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Molecular detection of hepatitis E virus among swine and poultry birds in Lagos, Nigeria

[1,2]Salu, O. B., [3,4]Mutiu, B. P., [1]Etok, M. J., [2]Orenolu, M. R., [2]Anyanwu, R. A., [2]Abdullah, M. A., [3,5]Saka, B. A., [3,6]Abdus-Salam, I. A., [7]Macaulay, R. M., [2,8]Oyedeji, K. S., and *[1,2,9]Omilabu, S. A.

[1]Department of Medical Microbiology and Parasitology, College of Medicine University of Lagos (CMUL), P.M.B. 12003, Lagos, Nigeria
[2]Centre for Human and Zoonotic Virology (CHAZVY), Central Research Laboratory, College of Medicine, University of Lagos (CMUL)/Lagos University Teaching Hospital (LUTH), Lagos, Nigeria
[3]Lagos State Bio-bank Laboratory, Mainland Hospital, Yaba, Lagos, Nigeria
[4]Department of Medical Microbiology and Parasitology, Lagos State University College of Medicine (LASUCOM), Ikeja, Lagos, Nigeria
[5]Centre for Biomedical Research Initiatives (CBRI), Nigeria
[6]Lagos State Ministry of Health (LSMOH), Alausa Ikeja, Lagos, Nigeria
[7]Lagos State Ministry of Agriculture (LSMOA), Alausa Ikeja, Lagos, Nigeria
[8]Department of Medical Laboratory Science, College of Medicine University of Lagos (CMUL), P.M.B. 12003, Lagos, Nigeria
[9]Department of Microbiology, Nigerian Institute of Medical Research, P.M.B. 2013, Yaba, Lagos, Nigeria
*Correspondence to: somilabu@unilag.edu.ng; Tel: +2348023119431

Abstract:

Background: Hepatitis E virus (HEV), the only hepatitis virus that replicates in humans and a wide range of animal hosts, is a significant public health enteric virus with a growing trend of infection globally. The public and environmental implications associated with HEV as a zoonotic transmitted virus remain to be fully elucidated. Thus, with the limited information on HEV in other species other than humans in Nigeria, this study aimed to detect by molecular methods HEV among some livestock in Lagos, Nigeria.
Methodology: A cross-sectional study of 172 (42.0%) poultry birds aged between 5 and 18 months, and 238 (58.0%) swine aged between 2 and 18 months purposively selected from Ojo, Ikorodu and Agege Local Government Areas (LGAs) of Lagos State, Nigeria between November 2017 and July 2019 was conducted. A total of 410 non-repetitive stool samples collected were analysed by molecular technique for the detection of HEV RNA. Descriptive statistics were computed for all relevant data. The association between gender and age with HEV RNA positivity was tested using Chi-square. All significant associations were recorded at $p \leq 0.05$.
Results: On the overall, 15 (3.7%) of the 410 stool samples were positive for HEV RNA with 5 (2.9%) and 10 (4.2%) of the 172 and 238 poultry birds and swine respectively. More female livestock (6.0%) had detectable HEV RNA than their male counterparts (1.0%) and the infection clustered majorly among age groups 1-6 months, and 7-12 months with a detection rate of 9.3%, 3.2% and 5.6%, 3.2% for both the swine and poultry birds respectively. Approximately 11.1% of the swine and 5.0% of the poultry birds' samples from Ikorodu LGA were positive for HEV RNA. Only 3.0% of the swine samples from Ojo LGA had detected HEV RNA. No sample from Agege LGA had detectable HEV RNA.
Conclusion: The detection of HEV in both the swine and poultry birds in Lagos, Nigeria further confirms the endemicity of HEV and a cause for public health concern regarding the epidemiology of HEV in Nigeria. There is an urgent need for active and continuous surveillance to further detect and subtype the circulating HEV among livestock to prevent the advent of virulent strains that may be transmitted to handlers and the community at large.

Keywords: Hepatitis E Virus, Swine, Poultry birds, Zoonotic Transmission, Polymerase Chain Reaction.

