



Expeditious detection of *Salmonella* species in newborn calves by polymerase chain reaction

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Abstract

Fecal samples collected from 85 diarrheic calves and 65 apparently healthy contact calves were examined for the presence of *Salmonella* species using bacteriological examination and *fimA* gene amplification assay (PCR). *Salmonella* were isolated from 43.53% of diarrheic calves and from 27.69% of apparently healthy contact calves. *Salmonella typhimurium* were isolated from diarrheic and contact calves in percentages of 17.65 and 15.38% respectively; whereas *Salmonella enteritidis* were isolated in percentages of 11.76 and 7.69%, respectively. *Salmonella dublin* were isolated from the diarrheic calves in a percentage of (8.24%) and contact calves in a percentage of (4.62%), but *Salmonella anatum* were isolated from diarrheic calves only in a percentage of (5.88%), The sensitivity and specificity of PCR were 100 and 95.8%, respectively during examination of fecal samples obtained from diarrheic calves, while during the examination of those obtained from contact calves the percentages were 100 and 89.4%, respectively.

Keywords: *Salmonella*, *fimA* gene, diarrheic calves, *Salmonella typhimurium*, *Salmonella enteritidis*.

INTRODUCTION

Bovine Salmonellosis is a worldwide bacterial disease causing great public health and economical problems. The most serious infection usually attack calves during the first ten weeks of life (Smith et al., 1980 and Hoiseth and Stocker, 1981). The most common host-adapted serotypes involved in bovine Salmonellosis are *Salmonella typhimurium*, *Salmonella enteritidis*, *Salmonella anatum*, *Salmonella newport*, *Salmonella agana* and *Salmonella dublin* (Moore et al., 1982; Konral et al., 1994; Veling et al., 2002 a and b). Culturing of *Salmonella* from fecal samples is a time consuming and laborious process therefore development of a rapid and sensitive method for the diagnosis of *Salmonella* species is desirable. Several techniques for improving the detection of *Salmonella* serovars in feces such as the use of selective culture medium and enzyme linked immunosorbant assay (ELISA) have been developed (Abshire and Neidhardt, 1993). However, problems remain

with sensitivity and specificity that have a limited Routine use of these procedures. Polymerase chain reaction (PCR) had been used to identify the presence of specific pathogens directly from clinical specimen. Fecal samples usually have inhibitory effect on PCR due to the presence of biliurbin and bile salts that hinder the PCR (Moore et al., 1982; Widjoatmodja et al., 1992; Swenson et al., 1994). *S. typhimurium fimA* gene had been cloned and sequenced (Nicols et al., 1990; Pollard et al., 1990).

The nucleotide sequence of the *fimA* gene of *Escherichia coli* and *Klebsiella pneumoniae* have a significant homology but are not fully homologous (Stoleru et al., 1996). Primers were designed specifically by Cohen et al. (1996 a and b) to amplify regions of the *fimA* gene. All *Salmonella* strains tested with the primers were positive, while all non *Salmonella* strain were

Table 1. Standard stains used for standardization of the PCR.

Bacterial strains	Source	Number
<i>S. typhimurium</i>	ATCC * 14028	2
<i>S. typhimurium</i>	Field isolate	2
<i>S. enteritidis</i>	ATCC 13076	1
<i>Salmonella typhi</i>	ATCC 9992	1
<i>E. coli</i> (O157:H7)	ATCC 35150	2
<i>Pseudomonas aeruginosa</i>	Field isolates	1
<i>K. pneumoniae</i>	Field isolate	1
<i>Staphylococcus aureus</i>	Field isolate	1

*ATCC: American type culture collection.

negative which confirm that *fimA* gene contain unique sequence to *Salmonella* strains and demonstrated that this gene is suitable for PCR targeted for detection of *Salmonella* serovars. Investigation of *Salmonella* infection in calves was the major strategy of our work in several localities, as well as to detect the most accurate sensitive and rapid diagnostic assay among different diagnostic procedures.

MATERIALS AND METHODS

Sample collection

A total number of 150 fecal swabs were collected from 85 beef calves suffering from mucoid and/or bloody diarrhea and from 65 apparently healthy contact calves. Calves age ranged from 1 to 6 months. Samples were collected during the period from October, 2008 to the end of June, 2009 from two farms one in the Menofia and the other in Kafr El-Sheikh Governorates, Egypt. Fecal samples were transferred to the laboratory in a cold chamber container to be cultured without delay.

Bacterial strains used as a control

A total number of 11 bacterial strains were used as a control, 6 strains belonging to *Salmonella* species and the other 5 strains belonging to bacteria other than *Salmonella* as described in Table (1).

Salmonella isolation and identification

Fecal samples were inoculated into selenit-F and tetrathionate broth for enrichment for 16 h at 37°C. A loop full of the broth were streaked onto XLD agar, MacConkey agar and SS. agar plates and incubated at 37°C for 37 - 48 h and the suspected colonies were identified morphologically, then biochemically using the API-20E kit system (Biomeraux, France) and serologically according to the Kauffman - white scheme by slide agglutination test using polyvalent and monovalent O antigen (Difco Laboratories, Detroit, Michigan, USA) and H antisera (Difco Laboratories, Detroit, Michigan, USA). Cultivation and identification were applied according to Morifnigo et al. (1986) and Chirino-Trejo (1999).

