TITLE
Antigenic and genetic characterisation of a divergent African virus, Ikoma lyssavirus

SHORT TITLE
Characterisation of Ikoma lyssavirus

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SUMMARY (213 words)

In 2009, a novel lyssavirus (subsequently named Ikoma lyssavirus, IKOV) was detected in the brain of an African civet (*Civettictis civetta*) with clinical rabies in the Serengeti National Park of Tanzania. The degree of nucleotide divergence between genomes of IKOV and other lyssaviruses predicted antigenic distinction from, and lack of protection provided by, available rabies vaccines. In addition, the index case was considered likely to be an incidental spill over event, and therefore the true reservoir of IKOV remained to be identified. The advent of sensitive molecular techniques has led to a rapid increase in the discovery of novel viruses. Detecting viral sequence alone however, only allows for prediction of phenotypic characteristics and not their measurement. In the present study we describe the in-vitro and in-vivo characterisation of IKOV, demonstrating that it is (1) pathogenic by peripheral inoculation in an animal model, (2) antigenically distinct from current rabies vaccine strains, and (3) is poorly neutralised by sera from humans and animals immunised against rabies. In a laboratory mouse model, no protection was elicited by a licensed rabies vaccine. We also investigated the role of bats as reservoirs of IKOV. We found no evidence for infection among 483 individuals of at least 13 bat species sampled across sites in the Serengeti and Southern Kenya.
INTRODUCTION

The discovery of novel viruses has flourished with the advent of highly sensitive molecular detection techniques (Bexfield & Kellam, 2011; Lipkin & Firth, 2013). These include metagenomic studies of the viral flora of healthy animals aimed at predicting transmission risks to other species, and the detection of pathogens in clinical samples and excreta for diagnosis (Bodewes et al., 2013; Ge et al., 2012; Li et al., 2011; Phan et al., 2011; van den Brand et al., 2012). Such studies provide valuable information on the presence and variability of viral pathogens in different animal populations. However, the detection of viral nucleic acid alone provides limited information on the zoonotic and pathogenic potential of such viruses.

Recent expansion of the genus Lyssavirus, within the family Rhabdoviridae, provides an example of rapid increase in the number of novel viruses identified, with the number of lyssavirus species doubling in the past ten years. There are 12 lyssavirus species classified by the ICTV, with two awaiting classification (Dietzgen, 2011) and genetic evidence for a further putative lyssavirus detected in Spain (Arechiga Ceballos et al., 2013). Lyssaviruses are divided into at least two phylogroups, based on genetic and antigenic distance (Badrane et al., 2001; Evans et al., 2012; Hanlon et al., 2005; Horton et al., 2010) (Table S1). All lyssaviruses cause rabies, which remains untreatable once clinical signs develop (Johnson et al., 2010) with a near 100% fatality rate. Therefore prevention of disease relies upon pre- and post- exposure prophylaxis through administration of vaccine and immune globulin. All licensed vaccines and immune globulin treatments are based on inactivated preparations or antibodies directed against “classical” rabies virus (RABV). These preparations elicit protective immunological responses to all RABV variants tested, but have variable efficacy against several other lyssaviruses (Badrane et al., 2001; Brookes et al., 2005a; Hanlon et al., 2005; Lodmell et al., 1995). Although difficult to quantify, this variation is related to antigenic distances of such lyssaviruses from vaccine strains (Badrane et al., 2001; Evans et al., 2012; Hanlon et al., 2005; Horton et al., 2010). A strong immunological response to standard rabies vaccine has been shown to protect against lyssaviruses that are classified within phylogroup I, but not against viruses classified within other phylogroups (Fooks, 2004).

In 2009, an African civet (Civettictis civetta), with clinical signs of rabies, was killed in the Serengeti National Park (SNP), Tanzania, after biting a child. The child received
appropriate wound care and post exposure rabies prophylaxis and remained healthy. Direct antigen-detection tests on brain samples from the civet confirmed infection with a lyssavirus, and genetic analysis demonstrated that the causative agent (Ikoma lyssavirus, IKOV) was highly divergent from all lyssaviruses characterised previously (Marston et al., 2012a; Marston et al., 2012b). This finding was particularly significant as dogs are not permitted in the SNP, and the area of the park where the civet was encountered had been free from canine rabies for over eight years (Lembo et al., 2008). Prediction of antigenic distance and by extrapolation, degree of protection, provided by rabies vaccine strains using sequence data alone is not precise, but the degree of genetic divergence suggested that currently available vaccines would likely not be able to confer protection against IKOV (Evans et al., 2012; Horton et al., 2010).

