Detection and genetic characterization of an avipox virus isolate from domestic pigeon (*Columba livia domestica*) in Morogoro region, Eastern Tanzania

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ABSTRACT

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

**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 by virus isolation, and confirmation by DNA extraction, polymerase chain reaction (PCR) amplification and sequencing of the P4b gene, gel electrophoresis of PCR products, purification of a selected PCR product, sequencing of a purified PCR product. Further the sequence was phylogenetically analyzed for its evolutionary relationship with other related viruses, and analysis of the sequence data using standard procedures.

**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 occurs in Morogoro region. However, these findings don’t rule out the disease in Pwani and Dar es Salaam regions because few samples were examined from these regions. The present study warrants the further surveillance/ molecular epidemiology of Detection and characterization of PGPV isolates in other regions is highly recommended in order to establish the genetic diversity (if any) of PGPV occurring in Tanzania in a large-scale.

**Keywords:** Pigeonpox virus, pigeon pox, cutaneous nodular lesions, virus isolation, PCR, 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), psittacinepox virus (PSPV), quailpox virus (QUPV), sparrowpox virus (SRPV), starlingpox virus (SLPV) and turkeypox virus (TKPV) (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].

Reports indicate that isolates of 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]. Avian pox 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 country region is of great value when planning for development of appropriate autogenous vaccines for control of avian pox. However, prior to this study no studies have been conducted on the Tanzanian isolates of PGPV, and as a result no data on genetic, antigenic, biological and evolutionary characteristics of field strain(s) of PGPV isolates currently circulating in the country Tanzania were available. The objective of this study was to determine molecular and evolutionary characteristics of the newly isolated Tanzanian isolates of PGPV currently occurring in the country in the Eastern Tanzania.

2. MATERIAL 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].
Fig. 1. Map of Eastern Tanzania showing study regions and sampling sites.
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 put in one plastic vial and were considered as one pooled as a single sample. Each sample was labeled and stored in a deep freezer at -20°C until required for investigation.

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.

2.3 Virus isolation

Inoculation for chorioallantoic membrane (CAM)

Inoculums for CAM were prepared from 17 samples of cutaneous nodular lesions, followed by inoculation of each inoculum in a 10 days-old embryonated chicken egg (ECE) through CAM 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. Nothing was inoculated in the negative control embryonated chicken eggs (ECEs). The eggs were incubated at room temperature for 5-7 days, thereafter they were examined for presence of nodular pock lesions on the chorioallantoic membranes (CAMs), or generalized thickening.
and haemorrhages of the CAMs. The inoculums were blind passaged in the CAMs four times.

2.4 DNA extraction

DNA samples were extracted from samples of CAMs containing virus cultures after the 4th passage by using ZR Genomic DNA™-Tissue MiniPrep Kit Catalog Numbers D3050 and D3051 (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 agarose gel as described by Masola et al. [30] with some modifications; in the present study 1% instead of 1.5% agarose gel was used. The amplicons were run in the gel for 40 instead of 45 minutes. Thereafter, gels were visualized under UV light using UVitec transilluminator and photographed using a digital camera.

Table 1. Primers used for PCR and sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Expected fragment size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>CAGCAGGTGCTAAACAACAA</td>
<td>578 bp</td>
<td>[1, 14, 18–22, 24, 25]</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>CGGTAGCTTAACGCCGAATA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6 Purification of a selected PCR product and sequencing of the purified PCR product

The PCR product was purified using ExoSAP-IT EXOSAP-amplicon Purification Kit (Affymetrix, USA) according to the manufacturer's instructions. The sequencing reaction was cleaned using ZR-96 DNA Sequencing Clean-up Kit™ Catalog Numbers D4052 and D4053 (Zymo Research Corp., USA) according to the manufacturer's instructions. A and purified sample was injected in the ABI 3500XL with P0P7 and a 50 cm array (Applied Biosystems, USA). Thereafter, the sample was sequenced using ABI V3.1 Big Dye Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions, using primers used for PCR. The obtained sequences (forward and reverse) were assembled using CLC Main Workbench version 6.7.1 software to generate 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 (excluding Tanzania) was investigated by blasting the sequence derived in this study in the GenBank using BLASTN [34]. Similarities between the Tanzanian APV isolate derived in the current study with each one of the other Tanzanian FWPV isolates derived in the previous study [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 [35] incorporated in MEGA 6.06 software [36]. Thereafter possible phylogenetic relationship and grouping of the Tanzanian APV isolate were investigated using MEGA 6.06 software [36], neighbour-joining method was used according to the maximum composite likelihood model. Molluscum contagiosum virus (MOCV) was used as an outgroup for the P4b gene.

