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Original Research Article

Prevalence, molecular identification and virulence attributes of *Salmonella* serovars isolated from feces of diarrheic cow and buffalo-calves

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A B S T R A C T

The aim of this work was to study the prevalence, virulence characteristics and molecular diagnosis of *Salmonella* in young diarrheic cow and buffalo-calves. A total of 591 diarrheic cow and buffalo-calves as well as 55 apparently normal cows and buffalo-calves were subjected to bacteriological examination for isolation of *Salmonella* species. The results revealed that 59 *Salmonella* species out of 646 fecal samples representing the percentage of 9.75%. *Salmonella typhimurium* was the major species among the isolates (25), then *Salmonella anatum* (14), *Salmonella entritidis* (8), *Salmonella meleagridis* (7), *Salmonella dublin* (6) and *Salmonella infantis* (4). Virulence tests were applied for detection of microorganism characteristics, including: motility test, invasion of Hela cells and haemaglutination pattern in RBCs of different animal species. Finally, polymerase chain reaction was applied to improve rapid, sensitive and specific diagnostic method for detection of such organism in calves. According to the results, all *Salmonella typhimurium*, *Salmonella dublin* and *Salmonella anatum* associated with diarrhea were motile. All *Salmonella* serovars were positively invasive to Hela cells after 3 hrs incubation. In addition, the majority of salmonella strains that had haemaglutination activity were mannose-resistant haemagglutinin type with bovine, sheep, guinea pig, chicken and human RBCs. Finally, the PCR products of *Salmonella typhimurium* include positive control, resulted in 243 bp amplified fragments and no amplified DNA fragments obtained from non *Salmonella* species. The application of PCR assay is important to develop a highly sensitive and specific diagnostic method for rapid detection of *Salmonella* species in calves.

Introduction

Salmonella: a genus of the family *Enterobacteriaceae*, is a primary etiological agent of infectious diarrhea and represents an important zoonotic pathogen worldwide (Hoelzer *et al.*, 2010). 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 (Callaway *et al.,* 2005). Calf hood diseases have a major impact on the economic viability of cattle operations (Lorenz *et al.,* 2011). Substantial economic losses were manifested through mortality and poor growth of infected animals as well as the hazard of transmission to humans either through the food chain or direct animal contact (Mohamed *et al.,* 2011). Calf diarrhea is the commonest disease in young calves and is the greatest single cause of death (Heinrichs and Radostits, 2001). It accounts for approximately 75% of the mortality of dairy calves fewer than three weeks of age. Calves are infected by ingestion of the organism from the environment which is contaminated with feces from infected animals. Six hours after ingestion, the organism multiplies in the intestine and can be found in the rectum (Peel *et al.,* 1990), the bacteria invade the intestinal mucosa and cause an inflammatory response, septicemia and pneumonia can follow. Signs of clinical disease are those of diarrhea and dehydration.

Salmonellosis is an important endemic disease of calves, which documented by an increase in incidence especially that caused by *S. typhimurium* in calves associated with the development of intensive rearing system (Hoelzer *et al.,* 2010). Calf morbidity and mortality are of great economic importance to all dairy producers (Curtis *et al.,* 1989). Calf death also causes a loss of genetic material for herd improvement and decreases the number of dairy heifers available for herd replacement and expansion. Economic losses resulting from calfhood mortality and morbidity can be easily recognized, but the effect on morbidity on future health and performance, which may constitute a loss of even greater importance, is difficult to estimate.

A dairy farm management system should employ a strategy that will reduce calf mortality and improve calf performance by controlling diseases. In a good management practice, annual mortality of calves less than one month of age can be reduced to below 3–5% and first calving age at around 24 months (Heinrichs and Radostits, 2001). 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- typhoid *Salmonella* species can pose a substantial risk to public health (Cummings *et al.,* 2010).

Culturing of *Salmonella* from fecal samples is a time consuming and laborious process therefore the development of a rapid and sensitive method for the diagnosis of *Salmonella* species is desirable. Several techniques for improving the detection of *Salmonella* serovars in faces such as the use of selective culture medium and enzyme linked immunosorbant assay (ELISA) have been developed (Abshire and Neidhardt, 1993). However, problems remain with sensitivity and specificity that have a limited routine use of these procedures. Polymerase chain reaction (PCR) had been used to identify the presence of specific pathogens (Swenson *et al.,* 1994). So the present study was, therefore, initiated with this background and has the following objectives: (1) Study the prevalence of

Salmonella in young calves. (2) Isolation of *Salmonella* microorganisms and identification of isolated strains. (3) Application of PCR technique as a rapid, sensitive and accurate method for diagnosis of *Salmonella*.

