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Prevalence of *Salmonella* and *E. coli* in neonatal diarrheic calves

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ABSTRACT

Neonatal calf diarrhea remains one of the most important problems faced by livestock, causing great economic losses. This study investigated the prevalence of *Salmonella* and *Escherichia coli*, especially enterotoxigenic *E. coli* (ETEC), in diarrheic calves. Fecal samples were collected from 127 diarrheic calves up to 3 months of age at 12 farms from different governorates in Egypt. 119 bacterial isolates (93.7%) were recovered and the prevalences of *Salmonella* and *E. coli* in diarrheic calves were 18.1% and 75.6%, respectively. Serotyping of *Salmonella* isolates revealed that *S.* Enteritidis and *S.* Typhimurium were the most prevalent serotypes, representing 60.9% and 30.4%, respectively, while *S.* Dublin was 8.7%. Serogrouping of *E. coli* isolates showed that 10 O-serogroups were obtained where O_{26} and O_{103} were the most prevalent (17.7% of each). *Salmonella* serotypes showed positive results with PCR test using oligonucleotide primer amplifying 521 bp fragment of *invA* gene of *Salmonella* while 70% of *E. coli* serogroups possessed ETEC virulent gene (K99). The *in-vitro* antibiotic sensitivity test indicated that *Salmonella* serotypes showed high sensitivity against enrofloxacin, spectinomycin and neomycin while *E. coli* isolates showed high sensitivities against marbofloxacin, spectinomycin and neomycin only.

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1. Introduction

Neonatal calf diarrhea (NCD) is one of the most common diseases in young animals, causing huge economic and productivity losses to bovine industry worldwide (Cho and Yoon, 2014). In 2007, The National Animal Health Monitoring System (NAHMS) for U.S. dairy reported that 57% of unweaned calf mortality was due to diarrhea especially in calves less than one month old (USDA Dairy, 2007). In Egypt, NCD continues to be the 1st cause of calf mortality, which ranges between 27.4% and 55% of the total deaths in young calves (Ahmed, 1980). The economic losses occur not only from mortality but also from other costs including treatment, diagnostics, labor, veterinary

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intervention and decreased number of herd replacements as well as subsequent chronic ill thrift and impaired growth performance (Bazeley, 2003).

NCD is a multifactorial syndrome including pathogen (infectious NCD) as well as non-infectious factors related to the animal (immunological and nutritional status), the environment or the management (Izzo et al., 2011). Because of the multifactorial nature of NCD, it is difficult to be controlled effectively (Cho and Yoon, 2014).

Infectious diarrhea is the most significant cause of morbidity and mortality in neonatal dairy calves throughout the world and it can be caused by many pathogens including viruses (coronavirus and rotavirus), protozoa (*Cryptosporidium parvum*) and bacteria (Izzo et al., 2011). Among bacteria, enterotoxigenic *Escherichia coli* (ETEC) and Salmonellae are the most economically important pathogens (Achá et al., 2004), although other bacteria have also been identified as causes of enteric disease and NCD, e.g. *Campylobacter* species (Myers et al., 1984) and *Clostridium* species (Cho et al., 2010). Although co-infection is considerably observed in diarrheic calves, sometimes single infection is recorded. Farm geographic location, management practices, as well as herd size affect considerably the prevalence of the pathogen. (Cho and Yoon, 2014). Identification of the possible causative agent in outbreaks of diarrhea is important to allow targeted preventative measures, such as vaccination, and identification of possible risk factors or sources of infection (Izzo et al., 2011).

Salmonella enterica colonizes the digestive of both adult cattle and calves, but the infection is often recorded in the first 3 months of age and often causing severe symptoms (Fossler et al., 2005). *S. enterica* serovar *typhimurium* (*S.* Typhimurium) and serovar *dublin* (*S.* Dublin) are the most common causative agents of salmonellosis in cattle causing acute and systemic diarrheal diseases, respectively (Cho and Yoon, 2014). Diarrhea due to *Salmonella* infection is watery and mucoid with the presence of blood and fibrin (Fossler et al., 2005). Calves can shed *Salmonella* for variable periods of time and intermittently depending on the degree of infection (Cho and Yoon, 2014). Furthermore, after the disappearance of the organism from the intestinal tract, up to 5% of recovered animals may become carriers shedding the organism in their feces (Jay, 2000). Infected cattle and carriers can serve as source of infection for other animals or even human through food-borne routes or direct contact and so the determination of *Salmonella* strains in fecal samples is not only important for the diagnosis of salmonellosis, but also essential to identify the carriers (Warnick et al., 2003).

