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DETECTION OF *Salmonella* IN CAPTIVE SNAKES BY TRADITIONAL ISOLATION AND SPECIFIC PCR

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The snakes constitute a group of reptiles used in different purposes, as pets, adornment, in the human feeding, skin exploitation and researches. The salmonelosis is an enteric illness of variable severity in humans and animals, with high morbidity and economic impact. The aim of this study were to estimate the prevalence of *Salmonella* spp. in captive snakes using the standard microbiological method and variations of Polymerase Chain Reaction (PCR). Thirty one fecal samples from snakes maintained in captivity in the Laboratory of Vertebrate Zoology of State University of Santa Cruz and in Serpentarium of the Executive Commission of Cocoa Crop Plan (CEPLAC) in Ilhéus/BA were collected. The samples were analyzed with traditional procedures for the prevalence of *Salmonella* spp. and molecular method using specific primers ST11 and ST15. Through PCR, using three types of samplings, (i) direct bacterial pool, (ii) enriched pool in peptone water buffered and (iii) amplification of the pure culture, were considered positive 55% in the direct bacterial pool, 65% in the enriched pool in peptone water buffered and 100% in the bacterial pure culture DNA. Using the standard method of isolating 58% were positive, being 39 *Salmonella* strains isolated.

Key words: Salmonellosis, reptile, microbial diagnosis, PCR.

Detecção de *Salmonella* em serpentes de cativeiro por isolamento tradicional e PCR específico. As serpentes constituem um grupo de répteis usado para diferentes finalidades, como animais de estimação, na alimentação humana, na exploração da pele e em pesquisas diversas. A salmonelose é uma doença entérica de gravidade variável nos seres humanos e animais, com alta morbidade e impacto econômico. O objetivo deste estudo foi estimar a prevalência de *Salmonella* spp. em serpentes cativas utilizando o diagnóstico microbiológico tradicional e variações da Reação em Cadeia da Polimerase (PCR). Foram coletadas trinta e uma amostras fecais de serpentes mantidas em cativeiro no Laboratório de Zoologia da Universidade Estadual de Santa Cruz e Serpentário da Comissão Executiva do Plano de Lavoura Cacaueira (CEPLAC), em Ilhéus/BA. As amostras foram analisadas com procedimentos tradicionais para a prevalência de *Salmonella* spp. e foram confirmados por métodos moleculares utilizando-se os primers específicos ST11 e ST15. Para a realização da PCR foram utilizadas três tipos de amostragem: (i) amostra direta, (ii) amostra enriquecida em água peptonada tamponada e (iii) amostra da cultura pura. Foram considerados positivos para *Salmonella* spp. 55% na amostra direta, 65% no pool enriquecido em água peptonada tamponada e 100% nas amostras de cultura pura. A partir do isolamento tradicional 58% das amostras analisadas foram positivas, sendo isoladas 39 cepas de *Salmonella* spp.

Palavras-chave: Salmonelose, répteis, diagnóstico microbiológico, PCR.

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Introduction

Cold-blooded animals harbor a wide variety of *Salmonella* serotypes in their gastrointestinal tracts, even simultaneously (Woodward et al., 1997; Burnham et al., 1998; Willis et al., 2002), being well-known as non-symptomatic carriers of *Salmonella* spp. (Chiodini, 1982; Ramsay et al., 2002). Over 90% of free-living as well as domesticated reptiles have been shown to be reservoirs of *Salmonella* and shed it in their feces (Chiodini & Sundberg, 1981; Woodward et al., 1997; Willis et al., 2002; Burnham et al., 1998), with different serotypes can be isolated from a single reptile (Ward, 2000). Researches indicate that *Salmonella* in reptiles is more virulent and invasive to humans than strains carried by others host (Scott & Foster, 1997; Mermin et al., 1997). However, it is still unclear whether the snakes become colonized by contaminated food or whether transmission occurs during pregnancy, birth and/or contact. Schröter et al. (2006), reported vertical transmission between captive newborn snakes fed with mice *Salmonella*-free, showing that just food is not the only factor for transmission of *Salmonella* in reptiles.

It is known there is a high proportion of reptiles carrying *Salmonella* spp. in the enteric tract without clinical manifestation and that elimination can be either continuous or intermittent (Chiodini & Sundberg, 1981; CDC, 1995; Scott & Foster, 1997; Geue & Löschner, 2002; Corrente et al., 2004). In some cases, the infection results in an invasive disease, septicemia and meningitis (CDC, 1995), but there are relatively few reports of these bacteria causing disease in reptiles (Ramsay et al. 2002; Chiodini, 1982). For this reason, it becomes a risk to the people who use the these animals as protein source in their diet or exotic pets (Ackman et al., 1995; Mermin et al., 1997; CDC, 1999; Vasconcellos, 2001).

