Characterization of a canarypox virus from an outbreak among canaries (Serinus canaria domesticus) in Lebanon

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### ABSTRACT

A canarypox outbreak resulted in the death of about 50% of canaries (Serinus canaria domesticus) in several breeding farms in Lebanon. Infected birds showed thickened eye lid and skin scab-like lesions at the beak, foot and caudal regions and died 5–6 days post disease symptoms onset. Out of seven sick birds that were autopsied for gross pathology evaluation, one bird demonstrated turbid airsacs with absence of any lesions in the trachea, oesophagus, liver and lung. Histopathological examination showed hyperplasia, heterophils infiltration and Bollinger body formation in the skin of five out of the seven autopsied canaries. Hyperplasia and infiltration of heterophils were also observed in the airsacs of one bird. PCR analysis of specimens taken from the skin and feet, targeting the fpv167 gene of the canarypox virus, was positive for all of the analyzed canaries. PCR analysis also revealed that two birds had concurrent infection with Mycoplasma gallisepticum. Sequencing and alignment of the amplified fpv167 gene of the canarypox showed 100% similarity with the Iranian canary pox isolate IR/H913/14. Smuggling of pet birds through the borders should be strictly controlled and biosecurity measures must be adequately applied to control the circulation of the two identified pathogens.

### Introduction

Canarypox disease is caused by a family of viruses called avipoxvirus. This virus infects many avian species including poultry, turkeys, and game birds such as fiches, canaries, parrots, parakeets etc. Host specificity plays a major role in the level of pathogenicity of avipoxviruses (Kim et al. 2003). In canaries, avipoxviruses cause a serious damage to both breeders and offsprings, resulting in high mortality that can reach 80% of the infected birds (Tripathy and Reed 1997). This virus colonizes mainly the skin, resulting in the thickening of the dermis and formation of scabs in the infected areas (Tripathy and Reed 1997; Williams et al. 2014). In diptheric avipoxviruses, lesions can develop internally, affecting the oral cavity, oesophagus, and other internal organs (Williams et al. 2014).

There is a scarcity of literature about circulation avipoxviruses, and specifically canarypox, in the Middle East and Asia. Few Asian countries reported the circulation of canarypox such as India and Iran. In Lebanon, information pertaining to the avipox outbreaks in avairy is almost absent. Barbour et al. (1997) reported the occurrence of fowl pox in one broiler breeder flock out of 29, and in one commercial layers flock out of 15, during a five years disease screening in poultry, namely from 1992 to 1996. The virus, though, was not isolated and identified.

As of March 2017, canaries suffered from a disease outbreak that caused the mortality of around 50% of breeders and offsprings in a breeder farm in Lebanon. Based on the observation of lesions and scabs on the feet and skin, the presumptive diagnosis was avipoxvirus infection. This study considers the confirmation, identification, and phylogenetic characterization of the causative canarypox virus for future control of this circulating pathogen.

### Materials and methods

#### Case history

As of the month of March 2017, a mortality of 50% of canaries (Serinus canaria domesticus) was reported in nine breeder farms, of 100–180 birds each, from different Lebanese regions, namely Beirut, Mount Lebanon, North and South Lebanon and the Bekaa valley. The outbreak affected young and adult birds that showed anorexia, weakness, thickened eyelid, skin and foot scab like lesions, and eventually died 5–6 days post symptoms onset. In a breeding farm, located in Jounieh, Mount Lebanon, the total number of dead birds was 85 Over 170 and the affected birds were treated with various antifungals (itraconazol), antibiotics (Doxycyclin, tetracyclin, enrofloxacin), anti-protozoans (Roniazidole) and even antivirals (Aciclovir) without significantly reducing the mortality rate. It is worth noting that none of the affected birds was vaccinated previously against avipox virus.

