Fecal Shedding of Non-typhoidal *Salmonella* Species in Dairy Cattle and their Attendants in Alexandria Suburbs

Osama N. Mohamed[1], Adel F. Farid[2], Amani F. Abaza[*1], and Rania F. Faltas[2]

[1]Microbiology Department, High Institute of Public Health, Alexandria University, Egypt
[2]Department of Bacteriology, Animal Health Research Institute, Ministry of Agriculture, Egypt
[*]amani_abaza@yahoo.com

Abstract: *Salmonella* infections in dairy cattle continue to be a major worldwide problem. Substantial economic losses were manifested through mortality and poor growth of infected animals as well as the hazard of transmission to humans either through food chain or direct animal contact. Our objective was the isolation and identification of *Salmonella* spp. shed in feces of dairy cattle and their attendants, together with the determination of their serotypes and antimicrobial susceptibility patterns. Fecal samples were cultured on non selective pre-enrichment broths, and selective enrichment broths and agar media. Serotyping of *Salmonella* spp. isolates was performed by slide agglutination tests and then screening for their antibiotic susceptibility. Seven *Salmonella* spp. (1.56%) were isolated from the 450 examined dairy cattle, while no *Salmonella* spp. were isolated from any of the examined attendants. *Salmonella* isolates were classified as serogroups B, C1, D1 and E1, with C1 as the most commonly observed serogroup (57.1%). Five different *Salmonella* serotypes were identified (Typhimurium, Anatum, Concord, Montevideo and Enteritidis). The 7 isolated *Salmonella* spp. showed no resistance to all tested antimicrobial agents except for trimethoprim-sulphamethoxazole and gentamycin. Application of optimal hygienic conditions and management strategies minimize the occurrence and spread of the *Salmonella* infections on dairy farms, as no *Salmonella* spp. were isolated from farm C, which had the proper hygienic conditions and management practices.
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1. Introduction

Salmonella; a genus of family Enterobacteriaceae, is a primary etiologic agent of infectious diarrhea (Collee *et al*., 1996). This organism can be pathogenic for both man and animals. Salmonellosis, the clinical form of *Salmonella* infection, is a costly disease to dairy producers on account of mortality, treatment expenses, reduced milk yield, and weight loss/decreased weight gain within the herd. Infected cattle can be either clinical or subclinical, shedding *Salmonella* in their feces; thus dairy producers need to be aware that *Salmonella* can be found on their farms within apparently healthy cows, which is important in terms of food safety risks (Callaway *et al*., 2005). Persistence of infection is an important epidemiologic feature of salmonellosis and can be related to serotype to which animal is infected (Van Kessel *et al*., 2007), (Heuvelink *et al*., 2007). Moreover dairy cattle infected with non- typhoidal *Salmonella* (NTS) spp. can pose a substantial risk to public health (Cummings *et al*., 2010). The global human health impact of NTS is high, with an estimated 93.8 million illnesses, of which 80.3 million are food-borne and 155,000 deaths each year (Majowicz *et al*., 2010). In Egypt, in a study done by Salman and Tanios (2004) to screen diarrheic cow calves for the isolation and identification of *Salmonella* serovars, *Salmonella* spp. were recovered from 11% of all calves tested (Salman and Tanios, 2004).

2. Material and Methods:

This study was carried out on 450 dairy cattle, 47 males and 403 females of various age groups and all their available attendants (only 12 attendants agreed to submit fecal samples for examination), from three different dairy farms A, B and C in Alexandria suburbs during the period from June 2007 to September 2008.

Questionnaire sheets including all the relevant information were filled for all studied cattle and their attendants.

The following samples were collected: (Warnick *et al*., 2003a; Fossler *et al*., 2005)

-Fecal samples were collected from all dairy animals of the three examined dairy farms included in this study, where 150 dairy animals had been randomly chosen from each farm and they were further categorized by age groups (50 from each age group) into the following:-

1. Pre-weaned calves; fed milk (0- <3 months of

age).
2. Growing calves & heifers; between weaning and before first calving (≥3 - <24 months of age).
3. Adult cattle; they had calved once or more times (≥ 24 months).

