



## Detection and Genetic Characterization of an Avipox Virus Isolate from Domestic Pigeon (*Columba livia domestica*) in Morogoro Region, Eastern Tanzania

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### Authors' contributions

Author SNM designed the study, did the field work (samples collection), laboratory work (data collection), data editing, analysis of sequence data, literature search and wrote the first draft of the manuscript. Authors AM and EDM were involved in some laboratory work (polymerase chain reaction and agarose gel electrophoresis) and phylogenetic analysis. Author EDM was also involved during sequence assembling. Authors CJK and PNW supervised the entire work and corrected the manuscript. All authors read and approved the final manuscript.

### Article Information

DOI: 10.9734/AIR/2015/13382

#### Editor(s):

(1) Anonymous.

#### Reviewers:

(1) Marcia Helena Braga Catroxo, Electron Microscopy Laboratory, Research and Development Center in Animal Health, Biological Institute, São Paulo, SP, Brazil.

(2) Anonymous, Institute of Experimental Morphology, Bulgaria.

(3) Anonymous, University of Hawaii at Hilo, USA.

(4) M. A. Ramakrishnan, Division of Virology Indian Veterinary Research Institute, Mukteswar, Uttarakhand – 263 138, India.  
Complete Peer review History: <http://www.sciencedomain.org/review-history.php?iid=757&id=31&aid=7060>

Original Research Article

Received 14<sup>th</sup> August 2014  
Accepted 1<sup>st</sup> October 2014  
Published 22<sup>nd</sup> November 2014

### ABSTRACT

**Aim:** To determine molecular and evolutionary characteristics of a newly isolated Tanzanian isolate of pigeonpox virus (PGPV).

**Study Design:** Experimental.

**Place and Duration of Study:** Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania; between November 2011 and May 2014.

**Methodology:** Samples of cutaneous nodular lesions were collected from 17 pigeons suspected to have pigeonpox in Morogoro (n = 11), Pwani (n = 2) and Dar es Salaam (n = 4) regions; followed

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by virus isolation, and confirmation by amplification and sequencing of P4b gene. Further the sequence was phylogenetically analyzed for its evolutionary relationship with other related viruses. **Results:** PGPV was detected in two samples, both from Morogoro region. Sequence analysis revealed that the Tanzanian PGPV isolate derived in this study was 90 – 99% identical to several avipoxvirus isolates from birds belonging to different species from several countries; for instance the Tanzanian PGPV isolate was 91% identical to each of the Tanzanian fowlpox virus isolates derived in the previous study, and 99% identical to all three PGPV isolates whose sequences were obtained in the GenBank i.e PGPV isolates from India (accession number DQ873811), Egypt (accession number JQ665840) and a PGPV (accession number AY530303) whose country of origin is unknown. Phylogenetic analysis revealed that the Tanzanian PGPV isolate belongs to clade A in subclade A2, sharing a recent common ancestor with members of subclade A3. **Conclusion:** Currently, pigeonpox virus is circulating in Morogoro region. The present study warrants the further surveillance/ molecular epidemiology of PGPV in Tanzania in a large-scale.

**Keywords:** Pigeonpox virus; cutaneous nodular lesions; virus isolation; amplification; sequencing; Tanzania.

## 1. INTRODUCTION

Avipoxviruses (APVs) are classified under the family *Poxviridae*, subfamily *Chordopoxvirinae* and genus *Avipoxvirus* [1-3]. APVs are usually named according to the species of the birds from which they were originally isolated [4]. To date the International Committee on Taxonomy of Viruses (ICTV) considers the genus *Avipoxvirus* to be comprised of only ten species namely canarypox virus (CNPV), fowlpox virus (FWPV), juncopox virus (JNPV), mynahpox virus (MNPV), pigeonpox virus (PGPV), psittacinopox virus (PSPV), quailpox virus (QUPV), sparrowpox virus (SRPV), starlingpox virus (SLPV) and turkeypox virus (TKPV) ([www.ictvonline.org/virusTaxonomy.asp](http://www.ictvonline.org/virusTaxonomy.asp)). The other three species namely peacockpox virus, penguinpox virus and crowpox virus are considered as tentative members of the genus *Avipoxvirus* [4].