Among *E. coli* pathogroups, the most common cause of NCD is ETEC stains that produce the K99 (F5) adhesion antigen (*E. coli* K99+) and heat-stable (*STa* or *STb*) and/or heat-labile (LT1 or LT2) enterotoxins (Kaper et al., 2004). *E. coli* causes a watery diarrhea and weakness in 1–4 day old newborn calves. Death usually occurred within 24 hours due to severe dehydration (Cho et al., 2010). The fimbrial adhesion F5 (K99) promotes the adhesion of bacterial cells to glycoproteins on the epithelial surface of the jejunum and/or ileum, and bacterial enterotoxin also causes damage to the epithelial cells, resulting in fluid secretion and diarrhea (Acres, 1985).

The present study aimed to investigate the prevalence of *Salmonella* and *E. col*, especially ETEC, in diarrheic calves in Egypt.

2. Material and methods

2.1. Samples

Fecal samples were collected every 4 weeks for a period of 5 months from the rectum of 127 cross-breed diarrheic calves up to 3 months of age at 12 farms from different governorates in Egypt along the period of April to November 2013. All samples were transferred in an ice box to the laboratory with minimal delay for bacteriological examination.

2.2. Bacteriological examination

One gram of each fecal sample was diluted in 3 mL of sterile saline. Samples were cultured and identified according to Quinn et al. (2002).

For isolation of *Salmonella* strains, a loopful from the diluted specimens was inoculated into selenite F broth with overnight incubation at 37 °C. Then, a loopful was streaked out onto MacConkey's agar, xylose lysine deoxycholate and *Salmonella–Shigella* agar media and incubated at 37 °C for 18–24 hours. Suspected colonies were subjected to biochemical testing according to Collee et al. (1996).

For isolation of *E. coli* strains, a loopful from the diluted specimens was inoculated into MacConkey's agar and incubated at 37 °C for 18–24 hours. Lactose fermenter (pink) colonies were streaked onto and eosin methylene blue agar and confirmed as *E. coli* using the standard biochemical tests according to Collee et al. (1996).

2.3. Serological identification

2.3.1. Serotyping of Salmonella

Salmonella serovars were identified serologically by slide agglutination test using diagnostic polyvalent and monovalent O and H *Salmonella* antisera according to Kauffman–White scheme (Kauffmann, 1974).

2.3.2. Serogrouping of E. coli isolates

E. coli serogroups were identified serologically by slide agglutination test using standard polyvalent and monovalent *E. coli* antisera according to Edwards and Ewing (1972).

2.4. Polymerase chain reaction

PCR was applied on 3 *Salmonella* isolates representing all the recovered serotypes as well as 10 *E. coli* isolates representing all the recovered serogroups. In case of *Salmonella*, oligonucleotide primers that amplify a 521 bp fragment for the *invA* (invasion A) gene in *Salmonella* serovars was used. Meanwhile, in case of *E. coli*, oligonucleotide primers that amplify a 314 bp fragment for the *K99* (F5) gene in *E. coli* species was used (Table 1).

2.5. Antibiotic susceptibility testing

All Salmonellae (23 isolates) and *E. coli* (96 isolates) recovered from diarrheic calves were tested for their antimicrobial susceptibility to 12 different antimicrobial discs including

Table 1 – Primers used in PCR.

Gene	Primer Sequence 5′-3′	Size of product	Reference
invA(F)	TTGTTACGGCTATTTTGACCA	521 bp	Swamy et al. (1996)
invA(R)	CTGACTGCTACCTTGCTGATG		
F5(F)	TATTATCTTAGGTGGTATGG	314 bp	Frank et al. (1998)
F5(R)	GGTATCCTTTAGCAGCAGTATTTC		

enrofloxacin (10 μg), marbofloxacin (10 μg), gentamycin (10 μg), erythromycin (15 μg), cefotaxime sodium (30 μg), amoxicillin (10 μg), penicillin (10 μg), tetracycline (30 μg), streptomycin (10 μg), trimethoprim-sulphamethoxazol (1.25 + 23.75 μg), spectinomycin (20 μg) and neomycin (20 μg) (Oxoid, Basing Stoke, UK). Antimicrobial susceptibility testing was performed using disc diffusion method on Muller Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) (2012). The antibiotic susceptibility was based on the induced inhibition zones according to the guidelines of the CLSI (2012).

