International Journal of Microbiological Research 8 (1): 01-08, 2017
ISSN 2079-2093

DOI: 10.5829/idosi.ijmr.2017.01.08

Detection of Salmonellae in the Feces of Feedlot Calves Farms in Egypt

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Abstract: *Salmonella* is one of the most common bacterial foodborn pathogen, that cause calvesillness, deaths and financial losse. A total of 200 fecal samples were collected from feedlot calves farms from Kaliobia, Sharkia and Monofia governorates of Egypt. All samples were subjected to bacteriological examination and biochemical identification using Vitek II Gram negative automated system. Due to more sensitive fluorescence-based technology of the VITEK 2 system, isolated strains revealed that all were identified as *Salmonella* species with accuracy up to 99% after 5 h. So this system is highly automated and accurate tool for the rapid identification of bovine salmonellosis. Seven *Salmonella* strains were serologically identified using specific antisera from 100 diseased calves (7%). On contrary no *Salmonella* could be isolated from apparently healthy calves.*Salmonella enteritidis* was the only predominant isolated serotype. All *Salmonella* strains were examined using real time PCR, DNA was extracted from *Salmonella* strains, Two oligonucleotide primers were used for amplification of a 150bp stretch of the *invA* gene of *Salmonella* spp. 40 PCR cycles were performed witdenaturation at 95°C/20 sec, annealing at 58°C/30 sec and extension at 72°C/30 sec using Agilent MX3005P. The*invA* gene was present in all detected bovine salmonellae and it can be used as a valuable diagnostic tool for detection of *Salmonellae*. Real time PCR can contribute to meet the need of fast identification and detection methods for use in monitoring and control measures programe of bovine salmonellosis.

Key words: *Salmonella* · PCR · *Inv*a Gene · Vitek II

INTRODUCTION

Bovine Salmonellosis is a worldwide bacterial disease causing great animal losses, public health and economic problems. *Salmonella* infections in calves have many impacts on animal and human health that are considered major worldwide problem [1]. Traditionally, detection and quantification of *Salmonella* have been largely based on the use of selective culture and standard biochemical tests.

Vitek II Gram negative identification method is an acceptable, highly promising accurate automated method for rapid identification of Gram negative bacteria [2].

The use of DNA based methods is becoming increasingly important in epidemiological survey and differentiations of *Salmonella* speciesSome real-time PCR-based assays for the detection of *Salmonella* have already been described [3,4].Therefore, the aim of this study was isolation and typing of *Salmonella* organisms using different bacteriological, biochemical methods and identification of *Salmonella* using RT PCR.

MATERIAL AND METHODS

Sampling: A total of 200 fecal samples were collected from 100 diarrheic calvessuffering from sudden onset of profuse yellow and bloody diarrhea with mucus and fever causing rapid, severe dehydration and 100 from apparently healthy contact calves. Calves were ranged from 1 month to 1 year old. Samples were collected during the years 2013 and 2014, from 7 private feedlot cattle farms in Kaliobia, Sharkia and Monofia governorates of Egypt. Samples were transported on ice and processed for bacterial isolation within 24 hours after collection.

Bacteriological Isolation: It was done according toISO[5] andDaves Davies*et al.*[6]. Thirty grams of fecal samples were first mixed with equal volumes of buffered

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peptone and placed in a sterile stomacher bag. The bags were placed in the stomacher and subjected to homogenization for at least 10 minutes at high speed. The whole content was sieved in sterile layers of cotton rapped with goose to remove the coarse particles, then 20 ml of the sieved product was then transferred to a sterile 50 ml centrifuge tubes and incubated at 37°C for 18 ± 2 hours. Using sterile tips, 0.1 ml of the buffered peptone was transferred to 10 ml of RVS (Rappaport-Vassiliadis with soya) broth and incubated at 41.5°C ±1°C for 24 hours ± 3 hours. The incubated RVS media was platted out onto 15 mm Ø Petri dishes containing XLD agar and Hektoen agar media and incubation was done at 37°C for 24h. The plates were examined for the presence of typical colonies of *Salmonella*.

