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Use of partial N-gene sequences as a tool to monitor progress on rabies control and elimination efforts in Ethiopia

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Abstract

Ethiopia is one of the African countries most affected by rabies. A coarse catalog of rabies viruses (RABV) was created as a benchmark to assess the impact of control and elimination activities. We evaluated a 726 bp amplicon at the end of the N-gene to infer viral lineages in circulation using maximum likelihood and Bayesian methods for phylogenetic reconstruction. We sequenced 228 brain samples from wild and domestic animals collected in five Ethiopian regions during 2010–2017. Results identified co-circulating RABV lineages that are causing recurrent spillover infections into wildlife and domestic animals. We found no evidence of importation of RABVs

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Declaration of Competing Interest

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Supplementary materials

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from other African countries or vaccine-induced cases in the area studied. A divergent RABV lineage might be involved in an independent rabies cycle in jackals. This investigation provides a feasible approach to assess rabies control and elimination efforts in resource-limited countries.

Keywords

Dog rabies; Monitoring elimination; Co-circulating lineages; Resource-limited countries

1. Introduction

Rabies virus (RABV), classified within the family *Rhabdoviridae* and genus *Lyssavirus*, represents a major global health threat due to complex measures needed for control (Fisher et al., 2018). In humans, RABV causes an acute fatal encephalomyelitis once it reaches the central nervous system, resulting in nearly 60,000 annual deaths worldwide (Hampson et al., 2015). Recent estimates place the annual cost for global human rabies prevention at about 1.7 billion USD, mostly invested to provide post exposure prophylaxis (PEP) (Hampson et al., 2015).

Ethiopia is one of the African countries most affected by rabies with an estimated national annual incidence rate of 1.6 human deaths per 100,000 inhabitants, which translates into 1483 human deaths (applying Word Bank population estimates for the year 2012), concentrated in the capital city Addis Ababa and its surroundings (The World Bank Group, 2019).

Information on how rabies control, prevention, and elimination efforts have impacted the number of human and animal deaths are likely underestimated due to inconsistent surveillance and reporting throughout Ethiopia, which also holds true for many developing countries (Taame et al., 2012).

There are regions of the world where multiple RABV variants circulate simultaneously in independently established, species-specific transmission cycles. However, such variants frequently spill over into other susceptible host species, often causing dead-end infections or short interspecific transmission chains. Alternatively, spillover infections may eventually result in the establishment of new transmission cycles in permissible host populations (Mollentze et al., 2014).

Circulation of heterogeneous viral populations of the same RABV variant in populations of the same host species during an epizootic or outbreak have been revealed through identification of nucleotide variation across genomic, subgenomic, cistronic, and sub-cistronic sequences (Brunker et al., 2015). This subtle level of genetic variation leaves either ephemeral or lasting nucleotide signatures at all levels of the genomic organization (Brunker et al., 2015; Tian et al., 2018). Phylogeographic/phylogenetic reconstructions have become instrumental tools to analyze and visualize such different levels of evolutionary complexity and to depict relationships among clades of viral sequences during epizootics, when adequate nucleotide sequence data and associated metadata are available (Dellicour et al., 2017; Tian et al., 2018).

The nucleoprotein (N)-gene is the second most conserved gene of the lyssavirus genome, yet it has been shown to preserve enough nucleotide diversity to characterize genetic clusters associated with rabies transmission chains, foci, and/or disease pockets circumscribed to specific geographic regions or animal species, which are consistent with what complete genomes have resolved with higher resolution (Brunker et al., 2015; Troupin et al., 2016). Partial or complete N-gene sequences have also shown to be suitable for carrying out coarse scale epidemiological studies to identify events of major impact such as vaccine- induced cases, incursions from other countries, to discriminate among wildlife-associated cycles, and to follow up on the impact of dog rabies elimination programs when epizootic metadata and follow up of rabies foci is feasible through established rabies control and laboratory-based surveillance programs (Paez et al. 2007; Tao et al., 2009; Favoretto et al., 2006).

The Africa 1, 1a, 1b, 1c (AF1, AF1a, AF1b, AF1c), Africa 2 (AF2) and Africa 4 (AF4) major and minor clades of the dog-variant are of special public health interest due to their main association with domestic dogs across Africa (Troupin et al., 2016). If applied continuously, effective control and elimination strategies should be able to contain disease dissemination and deplete the diversity and geographic distribution of specific variants and lineages in the target hosts (Velasco-Villa et al., 2008). Thus, identification and characterization of RABV viral diversity within target reservoir host populations, even at coarse resolutions, should be an adequate framework for the assessment of control and elimination progress.