#### Birds’ symptoms and gross pathology

Seven ill birds were brought from Jounieh’s breeding farm to the Animal Pathology and Physiology Lab at the American University of Beirut. The birds exhibited thickened eye lid, skin and foot scab like lesions, and eventually died 5–6 days post symptoms onset. In a breeding farm, located in Jounieh, Mount Lebanon, the total number of dead birds was 85. Over 170 and the affected birds were treated with various antifungals (itraconazol), antibiotics (Doxycyclin, tetracyclin, enrofloxacin), anti-protozoans (Roniazidole) and even antivirals (Aciclovir) without significantly reducing the mortality rate. It is worth noting that none of the affected birds was vaccinated previously against avipox virus.
The birds were between 3 months and 2 years of age. Skin was observed for different symptoms and lesions such as thickened eye lid, scab-like lesions while feet were observed for scab or crust-like lesions. Birds were humanely sacrificed with CO2 and autopsied for the observation of thickened/turbid airsacs, hepatic, tracheal, oesophageal and lung lesions.

**Histopathological observations of microscopic lesions**

Airsacs and skin specimens were fixed in 10% formalin in phosphate-buffered saline (PBS). Tissue sections of 4μm of thickness were fixed on slides and stained with H and E (Cardiff et al. 2014) for microscopic lesions observation namely, airsac thickening/hyperplasia and heterophil infiltration, skin’s dermis thickening/hyperplasia, heterophil infiltration, and Bollinger body formation.

**Fungal and protozoal staining**

Slides with impression smears from the internal side of the eyelids and skin scabs were prepared. Slides were stained with Giemsa (Carter and Cole 2012) and Lactophenol Cotton Blue (Jung et al. 2009) for the identification of protozoans and fungi, respectively.

**Serological analysis**

An amount of 500 μL of blood was collected from the hepatic vein of the seven ill birds. Blood centrifuged for 10 min at 520 xg. Sera were collected, and Hemagglutination Inhibition test was performed against H9N2 avian Influenza (AI) and Newcastle disease (NDV) (AAAP 1997). Slide agglutination test was applied to reveal antibody reaction to *Mycoplasma gallisepticum* (AAAP 1997).

**Table 1. Frequency of birds with specific symptoms or gross pathology.**

| Symptom/Gross pathology                  | Frequency/7 (%) |
|-----------------------------------------|-----------------|
| Thickened eye lid                       | 7 (100)         |
| Scab-like lesion on skin and/or feet    | 7 (100)         |
| Thickened/turbid airsac                 | 1 (14)          |
| Hepatic lesion                          | 0 (0)           |
| Tracheal lesion                         | 0 (0)           |
| Oesophageal lesion                      | 0 (0)           |
| Lung lesion                             | 0 (0)           |

university of Beirut for analysis. The birds were between 3 months and 2 years of age. Skin was observed for different symptoms and lesions such as thickened eye lid, scab-like lesions while feet were observed for scab or crust-like lesions. Birds were humanely sacrificed with CO2 and autopsied for the observation of thickened/turbid airsacs, hepatic, tracheal, oesophageal and lung lesions.

**Figure 1.** Thickened eye lids of affected birds.
PCR analysis for avipoxvirus, chlamydia psittaci, and mycoplasma gallisepticum

Skin, liver, lungs and airsac specimens were homogenized individually, in transport medium (Dufour-Zavala 2008) then the homogenates were subjected to freezing and thawing, three times. The DNA was extracted using the Qiagen DNA minikit (Qiagen GmbH, Hilden, Germany) and subjected to PCR amplification using the RedTaq Ready Mix (Sigma-Aldrich, 3050 Spruce Street, St Louis, MO, USA). The primers were P1 5′-CAGCAGGTGCTAAACAACAA-3′ and P2 5′-CGGTAGCTTAACGCCGAATA-3′ targetting a partial sequence (576 bp) of the gene encoding the P4b core protein (fpv167 locus) of avipoxvirus (Esteves et al. 2017; Lee and Lee 1997). The cycling conditions were as follows: 15 min at 95°C followed by 32 cycles of: 95°C for 1 min, 60°C for 2 min, and 72°C for 3 min. A final extension was performed at 72°C for 10 min.

The primers specific to Chlamydia psittaci were CPF: 5′-GCAAGACACTCTCTCAAAGCC-3′ and CPR: 5′-CCTTCCCACATAGTGCCATC-3′ were identical to bases −139 to −120 of the 5′ non-translated region and complementary to bases 106 to 125 of the C. psittaci GPIC MOMP gene (Circella et al. 2011; Hewinson et al. 1997; Zhang et al. 1989). The cycling conditions were similar to those of avipoxvirus, except for the annealing temperature that was 50°C. Amplicons were visualized on 2% agarose gel in TAE buffer, following electrophoresis at 100 V for 50 min and staining with Ethidium Bromide.

