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SHORT REPORT

Genetic diversity of rabies virus in different host species and geographic regions of Zambia and Zimbabwe

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Abstract
Rabies is endemic in Zambia and Zimbabwe. The previously investigated strains of rabies virus in central Zambia belong to the Africa 1b lineage, with similar circulating virus strains found in the various tested hosts and regions. However, prior work assessed only limited regions and host species. Thus, this study aimed to more comprehensively determine the genetic diversity of rabies virus across regions of Zambia and Zimbabwe. RNA ($n=76$) was extracted from positive direct fluorescent antibody test brain tissues from dog, cow, goat, cat, pig, human, and jackal collected from Zambia and Zimbabwe. The amplicons of the nucleoprotein and glycoprotein genes were obtained from all examined samples by nested RT-PCR and subsequently sequenced. A phylogenetic analysis of the N gene confirmed that all the endemic strains of rabies virus in Zambia and Zimbabwe belong to the Africa 1b lineage. The obtained viral gene sequences were phylogenetically divided into two clusters. Cluster II comprised only Zambian strains. In contrast, cluster I comprised both Zambia and Zimbabwe strains, with strains from Zimbabwe forming a distinct lineage from Zambian strains, implying viral genetic divergence due to geographical barriers. However, no evidence of clustering based on host or region was observed, implying the circulation of similar virus strains occurs in different hosts and regions of Zambia and Zimbabwe. The clustering of rabies virus strains from jackals with those from domestic animals provides evidence of similar virus strains circulating in both wildlife and domestic animals, and that the jackal might be one of the potential reservoirs of rabies virus infection. In this study, no strains circulating in Zimbabwe were detected in Zambia.

Keywords Zambia · Zimbabwe · Rabies · Nucleoprotein · Glycoprotein · Phylogenetic

Background

Rabies, a devastating and fatal disease of livestock and humans, is caused by a rhabdoviridae virus transmitted through the bite of a rabid animal. The disease has a worldwide distribution and caused 24,600 human deaths in 2010 alone [1]. Rabies is endemic in both Zambia and Zimbabwe, with prevention attempts focusing on the strict use of vaccination and population control of dogs. Several wildlife carnivores such as jackals [2–5] and mongooses [6–9] have been implicated as reservoirs of rabies. However, in contrast to work in other regions, a study on the Selous mongoose (*Paracynictis selousi*) in Zambia ruled out the possibility of that carnivore being a wildlife reservoir of rabies in Zambia [10]. Nonetheless, there is serological evidence suggestive of the presence of rabies in lions [11] in Zambia. In Zambia, rabies is established in both domestic dogs and wild foxes [12] while in Zimbabwe, it is established in domestic dogs, black striped Jackal (*Canis mesomelas*) and side-striped Jackal (*Canis adutus*) [13–15].

The application of reverse transcription polymerase chain reaction (RT-PCR) assays using the nucleoprotein (N) gene for lineage analysis and the glycoprotein (G) gene for genetic diversity analysis has revealed several lineages of rabies viruses (RABVs) in different regions of Africa and the world [16–20]. The strains circulating in eastern, central,

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and southern Africa were shown to belong to the Africa 1b lineage [16]. According to Muleya et al. [21], the RABV strains in central Zambia belong to the Africa 1b lineage, and similar RABV strains circulate in different hosts and regions of central Zambia. However, that study was limited to the Central, Lusaka, and Copperbelt provinces of Zambia, and the evaluated host range comprised only domestic dogs and cattle. Notably, the previous work left out the majority of the country, as well as many known host species, so the present study was conducted as a follow-up. In contrast to the previous study, the present report encompasses all the regions/provinces of Zambia and Zimbabwe. It also includes a wider host range allowing a more accurate determination of the lineage and genetic diversity of circulating RABV in both domestic animals and wildlife in Zambia and domestic animals in Zimbabwe. Zambia and Zimbabwe are close neighbours sharing a border created by the Zambezi river and Lake Kariba. The Zambezi river and Lake Kariba form a natural barrier preventing the easy movement of animals (Fig. 1a).

In this study, a total of 76 (59 and 17 from all regions of Zambia and Zimbabwe, respectively) direct fluorescent antibody test (DFAT)-positive brain tissues were examined (Table 1). Brain tissues were collected in Zambia from 46 domestic dogs, 1 cat, 8 cows, 1 pig, 1 human and 2 jackals (*Canis adutus*) over a period of 16 years (1999–2015), while similar samples were collected in Zimbabwe from 13 domestic dogs, 1 goat, and 3 cows in 2014. The University of Zambia Biomedical Research Ethics Committee provided ethical approval for the molecular epidemiological work described in this study (REF. NO. 012.11.18). RNA was extracted from brain tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific) and a PureLink RNA mini-kit (Ambion; Thermo Fisher Scientific) and stored at − 80 °C.

