SHORT COMMUNICATION

Molecular Detection of Hepatitis E Virus in *Rattus norvegicus* in Lagos, Nigeria

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Abstract: Hepatitis E virus (HEV) is an important cause of acute viral hepatitis globally which is mainly transmitted through fecal-oral routes. HEV has also been reported in animal hosts like swine, rabbit, mouse, and wild rodents from different parts of the world. Wild rodents trapped within human dwellings around Ikotun, Alimosho Local Government Area of Lagos State, Nigeria were screened to determine their contribution to HEV epidemiology in the country. In this cross-sectional study in an urban setting, twenty small mammals including, *Rattus norvegicus* (12) and *Crocidura dolichura* (8) were captured, anesthetized with chloroform and euthanized by cervical dislocation. Viral RNA was extracted from blood, liver, kidney, heart and lung tissues and amplified by Nested reverse transcription Polymerase chain reaction (RT-PCR) and amplicons detected by 1.8% agarose gel electrophoresis. Eight out of twenty rodents (40%) identified as *Rattus norvegicus* were positive for HEV. The viral genome was detected in various organs of the rat including blood (50%), kidney (25%), lungs (33.3%), liver (50%), and heart (41.7%). Non-parametric Kruskal-Wallis test shows no significant difference in HEV among the tissues (P=0.0790; α = 5%). HEV RNA was not detected from *C. dolichura*. The high prevalence (40%) of HEV RNA detected in *R. norvegicus*, makes rodents an obvious target for further investigations for their roles in HEV epidemiology in Nigeria. Genome sequencing and comparison with human HEV sequences will help explain whether these rodents pose zoonotic threats.

Keywords: Hepatitis E Virus, Rodents, RT-PCR, Lagos.

INTRODUCTION

Hepatitis E virus is a naked, single-stranded, positive sense RNA virus and classified in the family *Hepeviridae,* and genus *Orthohepevirus.* HEV can be transmitted through fecal-oral routes from ingestion of contaminated water or food (Emerson *et al.,* 2004; Lapa *et al.,* 2015). The virus is an important public health problem causing acute sporadic and epidemic diseases worldwide. According to WHO, HEV infects 20million people, with 3.3million acute cases and 56,600 deaths annually (WHO, 2018). In developing countries such as Indonesia, Malaysia, Philippines and others in Southeast Asia, and African countries like; Egypt, Burkina Faso and Nigeria, HEV is endemic and causes outbreaks of acute hepatitis, usually during flooding (Jary, 2005; WHO, 2010). However, in the developed countries like USA and European countries, HEV was formally judged to be peculiar to those with previous travel history to endemic countries (Webb and Dalton, 2019). However, this is not totally correct due to reported cases of HEV among patients who had no travel history to endemic countries (Aggarwal and Jameel, 2011; Lapa *et al.,* 2015). Among immunocompetent individuals, HEV only cause asymptomatic and self-resolving infection, but there is a more severe manifestation of infection in immunosuppressed individuals as well as pregnant mothers with high mortality (Meng, 2010).

Pigs are regarded as the most important animal reservoir for HEV, but the virus has been detected in other mammals like chickens, deer, rabbits, mongooses, sheep and cattle (Buisson *et al.,* 2000; Meng, 2011).