Materials and Methods

Samples

A total of 591 diarrheic cow and buffalo-calves 3 days to 4 weeks old were included in the present work as well as 55 apparently healthy cows and buffalo-calves. About 5 grams of feces were collected from 428 buffalo-calves and 218 cow-calves. 591 calves were diarrhea cases and 55 were normal. All fecal samples were subjected to bacteriological examination. Calves were of mixed breed and located at different *Salmonella* endemic farms in El-Kalubia, El-Sharkia, El-Behira, El-Giza, El-Fayoum, Bani Seuef, Kafr El- Sheikh, El-Menoufia and El-Menia Governorates. They were investigated for the presence of *Salmonella* species.

Bacterial culturing of feces (fecal culture)

A separate disposable plastic glove was used to collect fecal material from the terminal portion of each calf rectum. Each glove was inverted after sampling then tied and labeled. Samples were forwarded on ice with a minimum of delay to the laboratory.

About one gram of feces was inoculated into 10 ml amounts of Selenite-F broth. After incubation for 18 hrs at 37ºC, a loop full was streaked onto the surface of MacConkey, Xylose lysine deoxycholate (XLD) and *Salmonella Shigella* (SS) agar plates. The latter were incubated for an additional 24–48 hrs at 37ºC before examination for suspected *Salmonella* colonies.

Identification of isolates

Morphological examination (Motility and Biochemical)

Suspected *Salmonella* colonies were picked up and examined microscopically in a gram-stained film before being transferred into triple sugar iron agar. Motility was assured by the stab cultivation of an organism into a tube of semisolid Penassay agar and incubating at 37ºC. In such agar-motile wild type strains migrate rapidly and non motile fails to migrate even after 24 hours (Carsiotis *et al.*, 1984). For initial screening, attempts were made using the criteria which are described by Kreig and Holt (1984). Biochemical characterization is continued only for lactose negative isolates that gave negative oxidase reactions, positive catalase, positive citrate, negative urease, negative phenylalanine deaminase and showed non spreading surface growth on Triple Sugar Iron (TSI) agar with an alkaline slant and acid butt with or without gas and hydrogen sulfide (H_2S) production in addition to fermentation reaction of sugars via glucose, lactose, sucrose, maltose, dulcitol, salicin, inositol and adonitol. Suspected isolates that shown clearly the biochemical reactions and sugar fermentation were supposed as *Salmonella* and they were subjected to serological typing.

Serological identification

Isolates that were preliminarily identified biochemically as Salmonellae were subjected to serological identification according to Kauffmann-White Scheme as follows: Suspected *Salmonella* isolates were cultured on a nutrient agar slope for 24 hours at 37ºC. The slide agglutination technique was applied. A loop full was suspended in a drop of physiological saline solution on a slide, so as to make a homogenous suspension, only smooth

isolates were examined serologically and rough autoagglutinable isolates were discarded. A drop of *Salmonella* antisera was added to the suspension with a standard loop and thoroughly mixed to bring the organisms in close contact with antisera. Positive agglutination occurred within a minute and could be easily seen with the naked eye. A delayed or partial agglutination was considered as negative or false positive.

Determination of "O" somatic and "H" flagellar antigens:

Polyvalent "O" somatic and "H" antisera were first tried to assure that the suspected isolates were Salmonellae. Positive cultures were then tested with each of the "O" grouping sera followed by the respective monospecific "O" and "H" antisera factors in order to determine the complete antigenic formula.

Detection of virulence attributes

Motility test: It was carried out on all isolated *Salmonella* strains from diseased and normal newly born calves on Penassay semisolid agar as described before in the identification of isolates.

