Molecular diagnosis of *Salmonella* species in captive psittacine birds

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Cloacal swabs were collected from 280 captive psittacine birds belonging to 13 species. Samples of DNA were tested by PCR using a pair of primers that amplify a 284 base pair fragment of the *Salmonella* genus *inv*A gene, and the PCR-positive samples were tested by standard microbiological techniques. Thirteen per cent of the samples were positive by PCR, but negative by microbiological techniques. The infection rates were significantly different among the 13 species, the most commonly infected being *Amazona amazonica* (28 per cent) and *Amazona pretrei* (20 per cent). Specific tests for *Salmonella* Typhimurium *Salmonella* Enteritidis, *Salmonella* Pullorum and *Salmonella* Gallinarum did not produce positive results.

PSITTACINE birds are subject to many bacterial diseases, particularly those that affect the respiratory and gastrointestinal tracts. The main bacteria involved in gastrointestinal diseases are enterobacteria, especially *Escherichia coli*, *Salmonella* species and *Yersinia* species, all of which also affect other avian and mammalian species (Dorrestein and others 1985). *Salmonella* species are primary pathogens, with many serovars that are able to break the mucous membrane immune barrier and infect a great variety of birds, mammals and reptiles (Reavill 1996). Avian salmonellosis constitutes a group of acute or chronic diseases induced by *Salmonella enterica* subspecies *enterica*, which can be divided into three serovar groups: pullorum disease, caused by *S enterica enterica* serovar Pullorum; fowl typhoid, caused by *S enterica enterica* serovar Gallinarum; and paratyphoid salmonellosis, caused by a group of different *S enterica* serovars that are also related to those implicated in food contamination. Paratyphoid *Salmonella* serovars can infect a wide range of wild birds, but their susceptibility and the occurrence of asymptomatic carriers vary greatly among species (Gerlach 1994, Dorrestein 1997). Gast (2003) pointed out that information about the prevalence and distribution of *Salmonella* serovars is essential to identify reservoirs that could be responsible for the transmission of salmonellosis from populations of wild and domestic birds to other species, including human beings. Many paratyphoid *Salmonella* serovars are responsible for infections in wild birds, very young birds being mainly affected and suffering high mortality. Clinically normal and infected birds that have survived the disease can be carriers of these bacteria, with the serovar *Salmonella* Typhimurium being the most frequent both in pet birds (Dorrestein 1997) and wild birds (Reavill 1996).

This study was performed to detect the occurrence of *Salmonella* species in captive macaws and parrots by means of a PCR.

MATERIALS AND METHODS

Samples and DNA extraction

The birds sampled were kept in Rio Grande do Sul, the southernmost Brazilian state (30°02' S), in aviaries registered and licensed by the Brazilian Institute of Environment and Natural Renewable Resources. They were identified by numbered leg rings and kept in pairs (a male and a female) in suspended cages, which were not entered by caretakers; commercial food and water were available ad libitum. Cloacal samples were taken with sterile swabs from 280 birds of 13 species (Table 1). The bird were captured in a net and handled with leather gloves. The procedures were carried out according to the principles of veterinary medical ethics (Redevet 2001) and the international guiding principles for biomedical research involving animals (Council for International Organizations of Medical Sciences [CIOMS] 1985).

The samples were immediately placed in 10 ml 1 per cent buffered peptone water (Merck) as transport medium. They were enriched by incubation at 37°C for 18 to 24 hours, and 100 µl aliquots of the enriched medium were transferred to 10 ml selective Rappaport-Vassiliadis (RV) broth (Merck) and incubated at 42°C for 24 hours (Oliveira and others 2002). Two 1 ml aliquots were used for the extraction of DNA by the phenol-chloroform method (Sambrook and Russell 2001). Cultures of *Salmonella* Enteritidis and *S* Typhimurium were used as positive controls, and *Staphylococcus epidermidis* was used as a negative control.

