Molecular Epidemiology of Rabies Virus Isolates in Ethiopia

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Abstract

Rabies is a subject of public health concern in Ethiopia. The current rabies profile in Ethiopia shows 12 exposure cases/100,000 population and 1.6 rabies deaths/100,000 populations primarily because of canine rabies virus transmission exacerbated by uncontrolled stray dog population. The objective of this piece of work was to study the molecular epidemiology of rabies virus based on nucleotide sequence analysis of rabies virus (RABV) isolates originating from three selected Regional States of Ethiopia. Active surveillance of rabies suspected domestic animals that have demonstrated signs of encephalitis and abnormal behavioral change were subjected for Lyssavirus antigen detection using direct fluorescent antibody test (DFAT) positive and negative results. Ten DFAT positive brain samples comprising of three different animal species (Canine, Feline and Bovine) were sampled during 2013-2014 and subjected to reverse transcriptase PCR analysis. Ten brain tissue samples were collected from a Northern Regional State and Central and Western Regions of Ethiopia. The nucleotide sequences of these samples were studied to obtain an understanding of the epidemiological relationships of the RABV isolates in the various Ethiopian geographical locations. All samples submitted were positive by RT-PCR and no samples were negative. Ten amplified cDNA samples were sequenced and the sequence data was phylogenetically analyzed as indicated in the phylogenetic tree with RABV isolates from other countries. These results also indicate the postal transportation of FTA card is workable for the quality preservation of RNA samples under the specific period in time.

Key words: Molecular epidemiology, Phylogenetic analysis, Public health, Rabies virus, Surveillance

Introduction

Rabies is a highly fatal neurotropic viral disease of all warm blooded animals including humans. Disease is mainly transmitted from rabies affected animal to man through close contact with infected saliva via bites or scratches (Pal, 2007; Deressa et al., 2011); and is caused by rabies virus, which belongs to genus Lyssavirus, family Rhabdoviridae and possesses a single-stranded, non-segmented, negative-sense RNA approximately 12 kb in length (Tordo et al., 1986; Deressa et al., 2011; Pal et al., 2013). The viral genome encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA dependent RNA polymerase (L) (Mebatsion et al., 1999; Pal et al., 2013). The G protein controls major aspects of host cell infection, such as receptor binding, antigenicity, and host adaptation (Badrane et al., 2001). RNA viruses are characterized by a high mutation rate during replication due to the lack of proofreading and post replication error correction by the RNA polymerase (Bourhy et al., 1999). The genetic diversity seems to provide an adaptive potential for RV that can vary according to the natural history of the virus (Dean et al., 1996). Nucleotide sequence analysis permits the most precise definition of virus type and helps in understanding the transmission of rabies virus from the reservoir host to other hosts, including humans and domestic animals.

Rabies is a subject of public health concern around the globe because of its propagation among animal and human interface. The current scenario in Ethiopia shows primarily a serious threat of canine rabies virus transmission in the rapidly growing human population where a poorly controlled stray dog population is equally growing fast. The retrospective record review of Ethiopian Public Health Research Institute between 2001-2009 have disclosed a total number of 26399 rabies suspected animals which were clinically observed during 10 days quarantine period (Deressa et al., 2010). The epidemiological study of Ethiopian Wolf rabies have been reported, as out of 15 brain samples of wolves submitted to rabies diagnosis center at CDC Atlanta, USA,13 were diagnosed positive (Deborah et al., 2004). The report gave more emphasis on a rabies outbreak in a subpopulation of endangered Ethiopian wolves in the Bale Mountains between 2003 and 2004.

The estimates of animal and human rabies deaths occurred each year in Ethiopia needs rigorous epidemiological studies to update and validate national picture of human and animal rabies incidence. In this line, laboratory based assessment of human and animal virus variants circulating in the country can bridge the gap observed in the area of diagnostic tests that rely on detection of epitopes or gene sequences which might be reliable for some genotypes but not for others. To date, molecular epidemiological studies of RVs have been performed by sequencing different regions of RV genomes (Nadin-Davis et al., 1993; Kissi et al., 1995; Heaton et al., 1997; Bourhy et al., 1999; Johnson et al., 2002; Johnson et al., 2004; Hughes et al., 2004; Nadin-David et al., 2004; Bernardi et al., 2005; Nel, 2005; Paez, 2005). With this back ground,
the purpose of this study was rabies incidence surveillance with two years follow-up to analyze the prominent genotypes and history of rabies virus circulating in Ethiopia.