Invasion of Hela cells: It was studied for different *Salmonella* strains isolated from buffalo-calves and cow-calves using Hela cell test (Jones and Richardson, 1981). Strains were routinely grown in Brain-Heart Infusion Broth (BHIB). Suspensions were washed with saline. In brief, a monolayer of Hela cells were grown in Eagle's minimal essential medium (supplemented with 10% fetal calf serum, 20 µ mol of glutamine/ml, 50 units of penicillin G and 50 µg of streptomycin/ml) and maintained in a humified incubator with 5% CO_2 tension, cells were trypsinized to detach them from glass bottles, and these cells were used to prepare monolayer on cover slips. 4 sterile No.1 cover slips, 18 µm in diameter were placed on the bottom of a sterile plastic petri dish and each dish was seeded with approximately 10^5 Hela cell. The culture medium was changed at 18 hours and every 48 hours thereafter. Approximately 24 hours before and again immediately prior to infection, the culture medium was removed and replaced with antibiotic-free medium. The monlayers were infected by simple addition of washed bacteria to give a final concentration of approximately $3x10^7$ bacteria/ml. After incubation for 5 hours, the tissue-culture medium was removed, the monloayers were washed with Hank's balanced salt solution, and fresh antibiotic-free medium was added to minimize extracellular multiplication of Salmonellae. A sample was taken from cover slips 0, 3, 7 and 24 hours after inoculation, rinsed in three changes of saline, fixed in methanol, acetic acid mixture (3:1) and stained with Giemsa stain or stained by covering with conjugated with *Salmonella* antisera and examined. Positive and negative controls were always included.

Haemagglutination (HA) test

In this work all strains were examined for mannose sensitive haemagglutination (MSHA) and mannose resistant haemagglutination (MRHA) against 2% suspension of erythrocytes from humans, sheep, cattle, guinea pig and chickens. Different blood samples were taken from various origins on anticoagulant such as Ethylene Diamine Tetraacetic Acid (EDTA) then washed twice with Phosphate-Buffered Saline (PBS). Erythrocyte suspension stored at 4^oC remained stable and gave reproducible results up to one week. Each sample was divided into 2 parts, each first

part was used for HA and the second part was mixed with 2% D-mannose for MSHA. All erythrocytes were suspended as 2% in PBS. Two basic kinds of HA were used in the present work: (1) the rocked test performed with mechanical rocking of a porcelain tile or manual rocking of the glass slide. (2) A static settling test in which erythrocyte and bacteria in the presence or absence of D-mannose were allowed to settle at 4°C in plastic microtitter trays. An equal volume of the erythrocyte was mixed with a bacterial suspension for each sample (5–10 minutes) and the results were read. Haemagglutination was read as strongly positive (+++) or moderately positive (++) when coarse clumping occurred in 1–10 minutes and as weekly positive (+) when fine HA occurred in 10 minutes. The absence of HA in parallel test in which 2% D-mannose in saline was substituted for saline confirmed the mannose sensitive nature of HA activity. For the demonstration of MRHA activity 0.02 ml of D-mannose, saline, erythrocyte suspension and bacterial suspensions were mixed in the depression of a tile rocked mechanically for 20 minutes in a refrigerator at the 4ºC reaction with each of erythrocyte species were read immediately on removal from the refrigerator.

DNA extraction and molecular identification by PCR

25 *Salmonella* strains were isolated and identified serologically and subjected for molecular characterization using polymerase chain reaction. *Salmonella* strains were cultured onto Luria Bartani (LB) broth for 24 hours at 37ºC then extraction of DNA was done according to Sambrook *et al.* (1989). Amplification process was performed according to Singer *et al.* (2006). PCR was carried out in 25 µl reaction volumes 12.5 µl 2x PCR master mix 0.47µl invA1, 0.048 µl invA2, invA 0.48 µl and 1.7 µl NA template. The reaction was completed up to 25 µl with distilled water. The PCR system was programmed for denaturation at 94ºC, 30 cycles of denaturation at 94ºC for 30 seconds. Annealing at 56ºC for 30 seconds and extension at 72ºC for 2 minutes. Then the reaction was held at 72ºc for 7 minutes, and stored at 4ºC.

Agarose gel electrophoresis

This step was done according to Sombrook *et al.* (1989). PCR products were electrophoresed at 2% (wt/vol) agarose and 0.5 µg of ethidium bromide (Sigma-Aldrich). The samples were electrophoresed at 85 volt for 20–30 minutes. A 300 nm transillumination was used to detect the bands which were then photographed.

