Salmonella typhimurium in *Natrix natrix*: detection and identification by culture and multiplex PCR methods

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Key words:
water snake, *Natrix natrix*, *Salmonella typhimurium*, multiplex PCR.

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Received: 19 January 2011
Accepted: 9 July 2011

Abstract:

A water snake (*Natrix natrix*) was referred to the Small Animal Teaching Hospital, Faculty of Veterinary Medicine, University of Tehran. It had two subcutaneous masses. Radiographic and ultrasonographic images revealed the presence of two subcutaneous abscesses. The abscesses were removed surgically and specimens were examined by conventional microbial culture and multiplex PCR. Inv-A universal primers were selected for detection of *Salmonella* at genus level. In order to identify *Salmonella tphimurium*, specific primers of Rfbj, *Flj*b and Flic related on genes sequences of O4, H1:i and H2:1,2, respectively, were used. In the positive control for expected size, PCR products were amplified from the *fli*C, *inv*-A, *flj*B and rfbJ genes. According to the results, this study showed antigens can be useful for detecting and identifying *Salmonella typhimurium* and can be achieved by using specific primers of O4, H1:i and H2:1,2 antigen, because only *S. typhimurium* has this antigenic structure out of about 2668 *Salmonella* serovars.

Introduction

The water snake (*Natrix natrix*), a non-venomous snake (*Aglypha*), belongs to the family *Typhlopidae* and genus Natrix. This genus has a wide distribution from west Europe and north-west Africa to central and East Asia. It can be found in the west and north provinces of Iran (Latifi, 2ooo) (Fig.1). This is the most common snake which is kept as pet in Iran.

Salmonellosis, in both humans and animals, is one of the most common infectious diseases in the world. *Salmonella Enterica* serovar *typhimurium* is the most frequently isolated serovars in the world (Gay, 1995). Traditional *Salmonella* detection methods are based on cultures using selective media after overnight enrichment broth and characterization of suspicious colonies by chemical and serological tests. These methods are generally time-consuming and laborious procedures (Aabo et al., 1993) .Therefore, development of a rapid and sensitive method is desirable for identification of *Salmonella* serotypes from clinical samples. A number of rapid methods for the detection of *Salmonella* in clinical samples have been developed; however, there are still problems with their sensitivity and specificity (Aabo et al., 1993; Widjojoatmodjo et al., 1992). The polymerase chain reaction (PCR) is a sensitive and rapid technique which provides a way of overcoming these difficulties (Aabo et al., 1993; Blackburn, 1993; Kwang et al., 1996; Nguyen et al., 1994).

The aim of this study was to multiplex PCR (m-

Table 1: Primers sets used for detection of *Salmonella* serovars. and identification of *S. typhimurium.*

Primers sets and sequences	Length (bp)	Target gene	Amplified fragment size (bp)	Reference
ST139-s: 5'-GTGAAATTATCGCCACGTTCGGGCAA-3'	23	*inv*A	284	Rhan et al., 1992
ST141-as: 5'- TCATCGCACCGTCAAAGGAACC -3'	22			
Rfbj-s: 5'- CCAGCACCAGTTCCAACTTGATAC-3'	25	rfbJ	663	Lim et al., 2003
Rfbj-as: 5'- GGCTTCCGGCTTTATTGGTAAGCA-3'	24			
Flic-s: 5'- ATAGCCATCTTACCAGTTCCCCC-3'	22	*fli*C	183	Lim et al., 2003
Flic-as: 5'- GCTGCAACTGTTACAGGATATGCC -3'	23			
*Flj*b-s: 5'- ACGAATGGTACGGCTTCTGTAACC-3'	24	*flj*B	526	Lim et al., 2003
*Flj*b-as: 5'-TACCGTCGATAGTAACGACTTCGG-3'	24			

PCR) assay by selected specific primers to detect *Salmonella* at genus level and also to identify *Salmonella enterica* serovar *typhimurium* in Natrix natris samples.

Materials and Methods

Samples: A water snake (*Natrix natrix*) was referred to the Small Animal Teaching Hospital, Faculty of Veterinary Medicine, University of Tehran, with two masses on its body (Fig.2). Radiographic and ultrasonographic images revealed that the masses were located subcutaneously and had distinct capsule and contained echogenic materials. There was not any blood supply in them which indicated a subcutaneous abscess. The abscesses were removed surgically and two samples were sent to bacteriology laboratory.

Conventional microbiological method: For the conventional test, samples were inoculated into selenite-cystein broth (Merck) for overnight enrichment at 37°C, and later plated on MacConkey agar (Merck) for primary selection. After 24 hours of incubation at 37°C, typical colonies (non-lactose fermenter) were confirmed by biochemical tests. Serotyping of isolates was tested by a standard agglutination test using O and H antisera (Difco).

Oligonucleotide primers: For an m-PCR assay, four set primers were selected: 139-141, specific for the *inv*-A gene from *Salmonella* serovars (Rahn et al., 1992); RfbJ, *Fli*C and *Flj*B, specific for the rfbJ, *Fli*C and *flj*B genes for specific identification of *Salmonella typhimurium* or the other *Salmonella* serovars which have similar antigenic properties (Lim et al., 2003). The primers' sequences and their corresponding genes are shown in table 1.

