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Apparent lack of evidence on selected infectious agents in wild Yellow-naped Amazon parrots: implications for releasing attempts

Aparente falta de evidencia sobre agentes infecciosos seleccionados de loras frente amarilla: implicaciones para los procesos de liberación

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Abstract: Blood and feather samples, as well as oropharyngeal and cloacal swabs, were collected from nine free-ranging *Amazona auropalliata* between January and May 2009 in two highly modified landscapes in northern Costa Rica. Samples were tested for avian circovirus, avian polyomavirus, avian influenza, *Chlamydophila psittaci*, *Borrelia burgdorferi* sensu lato, and *Rickettsia* spp. All samples were negative. Results are discussed in terms of implications of using captive birds for reintroduction attempts into wild populations in Costa Rica.

Keywords: Amazona auropalliata, avian infectious diseases, Costa Rica, reintroduction.

Resumen: Muestras de sangre, plumas, hisopados orofaríngeos y cloacales fueron obtenidos de nueve *Amazona auropalliata* de vida libre entre enero y mayo de 2009 en dos paisajes muy modificados en el norte de Costa Rica. Estas fueron analizadas para circovirus aviar, poliomavirus aviar, influenza aviar, *Chlamydophila psittaci*, *Borrelia burgdorferi* sensu lato y *Rickettsia* spp. Todas fueron negativas. Los resultados se discuten en términos de las implicaciones de los programas de reintroducción en las poblaciones silvestres.

Palabras clave: Amazona auropalliata, enfermedades infecciosas de aves, Costa Rica, reintroducción.

INTRODUCTION

The release of individuals into the wild is rapidly becoming a common strategy to re-stock reduced populations of threatened species, and to reintroduce species in their historical ranges (IUCN 1998). The risk of introducing diseases into wild populations through these

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conservation efforts is well known, and may occur either by direct contact of individuals aimed for release when they are in contact with conservationists whose equipment is contaminated or when housed with other infected individuals during captivity. Furthermore, the use of individuals confiscated from illegal trade in release efforts could be a negative conservation strategy if it is considered that they are normally kept illegally as pets in cages with other species in poor conditions, and that the rescue centers have a high density population. All these situations can favor the transmission of infectious agents and affect immune response to them. Animal translocations can also involve the movement of animals and their 'biological packages', which include all the macro and micro-parasites co-existing with them (Kock et al. 2010; Sainsbury et al. 2012). Most of these parasites can be harmless, but some can pose a significant threat to the conservation of free populations if wild populations have not been previously exposed to these microbes. Travis et al. (2011) documented the transmission of over 60 pathogens, many of which were zoonotic, through the movement of wildlife or their parts. One example of this is the impact that the free population of the critically endangered Echo parakeet (*Psittaculus echo*) suffered due to the Psittacine Beak and Feather Disease (PBFD) when the invasive Rose-ringed parakeet (*Psittaculus krameri*) was introduced (Kundu et al. 2012). Considering this risk, it is necessary to screen for the different pathogenic animals that may be released (Labruna et al. 2011), as well as to investigate what infectious agents are present in the free populations.

One of the most important infectious agents that may have implications on parrots' health is avian circovirus, causing PBFD, which has been detected in over 60 species of psittacine birds, both free-ranging and captive (Kundu et al. 2012). This chronic disease is characterized by symmetric feather dystrophy, beak deformities and eventual death. This disease can be acquired by contact with other captive native or exotic psittacine species (Kundu et al. 2012; Massaro et al. 2012). A second infectious agent is avian polyomavirus (APV), which causes abdominal distention, feather abnormalities known as "French molt" in fledgling and young budgerigars, or even acute death (Hirai et al. 1984). However, the degree of disease susceptibility and severity seems to be dependent on the species infected (Enders et al. 1997). An important zoonotic bacterium previously reported in captive psittacines in Colombia and Costa Rica is *Chlamydophila psittaci*, which has been classified as a bioterrorism agent in the United States due to its high transmission rate and virulence (Rodolakis & Mohamad, 2009; Sheleby-Elías et al. 2013; Federation of American Scientists, 2014;). Influenza A virus is also a zoonotic virus that can infect psittacine birds, although the acquisition of this pathogen in the wild is rare (Hawkins et al. 2006; Kaleta et al. 2007).

As mentioned before, wild parrots may acquire some other disease-causing pathogens by means of the parasites they host. Some of these ectoparasites have been identified as vectors of various pathogens responsible for causing diseases in humans and animals, such as rickettsioses and borreliosis (Anderson et al. 1986; Anderson et al. 1990; Ogrzewalska et al. 2010). Both causative agents result from obligate intracellular bacteria and have been detected in Costa Rica in human clinical samples and in ticks collected from horses, dogs and rabbits (Labruna et al. 2004; Hun et al. 2009; Hun et al. 2011; Labruna et al. 2011); however, to the best of our knowledge, neither pathogen has been identified in birds in Costa Rica.

