Molecular detection of rabies virus strain with N-gene that clustered with China lineage 2 co-circulating with Africa lineages in Monrovia, Liberia: first reported case in Africa

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Abstract

Despite a long history of dog-transmitted human rabies outbreaks in Liberia, West Africa, no reports exist of molecular characterisation of the causative lyssaviruses. This study investigated Rabies lyssavirus (RABV) strains isolated at the dog–human interface in Monrovia, Liberia 2016 and 2017, by reverse transcription polymerase chain reaction, using primers specific for the nucleoprotein (N) gene. Out of 20 specimens (19 dog brain samples and one human saliva) tested as suspected rabies cases, three (15%) were positive. Purified amplicons from all three positive specimens were sequenced in both forward and reverse directions. Phylogenetic analysis was conducted in MEGA7 and PhyML3 to determine their relationship with RABV sequences accessioned in NCBI GenBank. The first of three RABV strains detected clustered with China lineage 2 RABVs of dogs (99% homology to KU963489 and DQ666322). The second strain segregated with Africa lineage 2 RABVs also of dog origin, and the third strain segregated with Africa lineage 3 RABVs of Southern Africa viverrids. Our results show a transcontinental strain of rabies virus co-circulating with Africa lineages in post-conflict Liberia. This finding should stimulate more effective sub-regional planning and execution of one-health actions, towards stepwise surveillance and elimination of rabies in West Africa by 2030.

Introduction

The goal of improving human–animal disease surveillance in West Africa remains an active concept note towards stepwise elimination of rabies in Africa by 2030 [1]. Although it has been more than seven decades since the first medical report of clinical diagnosis of rabies in dog bite victims (DBVs) in Liberia [2], the disease remains a neglected public health challenge associated with acute, progressive and highly fatal viral encephalomyelitis of warm-blooded animals (especially dogs) and humans [3, 4]. The extremely high rabies case fatality rate of nearly 100% makes it the deadliest of all known infections that are transmissible between animals and human beings. Yet, in post-conflict Liberia where dog-transmitted human rabies (DTHR) is enzootic, active surveillance for cases has been relatively neglected [3, 4], and the country is regarded as one of the remaining dark corners of rabies in Africa [5]. Periodic evaluation of the rabies status of many developing world countries such as Liberia is hampered by misdiagnosis, under-reporting, poor surveillance and unreliable data gathering at the local level [5]. However, with the emergence of Rabies in West Africa (RIWA) forum [6], there has been an increase in scientific studies and reports on rabies at the human–animal interface at various locations in Liberia, including Lofa County in the north, Bong County in the north-central and Grand Bassa County in the west-central region of the country [3], and also in Montserrado County, home to Monrovia the political capital of the country in the northwest region [4].

The high incidence of DTHR cases in Liberia has been attributed to poor vaccination coverage of owned dogs, the large population of stray dogs in urban areas, weak enforcement of dog control laws and difficulties in the cold-chain maintenance of vaccines in the post-conflict era [4]. Other factors include poor health care-seeking behaviour of DBVs, the high cost of rabies post-exposure prophylaxis, and a tendency of some DBVs to seek indigenous rather than orthodox healthcare [4]. While it is known that rabies is caused by all the 16 virus species

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currently listed under the order Mononegavirales, family Rhabdoviridae and genus Lyssavirus (bullet-shaped, single-stranded, negative-sense RNA viruses), [7] little has been done to characterise those that are in circulation in Liberia. Elsewhere, lyssaviruses that have been characterised include Aravan lyssavirus; Khujand lyssavirus; Irkut lyssavirus; Rabies lyssavirus (RABV); Duvenhage lyssavirus; European bat lyssavirus type 1; European bat lyssavirus type 2; Bokeloh bat lyssavirus; Australian bat lyssavirus; Lagos bat lyssavirus (LBV); Mokola lyssavirus (MOKV); Shimoni bat lyssavirus; Ikoma lyssavirus (IKOV); Gannoruwa bat lyssavirus; and Lleida bat lyssavirus [7]. Of these lyssaviruses, the prototype and most commonly incriminated species in rabies epizootics worldwide is RABV [8]. An estimated 60 000 human rabies deaths