Real-time PCR (q-PCR) was applied to detect Mycoplasma gallisepticum in the eye lid and airsac homogenates, according to Grodio et al. (2008). Briefly, DNA was extracted from tissue homogenates using Qiagen DNA minikit (Qiagen GmbH, Hilden, Germany). DNA was amplified using the iQ Supermix (Bio-Rad Laboratories Inc., 2000 Alfred Nobel Drive, CA, USA) and the following primers: mgc2F 5′-GGTCCTAATCCCCAACAAAGAAT-3′; mgc2R 5′-CTTGGTTCTATATTAGGCATTT and probe: 5′-6-FAM-CCACAGGGCTTGGTGGCCCA-BHQ-1. The cycling conditions were as follows: 95°C for 3 min, 40 cycles of: 95°C for 12 s and 60°C for 1 min.

Sequencing and phylogenetic analysis of the avipox fpv167amplicon

The DNA amplicon of 576 bp was excised from the gel and purified using the QIAquick Gel Extraction kit (Qiagen GmbH, Hilden, Germany). The nucleotide sequence was determined using 3100 Avant Genetic Analyzer: ABI PRISM (Applied Biosystems, Hitachi) and the CPF primer. Nucleotide sequences were compared, using a Cladogram, for similarity to internationally reported sequences of avipox strains using the National Centre for Biotechnology Information Website (www.ncbi.nlm.nih.gov).

Results and discussion

Birds symptoms and gross pathology

The frequency of birds showing specific symptoms and lesions is shown in Table 1.

All of the birds showed thickened eye lid (Figure 1) and scab like lesions on skin or feet (Figure 2). These symptoms and gross lesions are typical of pox as reported in previous literature and reference books (Moss 1996; Tripathy and Reed 1997). The thickened airsac lesion was observed in one bird only (14%), along with the absence of any hepatic, tracheal, oesophageal and lung lesions indicating that the pox infection was not diphtheric (Tripathy and Reed 1997).

Histopathological observations of microscopic lesions

The frequency of birds showing microscopic skin and airsac lesions is shown in Table 2. All of the birds showed skin hyperplasia and heterophil infiltration (Figure 3). Bollinger bodies were observed in the skin of 71% of the birds (Figure 3), confirming their infection with pox virus (Alehegn et al. 2014). Only one bird (14%) showed microscopic lesions in the airsac, namely hyperplasia and heterophil infiltration (Figure 4), meaning that the canarypox virus didn’t develop in the internal organs and was only limited to skin (Moss 1996; Williams et al. 2014).

Table 2. Frequency of birds with specific microscopic lesions in the skin and feet.

| Microscopic lesion                  | Frequency/7 (%) |
|------------------------------------|-----------------|
| Skin hyperplasia                   | 7 (100)         |
| Heterophil infiltration in the dermis | 7 (100)         |
| Bollinger body formation in the skin | 5 (71)          |
| Airsac hyperplasia                 | 1 (14)          |
| Heterophil infiltration in airsacs  | 1 (14)          |
Fungal and protozoal staining

Giemsa and Lactophenol Cotton Blue staining confirmed respectively the absence of protozoal and fungal agents in the inner side of the eye lid and skin scabs. The excessive treatment of birds by the canary breeders with antifungal and anti-protozoal drugs might have cleared or prevented the concurrent infection with fungi or protozoa. Ronidazol that was used in the treatment of sick birds is known for its high efficiency against a wide range of protozoans including Trichomonas (Brandao and Beaufrière 2013), and itraconazol is highly efficient against a wide range of fungal diseases (Proença et al. 2014).

Serological analysis

Hemaglutination inhibition test (HI) was negative for H9N2 Al and NDV, revealing the absence of a previous infection with any of these pathogens. These results confirm those obtained with the gross pathology observations that showed absence of any lesions in the tissues targeted by Al and NDV namely tracheas and lungs of the affected birds (Alexander 2003; Swayne and Halvorson 2008). However, the slide agglutination test was positive for Mycoplasma gallisepticum, namely in two out of the seven affected birds (29%). The presence of MG in the airsacs and inner side of the eye lid was further confirmed with qPCR analysis developed in the below paragraph.

