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Genotypic detection of the *fim*A gene and virulence associated genes of salmonellea in cattle

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Abstract

Bacteriological examination of 640 fecal samples collected from apparent healthy (250) and diarrheic cattle (390) for the presence of Salmonella organism revealed that 12(1.87%) Salmonella isolates were isolated. The highest percentage rate for Salmonella recovery was in diarrheic cow calves (3.6%) followed by apparent healthy cow (2.2%). Serological identification of Salmonella isolates revealed that the most prevalent serovers were *S*.Typhimrium(2), *S*.Belgdam(2) and *S*.Lomita(2). Followed by S.Larochelle, *S*.Virchow, *S*.Infantis, *S*.Derpy, *S*.Anatum and *S*.Berzany one isolate for each. The goal of this study was to evaluate the suitability of the *fim*A gene amplification by Real-time PCR as a specific method for detection of salmonellae from fecal samples. It was noticed that 3 fecal samples were positive by Rt-PCR more than cultural method. On the other hand 2 samples were positive by cultural method and could not be detected by *fim*A Rt-PCR. *pef* and *avr*A virulence genes were detected in 7 and 4 out of 12 culture positive samples. In conclusion, Rt PCR is rapid, reproducible, and robust method for detection of genus *Salmonella*. However it should be taken in consideration that 10-20% of isolates could be missed due to absence of *fim*A gene. This may be corrected by application of multiplex Rt-PCR using more virulence genes.

Introduction

Bovine salmonellosis is a costly disease to dairy producer on account of mortality, treatment expenses, reduced milk yield and decreased weight gain within the herd as well as the hazard of transmission to humans either through food chain or direct animal contact[9)]. Calf diarrhea is the commonest disease in young calves and is the greatest cause of death[15)].

The manifestation of salmonellosis in cattle varies with virulence of the strain, infections dose and immunity of the host[10)]. Culture technique was universally recognized as standard method for the detection salmonellae however several rapid and sensitive methods have been developed for identification of Salmonella organisms and serovars from clinical samples based on detection of DNA.

Although certain types of *Escherichia coli* fimbriae are known to be important for virulence, only type 1 fimbriae have been implicated in Salmonella pathogenicity. Expression of type 1 fimbriae is encoded by a cluster of genes. A single gene *fim*A, encoded the major fimbrial subunit, that contain sequances unique to Salmonella strains and it is a suitable PCR target for detection of the genus *Salmonella*[6)]. Pathogenesis of salmonellosis depends upon a large number of factors controlled by an array of genes responsible for the actual virulence of Salmonella[11)]. The virulence of

Salmonella related to their ability to invade and colonize at the site of infection, replicate and then produce bacterial products like toxins[5]. Therefore the purpose of the study reported here was initially to estimate the prevalence of salmonellae in apparent healthy and diarrheic cattle, provide update recognition of Salmonella serovares and detection of salmonellae using *fim*A gene and its virulence associated genes.

Materials and methods

Sampling:
A total of 640 fecal samples were collected from apparent healthy cattle (250) and diarrheic cattle (390).The distribution of animals in relation clinical status and kind of animal as shown in table (2).
Bacteriolgical examination:
About 10gm of feces was inoculated into 90 ml of both Selenite-F-broth and tetrathionate broth incubated at 37°C for 16-18 hrs, and 24hrs, respectively. Then a loopful from the incubated broth was streaked on the surface of Salmonella Shigella (S.S) agar and Xylose Lysine Deoxycolate (XLD) agar, incubated at 37 °Cfor 24 hrs. The suspected isolates were purified and identified according to Quinn *et al.*[13] and by API 20E according to manufacture instruction.
Serological identification:
According to White Kuffmann-Le Minor scheme described by Grimont and Weill[7] using SIFIN antisera Berlinar Allee 317-321, 13088.
Detection of genus *Salmonella* by *fim*A gene Rt-PCR and virulence associated genes *avr*A and *pef* by cPCR.
Bacterial DNA preparation for PCR:
A total of 20 fecal samples, 12 Salmonella culture positive and 8 Salmonella negative were collected from cattle with symptom of salmonellosis and/ or suspected to be Salmonella positive and missed on culture method pre-enriched in tetrathionate broth. DNA extraction was done according to ABIOpure Genomic DNA extraction Kit instructions.
Primers: were supplied from metabion (Germany) as well as PCR amplification cycles were illustrated in table (1).

