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Full Length Research Paper

Rapid detection of *Salmonella* species in newborne calves by polymerase chain reaction

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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 entertidis* 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.

Key words: *Salmonella, fimA* gene, diarrheic calves, *Salmonella typhimurium, Salmonella entertidis*.

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 mediurn 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 biliuribin and bile salts that hinder the PCR (Moore et al., 1982; Widjojoatmodja 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

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

Bacterial strains	Source	Number
S. typhimurium	ATCC * 14028	2
S. typhimurium	Field isolate	2
S. enteritidis	ATCC 13076	1
Salmonella typhi	ATCC 9992	1
E. coli (O157:H7)	ATCC 35150	2
Pseudomonas aerugenosa	Field isolates	1
K. pneumoniae	Field isolate	1
Staphylococcus aureus	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 monavalent O antigen (Difco Laboratories, Detroit, Michigan, USA) and H antisera (Difco Laboratories, Detroit, Michigan, USA). Cultivation and identification where 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 IA)*, 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	*S. typhimurium*	*S. entertidis*	*S. dublin*	*S. anatum*	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 entertidis* 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 (Rassmussen 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

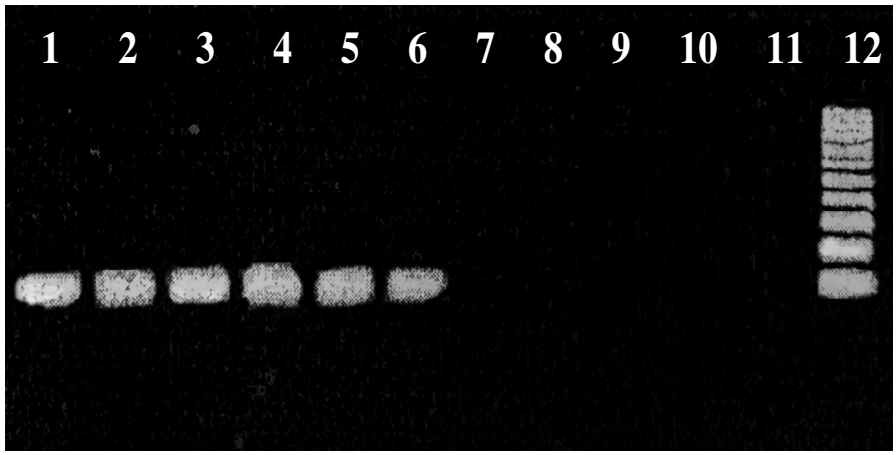

Figure 1. Showing positive *fimA* gene amplification of tested strains where *S. typhimurium* ATCC strains in lane1 and 2, while lanes 3 and 4 belong to local strains, lane 5 of *S. Enteritidis* lane 6 of *S. typhi*, while lanes 7, 8, 9, 10 and 11 are of negative samples concerning *Escherichia coli* (O157:H7) *Pseudomonas aerguenosa*, *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 contain sequences unique to *Salmonella* serovars and can be used as a target sequence for direct detection of *Salmonella* serovars. This results 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 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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REFFERENCES

Abshire KZ, Neidhardt FC (1993). Analysis of proteins synthesized by *S. typhimurium* during growth within a host macrophage. J. Bacteriol. 175: 3734-3743.

Chirino-Trejo JM (1999). A more sensitive procedure for the detection of *Salmonella* carriers in swine. Proc. Western Candian Assoc. Swine Pract. 1999: 50-53.

Chiu CH, Ou JT (1996). Rapid identification of *Salmonella* serovars in feces by specific detec¬tion of virulence genes, invA and spvC, by an enrichment broth culture-multiplex PCR combination assay, J. Clin. Microbiol. 34: 2619-2622.

Cohen HJ, Subbaiah M, Meehanda SM, Lin W (1996 a). PCR Amplification of the FimA Gene Sequence of *S. typhimurium*, a specific method for detection of *Salmonella* spp. Appl. Environ. Microbiol. 62: 4303-4308.

Cohen ND, Martin LJ, Simpson RP, Wallis DE, Neibergs HL (1996 b). Comparison of polymerase chain reaction and microbiological culture for detection of Salmonellae in equine feces and environmental samples. Am. J. Vet. Res. 57: 870-876.

Fang FC, Krause M, Roudier C, Fierer J, Guiney DG (1991). Growth regulation of a *Salmonella* plasmid gene essential for virulence. J. Bacteriol. 173: 6783-6789.