In addition, HEV has been reported among distinct rodent species from different parts of the world including: *Rattus rattus, Rattus norvegicus, Sigmodon hispidus, Peromyscus maniculatus* thereby bringing to fore the importance of small mammals in HEV epidemiology (Meng *et al.,* 2002; Emerson and Purcell, 2003; Hirano *et al*., 2003; Johne *et al*., 2010a; Kamar *et al.,* 2012; Kanai *et al.,* 2012; Dremsek *et al.,* 2012; Widen *et al*., 2014). Although, there is no study that has fully established a zoonotic transmission of the specie Orthohepevirus-A (HEV-A) from rodents to man, recently, the transmission of the specie Orthohepevirus-C (HEV-C) was reported in a liver transplant patient in China (Sridhar *et al.*, 2018). Therefore, rats remain a huge threat for zoonotic hepatitis E infections, most importantly as they forage in and around human dwellings both in urban and rural settings. Although genotype-2 HEV has been reported in Nigeria together with other sporadic outbreaks in other parts of the country (Buisson *et al.,* 2000; WHO, 2010). However, there is no previous report of HEV among rodents from Nigeria which could help advance knowledge of HEV epidemiology in the country. Thus, we show in this study that brown rats (*Rattus norvegicus*) trapped from different streets within Ikotun environs of Lagos State, Nigeria harbor hepatitis E virus. This could be a baseline for further investigations of the possible roles of wild rodents in HEV transmission in Nigeria.

MATERIALS AND METHODS

Ethics approval

This study on wild rodents received ethical approval from the Research Grants and Experimentation Ethics Committee (RGEEC) of the College of Medicine, University of Lagos, Nigeria (approved #CM/COM/08/VOL.XXVI). The body also gave the permission for trapping and collection of samples from wild rodents.

Study design

This was a across-sectional study carried out within an urban setting in Ikotun, Alimosho Local Government area of Lagos State, Nigeria. Two species of small mammals were trapped from six different streets in the study area. Sample size was calculated by using the method of Kanai *et al.* (2012). Although the estimated sample size is 97, we were only successful in trapping 20 rodents. Some rodents were lost either via escaping from the cage or died before dissection.

Trapping and Collection of Samples from Rats

Rats were caught with medium-sized dorvin traps (25 cm x 6 cm x 6 cm) and larger (32 cm x 12 cm x 12 cm) traps at distinct human residential locations within six streets (A-F) in Ikotun area of Lagos, Nigeria. Rat identification was done by a specialist at the Department of Forestry and Wildlife Management, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. A total of 20 small mammals in the order Rodentia and Eulipotyphla were captured. Briefly, traps containing baits like fish and biscuit were placed in specific locations at the kitchen, living room and gutters near to refuse collection bins at each household in the various streets. Five samples comprising blood, liver, kidney, heart and lungs were taken from each rat. In all, sixty samples were from *Rattus norvegicus* (order Rodentia) and 40 samples from *Crocidura dolichura* (order Eulipotyphla). Rodents were anesthetized with about 400µL of chloroform per rat, and euthanized by cervical dislocation. Samples were collected in sterile plain bottles from each rodent and stored at -80°C before analysis.

Extraction of RNA Using Spin Column

During the first round of the extraction process, the (heart, liver, kidney and lungs) tissue samples from each rat were pooled together. Viral RNA was extracted using Qiagen RNA extraction kit (Qiagen, Germany). Tissues from each of the positive pools after nested RT-PCR were thereafter subjected to separate RNA extraction at the second phase of the extraction process so as to ascertain which particular rat tissue is positive for HEV RNA. 50mg of each tissue was collected with sterile surgical blade into 1.5ml tube. Thereafter, 800 µL of phosphate buffer solution (PBS, pH = 7.4) was added

and homogenized with the aid of sterile glass rod to form the stock. Aliquot from the stock was mixed with 560 µL of lysis buffer (pH = 7.2), and for the blood samples, 100 µL of whole blood was mixed with 560 µL of lysis buffer and incubated at room temperature for 10 min. Viral RNA was extracted using Qiagen RNA extraction kit (Qiagen, Germany) according to manufacturer's instructions