Results

Bacteriological examination of 640 fecal samples collected from healthy and diarrheic cattle revealed isolation of 12 *Salmonella* isolates with an overall incidence 1.87%.The frequency of Salmonella isolates in healthy and diarrheic animals is reported in table(2)
Serological identification revealed that the most prevalent serovars was *S*.Typhimurium (2)&*S*. Belgdam(2) and *S*.Lomita(2). Followed by *S*. Larochelle, *S*. Virchow, *S*.Infantis , *S*.Derpy & *S*.Anatum and *S*.Berzany one isolate for each (Table,3).
Detection of geuns Salmonella using fimA gene by Real time- PCR:
Thirteen fecal samples were positive by SYBER green Rt-PCR. The specificity of the reaction was confirmed by melting temperature (Tm) which was consistently specific for ampilicon obtained; the mean peak Tm obtained with curve specific was 82.60. The negative control did not show peaks in the Tm when subjected to 40 cycles of amplification. It was noticed that 3 samples no. 13&14 and16 could be detected while it was negative by culture method. On the other hand 2 samples (19&20) were positive by culture method and could not be detected by Rt-PCR. (Table4 & Fig 1).
Detection of virulence gens pef and avrA using conventional PCR:
The used primer of the *pef* gene (plasmid encoding fimbriae) multiply a region of 700 bp . Seven out of 12 (58.3%) culture positive samples harbor *pef* gene. The avrA gene (virulence associated gene) amplified a region 422 bp in 4 samples out of 12 (33.3%) (Table4& Fig 2 and 3)

Table (1): Oligonucleotide primers sequence and cycling condition.

gene	Primer((5' –3')	Reference	Cycling condition
***fim*A**	CCTTTCTCCATCGTCCTG AATGGTGTTATCTGCCTGAC CA	[6]	One cycle Primary denaturation at94^0 c for 5 min. then,40 cycle Secondary denaturation at94^0 c for 30 sec; Annealing at50^{0}c for 30 sec. Extension at72^0 c for 1min. Dissociation curve Secondary denaturation 95^{0}c for 1min. Annealing at50^{0}c for1 min. Final denaturation at95^{0}c for 30 sec.
pef	TGTTTCCGG GCT TGTGCT CAGGGCATT TGCTGATTCTTCC	[11]	Primary denaturation. 1 cycle at 94^{0}c for 6 min then 35 cycles Secondary denaturation at 94^0 c for 55 sec; Primer annealing at 55^0 c for 55sec; Extension at 72^0 c for 55 sec and one cycle final extension at 72^0 c for 10 min.
***avr*A**	CCT GTATTG TTG AGC GTCTGG AGAAGAGCTTCGTTGAATGTCC	[8]	1- Primary denaturation and activation 1 cycle at 94^0 c for 6 min then 35cycles, secondary denaturation at 95^{0}c for 30 sec; Primer annealing at 58^{0}c for 30 sec; Extension at72^0 c for30 sec and 1 cycle at 72^{0}c,7 min for Final extension

Preparation of PCR Master Mix for Rt-PCR: according to Quantitect SYBR green PCR kit.

Preparation of PCR Master Mix for cPCR: according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit.

Amplified PCR products was electrophoresed in 1.5% agar by agarose gel electrophoreses according to Sambrook *et al*.[14] and visualized by u.v. transilluminator.

Table (2): frequency distribution of *Salmonella* organism in relation to age and cattle status.

Animal	Apparent healthy			Diarrheic animals			Total	
	No	+ve	%	No	+ve	%	no	%
Cow calves	50	1	2	140	5	3.6	6/190	3.2
Buffalo calves	30	0	0	115	2	1.7	2/145	1.4
Cow	90	2	2.2	65	1	1.5	3/155	1.9
Buffalo	80	1	1.1	70	0	0	1/150	0.7
Total	250	4	1.6	390	8	2.05	12/640	1.87

*The percentage is calculated according to the no. of each clinical case.

** The percentage is calculated to the total no. of each kind of animal.

*** The percentage is calculated to the total no. of animals.

Table (3): Serological identification of *Salmonella* isolates in relation to age and cattle status.