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Détection moléculaire du virus de l'hépatite E chez les porcs et les volailles à Lagos, Nigeria

[1,2]Salu, O. B., [3,4]Mutiu, B. P., [1]Etok, M. J., [2]Orenolu, M. R., [2]Anyanwu, R. A., [2]Abdullah, M. A., [3,5]Saka, B. A., [3,6]Abdus-Salam, I. A., [7]Macaulay, R. M., [2,8]Oyedeji, K. S., et *[1,2,9]Omilabu, S. A.

[1]Département de Microbiologie Médicale et Parasitologie, Faculté de Médecine Université de Lagos (CMUL), P.M.B. 12003, Lagos, Nigéria
[2]Centre de Virologie Humaine et Zoonotique (CHAZVY), Laboratoire Central de Recherche, Faculté de Médecine, Université de Lagos (CMUL)/Hôpital Universitaire de Lagos (LUTH), Lagos, Nigéria
[3]Laboratoire de Biobanque de l'État de Lagos, Hôpital Continental, Yaba, Lagos, Nigeria
[4]Département de Microbiologie Médicale et de Parasitologie, Collège de Médecine de l'Université d'État de Lagos (LASUCOM), Ikeja, Lagos, Nigéria
[5]Centre d'Initiatives de Recherche Biomédicale (CBRI), Nigéria
[6]Ministère de la Santé de l'État de Lagos (LSMOH), Alausa Ikeja, Lagos, Nigéria
[7]Ministère de l'Agriculture de l'État de Lagos (LSMOA), Alausa Ikeja, Lagos, Nigéria
[8]Département des Sciences de Laboratoire Médical, Faculté de Médecine Université de Lagos (CMUL), P.M.B. 12003, Lagos, Nigéria
[9]Département de Microbiologie, Institut Nigérian de Recherche Médicale, P.M.B. 2013, Yaba, Lagos, Nigéria
*Correspondance à: somilabu@unilag.edu.ng; Tél: +2348023119431

Contexte: Le virus de l'hépatite E (VHE), le seul virus de l'hépatite qui se réplique chez l'homme et chez un large éventail d'hôtes animaux, est un virus entérique important pour la santé publique avec une tendance croissante d'infection à l'échelle mondiale. Les implications publiques et environnementales associées au VHE en tant que virus transmis par des zoonoses restent à élucider pleinement. Ainsi, compte tenu des informations limitées sur le VHE chez d'autres espèces autres que les humains au Nigeria, cette étude visait à détecter par des méthodes moléculaires le VHE chez certains animaux d'élevage à Lagos, au Nigeria.
Méthodologie: Une étude transversale portant sur 172 (42,0%) volailles âgées de 5 à 18 mois et 238 (58,0%) porcs âgés de 2 à 18 mois, sélectionnés à dessein dans les zones de gouvernement local (LGA) d'Ojo, Ikorodu et Agege de L'État de Lagos, au Nigéria, a été mené entre novembre 2017 et juillet 2019. Au total, 410 échantillons de selles non répétitifs collectés ont été analysés par technique moléculaire pour la détection de l'ARN du VHE. Des statistiques descriptives ont été calculées pour toutes les données pertinentes. L'association entre le sexe et l'âge avec la positivité de l'ARN du VHE a été testée à l'aide du chi carré. Toutes les associations significatives ont été enregistrées à $p \leq 0,05$.
Résultats: Au total, 15 (3,7%) des 410 échantillons de selles étaient positifs pour l'ARN du VHE, dont 5 (2,9%) et 10 (4,2%) des 172 et 238 volailles et porcs respectivement. Un plus grand nombre de femelles (6,0%) présentaient un ARN du VHE détectable que leurs homologues mâles (1,0 %) et l'infection se concentrait principalement dans les tranches d'âge de 1 à 6 mois et de 7 à 12 mois avec un taux de détection de 9,3%, 3,2% et 5,6%, 3,2% respectivement pour les porcs et les volailles. Environ 11,1% des échantillons de porcs et 5,0% des échantillons de volailles de la LGA d'Ikorodu étaient positifs pour l'ARN du VHE. Seulement 3,0% des échantillons porcins d'Ojo LGA avaient détecté l'ARN du VHE. Aucun échantillon d'Agege LGA ne contenait d'ARN du VHE détectable.
Conclusion: La détection du VHE chez les porcs et les volailles à Lagos, au Nigeria, confirme en outre l'endémicité du VHE et une source de préoccupation pour la santé publique concernant l'épidémiologie du VHE au Nigeria. Il existe un besoin urgent d'une surveillance active et continue pour détecter et sous-typer le VHE en circulation parmi le bétail afin de prévenir l'avènement de souches virulentes susceptibles d'être transmises aux manipulateurs et à la communauté dans son ensemble.

