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HEALTH-SCREENING PROTOCOLS FOR VINACEOUS AMAZONS (*AMAZONA VINACEA*) IN A REINTRODUCTION PROJECT

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Abstract: Reintroduction is a growing field in the conservation of endangered species. The vinaceous Amazon parrot (*Amazona vinacea*) is extinct in several areas, and a project to release confiscated individuals to their former range is currently underway. The objective of this study was to evaluate and improve the selection and treatment of individual release candidates by detecting possible pathogen carriers using samples taken before and during release. As part of prerelease health protocols, samples were obtained from 29 parrots on three different occasions while in captivity and once after their release. Samples were screened for paramyxovirus type 1, avian influenza, poxvirus, coronavirus, psittacine herpesvirus 1, *Chlamydia psittaci*, enteropathogenic *Escherichia coli* (EPEC), *Salmonella* spp., and endoparasites. The majority of samples returned negative results, with the exception of two individuals that tested positive for *C. psittaci* in the first sampling and for *Ascaridia* spp. in the second pooled sampling. Treatments for *C. psittaci* and endoparasites were administered prior to release, and negative results were obtained in subsequent exams. The number of positive results for *E. coli* (non-EPEC) decreased during the rehabilitation period. Adequate quarantine procedures and health examinations greatly minimize disease risks. The protocols employed in this study resulted in acceptable health status in accordance with current environmental legislation in Brazil. Additionally, protocols allowed informed decisions to release candidates, minimized risks, and favored the selection of healthy individuals, thereby contributing to the recovery of this species. It is important to determine appropriate minimum health-screening protocols when advanced diagnostics may not be available or high costs make the tests prohibitive in countries where confiscations occur. We hypothesize that a minimum panel of tests of pooled samples can serve as an alternative approach that minimizes costs and overall workload and supports projects intended to restore and promote flagship species and hamper their illegal trade.

Key words: *Amazona vinacea*, bacteria, intestinal parasites, parrots, reintroduction, viruses.

INTRODUCTION

The increasing demand for natural resources, coupled with climate change and emerging diseases, has led to growing losses in biodiversity and the disruption of ecosystems. These changes have affected both wildlife and human health and have created new challenges for professionals working in the field of conservation.[1,56]

Brazil has the largest number of parrot species in the world, and 15 of these species are classified as vulnerable to critically endangered; the numbers of several species are rapidly diminishing.[46,49] Millions of wild animals have been seized in an effort to combat the illegal Brazilian wildlife trade, and as efforts to fight this trade are undertaken, the challenge of what to do with the growing number of seized animals becomes more acute.[40] Many animals are sent to zoos and rescue centers that are already overwhelmed with previously confiscated animals and that are limited by scarce resources, which results in high mortality rates and a low quality of life.[55]

Health survey protocols of confiscated animals are vital to determine whether the animals should be kept in captivity, reintroduced, or euthanized. As part of conservation efforts, species may also be relocated, which is defined as the introduction, reintroduction, reinforcement, or translocation of populations.[26] There is a danger that relocated animals may introduce diseases into native populations and pose a severe threat to the survival of the original population as well as other organisms in the ecosystem. To maximize the chances of success and prevent disease epidemics, the source population must be free of selected pathogens, and its sanitary status must be recorded prior to any animal release.[4,21,27]

The vinaceous Amazon parrot (*Amazona vinacea*) was once found throughout eastern South America. However, populations have become isolated due to heavy deforestation and capture for illegal trade, and approximately 2,000 individuals remain in Brazil.[11,49]

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Chicks are captured to supply the trade of wild-caught birds, which reduces population numbers and negatively affects annual recruitment. Consequently, the population ages progressively, with a lower reproductive output and fewer individuals to perpetuate the species. Relocation efforts that introduce new individuals can improve a declining population, and reintroducing juvenile and/or adult individuals bypasses a critical part of the development where predation and poaching could more easily occur.[25]

Several release projects have taken place in the last few years in Brazil, and one of the first to reintroduce confiscated psittacines dates back to 1969–1970, when conures (*Pyrrhura* spp.) were reintroduced into a national park. This project relied upon the very basic diagnostic methods available when avian medicine was in its infancy. Nevertheless, the species was established and breeding was reported.[10] Nowadays, with the availability of more diagnostic tools it is possible to lower the associated risks and increase success rates.