-All animal attendants were asked to provide stool samples.

-All collected samples were kept in an ice box and were transported to the laboratory within 2 hours. Each fecal sample was subjected to the following (World Health Organization, 2003):

1. Non selective pre- enrichment using buffered peptone water (BPW).
2. Selective enrichment using Tetrathionate (TT) broth and Rappaport Vassiliadis soy peptone (RVS) peptone broth.
3. Culturing on selective media; Xylose lysine desoxycholate (XLD) and Bismuth sulfite (BS) agar plates.

Isolated colonies (pink or reddish color with black centre on XLD and black colonies with black halo and metallic sheen on BS were identified morphologically by microscopic examination and biochemically to verify that they were *Salmonella* spp.

Biochemically identified isolates were then inoculated on nutrient agar and incubated at 37°C for 24 hrs for serotyping (World Health Organization, 2003). Serotyping of *Salmonella* spp. isolates was performed on the basis of somatic O and phase 1, phase 2 flagellar antigens by slide agglutination tests with antisera using Kaufmann– White scheme according to Popoff (Popoff, 2001). Then they were screened for their antibiotic susceptibility by single disc diffusion method described by Bauer *et al*. (1966). The test was done on Mueller Hinton agar plates, using the 8 selected antibiotic discs with various concentrations. Inhibition zones were measured and susceptibility was recorded as susceptible (S), Intermediate (I) and resistant (R) according to standard tables published by Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2007).

Statistical analysis (Altman, 1992; Armitag *et al*., 2002)**:**

The results of the present study were tabulated and statistical analyses were conducted using PC with the software: Statistical Package for the Social Sciences (SPSS) version 15 and Excel.

Statistical significance was set at 5% ($P < 0.05$).

The following tests were done:

Z- test,

Chi- square test (X^2)

Rater agreement (Kappa)

Agreement between the two tests =

$$\frac{\text{Positive results by both tests + Negative results by both tests}}{\text{Total number of samples}}$$

3. Results:

Results:

Of the 450 dairy cattle tested, *Salmonella* were isolated from 1.56% of them. No *Salmonella* spp. were isolated from any of the 12 examined attendants.

All isolated *Salmonella* spp. were recovered from pre-weaned calves and no *Salmonella* strains were isolated from either growing calves & heifers or adult cattle.

The highest percentages of isolated *Salmonella* spp. were recovered from diarrheic animals (7.69%) compared to only 0.97% from apparently healthy animals (Table 3).

Salmonella spp. were isolated from farms A and B in percentages of 2.00%, 2.67%, respectively but no *Salmonella* isolates were detected in farm C (Table 3).

The majority of *Salmonella* isolates were recovered by RVS broth (71.43%) compared to only 42.86% by TT broth, but they had very good agreement (Table 4).

XLD agar showed much higher efficiency in the isolation of the 7 *Salmonella* spp. (100%) compared to only (14.29%) by BS agar (Table 5).

Salmonella isolates were classified as serogroups B, C1, D1 and E1, with C1 as the most commonly observed serogroup, accounting for more than half of the *Salmonella* isolates (57.1%). Serogroups B, D1 and E1 were of 14.3% each. They were represented by serotypes Typhimurium, Anatum, Concord, Montevideo and Enteritidis (Table 6).

Salmonella serovars were isolated from diarrheic and apparently healthy dairy cattle with the following distribution and were elucidated namely, *S.* Typhimurium 2.56% and 0.00%; *S.* Montevideo 0.00% and 0.49%; *S.* Enteritidis 0.00% and 0.24%; *S.* Anatum 2.56% and 0.00%; while *S.* Concord was identified in 2.56% and 0.24% respectively (Table 7).

All of the 7 *Salmonella* isolates (100%) were susceptible to ampicillin, tetracycline, chloramphenicol, nalidixic acid, ciprofloxacin, ceftriaxone and resistant to trimethoprim-sulphamethoxazole, while only one isolate (14.3%) was resistant to gentamycin (Table 9).