Mots clés: Virus de l'hépatite E; porcs; volailles; transmission zoonotique; réaction en chaîne par polymérase

Introduction:

Globally, Hepatitis E virus (HEV) has received recognition as a significant public health enteric ribonucleic acid (RNA) virus that infects and replicates in humans and a wide range of animal hosts such as wild and domestic swine, deer, chicken, mongoose, rat, ferret, fish, and rabbits, with an ever-expanding host range (1-5). The peculiarity of HEV is that among all known major hepatitis viruses (A, B, C, E and G), HEV is the only one with animal reservoirs and is zoonotic (2,6-9).

Hepatitis E virus belongs to a group of non-segmented, non-enveloped and single-stranded RNA family Hepeviridae, which is divided into two genera, Orthohepevirus and Piscihepevirus (5,9,10-12). The genus, Orthohepevirus, where HEV is domiciled has four designated species known as Orthohepevirus A to D (9,12,13). Orthohepevirus A contains eight genotypes, HEV-1 to HEV-8 (9,13).

HEV-1 and HEV-2 are only implicated in human infections and are responsible for large hepatitis E outbreaks described in developing regions like Africa and Asia (5,9,14,15). HEV-3 and HEV-4 have been isolated in humans and other animals and are the main cause of sporadic infection among humans in develped countries. These two genotypes are considered zoonotic, and pigs and other animal species are reservoirs of these viruses which are transmissible to humans (5,9,12). HEV-5 and HEV-6 have been identified in Japanese wild boars; HEV-7 has been described recently in an immunocompromised transplant patient and dromedary camels, while HEV-8 was detected recently in Bactrian camels in China (5, 9,12). During an outbreak of HEV in 2017 in Nigeria, analysis of HEV genotypes responsible for the outbreak revealed the predominance of HEV genotypes 1 (HEV-1) and 2 (HEV-2), the generation of the second full-length HEV-1e genome available till date in the country (16, 17).

It has been estimated that about 20 million cases of HEV infections resulting in 3.4 million clinical cases, 30,000 stillbirths and

about 70,000 deaths, occur worldwide each year (5,18). In developing countries, HEV is generally considered a waterborne disease, transmitted by poor sanitation and faecal contamination of water supplies, and has also been associated with high morbidity and mortality in pregnant women (15,16,19,20). In the developed world, it is primarily a zoonotic disease and transmission is by consumption of undercooked infected meat, especially pork (5,9,11,14,15,19).

Hepatitis E virus is still regarded as an emerging pathogen in developing countries including sub-Saharan Africa, where information regarding the actual burden of the disease in humans and most particularly animals, is currently lacking due to inattention to the disease and limited public health responses (20). The public and environmental health concerns and risks associated with HEV infection, with more emphasis on zoonotic transmission, remain to be fully elucidated in this part of the world. However, documented evidence of HEV infections/outbreaks particularly in humans with few studies in animals have been described in Kenya, Sudan, Uganda, the Democratic Republic of Congo (DRC), Cameroon and the Central African Republic (14,15,21,22).

In Nigeria, varying prevalences of HEV infections have also been documented in different States; Jos-42.7% (23); Oyo-1.7% (24); Ekiti-13.4% (25); Osun-43% (26) and Lagos-17.8% (27). These infections have mostly been linked to human populations (9,17,28,29). Just a few studies have documented HEV in animals in Nigeria (17). Osanyinlusi et al., (30) in 2020 reported a high prevalence of HEV RNA detected in *Rattus norvegicus*, a rodent predominantly present within human dwelling, makes rodents an obvious target for further investigations for their roles in HEV epidemiology in Nigeria.

Based on the dearth of information on HEV in species other than humans in Nigeria, this study aimed to detect, using molecular methods, HEV among animals in Lagos State, the most populous and economic nerve center of Nigeria.

