Gairo virus, a novel arenavirus of the widespread *Mastomys natalensis*: Genetically divergent, but ecologically similar to Lassa and Morogoro viruses

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**A B S T R A C T**

Despite its near pan-African range, the Natal multimammate mouse, *Mastomys natalensis*, carries the human pathogen Lassa virus only in West Africa, while the seemingly non-pathogenic arenaviruses Mopeia, Morogoro, and Luna have been detected in this semi-commensal rodent in Mozambique/Zimbabwe, Tanzania and Zambia, respectively. Here, we describe a novel arenavirus in *M. natalensis* from Gairo district of central Tanzania, for which we propose the name “Gairo virus”. Surprisingly, the virus is not closely related with Morogoro virus that infects *M. natalensis* only 90 km south of Gairo, but clusters phylogenetically with Mobala-like viruses that infect non-*M. natalensis* host species in Central African Republic and Ethiopia. Despite the evolutionary distance, Gairo virus shares basic ecological features with the other *M. natalensis*-borne viruses Lassa and Morogoro. Our data show that *M. natalensis*, carrying distantly related viruses even in the same geographical area, is a potent reservoir host for a variety of arenaviruses.

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**Introduction**

Arenaviruses are single-stranded, negative-sensed RNA viruses with mostly rodents as natural hosts. Their bi-segmented genome consists of four open-reading frames (ORF) in ambisense orientation. The small (S) segment encodes for precursor glycoprotein (GPC) and nucleoprotein (NP), the large (L) segment contains genes for matrix protein Z and the L protein (L), respectively (Fig. 1). Most arenaviruses appear restricted to a single rodent species as natural host, but several host-switching events have likely occurred during their evolutionary history (Jackson and Charleston, 2004; Coulilbaly-N’Golo et al., 2011; Bowen et al., 1997). The majority are not pathogenic to humans, but seven arenavirus species are known to cause hemorrhagic fevers in humans, of which two in Africa (Lassa and Lujo viruses) and seven arenavirus species are known to cause hemorrhagic fevers in Africa (Bowen et al., 1997). The majority are not pathogenic to humans, but no human infections with *M. natalensis*-borne arenaviruses have been reported from southern or eastern Africa. Also none of the other arenaviruses detected in different African rodents have been linked to human diseases.

To extend our knowledge on the geographical distribution, diversity, and ecology of African arenaviruses, we screened the...
small mammal community in Central Tanzania for arenaviruses. We detected and characterize a novel arenavirus, Gairo virus (GAIV), in M. natalensis. The virus was found in close proximity to the distribution area of Morogoro virus and its ecology appears similar to that of other M. natalensis-borne arenaviruses, yet it is genetically divergent.

Results

We captured a total of 371 small mammals, 332 of which were M. natalensis. One animal died inside the trap and was not further processed; yielding a total dataset of 370 small mammals of which 331 were M. natalensis (Table 1). Morphological identification of animals as M. natalensis was confirmed by cytochrome b sequences. Of the 247 M. natalensis individuals for which kidney samples were available, 18 (7.3%) were arenavirus RT-PCR positive in both dried blood spots (DBS) and kidney samples while a further 6 individuals (2.4%) had only positive kidney samples (Table 1). Of the remaining 84 M. natalensis, 5 (6.0%) had RT-PCR positive DBS samples. Thirty out of 331 individuals (9.1%) carried anti-arenavirus antibodies, of which two were also RT-PCR positive in both blood and kidneys, and of which one was RT-PCR positive in kidneys alone (Table 1). Sequencing of the RT-PCR products of partial L and partial NP gene of which one was RT-PCR positive in kidneys alone (Table 1). Sequencing of the RT-PCR products of partial L and partial NP gene and the resulting phylogenetic analyses (Fig. 3A, Supplementary Fig. 1A) revealed that all 29 PCR-positive samples contained a novel arenavirus, for which we propose the name Gairo virus (GAIV). GAIV was not detected in any of the other sampled rodent species (Table 1). One Arvicanthis neumanni individual from Chakwale was arenavirus RT-PCR positive, but a GenBank BLAST search of the 340 nt partial L gene sequence indicated the presence of an arenavirus related to Ippy virus, which was previously isolated from Arvicanthis sp. in Central African Republic (Digoutte et al., 1970; Swanepoel et al., 1985).

