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Title:

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

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Short title: Avipox PCR sample type test comparison

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Abstract

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 only used 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. PCR was used to detect Avipox virus and avian papillomavirus (which also induces cutaneous lesions in birds). Four out of 14 tissue samples were positive but all 29 blood 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. Therefore these results 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 positives than blood samples, which are therefore not recommended for sampling these viruses.

Introduction

Avian poxvirus (Avipox) causes a mild to severe disease in birds which 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, though secondary bacterial infections may prove fatal (Hansen, 1999), while the diphtheritic form may cause mortality rates of 80 to 100% (Tripathy et al., 1997). The two forms may occur together (Weli et al., 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 C et al., 2007), using classical and molecular detection techniques (Bolte et al., 1999). Ten Avipox viral species are recognized (Buller et al., 2012), though only three major Avipox clades, Canarypox, Fowlpox, and Psittacinepox viruses (Carulei et al., 2009; Jarmin et al., 2006), 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 in the first half of the 20th century; and electron microscopy in the second half of the 20th 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 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 analysis of

sequences from a broader range of hosts can assist in this process. The purpose of this study is 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, that 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 C & Forrester DJ, 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 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 (Brower et al., 2010; Manarolla et al., 2010; Saito et al., 2009). In canaries the presence of Bollinger bodies in 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 monkeypoxviruses (Saijo et al., 2008), report detection from blood and swab samples (though 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, though 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 form 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, routine biometrics and blood, swab and / or tissue samples were collected. All birds were examined for cutaneous skin lesions. PBS soaked dacron-tipped swabs were rubbed repeatedly (30 times) against cutaneous lesions and stored in sterile tubes (N=22). Blood samples (approximately 50µl)

were collected from the jugular vein of each bird, and stored in 100% ethanol (N=29). Tissue biopsies were excised using a scalpel blade, 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 minimise the possibility of contamination during sample collection. Swabs were stored on ice in the field and transferred to -20ºC freezers until further analysis. Given the rarity of encountering symptomatic birds, field limitations and sample collection by seven experienced, field workers, sizes of different sample types were unequal: blood (N=29), swabs (N=22), 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), though all three were seldom available. These include 6 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 homogenised in lysis buffer containing proteinase K (Fermentas, Burlington, Canada) to a final concentration of 500 mg/ml and incubated at 60ºC for 2–3 hr. Swabs were also placed in lysis buffer and vortexed for 30 seconds. DNA from blood, swabs and 9 tissue samples was extracted using a standard ammonium acetate extraction technique (Sambrook et al., 2001). A phenol-chloroform-isoamyl alcohol technique followed by isopropanol precipitation was used for 6 tissue samples originally tested in an earlier study (8). DNA extracts were stored at -20ºC. Extracts were tested with Cytochrome *b* primers to verify adequate preservation of DNA (Kocher et al., 1989), and considered positive if they produced an agarose gel band of the anticipated size (c.300bp). All extracts were then tested using a multiplex PCR designed to distinguish Avipox virus and avian papillomavirus. In brief, amplification was performed in a

25 ml reaction mixture containing 0.625 U of 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 of each primer. The PCR mix was subjected to 45 cycles of 95ºC for 1min, 50ºC for 1min and 72ºC for 1 min, and a final extension step at 72ºC for 5 min (Pérez-Tris, et al., 2011). See supplementary materials for primer sequences. Samples positive using the multiplex PCR, or those that produced agarose gel bands of roughly the anticipated size for Avipox (c.250bp) were further tested with P4b primers for the detection of Avipox virus (Lee et al., 1997). Amplified DNA was visualized by subjecting 5µl of PCR product to gel electrophoresis through a 1.5% agarose 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 testing. Associations between positive (Avipox virus / avian papillomavirus) cases and sample type were tested using Fisher's exact test, and considered statistically significant if the p value was <0.05. All statistical analyses were carried out using SigmaPlot 11.0 (Systat Software Inc., IL, USA).