PCR analysis for chlamydia psittaci, avipox and mycoplasma gallisepticum

PCR analysis was negative for C. Psittaci, with absence of the 264 bp band in all of the tested tissues, namely the skin, liver, lung and airsac. PCR analysis showed positive results for the fpv167 gene of avipox virus with a band of 576 bp in the skin only (Figure 5). These results offer an additional confirmation that the etiologic agent is pox virus (Esteves et al. 2017). The absence of pox from the liver, lung and airsacs, confirms again the non-diphteric nature of this virus (Moss 1996; Williams et al. 2014).

The qPCR analysis revealed the MG colonization of the inner side of the eye lids of two birds (29% of the affected birds) and airsac of the bird that showed hyperplasia and heterophil infiltration (14% of the affected birds). Mycoplasmosis results in the development of conjunctivitis and ocular and nasal discharge of the affected cage birds without significantly increasing the mortality rates as the clinical manifestations are usually slow to develop (Duckworth et al., 2003). However, MG worsens any situation specifically in mixed viral infections, and might have increased the mortality rate, along with the concurrent canarypox infection. In mixed infections, MG usually

Figure 3. Skin hyperplasia and heterophils infiltration (a) (200x magnification) and Bollinger Body formation (b) (400x magnification).

Figure 4. Airsac hyperplasia and heterophils infiltration (400x magnification).

Figure 5. PCR amplification of the fpv167 gene encoding for the p4b core protein of avipox in tissue homogenates of seven affected birds. Lane 1 = 100 bp molecular ladder, Lane 2 = Negative control, Lanes A3-A9: DNA amplicons of skin homogenates, Lanes B3-B9 = DNA amplicons of liver homogenate, Lanes C3-C9 = DNA amplicons of airsac homogenate, Lanes D3-D9 = DNA amplicons of lung homogenates.
weakens the physical barriers of the upper and lower respiratory tracts such as tracheal deciliation and mucous disintegration, which paves the way for secondary pathogens to complicate the respiratory illness (Kleven 1998; Ley and Yoder 1997).

On the other hand, these results confirm the usefulness of the adopted PCR protocol, using the primers and probes indicated in this study, as a strong tool to screen and identify circulating canarypox viruses and MG in birds (Grodio et al. 2008; Hewinson et al. 1997).

Sequencing and phylogenetic analysis of the avipox fpv167 amplicon

Sequencing of the partial fpv167 gene coding for the p4b core protein of avipox and sequence alignment revealed 100% similarity of the field isolate to the Iranian Canarypox virus strain IR/H913/14 (Cladogram, Figure 6). These results indicate the rigidity of the P4b protein encoding gene to mutations, possibly due to the fact that these viruses have a double stranded DNA (Hewinson et al. 1997). In Iran, both diptheric and cutaneous viral forms circulate in the country (Nayeri Fasaei et al. 2013) and the geographic proximity of Iran to Lebanon suggests the circulation of the same cluster of canarypox viruses in the Middle East region. Unfortunately, the information about circulating avipox viruses in Middle Eastern neighbouring countries is scarce, prohibiting the comparison of the Lebanese isolate with them. However, it is worth noting that as the geographical distance increases, the similarity percentage decreases gradually to reach 98–99% with European isolates and 91% with South American isolates (Brazilian Penguin Pox isolate FLO 190 Clone 1).

In conclusion, canarypox with the concurrent infection with MG were the causative agents that resulted in the loss of about 50% of the canaries in several breeding farms in Lebanon. The same cluster of pox viruses is circulating among neighbouring countries of the Middle East, with high genetic similarity among each other. The smuggling of pets and animal feedstuff through the Syrian border causes a serious threat to the control of pathogen circulation in the country, specifically canarypox and MG. Therefore, the application of biosecurity measures should be seriously considered, including vaccination of pet birds for pox, which is not a common practice in Lebanon.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Conflict of interest

The authors declare no conflict of interests in this manuscript.

Ethical statement

This work was approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut.

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