Fig. 1. Map of Liberia in the West coast of Africa (a), Montserrat in the West coast of the country (b), and the four districts of Montserrat including Monrovia, the national capital (c).
### Table 1. Rabies diagnostic samples collected in Monrovia, Liberia for molecular and phylogenetic analysis

| Serial No. | Lab. ID      | Date of collection | Species                  | Source location                           | Types of sample | Other | RT-PCR          |
|------------|--------------|--------------------|--------------------------|-------------------------------------------|-----------------|-------|-----------------|
| 1          | CCPZUI/HS/35 | 22 March 2014      | Homo sapiens             | Paynesville, Monrovia, Liberia            | No              | Drool saliva\(^*\) | Negative |
| 2          | DBS/004      | 20 June 2016       | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 3          | DBS/01       | 15 October 2016    | Canis familiaris         | Duala, Monrovia North, Liberia            | Yes             | No    | Positive; GenBank Accession No. MF765758 |
| 4          | DBS/020      | 04 September 2017  | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 5          | DBS/030      | 13 September 2017  | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 6          | DBS/034      | 14 September 2017  | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 7          | DBS/039      | 15 September 2017  | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 8          | DBS/044      | 16 September 2017  | Canis familiaris         | Duala, Monrovia North, Liberia            | Yes             | No    | Negative        |
| 9          | DBS/052      | 21 September 2017  | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 10         | DBS/067      | 27 September 2017  | Canis familiaris         | Duala (Monrovia North), Liberia           | Yes             | No    | Negative        |
| 11         | DBS/069      | 29 September 2017  | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 12         | DBS/070      | 29 September 2017  | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 13         | DBS/071      | 29 September 2017  | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 14         | DBS/072      | 29 September 2017  | Canis familiaris         | Paynesville, Monrovia                    | Yes             | No    | Negative        |
| 15         | DBS/073      | 02 October 2017    | Canis familiaris         | Paynesville, Monrovia                    | Yes             | No    | Negative        |
| 16         | DBS/074      | 02 October 2017    | Canis familiaris         | Red light, Paynesville, Monrovia, Liberia | Yes             | No    | Negative        |
| 17         | DBT/075      | 02 October 2017    | Canis familiaris         | Paynesville, Monrovia                    | Yes             | No    | Positive; GenBank Accession No. MH507336 |
| 18         | DBS/076      | 02 October 2017    | Canis familiaris         | Paynesville, Monrovia                    | Yes             | No    | Positive; GenBank Accession No. MH507337 |
| 19         | DBS/077      | 03 October 2017    | Canis familiaris         | Red light, Paynesville, Liberia           | Yes             | No    | Negative        |
| 20         | DBS/078      | 03 October 2017    | Canis familiaris         | Paynesville, Monrovia                    | Yes             | No    | Negative        |

\(^*\) Three serial samples collected from the patient over a period of 6 h.

### Table 2. Primers used for RT-PCR assay for amplification of the \(N\) gene of lyssaviruses

| Primer (sense) | Detection | Position according to Pasteur virus nucleoprotein (N) gene\(^a\) | Nucleotide sequence 5′–3′ | Purpose                  | References |
|----------------|-----------|---------------------------------------------------------------|---------------------------|--------------------------|------------|
| 001Lys (+)     | RABV      | 1–15                                                          | ACCTCTAAAGAAA             | cDNA synthesis, PCR and sequencing | [12]       |
| JW12 (+)       | Pan-Lyssavirus | 55–73                                                   | ATGTAACACCTCTACAATG     | cDNA synthesis           | [13]       |
| 550B (−)       | RABV, LBV | 647–666                                                      | GTRCTCCATTAGCRCACAT       | PCR and sequencing       | [12]       |