Animal	Apparent healthy			Diarrheic animal			Total	
	Serovars	no	%*	Serovars	no	%*	no	%**
Cow calves	*S.*Belgdam	1/1	100	*S.*Lomita *S.*Belgdam *S.*Typhimurium *S.*Virchow	2/5 1/5 1/5 1/5	40 20 20 20	6	50
Buffalo calves	----------	0	0	*S.*Anatum *S.*Berzany	1/2 1/2	50 50	2	16.7
Cow	*S.*Larochelle *S.*Infantis	1/2 1/2	50 50	*S.*Typhimurium	1/1	100	3	25
Buffalo	*S.*Derpy	1/1	100	----------	0	0	1	8.3

*The percentage is calculated according to number of *Salmonella* serovars recovered in each kind of animal

** The percentage is calculated according to the total no of isolates (12)

Disscussion

Twelve Salmonella isolates were recovered from cattle with an overall incidence 1.87%. Four isolates were recovered from apparent healthy cattle with a percentage rate 1.6%. This finding is lower than that reported by[4] who said that 2.6% were positive for Salmonella with microbiological culture and declared that Salmonella infection can be present without clinical signs in cow and calves.

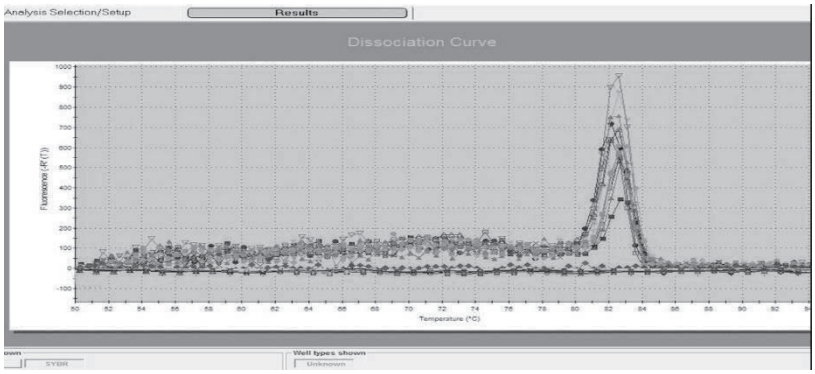

Fig. 1. Melting curve for salmonellae after 40 cycles

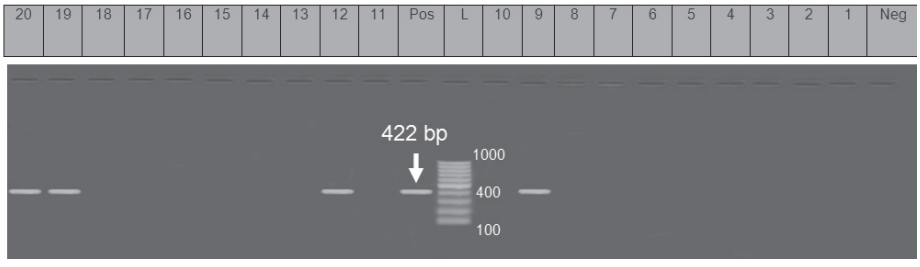

Fig. 2. Agar gel electrophoresis showing PCR with amplification of 422bp for *avr* A gene

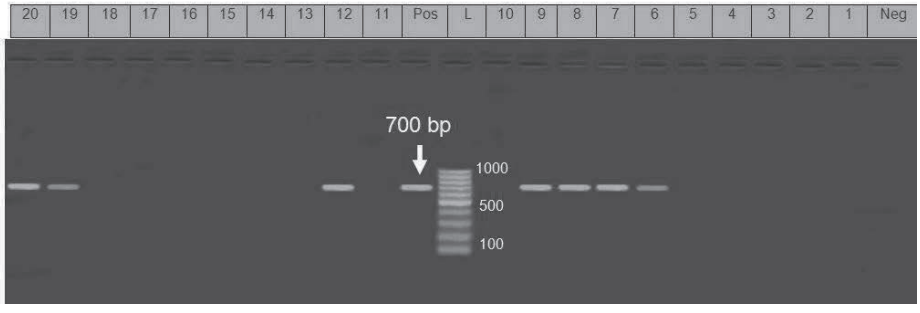

Fig. 3. Agar gel electrophoresis showing PCR with amplificaion of 700 bp for *pef* gene.

Table(4): Detection of *salmonellae* by *fim*A gene Rt-PCR and distribution of virulence genes *avr*A and *pef* among examined clinical samples.