3. Results

3.1. *Bacterial isolation*

Out of 127 collected fecal samples from diarrheic calves, 119 (93.7%) bacterial isolates were recovered, including 23 (18.1%) *Salmonella* serovars and 96 (75.6%) *E. coli* strains (Table 2).

3.2. *Serological identification*

3.2.1. *Serotyping of* Salmonella *isolates*

The 23 *Salmonella* isolates were serotyped as 14 *S.* Enteritidis (60.9%), 7 *S.* Typhimurium (30.4%) and 2 *S.* Dublin (8.7%).

3.2.2. *Serogrouping of* E. coli *isolates*

Out of 96 *E. coli* isolates, 10 O-serogroups were identified. The serogroups O_{26} and O_{103} were the most prevalent representing 17 isolates (17.7%) for each followed by serogroups O_{127}; 14 isolates (14.6%) and O_{119}; 13 isolates (13.6%). While the serogroups O_{86}, O_{111} and O_{157} represented 5 isolates (5.2%) for each, meanwhile O_{44} and O_{158} serogroups represented 4 isolates (4.2%) and finally serogroup O_{78}; 3 isolates (3.1%). Moreover, there were 9 (9.4%) isolates were untyped with the available antisera.

Table 2 – Prevalence of *Salmonella* and *E. coli* in diarrheic calves.

No. of samples	*Salmonella*		*E. coli*		Total		Negative isolation	
	No.	%	No.	%	No.	%	No.	%
127	23	18.1	96	75.6	119	93.7	8	6.3

%: was calculated according to the number of the collected samples.

3.3. *PCR results*

3.3.1. *PCR of* Salmonella *serovars*

PCR results revealed that all the tested isolates confirmed morphologically, biochemically and serologically as being *Salmonella* showed positive results with PCR assay with oligonucleotide primer that amplified a 521 bp fragment of *invA* gene (Fig. 1).

3.3.2. *PCR of* E. coli *serogroups*

PCR results revealed that out of the tested 10 *E. coli* serogroups, 7 serogroups (70%) possessed ETEC virulence gene (*K99*) including O_{26}, O_{44}, O_{78}, O_{86}, O_{111}, O_{119} and O_{127} while O_{103}, O_{157} and O_{158} not possessed *K99* gene (Fig. 2).

3.4. *Antibiotic susceptibility testing*

Results of *in-vitro* sensitivity tests of *Salmonella* isolates against 12 antimicrobial agents revealed that *S.* Typhimurium and *S.* Enteritidis isolates showed high sensitivities against marbofloxacin, enrofloxacin, trimethoprim-sulphamethoxazol, spectinomycin and neomycin. On the other hand, high resistances were observed against amoxicillin, gentamycin, cefotaxime sodium, streptomycin, erythromycin, tetracycline and amoxicillin. Meanwhile, *S.* Dublin isolates were sensitive to enrofloxacin, cefotaxime sodium, penicillin, tetracycline, streptomycin, spectinomycin and neomycin. On the contrary, they were resistant to marbofloxacin, gentamycin, erythromycin, amoxicillin and trimethoprim-sulphamethoxazol. Concerning *E. coli* isolates, the different serogroups as well as the untyped group showed different degrees of sensitivity against the tested antibiotics. Mostly, *E. coli* isolates showed high resistances against the majority of antimicrobials, meanwhile, high sensitivity was observed against marbofloxacin, spectinomycin and neomycin only.

4. Discussion

Neonatal calf diarrhea remains as one of the most important problems faced by livestock, causing great economic losses. Calves are at greatest risk of diarrhea within the first month of life and the incidence of diarrhea decreases with age (Garcia et al., 2000).

The multifactorial nature of NCD makes this disease hard to control effectively. Therefore, prevention and control of such disease must be based on a good understanding of those disease complexities during the calving period before disease outbreaks (Cho and Yoon, 2014). Identification of the possible causative agent in outbreaks of diarrhea is important to allow targeted preventative measures, such as vaccination, and identification of possible risk factors or sources of infection (Izzo et al., 2011). *E. coli* and *Salmonella* are the most common identified pathogens in scouring calves less than 2 months of age (Achá et al., 2004). Their prevalences vary by geographical location of the farms, farm management practices, and herd size (Cho and Yoon, 2014).