Materials and Methods

Study Design and Study Area

The laboratory based active surveillance was a risk based follow-up study designed using a multistage cluster sampling method with stratification by region and by urban strata. The surveillance was conducted in 3 regions of Ethiopia namely, Tigray, Amhara, Oromia, between 2013 and 2014 (Fig. 1).

Sample Collection

During the period between 2013 and 2014 rabies outbreak record was used for further laboratory based surveillance. Active surveillance of rabies suspected species of domestic animals that have demonstrated signs of encephalitis and abnormal behavioral changes, were subjected for Lyssavirus antigen detection using direct fluorescent antibody test (DFAT) positive and negative results.

![Figure1: Map Showing Epidemiological Surveillance for Lyssa Virus Antigen Detection in Ethiopia](image)

**Figure1:** Map Showing Epidemiological Surveillance for Lyssa Virus Antigen Detection in Ethiopia

Direct Fluorescent Antibody Test (DFAT)

All samples were tested for the presence of lyssavirus antigens using the DFAT as described by Dean and co-workers (1996). Accordingly, smears including all areas of the brain were prepared on the microscope slides, air-dried and acetone fixed for 30 minutes. A polyclonal fluorescein isothiocyanate conjugated immunoglobulin (Onderstepoort Veterinary Institute, Rabies Unit, South Africa) that is capable of detecting all lyssavirus genotypes was used at 1:20 dilution. Evans Blue counter stain (0.5% in PBS (0.01 M phosphate buffer, PH 7.4; 0.138 M NaCl; 0027 M KCl, Sigma Aldrich) was added to the working
dilution conjugate. After adding the conjugate, slides were incubated at 37 °C for 30 minutes in humidity chamber and then washed in PBS three times for 5 minutes, air-dried and mounted with 20% glycerol solution (0.05 M Tris-buffered saline pH 9.0 with 20% glycerol, Sigma-Aldrich). Slides were read using a fluorescent microscope with staining fluorescent intensity, and distribution was graded from + 4 to + 1. Samples demonstrating fluorescence were further characterized.

**Lyssavirus Antigen Sample Transportation to Advanced Rabies Laboratory**

**Sample Preparation**

Brain tissue was homogenated in mortar or microtube. In case of 1.5 mL microtube, approximately 1/3 volume of tube (brain tissue 0.2-0.3g) was put in a tube and then PBS (with antibiotics) was added up to total volume 1 mL to make a 20-30% (w/v) homogenate solution. This brain homogenate was centrifuged at 8000 x g for 3min. Supernatant was available for application to FTA cards.

**Sample Application to FTA Cards**

(100 μL [up to 125 μL] samples dropped per 1 inch circle) onto the Card in a concentric circular motion within the printed circle. The centrifuged brain homogenate solution was available as a fluid sample. The samples were allowed to dry completely for about 1 hour at room temperature. Afterwards, the samples became non-infectious.

**Storage of RNA on FTA Cards**

After the sample is to be archived, the card is placed in a multi-barrier pouch (with desiccant) and stored in humidity controlled, cool, dry environment. Samples collected for RNA purification were stored at room temperature prior to purification for different lengths of time depending on the sample type. Then, transported by airplane at room temperature for 2-3 days assuming that it may not affect the recovery of the virus while performing the RT-PCR analyses in the laboratory. It is also advisable to store the FTA cards at -20°C or -70°C to extend the storage life of the sample for RNA purification. This precaution was taken because RNA is less stable than DNA.

**Table 1: Nucleotide Sequence of Primers Used For Polymerase Chain Reaction**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sense</th>
<th>nt position</th>
<th>Sequence</th>
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<td>Nes-s</td>
<td>+</td>
<td>71-90</td>
<td>ATGGATGCCGACAAGATTGT</td>
</tr>
<tr>
<td>Nes-c</td>
<td>-</td>
<td>360-341</td>
<td>GCWATCAGGATCCATAGCT</td>
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Table 2: Rabies Virus Sequences Used As a Reference in This Study

<table>
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<th>Sequence</th>
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<th>Species</th>
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Table 3: Rabies Virus Sequences Analyzed in This Study

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<th>Isolate ID</th>
<th>Host</th>
<th>Country</th>
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<th>Year</th>
<th>Accession number</th>
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*** Not Applicable

Total RNA Extraction and RT-PCR

Total RNA was extracted directly from FTA cards originally infected animal brain material using TE buffer. The RNA elute was purified using QIAamp RNA Mini Kit (Qiagen) according to manufacturer’s instruction.