Figure (1): Flow diagram for detection of *Salmonella* from feces

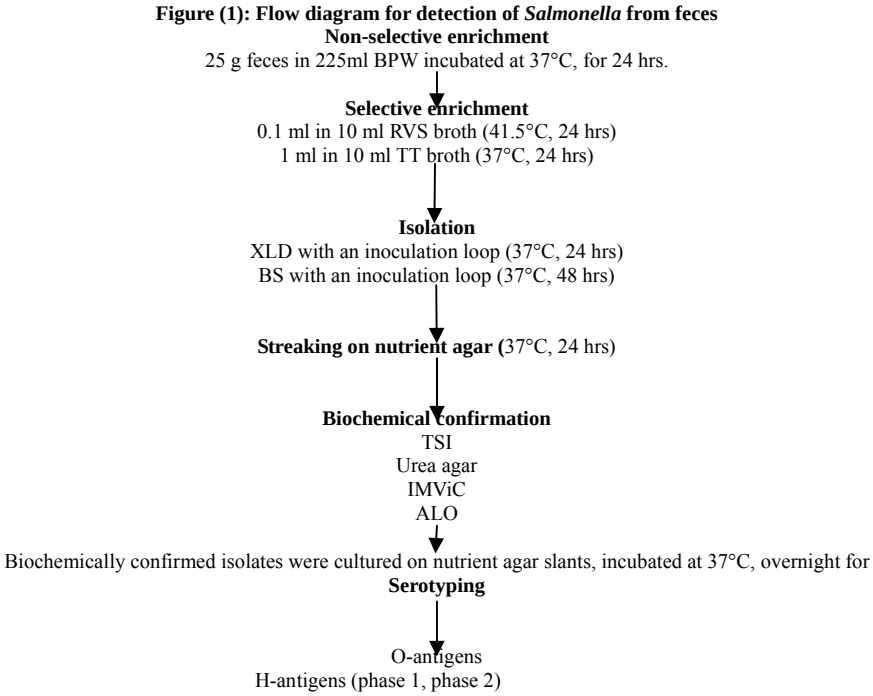

Table (1): General characteristics of the three examined dairy farms.

Examined farm / Farm characteristics	Farm A	Farm B	Farm C
Animals purchased in the past 12 months (replacement stock)	Yes	Yes	No
Quarantine of newly added animals	No	No	Not applied
Housing:			
Exclusive use of individual animal area(boxes) to house pre-weaned calves	Individual boxes & group housing	Group housing only	Individual boxes
Use of maternity housing as a hospital area for sick cows	No	Yes	No
Bedding type	Sand& crushed limestone	Concrete (maternity pen: sand)	Sand
Calf management & feeding			
Feeding colostrum only from their dams (times of feeding)	Yes (3 times daily/ 3 days)	No (pool colostrum)	Yes (3 times daily/ 3 days)
Type of milk routinely fed to pre-weaned calves	Whole milk +medicated milk replacer	Whole milk + waste milk	Whole milk
Suckling method	Training on calf milk bucket		Bucket teat
Calf milk buckets routinely washed between each feeding	Yes	No	Yes
Farm characteristics			
Water & feed systems:			
Water supply (source of drinking water)	Well	Municipal water	Municipal water
Primary water source for dairy cattle is chlorinated	No	Yes	Yes
Purchased feeds or feeds obtained from off farm sources	No	Yes	No
Manure management:			
Method used to remove manure from cow housing areas	Alley scraper (mechanical)	Hand fork or shovel	Alley scraper (mechanical)
Manure disposal on owned or rented land	Yes	Yes	Yes
Use loader bucket to move feed & manure (could be the same)	Yes	Yes	No
Other farm characteristics:			
Access of wild birds/ rodents	Yes (noted in feed storage area)		
Use of chemicals/ bait for rodents control	No	No	Yes
Presence of dogs/ cats	Yes	Yes	Yes

Table (2): Distribution of the 450 examined dairy cattle from the three selected dairy farms according to their health condition and age groups, Alexandria suburbs (June 2007- September 2008)