The Yellow-naped Amazon, *Amazona auropalliata*, is listed on Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2012), and is considered as threatened in the IUCN Red List. This species is highly valued as a pet, making it one of the most poached parrot species (Wright et al. 2001). This medium-size parrot can be found from Southern Mexico to Northern Costa Rica, being the tropical dry forest its most predominant habitat. Most of the habitat throughout its distribution range has been degraded, fragmenting populations in some areas and causing decline in others (CITES 2002). The significant modification of this key habitat for the species has made it one of the most threatened (Janzen 1988).

Despite the potential implications of introducing pathogens into the wild during the release of individuals for conservation purposes, our knowledge on the health status of recipient populations is still limited. To date, there have been few studies conducted to determine the health status of captive birds in Costa Rica (Herrera et al. 2001; Sheleby-Elías et al. 2013), and evaluations of health status in wild populations are even more scarce (Xenoulis et al. 2010). The need for more research on diseases affecting wild populations of threatened and endangered species has been emphasized recently because of their significance in global species extinctions (Smith et al. 2006). The aim of this survey is to identify some infectious agents in wild parrots that have been previously detected in captive parrots in Costa Rica (PBDF, APV, and *Chlamydophila psittaci*), or that have been found in other species (rickettsioses, borreliosis and influenza virus). This study would enable us to generate knowledge about the health status of the wild populations and to strengthen the sanitary protocols of the release programs in the country.

MATERIALS AND METHODS

The study was conducted in the southernmost part of the distribution range of the Yellow-naped Amazon in the Guanacaste province of Costa Rica. The rainy season is highly seasonal, occurring from June to November and accounting for 85% of the 1656 mm average total annual rainfall for 1980–2009 in Santa Rosa National Park in the northern region. The characteristic vegetation of this area is tropical dry forest, where the landscape has been largely modified due to human activities (Edelman 1992). The northern area is dominated by cattle-ranching, with remaining patches of natural vegetation of mainly tropical dry forest in long strips along creeks embedded in an extensive grassland matrix with scattered trees (Tosi 1969; Hartshorn, 1983). The southern region is characterized by clustered remnant patches of dense riparian forest surrounded by large areas of crop fields, a human settlement, and the original highlands sub-deciduous forest. Both regions are located about 30 km apart and separated by urban/industrial areas around the town of Liberia.

Fig. 1. Location of the two study regions in northern Costa Rica.

Birds were captured at large communal roosts located in Los Ahogados (between 10°52'00''N – 85°35'00''W and 10°42'00''N – 85°28'00''W) and El Pelón de la Bajura (between 10°30'00'' N – 85°26'00'' W and 10°26'00'' N – 85°22'00'' W); previous studies identified the ecology, conservation, and communication system of these birds (Fig. 1) (Wright 1996; Salinas-Melgoza et al. 2013). The roosting area of Los Ahogados is largely dominated by cattle pasture, while El Pelón de la Bajura is mostly represented by rice and melon agriculture (Salinas-Melgoza et al. 2013). These two sites present contrasting landscapes with more dispersed forest in the cattle pasture site and more concentrated patches in the crop fields (Salinas-Melgoza & Wright 2012). At each area, birds were captured using canopy mist-nets in trees adjacent to traditional roosting areas. Playbacks of recordings of local contact calls and duets of mated pairs were used to attract the birds to the mist-nets (Salinas-Melgoza & Wright 2012; Salinas-Melgoza et al. 2013). The capture and handling of individuals was conducted under NMSU-IACUC approval (protocol 2006-027).

Birds were carefully removed from the nets, placed in cages and transported to the processing site. Once birds had acclimated to the cages, a clinical examination was performed to look for physical abnormalities, and biological samples were collected and maintained at 4°C afterward. Approximately 1% of body weight of blood was collected from the jugular or basilic vein with a syringe using a 25 ga needle; the sample was immediately placed in

an EDTA-lined microvacutainer. In addition, a secondary feather was plucked with a clean hemostat by grasping it at the base of the shaft and pulling it swiftly, and oropharyngeal and cloacal swabs were collected from every bird. Once the samples were collected, all the birds were released. The samples were transported to the School of Veterinary Medicine, Universidad Nacional, within a period of approximately one week, and were kept at -20°C until they were analyzed. All the procedures were done using proper biosecurity methods to avoid pathogen transmission among birds and investigators.