Fang FC, Libby ST, Buchmeier N, Loewen F, Switala J, Guiney D (1992). Regulation of *Salmonella* virulence by an alternative sigma factor. Proc. Natl. Acad. Sci. 89: 11978-11982.

Fierer J, Eckamann L, Fang F, Pfeifer C, Finlay BBandGuiney D (1993). Expression of the *Salmonella* virulence plasmid gene spvB and cultured macrophages and non phagocytic cells. Infect. Immun. 61: 5231-5236.

Hoiseth SK, Stocker BA (1981). Aromatic-dependent *S. typhimurium* are non virulent and effective as live vaccines, Nature 291: 238-239.

Jones PW, Collins P, Aitken MM (1988). Passive protection of calves against experimental infection with S. *typhimurium*. Vet. Rec. 123: 536-41.

Konral H, Smith BP, Dilling GWandJohn KH (1994). Production of *Salmonella* serogroup D (O9) - specific enzyme-linked immunosorbant assay antigen. Am. J. Vet. Res. 55: 1647-1651.

Morifnigo MA, Borrego J JandRomero P (1986). Comparative study of different method for detection and enumeration of *Salmonella* spp. in natural waters Appl. Bacteriol. 61: 169-176.

Moore GR, Rothenbacher H, Bennett MV, Barner RD (1982). Bovine Salmonellosis. J. Am. Vet. Med. Assoc. 141: 841-844. Nicols WA, Clegg S, Brown MR (1990). Characterization of type I fimbrial subunit gene (FimA) of Serratia marcescens. Mol. Microbiol. 4: 2119-2126.

Pollard DR, Johnson WM, Lior H, Tyler SD, Rosee KR (1990). Rapid and specific detection of verotoxin genes in *E. coli* by the polymerase chain reaction. J. Clin. Microbiol. 28: 540-545.

Purcell BK, Pruckler J, Clegg S (1987). Nucleotide sequences of the genes encoding type I fimbrial subunits of *K. pneumoniae* and *S. typhimurium* J. Bacteriol. 169: 5831-5834.

Rassmussen SR, Kassmusstn HB, Larsen LR, Hoff-Jorgensen R, Cano R (1994). Combined polymerase reaction hybridization micro plate assay used to detect leukemia virus and *Salmonella*. Clint. Chern. 40: 200-205.

Sambrook J, Fritsch FF, Maniatis T (1989). Molecular Cloning: a Laboratory Manual. 2nd edition. Cold Spring Harbor. NY: Cold Spring Harbor Laboratory Press.

Segall T, Lindberg AA (1993). Oral vaccination of calves with an aromatic *S. dublin* (09, 12) hybrid expressing 04, 12 protects against *Salmonella* Dubillin (09, 12) bill not against *S. typhimurium* (04, 5, 12). Infect. Immun. 61: 1222-1231.

Seleim RS, Sahar R, Novert MH, Gohran RA (2004). *Salmonella* infection in calves: virulence proteins and its immunogenic properties. J. Vet. online.

Smith BP, Habasha FG, Reina-Guerra M and Hardy AJ (1980). Immunization of calves against Salmonellosis. Am. J. Vet. Res. 41: 1947-1951.

Stoleru GH, Le Minor AM, Lheritier AM (1996). Polynucleotide sequence divergence(' . among strains of *Salmonella* subgenus IV and closely related organisms. Ann. Insl. Pasteur Microbiol. 122A: 477-486.

Swenson DL, Clegg S Old DC (1994). Frequency of the *fimA* genes among *Salmonella* serovars. Microbiol. Pathol. 10: 487-490.

Timmreck TC (1994): An introduction to epidemiology. Jones and Bartlett publisher Boston. Loadon and Singapore.

Veling J, Barkema HW, Schans J, Zijderveld F, Verhoeff J (2002 a). Herd level diagnosis for *Salmonella* enterica subsp. enterica serovar *dublin* infection in bovine dairy herds. Prevo. Vet. Med. 53: 31-42.

Veling J, Wilpshaar H, Frankena K, Bartels C, Barkema HW (2002 b): Risk factors for clinical *Salmonella* enterica subsp. enterica . Prevo. Vet. Med. 54: 157-168.

Widjojoatmodjo MN, Fluit AC, Torensma R, Verdonk GP, Verhoef J (1992). The magnetic lmmuno polymerase chain reaction assay for direct detection of *Salmonella*e in fecal samples. J. Clin. Microbiol. 30: 3195-3199.