APVs have a double-stranded DNA genome, ranging from 260 to 365 kb [4]. The avipoxvirus (APV) genomes which have been completely sequenced, showing considerable divergence between them are genomes of FWPV [5] and CNPV [6]. APVs are worldwide distributed and cause pox in domestic, wild, and pet birds of several species [7]. They cause three forms of pox in birds; the cutaneous, diphtheritic, and systemic form [8,9], characterized by formation of proliferative lesions ranging from papules to nodules in the unfeathered parts of the body, which eventually hardens to form scabs; formation of fibrous necrotic proliferative lesions in the mucous membrane of the digestive and upper respiratory tracts [4,10]; and involvement of various body systems and tissues of an

infected bird [9], respectively. Pigeonpox is a slowly developing disease affecting pigeons of all age groups and both sexes. The disease may be complicated with parasitism or poor body condition of birds leading to high mortality rates [11].

APVs from different species of birds have been characterized in many countries based on their genetic, antigenic, biological or evolutionary properties [1,3,8,10,12-31]. Avianpox is mainly controlled by vaccination of susceptible birds using appropriate vaccines. Each one of the currently available vaccines against fowlpox, canarypox, pigeonpox and quailpox is developed using virus strains isolated from the respective avian group [3]. The understanding of genetic, antigenic, biological and evolutionary characteristics of field strains of APVs prevalent in a particular region is of great value when planning for development of appropriate autogenous vaccines for control of avian pox. However, no information is available on the genetic, antigenic, biological and evolutionary characteristics of field strain(s) of PGPV isolates currently circulating in Tanzania. The objective of this study was to determine molecular and evolutionary characteristics of a newly isolated PGPV in the Eastern Tanzania.

## 2. MATERIALS AND METHODS

### 2.1 Study Location

Field work was conducted in Morogoro, Pwani and Dar es Salaam regions; Eastern Tanzania (Fig. 1). It involved collection of samples of cutaneous nodular lesions from live pigeons suspected to have pox, or pigeon cadavers

suspected to have died of pox. Laboratory work was conducted at the Faculty of Veterinary Medicine (FVM), Sokoine University of Agriculture (SUA), Morogoro, Tanzania. Eastern Tanzania is located between latitudes 4° - 8° South, and longitudes 37° - 40° East [32].

## 2.2 Samples Collection and Storage

Between October 2012 and June 2013 samples of proliferative cutaneous nodular lesions (n= 17)

were collected from featherless or poorly feathered parts of pigeon cadavers suspected to have died of pox (Fig. 2), or live pigeons suspected to have pox; in Morogoro (n = 11), Pwani (n = 2) and Dar es Salaam (n = 4) regions of Eastern Tanzania. Pieces of cutaneous nodular lesions collected from the same bird were pooled as a single sample. Each sample was labeled and stored in a deep freezer at - 20°C.



Fig. 1. Map of Eastern Tanzania showing study regions and sampling sites

### 2.3 Virus Isolation

An initial polymerase chain reaction (PCR) assay on DNA isolated directly from the lesions produced negative results, so avipox virus DNA was isolated after virus growth on chorioallantoic membranes (CAMs).

#### Inoculation for CAMs

Inoculums for CAMs were prepared from 17 samples, followed by inoculation in 10 days-old embryonated chicken eggs (ECEs) through CAMs using procedures described by Wambura and Godfrey [33], except that in the current study the eggs were incubated at room temperature (25 – 28°C) instead of 37°C for 5 – 7 days; thereafter they were examined for presence of pock lesions on the CAMs, or generalized thickening and haemorrhages of the CAMs. The inoculums were blind passaged four times.

### 2.4 DNA Extraction

DNA samples were extracted from samples of CAMs containing virus cultures after the 4<sup>th</sup> passage by using ZR Genomic DNA<sup>TM</sup>-Tissue MiniPrep Kit (Zymo Research Corp., USA) according to the manufacturer's instructions.

### 2.5 PCR and Agarose Gel Electrophoresis

Conventional PCR was conducted in Takara PCR Thermal Cycler (Takara Bio Inc., Japan) using a set of primers designed to amplify the P4b gene (virus core protein) (Table 1). The components of each amplification reaction and the cycling parameters were as described by Masola et al. [30]. PCR products were run in 1.5% agarose gel and visualized under UV light using UVI tec transilluminator and photographed using a digital camera.



