Polymerase chain reaction detection of avipox and avian papillomavirus in naturally infected wild birds: comparisons of blood, swab and tissue samples

Richard A. J. Williams¹,²*, Clara Escudero Duch³, Javier Pérez-Tris¹ and Laura Benítez³

¹Department of Zoology and Physical Anthropology, Faculty of Biological Sciences, Universidad Complutense de Madrid, Madrid, Spain, ²Natural Sciences, Saint Louis University, Madrid, Spain, and ³Department of Microbiology, Faculty of Biological Sciences, Universidad Complutense de Madrid, Madrid, Spain

Avian poxvirus (avipox) is widely reported from avian species, causing cutaneous or mucosal lesions. Mortality rates of up to 100% are recorded in some hosts. Three major avipox clades are recognized. Several diagnostic techniques have been reported, with molecular techniques used only recently. Avipox has been reported from 278 different avian species, but only 111 of these involved sequence and/or strain identification. Collecting samples from wild birds is challenging as only few wild bird individuals or species may be symptomatic. Also, sampling regimes are tightly regulated and the most efficient sampling method, whole bird collection, is ethically challenging. In this study, three alternative sampling techniques (blood, cutaneous swabs and tissue biopsies) from symptomatic wild birds were examined. Polymerase chain reaction was used to detect avipoxvirus and avian papillomavirus (which also induces cutaneous lesions in birds). Four out of 14 tissue samples were positive but all 29 blood samples and 22 swab samples were negative for papillomavirus. All 29 blood samples were negative but 6/22 swabs and 9/14 tissue samples were avipox-positive. The difference between the numbers of positives generated from tissue samples and from swabs was not significant. The difference in the avipox-positive specimens in paired swab (4/6) and tissue samples (6/6) was also not significant. These results therefore do not show the superiority of swab or tissue samples over each other. However, both swab (6/22) and tissue (8/9) samples yielded significantly more avipox-positive cases than blood samples, which are therefore not recommended for sampling these viruses.

Introduction

Avian poxvirus (avipox) causes a mild to severe disease in birds that may manifest in two forms; diphtheritic pox, affecting mucous membranes of the respiratory and digestive tracts (wet pox); and cutaneous pox, typically presented as wart-like growths on the skin (dry pox). The cutaneous form is considered to be mildly pathogenic, although secondary bacterial infections may prove fatal (Hansen, 1999), while the diphtheritic form may cause mortality rates of 80 to 100% (Tripathy & Reed, 1997). The two forms may occur together (Weli & Tryland, 2011).

Increasing detection of avipox in wild birds and recent spatial and host taxonomic range expansion suggests avipox may be an emerging disease (Lawson et al., 2012). Avipox has been diagnosed in a broad range of avian species: from 278 species in 20 orders (van Riper & Forrester, 2007) using classical and molecular detection techniques (Bolte et al., 1999). Ten avipox viral species are recognized (Buller et al., 2012), although only three major avipox clades—canary poxvirus, fowl poxvirus, and psittacine poxvirus (Jarmin et al., 2006; Carulei et al., 2009)—and some minor clades (Gyuranecz et al., 2013) have been recovered by phylogenetic analysis. Several diagnostic techniques have been reported for avipox: histopathology was first reported in 1873, virus isolation was reported in the first half of the twentieth century and electron microscopy in the second half of the twentieth century (Bolte et al., 1999). Molecular techniques had not been used as a tool for diagnosis of avipox until 1987 (Binns et al., 1987). Sequence information for avipox strain identification is only available for viruses from less than one-half of known hosts, 111 species of 13 orders (Gyuranecz et al., 2013), leaving a gap in understanding of the diversity and host species range of strains. Obtaining and analysing sequences from a broader range of hosts can assist in this process. The purpose of this study was to establish the most effective method of collecting samples from wild bird hosts in order to fill that gap, with the least impact on the host. All samples were also tested for avian papillomavirus, which also causes cutaneous lesions but may be confused with pox lesions (Pérez-Tris et al., 2011).