The current standard laboratory procedure to *Salmonella* culture and identification is laborious and can last up to 7 days (Flowers et al., 1992). The Polymerase Chain Reaction (PCR) represents a major advance in terms of the speed, sensitivity and specificity, and has been increasingly used to identify several bacterial species from clinical and environmental samples (Aabo, et al. 1993; Lantz et al., 1994). The main obstacle to using PCR for the detection and identification of pathogenic organisms from clinical samples is the presence of substances that are inhibitory to PCR (Lantz et al., 1994; Stone et al., 1994). In addition, a number of molecular typing methods have also been used to try improve the identification of *Salmonella* and also to differentiate strains to the level of serotypes (Nair et al., 2002).

In this context, the aim of this study was to estimate the prevalence of *Salmonella* spp. in captive snakes through traditional isolation and molecular methods (specific PCR).

Materials and Methods

The species of snakes tested are listed in Table 1. Twenty-seven samples were collected from animals kept in Serpentarium of Executive Commission of Cocoa Crop Plan - CEPLAC (Ilhéus-BA-Brazil) and four samples for animals of Vertebrates Zoology Laboratory of State University of Santa Cruz (Ilhéus-BA-Brazil). All the snakes were healthy and they were tested by using sterile cloacal swabs. The samples were sent immediately to the laboratory and kept at 6ºC.

The samples were examined for the presence of *Salmonella* according to APHA (Flowers et al., 1992) with some modifications. Each swab was inoculated into 2 mL of buffered peptone water (BPW; Merck) and incubated at 37ºC for 24 h. One milliliter of this culture was transferred to 9 mL of Tetrathionate broth (TTB; Merck) and into 9 mL of Selenite-Cistine broth (SCB; Merck), and incubated at 43ºC for 24 h. Two differential media were used, Xylose-Tergitol-4 agar (XLT4; Merck) and *Salmonella-Shigella* agar (SS, Merck), with incubation 43ºC for 24 h. Five colonies with typical properties of *Salmonella*, as indicated by supplier's instructions, were selected from each plate and tested using a biochemical methods (TSI, LIA, MR, Urease and SIM) according APHA (Flowers et al., 1992). Positive samples grown in Trypticate Soy agar (TSA; Merck) and were sent to the Enterobacteria Laboratory of Fundação Oswaldo Cruz, Rio Janeiro-RJ-Brazil for specific identification using serogroup-specific antisera.

For molecular analysis, the cloacal swabs, after incubation in BPW, were streaked way in Petri dishes containing TSA and incubated at 37ºC for 24 h. Then,

Table 1. Snakes used in the experiments and Salmonella serotypes found by standard microbiological method

Snake specie	*Salmonella serotype*
Bothrops jararacussu	-
Bothrops jararacussu	-
Bothrops jararaca	*Salmonella enterica* subsp. *enterica* (O11:r:-)
Bothrops leucurus	-
Bothrops leucurus	*Salmonella enterica* subsp. *enterica* (O4,5:b:-)
Bothrops leucurus	*Salmonella* Newport
Bothrops leucurus	-
Bothrops leucurus	*Salmonella enterica* subsp. *enterica* (O4,5:b:-)
Bothrops leucurus	*Salmonella* Newport
Bothrops leucurus[a]	*Salmonella enterica* subsp. *enterica* (O4,5:b:-); *Salmonella* Newport; *Salmonella* Gaminara
Bothrops leucurus	-
Bothrops leucurus	*Salmonella* Newport
Bothrops pirajai[b]	*Salmonella enterica* subsp. *enterica* (O4,5); *Salmonella enterica* subsp. *enterica* (O4,5:b:-)
Bothrops bilineatus	*Salmonella enterica* subsp. *enterica* (O4,5:b:-)
Bothrops bilineatus	*Salmonella enterica* subsp. *houtenae* (O61:c:1,5)
Corallus hortulans	-
Crotalus durissus	*Salmonella* Gaminara
Crotalus durissus	-
Epicrates cenchria hygrophilus	*Salmonella enterica* subsp. *enterica* (O4,5:b:-)
Epicrates cenchria hygrophilus	*Salmonella enterica* subsp. *diarizonae* (O:47)
Epicrates cenchria hygrophilus	-
Epicrates cenchria hygrophilus	-
Lachesis muta rhombeata[b]	*Salmonella enterica* subsp. *enterica* (O4,5:b:-); *Salmonella enterica* subsp. *enterica* (O4,5)
Lachesis muta rhombeata[b]	*Salmonella enterica* subsp. *enterica* (O4,5:b:-); *Salmonella enterica* subsp. *enterica* (O4,5)
Lachesis muta	*Salmonella enterica* subsp. *enterica* (O4,5:b:-)
Lachesis muta	-
Lachesis muta	*Salmonella enterica* subsp. *enterica* (O4,5:b:-)
Lachesis muta	-
Lachesis muta	*Salmonella enterica* subsp. *enterica* (O4,5:b:-)
Lachesis muta	-
Spilotes pullatus	-