Materials and method:

Study area:

This study was conducted in three (Ojo, Ikorodu and Agege) selected local government areas (LGAs) of Lagos State, Nigeria, based on the locations having farms with mixed husbandry practice. Lagos is the commercial nerve centre of Africa and the most populous black city with over 21 million people (31). Lagos State is in South-Western region of Nigeria, with a total land area of 356,861 hectares (h), including 75,755 h of wetlands (31).

Lagos State is divided into five divisions consisting of Ikeja, Badagry, Ikorodu, Lagos Island and Epe (IBILE), with different farms spreading across the divisions where animals such as swine, chicken, cattle, and the likes are reared and sold in mixed husbandry practice. It shares its northern and eastern boundaries with Ogun State, its western boundary with the Republic of Benin, and its southern boundary with the Atlantic Ocean. It consists of 20 LGAs, with 37 local council development areas. It has a very diverse population due to heavy migration from other parts of Nigeria and surrounding countries.

Study design:

This was a cross-sectional study on molecular detection of the HEV among poultry birds, and swine in selected LGAs of Lagos State, Nigeria, between 2017 and 2019.

Ethical approval:

Approval for the study was obtained from the Health Research Ethics Committee (HREC) of the College of Medicine, University of Lagos with approval identification code CM/HREC/2/17/103, and the Lagos State Ministry of Agriculture.

Sample collection, transportation, & storage:

A total of 410 stool samples from active and inactive animals of both sexes and all ages, consisted of 172 chicken and 238 swine were collected using random sampling technique during the study period. The distribution of the samples within the LGAs sampled were; 164 from 101 chicken and 63 from swine in Ikorodu LGA; 171 from 71 chicken and 100 from swine in Ojo LGA; and 75 from 15 chicken and 60 from swine in Agege LGA.

Freshly voided droppings were collected using sterile universal sample containers with scoops and transported immediately in cold chain using triple-level packaging to the Centre for Human and Zoonotic Virology (CHA ZVY), College of Medicine of the University of Lagos, Idi-Araba, Lagos. All the samples were stored at -80ºC until required for RNA extraction, amplification, and detection by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

Viral RNA extraction, RT-PCR and nested PCR:

RNA extraction of HEV was done in a class II biosafety cabinet using the QIAamp Viral RNA Mini Spin kit (Qiagen, Germany), according to the manufacturer's instructions. In the first round, the RT-PCR was performed with the extracted RNA using the One-Step RT-PCR Master Mix Reagent (Jena Bioscience, Germany) in a total volume of 25µl comprising of 1µl each of the primers; HEV-cs 5'-TCG CGC ATC ACM TTY TTC CAR AA (23)-3'and HEV-cs 5-GCC ATG TTC CAG ACD GTR TTC CA (23)-3', 12.5µl one strength (1x) PCR Buffer, 1µl 1x enzyme mix, 5µl of extracted RNA and 4.5µl

molecular grade water. The RT-PCR cycling condition used was 30 minutes at 50^0C for reverse transcription reaction, followed by 5 minutes at 95^0C for reverse transcriptase inactivation/denaturation of the cDNA, followed by 40 cycles amplification of 15 seconds at 95^0C for denaturation and 15 seconds at 55^0C for annealing, with an extension of 20 seconds at 68^0C and a final extension at 3 minutes at 68^0C.

The first round of RT-PCR products was then prepared for nested PCR using 2µl of RT-PCR product, 5µl PCR master-mix buffer, 1µl forward and reverse primers (the nested primer HEV-csn neu 5-TGT GCT CTG TTT GGC CCN TGG TTY MG (26)-3'and HEV-casn 5-CCA GGC TCA CCR GAR TGY TTC CA (26) 3', and 16µl molecular grade water to make a final volume of 25µl. The nested PCR was carried out using the following cycling conditions; 5 minutes at 95^0C for initial denaturation, followed by 35 cycles amplification of 30 seconds at 95^0C for denaturation, and 30 seconds at 55^0C for annealing with an extension of 30 sec at 72^0C, and a final extension of 5 minutes at 72^0C.