This study aimed to screen confiscated and subsequently reintroduced vinaceous Amazons for selected pathogens (paramyxovirus type 1, avian influenza, poxvirus, coronavirus, psittacine herpesvirus 1, *Chlamydia psittaci,* enteropathogenic *Escherichia coli* [EPEC], *Salmonella* spp., and endoparasites). Animals must be screened for these agents to comply with part of the protocols established by the Brazilian Environmental Agency (IBAMA) when undertaking a release project. The objective of the study was to evaluate and improve the selection and treatment of individual release candidates by detecting possible carriers through sampling both before and after release. The importance of health-screening protocols and the challenges involved in the rehabilitation process are discussed and compared with similar efforts.

MATERIALS AND METHODS

Study area and background

Between July 2011 and February 2012, a group of 29 vinaceous Amazons were selected for reintroduction into a private, protected area of approximately 36 hectares, with 90% of the area composed of Atlantic Rainforest (23°55′57″S, 47°4′1″W, Juquitiba, Southeast Brazil). The species has been declared locally extinct in this area for at least 30 years, and an effort to re-establish a population in the species' historical range is currently underway.

This study was approved by the Animal Ethics Committee of the University of São Paulo, and permits were issued by the appropriate environmental authorities.

During the selection process, birds were chosen according to their behavior as well as physical and laboratory health screenings. All birds had been confiscated and had been previously maintained as illegal pets; the birds were of undetermined ages, but all were considered adults based on sexual behavior and their history of being kept in captivity for at least three years.

Sample collection

A sampling regimen was established to obtain paired samples and detect carriers using sterile swabs (CultureSwab, Becton Dickenson and Company, Sparks, Maryland 21152, USA) of cloacal contents and feces.

Individual cloacal swabs from all 29 individuals were obtained on three separate occasions while the animals were still in captivity (at 120 days, 60 days, and 14 days before release). Fecal samples were collected in an enclosure and after release (feces shed over a sheet cover under the acclimation flight and around supplemental feeders).

Positive controls

Positive controls for the polymerase chain reactions (PCR) consisted of available vaccines for farm and pet animals (paramyxovirus type 1, influenza type A, coronavirus, poxvirus, *Chlamydia psittaci*; Merial, Campinas, São Paulo 13091908, Brazil) and field strains available from PCR positive clinical cases (*Salmonella* spp. and psittacine herpesvirus 1). Strain E2348/69 was used as the positive control for EPEC. PCR amplifications were performed using a 2720 Thermal Cycler (Applied Biosystems, São Paulo, São Paulo 04311900, Brazil).

Bacterial testing

Samples for bacterial testing were stored at 10°C until processing (within 2 wk of collection), during which a swab was dipped in 10 ml tetrathionate broth (Difco, Becton Dickenson and Company, Sparks, Maryland 21152, USA) to test for *Salmonella* spp. or 5 ml brain and heart infusion broth (Difco, Becton Dickenson and Company, Sparks, Maryland 21152, USA) to test for *E. coli*; the samples were then incubated at 37°C for 24 h.

To test for *E. coli,* samples were streaked onto MacConkey agar plates (Difco) and incubated at 37°C for another 24 h. Isolated colonies were then identified using a biochemical kit (Newprov, Pinhais, Paraná 83323020, Brazil). A suspension of three *E. coli* colonies was prepared in 200 µl phosphate-buffered saline (PBS, pH 7.2) and stored at –20°C.

No positive cultures were obtained during *Salmonella* testing; therefore, no subsequent isolated colonies were retrieved. However, PCR analysis was also performed on negative samples cultured in tetrathionate broth to increase sensitivity and possibly detect organisms missed by the standard culture technique.[44] After incubation, samples were extracted from the broth, and 1 ml was centrifuged at 12,000 *g* (5 min). Finally, 200 µl of the supernatant was selected for DNA extraction and stored at –20°C.

