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Research Article

Herd problems and occupational zoonoses of *Salmonella enterica* serovars Typhimurium and Enteritidis infection in diarrheic cattle and buffalo calves in Egypt

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Abstract. This study aimed to investigate *Salmonella enterica* serovar Typhimurium (ST) and *Salmonella enterica* serovar Enteritidis (SE) infections of diarrheic calves in bovine dairy herds and their farm workers. Antimicrobial drug resistance profile of the isolates was determined by standard disc diffusion assay. Fecal swabs were collected from diarrheic calves aged from one week to three months (Median age 33.5 days); from three farms representing cattle, native breed buffalo and cross-breed buffalo farms. Each farm was visited every three months for one year, from March 2011 to February 2012. Collected Swabs were examined by conventional bacterial culture and serotyped by multiplex PCR. Results showed that *Salmonella* was isolated from 42/255 (18.66%) diarrheic calves and 27/35 (77.14%) calves died from diarrhea. Incidence of ST was significantly higher in calves died from diarrhea than diarrheic calves (P>0.001). On farm level, isolation rate of *Salmonella* was significantly higher in dead calves in cross-breed buffalo farm than native breed buffalo and cattle farms (P>0.001). On other hand, the incidence of *Salmonella* among stool swabs of farm workers caring the calves was 8.13%; with isolation rate of (2.32%) ST, (1.16%) SE and (4.65%) other *Salmonella* serovas. Isolates showed a high percentage of multi-drug resistant profile (63.88%). All isolates were completely resistant to oxytetracycline, danofloxacin and oxacillin. Enrofloxacin was the drug of choice. In conclusion, a high incidence of mutli-drug resistant *Salmonella* infections was detected in cattle and buffalo calves that might be associated with high mortality rate in calves and zoonotic potential to the farm workers. Adequate hygienic measures, proper immunization programs and regular training to the labor should be applied.

Key Words: Salmonellosis, Typhimurium, Enteritidis, Calves, Zoonoses

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Introduction

Salmonella infections in dairy calves have many impacts on animal and human health that are considered major worldwide problem. Substantial economic losses were manifested through mortality and poor growth of infected animals as well as the potential of zoonotic transmission (Smith *et al* 2004). Many outbreaks with high prevalence of clinical and subclinical *Salmonella* infections have been reported in cattle and calves worldwide, which encountered many isolated serovars that considered host-adapted *Salmonella* for cattle (Lance *et al* 1992; Radke *et al* 2002; Veling *et al* 2002; Berge *et al* 2008; Daly & Neiger 2008). Herikstad *et al* (2002) reported that *Salmonella enterica* serovar Typhimurium (ST) is the most frequently isolated from foodborne outbreaks throughout the world. *Salmonella enterica* serovar Enteritidis (SE) also is a major cause of foodborne diseases (Rodrigue *et al* 1990). In Egypt, a varying prevalence of *Salmonella* infections in calves was recorded with predominance of ST and SE serovars (Seleim *et al* 2004; Younis *et al* 2009; Moussa *et al* 2010, 2012).

It was reported that over 90% of the farms had at least one *Salmonella*-positive culture (Fossler *et al* 2004). Considering these high herd-level prevalence values, it would seem logical that fecal shedders within a herd represent a potential point of intervention to mitigate public health risk. In particular, those occupationally work or otherwise interact with livestock are also at risk of zoonotic infection (Vanselow *et al* 2007). In addition, indirect transmission can be caused by either contamination of crops as well as water by manure contamination used as a fertilizer or during manure run-off (Islam *et al* 2004). Food chain contamination during slaughtering animals and contamination in animal markets are additional sources of transmission (Mead *et al* 1999). All these potentials of zoonotic infections are possible to occur in Egypt. However, there is lack of reported enteric non-typhoid *Salmonella* human infection.