Virolological characterization

GAIV was isolated from four M. natalensis samples in Vero E6 cell culture. The culture supernatants reached infectious particle titers of $3.5 \times 10^4$ FFU/mL (TZ-27317), $2.1 \times 10^4$ FFU/mL (TZ-27370), $5.1 \times 10^4$ FFU/mL (TZ-27421) and $2.5 \times 10^4$ FFU/mL (TZ-27457) 13 days post inoculation. Virus particles in the supernatant of the second passage of TZ-27421 (2.1 $\times 10^3$ FFU/mL) were used for 454 sequencing. Of the 110,822 reads generated by 454 GS-FLX, 1.36% mapped to the preliminary GAIV consensus genome generated in Geneious 6.1.7. The average coverage was 88.5 (SD = 33.0) for the GPC, 54.6 (SD = 15.5) for the NP, 44.3 (SD = 8.6) for the L and 23.7 (SD = 3.4) for the Z gene.

Assembly allowed reconstruction of the entire Gairo virus S and L segments, apart from 17 non-coding nucleotides at the 3’ end of the S segment, 8 non-coding nucleotides at the 5’ end of the L segment and 17 non-coding nucleotides at the 3’ end of the L segment.
While the identity percentages of the entire gene/protein sequences between GAIV and MOBV were substantially higher than between GAIV to other OWAs, for the G1 part of GPC, which is involved in host receptor recognition (Cao et al., 1998), the 64.3/68.2% nucleotide/AA identity were similar to comparisons between GAIV and other *M. natalensis*-borne arenaviruses and Gbagroube (Supplementary Table 2). Also in the phylogenetic tree of only the G1 part of the GPC gene (Fig. 3C), GAIV failed to form a monophyletic group with MOBV, and instead the position of neither GAIV nor Mobala virus can be resolved. The phylogenetic tree of the G2 part of the GPC was on the other hand well resolved (Fig. 3D).

**Ecological characterization**

The probability of GAIV infection significantly decreases with the age (as measured by eye lens weight) of the animals ($\chi^2 = 4.95$, $p = 0.026$), but none of the other host characteristics (sex, weight, sexual maturity, BMI) was significantly associated with GAIV RT-
$252$

sub-adults/juveniles (vs. 12/121 female) or RNA (10/109 male vs. 14/121 female) rodent

M. natalensis can be found in the Supplementary Table 3.

each of the 4 entire gene sequences revealed that the novel

no infections in humans have ever been reported. Phylogenies of

clade thus appear to include several host-switches. Adaptation to

al., 1983). Other relatives of MOBV have previously been geneti-

cence of amino-acid sequences (Emonet et al., 2006). While there

highlands (Meheretu et al., 2012)( Figs. 2 and 3).

recognized (Emonet et al., 2006; Bowen et al., 2000). We therefore

interspecies cut-off divergences of 10.2% or 12% are generally

different host species. The nucleoprotein AA sequence of GAIV

is no published data on the occurrence of GAIV and MOBV and its

The evolutionary histories of the arenaviruses within this MOBV-

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novel hosts is likely at least partially mediated through substitutions in the G1 part of GPC, which is known to be involved in host cell

receptor recognition. Indeed, the G1 AA sequence of GAIV and MOBV were only 68% identical, and in the G1 phylogenetic tree the relationship between GAIV and all other non-LCMV OWAs remained unresolved (Fig. 3C). The discrepancy between trees of G1 and other genes was also apparent for some other OWAs, but the tight monophyletic cluster of Morogoro and Mopeia virus (that have a phylogenetic distance similar to that between GAIV and MOBV virus but both infect M. natalensis) is just as well resolved in the G1 tree as in the other gene trees. While G1 phylogenies thus do not accurately reflect the evolutionary histories of the arenaviruses, they may contain information on current host species. Rapid adaptation of arenaviruses to novel hosts through substitutions in G1 region might also explain the 5 polymorphic positions observed in this region in the GAIV strain cultured in Vero E6 cells (originally derived from African green monkeys), of which 3 were non-synonymous.