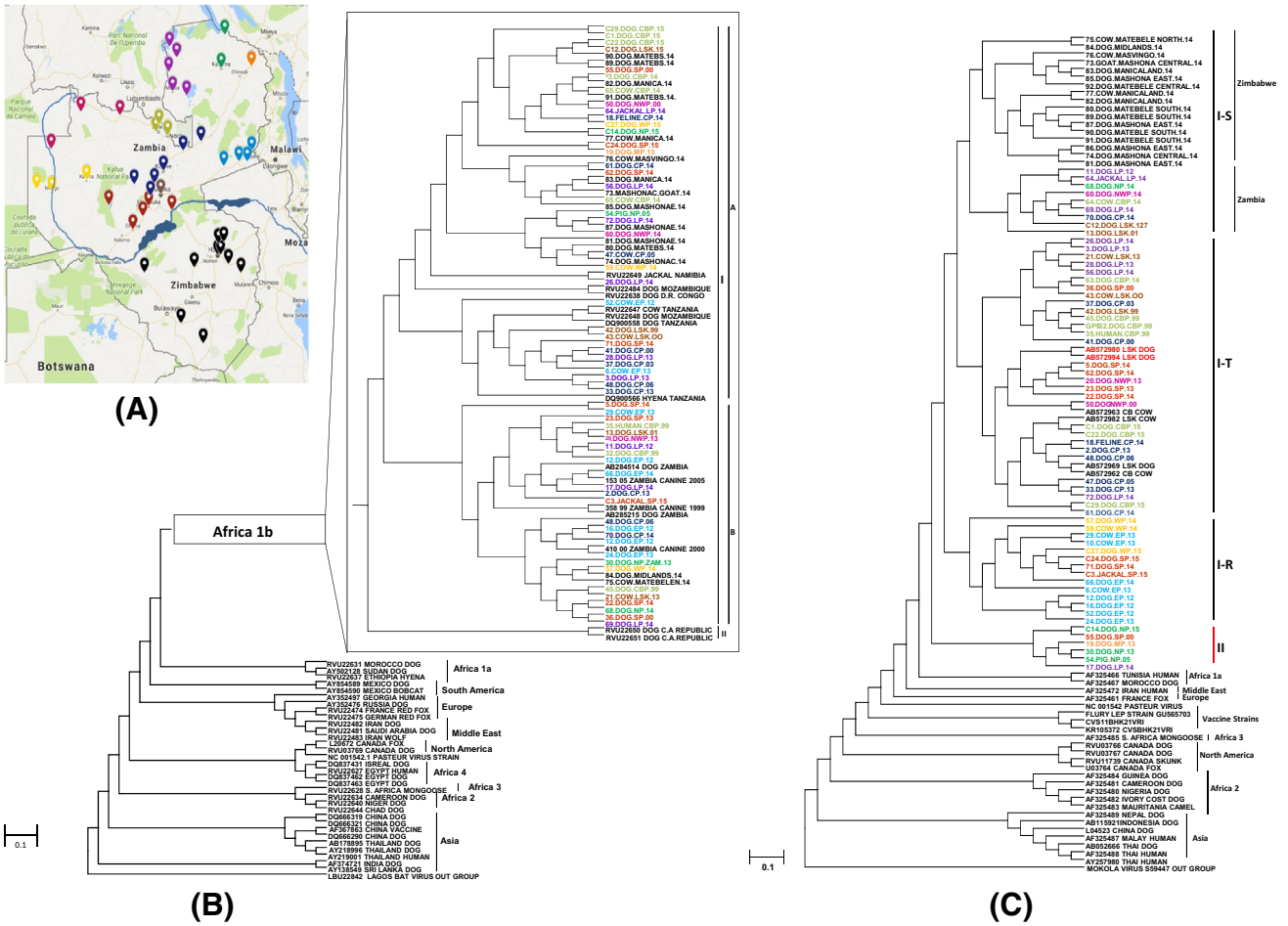

Fig. 1 **a** Map of Africa showing the sampling locations in Zambia and Zimbabwe. The sampling location of each sample is indicated by a location pointer on the map. Black location pointers indicate sampling locations in Zimbabwe, and different coloured location pointers indicate sampling locations in different Zambian provinces. Zambia and Zimbabwe are geographically separated by the Zambezi river (blue line) and the Kariba dam. Map adapted and edited from Google maps. **b**, **c** Nucleoprotein gene (**b**) and Glycoprotein gene (**c**) phylogenetic trees based on 461 and 609 nucleotide sequences, respectively. Sequences from Zambia are shown in colours similar to the location pointers on the map, whereas all reference sequences as well as sequences from Zimbabwe are shown in black