\(^a\) Pasteur virus (NCBI GenBank Accession Number M13215).
| GenBank Accession No. | Collection (year) | Country | Host species | Lineage |
|-----------------------|-------------------|---------|--------------|---------|
| AB094438.1            | 2002              | Kyrgyzstan | Myotis blythi (bat) | Outgroup Lyssavirus |
| KC169985.1            | 2012              | France   | Myotis nattereri (bat) | Outgroup |
| AYS73943.1            | 2014              | Australia | Pteropus alecto (bat) | Outgroup |
| EU293120.1            | 1981              | Republic of South Africa | Miniopterus (bat) | Outgroup Lyssavirus |
| KF155004.1            | 2004              | UK       | Myotis daubentonii (bat) | Outgroup Lyssavirus |
| MF187859.1            | 1989              | France   | Eptesicus serotinus | Outgroup Lyssavirus |
| KX874609.1            | 2013              | Nigeria  | Canis familiaris (domestic dog) | Africa 2 |
| KX874612.1            | 2010              | Nigeria  | Canis familiaris (domestic dog) | Africa 2 |
| KX874613.1            | 2013              | Nigeria  | Canis familiaris (domestic dog) | Africa 2 |
| MH507336 (this study) | 2017              | Liberia  | Canis familiaris (domestic dog) | Africa 2 |
| MH507337 (this study) | 2017              | Liberia  | Canis familiaris (domestic dog) | Africa 3 |
| JX193798.1            | 2009              | Tanzania | Civettictis civetta (civet cat) | Ikoma lyssavirus (outgroup) |
| JX197457.1            | 2012              | China    | Murina leucogaster (bat) | Irkut virus (outgroup) |
| EF614261.1            | 2013              | Tajikistan | Bat | Khujand lyssavirus (outgroup) |
| EU293108.1            | 1985              | Senegal  | Eidolon helvum (bat) | Lagos bat virus (outgroup) |
| KY006983.1            | 2011              | Spain    | Miniopterus schreibersii | Lleida bat lyssavirus (outgroup) |
| GU170201.1            | 2009              | Kenya    | Hipposideros commersonii | Shimoni bat virus (outgroup) |
| EF614258.1            | 2018              | Liberia  | Dog | Africa 2 |
| MH481711.1            | 2018              | Liberia  | Dog | Africa 2 |
| MH481712.1            | 2017              | Liberia  | Dog | Africa 2 |
| EU853577.1            | 1986              | Tunisia  | Human | Africa 1a |
| EU853580.1            | 1987              | Ethiopia | Bovine | Africa 1a |
| U22643.1              | 1982              | Algeria  | Dog | Africa 1b |
| U22852.1              | 1982              | Morocco  | Dog | Africa 1b |
| KX148209.1            | 2004              | Madagascar | Dog | Africa 1c |
| KX148210.1            | 1998              | Madagascar | Human | Africa 1c |
| U22640.1              | 1990              | Niger    | Dog | Africa 2 |
| U22639.1              | 1995              | Cote D’ Voire | Dog | Africa 2 |
| EU718771.1            | 2006              | Chad     | Dog | Africa 2 |
| KX148242.1            | 1994              | Cameroon | Dog | Africa 2 |
| KF022180.1            | 2005              | Nigeria  | Dog | Africa 2 |
| EU888728.1            | 2006              | Nigeria  | Dog | Africa 2 |
| AF467949              | 2002              | Republic of South Africa | Mongoose | Africa 3 |
| DQ837431.1            | 1950              | Israel   | Dog | Africa 4 |
| DQ837461.1            | 1998              | Egypt    | Dog | Africa 4 |
| KU963489.1            | 2005              | China    | Dog | Asia (China 2) |
| DQ666322              | 2008              | China    | Dog | Asia (China 2) |
| MF765758 (this study) | 2006              | Liberia  | Dog | Asia |
| U22482                | 1986              | Iran     | Dog | Asia |
| U22918                | 1989              | Nepal    | Dog | Asia |
| U22480                | 1990              | Oman     | Red fox | Asia |
| U22654                | 1981              | Greenland | Arctic fox | Oceania |

(Continued)
occur each year, mostly among DBVs who are below the age of 15 years and resident in developing countries of Africa and Asia where cases are grossly under-reported [9].