DNA analysis

Three sets of primer pairs were used. All the samples were first tested to identify *Salmonella* species by using PCR primers based on the *inv*A gene (Rahn and others 1992). The positive samples in the first test were then analysed to detect the *fli*C gene of *S* Typhimurium (Soumet and others 1999) and the *sef*A gene of *S* Enteritidis, *S* Pullorum and *S* Gallinarum (Doran and others 1996). The PCR was carried out according to the protocol described by Oliveira and others (2002). The amplification products were analysed by electrophoresis in 1·2 per cent agarose gels containing 5 mg/ml ethidium bromide and using a 100 base pair (bp) ladder as a molecular weight marker.

Sequencing of amplification products

To confirm the positive PCR results, the amplicons obtained from one *Ara ararauna*, one *Amazona aestiva* and one *Amazona amazonica* were sequenced (both strands) by using the ABI PRISM 310 system (Perkin-Elmer). The products were prepared with the ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit with the appropriate PCR primers, and were purified with the PCR Product Pre-Sequencing Kit (Amersham Pharmacia Biotech Life Science). Clustal software (Thompson and others 1997) was used for the alignment of the sequences (284 bp fragment) with one *S enterica enterica* group 1 DNA sequence (GenBank accession number U43238).

Microbiological tests

The PCR-positive samples were tested by standard microbiological techniques as described by Oliveira and others (2002).

Resampling of birds

Three months later, the birds whose samples were PCR-positive for *Salmonella* species were resampled to determine the presence of any persistently infected individuals by tak-

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TABLE 1: Numbers and percentages of different species of psittacine birds that were PCR-positive for salmonellae

Species	Popular name	Number sampled	Number PCR-positive for *Salmonella* (%)
Anodorhynchus leari	Lear's macaw	4	1 (25)
Ara ararauna	Blue and yellow macaw	56	4 (7·1)
Ara macao	Scarlet macaw	3	0
Ara chloroptera	Red and green macaw	8	1 (12·5)
Amazona aestiva	Blue-fronted parrot	163	22 (13·5)
Amazona amazonica	Orange-winged parrot	25	7 (28)
Amazona pretrei	Red-spectacled parrot	10	2 (20)
Amazona vinacea	Vinaceous parrot	1	0
Amazona farinosa	Mealy parrot	4	0
Myiopsitta monachus	Monk parrot	1	0
Pyrrhura frontalis	Maroon-bellied parrot	3	0
Pionus maximiliani	Scaly-headed parrot	1	0
Pionus menstrus	Blue-headed parrot	1	0
Total		280	37 (13·2)

ing fresh cloacal swabs by the same methods and reanalysing them by PCR and standard microbiological techniques.

Statistical analyses

The proportions of positive samples obtained from the different species of birds were compared by Fisher's exact test, using SPSS for Windows software (version 10.0.5).

RESULTS

Thirty-seven of the 280 birds (13·2 per cent) were PCR-positive for *Salmonella* species (Fig 1, Table 1), but none of them was positive by standard microbiological techniques. The PCR-positive samples were retested by using a duplicate DNA sample, and two of these samples produced negative results. The proportions of the different species of birds that were infected differed significantly (P=0·015); the most commonly infected were *A amazonica* (28 per cent) and *Amazona pretrei* (20 per cent). Very few specimens of some species were tested, and these were PCR-negative except for one of four specimens of *Anodorhynchus leari*.

The specific analyses for *S* Typhimurium, *S* Enteritidis, *S* Gallinarum and *S* Pullorum applied to the 37 PCR-positive samples gave negative results.

The sequence analyses of the *inv*A gene applied to one *Ara ararauna*, one *A aestiva*, and one *A amazonica* were 100 per cent homologous with *S enterica enterica* group I sequences (*S* Gallinarum [U43273], *S* Dublin [U43272] and *S* Typhimurium [AE016843]).

When the 37 PCR-positive birds were resampled three months later, the PCR test was negative in 36 of them and positive in one, but all the birds were negative by standard microbiological tests.