RT-PCR

RT-PCR was conducted using Superscript One-Step RT-PCR System with Platinum Taq DNA kit as described previously. The primers used for the RT-PCR were Nes-s (ATGGATGCGACAAGATTGT) and Nes-c (GCWATCAGGATTCCATAGCT) that amplify 606bp of nucleoprotein (Table 1). Amplified products were visualized on 1.5 % agarose gel with ethidium bromide.

Sequencing of RT-PCR Products

All PCR products were purified using PCR purification kit (Promega). PCR product was used in a sequencing reaction with either the Big Dye sequencing kit (Applied Biosystems) or the primers of Nes-s and Nes-cas described previously forwarded, and reverse sequences for each isolate were combined using ATCG software (GENETIX).

Phylogenetic Analysis

The rabies sequences used as a reference in the current study is mentioned in Table 2, and the rabies virus sequences analyzed in the study is presented in Table 2. The isolate sequences (n=10) were assigned Gene bank accession numbers and compared to other previously published African sequences (Table 2 and 3).
The nucleotide sequences of rabies isolated in the other countries in Africa were collected from the database of NCBI. The multiple alignments were made using the MUSCLE software implemented in MEGA software. Phylogenetic tree was generated using the maximum likelihood method with boot strapping re-sampling of 100 replicates as described previously (Johnson et al., 2002).

**Results**

Rabies is a major public health and veterinary concern in all parts of Ethiopia. Dogs (owned and stray) were the major animals responsible for the spread and transmission of rabies to other animals including humans in Ethiopia. The results of this study were presented in Table 4, 5, and 6. The risk based district rabies survey in Tigray, Amhara and Oromiya regional states in particular across the nation in general (Table 4 and 5) revealed a wide range of outbreaks, and deaths were recorded between 2013 and 2014 (Table 4) and higher proportion of rabies cases were laboratory confirmed and reported across the nation in dogs 303, and 200 followed by other domestic animals 35 and 21, respectively. Of the total animal rabies cases reported, only 4 and 2 were in wild animals. Both the dog population management and vaccination efforts were not significant to control the spread of rabies in domestic and wild animal population (Table 5). In this regard, the total number of positive rabies cases diagnosed every year across Ethiopia showed significant burden of animal rabies across the nation (Table 5).

**Table 4: Animal rabies outbreaks and deaths in three regional states of Ethiopia during 2013-2014**

<table>
<thead>
<tr>
<th>Region</th>
<th>Number of Outbreaks</th>
<th>Number of Deaths</th>
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<td>2013</td>
<td>2014</td>
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<tr>
<td>Tigray</td>
<td>39</td>
<td>31</td>
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<tr>
<td>Amhara</td>
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<td>26</td>
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<td>Oromiya</td>
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**Laboratory Based Active Surveillance Using Direct Fluorescent Antibody Test (DFAT)**

Laboratory based active surveillance of DFAT brain samples held at the EPHI rabies referral laboratory in Ethiopia comprising three different animal species (dog, cat and cattle) collected between 2013-2014 were strongly positive (Table 6), and subjected to RT-PCR for further molecular study.

**Discussion**

Rabies is a dreadful and fatal viral anthrozoanosis, which infects humans, domestic and wild animals (Pal et al., 2013). It is estimated that rabies is responsible for nearly 55,000 deaths mostly in children every year, and about 10 million people annually receive post-exposure treatments after being exposed to rabies suspected animals (Pal et al., 2013). Dog is the principal transmitter of rabies to humans as well as other animals, with 99 % of human rabies cases attributed to dog bite (Pal et al., 2013). In this current
study, ten brain tissue samples were collected from a Northern Regional State and Central and Western Regions of Ethiopia. The nucleotide sequences of these samples were studied to obtain an understanding of the epidemiological relationships of rabies virus isolates in the various Ethiopian geographical locations. All samples submitted were positive by RT-PCR and no samples were negative. Ten amplified cDNA samples were sequenced and the sequence data was phylogenetically analyzed as indicated in the phylogenetic tree with rabies virus isolates from other countries (Fig.1). Phylogenetic analyses showed that African rabies virus sequences group in to the previously described 1 (a and b), 2, 3 and 4 (Bourhy et al., 1990; Johnson et. al., 2004).