The African civet, a solitary scavenger, does not fill the ecological niche nor have the population dynamics typical of a carnivore rabies host (Cleaveland & Dye, 1995; Estes, 1992). The few published reports of rabies in civets have been either dog or mongoose associated RABV variants, suggestive of spill over rather than maintenance of infection (Sabeta et al., 2008). Despite dogs being responsible for the majority of human rabies cases worldwide, a variety of RABV lineages and numerous diverse lyssavirus species have been detected in wildlife, and particularly in a range of bat species (Banyard et al., 2011; Hayman et al., 2012; Kuzmin et al., 2010). Antibodies to a phylogenetically related lyssavirus, West Caucasian bat virus (WCBV), have been detected in Miniopterus spp. bats in Kenya close to the border with Tanzania (Kuzmin et al., 2008a), but there are no published studies showing evidence for lyssavirus antibodies in bats in the SNP. Therefore, with evidence for bat reservoirs for closely related viruses, it is possible that the natural host and reservoir for IKOV is a bat species.

The detection of genetic material from a new virus causing rabies in a potential spill over host, with no known reservoir, requires further investigation. Here we describe the isolation and characterisation of viable IKOV in-vivo and in-vitro, assess the degree of serological cross neutralisation of IKOV with RABV and WCBV, and assess the efficacy of rabies vaccination against IKOV in an animal model. We also present results of surveillance for IKOV in bats living in close proximity to the location of the index case.

RESULTS
**In-vitro** characterisation

IKOV was not easily isolated from clinical material. Repeated blind passage of civet brain homogenate in neuroblastoma (NA) cell culture failed to amplify viable virus. IKOV was initially isolated through intracranial inoculation of four week old CD-1 mice and stocks of virus were then generated by six serial passages in murine fibroblast (baby hamster kidney, BHK-21) cells. The fluorescent antibody test (FAT) was performed on brain smears post mortem. Viral antigens were detected using fluorescein isothiocyanate (FITC)-conjugated anti-rabies nucleocapsid protein antibodies (FITC Anti-Rabies Monoclonal Globulin, Fujirebio diagnostics, Malvern USA). This preparation of antibodies has demonstrated high sensitivity and specificity in detecting lyssaviruses (Robardet *et al*., 2012). Virus titres at passage five (10^{4.42}, 50% tissue culture infectious doses (TCID\_50)/ml) and passage six (10^{4.8} TCID\_50/ml) were comparable to those seen with other lyssaviruses (Brookes *et al*., 2005a; Horton *et al*., 2010; Koraka *et al*., 2012).

A modified fluorescent antibody virus neutralisation (mFAVN) test was used to assess the degree of cross neutralisation between IKOV and other lyssaviruses (Brookes *et al*., 2005b; Cliquet *et al*., 1998). Sera from four humans and ten dogs vaccinated with commercial rabies vaccines demonstrated negligible levels of cross neutralization against IKOV (Table 1). Three of these individuals had exceptionally high reciprocal antibody titres against RABV (Challenge Virus Standard, CVS) of over 1:30,000, and yet had reciprocal titres of less than 1:8 against IKOV, indistinguishable from negative controls and therefore effectively negative. In addition, neat human rabies immune globulin (HRIG), at a concentration of 270 International Units (IU)/ml, showed no detectable neutralisation of IKOV. Sera from mice inoculated with IKOV that showed reciprocal antibody titres of 1:27 to 1:420 against IKOV, failed to neutralise CVS in standard FAVN tests. Furthermore, rabbit serum with a neutralising antibody titre of over 1:1000 against WCBV failed to neutralise IKOV (Table 1). This lack of detectable cross neutralisation precluded accurate positioning of IKOV on an antigenic map developed previously (Horton *et al*., 2010).

