Detection and Genetic Characterization of an Avipox Virus Isolate from Domestic Pigeon (Columbia 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

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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
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 (CPNV), fowlpox virus (FWPV), juncopox virus (JNPV), mynahpox virus (MNPV), pigeonpox virus (PGPV), psittacinepox virus (PSPV), quailpox virus (QUPV), sparowpox 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].

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 4º - 8º South, and longitudes 37º - 40º 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 -20ºC.

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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 4th passage by using ZR Genomic DNA™-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 UVitec transilluminator and photographed using a digital camera.

Table 1. Primers used for PCR and sequencing

| Primer        | Sequence (5’ → 3’)         | Expected fragment size | Reference |
|---------------|----------------------------|------------------------|-----------|
| Forward primer| CAGCAGGTTGCTAAACAACAA     | 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

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

Sequence of a Tanzanian PGPV isolate derived in this study, *Reference sequences - Source: Manarolla et al. [1], †Reference sequences - Source: Gyuranecz et al. [3]*

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

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

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 A 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 Gruiiformes 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 EFO16108) 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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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