Despite the high genetic divergence between M. natalensis

arenaviruses, infection by these viruses seems to result in similar ecological patterns in their respective M. natalensis populations. The general prevalences of GAIV RNA and anti-arenavirus antibodies in this study were similar than reported previously for other M. natalensis-borne arenaviruses (Lassa, Morogoro and Luna) in differ-

ent regions (Ishii et al., 2011, 2012; Fichet-Calvet et al., 2007; Demby et al., 2001) and Morogoro virus in Tanzania (Borremans et al., 2011), as well as MOBV in Praomys sp. Gonzalez et al., 1983). In contrast, ecological studies on the New-World arenaviruses Junin (Mills et al., 1994), White-Water Arroyo (Fulhorst et al., 2002), Bear-Canyon (Fulhorst et al., 2002) and Catarina virus (Milazzo et al., 2013) show that most rodents with an active arenavirus infection simultaneously carry the antibodies against it (and vice versa), suggesting that these arenaviruses get cleared later from rodent host bodies after the antibody response than the studied M. natalensis and Praomys-borne viruses.

## Discussion

Besides the well-known pathogen Lassa virus, the African rodent M. natalensis carries several other arenaviruses of which no infections in humans have ever been reported. Phylogenies of each of the 4 entire gene sequences revealed that the novel M. natalensis-borne Gairo virus (GAIV) forms a monophyletic group with Mobala virus (MOBV), which was previously isolated from Praomys sp. in Central African Republic (CAR) (Fig. 3) (Gonzalez et al., 1983). Other relatives of MOBV have previously been genetically
c
dected in M. awashensis and S. albipes in the Ethiopian highlands (Meheretu et al., 2012) (Figs. 2 and 3).

clineating species in the genus Arenavirus follows multiple criteria that are based on antigenic cross-reactivity, geographic distribution, host species, pathogenicity to humans, and diver-
gence of amino-acid sequences (Emonet et al., 2006). While there is no published data on the occurrence of GAIV and MOBV and its relatives outside of the study areas in CAR, Ethiopia and Tanzania, they were each detected thousands kilometers apart and each in different host species. The nucleoprotein AA sequence of GAIV differs 15.4% from that of MOBV, while for Old-World arenaviruses interspecies cut-off divergences of 10.2% or 12% are generally recognized (Emonet et al., 2006; Bowen et al., 2000). We therefore propose that GAIV be recognized as a distinct arenavirus species within the MOBV clade.

The evolutionary histories of the arenaviruses within this MOBV-clade thus appear to include several host-switches. Adaptation to

PCR positivity. The probability of antibody presence significantly increases as animals get older ($\chi^2 = 19.22, p = 0.002$) and heavier ($\chi^2 = 21.53, p < 0.001$; BMI: $\chi^2 = 12.93, p < 0.001$). Sexually mature animals were 1.98 times as likely to carry antibodies than sub-adults/juveniles ($\chi^2 = 15.92, p < 0.001$). Antibody (13/109 male vs. 12/121 female) or RNA (10/109 male vs. 14/121 female) prevalence did not differ significantly between sexes ($\chi^2 = 0.21, p = 0.65$). An overview of prevalences in categories of the different host characteristics is given in Table 2. Summaries of the statistics can be found in the Supplementary Table 3.