DISCUSSION

To the authors' knowledge, this is the first report of the use of PCR to detect *Salmonella* species in captive psittacine birds. The prevalence of *Salmonella* (13·2 per cent) was higher than the prevalences reported for other captive psittacine and non-psittacine birds by using different methods. The high value in these clinically healthy birds was probably due to the greater sensitivity of PCR than standard bacteriological techniques, which gave consistently negative results in the PCR-positive birds. The results agree with the results of Tuchili and others (1995) and Oliveira and others (2002) who found that PCR was more sensitive than microbiological techniques for detecting *Salmonella* in poultry.

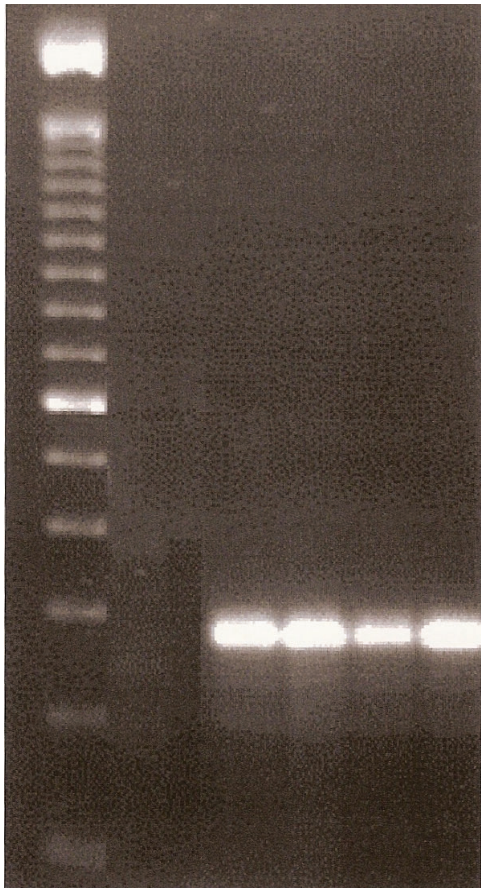

FIG 1: Agarose gel electrophoresis of PCR amplification products. Lane 1 100 base pair (bp) DNA ladder, Lane 2 negative control, Lane 3 DNA-positive control: *Salmonella* Typhimurium, Lanes 4 to 6 Positive psittacine samples

Studies using microbiological methods to determine the prevalence of *Salmonella* in the viscera of wild non-psittacine birds with clinical signs of salmonellosis detected the bacteria in between 0·6 per cent and 27·3 per cent of the samples (Wilson and MacDonald 1967, Goodchild and Tucker 1968, MacDonald and Brown 1974, Panigrahy and others 1979, Okoh and Onazi 1980, Euden 1990, Quessy and Messier 1992, Mikaelian and others 1997, Hudson and others 2000, Ward and others 2003, Pennycott and others 2006). In a study with postmortem samples, Panigrahy and others (1979) reported a *Salmonella* prevalence of 7·8 per cent in psittacines, while Dorrestein and others (1985) reported 1·7 per cent with the same sample design. In cloacal samples from live birds, Goodchild and Tucker (1968), Kirkpatrick and Trexler-Myren (1986), Brittingham and others (1988), Palmgren and others (1997), Reche and others (2003), and Huybens and others (2006) detected *Salmonella* by standard microbiological techniques in between 0 and 7 per cent of clinically healthy birds.

Using serological methods in live captive psittacine birds, Grimes and Arizmendi (1992) detected 1·6 per cent seropositive for *S* Typhimurium, while Karesh and others (1997) found 32 per cent of the live wild macaws seropositive for *S* Pullorum. Allgayer and others (2002) found no *S* Pullorum-seropositive birds among 60 captive live psittacines, but when they were analysed by PCR three of them were positive for *Salmonella* species. These differences in prevalence could be due to various factors, such as differences in susceptibility, nutritional and clinical status (diseased or healthy), habitat (urban or natural, captive or wild), whether the birds were alive or dead, or the type of sample analysed (viscera, cloacal swabs or sera), and the specificity and sensitivity of the diagnostic method.