Extraction of bacterial DNA was performed according to previously described protocols, with 200 µl from each sample.[6] PCR was used in accordance with previous studies for detecting adhesions (*eae*/*bfp* genes) of EPEC and the invasion gene (*inv*A) of *Salmonella* spp.[3,38]

Viral and chlamydial testing

A 200 µl PBS suspension was made from the second swab of all participants in the study. The suspension was then used to test for viral particles and *Chlamydia* using reverse transcriptase PCR (RT-PCR) and PCR. Samples were centrifuged at 12,000 *g* (30 min), and 100 µl of the supernatant was selected for DNA extraction (*C. psittaci,* poxvirus, psittacine herpesvirus 1) according to previously described protocols.[6] Additionally, 100 µl was used for RNA extraction (avian influenza, paramyxovirus type 1, coronavirus) using TRIzol© (Invitrogen, Carlsbad, California 92008, USA).

Poxvirus PCR was performed using the methodology for detecting the conserved region of the 4b gene.[30] Seminested protocols were performed to detect psittacine herpesvirus UL16/17 genes and the conserved MOMP gene of *C. psittaci*.[9,54] RT-PCR was used to detect the conserved matrix gene of type A influenza, the cleavage fusion protein of paramyxovirus type 1, and the adapted PCR conditions for the conserved gene region shared by coronavirus types 1, 2, and 3.[17,37,53] Amplified PCR products were separated using agarose gel electrophoresis (1.5%) with ethidium bromide, and fragments were visualized using UV transillumination.

Fecal testing

Pooled fecal samples were collected by spreading plastic sheets below preferred perches while birds were in captivity and around supplemental feeders after release. The researcher remained at an observation point while samples were collected during the first feeding of the day (approximately 40 min in the early morning); care was taken to differentiate feces shed by the reintroduced vinaceous Amazons and not from small passerines or other parrot species (determined by assessing consistency and size). Prior to this project, the vinaceous Amazon was locally extinct in this area, and the only other parrot species visiting the region at the time of this study was the maroon-bellied parakeet (*Pyrrhura frontalis*), a species that passes very distinguishable small-sized feces and did not use the supplemental feeders. Generally, the number of retrieved feces samples was related to the number of birds visiting the feeders (postrelease) or remaining in the sampling area; an average pool of 10 to 15 fecal samples could be obtained each day.

To detect endoparasites both during acclimation and after release, samples were stored in a 10% formaldehyde solution and maintained at room temperature for a maximum of 2 days (until they arrived at the laboratory). Standard methods involving Sheather's sucrose and zinc sulfate fecal flotation with centrifugation were used to screen for the presence of parasites and ova.[47]

Pooled feces were collected for bacteriological and viral testing postrelease using sterile swabs. The samples were stored for up to 2 wk at 10°C for bacteriological testing and –20°C for viral testing and were processed using the same bacteriological and molecular methods used for individual cloacal swabs, as described above.

RESULTS

A total of 87 individual cloacal samples and three nonindividually characterized pooled fecal samples were obtained during captivity, and one pooled fecal sample was obtained postrelease.

Viral diseases or enteropathogenic *E. coli* were not identified. *Salmonella* cultures and PCR of the cultured tetrathionate broth yielded negative results. Appropriate-sized amplicons were amplified from the positive control samples.

Eight samples tested positive for *E. coli* (non-EPEC) in the first sampling, seven in the second sampling, and two at 14 days before release. No samples tested positive after release (Table 1).

Table 1. Results of pathogen testing during the rehabilitation period and postrelease of vinaceous Amazons (*Amazona vinacea*) in Brazil. Numbers denote positive test results and the total number of samples tested.