To our best knowledge, the species, strains and phylogeny of lyssaviruses responsible for human and animal rabies in Liberia have not been previously identified or reported in medical literature. This was evident in the complete absence of accessioned rabies virus genes from Liberia in the NCBI or any other gene bank, before the three deposited from this study. Since a majority of the reported human rabies cases in Liberia are traceable to the bites of suspected rabid dogs [2–4], we hypothesised that dog-adapted RAVB variants are responsible for enzootic rabies in the country. In this study, we aimed to characterise the causative strains and phylogeny of rabies viruses at the dog–human interface in Liberia for the first time.

Methods

Ethical approval

The ethical approval (EC/LIBR/014/039) entitled ‘Ethical approval for spatio-temporal epidemiology of suspected human cases of rabies, habitat suitability for rabies virus circulation, and molecular investigations, Liberia’ was granted by the Liberian Biomedical Research Institute (LIBR).

Study locations

The study was primarily conducted in Monrovia city (also known as Greater Monrovia), in Liberia (Fig. 1). Monrovia (population: 970,824) is located on latitude 6°18′ N and longitude 10°48′5″ W in Montserrado, the oldest of the 15 Counties of the government of the Republic of Liberia. Monrovia is the administrative and financial capital of Liberia, and also the most densely populated location in the entire country [10]. The climate of the area is tropical with a distinct wet season that lasts from May to November and a dry season that lasts from December to April, each year. A Central Veterinary Laboratory and Clinic (CVLC) is located in Fendel, a small town, East of Monrovia within Montserrado County.

The CVLC kept an archive of specimens that comprised brain tissues (mainly brain stem and hippocampus) of suspected rabid dogs (n = 110), killed during outbreaks of canine rabies within and around two Monrovia communities – Paynesville, including the Red Light neighbourhood, and Duala – from June to October 2016 and from September to October 2017.

Passive surveillance of human–animal rabies in Liberia

The chronic neglect of rabies research in Liberia [11] received decisive attention by January 2012, when the University of

| GenBank Accession No. | Collection (year) | Country | Host species | Lineage |
|-----------------------|-------------------|---------|--------------|---------|
| U22475                | 1991              | Germany | Red fox      | Europe/Mid. East |
| U42706                | 1986              | Bosnia-Herzegovina | Red fox | Europe/Mid. East |
| EU293117              | 1974              | Cameroon| Crocidura sp. (shrew) | Mokola lyssavirus (outgroup) |
| GU992322              | 1993              | Morocco | Rabies virus strain Pasteur (PV) |
| GU992319              | 1995              | Czech Republic | Vaccine strain SAD Vnukovo |

Active surveillance for Lyssavirus species in Liberia

Active surveillance for Lyssavirus species in Liberia commenced in March 2014. At the time, there were no trained personnel and laboratory facilities for rabies diagnosis in Liberia. There were also no cold storage facilities available for the preservation of rabies suspect tissues. Against this backdrop, we decided to use Whatman™ FTA™ (Flinders Technology Associates) cards to collect and preserve rabies diagnostic samples including saliva, oro-pharyngeal secretions and brain tissues, for analysis at a later date. FTA cards have a filter paper component that is laced with proprietary chemicals that lyse cells and stabilise nucleic acids on contact, ensuring safe and long-term storage even at room temperature.