Collecting samples from live wild birds is challenging for two major reasons. Firstly, prevalence of avipox infection is low in wild birds. Although prevalence of up to 50% has been reported in susceptible hosts, particularly those from...
remote islands (e.g. Canaries, Galapagos, Hawaii), modal prevalence of avian pox lesions in wild birds in regions where avipox and its hosts have had a long co-evolutionary history varies between 0.5 and 1.5% (van Riper & Forrester, 2007). Typically, collecting a small number of positive avipox samples requires sampling a large number of birds, which is ideally achieved in collaboration. Secondly, permits for testing wild birds are tightly regulated, and the least intrusive sampling methods are more likely to facilitate obtaining sampling permits. It is also easiest to persuade non-researchers to collect non-invasive samples. Obtaining samples lends itself to “citizen science”—potentially using networks of licensed bird ringers to collect samples from symptomatic birds.

To date, most polymerase chain reaction (PCR)-based studies of avipox have detected viral DNA from tissue samples (Shivaprasad et al., 2009; Weli & Tryland, 2011), typically from cutaneous lesions. To our knowledge only one study has detected viral DNA in superficial skin swabs of cutaneous lesions of birds (Pérez-Tris et al., 2011), while another detected viral DNA from blood samples using the Taqman real-time PCR described before (Farias et al., 2010). It seems plausible that avipox can induce viraemia, and thus should be detectable in blood. Intracytoplasmic inclusions (Bollinger bodies) have been described in mucous membranes, particularly from the oropharynx and respiratory tract, and sometimes extending from cutaneous lesions (Saio et al., 2009; Brower et al., 2010; Manarolla et al., 2010). In canaries the presence of Bollinger bodies in the lung, heart, spleen, bone marrow and peritoneum suggests viral circulation (Shivaprasad et al., 2009). However, no specific PCR was used to detect virus in blood in these studies.

By contrast, studies of related, non-avian capripoxviruses (Babiuk et al., 2008) and monkey poxviruses (Saijo et al., 2008) report detection from blood and swab samples (although not from cutaneous lesions) from experimentally infected individuals, using more sensitive real-time PCR techniques.

We compared three methods of collecting avipox samples from symptomatic wild birds (n = 30 from 10 different bird species, out of 1944 individuals examined) captured from 43 sites across Spain between 2007 and 2011 to determine whether the sensitivity of sampling methods varied. Swabbing cutaneous lesions was the least intrusive method and required the least training; collecting blood samples from brachial or jugular veins or collecting tissue biopsies from suspect pox lesions were more intrusive methods, although not risky when carried out by experienced field workers. Collecting blood samples, in particular, required practice. Occasionally suspect lesions proved too small to excise with a scalpel, necessitating the collection of swab samples. All samples that formed a part of this study were collected by experienced field workers.

Materials and Methods

Field methods. Wild birds were captured using mist nets, as part of routine bird ringing activities or as part of other studies. After fitting leg rings, birds were examined for cutaneous skin lesions. Phosphate-buffered saline-soaked dacron-tipped swabs were rubbed repeatedly (30 times) against cutaneous lesions and stored in sterile tubes (n = 22). Blood samples (approximately 50 µl) were collected from the jugular vein of each bird, and stored in 100% ethanol (n = 29). Tissue biopsies were excised using a scalpel blade, and were then stored in sterile tubes containing ethanol (n = 14). Birds were then released unharmed. Field workers changed gloves each time they sampled a symptomatic bird, and used sterile hypodermic syringes, scalpel blades or swabs for the collection of each sample in an effort to minimize the possibility of contamination during sample collection. Swabs were stored on ice in the field and transferred to −20°C freezers until further analysis. Given the rarity of encountering symptomatic birds, field limitations and sample collection by seven experienced, field workers, the sizes of different sample types were unequal: blood (n = 29), swabs (n = 22), and tissue (n = 14) (Table 1). Samples tested in this study are all symptomatic individuals (n = 30) for which two or more sample types (blood, swab or tissue) were available (from a total of 1944 birds examined), although all three were seldom available. These include six positive tissue samples discussed previously (Pérez-Tris et al., 2011), collected using the field protocol outlined above, for which previously untested blood samples were available.

DNA extraction and viral characterization. Tissue biopsies were homogenized in lysis buffer containing proteinase K (Fermentas, Burlington, Canada) to a final concentration of 500 mg/ml and incubated at 60°C for 2 to 3 h. Swabs were also placed in lysis buffer and vortexed for 30 sec. DNA from blood, swabs and nine tissue samples was extracted using a standard ammonium acetate extraction technique (Sambrook & Russell, 2001). A phenol-chloroform-isooamyl alcohol technique followed by isopropanol precipitation was used for six tissue samples originally tested in an earlier study (Pérez-Tris et al., 2011). DNA extracts were stored at −20°C.

