SOME BACTERIOLOGICAL STUDIES ON SALMONELLA INFECTION IN BUFFALO CALVES USING PCR TECHNOLOGY

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Abstract

In this study, out of 195 fecal samples collected from diarrheic calves at different localities from a private farm located in Kafr el-Sheik and Al Qualubia provinces, 120 of them were from Kafr el-Sheik and 75 were from Al Qualubia provinces. 12 were positive for *Salmonella* with a percentage of 6.1%. The typing of *Salmonella* isolates include *S.typhimurium*, *S. typhimurium* Lac+, *S.entertidis*, *S.dublin*, *S.gaillac*, *S.derby*, *S.virginia*, and *S.wingrove* which is one strain for each. Antibiogram for isolated strains revealed that all serogroups of Salmonella isolates were highly sensitive to enrofloxacin, levomicin, and neomycin. However, for tobramycin, amoxicillin, oxytetracycline, colistinsulphate and ampicillin, the total isolates were resistant. Variable results were recorded with the remaining tested antimicrobial agents. Real-time PCR test is a rapid, reproducible, and is a robust method for detecting *Salmonella enteritidis* and *Salmonella typhimurium* using primers SEFA-2 and SEFA-4 *for S.enteritidis*, fli 15, and tym for *S.typhimurium*. The specificity of the reaction was confirmed by the Tm, which was consistently specific for the amplicon obtained. The mean peak Tm obtained with curves specific for serotype *S. enteritidis* was 83.03°C, and was 84.04°C for *S.typhimurium*. The results of this study demonstrate that the SYBR Green I real-time PCR constitutes an effective and easy-to-perform method for detecting *S. enteritidis* and *S. typhimurium* in clinical samples.

Keywords: Buffalo calves, Diarrhea, *Salmonella*, SYBR Green dye

Introduction

Salmonella is a genus of Gram-negative rod-shaped bacteria of the family *Enterobateriaceae* which causes a wide production range of human diseases **(Forough et al., 2013). EFSA (2012)** published that *Salmonella* is one of the pathogens most often implicated in foodborne disease. Nevertheless, *Salmonella* has continued to be an important threat to public health. *Salmonella spp*. infection occurs when a susceptible animal ingests feed or water contaminated with faeces from animals shedding the organism (**Sheila and Simon, 2003).**

Consequently, calf diarrhea is commonly reported in young animals and is still a major cause of productive and economic loss to cattle producers worldwide. In the report of the 2007 National Animal Health Monitoring System for U.S dairy, half of the deaths among un-weaned calves were attributed to diarrhea (**Yong-il Cho and Kyoung-jin Yoon, 2014).**

Salmonella spp. was isolated in only 2% of all calves. *Salmonella*-infected calves have symptoms associated with endotoxemia. Thus, they are usually severely affected, do not drink milk, becomes severely dehydrated, and usually have a high fever. Feces are watery and are often tinged with blood. In addition, there is a high mortality rate among infected calves, with death occurring within 12-48 hours after the first signs appear. Salmonella can be infected at any age, but they usually affect calves that are over 10 days old. In some European countries, Salmonella has been identified as a widespread diarrhoeal agent in dairy calves (**Anonymous Trends and Sources of Zoonotic Agents in Animals, Feedstuffs, Food, and Man in the European Union, 1997).**

The diarrhoeal syndrome has a complex etiopathogenesis. However, this is because various infectious agents, either alone or in combination, may be associated with many field outbreaks. In addition, environmental, management, and nutritional factors influences the severity and outcome of the disease. Consequently, rotavirus, coronavirus, enterotoxigenic *E. coli*, and *Crytosporidium parvum* are the four major pathogens associated with neonatal calf diarrhoea worldwide. Thus, these organisms are responsible for the vast majority (75%–95%) of enteric infections in neonatal calves worldwide (**Tzipori S, 1985).**

Furthermore, serotypes which are significantly associated with animal and human disease include *S.Typhimurium*, *S.Enteritidis*, *S.Newport* **(Dunkley *et al*., 2009)** and *S. Kentucky* **(Majtán *et al*., 2006).** Thus, some of these serotypes include *S.Typhimurium* and *S.Enteritidis* which can be responsible for disease outbreaks leading to severe economic losses (**Calenge et al., 2010).**

Concerning the drug sensitivity, **Pantozzi et al. (2010)** reported that *Salmonella gaminara* (16:d:1,7) showed a greater percentage of resistance and multi-resistance in intensive breeding animals.

