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Posted Date: 11 June 2024

doi: 10.20944/preprints202406.0734.v1

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Isolation and Molecular Detection of Pigeon Pox Virus in a Pigeon with Both Cutaneous and Diphtheritic Forms of Pigeon Pox in Ghana

Richard Kwamena Abbiw [1,*], Kingsley Kwabena Amoako [2], Ben Enyetornye [2], Theophilus Odoom [2,3,*], Pius Agyenim Boateng [1], Stacy Magdaline Abbang [1], Collins Moranga [1], Tawiah-Yingar Nana Yaw [3], Patrick Tetteh Ababio [3], Godwin Dogbe [4], Richard Suu-Ire [2], Osbourne Quaye[1]

[1] West African Centre for Cell Biology of Infectious Pathogens, University of Ghana
[2] School of Veterinary Medicine, University of Ghana, Legon
[3] Accra Veterinary Laboratory, Veterinary Service Directorate
[4] University of Development Studies, Ghana
* Correspondence: Abbiw.richardk@gmail.com; theodoom@yahoo.com

Abstract: Unlike fowl pox, pigeon pox virus (PPV) has not been described in Ghana. This is the first report of the disease and virus in Ghana based on gross necropsy, histopathological analysis and molecular techniques. This index case was presented as the occurrence of both cutaneous (dry) and diphtheritic (wet) forms of the pigeon pox disease in a 5-month-old female pigeon. Although the observation of Bollinger bodies during histology is confirmatory of pox viruses, the amplification of the p4b gene using polymerase chain reaction and its visualisation by gel electrophoresis confirmed the presence of an avian pox virus. Sequencing and phylogenetic analysis of the isolates from this case showed 100% relatedness to the reference PPV isolate FeP2. Thus, confirming the presence of PPV. The discovery of this virus in the Ghana raises the need for surveillance of both wild and domestic avian populations in preparation for any future outbreak, protection of the production birds and decision on the need for other pox viruses in the country.

Keywords: Pigeon pox; Pigeonpox virus; Bollinger bodies; Ghana

1. Introduction

Pigeon pox (PPV) is a poxvirus in the family Poxviridae and genus Avipoxvirus [1–4]. The genus is made of three main strains: Fowl pox virus (FPV), Canary pox virus (CPV) and PPV. Pigeon pox virus is a double-stranded enveloped DNA virus causing mild to severe slow-spreading disease in infected pigeons [5–7]. Similar to poxviruses in other species, PPV is generally self-limiting, but can cause more severe disease in young or immunocompromised animals [2].

Transmission of the virus is usually by direct contact through skin abrasions, contaminated feed, water and eggs [8,9]. Mosquitoes, other insects and cannibalism have been implicated in mechanical transmission of the virus [2,8]. Non-specific clinical signs include dullness, depression, dehydration, emaciation and ruffled feathers, and only a few birds developing lesions at a time [10]. PPV leads to the formation of visible wart-like lesions known as pox scabs on mucous membranes and non-feathered skin [10–13], and rarely neurologic signs [9]. The scabs can be the source of aerosol infection in poultry houses, resulting in respiratory tract infection [2]. The lesions may be described as dry/cutaneous or wet/diphtheritic. The cutaneous form is predominant in most outbreaks and is characterized by scabs on non-feathered skin, unthriftiness and reduction in egg production [10–12,14]. The relatively severe diphtheritic form produces firmly attached, caseous pseudomembranous deposits on and in the mucous membranes of the mouth, tongue, entrance to the trachea, eye and/or nasal cavity, thereby interfering with feeding or breathing [15]. Gross internal pox lesions do not usually appear in pigeons even though the virus may produce a systemic reaction

 doi:10.20944/preprints202406.0734.v1

and occasionally results in a viremia [16,17]. Latent infection is possible for years with reactivation usually through non-specific stress factors [4,14].