Extracts were tested with cytochrome b primers to verify adequate preservation of DNA (Kocher et al., 1989), and were considered positive if they produced an agarose gel band of the anticipated size (approximately 300 base pairs). All extracts were then tested using a multiplex PCR designed to distinguish avipoxvirus and avian papillomavirus. In brief, amplification was performed in a 25 µl reaction mixture containing 0.625 µM AmpliTaq DNA polymerase (Applied Biosystems, Warrington, UK), GeneAmp II PCR buffer supplemented to a final 4.0 mM MgCl₂, 0.8 mM concentration of each deoxynucleotide triphosphate (Fermentas), and 50 pmol/ml each primer. The PCR mix was subjected to 45 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, and a final extension step at 72°C for 5 min (Pérez-Tris et al., 2011). See supplementary materials for primer sequences. Samples positive using the multiplex PCR or those that produced agarsose gel bands of roughly the anticipated size for avipox (approximately 250 base pairs) were further tested with P4b primers for the detection of avipoxvirus (Lee & Lee, 1997). Amplified DNA was visualized by subjecting 5 µl PCR product to gel electrophoresis through a 1.5% agarsose gel, and staining with Gel Red. Products were sequenced from both ends on an ABI Prism 3730 automated sequencer, and sequences were compared with known avipox and avian papillomavirus sequences available in GenBank. Samples were considered positive if PCR amplicons yielded sequences homologous to the avipox (P4b) and avian papillomavirus (L1).

Statistical analysis. Associations between positive (avipoxvirus/avian papillomavirus) cases and sample type were tested using Fisher’s exact test, and were considered statistically significant if P < 0.05. All statistical analyses were carried out using SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA).

Results

Polymerase chain reaction. All samples tested positive for cytochrome b amplification. In total, 12/30 (40%) individuals were avipox positive—in both multiplex and P4b, 4/30 (13.3%) individuals were positive for avian papillomavirus. Sixteen out of 30 (53.3%) individuals were positive for one or other virus. However, results varied by sample type (Table 1), with 0/29 blood samples, 8/22 (36.4%) swab samples, and 9/14 (64.3%) tissue samples avipox-positive in multiplex PCR (Pérez-Tris et al., 2011). All tissue samples but only 6/8 (27.3%) swab samples tested in the multiplex were confirmed positive using the P4b PCR. Four tissue samples were papillomavirus positive. All blood and swab samples were papillomavirus negative. All positive samples in this study produced a readable sequence of the corresponding virus.
**Table 1. Summary of PCR tests for cytochrome b, avipox/avian papillomavirus multiplex and simple avipox.**

| Species                          | Blood                          | Swab                           | Tissue                          |
|----------------------------------|-------------------------------|--------------------------------|---------------------------------|
|                                  | Multiplex (APV/PV) | Simple (APV) | Multiplex (APV/PV) | Simple (APV) | Multiplex (APV/PV) | Simple (APV) |
| Carduelis carduelis              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Carduelis carduelis              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Cyanites caeruleus               | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Emberiza rubecula                | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Fringilla coelebs 1              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Fringilla coelebs 2              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Fringilla coelebs 3              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Fringilla coelebs 4              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Garrulus glandarius              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Passer domesticus 1              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Passer domesticus 2              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Passer domesticus 3              | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Parus major                      | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sittella nolitina                 | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sittella nolitina                 | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 1             | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 2             | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 3             | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 4             | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 5             | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 6             | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 7             | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 8             | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 9             | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 10            | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 11            | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Sylvia atricapilla 12            | (+)                          | (+)                  | (+)                            | (+)            | (+)              | (+)             |
| Total tested                     | 29 (100%)                    | 22 (100%)            | 14 (100%)                     | 8 simple PCR (36%) | 9 simple PCR (64%) | 4 (29%)  |

APV, avipox; Cyt b, cytochrome b; PV, avian papillomavirus.

**Statistical analysis.** Sample groups were poorly paired, and corroborating evidence that symptomatic individuals were positive/negative was not always available. We thus compared paired sample types; for example, the nine tissue samples for which there were also blood samples, and so forth (Table 2). Eight out of nine tissue samples were avipox-positive, significantly more positive than the 0/9 blood samples (<0.001). Similarly, 6/22 avipox-positive swab samples yielded significantly more positive results than the 0/22 blood samples (<0.01). No significant difference was detected between 4/6 positive swab samples, and 6/6 positive tissue samples from the same individuals. Papillomavirus results did not differ significantly by sampling treatment.

