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COMPARATIVE STUDY OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION ASSAY AND REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION TECHNIQUE IN THE DIAGNOSIS OF EBOLA VIRUS IN HUMAN BLOOD SAMPLES IN LAGOS, NIGERIA.

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ABSTRACT

The not too distant past outbreak of Ebola viral disease (EVD) in West African countries was reported to be the worst since its emergence in 1976. A major challenge to the management and control of EVD is lack of rapid and accurate diagnosis. Therefore, this study was conducted to compare the Loop Mediated Isothermal Amplification (LAMP) assay to the Reverse transcription-polymerase chain reaction (RT-PCR) technique for the diagnosis of Ebola virus (EBV) in human blood samples. EBV was confirmed in 5 (33%) and 16(12%) human blood samples by LAMP and RT-PCR, respectively. The sensitivity of LAMP assay was 83.3% while the specificity was 100% for LAMP when compared to RT-PCR. Findings from this study indicate that LAMP may be a rapid diagnostic tool that requires minimal expertise in developing countries in the diagnosis of this disease, which compares favourably to the gold standard RT-PCR.

Findings from this study indicate that LAMP with necessary diagnostic improvements may be a rapid diagnostic tool that requires minimal expertise in developing countries in the diagnosis of this disease, if it is to compare favourably to the gold standard RT-PCR.

Keywords: Human blood samples, Ebola virus, LAMP, RT-PCR, Diagnosis.

INTRODUCTION

Ebola virus disease (EVD) or Ebola haemorrhagic fever (EHF) is a disease of man and animal caused by Ebola virus. It is from the genus of *Filoviridae* family and *Mononegavirales* order. Filoviridae. The family members are characterized by filamentous enveloped particles with a negative sense single-stranded RNA genome (Kuhn *et al.,* 2010).

The transmission of EVD is still unclear. However the fruit bat is thought to be the natural reservoir of Ebola virus. Transmission can be from bats to bats or to other wild animals. It can be transmitted to human, from contact with an infected animal (Leroy *et al.,* 2005). The length of time between exposure to the virus and the development of symptoms (incubation period) is between 2 and 21 days (Charles, 2014).

The first outbreak occurred in Yambuku, Zaire in 1976 with a high mortality rate of 88%. Since then, series of outbreaks had affected the Republics of Guinea, Sierra Leone, Democratic Republic of Congo (DRC), Gabon, Liberia, South Sudan, Uganda, Ivory Coast and South Africa (Haradhan, 2014). The latest and largest outbreak occurred between 2013 and 2016, in West African countries including Guinea, Sierra Leone, Liberia, and Nigeria. This outbreak was reported to be the highest with 28,637 cases and 11,315 deaths reported worldwide with majority of the outbreak from Guinea, Liberia and Sierra Leone (WHO, 2015). The lack of rapid diagnosis for EVD means that infected people will unknowingly transmit the virus to others since the disease has similar symptoms with other common infectious disease such as malaria, dengue, and typhoid (Silvia *et al.,* 2015; WHO, 2015). Whereas, the reverse transcription-polymerase chain reaction (RT-PCR) which is the gold standard is very costly, time consuming and needs highly skilled technician to operate the equipment.

However, the loop mediated isothermal amplification (LAMP) assays have been developed for the diagnosis of other important pathogens, including measles virus, human papilloma virus, mumps virus, Human African Trypanosomiasis (HAT). Whereas, the reverse transcription-polymerase chain reaction (RT-PCR) which is the gold standard is very costly, time consuming and needs highly skilled technician to operate the equipment; the loop mediated isothermal amplification (LAMP) assays on the other