Although avian pox has been described worldwide with the exception of Antarctica and Arctic regions [18,19], reports of PPV in Africa are rare [1,20–22]. While fowl pox has been documented in Ghana [23] and is relatively common, PPV has not yet been reported [19]. Here we describe for the first-time gross necropsy, histopathology, and molecular diagnostic findings of poxvirus in a pigeon (*Columbia livia*) in Ghana.

2. Materials and Methods

2.1. Case

A 5-month-old female pigeon weighing 0.14 kg kept in quarantine prior to joining 12 other pigeons, developed bumps**,** and was presented for veterinary was presented care. All the birds were fed mixed grains of maize, rice, millet, and soya beans. Physical examination revealed multiple nodular lesions of about 0.3 to 1 cm in diameter. The nodules were unevenly distributed chiefly on non-feathered areas including around the eyes and the beak. The lethargic bird had droopy and ruffled feathers, greenish stained vent, and was emaciated. The left eye was completely sealed by periocular nodules. The patient was euthanized using cervical dislocation and a post-mortem examination was performed after duly informing the client of the risk to the other birds, success of treatment and seeking the consent of the owner.

2.2. Postmortem and Histopathology

Necropsy of the bird was performed as described Butcher and Miles [24] and Dharanesha [15]. Briefly, the bird was first dipped in water containing disinfectant to reduce the chances of transmission of psittacosis [24] and then examined externally. The internal organs were then regionally examined; the cranial, thoracic, and then abdominal regions. Images of gross lesions were captured and recorded. Nodules on the head and in the oral cavity was sectioned for histopathological processing [15]**.**

2.3. Sample Collection and Preparation

Portions of the trachea, feather stalk, oesophagus, lung, cutaneous lesions, and gastrointestinal tract were aseptically sampled into sterile tubes and transported on ice to both the Accra Veterinary Laboratory, Veterinary Service Directorate (VSD). Viral inocula were prepared as described by Sultana et al. [5]. Briefly, a 10 % (w/v) viral suspension was made by adding phosphate buffered saline (PBS) to the ground samples. The viral suspension was then treated with 300µL of gentamicin for an hour and cultured on blood agar for 24 hours. 0.5 ml of the sterile inocula were used for inoculating embryonated eggs for viral extraction while the rest were stored at -20 ℃ for future use.

2.4. Virus Isolation

The virus was propagated by inoculating the prepared inocula into 10 -12 days old embryonated chicken hen through the chorioallantoic membrane (CAM) route as described by Rahman et al. [25]. That is, 0.5 ml of the sterile inocula was inoculated into the centre of the CAM using sterile 1 ml, 1.5 inches needle tuberculin syringe, and the opening of the air sac and shell sealed with melted wax. The inoculated eggs were placed in trays with the CAM upwards and incubated at 37 ℃ for 5 days in an egg incubator. The inoculated eggs were then chilled at 4 – 8 ℃ for 2 hours. Afterwards, the inoculated eggs were coated with iodine tincture, cracked open and the thickened CAM harvested for preparation of inocula as described above. The viral concentration was increased by performing three passages and the CAMs with pock lesions collected and transported to the Virology Laboratory at the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP) for further analysis.

2.5. DNA Extraction, PCR and Gel Electrophoresis

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DNA of the virus was extracted using Quick-gDNA™ Miniprep Kit as per the instruction of the manufacturer. PCR technique targeting pox virus p4b gene with the primer set p2fPF- 5′ CAGCAGGTGCTAAACAAACAAA 3′ and p2fPR- 5′ CGGTAGCTTAACGCCGAATA 3′ with an amplification size of 578 bp [1,18] was employed. Over here, PCR reaction mixture containing 12.5 µL One Taq 2X master mix, 1 µL each of forward and reverse primers, 6.5 µL Nuclease-free water and 4 µL DNA template was prepared. PCR was done in PCRmax alpha cycler with cycling conditions as follows: initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 45 sec, annealing at 48 ℃ for 1.5 min, elongation at 60 ℃ for 2 min, and final extension at 60 ℃ for 10 min. Then, a 1.5% agarose gel was prepared, and electrophoresis of the PCR products was done with a follow-up visualization using Amersham Imager 600.