Identification of the Isolated Strains

Biochemical Reactions: It was done using Vitek II automated system according to the manufacture instructions. Briefly, a sterile swab was used to transfer a sufficient number of colonies of a pure culture and to suspend the isolates in 3.0 ml of a sterile saline. The turbidity of the resuspended isolates was adjusted at 0.5- 0.63 Angstom using a Densichek turbidity meter.

The Gram-negative identification cards were inoculated with microorganism suspensions using an integrated vacuum apparatus. All cards types were incubated at 35.5°C. Each card was removed automatically from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings and then returned to the incubator until the next read time. Data were collected at 15 minutes intervals during the enteric incubation period.

Serotyping: It was performed in the Central Laboratories of Ministry of Health and Population using serials of monoclonal antibodies against different *Salmonella* species.

Identification by Real time qPCR: Isolates grown onto Hekton enteric agar were collected in 1mL TE buffer pH8.0 and washed twice with cooled TE buffer. The bacterial pellets were subjected to enzymatic digestion using 180 µl of enzymatic lysis buffer and incubation took place at 37°C for 30 min. then the genomic DNA was extracted using QIAamp DNA Mini Kits (Qiagen cat # 51304) according to the manufacture instruction. The eluted DNA was electrophoresed on 1% agarose to check for its purity and then quantified using nanodrop (Nanodrop 8000, USA) stored at -20°C till used. Two µL of the eluted DNA was mixed with 12.5 µL of Brilliant II QPCR master mix (Agilent cat # #600806) and 100nM of each of the sense primer (GCGTTCTGAACCTTTGGTAATAA) and antisense primer (CGTTCGGGCAATTCGTTA) and 200 nM of the FAM-TAMRA labeled probe (TGGCGGTGGGTTTTGTTGTCTTCT) these primers amplify a 150bp stretch of the *invA* gene of Salmonella spp. The primers were designed using CLC main workbench V6.7.1. the reaction was adjusted at 95°C/10 min for initial denaturatione and 40 cycles of denature at 95°C/20 sec, annealing at 58°C/30 sec and extension at72°C/30 sec using Agilent MX3005P which was programmed as follow:

Taq enzyme activation step	95°C/10 min	1 cycle
PCR step		
Denature	95°C/20 sec	35 cycle
Annealing	58°C/30 sec	
Extension	72°C/30 sec	

Program was adjusted so that the florescent data was collected at the end of the extension step.

RESULTS

Bacterial Isolation: Positive *Salmonella* colonies appeared green with black center on Hektoen agar (Fig. 1) and Had a black center and a highly transparent zone of reddish color on XLD media). Out of the 200 samplestested (Collected from the diseased and apparently healthy calves),seven samples were positive onto these selective media (3.5%). However, concerning the samples taken from the healthy contact calves no positive *Salmonella* growth could be detected.

Biochemical Identification: Vitek II analysis of the isolated strains revealed that all were identified as *Salmonella* species with accuracy up to 99%.

The biochemical identification of the isolated 7 strains showing the governorates from which the sample was collected, the organism identification the percentage of the probability and detailed biochemical reactions areshown in tables (1) to (7).

Serological Identification: The Serotyping of the 7 isolates revealed that they were identified as *S. Enteritidis*.

Identification by Real time qPCR: The invA gene was present nearly in all *Salmonella* species and it was used as a target for identification of the isolated strains. All the seven isolates gave positive amplicon, confirming both the biochemical and serological identification of the strains(Fig2).

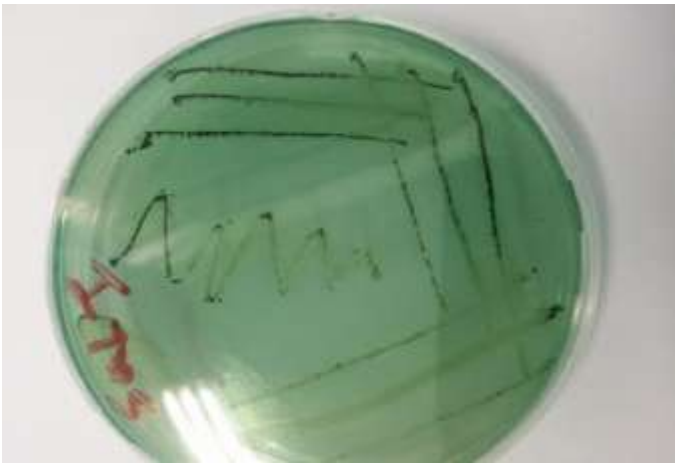

Fig. 1: Hektoen agar plate showing growth of the isolate showing a black precipitation of iron in the middle of the colonies.