Specific polymerase chain reaction (PCR) protocols were employed for the detection of avian circovirus (Raue et al. 2004) and avian polyomavirus (Johne & Müller 1998) using whole blood and feathers. For *Chlamydophila psittaci* (Kaltenboeck et al. 1991) and influenza virus (Albonik et al. 2010), oral and cloacal swabs were analyzed using a nested PCR and a real time PCR protocol, respectively. Whole blood was used to determine the presence of *Borrelia burgdorferi* sensu lato (Rijpkema et al. 1995) and *Rickettsia* spp. (Labruna et al. 2004) by PCR.

DNA and RNA extraction of all samples was performed using the DNeasy® Blood & Tissue Kit (QIAGEN), according to the manufacturer's instructions. For detection of PBFD virus, a PCR protocol was used as described by Raue et al. (2004), which amplifies a region of 202 bp of the highly conserved orfC1 gene. Primers used were: circo-s: 5`-CGG TGC CAG AAA ATG GTA TGT TAG-3` and, circo-as: 5`-GAA GCT GAA GCC AAT GCC GTA-3`. The protocol described by Johne and Müller (1998) was used to detect APV. This protocol amplifies a segment of 310 bp of the antigen t/T of the genome, using the following primers: 5`-CAA GCA TAT GTC CCT TTA TCC C-3` and: 5`-CTG TTT AAG GCC TTC CAA GAT G-3`. PCR reactions were prepared in a final volume of 25 µl using Dream Taq™ PCR Master Mix 2X (Fermentas ®), 0.1 µM of each primer and 20 ng of DNA. Amplification program consisted of an initial denaturalization at 95˚C for 5 min, followed by 35 cycles of denaturalization (94˚C for 30 s), alignment (60˚C for 30 s), extension (72˚C for 30 s) and a final extension at 72˚C for 10 min.

For the detection of *C. psittaci*, a nested PCR described by Kaltenböck et al. (1991) and modified by Theegarten et al. (2004) was used. This procedure amplifies partially gene omp A (outer membrane protein A) to identify the genus *Chlamydia spp*. Primers used were 191CHOMP (5′-GCI YTI TGG GAR TGY GGI TGY GCI AC-3′) and CHOMP371 (5′-TTA GAA IC [GT] GAA TTG IGC [AG] [TC] IA GTG IGC IGC TT-3′). Reactions with 18.9 µL Dream Taq PCR Master Mix 2X (Fermentas), 1.0 µL of each primer (0.1 µM), 0.5 µL DNA (~20 µg), and 4.6 µL water (molecular biology grade, Fermentas) were prepared to a final volume of 25 µL. Amplification protocol consisted of an initial denaturalization at 95°C for 30 s, followed by 35 cycles of denaturalization (95°C for 30 s), alignment (50°C for 30 s), extension (72°C for 30 s), and a final extension at 72°C for 7 min. PCR products were visualized using agarose gel electrophoresis (1.4%) in TBE (Tris Base, boric acid, EDTA, pH8, 0.5 M), stained with ethidium bromide (0.5 µg/mL). GeneRuler 100 bp DNA Ladder Plus (Sm0321, Fermentas) was used as marker. All amplification products were subjected to a second PCR to identify*C. psittaci*, using the primers CHOMP 336 s (5′-CCR CAA TTT CTR GAY TTC AWY TTG TTR en GMT-3′) and 218PSITT (5′-GTA ATT TCI AGC CCA GCA CAA TTY GTG-3′), in a reaction in a similar way as described above, varying the conditions of the cycler: 95°C for 30 s followed

by 20 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 7 min. The second PCR products were visualized using agarose gel electrophoresis as described above. Samples that showed bands with weights 389–404 bp were considered positive.

In the case of influenza virus, the reverse transcription and amplification was done in one step by 7500 Real-Time PCR System (Applied Biosystems, USA), using the TaqMan, One Step RT-PCR Master Mix Reagents (Applied Biosystems, USA), with the following primers: AGA TGA GTC TTC TAA CCG AGG TCG and TGC AAA AAC ATC TTC AAG TCT CTG, and the probe FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA. Thermocycling RT-qPCR conditions were as follows: Reverse transcription reaction for 50 °C during 30 min, followed by an initial denaturalization step (95 °C for 15 min) and 40 cycles of 95 °C for 10 s, and 60 °C for 20 s, with reading of fluorescence in this step (Albonik et al. 2010). The final volume of the reaction was 25 µL, including 8 µL template, 12.5 µL master mix, 0.6 µL 40X Multiscribe, 0.5 µL of each primer (final concentrations of 400 nM), 0.5 µL of the probe (final concentration of 120 nM), and 2.4 µL water.