Health condition / Age Groups	Farm A (150)				Farm B (150)				Farm C (150)			
	Diarrheic		Apparently healthy		Diarrheic		Apparently healthy		Diarrheic		Apparently healthy	
	No	%	No	%	No	%	No	%	No	%	No	%
Preweaned- calves	11	64.7	39	29.32	4	50.0	46	32.39	6	42.9	44	32.4
Growing calves & Heifers	2	11.8	48	36.09	2	25.0	48	33.8	5	35.7	45	33.1
Adult cattle	4	23.5	46	34.59	2	25.0	48	33.8	3	21.4	47	34.6
Total	17	11.3	133	88.67	8	5.3	142	94.67	14	9.33	136	90.7
Test of significance	X^2=8.89		P= 0.011		X^2= 1.06		P=0.589		X^2= 1.1		P=0.576	

Table (3): Distribution of the 7 isolated *Salmonella* spp. according to health condition of the 450 examined dairy cattle, Alexandria suburbs (June 2007- September 2008)

Examined farms	Health condition	No. of *Salmonella* isolates	% of *Salmonella* isolates	Total No. (%)	Test of significance
A (150)	Diarrheic (17)	1	5.88	3 (2.00)	
	Apparently healthy (133)	2	1.50		
B (150)	Diarrheic (8)	2	25.00	4 (2.67)	
	Apparently healthy (142)	2	1.41		
C (150)	Diarrheic (14)	0	0.00	0 (0.00)	
	Apparently healthy (136)	0	0.00		
Total (450)	Diarrheic (39)	3	7.69	7 (1.56)	X^2= 10.5
	Apparently healthy (411)	4	0.97		P= 0.001

Table (4): Comparative efficiency of RVS and TT enrichment broths as regards the isolation of 7 *Salmonella* spp., Alexandria suburbs (June 2007- September 2008)

Selective enrichment / *Salmonella* isolation	RVS broth		TT broth		Test of agreement			
	No.	%	No.	%	A	B	C	D
Positive	5	71.43	3	42.86	0.987	0.244	4.420	0.0001
Negative	2	28.57	4	57.14				

A= Observed agreement B= Kappa coefficient C= Z of Kappa D= P value

Agreement between the two enrichment broths= $\frac{\text{Positive results by both broths + negative results by both broths}}{\text{Total number of samples}}$

Table (5): Comparative efficiency of XLD agar and BS agar as regards the isolation of *Salmonella* spp., Alexandria suburbs (June 2007- September 2008)

Differential plating / *Salmonella* isolation	XLD agar		BS agar		Test of agreement			
	No.	%	No.	%	A	B	C	D
Positive	7	100	1	14.29	0.987	0.247	5.07	0.0001
Negative	0	0.00	6	85.71				

A= Observed agreement B= Kappa coefficient C= Z of Kappa D= P value

Agreement between the two enrichment broths= $\frac{\text{Positive results by both broths + negative results by both broths}}{\text{Total number of samples}}$

Table (6): Frequency of O- antigen groups of the 7 *Salmonella* spp. isolated from examined dairy cattle, Alexandria suburbs (June 2007- September 2008).

Frequency / O- antigen group	No.	%
B	1	14.3
C1	4	57.1
D1	1	14.3
E1	1	14.3
Total	7	100.0

Table (7): Frequency of the 7 *Salmonella* serotypes isolated from examined dairy cattle, Alexandria suburbs (June 2007- September 2008).

Frequency / Serotype	No.	%
Enteritidis	1	14.3
Montevideo	2	28.6
Concord	2	28.6
Anatum	1	14.3
Typhimurium	1	14.3
Total	7	100.0

Table (8): Antigenic formulae of the 7 *Salmonella* serovars isolated from examined dairy cattle, Alexandria suburbs (June 2007- September 2008).