Table 1 Description and origin of samples used in this study

Sample ID	Country of origin	Year of collection	Province	Region/town	Host	Accession N gene	Accession G gene
32	Zambia	1999	Copperbelt	Ndola	Canine	LC380088	LC380161
45	Zambia	1999	Copperbelt	Ndola	Canine	LC380090	LC380163
C1	Zambia	2015	Copperbelt	Kalulushi	Canine	LC380093	LC380166
C22	Zambia	2015	Copperbelt	Mufulira	Canine	LC380094	LC380167
C29	Zambia	2015	Copperbelt	Ndola	Canine	LC380095	LC380168
35	Zambia	1999	Copperbelt	Ndola	Human	LC380089	LC380162
63	Zambia	2014	Copperbelt	Ndola	Canine	LC380091	LC380164
65	Zambia	2014	Copperbelt	Ndola	Bovine	LC380092	LC380165
2	Zambia	2013	Central	Chibombo	Canine	LC380096	LC380169
18	Zambia	2014	Central	Shibuyunji	Feline	LC380097	LC380170
37	Zambia	2003	Central	Kabwe	Canine	LC380099	LC380172
41	Zambia	2000	Central	Kabwe	Canine	LC380100	LC380173
48	Zambia	2006	Central	Kabwe	Canine	LC380102	LC380175
33	Zambia	2013	Central	Mumbwa	Canine	LC380098	LC380171
47	Zambia	2005	Central	Serenje	Bovine	LC380101	LC380174
61	Zambia	2014	Central	Mkushi Central	Canine	LC380103	LC380176
70	Zambia	2014	Central	Kabwe	Canine	LC380104	LC380177
6	Zambia	2013	Eastern	Katete	Bovine	LC380105	LC380178
10	Zambia	2013	Eastern	Katete	Bovine	LC380106	LC380179
12	Zambia	2012	Eastern	Petauke	Canine	LC380107	LC380180
29	Zambia	2013	Eastern	Katete	Bovine	LC380109	LC380183
52	Zambia	2012	Eastern	Chipata	Canine	LC380110	LC380184
16	Zambia	2012	Eastern	Chadiza	Canine		LC380181
24	Zambia	2013	Eastern	Petauke	Canine	LC380108	LC380182
66	Zambia	2014	Eastern	Chipata	Canine	LC380111	LC380185
11	Zambia	2012	Luapula	Mwense	Canine	LC380113	LC380187
17	Zambia	2014	Luapula	Mbala	Canine	LC380114	LC380188
26	Zambia	2014	Luapula	Nchelenge	Canine	LC380115	LC380189
28	Zambia	2013	Luapula	Mansa	Canine	LC380116	LC380190
3	Zambia	2013	Luapula	Nchelenge	Canine	LC380112	LC380186
56	Zambia	2014	Luapula	Mansa	Canine	LC380117	LC380191
64	Zambia	2014	Luapula	Samfya	Jackal	LC380118	LC380192
69	Zambia	2014	Luapula	Kawambwa	Canine	LC380119	LC380193
72	Zambia	2014	Luapula	Samfya	Canine	LC380120	LC380194
42	Zambia	1999	Lusaka	Lusaka	Canine	LC380123	LC380197
43	Zambia	2015	Lusaka	Lusaka	Bovine	LC380124	LC380198
C12	Zambia	2015	Lusaka	Lusaka	Canine	LC380125	LC380199
13	Zambia	2001	Lusaka	Lusaka	Canine	LC380121	LC380195
21	Zambia	2013	Lusaka	Lusaka	Bovine	LC380122	LC380196
54	Zambia	2005	Northern	Kasama	Porcine	LC380128	LC380202
C14	Zambia	2015	Northern	Kasama	Canine	LC380130	LC380204
30	Zambia	2013	Northern	Kasama	Canine	LC380127	LC380201
68	Zambia	2014	Northern	Kasama	Canine	LC380129	LC380203
T19	Zambia	2013	Muchinga	Isoka	Canine	LC380126	LC380200
20	Zambia	1999	North-Western	Mwinilunga	Canine	LC380131	LC380205
50	Zambia	2000	North-Western	Zambezi	Canine	LC380132	LC380206
60	Zambia	2014	North-Western	Solwezi	Canine	LC380133	LC380207
5	Zambia	2014	Southern	Mazabuka	Canine	LC380134	LC380208
23	Zambia	2013	Southern	Chirundu	Canine	LC380136	LC380210