Pathogen	Test	Captivity (individual sample)			Release (pooled sample)
		4 months before release	2 months before release	2 weeks before release	
Escherichia coli	Culture	8/29	7/29	2/29	0
Salmonella spp.	Culture	0/29	0/29	0/29	0
Chlamydia psittaci	PCR	2/29	0/29	0/29	0
Avian influenza, paramyxovirus type 1, psittacine herpesvirus 1, coronavirus, poxvirus	PCR	0/29	0/29	0/29	0
Endoparasites (pooled sample)	Fecal flotation and concentration	0	Positive for *Ascaridia* spp.	0	0

C. psittaci was detected in two samples, and only in the first sampling. These birds were treated with doxycycline (200 mg tablets, Ourofino, Cravinhos, São Paulo 14140000, Brazil; 50 mg/kg p.o. q.d., for 40 days[32]), and their samples tested negative after the second testing.

Of the pooled fecal samples obtained to test for intestinal parasites, *Ascaridia* sp. was detected during the second sampling while the birds were in captivity. All Amazons were subjected to a single course of ivermectin (10 mg/ml suspension, Merial, Campinas, São Paulo 13091908, Brazil; 0.2 mg/kg p.o., once[32]), and subsequent testing during the acclimation period and postrelease revealed no further positive results.

The results of bacterial, viral, and endoparasite testing are described in Table 1.

DISCUSSION

Throughout the world, the release of many species of endangered parrot has been documented, with most projects reporting successful results demonstrating the adaptation of released individuals and several cases of reproduction, thereby contributing to species recovery.[5,12,28,29,34,43,48,50] Even projects that were not successful provided experience and information that improved future attempts. Unfortunately, health-screening protocols are not usually described in detail or often differ among projects, which makes it difficult to compare the methodology used and results obtained in this study with those of previous studies.

One well-described report involving the release of Amazon parrots included yellow-headed Amazons (*Amazona oratrix*) and red-crowned Amazons (*Amazona viridigenalis*).[35] The released group was composed of captive-bred and confiscated birds. The birds were tested for avian influenza, paramyxovirus type 1 (using serological assays), psittacine herpesvirus 1 and *C. psittaci* (using both DNA and serological tests), endoparasites (using fecal exams), and cloacal cultures, as well as other examinations. The results were negative for avian influenza, paramyxovirus type 1, psittacine herpesvirus 1, and endoparasites. Nonsignificant titers were initially detected for *C. psittaci* (based on the low titers obtained and for a handful of individuals), but the results of a paired test 3 wk later were negative. *Escherichia coli* of an undetermined strain was detected in one sample. The survival of several individuals also included the reproductive success of a pair of *A. oratrix*.[35] Similar to the approach used here in the present study involving *A. vinacea,* the previous study also selected birds based on their health status and provided guidance for the re-evaluation of certain individuals when deemed necessary, after which these individuals were retested. The protocols employed here for *A. vinacea* ensured an acceptable health status of the birds according to the current environmental legislation in Brazil prior to their release, minimizing the risk of disease spread and favoring the selection of healthy individuals. Because carrier birds under stressful conditions, such as release, can manifest clinical signs more easily (particularly during the breeding season), we hypothesize that the selection of healthier candidates contributed to higher survival and success rates after reintroduction and, as a result, contributed to the recovery of the species in the area, as evidenced by the breeding attempts by two pairs within 8 mo of release; three fledglings from one pair have also been reported.

In Brazil, several releases have been performed, a number of which followed the requirements of the Brazilian legislation, which includes testing for the pathogens surveyed in the present study.[24,29,45] The results of this study and several

others that have not been published in peer-reviewed journals suggest that basic testing protocols (such as those used in the present study) and good husbandry standards are sufficient to prevent outbreaks or the introduction of diseases in wild populations in most Brazilian projects. However, some projects have not followed even basic health-screening protocols, which are important to avoid exposing populations of existing species to possible pathogens and preventing negative attitudes toward such efforts.[13]

Although animals previously kept as pets, and confiscated birds in particular, are considered by some to be less desirable participants in relocation projects, several studies have determined that when the causes for a species' vanishing numbers are appropriately addressed, behavioral, genetic, and health issues can be overcome as part of a reintroduction project.[7,29,35,43,49,50]

In the present study, all birds had been poached as chicks and maintained as pets, possibly exposing them to pathogens, such as those commonly found in the clinical avian practice, while in captivity. The present study included tests for common pathogens in an attempt to minimize potential risks; the results suggest that as long as these questions are properly approached, reintroduction remains a viable conservation tool.