[a] One animal was carrier of three serovars: *S. enterica* subsp. *enterica* (O4,5:b:-); *S.* Newport and *S.* Gaminara.
[b] Three animals were carriers of two serovars: *S. enterica* subsp. *enterica* (O4,5:b:-); *Salmonella enterica* subsp. *enterica* (O4,5).

were added 3 mL of Phosphate Buffered Saline (PBS; pH 7.2) to promote the bacteria solubilization. Tree samples were taken from this procedure. *Bacterial pool:* 200 ì L were pipetted from bacterial suspension in 1.5-mL microtubes. This sample was centrifugated at 7000 x g for 3 min and the pellet washed by adding 200 ì L of sterile Milli-Q® water, shaken vigorously and centrifugated at 7000 x g for 3 min. The supernatant was discarded and this procedure was repeated twice. In the last step, the samples were placed in boiling water for 5 min to bacterial lyses. *Enrichment with BPW:* 100 ì L from bacterial pool were inoculated in 10-mL tubes containing 2 mL of BPW and incubated at 37°C for 24h. After this period, 1.5 mL was transferred to 2-mL microtubes and centrifuged at 7000 x g for 200 seconds, the supernatant was discarded and were added 200 ì L of sterile Milli-Q® water, this procedure was repeated twice. In the last step, the samples were placed in boiling water for 5 min to bacterial lyses. *Pure culture:* 100 ì L of pure cultures of *Salmonella* from positive snakes in traditional isolation were inoculated into 10-mL tubes containing 2 mL of TSB. The cultures were incubated at 37°C and 180 rpm for 24 h. After this period, 1.5 mL were transferred to 2-mL microtubes and centrifuged at 7000 x g for 3 min and the pellet was washed by adding 200 ì L of sterile Milli-Q® water, shaken vigorously and centrifugated at 7000 x g for 3 min. The supernatant

was discarded and this procedure was repeated twice. In the last step, the samples were placed in boiling water for 5 min to bacterial lyses.

Four microliters of the each lysate sample was transferred to a PCR tube containing 46 µL of a mixture of 35.25 µL sterile Milli-Q® water, 10 mM *Taq* polymerase buffer, 2.5 mM $MgCl_2$, 10 pmol each primer, 200 µM each dNTP, 1.25 U *Taq* polymerase. Table 2 shows the sequence of primers and PCR conditions. PCR products were electrophoresed on a 1% (w/v) agarose gel in 1X Tris/Acetate/ EDTA (TAE) buffer at 100 V for 30 min and stained with ethidium bromide (0.5 µg mL^{-1}). The amplified products were visualized under UV illumination.

Results and Discussion

Sa & Solari (2001) confirmed the presence of *Salmonella* spp. in fecal samples from 97 pet reptiles in Brazil. Snakes reached the second place in this survey with 53.3% of prevalence, demonstrating its epidemiologic importance. In the present study by using the standard microbiological method, *Salmonella* spp. was detected in 58.07% of the analyzed snakes. This result was similar to Sa & Solari (2001). However, we must keep in mind that negative animals at our diagnosis can be within the group in which *Salmonella* elimination is intermittent, did not releasing in their feces, as shown by Burnham et al. (1998) and Argôlo Filho (2007).