This research seeks to apply partial and complete N-gene sequencing as a coarse screening tool to build a baseline RABV catalog (given current surveillance efforts in Ethiopia) for subsequent follow up on rabies elimination and control strategies, as well as to monitor the occurrence of rabies vaccine-induced cases, look for alternative rabies cycles associated with wildlife, and identify conspicuous introductions of RABV lineages from other African countries.

2. Materials and methods

2.1. Samples and associated metadata

A total of 384 brain specimens from wild and domestic animals that had either potentially exposed people to RABV or were collected during conspicuous rabies outbreaks as part of surveillance activities within the Amhara, Oromia (includes Addis Ababa), Somali, Southern Nations Nationalities and People's (SNNP), and Tigray regions over the period 2010–2017 were confirmed positive by the direct fluorescent antibody test (DFA) at the Ethiopian Public Health Institute (EPHI). These samples were submitted in two different batches to the U.S. Centers for Disease Control and Prevention (U.S. CDC), one in 2010 and the other in 2018, for subsequent analyses. Forty-five samples received in 2010 were sequenced for the complete N-gene and the remaining 339 specimens (received in 2018) were screened by the real time RT-PCR LN34 assay to confirm positives and gage quality of the specimens for further partial N-gene sequencing at the CDC (Table S1).

2.2. Species identification of wildlife samples

DNA was extracted from the only 10 samples labeled at EPHI as wildlife (Table S1). Total DNA was extracted from brain tissues and a locus of 400 bp within the mammalian mitochondrial cytochrome b gene was PCR amplified and sequenced using primers MVZ05 and 400R (Table S2). Sequences were then compared with those in GenBank using the BLAST (blastn) tool available at NCBI.

2.3. RT-PCR amplification and cycle sequencing of partial and complete N genes

Brain tissues were suspended in 1 mL of Trizol (Invitrogen, San Diego, CA, formerly, GIBCOBRL, Inc.) for further total RNA purification with Direct-zol RNA MiniPrep (Zymo Research Corp. Irvine, CA 2016). An 848 bp amplicon at the end of the N-gene and beginning of the P-gene was obtained using primers 550F - 304B (Table S2). To obtain complete N-gene sequences, three overlapping amplicons were generated using primer sets Lys001F - 550B, Lys001F - N921B, and 550F – 304 (Table S2). Amplicons were purified for cycle sequencing using the ExoSapIt enzyme (Affymetrix Santa Clara, CA 2016), cycle sequenced with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc. Foster City, CA 2016), and subsequently separated/read on DNA Analyzer 3730 (Applied Biosystems Inc. Foster City, CA).

2.4. Sequence analyses

Using DNAStar Lasergene software (DNAStar, Inc., Madison, WI), complete N-gene sequences were assembled, or partial ones edited, to a 726 bp fragment at the end of the N-gene coding region (positions 695 to 1420 according to the SAD-B19 reference genome). This 726 bp portion of the N-gene is an extended version of the 320/264 bp (position 1157 to 1420 as compared with SAD-B19) sequence region extensively used in Latin American countries to identify RABV genetic variation in dogs and wildlife and to follow up on progress of dog-maintained rabies elimination in the region (Velasco-Villa et al., 2005; Favoretto et al., 2007; Jaramillo-Reyna et al., 2020).

2.5. Designation of lineages by phylogenetic reconstructions and genetic distance analyses

A total of 228 Ethiopian sequences (GenBank accession numbers MZ417876 - MZ418103) plus 100 reference sequences representing major/minor RABV clades, variants, and lineages circulating in Africa, vaccine strains used to formulate dog rabies vaccines worldwide (Hurisa et al., 2013), and an Ethiopian strain used to produce a domestic cell culture vaccine (JQ944709EPHvaccine Ethiopia2008) (Table S3) were aligned using ClustalX version 2.0 and trimmed to a 726 bp stretch using BioEdit to create a 328 taxa data set. MEGA7 was used to select the best nucleotide substitution model based on the best likelihood ratio and Akaike information criteria scores for 24 models tested (Table S4), applying 1000 bootstrap iterations. Samples from the 328 taxa data set were assigned to different lineages by observing their clustering patterns using phylogenetic reconstructions created in PhyML 3.0 and MrBayes V3.2 (Guindon et al., 2010; Huelsenbeck and Ronquist, 2001). These are robust and widely used algorithms for phylogenetic reconstruction that implement statistically sound methods for branch support evaluation (Guindon et al., 2010). ML