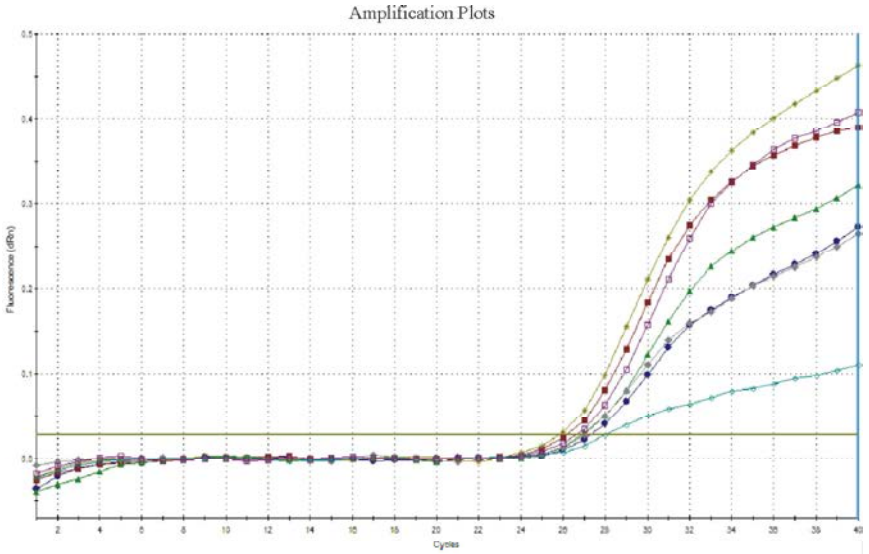

Fig. 2: Amplification plot of the 7 *Salmonella* spp isolates

Table 1: The biochemical identification and biochemical tests on Gram negative card of the isolate No. 1 collected from Sharkia governorate:

Sample No																	1
Location of sample																	Sharkia
Organism																	Salmonella spp
Probability																	96%
Confidence																	Excellent identification
Biochemical details																	
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	±	11	BNAG	-	12	AGLTp	-	13	dGLU	±	14	GGT	-	15	OFF	±
17	BGLU	-	18	dMAL	±	19	dMANE	±	20	dMNE	±	21	BXYL	-	22	BALAp	-
23	ProA	-	26	LIP		27	PLE		29	TyrA	±	31	URE	-	32	dSOR	±
33	SAC	-	34	dTAG	±	35	dTRE	±	36	CIT	±	37	MINT	-	39	5KG	-
40	ILATK	±	41	AGLU		42	SUCT	±	43	NAGA	-	44	AGAL	±	45	PHOS	±
46	GlyA	-	47	ODC	±	48	LDC	±	53	IHISA	-	56	CMT	±	57	BGUR	±
58	O129R	-	59	GGAA	-	61	IML Ta	-	62	ELLM	-	64	ILATa	-			