Serial saliva samples were obtained from a 10-year-old female DBV who was admitted to the Referral Clinic for Rabies Exposure in Monrovia, 2 months post-exposure with signs of clinical rabies. In April of 2014, the collection of rabies diagnostic samples was suspended in Liberia due to Ebola Virus Disease epidemic in the country. By the time sampling resumed in June 2016, the Liberian Government had set in motion a plan to upgrade the CVLC in Fendel, Monrovia. A solar-powered deep freezer facility and an archive of frozen dog brain tissue samples were soon established.

From the archive, a total of 19 dog brain samples were randomly selected for molecular tests to detect Lyssavirus infections. The samples were preserved in Ethanol 99% concentration, making them harmless and non-infections materials during shipment to laboratories in Nigeria where investigations aimed at detecting and determining the species, strain and phylogeny of the causative Lyssavirus were conducted at the Molecular Biology Laboratories of the Center for Control and Prevention of Zoonoses (CCPZ), University of Ibadan, Nigeria, and the National Veterinary Research Institute (NVRI), Vom, Plateau State, Nigeria. Sequencing of amplicons were conducted at the Biosciences Laboratory [11], International Institute of Tropical Agriculture (IITA),
Laboratory investigations

(1) RNA extraction

One gram of brain tissue was homogenised and 9 ml of PBS was added and centrifuged in a refrigerated centrifuge at 10,000 rpm for 5 min to make 10% tissue suspension. Total RNA was added and centrifuged in a refrigerated centrifuge at 10,000 rpm. The sample was immediately stored at −20 °C until further analysis.

(2) Detection of the nucleoprotein (N) gene of lyssaviruses by reverse transcription polymerase chain reaction (RT-PCR)

Detection of the N gene of lyssaviruses by RT-PCR was performed using the GeneAmp® Gold RNA PCR Core Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s guidelines. Three oligonucleotide primers namely JW12 (+), Lys001 (+) and 550B (+) were selected for use in this study, based on their annealing positions on Pasteur Virus (GenBank Accession Number M13215) (Table 2), as previously described [12, 13]. The primers were synthesised at Inqaba Biotechnical Industries (Pretoria, South Africa). The stages of the RT-PCR assay were as follows:

(a) Reverse transcription (RT)

Complementary DNA (cDNA) was produced by RT reaction using the following protocol: 1.0 µl of forward primer JW12 (20 µM) was added to 1.0 µl of forward primer 001lys (20 µM), 7.0 µl of nuclease-free water (NFW) and 2 µl of total RNA. The mixture was incubated at 65 °C for 2 min and cooled on ice for 5 min, to heat denature and anneal it with the primers. This was followed by RT at 37 °C for 60 min, 85 °C for 5 min, 37 °C for 10 min, 72 °C for 5 min and cooling at 4 °C, in a final volume of 20 µl containing 2 µl. A 1X reverse transcriptase buffer, 4.0 µl of deoxynucleoside triphosphate (dNTP) mixture (10 mls), 0.5 µl MultiScribe™ Reverse Transcriptase (15 U/µl) and 0.5 µl of Ribonuclease inhibitor (20 U/µl).

(b) PCR assay

Primary and secondary amplification of the cDNA (template) was performed using the primers 001lys and 550B, as previously described [12]. Briefly, a lyophilised anti-rabies vaccine (low egg passage Flurry Strain) from NVRI served as the positive control in the PCR. A non-template blank served as the negative control. Primary amplification of 5.0 µl of the cDNA template was performed in a final volume of 50 µl containing 4.0 µl of 001lys (20 µM), 4.0 µl of 550B (20 µM), 24.75 µl of NFW, 5.0 µl of 10X buffer, 3.0 µl of MgCl₂ (25 mls), 4.0 µl of dNTP mixture (10 mls) and 0.25 µl of AmpliTaq polymerase (5 U/µl).