Detection of *Salmonella* using polymerase chain reaction (PCR)

Preparation of fecal samples for PCR assay: Fecal samples were diluted 10 fold in phosphate buffered saline pH 7.2, 0.5 - 1 ml of the diluted sample were inoculated into selenit-F broth and incubated at 37°C for 6 h without shaking as described by Chiu and Ou (1996). 1 ml of each culture was centrifuged and then the sediment was washed five times with sterilized water and finally suspended in 1.0 ml of sterilized water. The suspension was kept at 95°C for 15 min and after centrifugation at 5,000 rpm for 5 min, 10 µl or the supernatant was directly used for PCR.

PCR design and amplification conditions

The oligonucleotide primers for PCR were synthesized according to Cohen et al. (1996 a and b) considering reported nucleotide sequence of the *fimA* gene (EMBL and Gen Bank accession number M18283 and sequence name F2M47) of *S. Typhimurium*. The 20-mer forward primer (*Fim 1A*), 5' - CCT TTC TCC ATC GTC CTG AA-3', has a calculated annealing temperature of 60°C and is located between bp 586 and 605 on the *fimA* gene of *S. Typhimurium*. The 20-mer reverse primer (*Fim 2A*), 5'-TGC TGT TAT CTG CCT GAC CA-3', has a calculated annealing temperature of 60°C and is located between bp 651 and 670 on the *fimA* gene of *S. typhimurium*. The 85-bp fragment was specifically amplified using this set of primers. From pure cultures or genomic DNAs of *Salmonella* strains, amplification of *fimA* gene was achieved on the thermal cycler as follows: The reaction mixtures consisted of 5 µl of the DNA template, 5 µl 10x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂SO₄), 1 µl dNTPs (40 µM), 1 µl (1 U Ampli Taq DNA polymerase), 1 µl (25 pmol) from the forward and reverse primers of both primer pairs and the volume of the reaction mixture was completed to 50 µl using DDW. The thermal cycler was adjusted as follows: Initial denaturation at 94°C for 5 min., followed by 35 cycles of (denaturation at 94°C for 1 min, annealing 55°C for 1 min and extension at 72°C for 1 min). Final extension was carried out at 72°C for 10 min and the PCR products were stored in the thermal cycler at 4°C until they were collected. The PCR products were visualized by agarose gel electrophoresis previously reported by (Sambrook et al., 1989) using suitable molecular weight markers.

Analysis of data

The sensitivity and specificity of PCR were calculated according to

Table 2. *Salmonella* isolates obtained from fecal samples obtained from diarrheic and contact calves.

Animals	Number	<i>S. typhimurium</i>	<i>S. enteritidis</i>	<i>S. dublin</i>	<i>S. anatum</i>	Total
Diarrheic calves	85	15 (17.65%)	10(11.76%)	7 (8.24%)	5 (5.88%)	37(43.5%)
Contact calves	65	10 (15.38%)	5(7.69%)	3 (4.62%)	0	18(27.6%)

Table 3. Results of PCR in comparison with bacteriological examination of 85 fecal samples obtained from diarrheic and apparently normal calves.

PCR results	Bacteriological examination		
	Positive	Negative	Total
Diarrheic calves			
Positive PCR	37	2	39
Negative PCR	0	46	46
Total	37	48	85
Apparently normal			
Positive PCR	18	5	23
Negative PCR	0	42	42
Total	18	47	65

Timmreck (1994) taking the bacteriological isolation as a gold standard.

RESULTS

Isolation and identification of *Salmonella* species

Bacteriological examination of the fecal samples collected from diarrheic and contact apparently healthy calves revealed the presence of *Salmonella* organisms in both of them. *Salmonella* were isolated from 37 (43.53%) out of 85 diarrheic calves and from 18 (27.69%) out of 65 apparently healthy calves. *S. typhimurium* strains were isolated from fecal samples of diarrheic and contact apparently healthy calves in percentages of 17.65 and 15.38%, respectively; whereas *Salmonella enteritidis* were isolated in percentages of 11.76 and 7.69%, respectively. *S. dublin* were isolated from the diarrheic calves (8.24%) and contact healthy calves (4.62%), but *S. anatum* were isolated from diarrheic calves only (5.88%) as shown in Table (2).

All *Salmonella* strains "standard strains and isolated strains" from fecal samples were positive for the amplification of the *fimA* gene and the specific PCR product "85 bp fragment" was visualized by agarose gel electrophoresis and ethidium bromide staining (Figure 1).

No amplification could be observed with all non *Salmonella* strains. All bacteriologically positive fecal samples were positive with PCR, whereas PCR detected 7 bacteriologically negative samples (two samples from diarrheic calves and 5 from apparently healthy one) as

shown in Table (3). The sensitivity and specificity of PCR were calculated according to Timmreck (1994) and they were found as 100 and 95.8%, respectively during examination of fecal samples obtained from diarrheic calves, while during the examination of those obtained from apparently healthy contact calves the percentages were 100 and 89.4 %, respectively.