The nested PCR amplicons were examined by electrophoresis on a 2.5% agarose gel, stained with 1.5µl 1x SYBR safe (Invitrogen, Carlsbad, California, United States) at 120 volts for 30 minutes, and the images of the amplicon band size of 345 base pair (bp) were visualized under ultraviolet (UV) illumination and documented with BioDoc Analyze 2.0imager (Biometra, Goettingen, Germany).

Data analysis:

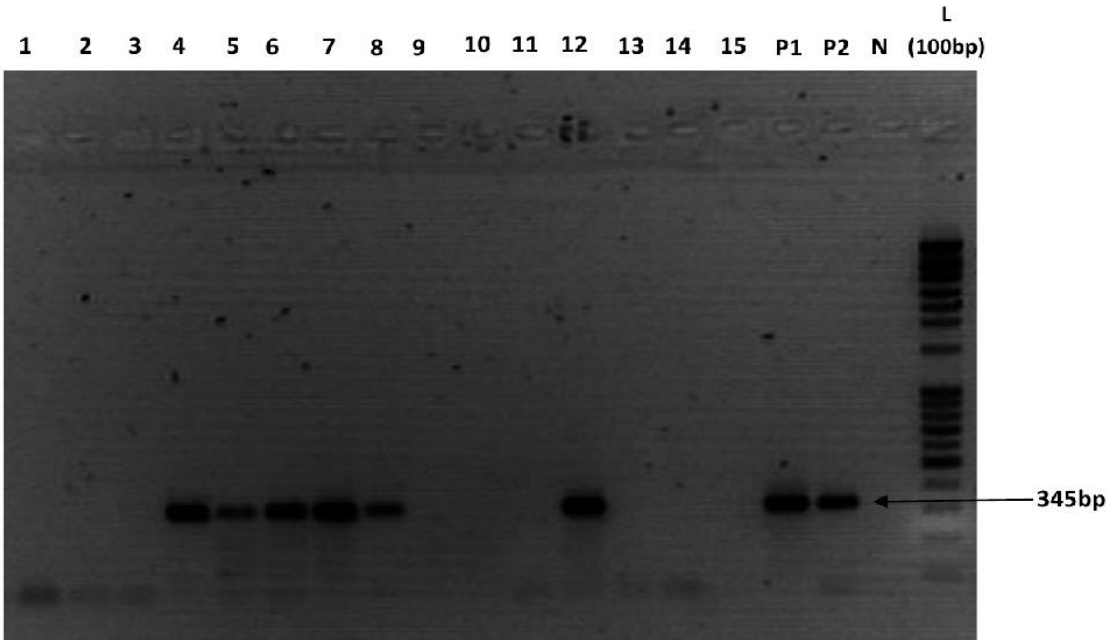
All qualitative/quantitative data were entered in the computer and analysed using SPSS version 26.0 for Windows (IBM Corp, 2021). Descriptive statistics were computed for all relevant data. The association between gender and age with HEV RNA positivity was tested using Chi-square. All significant associations were recorded at $p \leq 0.05$.

Results:

The analysis of all the 410 stool samples from 172 (42%) poultry birds aged between 5 and 18 months and 238 (58%) swine aged between 2 and 18 months, respectively, by the RT-PCR and the nested PCR revealed positive amplicons with band size of 345 bp for HEV RNA target and corresponding with the positive control band as shown in the image of the agarose gel electrophoresis (Fig 1.0). On the overall, 15 (3.7%) of these stool samples were positive for HEV RNA. Based on the different livestock sampled, 5 (2.9%) and 10 (4.2 %) of the 172 and 238 poultry birds and swine respectively, were positive for HEV RNA with an infection ratio of 1:2 between the poultry birds and swine (Table 1).

The detected HEV RNA were more prevalent in the female livestock (6.0%) than the male counterparts (1.0%) (Tab 1). The detected HEV RNA were mainly clustered among age groups 1-6 months, and 7-12 months for both animal groups, with a detection rate of 9.3%, 3.2% and 5.6%, 3.2% for both the swine and poultry bird samples respectively (Table 1). However, there was no statistically significant difference observed in the detection rate of HEV RNA across the gender (p=0.27) and age groups (p=0.20 and p=0.28) sampled for both livestock samples (Table 1). The HEV RNA was not detected in any sample from age group 13-18 months for both the swine and poultry bird samples (Table 1).