Antimicrobial resistant strains of *Salmonella* pose a severe and costly animal health problem, by prolonging illness and decreasing productivity through higher morbidity and mortality rates. Moreover, it impaires treatment of human in case of zoonotic infections. Strains of *Salmonella* isolated from animals and human that are resistant to antimicrobial agents have been reported. (Angulo & Griffin 2000; Breuil *et al* 2000, Koirala, 2011; Sugawara *et al* 2012). In Egypt, multidrug resistant isolates of ST and SE from diarrheic calves was detected and

molecular basis of drug resistance of these isolates were identified (Ahmed *et al* 2009).

Generally, buffalo species are genetically divided into the Egyptian, Greek and Italian phenotypes (Moioli *et al* 2001). The buffalo in Egypt belongs to one phenotype (Hassanane *et al* 2000). The buffalo population in Egypt was estimated to account about three million head, slightly higher in numbers than cattle and; over 90% of the population are reared in small herds (1–3 animals) (Nigm 1992). Recently, many farms started to cross breed the Egyptian native buffalo with Italian breed; the new cross-breed differs in its immunity to diseases. However, little systematic work has been done to obviate the impact of *Salmonella* in diarrheic cattle and buffalo calves including the cross-breeds buffalo in Egypt.

Salmonella is detected and identified by standard bacteriological, biochemical and serological tests. These tests are generally time-consuming, tedious and costly as they require hundreds of antisera as well as well-trained technicians (Echeita *et al* 2002). However, multiplex PCR provides a specific method and superior ability to detect *Salmonella enterica* serovars in the presence of other bacteria simultaneously (Moussa *et al* 2012). Therefore, in the current study multiplex PCR was used for identification of ST and SE.

The overall objective of this study was to investigate ST and SE infection of diarrheic calves in different bovine dairy herds and to identify its occupational associated infections and to determine the antibiotic sensitivity pattern of the isolates.

Material and methods

Study populations and Herd problems

In this study, three dairy farms were included and identified as A, B and C. Farm A was Holstein-Friesian cattle, farm B was native breed buffalo, and farm C was cross- breed native and Italian breed buffalo. The total number of animals in the three farms was 4050; the number of calves was 800. Fecal swabs were collected from diarrheic calves aged from one week to three months (Median age 33.5 days); each farm was examined once every three months for one year (March 2011 to February 2012). The three farms administered scour guard 3 to the pregnant cows; anti-parasitic drugs were regularly administered. Clinical and necropsic examination of calves died from severe diarrhea were carried out.

Sample collection

A total of 255 fecal swabs from diarrheic calves and 57 swabs from intestinal content and gall bladder of calves died from diarrhea were collected. In addition, 84 stool swabs were also collected and examined for *Salmonella* from 24 farm workers, especially those suffering from gastric upset, with fever and diarrhea. Specimens were transported to the laboratory in ice box with minimum delay.

***Salmonella* isolation and identification**

Samples were cultured and identified according to Edward and Ewing (1972). 10 ml of buffered peptone water was added to the swabs followed by incubation at 37°C for 18 h. 0.1 ml of each incubated swab was added to 10 ml of Rappaport-Vassiliadis broth and incubated for 24 h. Following incubation, samples were plated on both *Salmonella – Shigella* and xylose lysine desoxycholate agar for 24-48 h. Suspected *Salmonella* spp., based on colony morphology on the selective media, was identified by standard metabolic and biochemical procedures using Triple Sugar Iron Agar (TSI), Urea agar (Christensen), L-lysine decarboxylase, b-galactosidase (ONPG), Voges Proskauer and Indole tests were performed.

Multiplex PCR for identification of ST and SE serovars

Salmonella isolates were serotyped as ST and SE using multiplex PCR. Three sets of primers were used in multiplex PCR for identification of *Salmonella* spp., ST and SE as described previously by Alvarez *et al* (2004).