Ahmed *et al.* (2009) isolated nine *Salmonellae* from 220 fecal samples from neonatal calf diarrhea in Egypt. Six of these showed that the multidrug resistance and the isolated serovar were *S. Typhimurium* and *S. Enteritidis.*

Salmonella spp. can be detected by various methods such as conventional bacteriological culture, serological assays, polymerase chain reaction (PCR), and more recently, real-time PCR methods **(Lee et al., 2009).**

Real-time PCR test was made to develop a rapid, reproducible, and robust method for detecting *Salmonella enteritidis* and *Salmonella typhimurium.* **Marwa (2014)** reported that the results of the study demonstrated that the real-time PCR constitutes an effective and easy-to-perform method of detecting *S. enteritidis, S.typhimurium,* and *S.kentucky* in poultry samples.

Consequently, the aim of this study is to throw light on the prevalence of *Salmonellae*, from diarrheic calves by the isolation of *Salmonellae* from fecal samples of diarrheic buffalo calves, cultural and biochemical identification of the isolates, serological identification of the isolated *Salmonellae* using specific antisera, detection of antibiogram of antimicrobial sensitivity of the isolates, as well as detection of the virulence factors by SYPER Green dye.

Material and Methods

Sampling and Cultivation

For isolation of *Salmonella* strains, a small portion of 195 fecal samples collected from diarrheic calves in a private farm located at Kafr el-Sheik and Qualubia provinces, was inoculated into Selenite-F and Mullar Kauffman Tetrathionate novobiocin broths. Thus, it was streaked onto XLD agar, brilliant green agar, and Bithmus Sulfite agar after overnight incubation at 37°C for 24 h. Suspected colonies were subjected to microscopical examination and identification using API 20E system (Bio Merieux) Biochemical testing according to **Iso 6579 (2002).**

Serological Identification

Slide agglutination test was used for the identification of different serovars of isolated strain according to **Kauffmann-White le Minor Schema (2007) (Iso 6579-2002** part ш).

Antisera were obtained from **DENKA SEIKEN CO., LTD and Pro-lab diagnostic U.K,** polyvalent O, O1, polyvalent H (phase 1 and phase 2), Monovalent *Salmonella* O, and Mono H antisera.

Antibiogram

Antimicrobial susceptibility testing of all isolates was done by agar disk diffusion technique according to **Quinn et al. (2002).** However, Muller Hinton agar plates and (14) chemotherapeutic agents (Oxoid) were used during the testing process. The results were interpreted according to **NCCLS (2001).**

Materials Used for Extraction of DNA

1. QIAamp DNA Mini Kit- Catalogue no. 51304
2. Ethanol 96% Applichem

Materials Used for Mastermix Preparation for Real Time PCR

1. Quantitect SYBR green PCR kit- Cat. No. 204141 Containing 1 ml 2x QuantiTect SYBR Green PCR Master Mix, 2 ml RNase-Free Water.
2. Oligonucleotide primers and probes used in SYBR Green real-time PCR *(Source: Metabion- Germany)*

Table (1). Primers and Probes used in SYBR Green real-time PCR

Agent	Primer	Target gene	Primer sequence (5'-3')	Length of amplified product	Reference
S. Typhimutium	*fli 15*	*fli*C	CGGTGTTGCCCAGGTTGGTAAT	559 bp	(Soumet *et al.*, 1999)
S. Enteritidis	*SEFA2*	*sefA*	GCAGCGGTTACTATTGCAGC	310 bp	(Akbarmehr *et al.*, 2010)
	SEFA4		TGTGACAGGGACATTTAGCG		