DNA amplification: PCRs were performed with 10 µl of DNA sample, 5 mM KCl, 1.5 mM MgCl2,10 mM Tris-HCl pH 8.5, 1 µM of each primer, 200 µM dNTPs (Fermentas) and 1U of Taq DNA polymerase (Fermentas) in a final volume of 25 µl. Amplifications were performed in a DNA thermocycler (Model TC-512., Techne, UK). The m-PCR protocol consisted of the following steps: (i) The initial denaturation step of 5 minutes at 95°C; (ii) 30 cycles, consisting of 1 minute at 95°C, 1 minute at 65°C and 30 seconds at 72 °C, and (iii) a final extension step of 7 minutes at 72 °C. The PCR products were electrophoresed in 1.2% agarose (Fermentas), and after staining with ethidium bromide (Sigma), photographed under a UV transilluminator. The positive control was *Salmonella typhimurium* (ATCC14028). In the negative control, the template DNA was replaced with 10 µl sterile distilled water.

Results

Serotyping: The two samples tested in this study, were culture positive for *Salmonella enterica* serovar

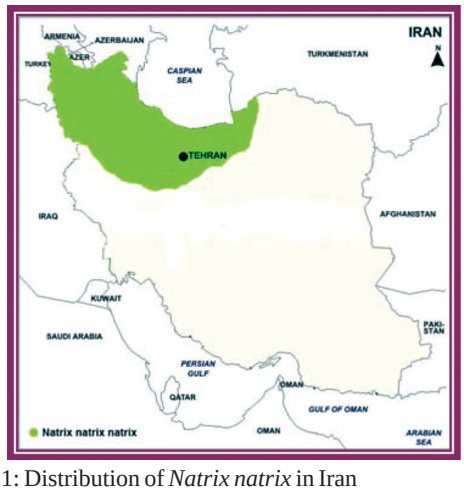

Figure 1: Distribution of *Natrix natrix* in Iran

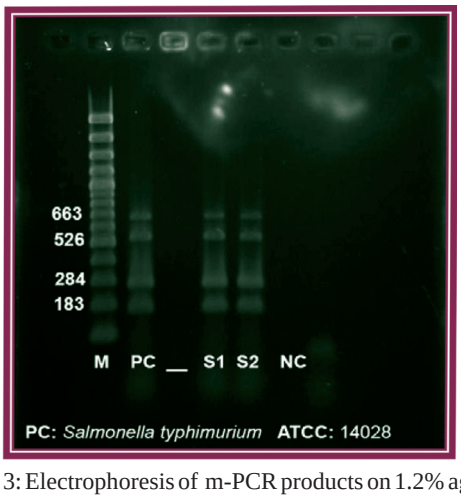

Figure 3: Electrophoresis of m-PCR products on 1.2% agarose gel stained with ethidium bromide: M,100 bp molecular weight marker; NC, negative control; PC, positive control (*Salmonella typhimurium* ATCC14028); 3 and 4 serovar *typhimurium* (1,4,5,12:i:1,2).

typhimurium.

Detection of *Salmonella*e by Multiplex PCR: To detect and identify *Salmonella* at genus level and *Salmonella enterica* serovar *typhimurium* using mPCR, four amplified products (663, 526, 284 and 183 bp) were found in two specimens which had serovar *typhimurium* (4,5,12:i:1,2) from rfbJ, *flj*B, *inv*-A and *Fli*c genes respectively (Fig.3). In the positive control (*Salmonella enterica* serovar *typhimurium*; ATCC14028) the expected size of PCR products was amplified from the *Fli*C, *inv*-A, *flj*B and rfbJ genes (Fig.3, lane2).

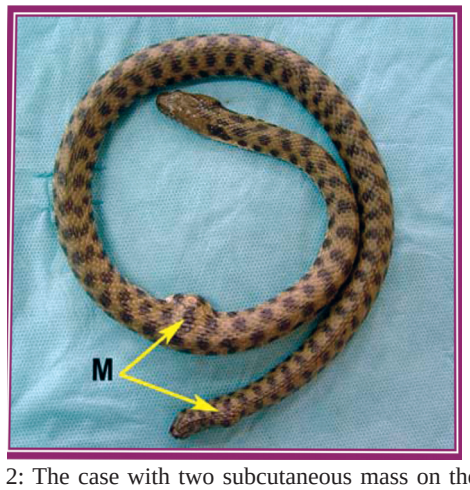

Figure 2: The case with two subcutaneous mass on the body (Arrows).

Discussion

The majority of bacteria identified from infections in captive snakes are Gram-negative aerobic micro-organism (Meredith and Redrobe, 2002). *Salmonella* spp. is frequently isolated from snakes (Beynon, 1992). Regarding the serovar level, the multiplex PCR assay for the identification of *S. typhimurium* was very specific. The results obtained in the present assay are in agreement with Lim et al., (2003) which used the similar target genes and primers. In conclusion, the results of this study showed which detection and identification of *Salmonella typhimurium*, using specific primers of O4, H2:1,2 and H1:i antigens, can be useful, because only *S. typhimurium* has this antigenic structure out of about 2668 *Salmonella* serovars.

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