**Fig. 2. A pigeon cadaver found in one of the pigeon flocks in Morogoro Municipality, Tanzania, presenting proliferative cutaneous nodular lesions at the base of the beak and featherless parts of the head. Note obstruction of vision caused by a complicated eye lesion with caseous material**

**Table 1. Primers used for PCR and sequencing**

Primer	Sequence (5' → 3')	Expected fragment size	Reference
Forward primer	CAGCAGGTGCTAAACAACAA	578 bp	[34]
Reverse primer	CGGTAGCTTAACGCCGAATA		

## 2.6 Purification of a Selected PCR Product and Sequencing of the Purified PCR Product

The PCR product was purified using ExoSAP-IT (Affymetrix, USA). The sequencing reaction was cleaned using ZR-96 DNA Sequencing Clean-up Kit™ (Zymo Research Corp., USA) and purified sample was injected in the ABI 3500XL with POP7 and a 50 cm array (Applied Biosystems, USA). Thereafter the sample was sequenced using ABI V3.1 Big Dye Sequencing Kit (Applied Biosystems, USA) using primers used for PCR. The sequences (forward and reverse) were assembled using CLC Main Workbench version 6.7.1 to generate a consensus sequence.

## 2.7 Analysis of Sequence Data

Sequence homology between the P4b gene of an APV isolate derived in the present study and the P4b gene of APVs isolated from various bird species in several countries was investigated by blasting the sequence derived in this study in the GenBank using BLASTN [35]. Similarities between the Tanzanian APV isolate derived in the current study with other Tanzanian FWPV isolates [30] were investigated using BLAST two sequences programme which gives alignment of two sequences of interest [25].

In order to establish evolutionary characteristics of the Tanzanian APV isolate, the nucleotide sequence derived in this study was aligned with reference sequences obtained from the GenBank (Table 2) using Clustal W programme [36] incorporated in MEGA 6.06 software [37]. Thereafter possible phylogenetic relationship and grouping of the Tanzanian APV isolate were investigated using MEGA 6.06 software [37], neighbour-joining method was used according to the maximum composite likelihood model. *Molluscum contagiosum virus* (MOCV) was used as an out group for the P4b gene.

## 3. RESULTS AND DISCUSSION

### 3.1 Results

Initial PCR assay on DNA isolated directly from samples of cutaneous nodular lesions indicated that all samples were negative for APV. This could be attributed to low concentration of APV DNA in the lesions. Therefore, inoculums prepared from the lesions were inoculated in the ECEs though CAMs so as to let the APV (if any) multiply and grow on CAMs in order to increase

the concentration of APV DNA to levels which are detectable by PCR.

### 3.1.1 Virus isolation

#### *Inoculation for CAMs*

Grossly, signs of embryopathic effects characterized by focal proliferations started to be visible during the third passage. Two to three pocks about 1 mm in diameter were observed on the CAMs. Marked proliferative pock lesions were observed at the fourth passage and the pocks were increased in number and size ranging from 1 – 2 mm in diameter. Most of them had coalesced to form large mass. The number of samples examined, number of PGPV-positive samples and percentage of PGPV-positive samples for each region were as tabulated in Table 3.

### 3.1.2 PCR

Positive results were indicated by migration of PCR products to approximately 578 bp, an expected fragment size for the P4b gene amplicon for APVs. Out of 17 samples tested 2 (11.76%) were positive.

### 3.1.3 Analysis of sequence data

Sequence analysis revealed that the nucleotide sequence of a Tanzanian APV isolate derived in the present study was 90 – 99% identical to sequences of the P4b gene of several APV isolates from birds belonging to different avian species from several countries. For instance, the Tanzanian APV isolate derived in this study was 91% identical to the sequence of the P4b gene of each of the Tanzanian FWPV isolates (GenBank accession numbers KF032407, KF722858, KF722859, KF722860, KF722861, KF722862, KF722863, KF722864, KF722865 and KF722866) derived in the previous study [30], and 99% identical to the P4b gene sequences of all three PGPV isolates obtained in the GenBank i.e an Indian PGPV isolate (accession number DQ873811); an Egyptian PGPV isolate, strain ELshargyia\_PGPV (accession number JQ665840); and a PGPV strain TP-2 (accession number AY530303) whose country of origin is not available.