Table 1 (continued)

Sample ID	Country of origin	Year of collection	Province	Region/town	Host	Accession N gene	Accession G gene
C3	Zambia	2015	Southern	Choma	Jackal	LC380140	LC380214
C4	Zambia	2014	Southern	Namwala	Canine	LC380141	LC380215
C24	Zambia	2015	Southern	Monze	Canine	LC380142	LC380216
22	Zambia	2014	Southern	Mazabuka	Canine	LC380135	LC380209
36	Zambia	2000	Southern	Monze	Canine	LC380137	LC380211
55	Zambia	2000	Southern	Monze	Canine	LC380138	LC380212
62	Zambia	2014	Southern	Namwala	Canine	LC380139	LC380213
C27	Zambia	2015	Western	Mongu	Canine	LC380145	LC380219
57	Zambia	2014	Western	Kalabo Central	Canine	LC380143	LC380217
59	Zambia	2014	Western	Kaoma Central	Bovine	LC380144	LC380218
81	Zimbabwe	2014	Mashonaland East	Acturus	Canine	LC380146	LC380220
85	Zimbabwe	2014	Mashonaland East	Harare	Canine	LC380147	LC380221
86	Zimbabwe	2014	Mashonaland East	Harare	Canine		LC380222
77	Zimbabwe	2014	Manicaland	Rusape	Bovine	LC380148	LC380223
82	Zimbabwe	2014	Manicaland	Rusape	Canine	LC380149	LC380224
83	Zimbabwe	2014	Manicaland	Rusape	Canine	LC380150	LC380225
87	Zimbabwe	2014	Mashonaland East	Marondera	Canine		LC380226
73	Zimbabwe	2014	Mashona Central	Glendale	Caprine	LC380151	LC380227
74	Zimbabwe	2014	Mashonaland Central	Mt. View Bindura	Canine	LC380152	LC380228
76	Zimbabwe	2014	Masvingo	Mwenezi	Bovine	LC380154	LC380229
92	Zimbabwe	2014	Matebeland Central	Glendale	Canine		LC380230
75	Zimbabwe	2014	Matebeleland North	Mapani Village	Bovine	LC380155	LC380231
80	Zimbabwe	2014	Matebeleland South	Filabusi	Canine	LC380156	LC380232
89	Zimbabwe	2014	Matebeleland South	Filabusi	Canine	LC380157	LC380233
90	Zimbabwe	2014	Matebeleland South	Filabusi	Canine	LC380158	LC380234
91	Zimbabwe	2014	Matebeleland South	Filabusi	Canine	LC380159	LC380235
84	Zimbabwe	2014	Midlands	Kadoma	Canine	LC380160	LC380236

A OneStep RT-PCR kit (Qiagen) was used to amplify the whole lengths of the N and G genes using primers described by Kamolvarin et al. [22] and Hyun et al. [23], respectively. Inner partial fragments of the N and G genes were amplified by means of Ex Taq HS polymerase (Takara Bio) using primers described by Kamolvarin et al. [20] and Yamagata et al. [24], respectively. Amplicons were visualized on 1.5% agarose gel coated with ethidium bromide. The PCR-positive products were purified using a Monofas Purification kit (GL Science) according to the manufacturer's instructions.

Cycle sequencing was then performed on the purified PCR products according to the instructions provided by the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Foster City, CA). The unincorporated dNTPs were removed from the cycle sequence products using the ethanol precipitation method. The purified sequence products were then subjected to capillary electrophoresis using the ABI 3130 Genetic Analyzer (Applied Biosystems) followed by blast analysis of all the obtained sequences. Clustal W1.6 was used to align sample sequences with reference sequences, followed by the creation of a Mega file format and construction of neighbour joining phylogenetic tress with 1000 bootstrap replicates as the confidence level using MEGA ver. 6 [25]. All sequences described in this study have been deposited in GenBank with accession numbers LC380088 to LC380236.