Results

PCR Results. All samples tested positive for Cytochrome *b* amplification. In total, 12/30 (40%) individuals were Avipox positive – in both multiplex and P4b and 4/30 (13.3%) individuals were positive for avian papillomavirus. 16/30 (53.3%) individuals were positive for one or other virus. However, results varied by sample type (Table 1) with 0/29 blood, 8/22 (36.4%) swab, 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.

Statistical Testing. Sample groups were poorly paired, and corroborating evidence that symptomatic individuals were positive / negative was not always available. We thus compared paired sample types – e.g. the nine tissue samples for which there were also blood samples, etc. (Table 2). 8/9 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.

Discussion

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

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), though circulating virus is short-lived compared to skin lesions in some non-avian poxvirus strains (Bowden et al., 2008; Murphy et al., 1999). 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 capripoxviruses (Babiuk, et al., 2008). Furthermore pox virions survive for up to a year in the environment (Murphy, et al., 1999; Tripathy & Reed, 1997). 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 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 lab. 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. 2/6 swab samples collected from tissue positive hosts were negative. No swab tested positive for avian papillomavirus (though 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 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 (27.3-92.8%) 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.

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Table 1. Summary of PCR challenges for Cytochrome ***b*** (Cyt b)**,** Avipox (APV) / Avian Papillomavirus (PV) multiplex and simple Avipox.

	Blood			**Swab**			**Tissue**		
Species	**Cyt b**	**Multiplex (APV/PV)**	**Simple (APV)**	**Cyt b**	**Multiplex (APV/PV)**	**Simple (APV)**	**Cyt b**	**Multiplex (APV/PV)**	**Simple (APV)**
Carduelis carduelis	(+)	(-) / (-)		(+)	(-) / (-)				
Carduelis carduelis	(+)	(-) / (-)		(+)	(-) / (-)				
Cyanistes caeruleus	(+)	(-) / (-)					(+)	(+) / (-)	(+)
Erithracus rubecula	(+)	(-) / (-)		(+)	(+) / (-)	(+)			
Fringilla coelebs	(+)	(-) / (-)					(+)	(-) / (+)	(-)
Fringilla coelebs	(+)	(-) / (-)					(+)	(-) / (+)	(-)
Fringilla coelebs	(+)	(-) / (-)					(+)	(-) / (+)	(-)
Fringilla coelebs	(+)	(-) / (-)					(+)	(-) / (+)	(-)

Garrulus glandarius	(+)	(-) / (-)		(+)	(-) / (-)				
Parus major	(+)	(-) / (-)		(+)	(-) / (-)				
Parus major	(+)	(-) / (-)		(+)	(-) / (-)				
Passer domesticus 1	(+)	(-) / (-)		(+)	(-) / (-)	(-)	(+)	(+) / (-)	(+)
Passer domesticus 2	(+)	(-) / (-)		(+)	(-) / (-)				
Passer domesticus 3	(+)	(-) / (-)		(+)	(-) / (-)	(-)	(+)	(+) / (-)	(+)
Passer domesticus 4	(+)	(-) / (-)		(+)	(+) / (-)	(+)			
Prunella modularis	(+)	(-) / (-)		(+)	(-) / (-)				
Sitta europea	(+)	(-) / (-)		(+)	(-) / (-)				
Sitta europea	(+)	(-) / (-)		(+)	(-) / (-)				
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	(+)	(-) / (-)					(+)	(+) / (-)	(+)
TOTAL TESTED	29			22			14		
CYT b POSITIVE	29 (100%)			22 (100%)			14 (100%)		
APV POSITIVE	0 (0%)			8 MULTIPLEX PCR (36%) 6 SIMPLE PCR (27%)			9 MULTIPLEX PCR (64%) 9 SIMPLE PCR (64%)		

PV POSITIVE	0 (0%)	0 (0%)	4 (29%)

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

Sample type		*k* (positive individuals)	*N* (Total individuals)	P-value[a]
Paired sample tests				
Paired Tissue		8	9	
	v. Paired Blood	0	9	<0.001
Paired Tissue		6	6	
	v. Paired Swab	4	6	NS
Paired Swab		6	22	
	v. Paired Blood	0	22	<0.01

[a] P value <0.05 was considered to be statistically significant.