Phylogenetic analysis revealed that the Tanzanian PGPV isolate clustered together with members of clade A in subclade A2, sharing a recent common ancestor with members of

**Table 2. Details of APVs used in this study**

<b>Virus name</b>	<b>Acronym</b>	<b>Host</b>	<b>P4b locus GenBank accession numbers</b>
Pigeonpox virus PGPV-TZ	PGPV-TZ	Pigeon	KJ913659 <sup>a</sup>
Fowlpox virus VR250	FWPVVR250	Chicken	AY453172 <sup>b</sup>
Fowlpox virus HP-B	FWPVHPB	Chicken	AY530302 <sup>b</sup>
Turkeypox virus GB 134/01	TKPV13401	Turkey	AY530304 <sup>b</sup>
Pigeonpox virus TP-2	PGPVTP2	Pigeon	AY530303 <sup>b</sup>
Pigeonpox virus Peekham	PGPVP	Pigeon	AM050385 <sup>b</sup>
Ostrichpox virus GB 724/01-20	OSPV	Ostrich	AY530305 <sup>b</sup>
Falconpox virus 1381/96	FLPV1381	Falcon	AM050376 <sup>b</sup>
Albatrosspox virus 353/87	ABPV	Black-browed albatross	AM050392 <sup>b</sup>
Falconpox virus GB362-02	FLPV36202	Falcon	AY530306 <sup>b</sup>
Sparrowpox virus 9037 31/5/66/23	SRPV23	Sparrow	AM050390 <sup>b</sup>
Great titpox virus GTPV-256	GTPV256	Great tit	AY453175 <sup>b</sup>
Canarypox virus GB 724/01-22	CNPV72401	Canary	AY530309 <sup>b</sup>
Pigeonpox virus 950 24/3/77	PGPV950	Pigeon	AM050386 <sup>b</sup>
Pigeonpox virus B7	PGPVB7	Pigeon	AY453177 <sup>b</sup>
Starlingpox virus /27	SLPV	Starling	AM050391 <sup>b</sup>
Macawpox virus 1305/86	MCPV	Macaw	AM050382 <sup>b</sup>
Parrotpox virus 364/89	PRPV	Parrot	AM050383 <sup>b</sup>
Agapornis virus APIII	AGPV	Agapornis	AY530311 <sup>b</sup>
Avipoxvirus P31		Trumpeter swan	KC017990 <sup>c</sup>
Avipoxvirus P32		Mottled duck	KC017991 <sup>c</sup>
Avipoxvirus P34		Redhead duck	KC017993 <sup>c</sup>
Avipoxvirus P38		Mourning dove	KC017997 <sup>c</sup>
Avipoxvirus P42		Rock dove	KC018001 <sup>c</sup>
Avipoxvirus P43		Canada goose	KC018002 <sup>c</sup>
Avipoxvirus P44		Bald eagle	KC018003 <sup>c</sup>
Avipoxvirus P51		Red kite	KC018010 <sup>c</sup>
Avipoxvirus P54		Mallard duck	KC018013 <sup>c</sup>
Avipoxvirus P109		American robin	KC018068 <sup>c</sup>
Avipoxvirus P29		Peregrine falcon	KC017988 <sup>c</sup>
Avipoxvirus P30		Red-footed falcon	KC017989 <sup>c</sup>
Avipoxvirus WCV34-03		American crow	DQ131893 <sup>c</sup>
Avipoxvirus WCV52-04		Great blue heron	DQ131898 <sup>c</sup>

<sup>a</sup>Sequence of a Tanzanian PGPV isolate derived in this study, <sup>b</sup>Reference sequences - Source: Manarolla et al. [1], <sup>c</sup>Reference sequences - Source: Gyuranecz et al. [3]

**Table 3. Detection of PGPV in cutaneous nodular lesions from pigeons by virus isolation**

<b>Region</b>	<b>Number of samples examined for PGPV</b>	<b>Number of PGPV-positive samples</b>	<b>Percentage of PGPV-positive samples</b>
Morogoro	11	2	18.18
Pwani	2	0	0.00
Dar es Salaam	4	0	0.00
<b>Total</b>	<b>17</b>	<b>2</b>	<b>11.76</b>

subclade A3 (Fig. 3). After analysis the sequence of the Tanzanian isolate was deposited in the GenBank under accession number KJ913659.