Prior to their transfer to a new facility, the birds in this study were fed from food bowls on the floor of an enclosure with a sand substrate. This feeding approach may have contributed to the positive *E. coli* results (not of the EPEC pathotype) at the beginning of the study. Before the birds in the study group were transferred to the present project, there had been many mortalities in the group from which they were taken, which originally included over 40 individuals. The deaths were determined by necropsy and laboratory analysis to be mainly related to a massive infestation of intestinal parasites and enterobacterial infections caused by *E. coli,* which is why these pathogens were given special attention in this study. It is well known that outdoor aviaries in which birds have access to the floor can allow organic material to build up and act as a route of transmission for reinfection. This is especially important for grain- and/or fruit-eating birds such as parrots, which can be severely affected by opportunistic infections and whose intestinal microflora do not normally harbor microorganisms related to decomposing organic material, such as *Enterobacteriaceae*.[22,33] At the new aviary, the birds were kept in a large suspended flight aviary and fed using hanging food bowls.

As long as appropriate husbandry procedures are employed, healthy parrots are able to eliminate gram-negative bacteria on their own.[19] In the present study, we have observed a similar occurrence with only two samples testing positive for *E. coli* during the third sampling and all pooled samples testing negative during the release period. However we do not know if these samples are representative of the whole released flock and therefore cannot state if the final results showed a significant *E. coli* reduction. Another study chose to treat birds testing positive for *E. coli* and *Salmonella* spp. before release (although for the latter, the positive bird was not released), and the results suggest that the treatment was effective and appropriate.[31]

Reports on endoparasites in Neotropic free-ranging parrots suggest that wild parrots are seldom parasitized and that the presence of parasites not found in a wild population should be eliminated using appropriate treatment prior to relocation.[2,52,57] The positive *Ascaridia* sp. result obtained during our second sampling also shows the importance of paired sampling and led to the decision to administer therapeutic treatment.

No positive viral PCR results were obtained in the current study, indicating that the birds were not shedding these pathogens from the intestinal tract at the moment of sampling. When tested using serological and DNA tests, healthy free-ranging parrots show a very low prevalence (or absence) of several of the viral agents surveyed in this study, although the two positive *C. psittaci* tests indicate that the birds may still be carriers for other agents.[14,18,52]

PCR results testing for *C. psittaci* in cloacal samples of healthy wild hyacinth macaw (*Anodorhynchus hyacinthinus*) and blue-fronted Amazon (*Amazona aestiva*) chicks were positive in 26.7% and 6.3% of samples, respectively.[39] A continuation of this study using wild hyacinth macaw chicks reported that 12.4% were carriers of psittacine herpesvirus.[20] In a separate study using Lear's macaws (*Anodorhynchus leari*), one sample tested positive for an EPEC strain in an asymptomatic chick.[42] A reintroduction project must determine the importance of such positive results and evaluate whether an individual would remain as a release candidate after treatment (if available for that agent) and retesting. In the current study, the birds that tested positive for *C. psittaci* were negative after the treatment course, and pooled samples after release were also negative. Generally, during stressful situations (such as those faced by newly released animals), there is a higher

risk of pathogens spreading and microorganisms shedding. In this study, therefore, birds testing positive for *C. psittaci* were treated even though wild parrot populations can include *C. psittaci* carriers.[39] The same decision was made with respect to the positive endoparasite pooled samples.[14] We decided that removing asymptomatic individuals that had positive initial test results (and tested negative after treatment) would decrease the founder population, and these individuals could not be easily replaced.

Previous surveys to detect potential pathogens in parrots in the release area were unavailable due to the inaccessibility of the region and the general scarcity of nest sites identified for parrot species. There were samples from newly confiscated wild-caught parrots close to the release area, but the information was limited. Therefore, the data available on other regions and species of parrots in Brazil and abroad were used as a basis for both the current study and several related government regulations.

Serological surveys, such as supplementary testing to determine previous exposure to viruses and *C. psittaci* and comparisons of paired titers, could be added to the screening protocols. However, these tests are not currently available in Brazil for some of the tested agents or have not been officially validated on nonpoultry species.