For the detection of *Rickettsia spp.*, a PCR protocol described by Labruna et al. (2004) was used, which amplifies a region of 401 bp of gltA gene. The primers used were *CS-78* GCAAGTATCGGTGAGGATGTAAT and CS-323 GCTTCCTTAAAATTCAATAAATCAGGAT. PCR reactions were prepared in a final volumen of 25.0 µl, using 12.5 µl Dream Taq™ PCR Master Mix 2X (Fermentas ®), 0.5 µl (10 µM) of each primer, 2 µl of DNA, 2 µl $MgCl_2$, and 7.5 µl molecular biology grade water (Fermentas®). The Amplification program consisted of an initial denaturation at 95˚C for 3 min, followed by 35 cycles of denaturation (95˚C for 20 s), alignment (50˚C for 60 s), extension (72˚C for 60 s) and a final extension at 72˚C for 7 min.

Detection of *Borrelia burgdorferi* sensu lato was performed by a nested PCR as described by Rijpkema et al. (1995), which amplifies the spacer region between the 5S and 23S rRNA genes (rDNA). The primers used were 23SN1 5´-ACCATAGACTCTTATTACTTTGAC and 23SC1 5´-TAAGCTGACTAATACTAATTACCC. Reactions with 12.5 µL Dream Taq PCR Master Mix 2X (Fermentas™), 1.0 µL of each primer (10 µM), 2 µL DNA (~20 µg), and 8.5 µL water (molecular biology grade, Fermentas®) were prepared to a final volume of 25 µL. Amplification protocol consisted of an initial denaturation at 94°C for 60 s, followed by 25 cycles of denaturation (94°C for 30 s), alignment (52°C for 30 s), extension (72°C for 60 s), and a final extension at 72°C for 5 min. All amplification products were subject to a second PCR using primers 23SN2 5´-ACCATAGACTCTTATTACTTTGACCA and 5SCB 5´-GAGAGTAGGTTATTGCCAGGG, in a reaction with proportions of reactants as described above, varying the conditions of the cycler: 95°C for 30 s followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, and 72°C for 5 min. Samples that showed bands with weights 226 bp in the second PCR by agarose gel electrophoresis were considered positive.

All PCR products were visualized by agarose gel electrophoresis (2%) in TBE (Tris Base, boric acid, EDTA, pH 8, 0.5 M) and stained with ethidium bromide (0.5 µg/ml). GeneRuler 100 bp DNA Ladder Plus (Sm 0321, Fermentas®) was used as a size marker. Samples that showed bands with the expected size were considered positive.

PBFD virus, APV and *C. psittaci* DNA were kindly provided by the Clinic of Birds, Reptiles, Amphibians and Fish, at the Justus Liebig University, Giessen, Germany and used as positive controls, while influenza virus positive control was kindly donated by the Servicio Nacional de Salud Animal de Costa Rica (SENASA), *Rickettsia felis* DNA by Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán, Mérida, Mexico and *B. burgdorferi* s.l. DNA by Centro de Investigación Biomédica de La Rioja, Spain. Molecular biology grade water (Fermentas®) was used as negative control in all the cases.

RESULTS AND DISCUSSION

Nine *Amazona auropalliata* individuals were captured between January and May 2009. The external clinical exam did not detect any abnormalities. No antigens were detected for any of the infectious agents studied.

Our results suggest that wild Yellow-naped Amazon parrots in Los Ahogados and El Pelón de la Bajura are not infected with PBDF virus, avian polyomavirus, *C. psittaci*, influenza virus, *Rickettsia* spp. nor *B. burgdorferi* s.l. These results could have implications for translocation or reintroduction as a potential conservation strategy to replenish wild *Amazona auropalliata* populations, taking into account that some of these infectious agents have been previously diagnosed in captive psittacine birds in Costa Rica (Hirai et al. 1984; Herrera et al. 2001; Dolz et al. 2013). These agents could be present in other captivity centers in Costa Rica putting in danger reintroduction attempts of these and other threatened and endangered species. In addition, factors such as the increased release of pet birds establishing feral parrot populations (Butler 2005) and potential vagrants of Yellow-naped Amazon parrots (Salinas-Melgoza et al. 2013) may increase the likelihood of putting pathogen-free populations in contact with infected individuals or even facilitating the spread of diseases to other geographic areas. If efforts to conserve species are oriented to the release of captive birds (captive-born or confiscated animals) to repopulate natural populations in Costa Rica, then appropriate protocols should be developed to ensure that infectious agents do not spread to wild populations.

As a small number of birds were captured in the present study, we were unable to establish the prevalence or absence of any infectious agent in the wild population. However, given that few disease studies are conducted in the wild because of the high costs involved, our results are valuable in providing some information to rescue centers to improve and standardize their release protocols to maintain wild populations free of infectious agents, especially considering that every center has its own protocol and that many of them work illegally in Costa Rica. The situation is even of more concern in Costa Rica since authorities do not have an updated list of the rescue centers currently in operation or actual data about the number of birds released into the wild. In the same way, this survey highlights the need to continue screening wild parrot populations to determine their health status.

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