### Table 1

<table>
<thead>
<tr>
<th>Number of captures and blood samples screened (% RT-PCR positive–% IFA positive)</th>
<th>Number of kidney samples screened (% RT-PCR positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acomys</strong></td>
<td><strong>Mastomys natalensis</strong></td>
</tr>
<tr>
<td><strong>sp.</strong></td>
<td><strong>sp.</strong></td>
</tr>
<tr>
<td>Berega total</td>
<td>6 (0–0)</td>
</tr>
<tr>
<td>2012_01</td>
<td>5 (0–0)</td>
</tr>
<tr>
<td>2012_02</td>
<td>5 (0–0)</td>
</tr>
<tr>
<td>2012_03</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>2012_04</td>
<td>0 (0–0)</td>
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<tr>
<td>2012_05</td>
<td>1 (0–0)</td>
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<tr>
<td>2012_06</td>
<td>0 (0–0)</td>
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<td>2012_13</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>2012_14</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td><strong>Chakwale total</strong></td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>2012_07</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>2012_08</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td><strong>Majawanga total</strong></td>
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</tr>
<tr>
<td>2012_09</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>2012_10</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>2012_11</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>2012_12</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>6 (0–0)</td>
</tr>
<tr>
<td>2012_01</td>
<td>2 (0–0)</td>
</tr>
<tr>
<td>2012_02</td>
<td>3 (0–0)</td>
</tr>
<tr>
<td>2012_03</td>
<td>3 (0–0)</td>
</tr>
</tbody>
</table>

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*Chi-square test*
Nevertheless, for nearly all ecologically studied arenaviruses (both New-World: Junin Mills et al., 1994, Oliveros Mills et al., 2007 and Old-World: Lassa Demby et al., 2001, Morogoro Borremans et al., 2011) the probability to carry anti-arenavirus antibodies increases with the age of the host individuals, and we found this also for GAIV (Table 2). In other words, the older an animal becomes, the higher chance it has to have acquired an arenavirus infection at some point in its life. While not excluding sexual or vertical transmission, this observation suggests that horizontal transmission is important for most arenaviruses. It also implies that relatively higher proportions of animals in younger age categories are still susceptible; and indeed we found a significant negative relationship between GAIV RT-PCR positivity and age. This was previously also observed in M. natalensis populations with Lassa (Fichet-Calvet et al., 2008) and Morogoro viruses (Borremans et al., 2011).

**Fig. 3.** Phylogenetic trees from nucleotide sequences of 340 nt of the L gene (A), entire NP gene (B), G1 part of GPC gene (C) and G2 part of GPC gene (D). Numbers on the branches represent Bayesian posterior probabilities that the taxa form monophyletic groups and the scale bar represents the number of substitutions per site. In the tree of the partial L gene (A), all GAIV positive samples from this study are included, as well as one representative of each published arenavirus taxon (if the L sequence was available). For the other trees (B–D), all available entire gene sequences of published OWA taxa described from their natural hosts (apart from Lujo for which the natural host is unknown) are included. See Supplementary Table 1 for a list of the arenavirus sequences used and their GenBank Accession numbers.
In conclusion, the discovery of GAIV in M. natalensis highlights the great arenavirus diversity a single rodent species can harbor. This is clearly not solely due to the wide, near pan-African distribution of the rodent, as we detected GAIV in M. natalensis populations just 90 km from the locality where previously Morogoro virus, an outlier strain of Mopeia virus from Mozambique, was described. M. natalensis’ arenavirus diversity is also markedly distinct from that of the house mouse, Mus musculus, from which only one arenavirus species (Lymphocytic choriomeningitis virus—LCMV) has been described across its cosmopolitan range. A more detailed sampling across the transition zone between two divergent arenaviruses of M. natalensis should allow a better understanding of the ecological processes that keep the occurrence of its arenaviruses limited to distinct geographical regions.