***Salmonella* serotypes & serogroups**	**No. of serovars**	**Somatic (O) antigen**	**Flagellar (H) antigen**	
			Phase 1	**Phase 2**
***S.* Enteritidis** Group O:9 (D1)	1	1,9,12	g,m	–
***S.* Montevideo** Group O:7 (C1)	2	6,7,14	g,m,[p],s	[1,2,7]
***S.* Concord** Group O:7 (C1)	2	6,7	l,v	1,2
***S.* Anatum** Group O:3,10 (E1)	1	3,10	e,h	1,6
***S.* Typhimurium** Group O:4 (B)	1	1,4,[5],12	i	1,2

Table (9): Antimicrobial susceptibility pattern of the 7 *Salmonella* isolates from examined dairy cattle, Alexandria suburbs (June 2007-September 2008)

Antimicrobial agent	**S**		**I**		**R**	
	No.	**%**	**No.**	**%**	**No.**	**%**
Ampicillin	6	85.7	1	14.3	0	0.0
Tetracycline	6	85.7	1	14.3	0	0.0
Chloramphenicol	7	100.0	0	0.0	0	0.0
Gentamycin	6	85.7	0	0.0	1*	14.3
Trimethoprim - Sulphamethoxazole	0	0.0	0	0.0	7	100.0
Nalidixic acid	6	85.7	1	14.3	0	0.0
Ciprofloxacin	6	85.7	1	14.3	0	0.0
Ceftriaxone	6	85.7	1	14.3	0	0.0

S= Susceptible * = *S.* Concord I= Intermediate SXT= Trimethoprim-Sulphamethoxazole R= Resistant

4. Discussion:

Salmonella infections in dairy cattle continue to be a major worldwide problem. Substantial economic losses were manifested through mortality and poor growth of infected animals as well as the hazard of transmission to humans either through food chain or direct animal contact. Identification of infected animals is fundamental to on-farm *Salmonella* control (Smith *et al*., 2004; Younis *et al*., 2009).

In the present study, Out of the 450 examined dairy cattle, 7 (1.56%) *Salmonella* spp. were isolated. Nearly similar results were reported by García *et al*. (2000) in Spain and Achá *et al*. (2004) in Mozambique, where *Salmonella* spp. were detected in the feces of neonatal diarrheic dairy calves and were of 1.8% and 2% respectively (García *et al*., 2000; Achá *et al*., 2004).

In the U.S., much higher percentages were documented by Warnick *et al*. (2003) in New York, and Cummings *et al*. (2009) in the northeastern United States, where *Salmonella* spp. were isolated from 9.9% and 22.5%, respectively. This was explained by the fact that sampled animals were chosen with priority given to previously *Salmonella* positive ones, sick pre-weaned calves and cows, cows within 2 weeks after calving and then other animals, in addition to the frequent sampling from the same animal, thus covering intermittent nature of *Salmonella* fecal shedding; which was not feasible in our study (Warnick *et al*., 2003a; Cummings *et al*., 2010).

In the present study and since *Salmonella* spp. can be transmitted by direct animal or fecal contact, animal attendants were asked to provide stool samples for culturing, but obtaining samples from all of them was not possible except for only 12; who

agreed to submit fecal samples for examination. No *Salmonella* spp. were isolated from any of the examined attendants. In contrast to what was reported in the present study, Hagagg *et al*. in Egypt (2005) reported *Salmonella* spp. in 7% of dairy farm workers and were of serotypes Typhimurium and Enteritidis.

Although *Salmonella* can cause illnesses in adult cattle, bovine salmonellosis is predominantly seen in young calves (Callaway *et al*., 2005). In this study, all isolated *Salmonella* spp. were recovered from pre-weaned calves (1.56%) and no *Salmonella* strains were isolated from either growing calves and heifers or adult cattle. These findings agreed with those reported by Gay and Hunsaker (1993), Pacer *et al*. (1989), Warnick *et al*. (2003) and Pangloli *et al*. (2008); who identified pre-weaned calves as one of the higher probability cattle group from which *Salmonella* spp. were isolated. (Pacer *et al*.; 1989, Gay and Hunsaker, 1993; Warnick *et al*., 2003b and Pangloli *et al*., 2008)