**In-vivo** pathogenesis

All mice inoculated intracranially (IC) with 0.03ml tissue culture passaged IKOV at high dose (10^{4.8} TCID\_50/ml) or a 10-fold dilution (10^{3.8} TCID\_50/ml) succumbed to challenge
with an incubation period of between 4.5 and 6 days, with no apparent dose effect. There was, however, a detectable dose effect (albeit not statistically significant at the 95% level) for the intramuscularly (IM) challenged mice, with 5/5 of the mice challenged IM with 0.03ml IKOV at $10^{4.8} \text{TCID}_{50}/\text{ml}$ succumbing between 6.5 and 9 days, but only 2/5 of the group receiving IKOV at $10^{3.8} \text{TCID}_{50}/\text{ml}$ succumbing, with incubation periods of 7 and 11 days (Fischer’s exact test, $p=0.08$) (Fig. 1). The remaining three mice inoculated IM with the lower dose had no detectable lyssavirus antigens in the brain post mortem, but had seroconverted when subjected to euthanasia at 28 days (Table 1). Prodromal clinical signs were similar to those recorded for other lyssaviruses in mice, including reduced appetite, ruffled fur and hunched posture (Healy et al., 2013). Mice then progressed to either hind limb paralysis, or hyperexcitability and convulsions, with a predominance of the latter, and were subjected to euthanasia.

Pathology

Mice inoculated with IKOV and subject to euthanasia at a clinical score 2-3 had developed non-suppurative encephalitis (Fig. 2a). The inflammatory changes were very mild, with occasional perivascular cuffing but without notable gliosis or degenerative changes in neurons. IKOV antigens were observed using immunohistochemistry in the perikaryon and neuropil in all brain regions examined, with the majority of staining in the medulla and only rare and dispersed antigens in the cortex and thalamus (Fig. 2b).

Vaccine challenge experiments

Nineteen mice were vaccinated with one dose of a commercial rabies vaccine, and eighteen of these had seroconverted against RABV by day 21 (Titre range 0.70-256 IU/ml), confirming an adequate response to vaccination. Mice vaccinated under identical conditions were previously demonstrated to be protected against IC challenge with RABV (Brookes et al., 2005a). All nineteen mice challenged IC with IKOV in a modified NIH test developed rabies, as did all the unvaccinated controls. The presence of IKOV antigens and RNA was confirmed in the brains of challenged mice.

Genetic characterisation
Comparison of the full genome of IKOV, with the full genome of representatives of other lyssaviruses allows interpretation of genetic relationships among the lyssaviruses (Fig. 3). Analysis of the full genomes supports the previously reported relationship of WCBV and IKOV. Lagos bat virus (LBV), Shimoni Bat virus (SHIBV) and Mokola virus (MOKV), all previously characterised as phylogroup II, comprise a strongly supported separate monophyletic group. The European bat lyssaviruses have separate ancestors. European bat lyssavirus type 1 (EBLV-1) shares a common ancestor with Duvenhage virus (DUVV) and Irkut virus (IRKV), separate from the ancestor common to European bat lyssavirus type 2 (EBLV-2) and the remaining lyssavirus species (RABV, Aravan, Khujand, Bokeloh, and Australian bat lyssavirus). IKOV and WCBV form a monophyletic group outside of the Phylogroup I and Phylogroup II lyssaviruses with significant bootstrap support, although they are separated by long genetic distance (63.4% nucleotide identity for the concatenated coding gene sequences).

Sampling of bats in Africa for antibodies to IKOV

The common and ubiquitous free-tailed bats (*Chaerephon pumilus*) in the immediate vicinity of the index IKOV case in the SNP were roosting in the roof space of the few human dwellings, used by park employees and research scientists. These were therefore the priority for sampling in Tanzania, and were the only species encountered during sampling in SNP in 2012. Sera were available from 25 of these free-tailed bats (*C. pumilus*) sampled within SNP, and in two settlements in close proximity to the northwestern boundary of the SNP. Two other species (*Hipposideros* sp., n=16 and *Nycteris* sp. n=1) were also sampled in these settlements. In Kenya, 441 individuals from at least ten different bat species were caught in multiple locations during 2011, in the framework of the Global Disease Detection Program of the US Centers for Disease Control and Prevention (CDC) (Table S2). These included 229 *Miniopterus* spp., of which 48 neutralised WCBV (titre range 1.3-3.1 log10 ED50). All 483 of these sera from Tanzania and Kenya failed to show any neutralising activity against IKOV (see supplementary information).
DISCUSSION