Material and methods

Rodent trapping and sample collection

We captured rodents at three localities in central Tanzania: Berega (6.18S, 37.14E), Majawanga (6.11S, 36.82E) and Chakwale (6.05S, 36.6E) (Fig. 2) between 18/08/2012 and 28/08/2012. In each locality, respectively 8, 4, and 2 grids of live traps (Sherman Live Trap Co., Tallahassee, FL, USA) were set for one night in harvested maize fields. Each grid consisted of 100 traps, baited with peanut butter mixed with maize flour, and placed at 10 m intervals. At least 500 m separated each grid from others. All 370 live captured animals were weighed, their blood was collected from the retro-orbital sinus (for released animals) and from the heart (for sacrificed animals) and dried on filter paper. Their reproductive status (sexual maturity) was examined following Leirs et al. (1993), with males recorded as immature when their testes were abdominal and not externally visible, and as mature when their testes were externally visible (in either abdominal or scrotal position); sexual maturity of females was based on vaginal perforation. A random subset of animals was sacrificed (a total of 283, of which 247 M. natalensis), their body length measured, and kidney, spleen, heart and liver samples were taken after dissection and immediately frozen in liquid nitrogen, as well as preserved in RNAlater (kidney and spleen only) and ethanol (liver only). M. natalensis eyes were preserved in 10% formalin. Dissected lenses were cleaned, dried at 80 °C for >36 h and weighed; their weight serving as a relative estimate of the age of the animals (Leirs et al., 1990). The remaining animals (a total of 87) were released after weighing, reproductive status examination, blood collection and clipping of two toes (preserved in ethanol) under anesthesia. All animal work was approved by the University of Antwerp Ethical Committee for Animal Experimentation (2011–52), and followed regulations of the Research Policy of Sokone University of Agriculture as stipulated in the “Code of Conduct for Research Ethics” (Revised version of 2012).

Genetic characterization of rodent species

For those animals morphologically identified as M. natalensis in the field, DNA was extracted from liver or toe samples with DNeasy DNA extraction kit (Qiagen), according to manufacturer’s instructions. Species identification was then confirmed by sequencing a portion of the cytochrome b gene, after a PCR using primers L14723 and H15915 (Lecompte et al., 2002) in a 0.02 μM concentration, among 1 × Buffer (GoTaq® Flexi Buffer, Promega), 0.1 μM MgCl2, 0.02 μM dNTP (Fermentas) and 1.35 units GoTaq® DNA Polymerase (Promega). Sequencing was done using the L14723 primers at the Genetic Sequencing Facility of the Vlaams Instituut voor Biotechnologie (Belgium). Cytochrome b sequences were subsequently entered in the BLAST search engine (http://blast.ncbi.nlm.nih.gov/) and compared with known sequences. The sequences for which at least 700 base pairs could be reliably called were deposited in GenBank (Accession numbers KP140966–KP141215).

Assay for anti-arenavirus antibody

All 370 dried blood spots (DBS, pre-punched spaces on the filter paper that each absorb approximately 10 μL of whole blood) were screened for anti-arenavirus IgG antibodies using an indirect immunofluorescence assay (IFA) as described in (Gunther et al., 2009). Each DBS was eluted in 300 μL PBS overnight, of which 10 μL was pipetted on slides covered with Vero cells infected with Morogoro virus. All IgG antibodies against known arenaviruses from the Lassa virus complex cross-react (Wulff et al., 1977; Gunther et al., 2009; Gonzalez et al., 1983). On each slide, PBS was used as negative control and a DBS sample known to be anti-MORV antibody positive was used as a positive control. After incubating at 37 °C for 1 h and washing thrice in PBS, FITC-conjugated rabbit anti-mouse IgG secondary antibodies were added. After another incubation and washing round, slides were manually verified for fluorescent labeling at 10 × 40 magnification.

Table 2

Prevalence of anti-arenavirus antibodies, of GAIV RNA in blood samples (viremic) and of GAIV RNA in kidney samples, given as percentages. Between brackets: 95% confidence intervals of the binomial probability (using the profile method), calculated in the R binom package. N: sample size per category (note that not all host characteristics could be determined for each of the total of 331 captured M. natalensis).