Reverse Transcription of RNA and PCR Amplification

One step reverse transcription of the first-round amplification using primer sequences in Table 1 was performed using the One Step RT-PCR Kit (Jena Bioscience, Germany). The components of the Master-mix in PCR include: 1x RT-PCR Buffer (pH = 8.7); 0.4 µM each of the sense and anti-sense primers as well as 1xScript RT-Enzyme-mix. In each reaction tube, 5 µL aliquots of viral RNA was used and polymerase chain reaction was performed in an Eppendorf Master cycler (Eppendorf, Hamburg, Germany) under the following conditions; RT at 50°C for 30 min, inactivation at 95°C for 15 min each, 40 cycles of denaturation at 95°C for 15 sec each, annealing at 55°C for 30 sec, and elongation at 70°C for 30 sec; and final extension at 72°C for 5 min. Blanks/negative controls as well as positive control (Swine HEV) were included in all experiments. After the first round of PCR, nested RT-PCR was performed. The Master-mix in PCR contains: 1 x Buffer, 10 mM dNTPs, 0.4 µM each for sense and antisense primers and 0.004µ/ml Taq. 1 µL of the PCR product was amplified with the nested PCR primers (Table 1) under the following cycling conditions; 35cycles of denaturation for 15sec each at 95°C, annealing for 30s at 60°C, elongation for 15sec at 72°C, and final extension for 5min at 72°C. The amplified nested PCR products were detected using 1.8% Agarose gel electrophoresis.

Statistical Analysis

Graph pad Prism (ver5) was used for statistical analysis. Differences in HEV RNA from different organs were computed using non-parametric Kruskal-Wallis test. *P* values less than 0.05 were considered statistically significant. Other variables were reported as percentages and Mean+SD.

RESULTS

Rattus norvegicus harbors Hepatitis E virus. By using a broad-spectrum primer targeting the conserved region of the ORF-1 of HEV genome, a nested RT-PCR protocol was used to screen for HEV RNA among wild rodents captured within Ikotun environs of Lagos State, Nigeria. A total of 12 *Rattus norvegicus* were trapped across the six streets with eleven males and one female. StrA has the highest number of HEV positive tissues (60%), whereas the lowest (13.3%) was from StrE (Table 2). Pooled tissues of 8 out of the 20 (40%) captured rats were positive for HEV at the expected product length of 331bp (Figure 1 A&B). The eight rodents from which we had the positive pools were all *Rattus norvegicus*. In order to verify which of the tissues in the pools were positive for HEV, HEV RNA was extracted from each of the tissues singly and carry-out a nested RT-PCR. Overall, twenty-four different tissues (40%) of sixty samples from *R. norvegicus* were positive for HEV RNA by nested RT-PCR (Table 2).The prevalence of HEV in each sample type include; blood (50%), kidney (25%), lungs (33.3%), liver (50%), and heart (41.7%) (Figure 2). There was no significant difference in the proportion of HEV RNA among the different tissues ($P = 0.0790$; $\alpha = 5\%$). HEV RNA was not detected from *Crocidura dolichura.*

DISCUSSION

In an attempt to investigate the contributions of small mammals to hepatitis E virus epidemiology in our environment, we screened for the presence of HEV RNA among *Rattus norvegicus* and *Crocidura dolichura* that were trapped within human habitations in Ikotun, Lagos-Nigeria. Eight out of the twenty (40%) captured rats identified as *Rattus norvegicus* were positive for HEV at the expected product length of 331bp.