analysis was run in PhyML 3.0, applying the Subtree Pruning and Regrafting (SPR) branch swapping algorithm under the GTR+G + I model, and using the approximate likelihood ratio test (aLRT) coupled with Shimodaira-Hasegawa-like procedure (SH-like) with 1000 iterations to evaluate branch support. Bayesian analysis was run in MrBayes V 3.2 with two independent runs under the GTR+G + I substitution model with 15 million generations, 4 chains each run, sampling for tree's parameters every 1000 generations to assess branch support. Samples in Table S1 were placed in their respective lineages as they appeared in the 328 taxa preliminary trees (available upon request).

Identical sequences forming polytomies from the same year, location, or animal species within robust clusters were removed from the 328 taxa data set trees to improve visualization for final figures. Final trees were created with a 117 taxa data set comprising 20 Ethiopian sequences representing five putative lineages and 97 African reference sequences (Fig. 2A and Figure S1 A).

To appreciate the extent of sequence divergence (genetic distance) within and among Ethiopian lineages assigned in ML and Bayesian reconstructions, pairwise p-distance analyses were conducted in MEGA 7. We searched for a genetic distance cut-off value that may indicate a lineage demarcation criterion.

3. Results

From the 384 samples collected at EPHI, 53 were confirmed negative by the LN34 assay and another 103 were either decomposed, had limited amount of tissue, yielded poor quality/limited amount of RNA, or had species, temporal and/or geographic overlap with samples already chosen for sequencing. The temporal and species distribution of all available samples is shown in Fig. 1. It is noteworthy that most cat samples were either confirmed negative by the LN34 assay or had poor quality/limited amounts of RNA, which precluded their use in this study. As for the remaining samples (n = 228), dogs dominated Ethiopia's sampling efforts (n = 115), albeit we were able to obtain samples from other domestic and wildlife species such as donkeys (n = 3), Ethiopian wolf (*Canis simensis* n = 1 ETH1549), and side stripped jackals (*Canis adustus* n = 4, all lineage E), Table S1. Overall, the 228 samples chosen for sequencing covered: 1) 10 to 50 samples from dogs per year sampled; 2) 1 to 5 cats per year; 3) all available samples from species other than dogs; 4) a maximum of 40 samples from Addis Ababa per year; 5) a minimum of two samples from all available regions outside of Addis Ababa and Oromia per year; 6) a minimum of two samples from each lower-level administrative division, if defined, within Addis Ababa and Oromia; 7) samples well-preserved upon visual inspection (red-pink vs brown greenish, solid consistency vs liquified); and, 8) robust content of RABV-RNA as indicated by the LN34 amplification thresholds, CT = 25 or lower, which warrants amplification of cDNA fragments longer than 700 bp in a single RT-PCR round and yields sufficient cDNA to be sequenced with no further problems.

The ten samples labeled as wildlife at EPHI and identified to the species level by sequencing of the mitochondrial Cytochrome b gene indicated a number of previously misidentified samples (Table 1). Nearly all fox and hyena samples were confirmed as domestic dogs.

However, samples ETH1549BTORODENDI15 and ETH1560FXAM-HARANWOLO16 (Figs. 2 and S1), originally labeled as a bat and a fox, were identified as a cow and Ethiopian wolf, respectively (Table S1). As our importation permits did not allow for cloven hooved animals [Foot-and-Mouth Disease Virus (FMDV) risk] or endangered species, these adjusted species identifications required reporting of results to the United States Department of Agriculture (USDA) to comply with import permit regulations. As both FMDV viral RNA or viable virus are select agents, the entire batch of 384 samples (including extracted RNA and cDNA libraries for complete genome sequencing using the Illumina platform) were required to be destroyed. Unfortunately, this precluded the generation of longer sequences spanning from complete concatenated N, P, G, and L cistrons to complete genomes.

Ethiopian RABV lineages were defined as groups of monophyletic sequences that were consistently observed across ML and Bayesian reconstructions (only lineages with posterior probabilities ≥ 0.95 or aLRT SH-like values ≥ 80 were considered consistent/robust), predominantly grouped more than one sequence from either the same animal species and/or from same Ethiopian region, and pertained to the same lineage for one year or more.