hand has been developed for the diagnosis of other important pathogens, including measles virus, human papilloma virus, mumps virus, Human African Trypanosomiasis (HAT). Tuberculosis, SARS *and* Dengue virus (Tsugunori *et al.*, 2015*).* Furthermore, the LAMP technology has been developed into commercially available detection kits for a variety of pathogens including bacteria and viruses and is acclaimed to be fast, accurate and employs the use of nucleic assay amplification. LAMP assay technique is based on generation of artificial stem-loop DNA structures flanking the target sequences. Cyclic strand displacement is performed at constant temperature, at which double-stranded DNA remains in the dynamic equilibrium. This allows primers to anneal to the complementary sequence of DNA, so DNA polymerase (with strand-displacing activity) can start DNA synthesis (Dorota and Milena, 2013). The LAMP method is adjudged to be accurate, time-saving and cost-effective compared with a conventional fluorescence based DNA chip because it does not require fluorochrome labelling and expensive fluorescence analysis equipment (Tsugunori *et al.,* 2000). These improvements takes diagnosis from the central laboratory to the needed environments such as the clinics, hospital emergency rooms, farms and other remote areas of need, but still requires improvement before fulfilling their potential (Yogesh *et al.,* 2014). LAMP method has yet to be documented for use in the diagnosis of EVD; but adopting its principles, we may probably achieve high performance results for EVD, because it has been documented for the past 10 years successes recorded in the used of LAMP based methods for detection of various pathogens (Parida *et al*., 2008; Fu *et al*., 2011; Yasuyoshi and Tsugunori, 2013), since the method does not require extensive training or equipment (Notomi *et al*., 2000; Yasuyoshi and Tsugunori, 2009; Yasuyoshi and Tsugunori, 2013). LAMP method is highly amenable to isothermal detection and best suited to overcome some of the disadvantages of other nucleic amplification methods such as PCR and real time PCR PCR (Yogesh *et al*., 2014). Therefore, the importance of accurate diagnosis in the management of the disease cannot be overemphasized. Hence, the aim of this study is to determine the effectiveness of LAMP assay in the diagnosis of EVD in blood samples.

MATERIALS AND METHODS

Ethical Review

The study protocol for the initial diagnosis was not reviewed because there was outbreak of the disease in the country as at the time of sample collection. However, exemption of study was received for the protocol for the validation of the LAMP assay where the research was conducted at the African Centre of Excellence for Genomics and Infectious Diseases (ACEGID) Redeemer's University, Ede. Osun State.

Study Sites and Patients Selection

The study was conducted in Nigeria during the EVD outbreak between July and October 2014. Samples were obtained from all suspected individuals during this period. All cases were made up of the first index case and contacts that later became indexes to other contacts. In addition other suspected cases with high fever and other symptoms of EVD were considered for enrolment.

Sample Collection

Venous blood samples obtained from all cases and suspected patients were collected from the Lagos State University Teaching Hospital (LUTH) during the outbreak and were sent to the African Centre of Excellence for Genomics of Infectious Diseases (ACEGID) laboratory in cold chain for the diagnosis.

Sample Handling and Sample Processing

Samples collected in the laboratory were handled with all the personal protective equipment (PPE's) properly put on. The Biosafety Lab 3 (BSL3) was sterilized continuously with chlorine as disinfectant.

One hundred and forty microliter (140 µl) of whole blood sample for RT-PCR and two dilutions (14 µl and 21 µl) for LAMP assay were inactivated by adding Qiagen buffer AVL to each tube and incubated at room temperature for 30 minutes in a laminar flow hood

RNA Isolation for Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and LAMP Assay

Qiagen Viral RNA extraction kit was used to isolate RNA from whole blood obtained during the EVD outbreak according to the manufacturer's protocol. Briefly, 560µl of ethanol was added to the inactivated samples and vortexed for 1 minute. The mixture was transferred into the Qiagen spin column and

centrifuged for 2 minutes at 800 rpm. The spin column was washed twice with 500 µl buffer AW1 and 500 µl AW2 and the RNA was eluted from the spin. Column with 60 µl of buffer AE was incubated at room temperature for 1 minute and centrifuged at 800 rpm for 2 minutes.

Diagnosis of Ebola Viral Disease

All samples received in the ACEGID laboratory were analysed for the presence of the Ebola virus using the RT-PCR technique while subset of the samples were selected for diagnosis using the novel LAMP assay technique.

Detection of Ebola Virus using the RT-PCR technique

The RT-PCR technique was used to detect the presence of Ebola virus in the samples obtained from the patients and suspected cases. The reaction was performed in a final volume of 25 µl containing 10 µl of Qiagen one-step buffer, 2.5 mM $MgCl_2$, 2 µl of dNTPs, 2 µl Qiagen one-step RT-PCR enzyme mix, 1 µl of forward and reverse primers and 2 µl of RNA template obtained from the samples obtained from the patients and suspected cases. The reaction was carried out in the MJ Research thermal cycler (Biorad, USA) set at 50 °C for 30 minutes and 15 minutes at 95 °C followed by 30 cycles of denaturation for 1min at 94 °C, annealing for 1 minute at 68 °C, extension at 72 °C for 1 minute and final extension of 72 °C for 10 minutes.