The discovery of a novel lyssavirus (IKOV) causing rabies in an African civet, in a wildlife-rich area with potential for wildlife-human interaction, required further investigation to assess its public and animal health significance. Here we have demonstrated that peripheral pathogenicity of IKOV is comparable to that of RABV in a rodent model. The virus is antigenically distinct to all other lyssaviruses, and vaccination with rabies vaccine produced no cross neutralising antibodies in humans or animals, and did not elicit protection in an animal challenge model.

The G protein is the immunodominant lyssavirus protein, and comparison of the G coding sequence of IKOV with other lyssavirus species demonstrates 46-50%, and 52-55% similarity at the nucleotide and amino acid levels, respectively (Evans et al., 2012). Previous quantitative assessments of the effect of these genetic differences on antigenicity suggested that 72% amino acid identity along the G ectodomain is a threshold for efficient cross neutralization (Badrane et al., 2001), and a 4.8% difference in amino acid identity over the ectodomain region would cause, on average, a two-fold difference in antibody titre against a virus (Horton et al., 2010). Therefore, with only 47% amino acid identity with CVS, we would expect to see no cross neutralization between RABV and IKOV. Here we were able to confirm this hypothesis, demonstrating a complete lack of cross neutralization in sera of humans and animals vaccinated from rabies, even if they demonstrated very high neutralizing titres against CVS. Similar studies of phylogroup I lyssaviruses showed reduced but significant neutralization of EBLVs relative to CVS (Brookes et al., 2006; Malerczyk et al., 2009), and even studies of the most divergent lyssavirus prior to this discovery, WCBV, showed a limited neutralization by sera of rabbits that had high neutralizing titers against CVS (Hanlon et al., 2005; Horton et al., 2010). Previous studies suggested that WCBV is highly divergent from other lyssaviruses at the genetic and antigenic level (Kuzmin et al., 2005). In this study we have demonstrated a lack of cross-neutralization between IKOV and WCBV, despite the viruses having a monophyletic relationship in phylogenetic reconstructions. These observations suggest that demarcation of lyssaviruses into phylogroups may be more complex than a comparison of glycoprotein identity values and amount of cross-neutralization as
proposed initially (Badrane et al., 2001). Crucially, HRIG at 270 IU/ml failed to show significant neutralization of IKOV (Table 1). In all category 3 exposures, HRIG is recommended as defined by the WHO (Anon, 2009), but these data demonstrated it is unlikely that HRIG would be effective against IKOV. This lack of protection afforded by rabies biologics reinforces the value of thorough wound cleaning and antiseptic treatment in cases of exposure to carnivores and bats (Anon, 2009).

The current gold standard test for assessment of rabies vaccine potency is the National Institutes of Health (NIH) test, which uses an intracranial challenge in mice vaccinated with serial dilutions of vaccine (Wilbur & Aubert, 1996). The test has been successfully modified for the assessment of protection against other viruses by using a single vaccine dilution, and mice vaccinated using this technique have previously been protected against intracranial challenge with RABV (Brookes et al., 2005a; Wilbur & Aubert, 1996). However, all 19 mice vaccinated with commercial rabies vaccine and challenged with IKOV succumbed to disease, demonstrating that no protection was conferred. These results confirm the predictions obtained from the genetic and in-vitro antigenic studies that rabies vaccines do not elicit protection against IKOV in mice and are therefore unlikely to provide protection in other mammals, including humans.

Analysis of full concatenated coding gene sequences of representative lyssaviruses showed similar topology to previous analyses based on partial N-gene sequences and glycoprotein sequences, suggesting that IKOV and WCBV form a monophyletic group, albeit with deeply rooted divergence (Evans et al., 2012; Marston et al., 2012a). One difference seen here is that in the previous partial N-gene analyses, MOKV, WCBV and IKOV formed a monophyletic group separate from LBV, which is not the case in this full genome comparison.