The number of identical sequences observed across all putative lineages shows the relatively low resolution of the 726 bp stretch at the end of the N gene. Nonetheless, our analyses revealed at least 5 consistent RABVs lineages with overlapping geographic and temporal distributions, mostly around Oromia and Addis Ababa (Fig. 2 and Figure S1). African minor (AF1b, AF1c) and major clades (AF2, AF3 and AF4) were not detected within Ethiopia, given that segregated out of Ethiopia's AF1a sequences consistently (Figs. 2 and S1). Similarly, we observed AF1a sequences from other countries segregating out of the Ethiopian lineages in most trees (Fig. 2A, Figures S1).

Lineage A showed the widest geographic and temporal distribution, with most cases in domestic dog populations that likely caused spillover infections into multiple domestic (cats), largely concentrated around Addis Ababa and Oromia during the period 2010–2017 (Fig. 2). Lineages B, C and D that likely were also maintained and transmitted by dogs, occurred mostly in regions outside of Addis Ababa. Thus, A, B, C and D formed part of a larger clade we called Ehiopia's AF1a dog rabies epizootic. Lineage B spanned over a period of two non-consecutive years, lineage C was only identified in 2010, and D spanned over two consecutive years (Fig. 2 panel B). Lineage E was detected over two consecutive years in the SNNP region, with one sample found in Addis Ababa. (Fig. 2 panel C). Samples obtained in 2010 only grouped within lineages A and C. Because 2010 samples did not have collection date listed by month, they could not be divided into yearly quartiles and were thus omitted from Fig. 2C.

Ethiopia's RABV average pairwise distance was 1.8%, ± 0.3% standard error, whereas AF1a sequences circulating in Cameroon, Somalia and Gabon were less divergent, ranging from 0.2 to 0.4%. Sequences from Morocco presented an average pairwise divergence of 1.4% (± 0.4% standard error), similar to the RABV divergence observed in Ethiopia (Table S5). The average divergence for sequences pertaining to the same Ethiopian lineage (A to E) ranged from 0.2% to 0.6%, with the highest within-lineage divergence observed for sequences within lineage A (Table S6).

Lineage E showed the highest average pairwise distance (2.9%) when compared to all other Ethiopian lineages, while lineage D was the least divergent (average pairwise divergence of 1.7%). The lowest divergence between sequences pertaining to different lineages was observed between lineages D and B (1.3% ± 0.4%), with the highest divergence observed between sequences of A and E (3.3% ±0.6%), Table S7. Thus, sequences diverging more than 1.3% (±0.4) across our 726 bp N-gene fragment could be considered as pertaining to different Ethiopian RABV lineages. When AF1a minor clade sequences were compared among different African countries (Table S8), the overall average pairwise distance among countries was 3.5% ± 0.6% while the average pairwise divergence for Ethiopia with other countries was 4.2% ± 0.7% (Table S8).

Finally, we contrasted AF1a pairwise divergence values with those observed for AF1b across different African countries. Intra-lineage and inter-lineage pairwise values were similar to those observed for Ethiopia Tables S9 and S10.

GenBank reference sequence AY500827, sample ETH1560FXAM-HARANWOLO16 (both obtained from rabid Ethiopian wolves), and KP723638 obtained from a dog, segregated out of Ethiopia's lineages A to E, but still embedded inside Ethiopia's AF1a (A-E) clade (Figs. 2 and S1). The overall average pairwise distance between each of these ungrouped taxa with lineages A-E was 1.8% with a range between 1 and 3.1% (Table S11).

Reference sequence KX148200 from a dog collected in Ethiopia in 1988 pertains to lineage B (Table S1, Figure S1 A and C). Therefore, most recent members of lineage B, mostly recovered from the SNNP region, share a common ancestry with RABVs circulating at least 32 years ago (Figs. 2 and S1)

Our phylogenetic inferences indicate that RABVs found in rabid dogs throughout Ethiopia were genetically distant from all vaccine strains, including the one used by Ethiopia (EPH vaccine Ethiopia 2008 accession number JQ944709) for production of local vaccine (Figs. 2 and S1). All vaccine strains segregated out of Ethiopia's AF1a sequences consistently. Thus, no major or detectable, RABV vaccine-induced outbreaks have occurred in Ethiopia during the last three decades, at least under the current laboratory-based surveillance efforts conducted in Ethiopia (Figs. 2A and S1).