**Table 2. Associations between virus-positive samples and sample type tested using Fisher’s exact test.**

| Paired sample test          | k (positive individuals) | n (total individuals) | P value* |
|-----------------------------|--------------------------|-----------------------|----------|
| Paired tissue versus        | 8                        | 9                     | <0.001   |
| Paired blood                | 0                        | 9                     | NS       |
| Paired swab versus          | 6                        | 6                     | <0.01    |
| Paired swab versus          | 6                        | 22                    |           |
| Paired blood                | 0                        | 22                    |          |

*P value <0.05 was considered statistically significant. NS, not significant.

**Discussion**

We were unable to detect avian papillomavirus in blood samples, including four samples paired to positive tissue samples. This was anticipated as papillomavirus infects squamous epithelial cells (de Villiers et al., 2004; White & Howley, 2013) and is often considered to be non-circulatory (Howley & Lowy, 2007), although some authors report the detection of papillomavirus in blood and other non-epithelial cell types (Yaguita et al., 2008; Chen et al., 2009).

Blood samples were also negative for poxvirus. Viraemia for poxvirus is to be expected; a putative route for avipox...
transmission is via biting insects (Tripathy & Reed, 1997), although circulating virus is short-lived compared with skin lesions in some non-avian poxvirus strains (Murphy et al., 1999; Bowden et al., 2008). Virus DNA is also expected to be detectable in cutaneous lesions, and high tropism for and long duration of viral presence in skin has been described for capriopoxviruses (Babiuk et al., 2008). Furthermore, poxvirions survive for up to a year in the environment (Tripathy et al., 1999; Murphy et al., 1999). We were unable to detect avipox in blood samples from symptomatic individuals, even from 12 hosts with avipox-positive tissue or swab samples. Avipox has been detected in blood samples, suggesting the virus can circulate in blood (Farias et al., 2010). However, in that study only 1/7 (14.3%) of blood samples were confirmed positive from tests on paired tissue samples, 13/28 (46.4%) of blood samples from symptomatic hosts tested positive for avipox using Taqman real-time PCR, and reproducible results were obtained in less than 40% of samples (Farias et al., 2010).

The proportion of avipox-positive samples was higher for tissues (64.3%) than swabs (27.3%). However, this difference was not significant and therefore does not indicate that one sampling method outperforms the other. In many cases swabs were collected but tissues were not, as lesions were too small to safely obtain a biopsy. Small lesions may yield little viral DNA, thereby reducing the likelihood of amplifying viral DNA from them. Furthermore, the DNA collected by swabs may be affected by the collection technique depending on how vigorously lesions are swabbed, or by storage from the field to the laboratory. It was difficult to control for these variables given that samples were collected by seven field workers, albeit experienced ornithology researchers. Alternatively, it is possible that some of the (particularly smaller) lesions may have been misidentified as avipox lesions. Where samples were paired, there was no significant difference in the proportion of paired swab/tissue testing positive. Two out of six swab samples collected from tissue-positive hosts were negative. No swab tested positive for avian papillomavirus (although nor did any blood or tissue samples paired to negative swabs).

Ethical, licensing, or practical concerns (that the suspect lesion is too small to yield tissue samples) favour swab sampling, which can be conveniently collected. Moreover, given the generally low prevalence of these viruses in wild birds, broad collaboration may be required to generate sufficient samples, and swab collection may best fit with the aim of developing a protocol for best field methods with a view to stimulating “citizen science” collaboration. We believe that “citizen science” collaboration with non-researchers is a good way to generate large numbers of samples, and possibly novel sequences. However, we acknowledge that use of non-researchers may introduce inconsistencies in a study if due care is not taken, particularly worries about contamination in the field and poor sample collection and preservation in inexperienced field workers. We thus propose an intermediate between true citizen science and scientific practice, as it takes advantage of the existence of well-trained people (licensed bird ringers) who will be able to do a conscientious job if they are given appropriate instructions.

We found a higher proportion (27.3 to 92.8%) of tissue and swab samples positive for avipox or avian papillomavirus than blood (0%) samples. Tissue and swab samples were effective for the detection of virus in birds, but blood samples of the same birds were not useful, at least using the PCR methodology used in this study.