It must be recognized that there is no realistic way of ensuring that relocated wild animals are free of pathogens, completely removing the possibility of occurrence of diseases, yet having zero tolerance prevents important programs from progressing.[4,35] For instance, for some time, captive parrot breeding programs designed to boost declining populations were considered to carry too many risks, with the main fear being that diseases contracted in captivity would spread to the endangered wild counterparts.[15] This was especially true with the Puerto Rican Amazon (*Amazona vittata*), whose wild population reached an all time low of 13 individuals in the early 1970s.[51] A captive breeding and release program was initiated even though there were disease risks, and the extreme concern proved unnecessary, provided that basic husbandry standards and periodic health testing (and improvements based on prior results) were applied for the groups of release candidates. As a result of these efforts, the Puerto Rican Amazon species numbers have increased considerably and the successful project is ongoing.[16] Other examples of successful reintroduction utilizing confiscated and/or captive bred parrots include the yellow-shouldered Amazon (*Amazona barbadensis*) and golden-capped parakeet (*Aratinga auricapillus*), which involved similar practices and reported no disease outbreaks.[29,43]

The success observed in the present project is encouraging. It has resulted in the recolonization of the area and has promoted continuity in the release program by reinforcing the now-existing initial population. The project also creates new opportunities for further expanding the project size and its influence on further habitat protection; there are plans for the acquisition of additional private lands for conservation.

Some authors have developed risk-assessment strategies for reintroductions based on statistical computer models designed to evaluate disease risks (among others). Although we have not tested these models in the present study, population viability analysis (PVA) can be a valuable tool in determining the risk of disease epidemics.[4] However, PVA provides limited data information accuracy due to the complexity of the interactions among the several risk factors involved. Nonetheless, these models can be used to inform management decisions, as well as adequate quarantine procedures, and appropriate health examinations, which greatly minimize disease risks on managed metapopulations, as observed in the present study.[23,57]

It should be noted that "diseases" is used as a broad and common term, but there should be a more specific assessment regarding whether a certain microorganism can become pathogenic; all environments contain microorganisms that can potentially cause disease if the conditions are favorable.[36] Thus, careful risk analysis should be an inherent component of all conservation programs.[4]

Other studies on the health screening of confiscated parrots have chosen particular protocols based on accessibility, pathogen prevalence, and available resources, which have worked well for these release efforts.[8,31,35,41] In the current project, basic husbandry improvements, quarantine, and testing were sufficient to meet the required standards and reduce associated risks.

The details of a minimum satisfactory health-screening protocol, in terms of the information needed to select, medicate, and reject individuals, must be considered. The tests employed in the present study were considered appropriate according to the Brazilian environmental legislation and provided guidance for improving husbandry and determining medical treatment while minimizing the possibility of pathogen introduction.

We propose that a minimum number of tests screening for the reportable diseases of agricultural and/or zoonotic importance that are generally required by national authorities (influenza, paramyxovirus type 1, *Salmonella* spp.) and basic screening for common pathogens affecting confiscated parrots (*C. psittaci*, psittacine herpesvirus, *E. coli,* and endoparasites) provide a relatively fast and economical means (providing they are performed on pooled samples and, if needed, retested individually in the case of positive results) of evaluating many individuals for reintroduction projects. Other agents that are not commonly encountered and/or are tested mostly for scientific interest (e.g., poxvirus, coronavirus) or that are known to be endemic to or more common in a certain geographical region or species (e.g., bornavirus, circovirus) may or may not be included depending on the importance and local characteristics of each project.

The authors also recognize that most confiscations take place in countries where state-of-the-art laboratories for molecular tests are not readily accessible or where even the basic associated costs make such testing impossible. By connecting local universities, researchers, and conservationists, we were able in this study to decrease costs and the overall workload, enabling a combined effort to support a continuous project intended to restore a species and hamper its illegal trade. This protocol might be applied in other South American countries and elsewhere where parrot populations are declining and their restoration as a flagship species can spearhead the protection of an entire habitat that contains many other endangered species.

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