For the secondary amplification reaction mixture (50.0 µl), 5.0 µl of primary PCR product (1:50 dilution) was used as a template and the conditions repeated as for the primary amplification. Both amplification reactions were performed on a GeneAmp® PCR System 9700 (Applied Biosystems). After denaturation at 95 °C for 1 min, reactions were cycled 40 times at 94 °C for 30 s, 37 °C for 30 s and 72 °C for 90 s, with final extension at 72 °C for 10 min. False-positive results were avoided by following the standard precautionary measures for PCR [14].

(3) Electrophoresis and gel documentation

The final PCR products/gene were visualised on 1% agarose gel stained with ethidium bromide using ultraviolet light, following electrophoresis at 100 volts for 40 min.

(4) Sequence determination

The PCR product was purified using ExoSAP-IT™ (Affymetrix Inc., Santa Clara, California, USA), according to the manufacturer’s guidelines. The purified product was sequenced in both forward and reverse direction using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer’s guidelines. The labelled product was then cleaned with the Zymo DNA Clean & Concentrator™−5 kit (Zymo Research Corporation, Irvine, California, USA), with subsequent analysis on an ABI3500XL genetic analyser with a 50 cm array (Applied Biosystems Inc.), using POP-7™.

(5) Phylogenetic analysis

The forward and reverse complement sequence reads (derived from the reverse sequence file using http://www.bioinformatics.org/sms/rev_comp.html) were combined into one contiguous sequence at the region of overlap of the two, using CAP3 sequence assembly program [15]. The sequence was edited manually using BioEdit Sequence Alignment Editor Version 7.2.6.1 software [16]. The edited sequence was used for a Megablast search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for highly similar nucleotide sequences in GenBank® database. The N genes of three vaccine strains SRV9 (AF499686), PV (GU992322) and SAD Vnukovo (GU992319) were also included in the list used for phylogenetic analysis of the Monrovia virus. Detailed information about all the sequences included in the phylogenetic analysis is provided in Table 3. The sequences were aligned using the Multiple Sequence Comparison by Log- Expectation (MUSCLE) option provided in the Molecular Evolutionary Genetics Software Version 7 (MEGA7) [17]. Pairwise genetic distances between sequences were estimated using the Kimura two-parameter substitution model [18], (Table 4). The results were used to construct a maximum likelihood tree using MEGA7. The phylogenetic tree was rooted to MOKV genotype 3 (EU293117) and other rabies-related lyssaviruses. Bootstrapping of 1000 replicates was used to statistically evaluate the branching order of the phylogenetic trees. The percentage of replicate trees in which the various strains clustered together is displayed next to the branches. A bootstrap support of 70% was considered significant and sufficient evidence for phylogenetic grouping [19].

In view of limitations encountered in the use of MEGA for phylogenetic analysis of particularly short sequences, a complementary phylogram of the detected RABV N-sequences was generated using the Maximum Likelihood algorithm and
| Replication Type/Gene | MF765758 | MF765759 | MF765756 |
|----------------------|----------|----------|----------|
| MF765763             | MF765764 |          |          |
| MF765761             | MF765762 |          |          |
| MF765760             | MF765761 |          |          |
| MF765759             | MF765760 | MF765762 |          |
| MF765758             | MF765759 | MF765761 | MF765763 |

Table 4. Estimates of evolutionary distances between N gene sequences of selected rabies lyssaviruses available in NCBI GenBank and Monrovia rabies lyssavirus isolate MF765758.
subtree-pruning-regrafting branch-swapping option in PhyML version 3.0 (PhyML3) software [20].

Results

Gel documentation imagery

Three (15%) of 20 specimens (19 brain samples of suspected rabid dogs and one human saliva) tested were positive for RABV N gene by RT-PCR. The gel documentation of PCR products of the three positive genes are shown in Figures 2 and 3. Each gene was within 550–650 bp size range. The DBS 01, DBS 075 and DBS 076 sequences were 554, 171 and 126 bp ORF, respectively.