**Table 5:** Total number of animal cases in Ethiopia during 2013-2014

<table>
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<th>Animal test</th>
<th>Animal diagnosis/year</th>
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<tr>
<td>Number of dogs tested</td>
<td>303</td>
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<tr>
<td>Dogs rabies positive</td>
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<td>Dogs rabies negative</td>
<td>129</td>
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<tr>
<td>Number of other domestic animals tested</td>
<td>35</td>
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<td>Animals rabies positive</td>
<td>16</td>
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<td>Animals rabies negative</td>
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<td>Number of wildlife tested</td>
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<td>Total number of positive rabies cases</td>
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</tbody>
</table>

All ten Ethiopian rabies virus isolates were belonged to genotype 1 African lineage 1a and most of Ethiopia samples form a monophyletic cluster with Sudan rabies virus isolates together with the isolates from other African countries surrounding Ethiopia. This cluster is further divided into two groups (Ethiopia group and Ethiopia/Sudan group). It seems that the contents of the host species and isolated areas are different between these groups. In Ethiopia, rabies is endemic not region specific and host specific, as is apparent from the phylogenetic tree (Fig.2). The molecular results indicated that the Ethiopian rabies virus isolates studied are genetically related to one another (average nucleotide similarity, 95%). However, in these phylogenetic analyses only “289” strain was located in different position of Africa 1a cluster. All nine Ethiopian sequences: RV255; RV027, RV322; RV171; RV089; RV308; RV0296; RV244 and RV001 were stand phylogenetically closer to the Sudanese RABV (Fig.2). This association with Sudan RABV supports previously published data (Johnson et al., 2004). This panel of rabies viruses facilitated the study of rabies molecular epidemiology in Ethiopia. Our phylogenetic analysis confirmed that Ethiopian rabies virus cluster within the Africa 1a group (Fig. 2) and supports previous reports that Africa 1a is a highly dispersed group of viruses detected throughout the African continent (Holmes et al., 2002; Johnson et al., 2004).
Thus, the finding of these phylogenetic analyses is the first report in identifying the predominant genotype 1 and lineage 1a of rabies virus isolates in Ethiopia. Similarly, a monophyletic cluster with Sudan rabies virus isolates and other African countries is also remarkable epidemiological survey report for the intervention of pan African rabies elimination program. The“289” strain location in different position of Africa 1a cluster sugessted that rabies virus from high grade dairy cow introduced into Africa from European countries during the importation of exotic breeds. This is in agreement with previous phylogenetic analyses of carnivore related rabies virus sequences in Brazil (Yuki et al., 2011). The overall sequence analyses and subsequent pylogenetic analyses of this study significantly contributed to the understanding of the epidemiology of rabies in Ethiopia.

Table 6: Positive samples for the presence of Lyssavirus antigen with direct florescent antibody test (FAT)