Acknowledgements

The authors thank bird ringers from Alula and Monticola, especially Alfredo Ortega and Chechu Aguirre, for help with the capture and ringing of birds, which made this project possible. Thanks to Alvaro Ramirez for samples. This research was funded by the Ministerio de Ciencia e Innovacion, Spain (grant number: CGL2010-15734/BOS). R.A.J.W. was supported by the Programa Internacional de Captación de Talento (PICATA) de Moncloa Campus de Excelencia Internacional while writing the manuscript.

Supplemental data

Supplemental data for this article can be accessed here.

References

Babiuk, S., Bowden, T., Parkyn, G., Dalman, B., Manning, L., Neufeld, J., Enzmbury-Hyatt, C., Copps, J. & Boyle, D. (2008). Quantification of lumpy skin disease virus following experimental infection in cattle. Transboundary and Emerging Diseases, 55, 299–307.

Binnis, M., Stenzler, L., Tomley, F., Campbell, J. & Boursnell, M. (1987). Identification by a random sequencing strategy of the Fowlpoxvirus DNA-polymerase gene, its nucleotide-sequence and comparison with other viral-DNA polymerases. Nucleic Acids Research, 15, 6563–6573.

Bolte, A., Meurer, J. & Katale, E. (1999). Avian host spectrum of avipoxviruses. Avian Pathology, 28, 415–432.

Bowden, T., Babiuk, S., Parkyn, G., Copps, J. & Boyle, D. (2008). Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats. Virology, 371, 380–393.

Brower, A., Cigel, F., Radi, C. & Tookey-Kurth, K. (2010). Beak necrosis in Hungarian partridges (Perdix perdix) associated with beak-bits and avian poxvirus infection. Avian Pathology, 39, 223–225.

Buller, R., Arif, B., Black, D., Dumbell, K., Esposito, J., Lefkowitz, E., Fadden, G., Moss, B., Mercer, A., Moyer, R., Skinner, M. & Tripathy, D. (2012). Poxviridae. In A.M.Q. King, M.J. Adams, E.B. Carstens. & E.J. Lefkowitz (Eds.). Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses (pp. 291–309). San Diego, CA: Elsevier Academic Press.

Caruelli, O., Douglass, N. & Williamson, A. (2009). Phylogenetic analysis of three genes of Puppoxvirus virus corresponding to Vaccinia virus GSR (VLTF-1), A3L (P4b) and H3L reveals that it is most closely related to Turkeycpox virus, Ostrichpox virus and Pigeonpox virus. Virology, Journal, 6, 52.

Chen, A., Keleher, A., Kedda, M., Spurdle, A., McMillan, N. & Antonsson, A. (2009). Human papillomavirus DNA detected in peripheral blood samples from healthy Australian male blood donors. Journal of Medical Virology, 81, 1792–1796.

de Villiers, E., Fauquet, C., Broker, T., Bernard, H. & zur Hausen, H. (2004). Classification of papillomaviruses. Virology, 324, 17–27.

Farfias, M., LaPorte, D., Atkinson, C., Czerwonska, C., Shrestha, R. & Jarvi, S. (2010). Taqman real-time PCR detects avipoxvirus DNA in blood of Hawai`i Amakihì (Hemignathus virens). PLOS One, 5, e01745.

Gyurancz, M., Foster, J., Dán, A., Ip, H., Egstad, K., Parker, P., Higashiguchi, J., Skinner, M., Höfle, U., Kreizinger, Z., Dorrestein, G., Solt, S., Sós, E., Kim, Y., Uhurt, M., Pereda, A., González-Hein, G., Hidalgo, H., Blanco, J. & Erdélyi, K. (2013). Worldwide phylogenetic relationship of avian poxviruses. Journal of Virology, 87, 4938–4951.

Hansen, W. (1999). Avian pox. In M. Friend & J. Franson (Eds.). Field Manual of Wildlife Diseases General Field Procedures and Diseases of Birds (pp. 163–170). Biological Resources Division, U.S. Geological Survey, Madison, WI, USA.

Howley, P. & Lowy, D. (2007). Papillomaviridae. In D. Knipe & P. Howley (Eds.). Fields’ Virology (pp. 2299–2354). Philadelphia, PA: Wolters Kluwer Health / Lippincott Williams & Wilkins.

Supplemental data for this article can be accessed here.