In the present study, the prevalences of *E. coli* and *Salmonella* in neonatal diarrheic calf were 18.1% and 75.6%, respectively (Table 2). The negative bacterial isolation in some

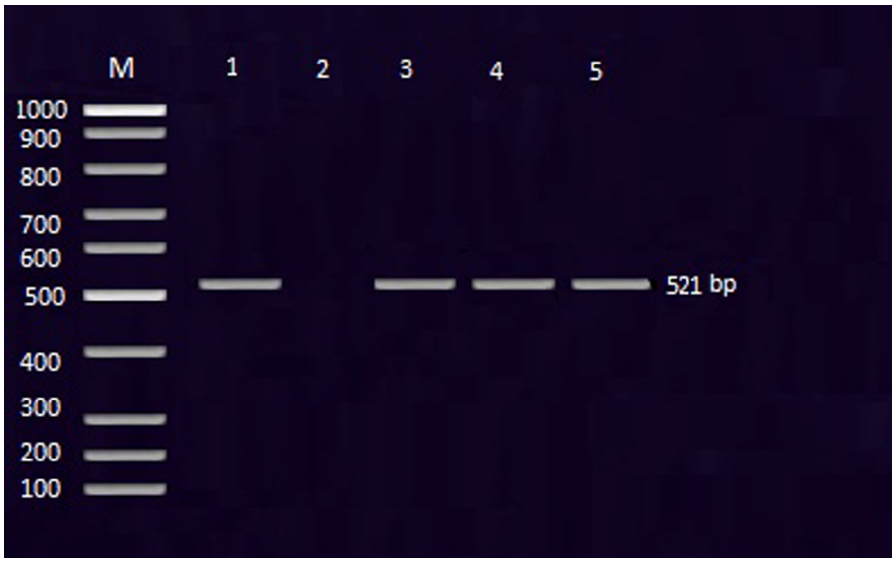

Fig. 1 – The PCR amplification of *invA* gene of *Salmonella* serovars showing positive amplicons at 521 bp. DNA size marker (M). Lane 1: positive control (S. Typhimurium strain). Lane 2: negative control. Lane 3: S. Enteritidis. Lane (4): S. Typhimurium. Lane (5): S. Dublin.

fecal samples (6.3%) did not mean absence of microbial infection but may attribute to infection with bacteria requiring specific or enriched culture media and can't grow on the used culture media such as *Clostridium* species (Cho et al., 2010) and *Campylobacter* species (Myers et al., 1984) or viral infection; requiring tissue culture, or even protozoal infection. Fricker (1987) elucidated that bacteria were not detectable due to the number of colony forming units are below the detection limit of the assay (especially in case of *Salmonella* in feces). Additionally, the presence of antibiotic residues may explain falsely negative bacteriological results because the withdrawal time is not regarded in our herds.

The prevalence of *Salmonella* in the present study was similar to those of other studies in Egypt (Riad et al., 1998: 18.2%; Seleim et al., 2004: 17.5%; and Youssef and El-Haig, 2012: 18.66%) while higher than that reported by Haggag and Khaliel (2002) (4%), and Younis et al. (2009) (4.09%). On contrary, this result was much lower than that reported by Moussa et al. (2010) (43.53%). Varying prevalences of salmonellosis in calves were recorded in diarrheic calves in African countries such as Mozambique (5%; Achá et al., 2004) and Algeria (66.6% in calves at the end of the first month; Akam et al., 2004), and other countries such as India (5%; Joon and Kaura, 1993) and Australia (23.8%; Izzo et al., 2011). Differences of the prevalence rates of *Salmonella* in diarrheic calves in comparison to the previous studies could be explained in the light of species and geographical locations and hygienic measures, and these factors significantly influence the prevalence of salmonellosis in calves (Younis et al., 2009).