Our findings at best shows that these rodents which are normally found within human dwellings in the study location harbor hepatitis E virus, although we could not determine whether they pose any risks for zoonotic transmission to humans. Hepatitis E is an important cause of acute viral hepatitis in many parts of the world with severe manifestations in immunosuppressed individuals such as organ transplant patients and pregnant mothers (Meng, 2010). Although the virus has been reported among different animals including rodents, the possibility of zoonotic transmission of the specie *Orthohepevirus-A* to humans is yet to be fully established (Haqshenas *et al.,* 2001; van der Poel *et al.,* 2001; Zhao *et al.,* 2009; Schielke *et al*., 2009), but that of the specie *Orthohepevirus-C* from rat to humans was recently published (Sridhar *et al.,* 2018). Already, there are a number of reports on the detection of HEV-specific antibodies in many rat species including *Rattus rattus* and *Rattus norvegicus* among others (Meng *et al.,* 2002; Hirano *et al*., 2003; Johne *et al*., 2010a; Kanai *et al.,* 2012; Dremsek *et al.,* 2012; Widen *et al*., 2014) and in the United States, around 40% of all rats have been reported to be seropositive for HEV (Meng *et al.,* 2002). HEV RNA has been reported in rats *(R. norvegicus)* from Germany (Johne *et al.,* 2010a), France (Widen *et al.,* 2014) as well as Norway (Kanai *et al.,* 2012). Direct contact with infected domestic animals such as pigs or contaminated sewage may serve as the source of HEV infection in rats (Arankalle *et al.,* 2001). In this study, HEV RNA was detected from *R. norvegicus* trapped within Ikotun environs of Lagos State, Nigeria. This agrees with previous reports by Johne *et al.*(2010a&b); Widen *et al.*(2014); and Kanai *et al.*(2012) who all detected HEV RNA from this specie of rodent. Although, the 40% prevalence rate of HEV RNA recorded in our study was much higher compared to the prevalence rates of 6.7%, 17.9% and 14.8% respectively from their studies. This might be due to our lower sample size. Our findings show the need for further investigations on the roles these rodents could possibly play in inter-species transmission of HEV in our environment, although this mode of transmission is yet to be totally proven except for species *Orthohepevirus-C* (Sridhar *et al.,* 2018). Genome sequencing and comparison with human HEV sequences will help explain whether rodents pose zoonotic threats to humans in our environment. HEV infection is very common in Nigeria, although with limited information on its detection from rodents. Yet, the virus has been reported to be widespread among Nigerian pigs and domestic animals with seroprevalence rates of 76.7% and 24.1% respectively as reported by Junaid *et al.* (2014) and Owolodun *et al.*(2014). In this study, HEV RNA was detected from the blood (50%), kidney (25%), lungs (33.3%), liver (50%), and heart (41.7%) samples of *Rattus norvegicus*, suggesting that HEV infects and replicates in wild rodents. The highest percentage of HEV RNA was recovered from the blood and liver samples, although there was no statistically significant difference in the concentration of HEV RNA between all the different tissues ($P > 0.05$, $\alpha = 5\%$). This study reveals that different tissues of *Rattus norvegicus* (blood, kidney, heart, lungs and liver) harbor HEV genome to almost equal degree. Our findings could be a base-line for future investigations which should seek to determine whether HEV genome detected in wild rodents in Nigeria have any similarity with human HEV sequences. This will help determine any potential threat for zoonosis. The small sample size as well as the lack of phylogenetic analysis of the obtained sequences is a limitation to this study.

In conclusion, a high prevalence of hepatitis E virus RNA was detected in *Rattus norvegicus* in Lagos-Nigeria. This gives a scientific proof that this rodent specie which is wide spread in every part of the study location harbors HEV. Thus, the activities of HEV in rodents require further investigations in our environment.

Table 1: Primers used in HEV PCR

Step	Primer designation	Sequence
RT-PCR (331bp)	HEV-cs	(5'- TCGCGCATCACMTTYTTCCARAA -3')
	HEV-cas	(5'- GCCATGTTCCAGACDGTRTTCCA-3')
Nested PCR (331bp)	HEV-csn	(5'- TGTGCTCTGTTTGGCCCNTGGTTYCDG-3')
	HEV-casn	(5'- CCAGGCTCACCRGARTGYTTCTTCCA-3')

Note: D=A, G or T; M=A or C; N=A, C, G or T; R=A or G; Y=C or T.*(adapted from Johne et al., 2010b)*

Table 2: Summary of rodents' species captured from each street &numbers of HEV RNA detected from each tissue