SYBR Green Real Time PCR Method

1. Extraction of DNA according to QIAamp DNA mini kit instructions.
2. Preparation of PCR Master Mix according to Quantitect SYBR green PCR kit

Table (2). Preparation of PCR Master Mix

Component	Volume/Reaction
2x QuantiTect SYBR Green PCR Master Mix	12.5 μl
Forward primer (50 pmol)	0.5 μl
Reverse primer (50 pmol)	0.5 μl
RNase Free Water	4.5 μl
Template DNA	7 μl
Total	25 μl

Cycling Conditions for SYBR Green Real-time PCR

Cycling conditions for SYBR Green Real time PCR of *S. enteritidis* according to Quantitect SYBR Green PCR kit is as shown below (**Akbarmehr *et al*., 2010**).

Table (3). Cycling conditions for SYBR Green Real-time PCR of S.enteritidis

Stage	Temperature	Time	Cycles
Primary denaturation	94 ^oC	5 min.	1
Amplification a) Secondary denaturation	94 ^oC	30 sec.	40
b) Annealing	52 ^oC	30 sec. (optics on)	
C) Extension	72 ^oC	30 sec.	
Dissociation curve a) Secondary denaturation	95 ^oC	1 min.	1
b) Annealing	52 ^oC	1 min. (optics on till final denaturation)	
a) Final denaturation	95 ^oC	30 sec.	

* Cycling conditions for SYBR green real time PCR of S. Typhimutium according to Quantitect SYBR green PCR kit is as shown below (Soumet et al., 1999).

Table (4). Cycling conditions for SYBR Green Real-time PCR of S.typhimurium

Stage	Temperature	Time	Cycles
Primary denaturation	94 ^oC	10 min.	1
Amplification a) Secondary denaturation	94 ^oC	45 sec.	40
b) Annealing	56 ^oC	45 sec. (optics on)	
C) Extension	72 ^oC	45 sec.	
Dissociation curve a) Secondary denaturation	95 ^oC	1 min.	1
b) Annealing	56 ^oC	1 min. (optics on till final denaturation)	
a) Final denaturation	95 ^oC	30 sec.	

Results

Table (5). Prevalence of isolated *Salmonellae* from diarrheic buffalo calves

Source of Samples	No of Samples	No of positive	%
Kaffer el Shikh	120	7	5.8
Qalubia	75	5	6.6
Total	195	12	6.1

Table (6). Serological identification of isolated *Salmonellae* from diarrheic buffalo calves

Serovars	No	%	Antigenic Structure
S.Typhimurium	3	14.26	O1,4 [5],12, H1:I ,H2 1,2
S. Typhimurium Lac+	2	14.26	O1,4 [5],12, H1:I ,H2 1,2
S.Entertidis	2	14.26	O1,9,12,H1 g,m H2:-
S.Dublin	1	14.26	1,5,12[Vi] H1:g,P H2:-
S.Gaillac	1	14.26	O8 ,20 ,H1c ,H2 1,5
S.Derby	1	14.26	1,4[5],12,H1:F,g H2[1,2]
S.Virginia	1	14.26	O8,H1:d, H2: 1,2
S.Wingrove	1	14.26	O6,8,H1:C,H2:1,2
Total	12	100	

Table (7). Antimicrobial discs used for *Salmonellae.* The result of sensitivity test of *Salmonella isolates*