Knowledge of the reservoir of zoonotic diseases is important, to inform prevention of spill-over infections. IKOV was detected in an African civet, an unlikely reservoir for a lyssavirus. Civets are mostly solitary scavengers, with presumed low contact rates (Estes, 1992). Consistent with this ecology is the detection of varied canid and mongoose variants of RABV in the few reported cases of rabies in civets, suggesting that civets are spill-over hosts (Sabeta et al., 2008). No further cases caused by IKOV have been detected, despite high levels of tourist and research related activity in the area that have been sufficient to detect RABV in another wild carnivore since the index
IKOV case. This suggests that the civet case did not result in a sustained chain of transmission. Nonetheless, a civet reservoir cannot be ruled out definitively, so vigilance for rabies in civets and other wild carnivore species still needs to be maintained, including virus characterisation of any positive cases.

In light of the association of lyssaviruses with bats (Banyard et al., 2011), attention has inevitably focused on bat populations in and around SNP to find the reservoir of IKOV. The IKOV-positive civet was encountered on the edge of the Serengeti plains, with few suitable roosting places for bats. Previous studies demonstrated up to 70% seroprevalence of bats to various lyssaviruses (Harris et al., 2009; Hayman et al., 2012; Kuzmin et al., 2010; Kuzmin et al., 2008a, b; Turmelle et al., 2009) but all bat sera tested in this study were negative for antibodies to IKOV. The limited sample size, species coverage and narrow timeframe preclude robustly ruling out IKOV infection in bats. However, based on these data, and seroprevalence levels demonstrated in reservoir bat populations to other lyssaviruses, it is unlikely that IKOV was circulating in the local free-tailed bat population at the time of sampling. However, the presence of other bat species, the variable success of bat capture techniques for different species, and the possibility of long distance bat movements, suggests that the reservoir of IKOV is still likely to be in a bat species. The most recently detected putative lyssavirus species, Lleida bat lyssavirus, was detected in a Miniopterus schreibersii bat in Spain, and is phylogenetically related to WCBV and IKOV (Arechiga Ceballos et al., 2013). Miniopterus spp. bats are also a putative reservoir for WCBV in Kenya (Kuzmin et al., 2008a). There was a notable absence of Miniopterus spp. captured in the SNP, despite the species being detected in large numbers in neighbouring Kenya. All Miniopterus spp. bat sera from Kenya were negative for IKOV antibodies, but there are at least 12 species of Miniopterus bats described in Africa (IUCN, 2013), and some species roost mainly in caves and can travel long distances to feed. Sampling of cave dwelling bats in and around the SNP, together with serological screening of other wildlife samples from the SNP, would be a logical next step to investigating potential reservoirs.

In the experimental animal model used in this study, the incubation period, clinical signs and pathology of IKOV infection were consistent with those caused by other lyssaviruses (Healy et al., 2013), and these data therefore confirm that IKOV causes rabies. The peripheral pathogenicity of IKOV concurs with recent studies showing that substitution at a key domain of the glycoprotein (K/R333D) which is shared by IKOV
and Phylogroup II lyssaviruses, does not result in lower peripheral pathogenicity (Badrane et al., 2001; Kgaladi et al., 2013; Kuzmin et al., 2010; Kuzmin et al., 2008b; Markotter et al., 2008), in contrast to initial data based on fewer isolates (Badrane et al., 2001). These results are corroborated by the fact that IKOV caused clinical rabies in the civet from which it was originally isolated, and therefore has potential to cause encephalitis in other hosts. This raises the question as to why spill over events like this do not happen more frequently. One obvious possibility is that they do, but remain undetected. IKOV was identified during dedicated studies on rabies dynamics in Tanzania, but virus characterisation is not routine in most regions of Africa, with positive cases assumed to be RABV. Alternatively, species constraints might limit the number of effective spill over infections with IKOV as was suggested for other bat lyssaviruses (Banyard et al., 2011). Continued enhanced surveillance, including laboratory-based confirmation of diagnosis and virus characterisation, is necessary to assess and mitigate the public and animal health threats posed by IKOV and other emerging viruses (Banyard et al., 2013).