In addition, considering the distribution of HEV among the livestock sampled across the three LGAs, most of the positive swine (11.1%) and all positive poultry birds (5.0%) were from samples collected within Ikorodu LGA. However, only 3.0% of the positive swine samples originated from Ojo LGA. No HEV RNA was detected from both the poultry birds and swine stool samples collected from Agege LGA. The study showed statistically significant association between HEV RNA detection and LGAs from which samples originated (x^2=10.835, p=0.0044)) with significantly higher HEV detection rate in Ikorodu (7.3%, 12/164) compared to Ojo (1.8%, 3/171) and Agege (0%) (Table 1).



Lanes 1 – 15 represent the samples (Lanes 4, 5, 6, 7, 8 & 12 positive for HEV RNA), P1 & P2 (positive controls), N (negative control) and L (100bp ladder)

Fig 1: Agarose gel electrophoresis image showing the PCR amplicon band size (~345 bp) for targeted HEV RNA

Table 1: Characteristics and molecular parameters of livestock from three Local Government Areas (LGAs) in Lagos, Nigeria

Parameters	Total samples (%)	No positive (%)	*p* value
Livestock			
Swine	238 (58.0)	10 (4.2)	0.35
Poultry Birds	172 (42.0)	5 (2.9)	
Gender			
Male	194 (47.3)	2 (1.0)	0.27
Female	216 (52.7)	13 (6.0)	
Age group (months)			
Swine age group (months)			
1-6	54 (22.7)	5 (9.3)	0.20
7-12	157 (66.0)	5 (3.2)	
13-18	27 (11.3)	0	
Poultry birds age group (months)			
1-6	36 (21.0)	2 (5.6)	0.28
7-12	95 (55.0)	3 (3.2)	
13-18	41 (24.0)	0	
Locations			
Ikorodu LGA			
Swine	63	7 (11.1)	0.0044*
Poultry birds	101	5 (5.0)	
Ojo LGA			
Swine	100	3 (3.0)	
Poultry birds	71	0	
Agege LGA			
Swine	60	0	
Poultry birds	15	0	

* = statistically significant

Discussion:

Hepatitis E virus (HEV) is generally considered a waterborne disease in developing countries and a zoonosis in developed countries. However, the actual burden and transmission dynamics of this emerging endemic disease found across Africa are yet to be fully elucidated. In Sub-Saharan African countries including Nigeria, the level of awareness reg-

arding the zoonotic transmission of HEV is still very low as the sources of infection require further investigations. The detection of HEV RNA in livestock within farms in this study suggests there is a salient circulation of HEV among livestock in our environment and these animals could serve as main reservoirs of HEV infection and an important driver of the animal-to-human and human-to-animal transmission chains of HEV.

Domestic pigs and wild boar have been confirmed by serologic and molecular techniques to be major reservoirs of HEV and potential zoonotic transmission sources (5,32-37). The interactions of the handlers of these HEV-infected animals remain a risk within our environment. The transmission chain of the virus could be enhanced when infected and may serve as source of infection to other members of the community where they live, thereby spreading the virus to humans and possibly animals in reverse zoonosis. Thus, active surveillance of HEV in both animals and humans directly in contact with these animals will help to elucidate the actual burden and transmission patterns of the virus within our environment.

The detection of HEV in swine and poultry birds as documented in this study is also of significant public health importance, particularly to the communities harboring farms. The waste and effluents from the farms could be another source of transmission for HEV within the communities if not properly decontaminated. HEV, based on its properties, has been documented to be highly stable and resistant to environmental stress such as heat, extreme pH and desiccation (38,39). This permits the virus to be maintained within an environment for a long time and as such could result in contamination of food and surfaces. Therefore, strict application of hygienic measures during food production is crucial to prevent HEV's persistence on surfaces and subsequent cross-contamination. Owing to the high population density of the LGAs sampled in this study and associated sanitary challenges, water contamination by HEV and foodborne transmission cannot be ignored as the possible means of HEV transmission these areas and in Nigeria.