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Case report</th>
<th>Result of FAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>001/14</td>
<td>Behavioral change of calf, and encephalitis was observed on September 01, 2013 in Shire district Tigray Regional State, Northern part of Ethiopia. The dog rabies was frequently reported from this region.</td>
<td>4</td>
</tr>
<tr>
<td>289/14</td>
<td>The signs and symptoms of rabies was observed in dairy cow on December, 02, 2013 in small scale dairy farm in Modjo town Oromia Regional State, Eastern part of Ethiopia.</td>
<td>3</td>
</tr>
<tr>
<td>027/13</td>
<td>Furious form of canine rabies was observed on October 3,2013 in Lemen district South West part of Oromia regional state, Ethiopia.</td>
<td>4</td>
</tr>
<tr>
<td>089/14</td>
<td>The signs of rabies was observed in dairy cow on December, 02, 2013 in small scale dairy farm in Modjo town Oromia Regional State, Eastern part of Ethiopia.</td>
<td>3</td>
</tr>
<tr>
<td>255/13</td>
<td>The case of canine rabies was observed as of 21st of November,2013 on owned and unvaccinated dog after biting 2 family members from the same household was killed and submitted for laboratory test from Menz district Amhara regional state, Ethiopia.</td>
<td>4</td>
</tr>
<tr>
<td>296/14</td>
<td>The case of canine rabies was seen as of 05December,2013 on unvaccinated stray dog after biting 3school children was killed and submitted for laboratory test from Adaberga district Oromia regional state, Western Ethiopia.</td>
<td>3</td>
</tr>
<tr>
<td>308/13</td>
<td>The case of canine rabies was observed as of 05December,2013 on unvaccinated stray dog after biting one child was killed and submitted for laboratory test from Selale-Kuyudistricta district Oromia regional state, North-Western Ethiopia</td>
<td>3</td>
</tr>
<tr>
<td>322/13</td>
<td>The case of canine rabies was observed as of 05 December, 2013 on un vaccinated stray dog after biting 1person was killed and submitted for laboratory test from Batu district Oromia regional state, Eastern Ethiopia.</td>
<td>3</td>
</tr>
<tr>
<td>171/14</td>
<td>The case of canine rabies was observed as of 09December, 2013 on unvaccinated stray dog after biting one old woman was killed and submitted for laboratory test from Ginchi town Oromia regional state, Western Ethiopia.</td>
<td>4</td>
</tr>
<tr>
<td>244/13</td>
<td>The case of canine rabies was observed as of 20November, 2013 on stray dog after biting more than 5 livestock and two persons,The dog was killed and submitted for laboratory test from Bako town Oromia regional state, Western Ethiopia.</td>
<td>4</td>
</tr>
</tbody>
</table>
In fact, most of the viruses originated from the central parts of Ethiopia, and were isolated from both reservoir and livestock species over a 2-year period, suggesting that Ethiopian rabies virus have either spread continuously into different parts of Ethiopia or have been established there for over two years. It is possible that uncontrolled animal movement in the country and traditional livestock trading between Sudan and Ethiopia can facilitate the spread of rabies virus in Ethiopia. In contrast to assumption that the currently circulating Ethiopian rabies virus might have an origin in Sudan (Johnson et al., 2004), however, data reported in this analysis, using a cohort of isolates, suggests that two sub lineages of Africa 1a are circulating, the first being unique to Ethiopia only (strain 289) and others, detected both in Sudan and Ethiopia. It is notable that in our panel of Ethiopian isolates only Africa 1a was identified. This observation is surprising considering the presence of Africa 2, Africa 3 and Africa 4 in other African countries (Morimoto et al., 1998; Bourhy et al., 1999).

The samples investigated in our study, however, were not distributed evenly across Ethiopia and instead originated in the Northern, central and a western region, which was in close proximity to the rabies referral laboratory. This reflected a common problem for most phylogenetic analyses in developing countries and
was recently highlighted for West Africa (Morimoto et al., 1998). This sample biasing was often due to the fact that limited infrastructure, capabilities to appropriately collect, store and ship samples were lacking in most parts of these countries. In addition, our observations further support the association with Sudanese rabies viruses shown with a discrete panel of canine rabies virus (Holmes et al., 2002). The Sudanese rabies virus sequences that group with the Ethiopian cluster reflected the close relationship of these viruses within the same lineage and related to the geographical proximity of the origin of these viruses (Fig. 2).

Conclusion

It is deduced that dogs are the principle reservoirs of the rabies virus transmission in Ethiopia. Rabies virus isolates tend to form genetic clusters based on the geographical region. Analysis of more samples is essential to identify the existence of other genetic clusters and also to identify the dominant genetic cluster. Studies of different genome targets (like the P gene and the N gene) must also be carried out to further characterize rabies virus isolates of Ethiopian origin. It is hoped that our encouraging result will help epidemiological survey work, and will contribute to the evidence based control of field rabies in Ethiopia. This study also indicated that the postal transportation of FTA cards is workable for the quality preservation of RNA samples under the specific period in time. It is emphasized that future analysis with domestic dog rabies virus, other domestic animal species and wild animal species genomes from Eastern and Southern parts of Ethiopia will further enhance our understanding of the cross-species transmissions that lead to epizootic cycles observed in the domestic and wild animals population.

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References