Interestingly, lineage E sequences, mostly associated with side-striped jackals (*Canis adustus*), segregated within Ethiopia's AF1a (A-E) clade, which has likely been either maintained or emerged from dog rabies epizootics that predated the period documented in this investigation (1988–2017) (Figs. 2A and S1 A and C). We also observed significant temporal and geographic overlap between lineage E and other lineages mainly detected in dogs (Fig. 2). Lineage C was mainly found in donkeys from Tigray, which may indicate a RABV outbreak in donkeys likely transmitted by rabid dogs in the region.

Considering our results altogether, we could redefine a lineage as a group of monophyletic sequences associated with a rabies focus (disease pocket or an independent transmission chain), whose taxa can be pre-dominantly associated with an animal species as well as temporally or geographically circumscribed or scattered with a genetic distance equal to or greater than 1.3% ± 0.4%. Although, we observed similar average pairwise

distance tendencies between concatenated genomes (as defined in Table S3) and partial N-gene sequences across major and minor African clades (matrices available per-request), estimations and interpretations of genetic distances using partial N-gene sequences should be taken with caution until corroborated with complete genomic sequences and more robust prospective samplings.

4. Discussion

Several countries in the Americas have used the variation in partial N-gene stretches to describe RABV diversity associated with domestic and wild animals at improved resolutions not previously attained by monoclonal antibody typing (Velasco-Villa et al., 2017). Over the last three decades, genetic typing with partial N-gene sequences has demonstrated not only to be a more sustainable technology to continue monitoring rabies in wildlife, but also to follow up on dog rabies elimination efforts by defining geographically circumscribed dog-maintained rabies foci (Favoretto, et., 2006; Paez et al., 2007; Carnieli et al., 2013; Garces- Ayala et al., 2017; Jaramillo-Reyna et al., 2020). However, one technical limitation of this approach is that phylogenetic reconstructions depicting putative lineages show low branch support values due to a limited number of informative sites compared to the large number of sequences analyzed at once (Guindon et al., 2010).

Continued surveillance of dog-maintained RABV lineages using partial, complete N-gene, or complete genome sequences was critical to validate Mexico free of human rabies mediated by dogs. Thus far, Mexico has had sustained RABV surveillance using partial N-gene sequences, reserving genomic approaches only for cases that require a higher level of resolution, such as persistent rabies foci on its border with central America (Garcés-Ayala et al., 2017). Applying a similar framework to Ethiopia, we observed that sequences pertaining to different lineages presented pairwise genetic distances greater than 1.3% ±0.4%, and that average pairwise genetic distances within-lineages increase as the spatial-temporal sampling increased across regions, consistent with studies in other African countries (Brunker et al., 2015, Bourhy, et al., 2016). However, these observations should be considered preliminary due to the uneven number of taxa obtained per lineage and the lack of correlation between lineages and their geographic region, year of collection or animal species that likely reflects a strong bias in surveillance efforts across Ethiopia.

Despite limitations, our results should serve to inform future sampling towards regions where lineages with fewer or ungrouped taxa were found (B, D, C, E) to verify their circulation and expand the phylogenetic and genetic distance analyses to corroborate the consistency of lineage demarcation threshold around 1.3% ± 0.4%. Thus far, there is no consensus on a genetic distance demarcation criterion to assign sequences to different lineages or transmission chains. However, considering the median substitution rate reported for RABVs (1.44 × 10–4 substitution/site/year), it would be unlikely to find 7 or more SNPs in sequences pertaining to the same lineage, transmission chain or rabies focus (Brunker et al., 2015; Troupin et al., 2016).

As a result of the coarse nature of the method used in this study, we could have missed a greater diversity of co-circulating RABV lineages in Ethiopia that could have been

detected using longer sequences. This might be the case for taxa KP723638, AY500827, and ETH1560FXAM-HARANWOLLO16, which our analysis could not resolve as part of any lineage, likely due to sequence size constrains and biased sampling. Similarly, lineage A could have borne more than one lineage or independent rabies foci that could be better defined with longer sub-genomic or genomic sequences. Thus, higher resolution attained with longer sequences could provide the ability to monitor the elimination of lineages with more sensitivity at finer temporal/spatial scales. Lineage B also highlights a possible limitation of this coarse approach, given that reference sequence KX148200 obtained in 1988 could have pertained to an extinct sub-lineage within lineage B. However, because sequences from distant years remain very similar across the 726 bps, we considered them as pertaining to the same lineage, despite they could have diverged from each other long ago.