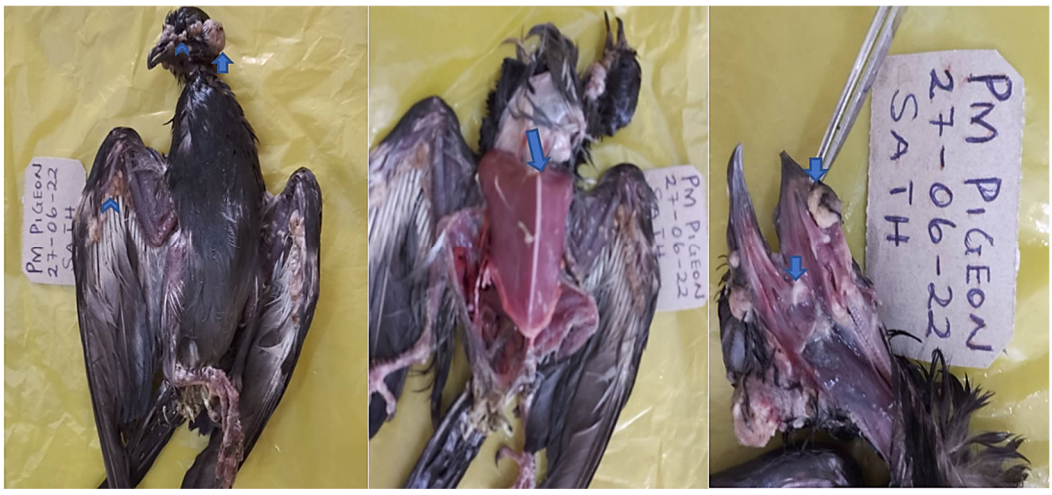
2.6. Sequencing and Sequence Analysis

PCR products were purified with Wizard® SV Gel and PCR Clean-up System. Sequencing was performed using MinION (nanopore) sequencing technology and long reads were obtained. Base calling and demultiplexing was performed using guppy basecaller and barcoder respectively. Quality control was assessed, and samples were trimmed using porechop before being mapped to the PPV isolate (FeP2) reference sequence, downloaded from NCBI (NC_024447.1:193678-195654), representing the region amplified by the PCR primers. Minimap2 was using for mapping and bcftools (mpileup), vcfutils.pl, and seqtk were used to map the fastq files and generate consensus sequences for the virus isolates. Alignment was performed using mafft and phylogenetic analysis using MEGA. A maximum likelihood tree was generated using 100 bootstraps and gamma distributed invariant sites.

3. Results

3.1. Postmortem

Physical examination revealed multiple nodular lesions of about 0.3 to 1 cm in diameter distributed primarily in non-feathered regions of the bird (Figure 1A), prominent keel bone and congested breast muscles (Figure 1B) and diphtheritic nodules in the oral cavity (Figure 1C). Other observed lesions were greenish pasty vent, air sacculitis and petechial haemorrhages on kidneys.



(a) **(b)** **(c)**

Figure 1. gross postmortem lesions on the pigeon. (a) Cutaneous nodular lesions of varying sizes Arrow shows nodules on the eye causing the left eye to be completely shut. Arrowhead shows areas of coalescing of the nodules (b) Congested carcass with quite prominent keel bone. (c) Diphtheritic nodular lesions in the oral cavity.