Serotyping is an important epidemiological tool for assessing the prevalence of certain strains and assist in a possible salmonellosis control program. A total of seven different serotypes of *Salmonella* were found among 39 strains of the 18 positive snakes, of which 42% represent *Salmonella enterica* (O4.5:b:-); 26% *S.* Newport, 13% *S. enterica* (O4.5); 8% *S. houtenae* (O61:c:1.5); 5% *S.* Gaminara and 3% *S. diarizonae* (O47) and *S. enterica* (O11:r:-) (Figure 1). In Table 1 these serotypes are listed according to snake species studied. Sa & Solari (2001), who studied the prevalence of *Salmonella* in reptiles imported into Brazil, also found three subspecies found in this study, *S. enterica, S. houtenae* and *S. diarizonae*. It was observed that three animals had more than one serotype simultaneously (Table 1), this is in agreement with those results obtained by Corrente et al. (2004) and Argôlo Filho (2007). Most of the isolates that are zoonotic belong to *S. enterica* subsp. *enterica*. The others subspecies are potentially virulent and are ocasionally associated with human infection. *S*. Newport was the third most common serovar of human salmonellosis in the U.S. (The Center..., 2005) *S*. Gaminara has also been associated to human salmonellosis outbreaks (Kim et al., 1998). These reports emphasize the risk in manipulate or keep snakes without proper care.

Three types of sampling (*Bacterial pool*, *Enrichment with BPW* and *Pure culture*) were analyzed by use specific primers (ST11 and ST15). The PCR amplification resulted in products of 429 bp (Figure 1). Aabo et al. (1993) found that this primers can be used successfully for *Salmonella* identification and no false-positive reaction was observed. The PCR with samples from "*Bacterial pool*" obtained 54.84% of positive *Salmonella,* result quite similar to that observed with the traditional method. However, the results from the "*Enrichmnet with BPW*" shown positivity of 64.52%, 10% more than previous results, suggesting an increase in the sensitivity of the diagnosis. Considering all tests,

Table 2. Sequence of ST11 and ST15 primers and PCR conditions (Aabo et al., 1993)

Primer	Sequence (5'-3')	PCR conditions	
		Size (bp)	Cycles
ST11	AGCCAACCATTGCTAAATTGGCGCA		1 cycle - 5 min - 94ºC
		429	30 cycles { 1 min - 94ºC; 2 min - 60ºC; 1 min - 72ºC }
ST15	GGTAGAAATTCCCAGCGGTACTG		1 cycle - 10 min - 72ºC

a total of 87.1% of all serpents were positive, reaching close to the results obtained by Schröter et al. (2006). The primers demonstrated its efficiency to detect 100% of *Salmonella* from pure cultures (Figure 2).

The PCR has been demonstrated to be more sensitive than standard isolation technique (Aabo et al., 1993; Lantz et al., 1994; Oliveira et al., 2002; Sibley et al., 2003). However, eight samples that were positive in standard microbiological test became negative in PCR from the *Bacterial pool* and tree positive samples in *Bacterial pool* became negative after the *Enrichment step* (Figure 2). A probable reason for this is some failure in DNA extraction or amplification is the presence of impurities that inhibited the PCR. It has been attempted to diagnose *Salmonella* directly from faeces without any cultivation step. However, there was great difficulty in getting a quality DNA for use in PCR (data not shown). Probably the presence of heterogeneous

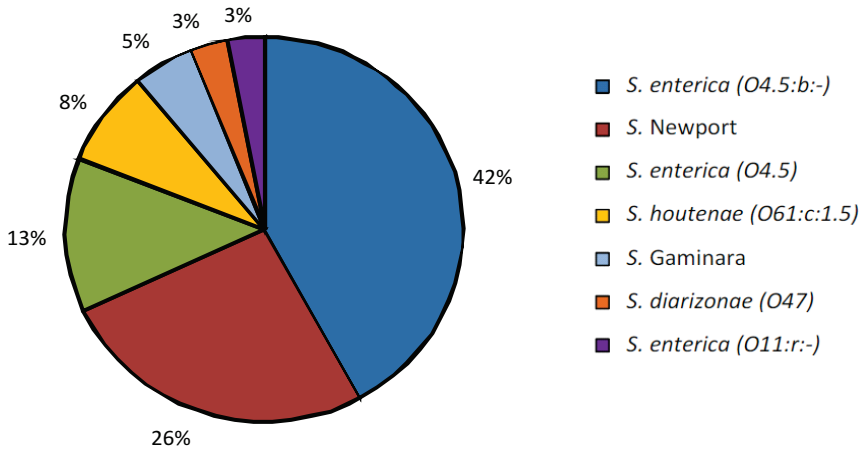

Figure 1 Percentages of *Salmonella serotypes* isolated from 18 positive snakes by standard microbiological method.