Through our course-scale approach, we were able to report no conspicuous introductions of other African RABV minor/major clades in Ethiopia and found no evidence of vaccine-induced rabies cases. China previously reported cases of rabid dogs infected with a RABV lineage closely related to a vaccine fixed strain used for local production of a dog RABV vaccine (Tao et al., 2009).

The sympatry of multiple lineages in Oromia and Addis Ababa may be driven by pastoralism that is a way of life for nearly 10 million Ethiopians (SOS-Sahel Ethiopia, 2008). In Tanzania, canine RABV dispersal showed evidence of long-distance spreading out of locally endemic areas as a result of pastoralism and other human activities (Brunker et al., 2015). Additionally, Ethiopia's vast road networks could in turn shape the distribution of RABV lineages by acting as barriers for viral spread or as facilitators for the translocation of infected animals (Talbi et al., 2010). Policies to restrict human-mediated movement of dogs should be considered throughout Ethiopia to contain rabies dissemination (Morters et al., 2014).

The description of a divergent lineage E circulating in side-striped jackals in Ethiopia may be similar to the one observed in black-backed jackals and bat-eared foxes in South Africa (Sabeta et al., 2007; Zulu et al., 2009). Lineage E, may be of public health significance considering dog-maintained lineages in Ethiopia have likely persisted for more than three decades. The consistent detection of lineage E in wildlife over two years may indicate local introduction of a divergent dog-maintained lineage that has gone unnoticed within Ethiopia or neighboring countries; or alternatively, may suggest a real host shift such as those observed in China, Taiwan, and Brazil (Carnieli et al., 2013; Chiou et al., 2014; Liu et al., 2010). If confirmed, this would reinforce that RABV host shifts from dogs to wildlife are likely to occur as consequence of longstanding rabies epizootics (Velasco-Villa et al., 2008). This finding also highlights the importance of earlier rabies control and eliminations interventions on dog populations to reduce the risk of future emergence of novel cycles in wildlife (Brunker et al., 2015).

Field adapted next generation sequencing (NGS) technologies are becoming less expensive and more appealing for implementation on the verge of global elimination of dog-maintained RABVs (Brunker et al., 2020) however, its sustainable implementation will depend on being both technologically and economically feasible for resource-limited

countries where the rabies problem persists. Ultimately, widespread implementation of RABV genomic sequencing, or variations of it, will be essential at late rabies elimination stages (Brunker, 2020). However, during the technological transition it is critically important to continue surveillance with coarse methods.