Salmonella infection can cause clinical disease in cattle, with the most common clinical signs being fever and diarrhea. However, it has been isolated from the feces of healthy dairy cattle, where it may exist as a normal or a transient member of the gastrointestinal population (Callaway *et al*., 2005, Fossler *et al*., 2005). In the present work, the highest percentages of isolated *Salmonella* spp. were recovered from diarrheic animals compared to apparently healthy animals (7.69% and 0.97% respectively). In Egypt (2005), in a study conducted in Alexandria and Beherra provinces, *Salmonella* spp. were isolated from 3.85% diarrheic and 1.43% apparently healthy dairy cattle (Hagagg *et al*., 2005).

In our work, BPW was used for pre-enrichment, RV and TT broths for selective enrichment and XLD and BS agar for plating. It was found that, 71.43% of recovered *Salmonella* spp. were isolated by RVS broth compared to only 42.86% by TT broth. This was in concordance with what was reported by Morinigo *et al*. (1993) and Hu *et al*. (1997) who considered RV broth as the most suitable enrichment broth, giving greater percentages of positive results, supporting the growth of more serotypes and increasing inhibition of more competing microflora than tetrathionate or selenite broths.

In the present findings, XLD agar showed much higher efficiency in isolation of all 7 *Salmonella* spp. (100%) compared to only one 14.29% by BS agar. Murinda *et al* reported that the best medium for isolation of Salmonella spp. was XLD agar followed by modified brilliant green, Eosin methylene blue (EMB), Brilliant green agar (BG), and BS agar. They did not seem to have much diagnostic value unless large numbers of colonies (> 10 per plate) were tested (Murinda *et al*., 2002). Also Rhodes and Quesnel (1986) and Hu *et al*. (1997) recommended XLD and reported that BS agar had several disadvantages. These include: *S.* Typhi is best isolated on fresh BS agar, which is inhibitory to other *Salmonella* serotypes. In addition plates must be incubated for 48 hrs for typical colonies to develop; moreover *Salmonella* spp. are often difficult to distinguish from competitors and reactions vary between strains (Rhodes and Quesnel, 1986; Hu *et al*., 1997).

In the present study, the identified *Salmonella* serogroups were B, C1, D1 and E1, with C1 was the most commonly observed serogroup; accounting for more than half of the *Salmonella* isolates (57.14%). Serogroups B, D1 and E1 were of 14.3% each. They were of serotypes Typhimrium, Anatum, Concord, Montevideo and Enteritidis.

These results were similar to those reported by Warnick *et al*. (2003) who found that the highest percentages of *Salmonella* isolates were of serogroup C1 followed by serogroups B, E3, C2 and E1. Common serotypes represented by these serogroups included Typhimurium, Newport, Anatum, Meleagridis, Muenster, Thomasville, Dublin, and Mbandaka. (Warnick *et al*., 2003b)

The results of this study described the contrasting picture of serovars distribution encountered with or without clinical signs of *Salmonella* infection. *Salmonella* serovars were isolated from diarrheic and apparently healthy dairy cattle with the following distribution and were elucidated namely, *S.* Typhimurium 2.56% and 0.00%, *S.* Montevideo 0.00% and 0.49%; *S.* Enteritidis 0.00% and 0.24%; and *S.* Anatum 2.56% and 0.00%; while *S.* Concord was identified in 2.56% and 0.24% respectively. These findings were supported by that described by several studies which reported that the serovar Montevideo was often isolated independently from the presence of clinical disease, whereas Typhimurium was the main serovar associated with bovine clinical cases of *Salmonella* (Edrington *et al*., 2004; Lailler *et al*., 2005).

In our study, *Salmonella* spp. were isolated from farms A and B with percentages of 2%, and 2.67% respectively, but no *Salmonella* isolates were detected in farm C. The differences in the prevalence of *Salmonella* infection between the three examined dairy farms may be due to variations in management strategies and hygienic measures that are followed. As in farm C, adequate hygienic measures and management system were strictly prevailed, represented by:

- Raising calves as a replacement stock (no newly added animals).
- Housing of pre-weaned calves in individual

boxes allowing less contact
- Colostrum feeding practices: calves were allowed to receive sufficient amount of colostrum from their dams.
- Frequent cleaning of calf milk bucket between feeding.
- Bedding type: sand (well drained ground).