Rattus norvegicus			**No of positive HEV RNA**						
Street ID	**No of catch**	**Sex**	**Blood**	**Kidney**	**Lungs**	**Liver**	**Heart**	**Total**	**%+ve**
StrA	4	M	3	2	2	2	3	12	60
StrB	1	F	1	0	0	1	0	2	40
StrC	2	M	1	0	0	1	1	3	30
StrD	0	-	0	0	0	0	0	0	0
StrE	3	M	0	0	1	1	0	2	13.3
StrF	2	M	1	1	1	1	1	5	50
Crociduradolichura									
StrA	3	M	0	0	0	0	0	0	0
StrB	1	M	0	0	0	0	0	0	0
StrC	0	-	0	0	0	0	0	0	0
StrD	2	F	0	0	0	0	0	0	0
StrE	1	M	0	0	0	0	0	0	0
StrF	1	M	0	0	0	0	0	0	0

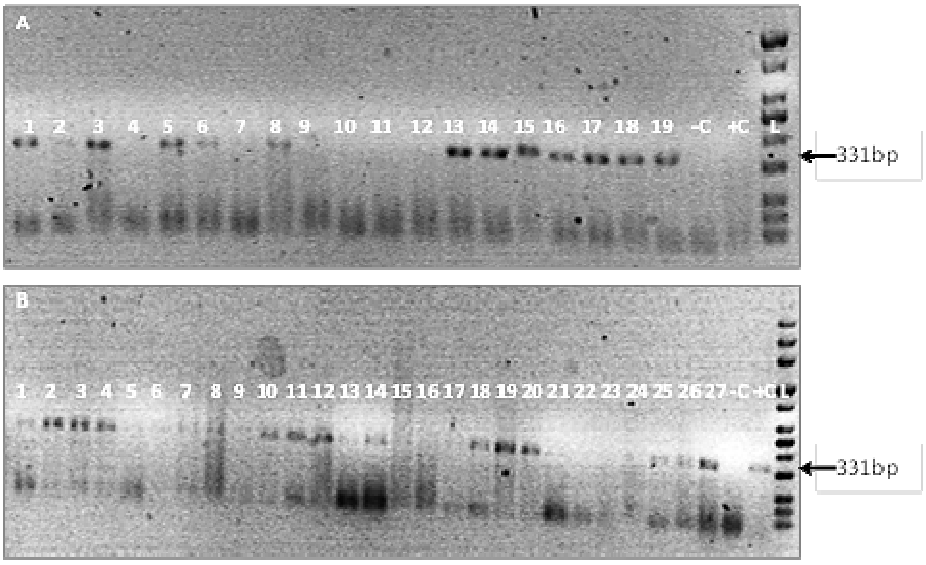

Figure 1: Agarose gel image of Nested RT-PCR of HEV RNA from rodent (*R. norvegicus*) from tissue pools-gel lanes 1,3,5,6,8,13,14,15,16,17,18, &19 show positive HEV RNA at the expected product length (331bp) from tissue pools (A); and rodent tissues in singles- gel lanes1,2,3,4,7,8,10,11,12,14,15,18,19,20,25,26 &27 show positive HEV RNA at the expected product length (B). RNase/DNase-free water was used as negative control (-C), while HEV positive sample recovered from swine in Lagos-Nigeria was used as positive control (+C). L= 100bp DNA Ladder (Qiagen, Germany).

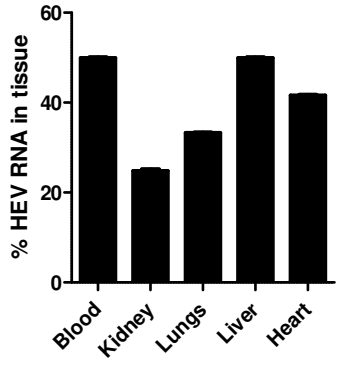

Figure 2: Percentage of HEV RNA recovered from different tissues *of Rattus norvegicus*. (Mean+SD of n=12 *R. norvegicus*)

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