<table>
<thead>
<tr>
<th>Host characteristic</th>
<th>Category</th>
<th>Antibody prevalence in blood samples (viremia)</th>
<th>N</th>
<th>GAIV RNA prevalence in blood samples</th>
<th>N</th>
<th>GAIV RNA prevalence in kidney samples</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female</td>
<td>9.4 (5.6–14.4)</td>
<td>170</td>
<td>7.6 (4.3–12.3)</td>
<td>170</td>
<td>10.8 (6.2–16.9)</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>8.8 (5.1–13.9)</td>
<td>158</td>
<td>6.3 (3.2–10.8)</td>
<td>158</td>
<td>8.6 (4.4–14.6)</td>
<td>116</td>
</tr>
<tr>
<td>Reproductive maturity</td>
<td>Immature</td>
<td>4.7 (2.4–8.1)</td>
<td>211</td>
<td>7.6 (4.5–11.6)</td>
<td>211</td>
<td>10.8 (6.5–16.5)</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>17.1 (11–24.6)</td>
<td>117</td>
<td>6.0 (2.6–11.2)</td>
<td>117</td>
<td>8.2 (3.8–14.7)</td>
<td>98</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>0–15</td>
<td>6.9 (1.2–19.8)</td>
<td>29</td>
<td>10.3 (2.7–24.7)</td>
<td>29</td>
<td>27.8 (11.0–50.5)</td>
<td>18</td>
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<tr>
<td></td>
<td>15–30</td>
<td>4.7 (2.3–8.3)</td>
<td>191</td>
<td>6.3 (3.4–10.3)</td>
<td>191</td>
<td>8.5 (4.7–13.9)</td>
<td>141</td>
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<tr>
<td></td>
<td>30–45</td>
<td>10.8 (5.4–18.7)</td>
<td>83</td>
<td>9.6 (4.5–17.2)</td>
<td>83</td>
<td>9.0 (3.7–17.2)</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>45–60</td>
<td>33.3 (15.9–54.6)</td>
<td>21</td>
<td>0 (0–14.4)</td>
<td>21</td>
<td>5.6 (0.6–23.3)</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>60–80</td>
<td>75 (27.7–98)</td>
<td>4</td>
<td>0 (0–51)</td>
<td>4</td>
<td>0 (0–71)</td>
<td>2</td>
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<tr>
<td>Eye lens weight (mg)</td>
<td>0–5</td>
<td>12.5 (1.2–44.5)</td>
<td>8</td>
<td>12.5 (1.2–44.5)</td>
<td>8</td>
<td>37.5 (11–71)</td>
<td>8</td>
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<tr>
<td></td>
<td>5–7.5</td>
<td>6.7 (2.4–13.8)</td>
<td>75</td>
<td>8.0 (3.3–15.6)</td>
<td>75</td>
<td>10.5 (7.5–18.9)</td>
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<tr>
<td></td>
<td>7.5–10</td>
<td>7.5 (3.5–13.6)</td>
<td>106</td>
<td>13.2 (7.7–20.5)</td>
<td>106</td>
<td>10.8 (5.8–17.8)</td>
<td>102</td>
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<tr>
<td></td>
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<td>18.2 (8.8–31.2)</td>
<td>44</td>
<td>4.5 (0.8–13.4)</td>
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<td>12.5–18</td>
<td>75 (27.7–98)</td>
<td>4</td>
<td>0 (0–51)</td>
<td>4</td>
<td>0 (0–51)</td>
<td>4</td>
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</table>
Arenavirus RNA screening