The prevalence of *E. coli* in the current study had nearly coincided with the findings of Awad et al. (1979: 80%) and Haggag and Khaliel (2002: 82%), while higher than those of Azzam et al. (2006: 5.4%), El-Shehedi et al. (2013: 35.83%), Osman et al. (2013: 63.6%), and Hassan (2014: 50%), and lower than that obtained by Ibrahim (1995: 100%). In comparison to other countries, the present results were similar to that of Hemashenpagam et al. (2009) in India (75%), while it was higher than those obtained by Joon and Kaura (1993) in India (23%), Viring et al. (1993) in Sweden (11.5%), Khan and Khan (1997) in Pakistan (54%), Steiner et al. (1997) in Germany (42%), Bendali et al. (1999) in France

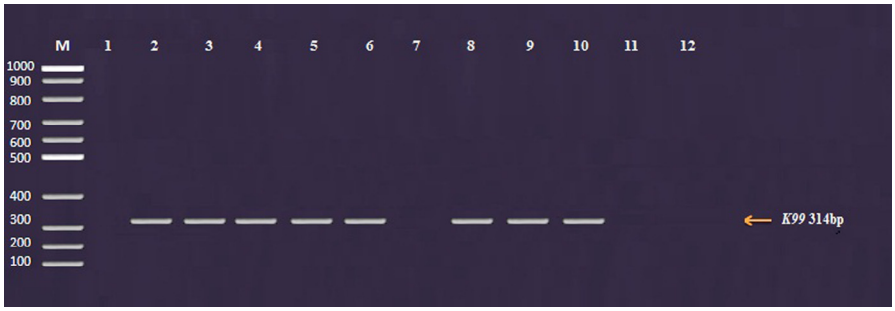

Fig. 2 – The PCR amplification of *K99* virulence gene of *E. coli* showing positive amplicons at 314 bp. M: DNA size marker (100–1000 bp). Lane 1: Negative control. Lane 2: Positive control (*E. coli* serogroup O_{86}). Lane (3–12): The tested serogroups (O_{26}, O_{44}, O_{78}, O_{86}, O_{103}, O_{111}, O_{119}, O_{127}, O_{157} and O_{158}, respectively).

(20.3%), Valdivia-Andy et al. (2000) in Mexico (63.7%), Oporto et al. (2008) in Northern Spain (35.9%), and Izzo et al. (2011) in Australia (17.4%). On contrary, this result was lower than that of Pourtaghi et al. (2013) in Iran (86.7%). The differences of the prevalence rates of *E. coli* in diarrheic calves may be attributed also to the geographical locations and management practice as well as hygienic measures where ETEC infection occurs mainly through ingestion of contaminated food or water (Cho and Yoon, 2014).

In the current study, serotyping of *Salmonella* isolates revealed that S. Enteritidis (SE) and S. Typhimurium (ST) were the most prevalent serotypes with 60.9% and 30.4%, respectively, while S. Dublin (SD) represented 8.7%. *Salmonella* serovars isolation frequencies vary from one location to another due to different management and hygienic measures as well as geographical, environmental and individual variations (Veling et al., 2002). The predominance of ST and SE serovars among diarrheic calves detected in the present study was supported by many previous reports in Egypt (Seleim et al., 2004; Younis et al., 2009; Moussa et al., 2010) and Youssef and El-Haig, 2012). In addition; this finding substantiated the reports from the other countries (Murray, 1994, in Australia, and Smith-Palmer et al., 2003, in Scotland).

S. Typhimurium and S. Enteritidis are non-host-adapted serovars and so they are less likely to establish a "carrier state" in the recovered animals. Infection with a non-host-adapted *Salmonella* results in transient shedding of organisms (3–16 weeks). The most common sources of infection are contaminated food and water. Approximately 40% of all animal-origin feed additives (bone or fish meals) are contaminated with *Salmonella*. Human sewage has also been tracked down as a source of infection in some herd outbreaks. Rodents, birds, and other animals spread infection through their feces and their carcasses (Donaldson et al., 2006). On contrary, S. Dublin is host-adapted in cattle, and commonly produces a carrier state in the recovered animals. It can survive intracellularly inside the mononuclear phagocytic cells in the face of high serum neutralizing antibody titers. Intermittent shedding occurs, which is maximized by stress factors. In outbreaks of S. Dublin infection, carriers are the most likely source. These asymptomatic carriers can shed organisms in their feces and milk for the remainder of their lives. Calves, which are fed raw, contaminated milk from the bulk tank, can be infected by this route (Lance et al., 1992).