Conclusions

This investigation reports the existence of a longstanding dog rabies epizootic in central Ethiopia, with potential divergence of a wildlife rabies cycle in side-striped jackals. Identifying variation in a partial 726 bp long fragment at the end of the N-gene through phylogenetic reconstruction proved to be an effective method to identify major RABV lineages potentially associated with ongoing rabies foci on a coarse scale. Variation in this sequence region also proved to be instrumental to reliably discriminate comparisons among country-specific AF1a RABV lineages, as well as to discriminate against other major RABV clades associated with rabies epizootics across Africa. The decentralization of RABV surveillance and diagnostic efforts will be necessary to inform the effectiveness of more systematic, long-term, intervention strategies. Reduction of the RABV genetic diversity by local extinction of circulating RABV lineages could be utilized as an objective parameter to measure progress in control and elimination strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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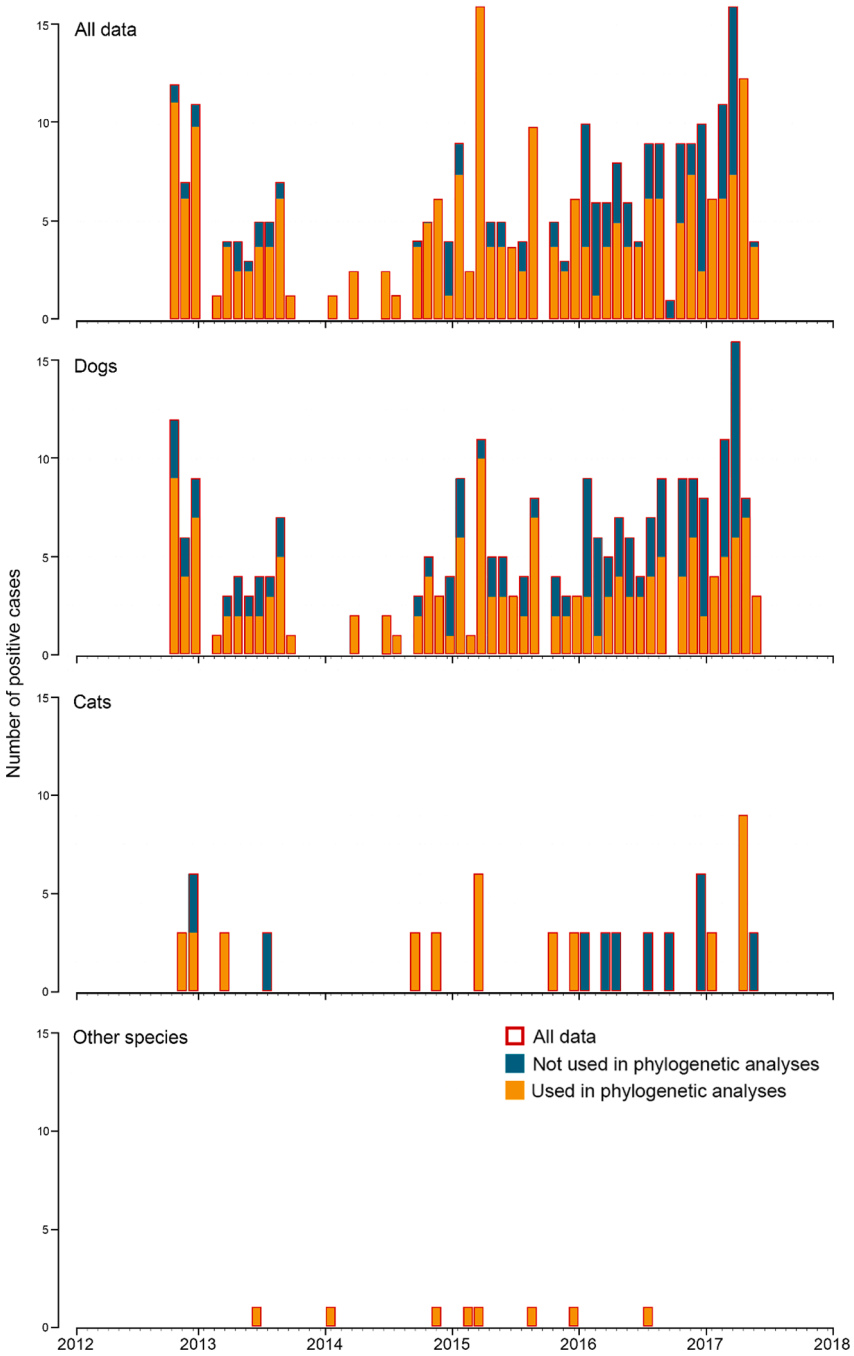

Fig. 1.

Temporal distribution of samples analyzed per quarter. Orange bars indicate the total number of samples included in the phylogenetic analyses. Green bars indicate total number of DFA positive samples confirmed at EPHI. The red border indicates total number of samples per quarter. From top to bottom we find the temporal distribution of all samples; temporal distribution of all dog samples; temporal distribution of cat samples; and temporal distribution of other species (jackals, and Ethiopian wolf).