METHODS

In-vitro

Civet brain material was stored frozen at the Tanzania National Park veterinary field laboratory in the Serengeti from 2009-2011 and then shipped to AHVLA for virus isolation and characterization. Brain homogenates from the first mouse passage were used to inoculate baby hamster kidney (BHK-21) cell culture, and the virus was passaged to a high titre for in-vivo analysis during six passages: 0.5ml clarified 10% mouse brain homogenate in PBS was added to 2 ml of a BHK-21 cell suspension at 2 x 10^5 cells/ml, and incubated at 37 degrees • for 20 minutes with intermittent agitation. The infected cells were then added to a cell culture flask with fresh Glasgow MEM supplemented with10% foetal bovine serum (FBS) and 10% tryptose phosphate broth. Every 3-5 days the cells were disrupted using antibiotic-trypsin-versene and transferred to a new flask with fresh media, and uninfected cells at 1:1 ratio of infected to uninfected cells. Virus titre was assessed at passages five and six using techniques described previously (Aubert, 1996) and calculated with the Spearman-Kärber method. Viral antigens were visualised in acetone-fixed cells by the direct fluorescent antibody test (FAT) using standard techniques (Dean et al., 1996) with FITC-conjugated antibody (FITC Anti-Rabies Monoclonal Globulin, Fujirebio diagnostics, Malvern USA).
A modified fluorescent antibody neutralization test (mFAVN) was developed and optimised for IKOV using tissue culture passaged virus supernatant (TCSN) (Horton et al., 2010). A standard quantity of virus (100 TCID₅₀/50µl) was added to serial 2-fold dilutions of serum in duplicate, with the quantity of virus checked by back-titration on each test. Sera and virus were incubated with BHK-21 cells for 48 hours before fixing in acetone and staining with FITC-conjugated antibody (Fujirebio diagnostics). The 50% endpoint serum dilution was calculated with the Spearman-Kärber method (Aubert, 1996). Mouse serum from the second mouse passage of IKOV was used as a positive control, and serum from uninfected mice was used as a negative control. A panel of sera from animals and human vaccinees, with proven high serum neutralizing antibody levels to RABV, were tested for their ability to neutralise IKOV (Table 1). A rabbit anti-WCBV serum (rabbit 821), which neutralised WCBV at a reciprocal titre of 1:1448, was also tested in the same assay (Horton et al., 2010).

In-vivo

All experimental work in animal models was undertaken under Home Office Licence after independent ethical review. Virus was first isolated from clinical material in four week old OF1 mice, after unsuccessful isolation attempts in cell culture despite repeated passages using the rabies tissue culture inoculation test RTCIT (Marston et al., 2012a). Clarified civet brain homogenate (30 l) was inoculated into mice (n=5) intracranially. Mice were monitored twice daily using a clinical scoring system from one to five (Healy et al., 2013) and subjected to euthanasia as soon as they progressed beyond clinical score one. Brains of mice were tested by FAT. To investigate the virulence of IKOV, four groups of five OF1 mice were challenged with either neat TCSN (high dose, neat 10⁴.₈ TCID₅₀/ml) or a 10-fold dilution (low dose 10⁻³.₈ TCID₅₀/ml) by either intracranial or intramuscular inoculation, followed by twice daily observation and euthanasia when clinical score progressed beyond one. Immunohistochemical demonstration of RABV antigens in post-mortem samples from brains of mice was performed as described previously (Hicks et al., 2009).

To assess protection provided by vaccination, a group of 19, four-week old OF1 mice were vaccinated with 0.5 ml of a commercially available vaccine (VERORAB, Sanofi Pasteur MSD) by intraperitoneal injection, as standard. A group of five control mice were left unvaccinated. After 21 days a blood sample was taken from the tail vein of each mouse and the level of RABV neutralizing antibodies was assessed using a CVS.
pseudotype assay (Wright et al., 2009). The mice were then challenged IC with IKOV TCSN at $10^{4.8}$ TCID$_{50}$/ml, observed twice daily and subjected to euthanasia at the first sign of disease.

Molecular analyses

Nucleic acids were extracted using Trizol (Invitrogen) according to the manufacturer’s instructions. A pan-lyssavirus real-time PCR assay using iScript (Bio-Rad) was then used to test for the presence of lyssavirus RNA in mouse brain (Hayman et al., 2011).