Bacterial DNA was extracted from cultured broth by centrifugation at 4°C at 3,000 x g for 10 min. The pellet was washed twice with phosphate-buffered saline and the cells were re-suspended in 800 µl of sterile distilled water and boiled for 10 min. The resulting solution was centrifuged at 3,000 x g for 10 min and the supernatant was used as the DNA template. Amplification reactions were carried out with 5 µl of boiled bacterial suspensions, 5 µl of 5X Taq Master Mix / high yield (Jena Bioscience, GMBH, Germany) and two pairs of each primer 50 pmol. Distilled water was added to bring the final volume to 25 µl. The PCR reaction products were subjected to electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and visualized and photographed under UV light. PCR protocol consisted of an initial denaturation step for 2 min at 95°C, followed by 30 cycles, with 1 cycle for 1 min at 95°C, 1 min at 57°C, and 1 min at 72°C, and a final elongation step for 5 min at 72°C. In each PCR run, a non-template control was included to detect possible external DNA contamination and control positive were used for confirmation.

Antibiotic sensitivity

Eighteen human and animal *Salmonella* isolates were selected and tested for antibiotic sensitivity using a Kirby- Bauer standard disk diffusion assay in Mueller-Hinton agar. Strains were screened for antibio-resistance using the following antibiotic discs (Bioanalyse): cefotaxime, ciprofloxacin, enrofloxacin, gentamicin, colistin, oxytetracycline, danofloxacin, amoxicillin, oxacillin, erythromycin, sulphamethoxazole-trimetoprim and neomycin. Concentrations were shown in Table 1. Results were recorded according to the zone-size and interpreted in accordance with the criteria of the Clinical and Laboratory Standards Institute 2006 (CLSI).

Statistical analysis

The significance of differences of *Salmonella* isolation rates and distribution of serovars among diarrheic and died calves were determined using a Chi- square contingency with Fisher's exact test. Statics were computed using GraphPad Prism (Version 5) software. P value of <0.005 was considered statistically significant.

Results

Clinical and Necrospy examination findings

By clinical examination, diarrheic calves suffered from profuse watery mucoid and/or bloody diarrhea, fever lasting one to seven days, anorexia, dehydration and evidence of endotoxemia. The

total mortality rate in the examined calves was 18.96% that was highest in farm B (25.71%), followed by farm C (20%) and farm A (6.15%). The necropsy findings of calves died by diarrhea showed intestine gas accumulation, inflammation with bluish coloration; mesentery lymph nodes were enlarged and congested. The most prominent post-mortem finding was enlargement of gall bladder (double or more the normal size).

Table 1. Antibiotic sensitivity of 18 *Salmonella* isolates to 12 antibiotics

Antibiotics, disc concentration	Sensitivity pattern		
	Resistant	Moderate sensitivity	High sensitivity
Cefotaxime (CTX) 30 mcg	5 (27.78%)	6 (33.33%)	7 (38.89%)
Ciprofloxacin (CIP) 5mcg	9 (50%)	7 (38.89%)	2 (11.11%)
Enrofloxacin (ENR) 5mcg	0	5 (27.78%)	13 (72.22%)
Gentamycin (CN) 10 mcg	11 (61.11%)	6 (33.33%)	1 (5.55%)
Colistin sulphate (CT) 10 mcg	10 (55.55%)	7 (38.89%)	1 (5.56%)
Oxytetracycline (T) 30 mcg	18 (100%)	0	0
Danofloxacin (DA) 5 mcg	18 (100%)	0	0
Amoxacillin (AM) 10 mcg	10 (55.56%)	6 (33.33%)	2(11.11%)
Oxacillin (OX) 1mcg	18 (100%)	0	0
Erythromycin (E)	16 (88.89%)	2 (11.11%)	0
Sulphamethoxazole-trimetoprim (CXT) 23.75/1.25ug	13 (72.22%)	5 (27.78%)	0
Neomycin (N) 30mcg	10 (55.55%)	8 (44.45%)	0
Total =216	138 (3.88%)	52 (24.07%)	26 (12.03%)

Total prevalence of *Salmonella* and serovar distribution

As shown in Table 2, *Salmonella* was isolated from 42 of 255 (18.66%) fecal swabs of diarrheic calves. However, *Salmonella* was isolated from 27 (77.14%) of 35 swabs collected from calves died after severe diarrhea. Among diarrheic calves, isolation rate of ST was significantly higher than isolation rate of SE and other types of *Salmonella* serovars (P<0.001). However, among calves died from diarrhea, isolation rate of ST (48.57%) was significantly higher than isolation rate of SE (11.42%) and other *Salmonella* serovars (17.14%) (P <0.001).