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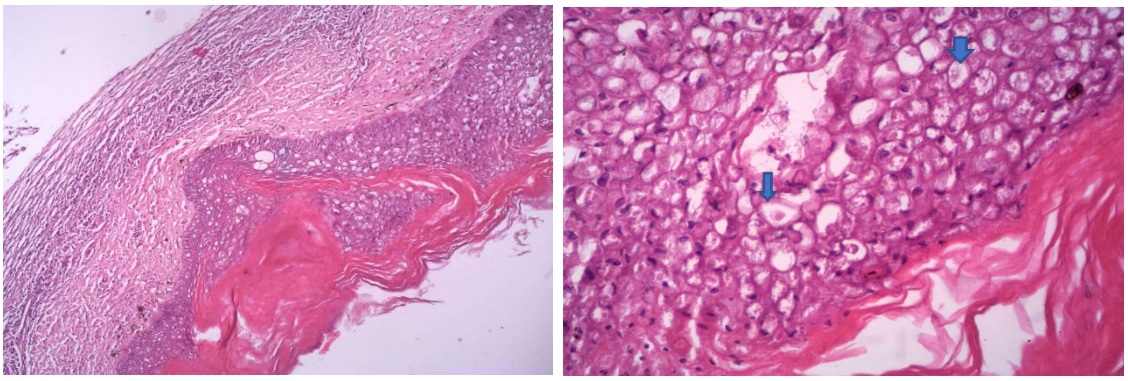
3.2. Histopathology

Infiltrating the epidermis were mixed inflammatory cells (macrophages and heterophils) and areas of necrosis. There is hyperkeratosis with the presence of eosinophilic intracytoplasmic inclusion bodies (Bollinger bodies) in markedly distended and vacuolated keratinocytes with pleomorphic and hyperchromatic nuclei. The presence of Bollinger bodies is an indication that the lesions were caused by a pox virus.



(a) **(b)**

Figure 2. Hyperkeratosis with hyperplasia of the basal epithelium. (a) Epidermis expanded by epithelial cells exhibiting vacuolar/ballooning degeneration. (b) Round eosinophilic intracytoplasmic inclusion bodies (Bollinger bodies).

3.3. PCR and Gel Electrophoresis

PCR products were observed in all samples except gastrointestinal tract indicating the presence or distribution of pox viruses in the trachea, lungs, cutaneous lesion, feathered stalk, and oesophagus. Band sizes of about 600 bp were observed. This was congruent with the band size expected for the p4b gene of pox virus.

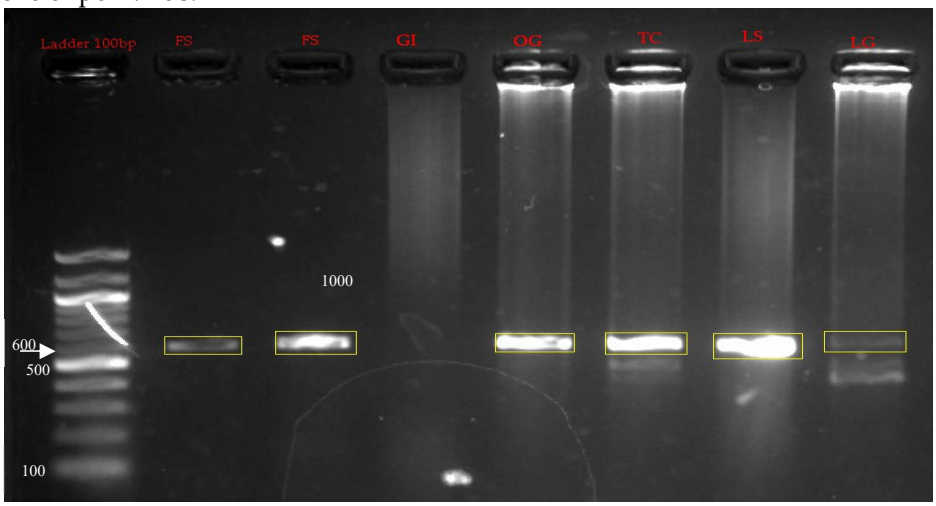


Figure 3. Agarose gel electrophoresis of the PCR products analysis of the P4b gene amplified from the isolated viruses. Lanes FS -feathered stock, GI- Gastrointestinal tract, OG- Oesophagus, TC- Trachea, LS- cutaneous lesions and LG- Lungs.

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3.4. Sequencing Data

Seven samples were sequenced, and they all mapped 100% to the NC_024447.1:193678-195654 Feral Pigeonpox isolate FeP2 sequence (Table 1). The sequences are phylogenetically related to the reference sequence (Figure 5).

Table 1. Mapping statistics.