DISCUSSION

The PCR technique provides a new strategy for rapid and sensitive detection of *Salmonella* strains (Rasmussen et al., 1994). In *E. coli* and *Salmonella* strains, the phenotypic expression of type 1 fimbriae is encoded by a cluster of genes (Nicols et al., 1990). A single gene, *fimA*, encodes the major fimbrial subunit (Purcell et al., 1987). The *S. typhimurium fimA* gene has been cloned and sequenced (Nicols et al., 1990; Swenson et al., 1994). Specific primers were designed by (Cohen et al., 1996 a and b) which have the ability to amplify region of the *fimA* gene from *Salmonella* strains but not from *E. coli* and *K. pneumoniae*, such two primers were used as specific primers for rapid detection of *Salmonella* serovars in feces of diarrheic calves and the apparently healthy contact calves. Fecal samples collected from diarrheic calves as well as the apparently healthy contact calves showed high incidence of *Salmonella* serovars 43.52 and 27.69%, respectively. *S. typhimurium* was the most predominant serovars (17.65 in diarrheic calves and 15.38% in contact apparently healthy calves) which indicate that *S. typhimurium* is the most predominant

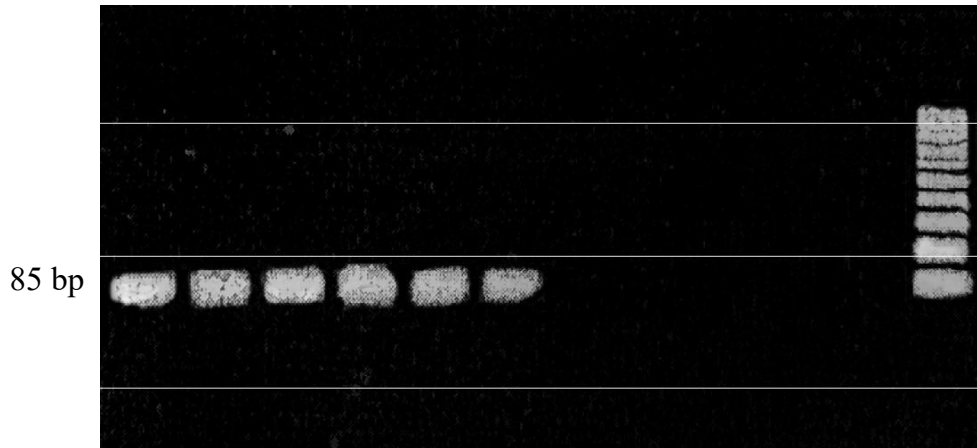


Figure 1. Showing positive *fimA* gene amplification of tested strains where *S. typhimurium* ATCC strains in lane 1 and 2, while lanes 3 and 4 belong to local strains, lane 5 of *S. Enteritidis* lane 6 of *S. typhi*, while lanes 7, 8, 9, 10 and 11 are of negative samples concerning *Escherichia coli* (O157:H7) *Pseudomonas aeruginosa*, *K. pneumoniae* and *S. aureus*, respectively. Lane 12 showing 100 bp ladder.

serovars causing enteritis in calves. *S. enteritidis* (8.24 and 4.62%) and *S. dublin* (8.24 and 4.62%) were also isolated from diseased and contact apparently healthy calves, respectively, while *S. anatum* were isolated from diseased calves only (5.88%). These results agree with the results of (Jones et al., 1988; Segall and Lindberg, 1993; Seleim et al., 2004). All *Salmonella* strains either standard or isolated from fecal samples were positive with PCR and the 85 bp PCR product was observed on agarose gel electrophoresis. No amplification could be observed with bacterial strains other than *Salmonella* strains which indicate that the *fimA* gene contains sequences unique to *Salmonella* serovars and can be used as a target sequence for direct detection of *Salmonella* serovars. This result confirms the result of Cohen et al. (1996 a and b).

All bacteriologically positive fecal samples were positive with PCR and the specific PCR product (85 bp fragment) could be observed. At the same time positive results were observed with 2 samples (2.35%) from diarrheic calves and 5 (7.69%) from apparently healthy contact calves which indicate the higher sensitivity of the PCR method, also indicate the ability of PCR to detect the carrier animals which secrete a very few number of the organism in the feces and which could not be detected by cultural methods (Fang et al., 1991; Fang et al., 1992; Fierer et al., 1993; Chiu and Qu, 1996). The sensitivity and specificity of PCR were 100 and 95.8%, respectively during examination of fecal samples obtained from diarrheic calves, while during the examination of those obtained from apparently healthy contact calves the percentages were 100 and 89.4%, respectively. Our results indicate that the PCR amplification of the *fimA* gene sequence of *S. typhimurium* could be used as a target sequence for rapid and sensitive method for direct

detection of *Salmonella* serovars in the fecal samples of diarrheic and contact apparently normal calves.

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