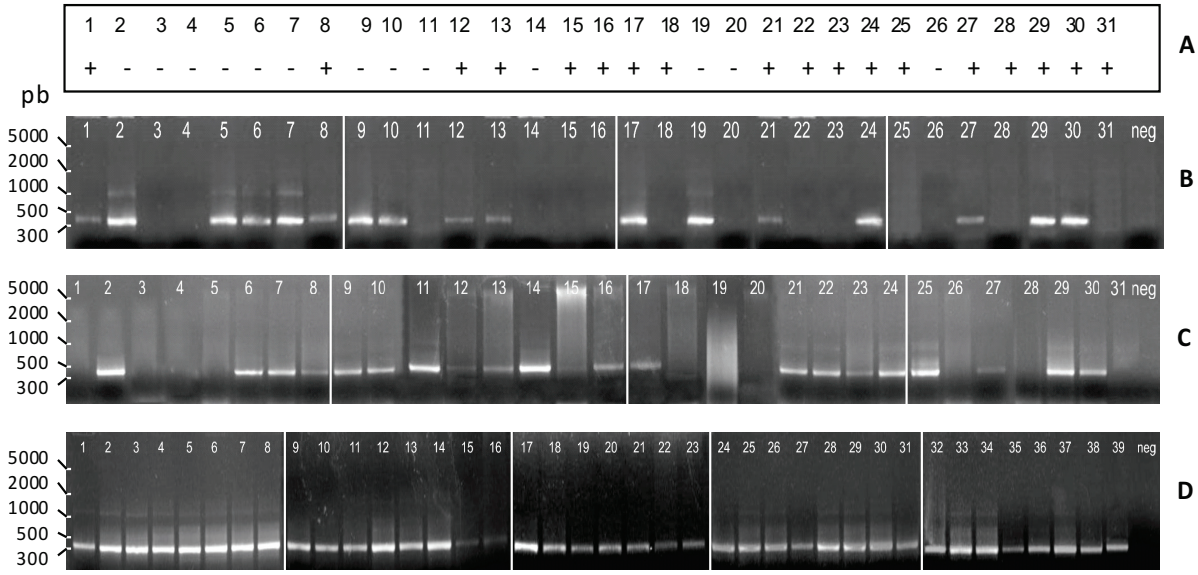

Figure 2. Detection of *Salmonella* by PCR using specific primers (ST11 - ST15) resulting in products of 429 bp. neg: negative control. (A) The results of standard microbiological method is defined as positive (+) and negative (-) to Salmonella. The presence of the band in PCR represents positive samples: (B) samples from Bacterial pool; (C) samples from Enrichment with BPW; (D) samples from Pure culture (the sample numbers of D do not coincide with A, B and C).

substances aggregated to fecal samples interfered in the processing of the sample as the result of DNA extraction (Fungaro, 2000). Diagnosis through the enrichment and subsequent PCR analysis did not show the same difficulties. The results showed that when promoting a pre-enrichment technique is more skilled to detect the presence of *Salmonella* in samples (Oste, 1988; Rahn et al. 1992; Aabo et al. 1993; Lantz et al. 1994 ; Oliveira et al. 2002; Agarwal et al. 2002; Sibley et al., 2003). Agarwal et al. (2002) propose that the ideal time for pre-enrichment is 6h in food. They noted that, despite the great sensitivity of PCR, without prior enrichment of the samples was not possible to detect *Salmonella*. In PCR there was no preferential amplification of one serotype in particular between those observed in this study. However, the development of a method for efficiently extracting DNA directly from fecal samples without enrichment is required for the diagnosis and monitoring of populations of pathogenic microorganisms and the celerity of animal treatment in hospitals and clinics, thus improving the prognosis.

Conclusions

The salmonellosis in reptile, especially in snakes, represents a important source of human illness and need prevention and control programs. In present study, 87.1% of all snakes were positive to *Salmonella*. PCR using specific primers ST11 and ST15 shown to be an effective and rapid method for the diagnosis of salmonellosis, although serotype does not become known by using these pair of primers. The use of enriched sample in buffered peptone water seems to be more effective than direct use of the fecal sample as DNA template in PCR, possibly due to greater selection and growth of bacterial cells. Due to the few studies conducted in Brazil on salmonellosis in reptiles, other works on this subject are of great importance, especially in molecular biology to carry out control programs more effective.

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