Results and Discussion

Prevalence of Salmonellae in examined cow and buffalo-calves

A total number of 591 diseased and 55 apparently healthy calves were included in the study. Bacteriological examination revealed the isolation of *Salmonella* organisms from 36 out of 393 diseased buffalo-calves with an incidence of 9.2% and 20 of 198 cow calves with an incidence of 10.1%, the overall incidence among examined buffalo and cow calves was 9.5% (Table 1). Examined cow-calves were belonged to some governors in Egypt, such as El-Gharbia (38 calves), El-Sharkia (46 calves), El-Giza (35 calves), El-Fayoum (50 calves) and Beni-Suef (29 calves). *Salmonella* organisms could be isolated from 0, 7, 4, 6 and 3 of these calves representing percentages of 0%, 15.2%, 11.4%, 12% and 10.3% in the above mentioned governorates, respectively (Fig. 1). Buffalo-calves were belonged to El-

Behira (80), El-Giza (65), Domiatt (63), kafr El-Sheikh (90), Bani-Suef (50) and El-Menia (45) govemorates and *Salmonella* organisms could be isolated from 9, 5, 5, 8, 4 and 5 of these calves representing percentage of 11.2%, 7.6%, 7.9%, 8.8%, 8% and 11% in these governorates, respectively (Fig. 2).

Identification of isolated strains

All isolated strains were identified morphologically and biochemically as *Salmonella* species according to Cruickshank *et al.* (1975) and Kreig and Holt (1984).

Serotyping and antigenic formula of isolated *Salmonella* strains

The isolated *Salmonella* strains (21 from cow-calves and 38 from buffalo-calves) were serotyped. Table 2 shows that these isolates were belonging to 6 serovars namely: *S. typhimurium* of group B with the antigenic formula 1, 4, 5, 12: i: 1,2, *S. anatum* group E l with the antigenic formula 3,10: eh: 1,6, *S. dublin* group D l with the antigenic formula 1, 9, 12: g.p, *S. entritidis* group Dl of the antigenic formula 1, 9, 12: g.m, *S. meleagridis* group El with the antigenic formula 3, 10, eh: 1, w and *S. infantis* group C l of the antigenic formula 6, 7: r; 1,5 with an incidence of 3.8%, 2.2%, 0.62%, 0.93%, 0.93% and 0.62% respectively.

Prevalence of *Salmonella* serovars regarding calf-species

Figure 5 shows the isolation rate of Salmonellae with regard to examine calf species, *S. typhimurium* and *S. anatum* were the most common species isolated from cow and buffalo-calves. *S. entritidis* and *S. meleagridis* were common in both calf species, but on the other hand, the isolation of *S. dublin* and *S. infantis* were restricted to buffalo-calves.

Prevalence of *Salmonella* serovars in calves regarding calf species and sex

There is no marked difference between males and females calves infected with *Salmonella* serovars. Regarding the species, there was some serovars that could not be isolated from cow calves as *S. dublin, S. infantis.* Table 3 also shows that *S. typhimurium* was almost the most predominant in both species and sexes, *S. anatum, S. entritidis* and *S. meleagridis* were also isolated from both species and sexes with variable percentages of isolation.

Motility test for *Salmonella* serovars regarding health status by examining calves

Motility test for *Salmonella* serovars resulted in 47/59 isolated strains were motile representing the percentage of 79.6%. All *S. typhimurium*, *S. dublin* and *S. anatum* associated with diarrhoea were motile. Other *Salmonella* serovars gave variable results.

Invasion of Hela cells by *Salmonella* species

Results in Table 4 and Figure 4, 5 and 6 revealed that all *Salmonella* serovars were positively invasive to Hela cells after 3 hrs incubation.

Haemagglutination patterns of Salmonellae in RBCs of different animal species.

The haemagglutination activity of isolated *Salmonella* strains was studied against bovine, sheep, guinea pig, chicken and human RBCs (mannose sensitive and mannose resistant) forms MSHA and MRHA. The results are illustrated in Table 5

and indicated that the majority of *Salmonella* strains that had HA activity were (MRHA) type. Of the 59 *Salmonella* strains isolated from newly born calves 21, 20, 37, 11 and 5 were MRHA with bovine, sheep, G. pig, chicken and human RBCs respectively.