2-APPA: Ala-Phe-Pro-ARYLAMIDASE, 3-ADO: ADONITOL, 4-PyrA: L-Pyrrolydonyl ARYLAMIDASE, 5-L-ARL: L-ARABITOL, 7-D-CELLOBIOSE, 9-BGAL: Beta Galactosidase, 10-H2S: H2S production, 11- BNAG: Beta N. Acetyl glucose aminidase, 12-AGLTp: Glutamyl arylamidasepNA, 13-Dglu: D.glucose, 14-GGT: gamma glutamyl transferase, 15-OFF: fermintation/glucose, 17-BGLU: beta-glucosidase, 18-Dmal: D maltose, 19-Dman: D mannitol, 20-d MNE: D mannose, 21-BXYL: Betaxylosidase, 22-BALAp: Beta-alaine arylamidase p NA, 23-Pro A: L-proline arylamidase, 26-LIP: lipase, 27-PLE: palatinose, 29-TyrA: tyrosine arylamidase, 31-URE: urease, 32-d SOR: D-sorbitol, 33-SAC: saccharose/ sucrose, 34-d TAG: D-tagatose, 35-d TRE: D-trehalose, 36-CIT: citrate (sodium, 37-MINT: malonate, 39-5KG: 5-keto-D-gluconate, 40-ILATK: L-lactate alkalinisation, 41-AGLU: alpha glucose glucosidase, 42-SUCT: succinate alkalinisation, 43-NAGA: beta N-acetyl galactose aminidase, 44-AGAL: alpha galactosidase, 45-PHOS: phosphatase, 46-GlyA: glycine arylamidase, 47-ODC: ornithine decarboxylase, 48-LDC: lysine decarboxylase, 53-IHISA: l-histidine assimilation, 56-CMT: coumarate, 57-BGUR: beta glucoronidase, 58-O129R: O/129 resistance, 59-GGAA: glu-gly-argarylamidase, 61-IMLTa: L-malate assimilation, 62-ELLM: ellman, 64-ILATa: L-lactate assimilation.

Table 2: The biochemical identification and biochemical tests on Gram negative card of the isolate No. (2) collected from Sharkia governorate:

Sample No																
														2		
Location of sample														Sharkia		
Organism														Salmonella spp		
Probability														99%		
Confidence														Excellent identification		
Biochemical details																
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGL Tp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BALAp	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MINT	-	39	5KG	-
40	ILATK	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	+
46	GlyA	-	47	ODC	+	48	LDC	-	53	IHISA	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IML Ta	-	62	ELLM	-	64	ILATa	-			

Table 3: The biochemical identification and biochemical tests on Gram negative card of the isolate No. (3) collected from Kaliobia governorate:

Sample No																
														3		
Location of sample														kalobia		
Organism														Salmonella spp		
Probability														99%		
Confidence														Excellent identification		
Biochemical details																
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGL Tp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BALAp	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MINT	-	39	5KG	-
40	ILATK	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	(+
46	GlyA	-	47	ODC	+	48	LDC	-	53	IHISA	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IML Ta	-	62	ELLM	-	64	ILATa	-			

Table 4: The biochemical identification and biochemical tests on Gram negative card of the isolate No. (4) collected from Kaliobia governorate:

Sample No	4
Location of sample	kalobia
Organism	Salmonella spp
Probability	99%
Confidence	Excellent identification

Biochemical details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	+	11	BNAG	-	12	AGL Tp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BALAp	-
23	ProA	-	26	LIP	-	27	PLE		29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MINT	-	39	5KG	(-)
40	ILATK	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	(-)
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISA	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IML Ta	-	62	ELLM	-	64	ILATa	-			

Table 5: The biochemical identification and biochemical tests on Gram negative card of the isolate No. (5) collected from Kaliobia governorate:

Sample No	5
Location of sample	kalobia
Organism	Salmonella spp
Probability	99%
Confidence	Excellent identification

Biochemical details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	+	11	BNAG	-	12	AGL Tp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BALAp	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	-	34	dTAG	+	35	dTRE	+	36	CIT	-	37	MINT	-	39	5KG	-
40	ILATK	-	41	AGLU		42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISA	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IML Ta	-	62	ELLM	-	64	ILATa	-			

Table 6: The biochemical identification and biochemical tests on Gram negative card of the isolate No. (6) collected from Monofia governorate:

Sample No	6
Location of sample	Monofia
Organism	Salmonella spp
Probability	99%
Confidence	Excellent identification

Biochemical details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	+	11	BNAG	-	12	AGL Tp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BALAp	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	-	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MINT	-	39	5KG	-
40	ILATK	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISA	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IML Ta	-	62	ELLM	-	64	ILATa	-			

Table 7: The biochemical identification and biochemical tests on Gram negative card of the isolate No. (7) collected from Monofia governorate:

Sample No	7
Location of sample	Monofia
Organism	Salmonella spp
Probability	99%
Confidence	Excellent identification

Biochemical details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	+	11	BNAG	-	12	AGL Tp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BALAp	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	-	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MINT	-	39	5KG	-
40	ILATK	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISA	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IML Ta	-	62	ELLM	-	64	ILATa	-			