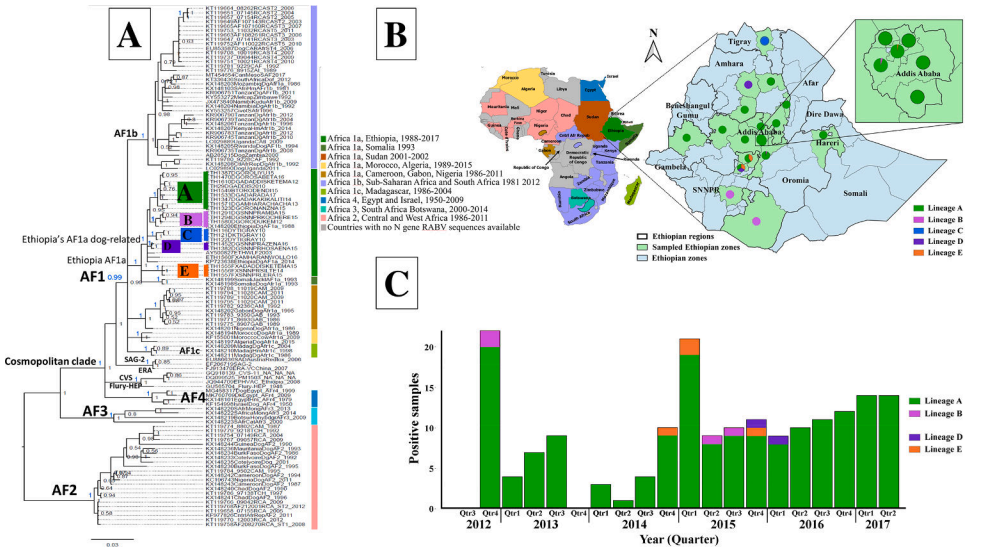

Fig. 2.

Designation of viral lineages and their geographic/temporal distribution per quarter. (A) Bayesian tree indicating robust branches with relevant posterior probabilities highlighted in blue. Main Ethiopian's AF1a lineages were highlighted with colored bars between branches and taxa names, indicating all taxa for each lineage included in final phylogenetic reconstruction. Wider colored lines on the right of the taxa names indicate minor and major African clades, whose coarse geographic distribution is shown in the same color on the Africa's map on panel B. (B) Map of Ethiopia showing the geographic distribution of all five Ethiopia's AF1a lineages displayed with same colors as shown in the Bayesian tree, panel A. Pie charts within Ethiopia's map depict centroid locations of lineages and their relative proportion, per location. Pie's size do not represent total number samples per lineage, only depict relative abundance. Projection from the center to the right upper side shows samples with overlapping distributions within Addis Ababa. The projection on the left shows the location of Ethiopia within the African continent as well as the distribution of major and minor lineages across the African continent. (C) Overlapping temporal distribution of all lineages per quarter. Lineage C was not depicted, since all 2010 do not have dates by day and month. Color codes for the lineages are the same displayed for the tree and map in A and B.

Table 1

Species identification by sequencing of cytochrome b.

	Gel results	PCR product conc	BLAST results			Field ID
Sample Name		**ng/uL**	**Fragment size**	**species ID Scientific name (common name)**	**% identity with BLAST species**	
ETH1549BTORODENDI15	bright	32.7	426	*Bos taurus* (cow)	99.5	Bat Oromia
ETH1551FXSNNPRDAWUR014 (A)	bright	21.9	406	*Canis lupus familiaris* (dog)	100.0	Fox SNNPR
ETH1551FXSNNPRDAWUR014 (B)	bright	23.2	377	*Canis lupus familiaris* (dog)	99.8	Fox SNNPR
ETH1552FXOROWAYU14	bright	25.2	376	*Canis lupus familiaris* (dog)	99.5	Fox Oromia
ETH1554FXSOMALIFILTU14 (A)	bright	27.9	376	*Canis lupus familiaris* (dog)	99.7	Fox Somali
ETH1554FXSOMALIFILTU14 (B)	bright	26.5	376	*Canis lupus familiaris* (dog)	99.7	Fox Somali
ETH1562HYOROCHANCHO14 (A)	bright	25.5	403	*Canis lupus familiaris* (dog)	99.8	Hyena Oromia
ETH1562HYOROCHANCHO14 (B)	bright	28.0	379	*Canis lupus familiaris* (dog)	99.7	Hyena Oromia
ETH1560FXAMHARANWOLLO16	present	20.0	369	*Canis simensis* (Ethiopian Wolf)	100.0	Fox Amhara
ETH1555FXADADDISKETEMA15	bright	24.7	375	*Canis adustus* (side striped jackal)	98.4	Fox SNNPR
ETH1556FXSNNPRSILTE14	bright	25.8	469	*Canis adustus* (side striped jackal)	98.5	Fox SNNPR
ETH1557FXSNNPRLERA15	bright	25.0	426	*Canis adustus* (side striped jackal)	98.4	Fox ADDIS
ETH1567UNKNOWN15	bright	26.6	377	*Canis adustus* (side striped jackal)	98.7	Fox Unknown

The (A) or (B) after some taxa's names indicate samples with two tubes available. Cytochrome b was run in both samples.