Antimicrobial agents	Disc code	Conc (µg)	S.Typhimurium	S. Typhimurium Lac+	S.Enteritidis	S.Dublin	S.Gaillac	S.Derby	S.Virginia	S.Wingrove
Levofloxacin	LEV	5	S	S	S	S	S	S	S	S
Tobramycin	TOB	10	S	S	R	I	S	I	R	I
Ceftriaxone	CRO	30	S	I	S	I	S	S	S	S
Enrofloxacin	ENR	5	S	S	S	S	S	S	S	S
Gentamicin	CN	10	S	S	S	I	S	I	S	S
Amikacin	AK	30	S	R	I	R	I	R	R	I
Tetracycline	TE	30	R	R	R	R	R	R	R	R
Sulphamethaxozle+Trimethoprim	SXT	25	S	S	S	I	S	S	S	S
Nalidixic acid	NA	30	R	R	R	R	S	R	I	I
Ampicillin	AMP	10	R	R	R	R	R	R	R	R
Neomycin	Neo	30	S	S	S	S	S	S	S	S
Levomicin	lev	5	S	S	S	S	S	S	S	S
Danofloxacin		30	S	S	S	S	S	S	S	S
Cefadroxil		30	R	R	R	R	R	R	R	R

N.B: S: Sensitive I: Intermediate R: Resistant

Detection of *S.typhimurium* and *S. enteritidis* by SYBR Green Real-time PCR

In the detection of *Salmonella enteritidis*, *Salmonella typhimurium* and using primers *SEFA*-2 and *SEFA*-4 for *S.enteritidis*, and *fli*15 and *tym* for *S.typhimurium* respectively, the specificity of the reaction was confirmed by the Tm. Hence, this was consistently specific for the amplicon obtained. The mean peak Tm obtained with curves specific for *S.* Enteritidis was 83.03°C, while it was 84.04°C and 83.99°C for *S.typhimurium* respectively. The negative controls and the samples contaminated with serotypes other than serotype enteritidis did not show peaks in the Tm when they were subjected to 35 cycles of amplification. However, they did show some peaks (for example, *S. typhimurium*) when they were subjected to 40 cycles.

Table (8). Dissociation curve for *S. enteritidis*

Well	Well Name	Dye	Tm Product 1 (-R'(T))
A1	Neg. *S. typhimurium*	SYBR	78.58
B1	*S. enteritidis*	SYBR	83.03
C1	Pos. *S. enteritidis*	SYBR	83.50

Table (9). Dissociation curve for *S. typhimurium*

Well	Well Name	Dye	Tm Product 1 (-R'(T))
A1	*S.* typhimurium lac+ve	SYBR	84.04
C1	*S.* typhimurium	SYBR	84.01