Total incidence and serovars distribution of *Salmonella* isolates in farms

The incidences of *Salmonella* among diarrheic calves were 8.42%, 19.23% and 27.88% for farm A, B and C respectively. There were no significant differences of isolation rates of *Salmonella* from diarrheic calves among the three farms (P<0.1). Relative proportion rates of ST were 62.5%, 60% and 58.62% in farm A, B and C, respectively. However, isolation rate of SE was 12.50%, 20%, and 17.74%, in the respective farms. Among the calves died of diarrhea, the incidence of *Salmonella* was significantly higher in farm C than farms A and B (P<0.001) (Table 3).

Table 2. Total incidence of serovars distributions of *Salmonella* isolates among diarrheic and died calves

	Total incidence	ST	SE	Other *Salmonella* spp. serovars
	No (%)	No (%)	No (%)	No (%)
Diarrheic calves No=255	42 (18.66%)	25 (11.11%)	7 (3.11%)	9 (4%)
Dead calves No=27	27 (77.14%)	17 (48.57%)	4 (11.42%)	6 (17.14%)

Table 3. Incidence and serovars distributions of *Salmonella* isolates among diarrheic and died calves in different farms

Calves Specimens	Farm	Isolation rates	ST No (%)	SE No (%)	Other *Salmonella* serovars No (%)
Diarrheic calves	**A**	8/95 (8.42%)	5 (62.5%)	1 (12.5%)	2 (25%)
	B	5/26 (19.23%)	3 (60%)	1 (20%)	1 (20%)
	C	29/104 (27.88%)	17 (58.62%)	5 (17.74%)	6 (20.68%)
Dead calves	**A**	7/10 (70%)	4 (57.14%)	1 (14.28%)	2 (28.57%)
	B	5/7 (71.42%)	3 (60%)	1 (20%)	1 (20%)
	C	15/18 (83.33%)	10 (66.66%)	2 (13.33%)	3 (20%)

***Salmonella* infections in farm workers**

Of 86 stool swabs of farm workers, 7 (8.13%) were positive to *Salmonella*. On the other hand, the detection rates of serovars were 2.32% ST, 1.16% SE and 4.65% other *Salmonella* spp. serovars (Table 4).

Table 4. Incidence and serovars distribution *Salmonella* isolates from stool swabs of farm workers

Total incidence	ST	SE	Other *Salmonella* spp. serovars
No (%)	No (%)	No (%)	No (%)
7/86 (8.13%)	2/86 (2.32%)	1/86 (1.16%)	4/86 (4.65%)

Antibiotic resistance

Results showed that the isolates were resistant to 138 of 216 antibiotic discs (63.88%) and was moderately sensitive to 52 (24.07%), and highly sensitive to 26 (12.03%) discs. All isolates were resistant to oxytetracycline, danofloxacin and oxacillin antibiotics. A high resistance of isolates was shown to

sulphamethoxazole-trimetoprim, neomycin and erythromycin; and moderate to high sensitivity of isolates to cefotaxime, ciprofloxacin, amoxacillin, colistin and gentamycin respectively. Isolates were highly sensitive to enrofloxacin which considered the drug of choice (Table 1). Moreover, three Typhimurium strains were resistant to all antibiotic discs used.

Discussion

Neonatal calf diarrhea remains an important cause of morbidity and mortality in young calves of dairy herds in Egypt. In the current study, the total incidence of *Salmonella* in diarrheic dairy calves of median age 32.5 days was nearly similar to the findings of Seleim *et al* (2004) (17.5%) and higher than that reported by Younis *et al* (2009) (4.09%). In contrary, isolation rate of *Salmonella* was much lower than that reported by Moussa *et al* (2010) (43.53%). Varying incidences of Salmonellosis in calves were recorded in African countries; the incidence of *Salmonella* spp. in diarrheic and non-diarrheic calves in Mozambique was 2% (Acha *et al* 2004). In Algeria, Akam *et al* (2004) reported that the incidence of *Salmonella* infection in calves at the end of the first month was 66.6%. Differences of the incidence rates of *Salmonella* in diarrheic calves than the previous reports in Egypt could be explained in the light of species and geographical locations and hygienic measures, these factors significantly influence the prevalence of *Salmonella* infections in calves (Snodgrass *et al* 1986; Younis *et al* 2009). In addition, this study focused on diarrheic calves at the age that calves were highly susceptible to *Salmonella* infections (Akam *et al* 2004).