Serogrouping of *E. coli* isolates showed that 10 O-serogroups were identified; of them O_{26} and O_{103} were the most prevalent groups (17.7% each) followed by O_{127} (14.6%) and O_{119} (13.6%). These results run hand to hand with those of Ibrahim (1972) and Abd-Elrahman (2013). The present findings were also nearly similar to that obtained by El-Shehedi et al. (2013) and Osman et al. (2013). Concerning the other countries, Tamaki et al. (2005) in Japan found that the most common *E. coli* serotypes isolated from diarrheic fecal samples of calves were O_{119}, O_{111}, O_{126}, and O_{78}. Similarly, Badouei et al. (2010) recovered $O_{157:}H_7$, O_{111}, and O_{26} serotypes from diarrheic and non-diarrheic calves and the most common serogroups was O_{26} (18.4%).

Salmonella invA gene has become one of the most popular PCR target sequences (Rahn et al., 1992) and its amplification now has been recognized as an international standard for detection of *Salmonella* (Malorny et al., 2003). The *invA* gene encodes a protein in the inner membrane of bacteria, which is responsible for invasion to the epithelial cells of the host (Darwin and Miller, 1999).

In the present study, PCR was applied on 3 isolates of *Salmonella* representing the recovered serovars to determine the virulence *invA* gene. The results revealed that all the tested isolates of *Salmonella* showed positive results with PCR assay using oligonucleotide primer that amplified a 521 bp fragment of *invA* gene of *Salmonella*. These results were similar to those obtained by Soliman (2014), who determined the virulence *invA* gene in all recovered *Salmonella* serogroups from bovine and ovine; S. Typhimurium, S. Enteritidis, S. Virchow, S. Montividio and S. Rubislow, by PCR.

Concerning *E.* coli, PCR was applied on 10 isolates representing all the recovered serogroups. Results revealed that 7 serogroups (70%) possessed ETEC virulent gene (*K99*) including O_{26}, O_{44}, O_{78}, O_{86}, O_{111}, O_{119} and O_{127} while O_{103}, O_{157} and O_{158} not possessed *K99* gene. Although Sherwood et al. (1983) confirmed the close correlation between enterotoxigenicity and the *K99* antigen presence and Pourtaghi et al. (2013) reported that most bovine ETEC produce *K99* fimbriae, although Moon et al. (1976) have reported *K99* antigen in non-enterotoxigenic *E. coli*.

Despite the increased availability of vaccines against ETEC and other pathogens associated with NCD and continued emphasis on optimizing colostral transfer of passive immunity, improved treatment protocols for calf diarrhea are necessary. Although the administration of intravenous fluids and oral electrolyte solutions plays the main role in treatment, the efficacy of antimicrobial drugs in treating calf diarrhea is argumentative (Constable, 2004). Diarrheic calves are more likely to have failure or partial failure of passive transfer, and so they are more likely to be bacteraemic (Constable, 2004) and this is an additional cause that antimicrobial agents might be indicated in the treatment of calf diarrhea. The type of antibiotic drug should better be selected on the basis of its sensitivity, which could be detected by laboratory examination, and the antimicrobial treatment of diarrheic calves should therefore be focused against bacteria in the two sites of infection: the small intestine and the blood (Constable, 2004).

In the present study, the results of *in-vitro* antibiotic sensitivity test showed that the different serotypes of *Salmonella* showed different degrees of sensitivity against the tested antibiotics but in general they all showed high sensitivities against enrofloxacin, spectinomycin and neomycin. On contrary, they were resistant to gentamycin, erythromycin and amoxicillin. On the other hand, the different *E. coli* serogroups as well as the untyped group showed different degrees of sensitivity against the tested antibiotics. Mostly, *E. coli* isolates showed high resistances against the majority of antimicrobials, meanwhile, high sensitivities were observed against marbofloxacin, spectinomycin and neomycin only. The high occurrence of resistance can be predicted since a large proportion of the animals are probably treated with antimicrobial drugs (de Verdier et al., 2012). Call et al. (2008) discussed the epidemiology of resistant *E. coli* in calves as multifactorial, complex and e.g. influenced by co-selection due to linkage of resistance genes. Walk et al. (2007) supposed that regardless of use of antimicrobials, antibiotic resistance in *E. coli* is co-selected in calves by an unknown "beneficial mutation."

5. Conclusion

Neonatal calf diarrhea remains one of the most important problems faced by livestock, causing great economic losses. The prevalences of *Salmonella* and *E. coli* in diarrheic calves were 18.1% and 75.6%, respectively. S. Enteritidis and S. Typhimurium were the most prevalent *Salmonella* serotypes while 70% of *E. coli* was ETEC.

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