Partial N gene characteristics

These three sequences were deposited in GenBank, under accession numbers MF765758, MH507336 and MH507337, respectively. The Monrovia RABV sequence MH765758 had very close resemblance (99% homology) with China lineage 2 RABV strains KU963489 (or SN2-62-CanineCHINA2005) and DQ666322 (or Jiangsu_Yc63), previously isolated from dogs in China (Table 4). The second RABV sequence obtained in Monrovia MH507336 clustered with Africa lineage 2 RABVs from Côte d’Ivoire, Nigeria, Chad, Cameroon and Egypt, while the third RABV sequence MH507337 clustered with Africa lineage 3 RABVs of Europe/Middle East lineage including U22480.1 (Oman), U22482.1 (Iran) U22475.1 (Germany) and U42706 (Bosnia-Herzegovina) (Fig. 5).

Discussion

This study was aimed at detecting and characterizing RABV strains circulating in Monrovia city and environs in Liberia, West Africa, 2016–2017, by RT-PCR testing of archived brain
specimens of suspected rabid dogs using primers specific for the N gene of lyssaviruses, followed by sequencing analysis of the amplicons obtained. Three strains of RABV sequences were detected and accessioned in NCBI GenBank. All three isolates were from the Red Light area of Paynesville, a commercial district and notable slum located in the suburb of Monrovia, the capital city of Liberia. While the first report of DTHR in Liberia dates back to the 1950s [2], there are no reports on molecular characteristics of RABVs from Liberia that predate our characterisation of the three Monrovia RABV strains that were identified in this study. The co-circulation of China lineage-2 with Africa lineage-2 and lineage-3 was probably due to inadvertent importation of rabies virus into Liberia from China or neighbouring countries, as a result of detection failure along import barriers. This allusion...

Fig. 4. Maximum likelihood (ML) phylogenetic tree of the first Monrovia RABV N-gene isolate (GenBank Accession No. MF756758) generated using ML algorithm (1000 bootstrap replications). The analysis involved 50 nucleotide sequences. All positions with <95% site coverage were eliminated. Evolutionary analyses were conducted in MEGA7 [17]. The bootstrap values (%) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method [18] and are in the units of the number of base substitutions per site.
is plausible with recent findings that show the predominance of China sourced rabies vaccines in Nigeria [22] and the all-time high Chinese presence in Liberia; while rabies vaccine quality compromise has been widely reported in China with resultant rabies outbreak following dog bite in humans [23, 24]. The direct importation of dogs considered vaccinated, yet incubating rabies is the most likely source of introduction of the China lineage 2 RABV into Liberia.

The phylogenetic results of this study revealed similarity of the Monrovia RABV strain MF765758 with DQ666322 or Jiangsu_Yc63 (China lineage 2) and other RABV lineages including Africa 1A-1C, Africa 4, Europe/Middle East and Asia, and vaccine strains PV (GU992322), SRV9 (AF499686) and SAD Vnukovo (GU992319) (Figs 4 and 5). The Chinese lineage C (especially C1) RABVs are closely related to rabies vaccine strains PV and SAD [25]. Incidentally, anti-rabies vaccines that are used for dog immunisations in Monrovia and environs in Liberia are the inactivated Czech strains SAD Vnukovo-32 and PV Op VB.

Clustering of the Liberian RABV strain MH507337 with RABV strain AF467949 regarded as a mongoose adapted strain previously isolated only in the southern African region [21], constitutes another unique finding of this study, and warrants further detailed investigation to ascertain its host species and transmission mechanisms in Monrovia. It is also necessary to determine whether MF765758 the Chinese strain discovered in a Monrovia dog resulted in a self-terminating outbreak or perpetual propagation.

The short strands of MH507336 and MH507337 genes accounted for their sub-optimal positioning on the phylogram. This limitation was further resolved in PhyML3 which presents a robust platform and ML algorithm applicable to the phylogenetic analysis of RABVs [26]. The recent discovery of three newly accessioned rabies N-genes from Liberia obtained from specimens collected through the CVLC in Monrovia, through an ongoing multi-national effort [27], further clarified the phylogenetic relationship of these sequences. These latest RABV sequences...
segregated along with others that belong to Africa lineage 2 (Figs 4 and 5). This corroborates with predominance of Africa lineage 2 strains that are indigenous to West Africa being in circulation in Liberia.