Table 2. Details of APVs used in this study

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Acronym</th>
<th>Host</th>
<th>P4b locus GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigeonpox virus</td>
<td>PGPV-TZ</td>
<td>Pigeon</td>
<td>KJ913659a</td>
</tr>
<tr>
<td>Fowlpox virus</td>
<td>VR250</td>
<td>Chicken</td>
<td>AY453172b</td>
</tr>
<tr>
<td>Fowlpox virus HP-B</td>
<td>FWPVHPB</td>
<td>Chicken</td>
<td>AY530302b</td>
</tr>
<tr>
<td>Turkeypox virus</td>
<td>GB 134/01</td>
<td>Turkey</td>
<td>AY530304b</td>
</tr>
<tr>
<td>Pigeonpox virus TP-2</td>
<td>PGPVTP2</td>
<td>Pigeon</td>
<td>AY530303b</td>
</tr>
<tr>
<td>Pigeonpox virus</td>
<td>Peekham</td>
<td>Pigeon</td>
<td>AM050385b</td>
</tr>
<tr>
<td>Ostrichpox virus</td>
<td>GB 724/01-20</td>
<td>Ostrich</td>
<td>AY530305b</td>
</tr>
<tr>
<td>Falconpox virus</td>
<td>1381/96</td>
<td>Falcon</td>
<td>AM050376b</td>
</tr>
<tr>
<td>Albatrosspox virus</td>
<td>353/87</td>
<td>Black-browned albatross</td>
<td>AM050392b</td>
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<tr>
<td>Falconpox virus</td>
<td>GB362-02</td>
<td>Falcon</td>
<td>AY530306b</td>
</tr>
<tr>
<td>Sparrowpox virus</td>
<td>9037</td>
<td>Sparrow</td>
<td>AM050390b</td>
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<td>Great titpox virus</td>
<td>GTPV-256</td>
<td>Great tit</td>
<td>AY453175b</td>
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<td>Canarypox virus</td>
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<td>AY530309b</td>
</tr>
<tr>
<td>Pigeonpox virus</td>
<td>950 24/3/77</td>
<td>Pigeon</td>
<td>AM050386b</td>
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<tr>
<td>Pigeonpox virus</td>
<td>B7</td>
<td>Pigeon</td>
<td>AY453177b</td>
</tr>
<tr>
<td>Starlingpox virus</td>
<td>/27</td>
<td>Starling</td>
<td>AM050391b</td>
</tr>
<tr>
<td>Macawpox virus</td>
<td>1305/86</td>
<td>Macaw</td>
<td>AM050382b</td>
</tr>
<tr>
<td>Parrotspox virus</td>
<td>364/89</td>
<td>Parrot</td>
<td>AM050383b</td>
</tr>
<tr>
<td>Agapornis virus</td>
<td>APIII</td>
<td>Agapornis</td>
<td>AM050391b</td>
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<tr>
<td>Avipoxvirus P31</td>
<td></td>
<td>Trumpeter swan</td>
<td>KC017990b</td>
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<tr>
<td>Avipoxvirus P32</td>
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<td>Mottled duck</td>
<td>KC017991b</td>
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<td>Avipoxvirus P34</td>
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<td>Redhead duck</td>
<td>KC017993b</td>
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<tr>
<td>Avipoxvirus P38</td>
<td></td>
<td>Mourning dove</td>
<td>KC017997b</td>
</tr>
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<td>Avipoxvirus P42</td>
<td></td>
<td>Rock dove</td>
<td>KC018001b</td>
</tr>
<tr>
<td>Avipoxvirus P43</td>
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<td>Canada goose</td>
<td>KC018002b</td>
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<tr>
<td>Avipoxvirus P44</td>
<td></td>
<td>Bald eagle</td>
<td>KC018003b</td>
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<tr>
<td>Avipoxvirus P51</td>
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<td>Red kite</td>
<td>KC018010b</td>
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<td>Avipoxvirus P54</td>
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<td>Mallard duck</td>
<td>KC018013b</td>
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<td>Avipoxvirus P109</td>
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<td>American robin</td>
<td>KC018068b</td>
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<td>Avipoxvirus P29</td>
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<td>Peregrine falcon</td>
<td>KC017988b</td>
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<tr>
<td>Avipoxvirus P30</td>
<td></td>
<td>Red-footed falcon</td>
<td>KC017989b</td>
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<tr>
<td>Avipoxvirus WCV34-03</td>
<td></td>
<td>American crow</td>
<td>DQ131893b</td>
</tr>
<tr>
<td>Avipoxvirus WCV52-04</td>
<td></td>
<td>Great blue heron</td>
<td>DQ131898b</td>
</tr>
</tbody>
</table>