Group housing of pre-weaned dairy calves, feeding pooled colostrum and disposal of manure in liquid form on owned or rented land were recorded as risk factors in farms A and B. This was in agreement with what was reported by Younis *et al.* (2009) in Egypt who recorded significant association between rearing calves in sandy areas, together with cleaning boxes daily and fecal shedding of *Salmonella* spp. in neonatal dairy calves. (Younis *et al*., 2009)

Veling *et al.* (2004) and Nielsen *et al*. (2007) analyzed risk factors related to the prevalence of *Salmonella* spp. in dairy herds and found that the main routes along which dairy herds can get infected with *Salmonella* spp. are the introduction of new infected animals into the herd, and their infected manure. (Veling *et al*., 2004; Nielsen *et al*., 2007) In addition, Berge *et al.* (2006) found that *Salmonella* prevalence in pre-weaned calves has been shown to be lower in herds that are closed (i.e., no cattle are introduced into the herd) (Berge *et al*., 2006). Other studies have shown that *Salmonella* spp. transmission can occur via contaminated feed or water, wild animals, equipment, fomites, improper manure management and human traffic (Bender, 1994; Evans and Davies, 1996and Kabagambe *et al*., 2000). Calves receiving colostrums from cows other than their dams and group housing were reported to be risk factors for *Salmonella* shedding by several studies (Evans *et al*., 1996; Popff *et al*., 2005 and Younis *et al*., 2009). From the aforementioned, it could be concluded that interaction of more than one factor could contribute to the occurrence of *Salmonella* spp. infection in dairy cattle.

Salmonella infection is of increasing concern due to emergence and increased prevalence of multi-antibiotic resistant strains (Sorensen *et al*., 2003). In the present study, all *Salmonella* isolates (100%) were susceptible to ampicillin, tetracycline, chloramphenicol, nalidixic acid, ciprofloxacin, ceftriaxone and were resistant to trimethoprim-sulphamethoxazole, while only one (14.3%) was resistant to gentamycin. These findings partly agreed with those reported by Blau *et al*. (2005) who found that *Salmonella* isolates from dairy cows had relatively little resistance to a number of antimicrobial agents; where 83.0% were susceptible to all antimicrobial drugs tested. All isolates were susceptible to amikacin, ciprofloxacin, nalidixic acid and trimethoprim-sulfamethoxazole. However resistance to tetracycline was most common (11.9% of all isolates) followed by resistance to streptomycin (9.5%) and resistance to ampicillin (4.4%) (Blau *et al*., 2005).

Conclusions:
- All isolated *Salmonella* spp. were recovered from pre-weaned calves.
- The highest percentages of the isolated *Salmonella* spp. were recovered from diarrheic animals.
- Either RVS broth or TT broth can be used for enrichment of *Salmonella* spp. as they both showed high agreement between them.
- XLD agar showed much higher efficiency in the isolation of the *Salmonella* spp. than BS agar.
- *Salmonella* isolates were classified as serogroups B, C1, D1 and E1, with C1 as the most commonly observed serogroup.
- Five different *Salmonella* serotypes were identified (Typhimurium, Anatum, Concord, Montevideo and Enteritidis).
- The 7 isolated *Salmonella* spp. showed no resistance to all tested antimicrobial agents except for trimethoprim-sulphamethoxazole and gentamycin.
- Application of optimal hygienic conditions and management strategies minimize the occurrence and spread of the *Salmonella* infections on dairy farms, as no *Salmonella* spp. were isolated from farm C, which had the proper hygienic conditions and management practices.

Corresponding author
Amani F. Abaza
Microbiology Department, High Institute of Public Health, Alexandria University, Egypt
amani_abaza@yahoo.com

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