Detection of Ebola virus using the Loop-Mediated Isothermal Amplification (LAMP) Assay

The loop-mediated isothermal amplification (LAMP) assay (Bio-innovation Solutions; Epicentre Technologies, USA) was used to detect the presence of Ebola virus in selected samples obtained from subset of the samples received during the epidemic according to the manufacturer's protocol. Five microliter (5 µl) of the RNA isolated from blood samples obtained from the patients was used in a 12.5 µl master mix reaction containing 2.5 µl of EBV primers (F3 and B3), 1µl enzyme mix (EM) and 3 µl of distilled water (DW). The reaction mix was placed in the MJ Research thermal cycler set at 63 °C for 45 minutes and 80 °C for 2 minutes. The product was observed for turbidity and resolved using the gel electrophoresis technique. Those that showed turbidity visually indicated the presence of the RNA virus, while those that were not turbid indicated the absence of the virus. Fluorescent band from UV trans illumination indicated presence of the RNA virus while absence of fluorescence indicated absence of the virus.

Gel Electrophoresis for RT-PCR and LAMP Assay Product

Two percent (2%) agarose gel was used to resolve the amplified RNA products from RT-PCR and LAMP assays. Two grams (2g) of agarose powder (SeaKem^{TM,} FMC Bioproducts, Rockland, ME, USA) was dissolved in 100 ml of 1X TBE and stained with 1 µl of ethidium bromide. Ten microliter (10 µl) of the amplified product was loaded into the wells of the gel and allowed to separate at 90 volts for 30 minutes. The gel was placed in UV light for fluorescence.

Data Analysis

The prevalence of Ebola virus using the 3 techniques was determined in percentages. The sensitivity and specificity of the LAMP assay in the detection of Ebola virus were determined using the formula:

$$\text{Sensitivity (\%)} = \frac{\text{True Positive} \times 100}{\text{True Positive + False Negative}}$$

$$\text{Specificity (\%)} = \frac{\text{True Negative} \times 100}{\text{True Negative + False Positive}}$$

Positive and negative predictive values were determined using the formula below

$$\text{PPV (\%)} = \frac{\text{True positive} \times 100}{\text{True positive + False positive}}$$

$$\text{NPV (\%)} = \frac{\text{True Negative} \times 100}{\text{True negative + False negative}}$$

Where True positives is defined as those correctly diagnosed with the disease while false positive is defined as those incorrectly diagnosed with the disease. True negatives are those correctly diagnosed as absence of the disease while false negative are those incorrectly diagnosed of having the disease. Positive Predictive Value (PPV) is to test how likely it is for a patient given a positive result to be positive while Negative Predictive Value (NPV) is to test how likely it is for a patient given a negative result to be negative.

RESULT

Demographic and Clinical Information

All the patients whose whole blood samples were obtained for the RT-PCR diagnosis of Ebola virus, had symptoms common to the viral haemorrhagic fever which includes high fever of temperature >38.3 °C headaches, diarrhoea, muscle pain, abdominal pain and vomiting. Most of the patients were Nigerians and living in Nigeria. A few were from other West African countries experiencing the EVD outbreak or just visited the countries.

Outcome of Diagnosis Using RT-PCR, and LAMP Assay Techniques

RT-PCR

RNA samples were successfully analyzed for the presence of Ebola virus by RT-PCR in 136 RNA isolated from blood samples obtained from suspected patients. Of all the 136 samples analyzed, 12% (16) were confirmed positive for the presence of Ebola virus while 88% (120) were confirmed negative for the absence of the virus.

LAMP Assay

RNA obtained from a subset of 15 blood samples were considered for Ebola virus diagnosis using the LAMP assay technique. According to reference 2.3 above, diagnosis of ebola virus disease (EBVD). All samples received at the African Centre of Excellence for Genomics of Infectious Diseases (ACEGID) laboratory, Lagos, Nigeria were analysed for the EBVD using the RT-PCR technique while the subsets of 15 LAMP were randomly selected from the positive samples of the RT-PCR which was used as the gold standard. Of the 15 samples analysed using the LAMP assay technique, 5 (33%) were confirmed positive for the presence of Ebola virus while 67% were negative for the absence of the virus.

Correlation Analysis of Diagnosis of Ebola Virus in Whole Blood Sample by RT-PCR and LAMP Assay

RT-PCR technique is the gold standard for diagnosis of Ebola virus. The outcome of LAMP assay in subset of 15 blood samples was therefore correlated with outcome by RT-PCR. Five (5) of 6 samples positive by RT-PCR were also positive by the LAMP assay. All samples confirmed negative by RT-PCR were also negative by the LAMP assay. One sample confirmed positive by RT-PCR was negative by the LAMP assay (Table 1). Sensitivity of the LAMP assay using the RT-PCR as the gold standard diagnostic technique for Ebola virus was 83% (95%CI 35.8 – 99.5%) while specificity was 100% (95%CI 66.4 – 100%). The agreement between the LAMP assay and RT-PCR as measured by Kappa is 0.85 (Table 1).