Results and discussion

This is the first extensive and comprehensive study on the lineage and diversity of RABV in Zambia that covers all regions and includes a wide host range comprising wildlife, domestic animals, and humans. Included samples, from both Zambia and Zimbabwe, were positive for RABV N and G genes based on nested RT-PCR. A phylogenetic analysis of the identified RABV N genes in this study revealed an Africa 1b cluster composed of the RABV strains from Zambia and Zimbabwe as well as the reference sequences; within the African 1b cluster, two major clusters, I and II,

were observed (Fig. 1b). Cluster I was divided into subclusters A and B; subcluster A comprised RABV strains from Zambia and Zimbabwe together with reference sequences, whereas subcluster B contained only the RABV strains from the present study. Cluster II contained only Africa 1b reference samples from the Central African Republic (CAR). In agreement with the earlier studies, all sequences from Zambia and Zimbabwe clustered with the Africa 1b strains from central and southern Africa (Fig. 1b) [21, 26]. In contrast with studies reporting two lineages of RABV in other regions [27], this study demonstrated the existence of only one lineage of RABV in Zambia and Zimbabwe, regardless of the host or geographical origin of the sample.

A phylogenetic analysis of the G gene sequences from the Zambia and Zimbabwe samples produced two main clusters, I and II (Fig. 1c). While clusters I-T, I-R, and II were composed exclusively of samples from Zambia that originated from different regions and hosts, cluster I-S contained two smaller individual subclusters, each of which contained only sequences from Zimbabwe or Zambia. When only Zambian samples from 2014 were analyzed with samples from Zimbabwe, the same level of clustering between the two countries was also observed (data not shown). In contrast with one previous study [28], this work found a lack of evidence for the clustering of sequences according to their hosts or geographical locations within both Zambia and Zimbabwe. Instead, similar viral sequences from different hosts and geographical areas formed common clusters, as was described in an earlier report [21]. The implication of this is that a common ancestor of these endemic RABV strains exists and that it is most likely maintained or transmitted by a common host animal in all regions of Zambia and Zimbabwe. The similarity of the viral sequence from the side-striped jackal (*Canis adutus*), a wildlife species, with the viral sequences from domestic dogs, cats, livestock and humans in Zambia indicates the transmission of RABV between side-stripped jackals and other mammalian species. In Zimbabwe, it is well known that jackals are potential reservoirs of infection in commercial farming areas [26]. However, the actual population size of jackals in Zambia is unknown, so it is unclear how significant a contribution this species makes to RABV prevalence; maintenance of RABV is most likely attained by domestic dogs. Nevertheless, there have been several reported incidences of jackals biting people in Zambia, and, in all cases, the jackal that attacked showed RABV-positive DFAT results when tested (personal communication). This finding highlights a serious public health concern and suggests that although mandatory dog vaccinations are performed, there remains a need to introduce wildlife vaccinations, like those implemented in other countries/regions [29, 30], to effectively control and prevent the direct transmission of rabies from jackals to humans and domestic animals in both countries.

The G gene sequences from Zambia were closely related to those from Zimbabwe. However, the lack of clustering of sequences from Zimbabwe with the sequences from Zambia, especially the sequence (Accession Number: LC380210) (Table 1, Fig. 1) collected from Chirundu, the closest Zambian town to Zimbabwe in this study, provides evidence of divergence according to country of origin. This level of divergence might be attributed to the Zambezi river and the Kariba dam, which are natural geographical barriers that have prevented the free circulation of RABVs between the two countries.

In conclusion, the RABV strains circulating in all regions of Zambia and Zimbabwe belong to the Africa 1b lineage and no evidence of clustering according to host or geographical origin with each country was observed. Although domestic dogs are reservoirs of RABV infection in Zambia, jackals are susceptible to RABV infection and could be potential reservoirs in Zambia. Rabies control programs in both countries based on mandatory dog/pet vaccination should continue and the vaccination of wildlife should be implemented as this is also critical to the improvement of control of rabies. Lastly, as of our sampling time, no rabies virus strains endemic in Zimbabwe were detected in Zambia.

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Author contributions WM and HS conceived and designed the study. WM, HMC and LG collected the samples. WM, NS, YQ, AM, MK, ZM, RM and HMC performed RNA extraction, nested RT-PCR and cycle sequencing. WM and MS analysed the data and WM wrote the manuscript. MS, LG, RM, EK, ASM, BM, AT and HS edited and approved the manuscript. HS and AT obtained funding for the study. The final manuscript was read and approved by all the authors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All the samples analysed in this work were obtained from stored direct fluorescent antibody test (DFAT) positive brain tissues. Authority was given by both countries to undertake this work while ethical approval for the molecular epidemiology research work presented in this study was provided by the University of Zambia Biomedical Research Ethics Committee (UNZABREC) (REF. NO. 012.11.18).

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