### 3.2 Discussion

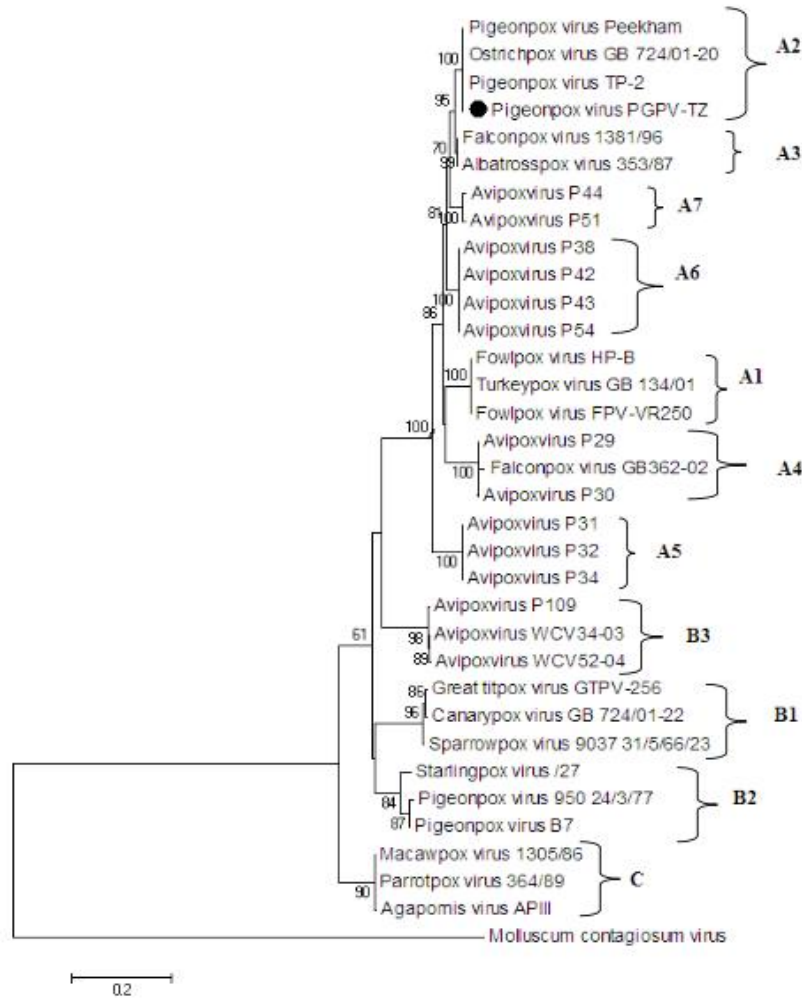
In the current study, PGPV was detected in samples of cutaneous nodular lesions from two

domestic pigeons in Morogoro region using virus isolation and PCR assay. To the best of our knowledge this is the first report confirming pigeonpox in Tanzania. However, the virus could not be isolated from other 15 samples. The possible reason for the failure in isolation could be due to incubating the embryonated chicken



eggs under room temperature instead of 37 °C. Alternatively, the lesions could be attributed to other diseases such as papillomatosis [38] and/or mange [39,40] which present clinical manifestations similar to those of the cutaneous form of pigeonpox. Although all samples from Pwani and Dar es Salaam regions were found to be PGPV-negative, this does not rule out pigeonpox in these regions because only few samples from Pwani (n = 2) and Dar es Salaam (n = 4) regions were examined.

High sequence identity (99%) of the Tanzanian PGPV isolate to sequences of all three PGPV isolates obtained in the GenBank demonstrates that the P4b gene is highly conserved [18,28,41]. A relatively low sequence homology (91%) of the P4b gene of a PGPV isolate derived in the present study to the previously derived Tanzanian FWPV isolates [30] indicates that the APV currently causing pigeonpox in Morogoro region is distinct from APVs currently causing fowlpox in chickens in Morogoro and other regions of Tanzania.



