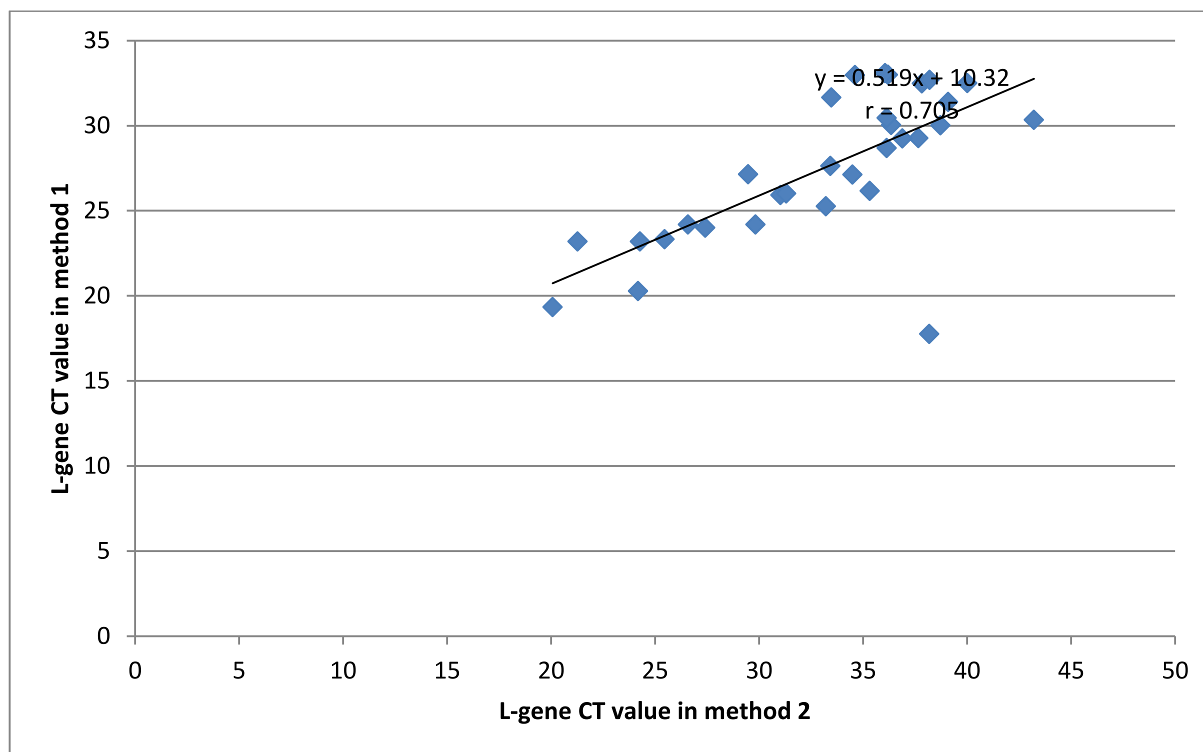

Fig 3: Scatter Diagram between L- gene CT Values of Method 1 and 2

The scatter diagram above showed that there is also a very strong positive correlation between L-gene Cycle Threshold value in Method 1 and L-gene Cycle Threshold in Method 2 ($r=0.705$, $p<0.05$). Also, in every unit of L-gene Cycle Threshold in Method 2, there is a corresponding increase in L-gene Cycle Threshold in method 1 by 0.519.