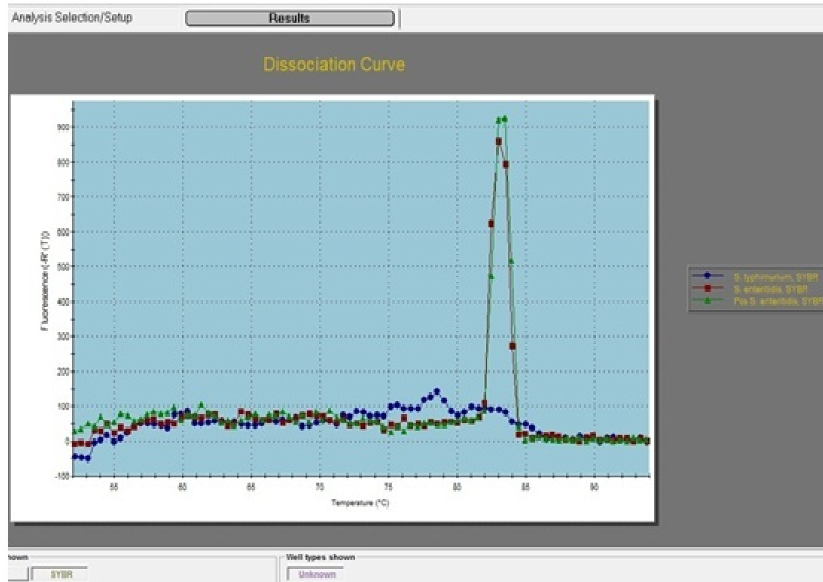

Fig. (1). Melting curve of *S. enteritidis* after 40 cycles

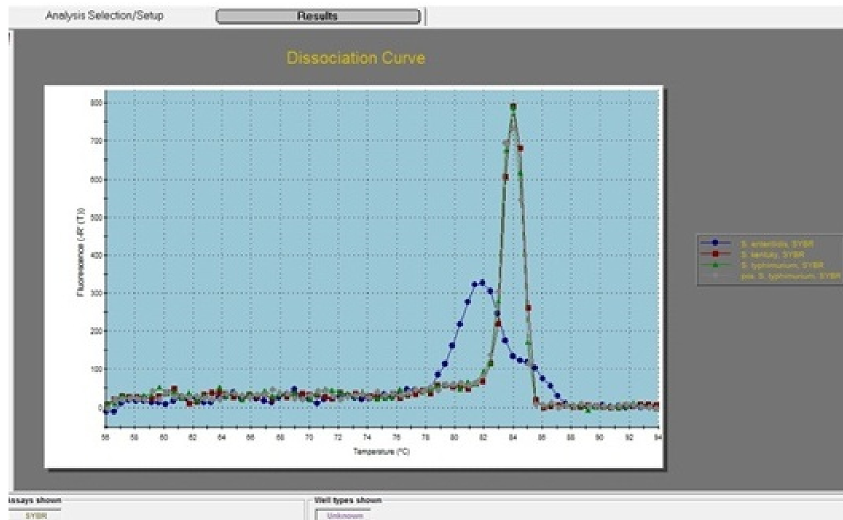

Fig. (2). Melting curve *of S. typhimurium* after 40 cycles

Discussion

Salmonella spp. is routinely detected in clinical, food, and environmental samples using microbiological culture after an enrichment step. Thus, it should be serotyped. Although this microorganism is non-fastidious and shows fast growth, up to 72 h or even more time is required to culture and type *Salmonella* isolates **(Woodward and Kirwan, 1996 and Youg, 2014).** In this study, a total of 40 fecal samples were examined from diarrheic buffalo calves for the detection of *Salmonella spp*., while 8(20%)

strains were isolated. Consequently, this result was almost coordinated through some researchers (**Anonymous, 1997).**

Salmonella is a zoonotic bacterial agent, while *S. enterica serotype typhimurium* is the most common serotype found in animals and in humans **(McDonough, 1986).** However, a child helping to manage calves became ill with an acute gastrointestinal disease manifested by vomiting, fever, diarrhea, and cramps. Stool specimens were cultured from this child, but *Salmonella* was not isolated in vitro. Its presumptive diagnosis, however, was Salmonellosis. This is partly based on the estimated incubation period of her disease, and on the knowledge of the Salmonellosis situation on her family farm.

S. Enterica Serotype Typhimurium Lac+

The epidemiological triad of disease consists of the host, the agent, and the environment, with all of its stress factors. For New York and Pennsylvania outbreaks, the host is the 3-day-old immunocompromised veal calf (hypogammaglobulinemic due to lack of colostrum or insufficient colostrum). However, this is trucked at least twice in its journey from the dairy farm of origin to the sales yard, and to the final destination at the veal farm. This calf potentially is exposed to the agent, i.e., *Salmonella*, in the environments of all the places it has been moved to. Calves tend to suckle and lick each other and their environmental surfaces during trucking **(Patrick et al., 2000).**

In each of these outbreaks, the strain of Lac+ *S. enterica serotype typhimurium* appeared to be more virulent, i.e., Death among calves within hours after clinical signs have been noted (**Patrick et al., 2000).**

In this present study, the isolate serotypes were sensitive to levofloxacin. However, most isolated serovars were resistant to tetracycline, nalidixic acid, ampicillin, and amikacin. Thus, based on the remaining use of chemotherapeutic, the results were variable. These results were nearly coordinating with many authors.