All 370 DBS were pooled by two before RNA extraction, using the QiAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and following the protocol described in (Goüy de Bellocq et al., 2010). RNA was also extracted from kidney samples of all 247 M. natalensis individuals for which these were available, also pooled by two, using the Nucleospin Total RNA Isolation Kit (Macherey Nagel) according to manufacturer’s instructions. Subsequently RT-PCRs were performed on both the blood and kidney extracts using the SuperScript One-Step RT-PCR System and universal African arenavirus primers targeting a 340-nt fragment of the L protein gene (Vieth et al., 2007). Positive pools were resolved by performing RNA extraction on separate kidney samples of the individuals represented in the positive pools, or, when no kidney samples were available, on the separate DBS samples. On positive resolved samples, an additional one-step RT-PCR was performed targeting a 558-nt fragment of the nucleoprotein (NP) gene (primers OWS2805-fwd, OWS2810-fwd, OWS3400-rev and OWS3400A-rev Ehichioya et al., 2011). All amplicons were Sanger-sequenced at the Genetic Service Facility of the Vlaams Instituut voor Biotechnologie (Antwerp, Belgium), and correspondingly material for next generation sequencing, Vero E6 cells in 10% FBS containing penicillin and streptomycin were used. All 370 DBS were pooled by two before RNA extraction, using the RNeasy Mini columns. Eluted RNA was also extracted using the QIAamp Viral RNA Mini Kit. Viral RNA concentration was monitored by quantitative RT-PCR using the QuantiTect kit. Vero E6 cells in 10% FBS containing penicillin and streptomycin were used. RT-PCRs were performed on the second passage of Gairo virus TZ-27421 were each mixed with 560 μL of water per column. Eluted RNA was combined into 3 extraction was performed according to manufacturer’s instructions. Subsequently RT-PCRs were performed on both the blood and kidney samples using the SuperScript One-Step RT-PCR System and universal African arenavirus primers targeting a 340-nt fragment of the L protein gene (Vieth et al., 2007). Positive pools were resolved by performing RNA extraction on separate kidney samples of the individuals represented in the positive pools, or, when no kidney samples were available, on the separate DBS samples. On positive resolved samples, an additional one-step RT-PCR was performed targeting a 558-nt fragment of the nucleoprotein (NP) gene (primers OWS2805-fwd, OWS2810-fwd, OWS3400-rev and OWS3400A-rev Ehichioya et al., 2011). All amplicons were Sanger-sequenced at the Genetic Service Facility of the Vlaams Instituut voor Biotechnologie (Antwerp, Belgium), and correspondingly sequencings deposited in GenBank (accession numbers KJ856580–KJ856623 and KP141216–KP141229).

Virus isolation

Virus isolation was attempted for 15 RT-PCR positive samples, under BSL-3 conditions at the Bernard-Nocht Institute for Tropical Medicine. An approximately 0.5 cm³ mixture of organ samples were lysed in 1 ml Dulbecco’s Modified Eagle’s medium (DMEM) containing 2% fetal bovine serum (FBS) using a bead mill. The lysed tissue was centrifuged for 2 min at > 10,000 × g, and 250 μL of the supernatant was used to inoculate a monolayer of 1 × 10⁵ Vero E6 cells in DMEM–2% FBS. After 1 h incubation at 37 °C, the medium was replaced with DMEM–10% FBS containing penicillin–streptomycin. Aliquots of supernatant were taken every 1 to 3 days, and RNA was extracted using the QiAamp Viral RNA Mini Kit. Viral RNA concentration was monitored by quantitative RT-PCR using the QuantiTect SyBR Green RT-PCR kit (Qiagen, Hilden, Germany) and primers GAIV_S287_F (TGCAACATGCCYCTCTCCTG) and GAIV_S5456_R (ATYGCCACATGGAARTGCT). Titers of infectious arenavirus particles was determined by immunofocus assay using Old World arenavirus N protein specific monoclonal antibodies for detection of infected cells, as previously described (Rieger et al., 2013). In order to produce sufficient material for next generation sequencing, Vero E6 cells in 75 cm²-flask were infected with supernatant of the first passage of strain TZ-27421, at an MOI of 0.01.