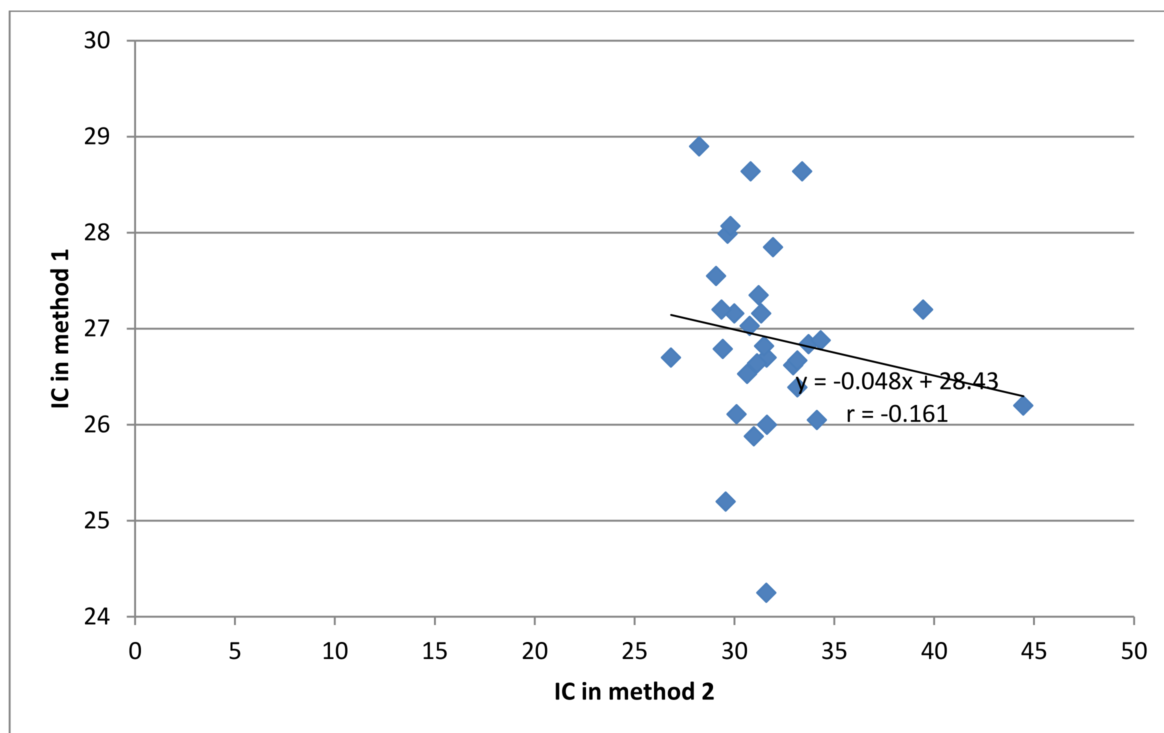

Fig 4: Scatter Diagram between Internal Control (IC) of L-genes Values of Method 1 and 2

The scatter diagram above showed that there is a negative correlation between IC value in Method 1 and IC value in Method 2 (r= -0.161, p>0.05). More so, in every unit of IC in Method 2, there is a corresponding increase in IC in method 1 by 0.048.

DISCUSSION

Lassa virus (LASV) is an Arenavirus belonging to the family Arenaviridae, which causes a viral haemorrhagic fever (VHF) known as Lassa Fever (LF).

In this study, two extraction methods that are used for Lassa fever diagnosis were compared in respect to their GPC-gene Cycle Threshold, Internal Control and L-gene Cycle.

From this study, data obtained for GPC- Gene using methods 1 and 2 showed no significant statistical difference. However, there was a statistical difference in the value obtained for L-gene using the two methods. In diagnostic application, GPC gene and L-gene data lower than 42 are predictors of good gene extraction. Based on this assertion, it might be inferred that while either method 1 or method 2 could be used for GPC gene extraction for molecular diagnosis, this might not be applicable to L-gene extraction. The differences observed in statistical levels between the two extraction methods could be attributed to the small sample size used for the study. The Sample volume used across methods is the cause of the increase in the GPC-gene CT value. The lower the

sample volumes, the higher the Cycle Threshold Value and the Higher the Sample Volume, the lower the Cycle Threshold Value.

In the vein, it might be hypothesized that the Sample volume (35 μl) used in Method 1 might cause an increase in the L -gene CT value of Method 1 and the Sample Volume (140 μl) used is Method 2 might also result in the Low Cycle Threshold of the L- gene for the samples. The lower the sample volumes, the higher the Cycle Threshold Value and the Higher the Sample Volume, the lower the Cycle Threshold Value.

Conversely, data obtained for internal control values for both GPC and L gene using method 1 and method 2 showed that values obtained using the two methods were statistically significant. This observation might be attributed to the small sample size used for the study. This observed difference might also be due to the fact that the Internal control was added at the Inactivation phase for Method 1 and there could have been inhibitions of the IC during the extraction process leading to an Increased IC output and the IC for Method 2 was added at the Master Mix phase without any occurrence of inhibition thus the low value of IC in method 2 this was revealed in (Table 1).

In analytical practice, IC data ranging between 25-28 are predictors of good internal control during the testing processes. The data obtained imply that using either of the methods could result in obtaining different values which might have impact on the testing outcome. The differences observed in the Internal controls for the GPC-gene for Method 1 and Method 2 is due to the Inhibition of the Internal control during the extraction process for Method 1 in which the IC is added at the Inactivation phase giving a higher IC output while the IC for Method 2 is added at the Master Mix phase and this gives a better result of the Internal control Output.

When the data obtained using the two methods were compared graphically (Figures 3.0-6.0) , it showed that there was a positive and weak correlation between values obtained using method 1 and 2 for the GPC gene (r,0.686) . There was a weak correlation in the values obtained for IC for the GPC gene (r, 0.486), IC2 for the L-gene (r, 0.705) using the two methods. However, graphical comparison between methods 1 and 2 for L-gene showed a negative and weak correlation (r, -0.161). This data could imply some level of agreement in the data obtained for GPC gene and IC-2 values for L-gene using the two methods; and and either might be used for the estimating the variables.

CONCLUSION

Lassa fever remains a major endemic disease among humans in developing country including Nigeria. This study has shown that: There is a great significant difference in the Internal Control values of Method 2 against Method 1 extraction procedures that were used in this study. The Cycle threshold of L-gene from Method 1 and Method 2 also showed a great difference in the values obtained. Method 2 Extraction procedure could be used for Lassa fever diagnosis because it gives comparable yield with Method 1 when used for GPC gene extraction assay.

RECOMMENDATION

Since Lassa fever remains endemic in the country, there is need to carry out more studies on the extraction procedures for Lassa fever diagnosis.

All reagents including extraction kits meant for diagnosis of Lassa fever should be retested before use to ensure that they will produce the desired result.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

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