Phylogenetic studies have shown that RABV isolates tend to form distinct groups and patterns of distribution that are each associated with specific geographical regions of the world [28]. Africa lineage 1 RABVs closely resemble RABVs from Europe and Asia and are thought to be recently introduced into Africa from Europe [29]. On the other hand, Africa lineage 2 RABVs comprise wild-type strains that originated from several central and eastern African countries, and are ancestral to Africa 1 and Eurasian RABVs [30]. The phylogenetic evidence gathered in this study suggests that the Monrovia RABV isolate (MF765758) has its origin in Asia, rather than it being an autochthonous (Librarian) RABV or an extant strain from another African country. Till date, only Africa lineages 1 and 2 RABVs have been isolated from rabid dogs in West and Central Africa [31–33], and to the best of our knowledge, this is the first report from any African country of a RABV isolate of domestic dogs that is phylogenetically closely related to RABVs circulating in China.

It is plausible that other lyssaviruses apart from RABV are involved in recent outbreaks of rabies in Monrovia and other parts of Liberia because more than 56 different species of bats have been documented in different ecological zones of the country [34], and all lyssaviruses with the exception of MOKV and IKOV are maintained in bats [35]. In neighbouring West African countries such as Nigeria and Ghana, enzootic LBV exposures have been detected in Megachiroptera bats especially Eidolon helvum [36, 37]. Although there are no reported spill-over of LBV into humans, LBV infections have been detected in dogs [38] and wildlife such as mongoose [12]. Such infections are of concern to public health authorities because of the likelihood that lyssavirus infections such as LBV may yet spill-over into the human population in future, and the available vaccines against RABV genotype 1 offer no protection against LBV.

While the goal of achieving a more effective and efficient detection, surveillance and stepwise elimination of animal and human rabies in West African cities and villages deserves stronger collaboration among African scientists, governments and beyond, current trends on epidemiological reports on one-health action against rabies presented at the 5th International Conference on Rabies in West Africa (RIWA), Bamako, Mali, 23–25 October 2018 show gradation in rabies one-health actions within the Economic Community of West African States (ECOWAS countries). Retrospective studies of DBVs presented for rabies exposure treatment in Buchanan, Suakoko and Voinjama, 2008–2013 showed that more than half of the DBVs were below 20 years of age, mostly male [3]. They were comparable to what obtained in Bamako city, Mali. Finally, while it was shown that canine rabies transmission to humans can be interrupted in an African city with currently available dog rabies vaccines, provided that the vaccination area includes larger adjacent regions, and local communities are informed and engaged [26], the higher education participation concept for developing more effective rabies surveillance and systematic elimination through mentorship programme in collaboration with one-health activities of ECOWAS governments and through Regional Rabies Control Strategies Elaboration and Validation Workshop is critically important to achieve the 2030 goal of elimination of rabies in Africa.

Conclusions

This study is the first to confirm RABV infection by molecular diagnostic technique in Liberia. A canine rabies virus variant likely to have been imported from Asia was found co-circulating with Africa lineages 2 and 3 RABVs at the dog–human interface in Monrovia, Liberia, during outbreaks of DTHR that occurred between 2016 and 2017. While there is a need to investigate the yet unknown involvement of wildlife in the maintenance and spread of rabies in Liberia, efforts should be focused on improving rabies surveillance and reporting in Liberia. An in-country rabies diagnostic laboratory that complies with the World Organization for Animal Health procedures should be established in Liberia. The current findings should stimulate more efforts towards rabies active surveillance in one-health mode within the Economic Community of West African States, particularly within the Regional Rabies Control Strategies Elaboration and Validation Workshop.

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