Phylogenetic analyses

The full genome of IKOV was previously determined directly from clinical civet brain material using a combination of next generation sequencing and Sanger sequencing methods (GenBank accession number (JX193798) (Marston et al., 2012b). Here concatenated gene sequences for all genes (N+P+M+G+L) from representatives from all lyssavirus species were compared to those of IKOV using neighbour joining analysis, thereby avoiding the potential issue of different evolutionary rates between genes. Complete concatenated sequences were aligned using the Clustal W algorithm in MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2006), a neighbour joining tree was constructed using p-distances with 1000 bootstrap replicates, and visualised using FigTree(v1.2).

Field sampling of bats in Africa

To investigate the possibility of a local bat reservoir for IKOV, after ethical review and under permit from local authorities, bats were sampled both in the immediate vicinity of the index IKOV case, and in areas of human habitation (Bunda town and Fort Ikoma Village) close to the northwestern border of the SNP in Tanzania (n=42) in July 2012. Sera were also analysed from bats sampled in multiple locations in Southern Kenya (n=441) as part of a separate study (Table S2). All 483 serum samples from at least 11 bat species were tested for the presence of neutralising antibodies to IKOV using mFAVN and modified rabies fluorescent focus inhibition test (see supplementary information).

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FIGURE LEGENDS

Figure 1. Survival chart showing percent survival in days post inoculation (p.i) for groups of five mice inoculated either intracranially (IC) or intramuscularly (IM) with neat IKOV ($10^{4.8}$ TCID50/ml) or a 1/10 dilution ($10^{3.8}$ TCID50/ml). Mice not clinically affected after 11 days were still healthy when euthanased at 28 days and negative for lyssavirus antigens post mortem.

Figure 2. Histopathological examination of brain from IKOV infected mice. 2a Perivascular cuffing in the cortex composed of few inflammatory cells. Inflammatory changes were minimal to mild and perivascular cuffs rare and not prominent, H&E 400x. 2b. Immunohistochemical demonstration of viral antigens in the medulla (brown labelling). IHC 400x.

Figure 3. Neighbour-joining phylogenetic tree of concatenated coding areas of five genes (N+P+M+G+L) of IKOV compared to representative lyssaviruses from all other species (Table S1). Branches are coloured by phylogroup; Green, phylogroup one, red phylogroup two and blue uncharacterised. Bootstrap values (proportion of 1000 replicates) are given at each node.
**TABLES**

Table 1. Cross neutralization of sera from rabies vaccinated humans and animals against IKOV

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<td>41</td>
<td>1263</td>
<td>&lt;8</td>
</tr>
<tr>
<td>HRIG</td>
<td>270</td>
<td>n.d.</td>
<td>&lt;16</td>
</tr>
<tr>
<td>Mouse 3</td>
<td>&lt;0.2</td>
<td>&lt;16</td>
<td>27</td>
</tr>
<tr>
<td>Mouse 4</td>
<td>&lt;0.2</td>
<td>&lt;16</td>
<td>420</td>
</tr>
<tr>
<td>Mouse 5</td>
<td>n.d.</td>
<td>n.d.</td>
<td>81</td>
</tr>
<tr>
<td>Rabbit 821</td>
<td>&lt;0.2</td>
<td>&lt;16</td>
<td>&lt;8</td>
</tr>
</tbody>
</table>

Neutralising antibody titres against RABV (Challenge virus standard, CVS) IKOV in reciprocal 50% endpoint titres. International units (IU) are given for rabies by comparison to a standard control (not applicable to IKOV). TCID50=50% tissue culture infectious dose. Mice 3, 4 and 5 were inoculated IM with IKOV at $10^{3.8}$ TCID50/ml and survived. Rabbit 821 is rabbit anti-WCBV serum with a reciprocal titre of 1448 against WCBV.
REFERENCES


Marston, D. A., Horton, D. L., Ngeleja, C., Hampson, K., McElhinney, L. M.,


Figure 1

Survival curve (IKOV)

- KOV IC Neat
- KOV IC 1:10
- KOV IM Neat
- KOV IM 1:10