Sampe ID	Barcode ID	QC Passed Reads	Mapped Reads	% Mapped
LS	NB26	162370	162370	100%
OG	NB27	6369	6369	100%
TC	NB28	54466	54466	100%
FS	NB29	26582	26582	100%
GI	NB30	1653	1653	100%
LG	NB31	97350	97350	100%

LS: pock lesion, OG: oesophagus, TC: trachea, FS: feather stalk, GI: Gastrointestinal tract, LG: lungs.

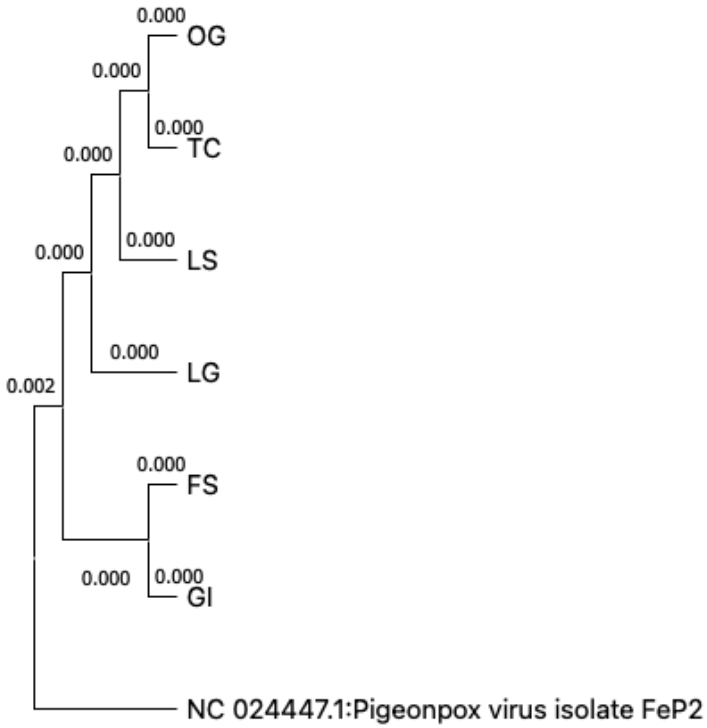

Figure 5. Maximum likelihood tree showing the relationship between the reference sequence and the 7 isolates. The numbers indicate the branch lengths and most of the samples have zero length which indicates that they are highly related to the reference sequence. LS: pock lesion, OG: oesophagus, TC: trachea, FS: feather stalk, GI: Gastrointestinal tract, LG: lungs.

4. Discussion

This is the first confirmation of pigeon pox virus in Ghana. The tentative diagnosis was made using observation of clinical signs coupled with the presence of Bollinger bodies and the hyperkeratosis or hyperplasia of the basal epithelium is diagnostic of avian pox [15,26]. Definitive diagnosis of PPV was by PCR confirmation as recommended [1,7,20,22,27]. Even though no specific lesions, as described by Audarya et al. [12], where observed from the CAM, the gel images confirmed the PCR amplification of the P4b gene. An identical observation was reported by Abd El-Samie et al. [1]. The infected pigeon presented with both the cutaneous and diphtheritic forms. While it relatively

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common for the cutaneous form to occur [2,6,7,11,12,21,25,28,29] and the diphtheritic form is uncommon and usually associated with young animals [27,30,31]. The occurrence of both forms in a bird is a rarity [17,32].

There is no known treatment for pigeon pox disease and attempts at treatment is usually not recommended since it might involve the disruption of the lesions which aids the spread of the virus [2,33]. Generally, attempts at treating birds with diphtheritic forms of pox are not successful. Wet forms of pigeon pox are complicated with inanition due to lesions in the oral cavity and respiratory tract [9]. The accompanying dysphagia associated with formation of the diphtheritic nodules in the oral cavity makes it impractical to give oral medicines. The combination of the digestive and respiratory factors result in higher mortality in pigeons with wet forms [11]. Euthanasia is recommended in severe cases, most instances of wet forms and in high-density colony situations as a means of preventing the spread of the disease [2]. The severity of the case, the possibility of spread to the other birds, and the admittance of the owner not to be able to follow through with the treatment regimen influenced the decision to euthanize the pigeon. Thus, the euthanasia allowed for the disinfection of the premises with the aim of preventing the perpetuation of a rather environmentally stable virus [34].