*a* Sequence of a Tanzanian PGPV isolate derived in this study.

*b* Reference sequences - Source: Manarolla et al. [1].

*c* Reference sequences - Source: Gyuranecz et al. [3].
3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Virus isolation

Inoculation for chorioallantoic membrane (CAM)

Grossly, signs of viral growth embryopathic effects characterized by focal proliferations (nodular lesions) were first observed started to visible during the third passage. Two to three nodules/pocks about 1 mm in diameter were observed on the CAMs. Marked proliferative nodules/pock lesions were observed at the fourth passage and, the nodules/pock lesions 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. No lesions were demonstrated on CAMs of negative control ECEs.

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

<table>
<thead>
<tr>
<th>Region</th>
<th>Number of samples examined for PGPV</th>
<th>Number of PGPV-positive samples (percentage positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morogoro</td>
<td>11</td>
<td>2 (18.18)</td>
</tr>
<tr>
<td>Pwani</td>
<td>2</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Dar es Salaam</td>
<td>4</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>2 (11.76)</td>
</tr>
</tbody>
</table>

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 known 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 subclade A3 (Fig. 3). After analysis the sequence of the Tanzanian isolate was deposited in the GenBank under accession number KJ913659.
Fig. 3. Phylogenetic tree of nucleotide sequences of the P4b gene of APVs and molluscum contagious 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.

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 diagnostic techniques. To the best of our knowledge this is the first report confirming pigeonpox in Tanzania.

However, the virus could not be isolated from was not detected in the 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, this implies that 15 pigeons from which the lesions were collected had no pigeonpox; the lesions could be attributed to other diseases such as papillomatosis [37] and/or mange [38, 39] 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 homology identity (99% identity) 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, 40]. A relatively low sequence homology (91% identity) 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.

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]. This implies that although these isolates (accession numbers KC821550, KC821555, KC821557, KC821558, KC821560, AM050386 and AY453177) were also isolated from pigeons; phylogenetically the Tanzanian PGPV isolate derived in this study is not as closely related to them as it is to APV isolates belonging to subclade A2, regardless of the species of birds from which they were isolated. Recently, an APV isolate from a rock dove (**Columba livia**) (accession number KCO18001) has been reported to cluster in subclade A6 together with APV isolates from mourning doves (**Zenaida macroura**) (accession numbers KCO17997, KCO17998, KCO17999 and KCO18000) belonging to the order **Columbiformes**, and an APV isolate from a canada goose (**Branta canadensis**) (accession number KCO18002) belonging to the order **Anseriformes**, the isolates were isolated from birds suspected to have pox in the USA [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 currently pigeonpox occurs in the Morogoro region, caused by Tanzanian PGPVs which 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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS’ CONTRIBUTIONS

Author SNM designed the study, did the field work (samples collection), laboratory work (data collection), data editing, sequence analysis, phylogenetic analysis, 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.

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