Several enteropathogens were recovered from neonatal calf with diarrhea; including viral, bacterial and parasitic agents (Snodgrass *et al* 1986). In all the studied herds, the pregnant dams vaccinated with Scour Guard 3 that was reported to provoke a significant immunization against enterotoxogenic Escherichia coli and other most frequent viral agents causing diarrhea to the newborn calves (Younis *et al* 2009). However, the lack of administration of any type of immunization to salmonellosis in the examined farms could be considered the main etiologic agent of diarrhea in calves of dairy herds with public health concern. Thus, *Salmonella* bacterins available commercially should be used in preventative and control measures.

The frequencies of *Salmonella* serovars isolation vary from one location to the other due to different managemental and hygienic regimes as well as geographical, environmental and individual differences (Ritchie *et al* 2001; Veling *et al* 2002). The predominance of ST and SE serovars among diarrheic young calves detected in this study was supported by many previous reports in Egypt (Seleim *et al* 2004; Younis *et al* 2009; Moussa *et al* 2010). In addition, this finding substantiates the reports from the other countries (Murray 1994; Smith-Palmer *et al* 2003). Moreover, the high predominance of ST and SE serovars isolated from calves died from diarrhea and also from their human contacts indicates that these serovars are circulating in animal husbandry in Egypt with potential foodborne infections.

In this study, the isolation rate of *Salmonella* was significantly higher in cross-breed buffalo calves than native breed buffalo and cattle calves ($P<0.001$). This finding indicates that the cross-breed buffalo calves were more susceptible to *salmonella* infection. Therefore, regular epidemiological investigations and regular immunization of cross-breed to Salmonellosis are important for preventation and control of salmonellosis in dairy farms. In Egypt, buffalo calves are preferred than cattle calves for slaughtering at young ages (1-2 months) for providing veal meat. From public health prospective, slaughtering newborn calves subclinically infected by *Salmonella* could be a source of contamination of veal meat and poses a risk of foodborne salmonellosis (Vanselow *et al* 2007).

In the present study, the high incidence of salmonellosis detected among calves died after diarrhea and severe dehydration, coupled with isolation of *Salmonella* from gall bladder indicate that *Salmonella* was a virulent pathogen to calves, and might be related to high mortality rate recorded in the studied farms. Moreover, ST was prevalent among calves died from diarrhea ($P<0.001$), indicating higher virulence of ST than the other serovars of *Salmonella*.

Salmonellosis on a farm is a potential zoonotic risk to farm workers and their families (Vanselow *et al* 2007). The organism spreads easily between operations, likely via manure contaminated clothing and footwear. In the present study, isolation of *Salmonella* including ST and SE serovars from workers caring calves was consistent with previous reports (Lyons *et al* 1980; Vanselow *et al* 2007). Therefore, precautions and awareness programs should be adopted with focusing on animal contacts.

The emergence of antibiotic-resistant bacteria has become an increasing global problem in both human and veterinary medicine (Busani *et al* 2004). Antimicrobial sensitivity pattern of *Salmonella* isolates in our study was nearly similar to previously recorded results for the same serovars by Ahmed *et al* (2009). Numerous resistance genes encoding these resistances were identified among *Salmonella* isolates from Egypt (Ahmed *et al*, 2009; Ahmed & Shimamoto, 2012).

Conclusion

A high incidence of multi-drug resistant *Salmonella* infections in cattle and buffalo calves might be associated mortalities in calves and zoonotic potentials. ST was the most common serovar among animal and human isolates. Adequate hygienic measures, proper immunization programs and regular training to the labor should be applied in dairy farms.

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