Table 1: Correlation of RT-PCR Results with LAMP Assay

Method	Positive	Negative	Sensitivity	Specificity		K
				(95%CI)	(95%CI)	
LAMP Assay						
Positive	5 (83%)	0 (0%)	83.3%	100% (35.8–99.5%)	(66.4–100%)	0.85
Negative		1 (17%)	9 (100%)			
Total		6 (100%)	9 (100%)			

DISCUSSION

The effectiveness of supportive treatment available for management of EVD depends on adequate, accurate and prompt diagnosis (Towner *et al*., 2004; Espy *et al*., 2006; Ratcliff *et al.*, 2007; Mori and Notomi, 2009; Kilgore *et al.*, 2015). In the recent outbreak of EVD in Nigeria, RT-PCR accurately detected the presence of the virus in blood samples obtained from suspected patients. The same technique was also used in the diagnosis of the disease in other West African countries during the 2013-2015 outbreaks (Silvia *et al*, 2015; WHO, 2015). Even though the LAMP diagnostic techniques have been used in the detection of other infectious diseases such as malaria, HIV and so on (Luc and Michel, 2010; Dhama *et al*., 2014); the WHO recommendation for sensitivity of a diagnostic tool when compared with the gold standard (RT-PCR) diagnostic technique should be >95% (WHO, 2010). The sensitivity of the LAMP assay in this study was 83%. Although the sensitivity reported for the diagnostic technique is below the WHO recommendation. There are however, potentials for the use of these

technique for the diagnosis of the disease if the techniques are modified and improved upon (Tsugunori *et al.*, 2015). The high sensitivity and specificity of the LAMP assay in whole blood sample as observed is consistent with reports in other infectious diseases confirming that the techniques are generally rapid, reliable, sensitive and specific (Ndao, 2009). However the technique needs to be improved upon in order to achieve the recommended sensitivity. The LAMP assay though has a high specificity (100%) in this study, the sensitivity conformity with the RT-PCR technique is low (83%). One possible reason in this case is the fact that the assay was designed for other biological fluid such as saliva or swab and not for whole blood. It is possible that other blood components such as the hemoglobin and other membrane proteins or cell components which are not present in saliva or swab may be interacting with the reagents particularly the enzyme in the assay, this is in agreement with the report of Tsugunori *et al.* (2015). Overall, this study has shown the ability of the LAMP assay to detect the presence of the Ebola virus in blood samples. This technique is rapid and requires little or no expertise for its deployment, one notable point of its advantage is the point of care application, there is however a need to improve on the sensitivity and specificity of this assay in order to achieve an overall diagnostic capability.

CONCLUSION

Accurate and rapid diagnosis play major role in the management and control of EVD thus there is a need for rapid, accurate and reliable diagnostic tools for the diagnosis of the disease. The LAMP assay evaluated in this study may be very useful in the successful diagnosis of the Ebola Virus Disease from whole blood testing if necessary improvement and further study is carried out on the assay.

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REFERENCES

Charles, N. H. (2014). On the Quarantine Period for Ebola Virus. *Public Library of Science Currents*, 5: 121-135 doi: 10.1371/currents.outbreaks.2ab4b76ba7263ff0f084766e43abbd88.

Dhama, K., Karthik, K., Chakraborty, S., Tiwria, R., Kapoor, S., Kumar, A. and Thomas, P. (2014). Loop-mediated isothermal amplification of DNA (LAMP): a new diagnostic tool lights the world of diagnosis of animal and human pathogens: a review. *Pakistan Journal of Biological Science*, 17: 151-166.

Dorota, D. and Milena, K. (2013) Loop-mediated Isothermal Amplification (LAMP) as a diagnostic tool in detection of infectious diseases Microbiology Department, Faculty of Chemistry, *PhD Interdisciplinary Journal* 11(12): 80-233.

Espy, M. J., Uhl, J. R., Sloan, L. M., Buckwalter, S. P., Jones, M. F., Vetter, E. A., Yao, J. D., Wengenack, N. L., Rosenblatt, J. E., Cockerill, F.R. and Smith, T. F. (2006). Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical Microbiology Review*, 19:165-256.

Fu, S., Qu, G., Guo, S., Ma, L., Zhang, N. and Zhang, S. (2011). Applications of loop-mediated isothermal DNA amplification. *Applied Biochemistry and Biotechnology*, 163: 845–850.