Sample	Serovar	*Pef* cPCR	*avr*A cPCR	*fim*A Rt-PCR	Tm product Rt - PCR
1	*S*.Belgdam	-	-	+	82.70
2	-	-	-	-	80.60
3	-	-	-	-	80.60
4	*S*.Belgdam	-	-	+	82.60
5	*S*.Larochelle	-	-	+	82.60
6	*S*.Berzany	+	-	+	82.60
7	*S*.Anatum	+	-	+	82.60
8	*S*.Typhimurium	+	-	+	82.15
9	*S*.Typhimurium	+	+	+	82.70
10	*S*.Lomita	-	-	+	82.70
11	*S*.Lomita	-	-	+	82.10
12	*S*.Infantis	+	+	+	82.60
13	-	-	-	+	82.60
14	-	-	-	+	82.60
15	-	-	-	-	80.60
16	-	-	-	+	82.15
17	-	-	-	-	80.60
18	-	-	-	-	80.60
19	*S*.Derpy	+	+	-	80.60
20	*S*.virchow	+	+	-	80.60
21	Positive control	+	+	+	82.60
22	Negative control	-	-	-	80.60

It was noticed that the rate of Salmonella isolation was somewhat higher in cow calves than buffalo calves 3.2% versus 1.4% and cow than buffalo 1.9% versus, 0.7 respectively(Table,2). The variable results of Salmonella incidence here could be attributed to several factors including difference in standard of nutrition, presence of stress factors, medication used and time of sampling as Salmonella organism are intermittently excreted by the affected animal in addition to collecting of fecal samples during the chronic phase also lead to negative culture results. *S. typhimurium* was recovered from both diarrheic calf and cow and *S.belgdam* were recovered from both diarrheic calf and apparently healthy cow while *S. lomita* were recovered from 2 diarrheic cow calves (Table 3). These may be due to recycling and shedding.

In the current study *fim*A gene amplification was used as specific method for the detection of salmonellae by real time PCR. *fim* gene play a critical role in the colonization by faciliting the initial attachment to specific host cells and tissue. This was explained by Tinker and Clleggs[16] who mentioned that type 1 fimbriae of S.Typhimurium are surface appendages that carry adhesion specific for mannosylated host glyconjugates and confirmed by Althouse *et al.*[2] who reported that the type1fimbriae were important for attachment to enterocytes and promoted intestinal colonization and Naravaneni and Jamis[12] declared that *fim*A gene demonstrates a high degree of sequence conservation among Salmonella serovars, however Salmonella serotypes form type 1 fimbriae , which bestow on bacteria diverse adhesive properties, strains of S. Gallinarum and S. Pullorum form type 2 fimbriae, which lack their adhesiveness. S. Gallinarum yields an amplicon (*fim*A), indicating that the gene is still present, which may be in an inactive form[6].

SYBER Green real time PCR was made for developing a rapid, reproducible, and robust method for detecting genus *Salmonella*. It was confirmed by melting temperature, determining the Tm has marked advantage of eliminating the phase of electrophoresis which is time consuming, and carries the risk of laboratory contamination

with nucleic acid due to post PCR manipulation and requires the use of ethidium bromide which is potent mutagenic agent. Thirteen samples were positive by Rt –PCR as shown in table (4) & fig (1). It was noticed that 3 samples were positive by Rt-PCR more than cultural method. The negative result by culture might be explained by loss of viability of bacteria with specimen handling or lack of sensitivity of culture method. On the other hand 2 samples (no. 19& 20) were positive by cultural method and could not be detected by *fim*A Rt-PCR. This in accordance with the finding of Aleslamboly[1] who reported that *fim*A gene was the most common in all isolates of *S*.Enteritidis recovered from different sources, as it was present in all cattle and human isolates (100%). In case of S. Typhimurium , it was present in all human isolates 100% and 75% of cattle isolates .

Pef gene is one of the fimbirial important surface structures that allow bacterial fixation to the cell and play a role in the elongation, adhesion and invasion of intestinal epithelial cell[5]. Seven out of 12 isolates possess *pef* gene (58.3%) that detected by the presence of 700 bp. Salmonella pathogeniciy islands(SPIS) encode a number of virulence factors and play an important role in the pathogenicity of *Salmonella*. Invasion related gene *inv*A and virulence associated gene *avr*A are located within SPI. The *avr*A gene, is involved in the induction of programmed cell death and the inflammatory response of hosts against infection[3]. Quinolone-resistance strains may be associated with lower virulence (*avr*A gene expression) than in quinolone susceptible strains[17]. In conclusion, although bovine salmonellosis affects cattle of all ages it was clear that calves are more susceptible to infection than adults. Subclinical infected dairy cattle act as asymptomatic shedders and could be source of infection for both animal and human. *fim*A gene is useful for rapid detection of salmonellae from clinical samples by Rt-PCR. However it should be taken in consideration that 10-20% of isolates could be missed due to absence of *fim*A gene. This may be corrected by application of multiplex Rt-PCR using more virulence genes.

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