Acknowledgements

The authors thank bird ringers from Alula and Monticola, especially Alfredo Ortega and Chechu Aguirre, for help with the capture and ringing of birds, which made this project possible. Thanks to Alvaro Ramirez for samples. This research was funded by the Ministerio de Ciencia e Innovacion, Spain (grant number: CGL2010-15734/BOS). R.A.J.W. was supported by the Programa Internacional de Captación de Talento (PICATA) de Moncloa Campus de Excelencia Internacional while writing the manuscript.

Supplemental data

Supplemental data for this article can be accessed here.
Jarmin, S., Manvell, R., Gough, R., Laidlaw, S. & Skinner, M. (2006). Avipoxvirus phylogenetics: identification of a PCR length polymorphism that discriminates between the two major clades. *Journal of General Virology, 87*, 2191–2201.

Kocher, T., Thomas, W., Meyer, A., Edwards, S., Paabo, S., Villablanca, F. & Wilson, A. (1989). Dynamics of mitochondrial-DNA evolution in animals—amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America, 86*, 6196–6200.

Lawson, B., Lachish, S., Colvile, K., Durrant, C., Peek, K., Toms, M., Sheldon, B. & Cunningham, A. (2012). Emergence of a novel avian pox disease in british tit species. *PLOS One, 7*, e40176.

Lee, L. & Lee, K. (1997). Application of the polymerase chain reaction for the diagnosis of fowl poxvirus infection. *Journal of Virological Methods, 63*, 113–119.

Manarella, G., Pisoni, G., Sironi, G. & Rampin, T. (2010). Molecular biological characterization of avian poxvirus strains isolated from different avian species. *Veterinary Microbiology, 140*, 1–8.

Murphy, F., Gibbs, E., Horzinek, M. & Studdert, M. (1999). Poxviridae *Veterinary Virology* (pp. 277–291). San Diego, CA: Academic Press.

Pérez-Tris, J., Williams, R., Abell-Fernández, E., Barreiro, J., Conesa, J., Figuerola, J., Martínez-Martínez, M., Ramírez, A. & Benítez, L. (2011). A multiplex PCR for detection of poxvirus and papillomavirus in cutaneous warts from live birds and museum skins. *Avian Diseases, 55*, 545–553.

Saijo, M., Ami, Y., Suzaki, Y., Nagata, N., Iwata, N., Hasegawa, H., Ogata, M., Fukushi, S., Mizutani, T., Izuka, I., Sakai, K., Sata, T., Kurata, T., Kurane, I. & Morikawa, S. (2008). Diagnosis and assessment of monkeypox virus (MPXV) infection by quantitative PCR assay: differentiation of Congo Basin and West African MPXV strains. *Japanese Journal of Infectious Diseases, 61*, 140–142.

Saito, K., Kodama, A., Yamaguchi, T., Gotoh, Y., Sakai, H., Fukushi, H., Masegi, T. & Yanai, T. (2009). Avian poxvirus infection in a white-tailed sea eagle (*Haliaeetus albicilla*) in Japan. *Avian Pathology, 38*, 485–489.

Sambrook, J. & Russell, D. (2001). *Molecular Cloning: A Laboratory Manual* 3rd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Shivaprasad, H., Kim, T., Tripathy, D., Woolcock, P. & Uzal, F. (2009). Unusual pathology of canary poxvirus infection associated with high mortality in young and adult breeder canaries (*Serinus canaria*). *Avian Pathology, 38*, 311–316.

Tripathy, D. & Reed, W. (1997). Pox. In B.W. Calnek, H. Barnes, C. Beard. & L. McDougald (Eds.). *Diseases of Poultry* 10th edn (pp. 643–659). Ames, IA: Iowa State Press.

van Riper, C. & Forrester, D.J. (2007). Avian pox. In N.J. Thomas, D.B. Hunter. & C.T. Atkinson (Eds.). *Infectious Diseases of Wild Birds* (pp. 131–176). Oxford, UK: Wiley Blackwell.

Weli, S. & Tryland, M. (2011). Avipoxviruses: infection biology and their use as vaccine vectors. *Virology Journal, 8*, 49.

White, E. & Howley, P. (2013). Proteomic approaches to the study of papillomavirus-host interactions. *Virology, 435*, 57–69.

Yagutu, A., Dagli, M., Birgel, E., Reis, B., Ferraz, O., Pitacco, E., Freitas, A., Becak, W. & Stocco, R. (2008). Simultaneous presence of bovine papillomavirus and bovine leukemia virus in different bovine tissues: in situ hybridization and cytogenetic analysis. *Genetics and Molecular Research, 7*, 487–497.