The prevalence of 4.2% among swine as compared to the 2.9% in poultry birds reported in this study is in line with the study of Adelabu *et al*. in 2016 [40] that reported an HEV prevalence rate of 4.4% among swine in South Africa. However, this prevalence rate is much lower than the prevalence rate of 76.7% of HEV detected by RT-PCR among swine in Plateau State, Nigeria (19). The disparities in this prevalence could be attributed to the fact that the stool samples used in this study were obtained from swine reared in confinement for commercial purposes and as such some levels of improved hygienic condition and animal health care service might have been put in place to avoid economic loss. In most situations in Nigeria, domestic animals like swine are managed in such a way that they are either housed or allowed to roam freely to scavenge for food and water. They visit waste heaps and stagnant or flowing water bodies thereby contaminating such areas with feces and urine. This system predisposes swine to diverse infections including HEV and thus facilitates subsequent transmission to humans, especially in environments where there are close association of swine and people (19,28). This detection in poultry birds could also result in commercial chicken farmers incurring losses in their businesses (41).

Considering gender, higher detection rate was recorded among the female compared to the male livestock, particularly in the swine population, although slightly more females were sampled in this study. This might not be unconnected with the rearing of offspring nature of the female swine. However, there was no statistically significant association between gender and HEV RNA detection among the livestock recruited in this study. Furthermore, the detection rate of HEV RNA in stool samples of both swine and poultry birds aged 1-6 months agreed with the findings of Martelli et al., (42), Owolodun et al., (19) and Abrantes et al., (43). However, both swine and poultry birds aged 7-12 months had a higher rate of HEV RNA in our study, suggesting that HEV could be circulating in all age groups and that swine that are close to slaughtering age can still be HEV-infected (44-49). It is important to note that in Nigeria, swine are usually slaughtered at 9 months of age, which raises concern about the potential zoonotic transmission of this virus by consumption and handling of raw or undercooked food products from pork meat which has been documented in the literature (5,50-52). However, there was no statistically significant association between age group and HEV RNA positivity among both livestock in this study.

There was a statistically significant difference (p=0.0044) in the association between the detection of HEV RNA among swine and poultry birds and the LGAs from which the samples originated. This finding is also of a significant public health concern in our environment, as the high population density and the high movement of people due to commercial and other purposes out of these LGAs could facilitate an enhanced salient transmission of this agent in Lagos State, Nigeria. There is a need for the implementation of comprehensive public health measures in educating farmers and other related workers such as abattoir workers, on infection prevention and

control by the public health policy makers, since there is no specific treatment for HEV infection (9,53-55). Although, the only currently available HEV vaccine was licensed in the year 2012 in China, a recommendation for its routine use has not been issued by the World Health Organization (WHO) (9). Therefore, the search for an effective vaccine against HEV for both humans and animals continue and requires urgent concerted efforts globally to minimize the risk of HEV transmission in endemic regions of the world (9).

Conclusion:

The detection of HEV in both the swine and poultry birds in Lagos, Nigeria further confirms the endemicity of HEV in the country and a cause for public health concern regarding the huge human population of the state and the country in general. HEV deserves increased monitoring and surveillance in humans and livestock within our locality for early detection and to forestall possible outbreaks. Also, further research needs to focus on the investigation of transmission chains from animal reservoirs to humans, including the food-borne route.

Implementation of a notification system for cases of HEV to help estimate the burdens and impact of infection in humans and animals, and an analysis of the genetic variability of HEV circulating in our environment is strongly advocated for in Nigeria. The analysis of the genetic variability of the strains of HEV circulating could not be analysed and reported in this study due to the high ambiguities and background noise of the generated nucleotide sequence data of HEV RNA and therefore remains a limitation.

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Contributions of authors:

SOB, EMJ, and OMR conceptualized the study and were responsible for the experimental and project design, analysis of data and writing the manuscript; MBP, ARA, AMA, and AIA made conceptual contributions, performed experimental analysis, and assisted in preparing the manuscript; SBA and MRM made conceptual contributions and assisted in preparing the manuscript; while OSA was the laboratory director, team lead of the Centre for Human and Zoonotic Virology and was responsible for the experimental and project design, analysis of data and writing of the manuscript. All authors read and approved the manuscript.

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No conflict of interest is declared.

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