However, in mild cases administration of supportive and preventive treatment including disinfection and administration of electrolytes, multivitamin and antibiotics have been found to reduce mortality [32]. For instance, the use of acyclovir (80mg/kg QID PO 8-10 days) [10] , azithromycin (20mg/kg BID) [10,11], or enrofloxacin (10 mg/kg IM for 14 days) [29] in conjunction with vitamin supplementation and topical application of turmeric and neem leave paste on the cutaneous lesions or liver tonic [10,11,14,35] proved useful in treating the cutaneous form of pigeon pox disease. To ensure the elimination of the viruses from the environment, 1% KOH, 2% NaOH or 5% phenol have been recommended [9]. This helps reduce the rate of transmission to naïve birds on the farm during an epornitic.

At best, the complete relatedness of all the isolates to the feral pigeon pox virus isolate (FeP2) which has been isolated globally including in South Africa [22,36,37], Egypt [31], India [38] and the general ubiquity of avipoxviruses [18,19,31,39] indicates the possible endemicity of the virus in Ghana. Furthermore, the isolation of other PPV in Nigeria [31,32], the only West African country with data on PPV [31], also gives some credence to the endemic hypothesis if not denote the risk of sharing the virus based on the fluid animal movement and trade between Ghana and Nigeria.

The impact of this discovery on the general avian population in Ghana is uncertain but appears to have a potential to negatively impact the pigeon population in the country. While pigeon farming is in its budding stage, it has been shown to provide a source of income to some individuals in the Northern Regions of Ghana [40] and a pigeon pox epornitic could be devastating to such an industry. Furthermore, most farmers prefer to keep other non-commercial birds including pigeons on their poultry farms [23]. The pigeons on such farms can be sources of other avipoxviruses to the commercial birds [8,41]. The established mantra that PPV is host specific incapable of infecting or causing disease in other avian species [7,31,37,42–44] might require a second look. This is due to the lack of a clear pattern of infection regarding species-specificity among avipoxviruses [38]. The existence of evidence of avipoxviral infection in not classically considered their host [6,18,30,45] hint at this possibility of a cross infections. Increasing evidence for the high relatedness of PPV to some avipoxviruses [31,36,37] and the establishment of a serological, antigenic and pathogenic relationship between PPV, FPV and CPV [46] also raise concern for a potential spill over into other potential avian hosts. Finally, chicken vaccinated with psittacine pox were shown to be susceptible to both fowl and pigeon pox [47] suggesting that under the right conditions, PPV can precipitate a disease in other avian species not considered the classical host of the virus. Hence, while this could be an isolated case, it highlights the need for surveillance in determining the distribution and potential hazard of the PPV viruses to pigeons and other avian species, especially, those of production significance to Ghana.

Author Contributions: Conceptualization, K.K.A, B.E, T.O and RKA; methodology, R.K.A, B.E, and T.O; validation, O.Q; formal analysis, C.M, R.K.A, and B.E; investigation, K.K.A, S.M.A, P.T.A, P.A.B, and T.Y.N.Y; resources, T.O, R.K.A, B.E and O.Q; data curation, K.K.A, B.E, and R.K.A; writing—original draft preparation,

B.E and R.K.A; writing—review and editing, G.D, T.O, and O.Q; visualization, R.K.A, C.M.; supervision, T.O, R.S and O.Q; funding acquisition, T.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research was primarily funded by the Accra Veterinary Laboratory and received additional support from the School of Veterinary Medicine and funds from a World Bank African Centres of Excellence grant (WACCBIP+NCDs: Awandare) and a DELTAS Africa grant (DEL-22-014: Awandare).

Data Availability Statement: Data is contained within the article or supplementary material.

Acknowledgments: the authors are grateful to the owner of the pigeonIn this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The authors declare no conflicts of interest.

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