Ahmad and Shimamoto (2012) reported that *Salmonella* isolates displayed multidrug resistance phenotypes, particularly against Ampicillin, Streptomycin, Spectinomycin, kanamycin, Tetracyclin, Chloramphenicol, Trimethoprim, and Sulphamethoxazole.

Akond *et al.* (2012) found that 76% of isolates were sensitive against Gentamicin. Moreover, **Boris *et al.* (2012)** found high sensitivity to Tetracycline. Also, **Abd-El Rahman *et al.* (2000)** reported that *Salmonella* species were sensitive to Enrofloxacin. These results were near to **Chashni *et al.* (2009)** who found that the antibiotic resistance analysis of most *Salmonella* isolates were sensitive to Norfloxacin (93%). They added that some of the isolates had intermediate sensitivity to Tetracycline, while the maximum antibiotic resistance was observed against Ampicillin (63%).

Thus, it also almost agrees with **Akter *et al.* (2007)** who revealed that the isolates were sensitive to Sulphamethoxazole/ Trimeoprim and Amoxycillin (50%), besides Tetracycline (60%). However, it differs from **Liu *et al.* (2010)** who revealed that there was no resistance to Gentamicin. Thus, most of the isolates were resistant to Doxycycline and Tetracycline. Also, it differs from that of **Oliveira *et al.* (2006)** who said that 100% of the isolated *Salmonella* strains showed resistance to Ampicillin and Tetracycline. Meanwhile, in this study, *Salmonella* isolates were highly resistant to Ampicillin and Nalidixic acid with a percentage of 84% and 69%, respectively. This agrees with **Yah and Eghafona (2007)** who reported that the isolates were highly resistant to Ampicillin and Tetracycline. Also, this is similar to **Munawwar *et al.* (2010)** who observed high degree of resistance for Ampicillin and Tetracycline. Furthermore, the results was close to that of **Ahmed (2011)** who found that the highest percentage of resistance (87.5%) was the Amoxicillin and Ampicillin. Thus, this differs from **Fallah *et al.* (2013)** who stated that 100% of *Salmonella* isolates were resistant to Nalidixic acid and Tetracyclin.

In the present study, isolated *S.derby* and *S. enteritidis* isolates were sensitive to Enrofloxacin and Gentamycin. Therefore, this agreed with **Balala *et al.* (2006)** who concluded that *S*. derby and *S*. enteritidis strains were sensitive to Norfloxacin, Gentamicin, and Cephalothin.

In addition, *S.typhimurium* isolates was sensitive to Enrofloxacine and Ceftriaxone. This agrees with **Nagappa *et al.* (2007)** who found that *Salmonella enterica serovar typhimurium* was sensitive to Chloromphenicol, Colistin, Polymixin, Enrofloxacin, and Ciprofloxacin. Also, **Begum *et al.* (2010)** identified that *S.typhi, S.typhimurium,* and *S.enteritidis* were found to be sensitive to Ceftriazone, Ciprofloxacin, Cephalexin, Gentamycin, and Chloramphenicol. On the other hand, some strains have shown resistance to Co-Trimoxazole, Nalidaxic acid, Ampicillin, Tetracyclin, and Kanamycin. Therefore, these results differ from **Chiu *et al.* (2010)** who stated that *S. typhimurium* was resistant to Ciprofloxacin and/or Enrofloxacin.

SYBR Green Real Time PCR Test

Consequently, this test was made for developing a rapid, reproducible, and robust method of detecting *Salmonella enteritidis and Salmonella typhimurium* in diarrheic buffalo calves samples using primers *SEFA*-2 and *SEFA*-4 (310 bp) for S.enteritidis, and *fli* 15 and tym (559 bp) for S.typhimurium, respectively.

Significance and Impact of the Study

SYBR Green RT-PCR is a powerful tool for rapid and accurate Salmonella monitoring in diarrheic buffalo calves samples together with standard bacteriological testing.

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