Haradhan, K. M. (2014). The most fatal 2014 outbreak of Ebola Virus disease in West Africa. *American Journal of Epidemiology and Infectious Disease*, 2(4): 101-108. Doi 10.12691/ajeid-2-4-4

Kilgore, P. E., Grabenstein, J. D. and Salim, A. M. (2015). Treatment of Ebola virus disease. *Pharmacotherapy*, 35:43-53.

Kuhn, J. H., Becker, S., Ebihara, H., Geisbert, T. W., Johnson, K., Kawaoka, M. Y., Lipkin, W. I. and Negredo, A. I. (2010). Proposal for a revised taxonomy of the family. Filoviridae: Classification, names of taxa and viruses, and virus abbreviations. *Archive of Virology*, 155: 2083–2103.

Leroy, E. M., Kumulungui, B., Pourrut, X., Rouquet, P., Hassanin, A., Yaba, P., Délicat, A., Paweska, J. T., Gonzalez, J. P. and Swanepoel, R. (2005). Fruit bats as reservoirs of Ebola virus. Ebola hemorrhagic fever outbreaks in Gabon, 1994-1997. Journal of Infectious Disease, *Journal of Infectious Disease*, 438: 575–576.

Luc, B. and Michel, G. B. (2010). Diagnosing Infections-Current and anticipated technologies for point-of-care diagnostics and home-based testing.

Clinical Microbiology and Infection, 16: 1044-1053.

Mori, Y. and Notomi, T. (2009). Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of Infectious Chemotherapy*, 15: 62–69.

Ndao, M. (2009). Diagnosis of Parasitic Diseases: Old and New Approaches. *Interdisciplinary Perspectives on Infectious Diseases*, Special Edition, Available at URL https://doi.org/10.1155/2009/278246

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K. and Amino, N. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28: 63-68.

Parida, M. S., Sannarangaiah, P. K., Dash, P. V. and Rao, K. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Review in Medical Virology*, 18: 407–421.

Ratcliff, R. M., Chang, G., Kok, T. and Sloots, T. P. (2007). Molecular diagnosis of medical viruses. *Current Issues in Molecular Biology*, 9: 87–102.

Silvia, D., Rosa, C., James, S. S., Hilde, D., Grazia, M. C., Anja, W., Kathryn, S., Gabriela, P., Raphael, B., Osman, B., Armand, S., Michel, V. H. and Rony, Z. (2015). Ebola outbreak in rural West Africa: epidemiology, clinical features and outcome. *Tropical Medicine and International Health*, 20: 448–454.

Towner, J. S., Pierre, E., Rollin, D. G. B., Anthony, S., Sharon, M. C., Martin, V., William, F. L., Christina, F., Spiropoulou, T. G., Ksiazek, M. L., Felix, K., Robert, D. and Stuart, T. N. (2004). Rapid Diagnosis of Ebola Hemorrhagic Fever by Reverse Transcription-PCR in an Outbreak Setting and Assessment of Patient Viral Load as a Predictor of Outcome. *Journal of Virology*, 78(8): 4330-4341.

Tsugunori, N., Hiroto, O.,Harrumi, M.,Toshihiro, Y., Keiko, W., ,Nobuyuki, A. and Tetsu, H. (2000). Loop-mediated isothermal amplification of DNA Nucleic Acids Res. *Clinical Microbiology of Infectious Disease*, 15:28(12).63-69.

Tsugunori, N., Yasuyoshi, M., Norihiro, T. and Hidetoshi, K. (2015) Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *Journal of Microbiology*, 53: 1-5.

WHO. (2010). WHO Recommendations on the diagnosis of HIV infection in infants and children:http://apps.who.int/iris/bitstream/handle/10665/44275/9789241599085_eng_Annexes.pdf, 2010 Accessed on 25th May, 2021.

WHO. (2015). World Health Organisation. Ebola Virus Disease: *Factsheet* http://www.who.int/mediacentre/factsheets/fs103/en/.2015. Accessed on 25th May, 2021.

Yasuyoshi, M. and Tsugunori, N. (2009) Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of infection and chemotherapy*. 15(2): 62-69.

Yasuyoshi, M. and Tsugunori, N. (2013). Loop-mediated isothermal amplification (LAMP): Recent progress in research and development *Journal of Infection and Chemotherapy*,19: 401-411.

Yogesh, C., Koelbl, J., Puckett, J., Moser, M. J., Klingele, A. J., Liles, M. R., Carrias, A., Mead, D. A. and Schoenfeld, T. W. (2014). A novel thermostable polymerase for RNA and DNA loop-mediated isothermal amplification (LAMP). *Frontiers in Microbiology* 5:395-408.