**Fig. 3. Phylogenetic tree of nucleotide sequences of the P4b gene of APVs and molluscum contagiosum virus (MOCV) orthologue sequence, rooted on MOCV, showing phylogenetic relationship of the Tanzanian PGPV isolate [marked with a black dot (●)] to APV isolates of other countries. The tree was obtained by the neighbour-joining method calculated with the maximum composite likelihood model. Bootstrap testing of phylogeny was performed with 1000 replications and values greater than 50 are indicated on the branches (as a percentage). The length of each bar indicates the amount of evolution along the horizontal branches as measured by substitution per site. APV subclades A1 to A7, B1 to B3, and clade C are labeled**

In the present study, the Tanzanian PGPV isolate clustered together with members of clade A in subclade A2 (Fig. 3). This implies that the isolate is phylogenetically closely related to APVs belonging to subclade A2, some of which include APV isolates from birds of the order *Columbiformes* such as rock doves (accession numbers KC017965, KC017966, KC017968, KC017969 and KC017971) from USA and Hungary, oriental turtle doves (accession numbers KC017972 and KC017973) from South Korea [3], a rock pigeon (accession number KC821559), racing pigeons (accession numbers KC821556, KC821552 and KC821557) and a feral pigeon (accession number KC821551) from South Africa, pigeons (accession numbers JX464827, DQ873811 and AM050385) from Egypt, India and UK, and a common wood pigeon (accession number HM481409) from India [28]; birds of the order *Accipitriformes* such as booted eagles (accession numbers KC017976 and KC017979) from Spain, an eastern imperial eagle (accession number KC017967) from Hungary and a red kite (accession number KC017978) from Spain; birds of the order *Gruiformes* such as great bustards (accession numbers KC017970 and KC017974) from Hungary and Spain; and birds of the order *Galliformes* such as an Indian peafowl (accession number KC017975) from Hungary, red-legged partridges (accession numbers KC017977 and KC017980) from Spain [3] and a grey partridge (accession number GQ180204) from Northern Italy [1]. Others include APV isolates from a canary (accession number GQ180208), a gyrfalcon (accession number GQ180210) and a common buzzard (accession number EF016108) from Northern Italy [1].

Although the Tanzanian PGPV isolate (accession number KJ913659) clustered together with APV isolates belonging to subclade A2, some APV isolates from pigeons (accession numbers KC821550, KC821555, KC821557, KC821558 and KC821560) have been reported to cluster together with APV isolates belonging to subclade A3 [28] and others (accession numbers AM050386 and AY453177) have been reported to cluster together with APV isolates belonging to subclade B2 [1,18,21]. Recently, an APV isolate from a rock dove (*Columba livia*) (accession number KC018001) has been reported to cluster in subclade A6 together with APV isolates from mourning doves (*Zenaida macroura*) (accession numbers (KC017997, KC017998, KC017999 and KC018000) belonging to the order *Columbiformes*, and an APV isolate from a

canada goose (*Branta canadensis*) (accession number KC018002) belonging to the order *Anseriformes* [3].

The grouping of the Tanzanian PGPV isolate in subclade A2 together with APV isolates from birds of the same species (*Columba livia*) and other APV isolates from birds not belonging to the species *C. livia*, while some APV isolates from birds belonging to species *C. livia* cluster in other subclades (subclades A3, A6 and B2) supports what was reported by Jarmin et al. [18] that evolutionary taxonomy of the host doesn't appear to have a major role in driving evolution of APVs.

#### 4. CONCLUSION

Our study demonstrated that Tanzanian PGPVs are phylogenetically closely related to APVs belonging to subclade A2, sharing a recent common ancestor with APVs belonging to subclade A3. Although none of the samples from Pwani and Dar es Salaam regions was found to contain PGPV, these findings do not rule out pigeonpox in these regions because only few samples from these regions were examined. More studies aimed at detection and characterization of PGPV in various regions and geographical locations of Tanzania are highly recommended so as to establish the genetic diversity (if any) of PGPV currently occurring in the country.

#### ACKNOWLEDGEMENTS

We thank Mr. Juma S. Marisa of Tanzania Veterinary Laboratory Agency (TVLA), Dar es Salaam; and Mr. Ernest C. Dimoso of Morogoro for their assistance during field work. Dr. Zacharia E. Makondo of TVLA, Dar es Salaam; and Mr. Jonas Fitwangile of FVM, SUA, Morogoro, Tanzania are thanked for their assistance during spatial Geographical Information System (GIS) analysis and virus isolation, respectively. This study was sponsored by the Tanzania Commission for Science and Technology (COSTECH).

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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