According to Gardner and others (1996), when a serological test validated for a domestic species is used on a wild species, differences in pathogenicity between strains and serovars of the same pathogen and cross-reactions that occur when a bird is exposed to a similar antigenic structure could produce false-negative or false-positive results; the test should therefore be validated in the wild species before it is used to investigate the prevalence of the infection. Grimes and Arizmendi (1992) and Karesh and others (1997) did not mention whether the serological tests they used had been validated for wild birds, and it is therefore possible that the constraints discussed above may have influenced their results. Several authors have suggested that *S* Pullorum is typical of domestic birds and could not be found in wild birds unless they were in close contact with infected domestic birds (Garcia and Schönhofen 1982).

In the present study, when the positive samples were retested in a second aliquot, the prevalence of *Salmonella* species decreased from 13·2 per cent to 12·5 per cent, indicating that although the birds were showing no clinical signs, most of them were excreting *Salmonella* species. This finding provides evidence of the reliability of the molecular methodology applied. The PCR test using specific primers for poultry disease produced negative results. According to Oliveira and others (2002), the PCR method based on the *inv*A gene can detect as few as two cells of *S* Typhimurium in positive samples, and eight cells of *S* Enteritidis, but requires $1{\cdot}2 \times 10^3$ cells of *S* Gallinarum and $1{\cdot}8 \times 10^5$ cells of *S* Pullorum. When using serovar-specific primers, the method would produce positive results with seven cells of *S* Typhimurium, $1{\cdot}2 \times 10^3$ cells of *S* Enteritidis, $4{\cdot}4 \times 10^7$ cells of *S* Gallinarum and $1{\cdot}8 \times 10^6$ cells of *S* Pullorum. However, Hudson and others (2000) demonstrated that the *sef*C fimbrial gene (present in the last three serovars) is absent from samples taken from wild birds, and that the strains of *S* Typhimurium isolated from such birds are similar to those of domestic birds in relation to their adherence and invasion genes. Furthermore, Reavill (1996) and Dorrestein (1997) have shown that *S* Typhimurium is the serovar most frequently isolated from the viscera of both pet and wild birds, while Garcia and Schönhofen (1982) have shown that *S* Enteritidis, *S* Gallinarum and *S* Pullorum were isolated only from wild birds with clinical signs that had had contact with either human or domestic birds' waste. The negative PCR results for these three serovars were therefore probably due to the absence of these bacteria in the psittacine birds examined, which had had no contact with human or domestic birds' waste; however, it is possible that it could be due to the low sensitivity of the specific PCR method. The failure to detect *S* Typhimurium was probably related to the fact that the birds tested were healthy.

The choice of primers for the detection of *Salmonella* species is supported by studies with domestic birds (Rahn and others 1992) and wild birds (Hudson and others 2000). By using the *inv*A gene primers, those authors were able to amplify the expected PCR product from all samples that were positive by standard microbiological techniques. Malorny and others (2003) proposed that this set of primers based on the *inv*A gene should be used as an international standard.

The negative results obtained from the samples collected three months later suggest that the bacteria may be being excreted intermittently, as indicated by Mikaelian and others (1997) and Gerlach (1994). According to these authors, non-virulent salmonellae can frequently colonise the intestines and cause subclinical infections during which the bacteria are released intermittently. Furthermore, paratyphoid aviary infections are almost always subclinical. The results of the sequence analyses of the three PCR-positive products obtained with the *inv*A gene primers confirm the presence of *Salmonella* species in the birds. The high prevalence of *Salmonella* species detected in these healthy psittacine birds suggests that the PCR method is more sensitive for detecting salmonellae than standard microbiological techniques (Oliveira and others 2002). The PCR method might be useful for verifying the presence of a *Salmonella* species infection in captive birds, reducing the number of false-negative results. This could help to avoid the infection of human beings and other animals with salmonellosis by carriers, as well as the reintroduction of potentially *Salmonella*-disseminating birds into their natural environment. Further studies will be needed to identify the serovars infecting captive psittacine birds and to clarify the dynamics of *Salmonella* species infections.

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