DISCUSSIONS

In this study the percentage of *Salmonella* isolated from diarrheic calves was 7%. Thisresult nearly agrees withEl-Shehedi*et al.*[7]who isolated *Salmonella* species with percent of 6.1% from fecal samples of diarrheiccalves in Egypt. Higher results were reported by Haggag and Khaliel [8] (4%), Younis*et al.* [9] (4.09%), Garcia *et al.*[10] (1.8%), Achá *et al.*[11] (2%) and Osama*et al.*[12] (1.56%) and lower results than reported were recorded by El-Seedy *et al.*[13] (18%), Youssef and El-Haig [1] (18.66%), Seleim *et al.* [14] (17%) and Riad *et al.*[15] (18.2%).

The variations inprevalence of *Salmonella* among apparently healthy and diarrheic calves reported in different countries reflect the effect of wide range of different management risk factors [16-18].

On contrary no *Salmonella* could be isolated from all apparently healthy calves,although *Salmonella* was found in only 7 calves of 200 calves that were sampled for this study the possibility exists that other calves were harboringthesebacteria but not shedding in their feces. These results may be attributed to different factors;*Salmonella* presents in low concentrations in fecal samples and it is more difficult to grow and detect *Salmonella* because of presence of other microorganisms. Non symptomatic ruminants shed *Salmonella* intermittently and therefore, infection is difficult to detect [19].

In this study all isolated *Salmonella* were recorded from young calves less than 3 months. Calves may be a greater risk of infection than adults due to their more naive immune system in addition to concurrent infection with multiple enteric pathogens (*E.coli*, rotavirus, corona virus) is a common scenario in calves [20].

In the current study, Vitek II analysis of the isolated strains revealed that all were identified as *Salmonella* species with accuracy up to 99%, Due to new, more sensitive fluorescence-based technology of the VITEK 2 system, final results were available after 5 h. Vitek II Gram negative identification method is a reliable and rapid automated method for accurate diagnosis of Gram negative bacteria. The identification results provided by Gram- negative cards are regarded as acceptable by numerous authors asFunke*et al.*[21],Jossartand Courcol [22] andLing*et al.*[23].

In the current study, serotyping of *Salmonella* isolates revealed that those were identified as *S. Enteritidis*. The predominance of *S. enteritidis* serovar among diarrheic calves detected in the present study was supported by many previous reports in Egypt [1-9-14-24, illustrate better]. In addition this finding substantiatedthe reports from the other countries [25, 26]. The predominance of S. *enteritidis* in our study might be due to the area of our study including the 3 governorates (Kaliobia, Sharkia and Monofia) which have the same policy of purchasing cattle and calves from dealers of cattle markets which facilitate the spread of the same source of infection between the 3 Governorates from the same geographic area.

The *invA* gene was present in all *Salmonella* species and it was used as a target for identification of the isolated strains. All the seven isolates gave positive amplicon, confirming both the biochemical and serological identification of the strains.

InvA target gene is located on *Salmonella*, pathogenicity island 1 (SPI1) which is essential for the invasion of epithelial cells by *Salmonella*. This gene is

highly conserved in almost all *Salmonella* species (Serotypes)and has been used as a potential target for *Salmonella* detection [27].

Real time PCR can contribute to meet the need of fast identification and detection methods for use in monitoring and control measures programe. However, it should not be used alone and be done in combination with conventional cultural methods for more accurate detection results of different *Salmonella* serovars.

The main benefits of real- time PCR are high speed, high sensitivity, high specificity, reduced amplicon size and no post PCR steps that reduce risks of cross contamination [28].

CONCLUSIONS

In conclusion, rapid and early diagnosis of bovine salmonellosis is very important for prevention of damages inflicted on livestock industry. So there is a need for more, reliable, accurate, acceptable and faster methods as Viteck II automated system and the diagnostic real time PCR for specific identification of *Salmonella* species. The VITEK 2 system identify Salmonella species with accuracy reach to 99% and Real time PCR used for amplification of a 150bp stretch of the *invA* gene was present in all *Salmonella*e species and can be used as a valuable tool for idetification and diagnosis bovine Salmonellosis.

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