Whole genome sequencing and assembly of Gairo virus isolate TZ-27421

Twenty-eight 140-μL supernatant samples of the second passage of Gairo virus TZ-27421 were each mixed with 560 μL AVL buffer (without carrier RNA) and in combination passed through a total of 12 QiAamp Viral RNA Mini Kit columns. RNA extraction was performed according to manufacturer’s instructions, and RNA was eluted in 27 μL of water per column. Eluted RNA was combined into 3 × 100 μL that were each cleaned up and concentrated to a volume of 14 μL using the RNeasy Mini Elute Clean up kit (Qiagen, Hilden, Germany). The cDNA library construction and 454 sequencing performed at Macrogen (Seoul, Korea). The cDNA library was constructed using cDNA Rapid Library Preparation Kit (GS FLX Titanium Series, Roche) according to manufacturer’s instructions. Preparation of template beads for 454 sequencing followed the emPCR method for rapid libraries (Lib-L) and for large volume emulsions (LV) (Roche Applied Science). Enriched DNA capture beads were then multiplexed with another cDNA library and sequenced on 1/8 region of a 454 GS FLX instrument (Roche Applied Science) according to manufacturer’s instructions.

Using PRINSEQ webserver (Schneider and Edwards, 2011), reads with a PHRED quality score < 20 were removed. First, reads were de novo assembled in Geneious 6.1.7. (Biomatters). The preliminary consensus sequences of the S and L segments were then subsequently used as a reference in bowtie2 (Langmead and Salberg, 2012) to map all reads of the sample to. The resulting assembly was then processed in Geneious 6.1.7. The final consensus nucleotide at each position of the sequence was called as the nucleotide represented by at least 75% of the reads (otherwise it was called as an ambiguity) and when coverage was at least 4 times. To obtain the sequence of the 3’ end terminal part of L segment, we performed a SuperScript One-Step RT-PCR with primers L3end_F (5’-GTCAGGGA-CAACCAAGGTGA-3’) and L3end_R (5’-GCTAGGATCCCCGTTGCG-3’) of which the amplicon was Sanger sequenced as indicated above. Genome segment sequences were deposited in GenBank under accession numbers KJ855308 (S) and KJ855307 (L). Potential N-glycosylation sites were searched for with NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Secondary structure of the intergenic regions was predicted on the RNAfold webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Phylogenetic analysis

Phylogenetic trees for the partial L (340 nt) and NP (518 nt coding region trimmed from 558 nt fragment) gene alignments were inferred in MrBayes (Huelsenbeck, 2001) with the parameters: general time reversible (GTR) model of nucleotide substitution and 6 categories of gamma distributed rate variation among sites (partitioned into the 3 codon positions); 2 Markov chain Monte-Marlo (MCMC) chains with 2000,000 generations. The nucleotide sequences of the complete genes (GPC, NP, Z and L) of GAIV were aligned with available entire gene sequences of other Old World arenaviruses (OWA) using the TranslatorX server (Abascal et al., 2010). Phylogenetic trees for each gene besides Z, as well as the parts of GPC gene encoding for G1 and G2, were inferred by MrBayes (Huelsenbeck, 2001). For all genes, we used a GTR substitution model with 6 categories of gamma distributed rate variation among sites and a birth-death model as prior on relaxed clock. Trees were rooted using the GPC gene of the Old World arenavirus Lassaravirus (Lassa virus (LASA, GenBank accession number DQ447803), and the GPC gene of the New World arenavirus Lassa virus (LASA, GenBank accession number DQ447803). For all genes, 2,000,000 generations were run on the 3 MCMC chains, after discarding the first 1,000,000 as burn-in. 2 MCMC chains were run between 10,000,000 and 30,000,000 generations and convergence was visualized in Tracer (Rambaut and A., 2007). All sequences used from GenBank are listed in Supplementary Table 1.

Ecological statistics

Presence of antibody and Gairo virus RNA in wild-caught M. natalensis individuals was related to several host characteristics (sex, weight, body mass index — BMI[weight/body-length²], reproductive status and lens weight: an estimate for the age of the animals Leirs et al., 1990) using separate generalized linear mixed models with logit link function in the lme4 package in R (R Core Team, 2014), always including trapping locality as a random effect. Likelihood ratio testing was used to assess statistical significance of a parameter by comparing full and reduced models. For these analyses, a subset of the dataset (n=230) was used for which the necessary data was available (RT-PCR of kidney samples, eye lens weight, body length).
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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.12.011.

References


Diagnoses associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.12.011.