Results of PCR assays

The obtained results of PCR showed that, the ability of *Salmonella*- specific primers to detect *Salmonella* species is primarily due to that the primer sequences are selected from the invA gene of serovar typhimurium. The invA gene encodes for proteins found in the inner membrane of the microorganism and necessary for invasion in intestinal epithelial cells. The results showed that all PCR products of isolates (*S. typhimurium*) include positive control, screened by PCR, resulted in 243 bp amplified fragments. No amplified DNA fragments were obtained from non *Salmonella* species (Fig. 7).

Salmonellae are thought to be major pathogens leading to serious economic losses in the animal industry and cause devastating disease conditions ranging from gastroenteritis to septicemia and death, depending on the serovar of the bacterium and the nature of the infected host (Kusters et at., 1993). Bovine salmonellosis has special importance in the spread and persistence of such pathogen potentially capable of causing human infection (Anderson *et al.*, 1990). Bovine salmonellosis affects cattle of all ages; calves are more susceptible to infection than adults (Wray and Sojka, 1981). In the present study, a total number of 591 diarrheic calves from *Salmonella* endemic farms and belonging to various localities in Egypt were surveyed (Figure 1). The overall prevalence of Salmonellae was 9.5% and this percentage of *Salmonella* isolation seems to be quite high. However, this would not represent the incidence of *Salmonella* infection due to the small proportion of calves, all governorates were not covered and the investigation was restricted to farms that had previous history of salmonellosis. Lower incidences were reported in Egypt by Farid *et al.* (1987) (4.17%) and Zaki (1994) (1.14%), while a relatively higher rate was reported by Hafez, (1989) (15.1%) and Eid (2010) (6.2%). *Salmonella* could be isolated from cow-calves in a higher rate than buffalo-calves (10.1% and 9.2%). This variation may be attributed to the variation in susceptibility between the two animal species. The increased susceptibility in cow-calves than buffalo-calves in Egypt was reported by Gaber (1995), while a contradictory result was recorded by Hafez (1989) who reported a higher rate of *Salmonella* infection in buffalo- calves.

Regarding the prevalence of *Salmonella* infection in the location of farms under study, results obtained in Figure 1 showed that the higher percent of isolates from cow-calves was in farms belonging to El-Sharkia and El-Fayoum (15.2% and 12%), respectively and lower percentages were in noticed El-Giza and Beni-Suef (11.4% and 10.3%), respectively; while no *Salmonella* organisms could be isolated from El-Gharbia governorate. The variation of *Salmonella* infection rates with regard to location of study farms was observed also with buffalo-calves (Figure 2). The highest percentages were observed in El-Behira and El-Menia (11.2% and 11%), respectively; while lower percentages were noticed in Kafr El-Sheikh, Bani-Suef, Domiatt and El-Giza in descending manner (8.8%, 8%, 7.9% and 7.6%). Inspite of the unequal and small proportions of examined calves, the variation in results could be explained as a result of several factors, including the difference in standard of nutrition as reported by MacCay (1973), association of

stress factors and the currently used medications, and the difference in the isolation techniques and laboratory workers as well as the time of sampling as reported by Richardson (l973) who concluded that *Salmonella* organisms are intermittently excreted by diseased animals. Hygiene and food management measures may play an additional role. Mclaren and Wray (1991) reported that enforcement of strict hygienic measures may be desired to prevent dissemination of *Salmonella* among infected animals as well as protecting man and food producing animals from contaminated food ingredients. Previously, reported incidences of *Salmonella* infection among calves in Egypt and worldwide confirmed such explanations as Nakamura *et al.* (1989) who reported Salmonellae incidences of 2.3%, 26.5% and 2.5% in cow calves respectively. While in buffalo-calves variable percentages were recorded at 19.4% (Hafez, 1989) and 18.2% (Riad *et al.,* 1998).

Serotyping of the isolated *Salmonella* strains (Table 2) revealed the existence of *S. typhimurium* (25), *S. anatum* (14), *S. dublin* (4), *S. entritidis* (6), *S. meleagridis* (6) and *S. infantis* (4) representing incidences of 3.8%, 2.2%, 0.62%, 0.93%, 0.93% and 0.62%, respectively. The flagellar antigens in *S. typhimurium, S. anatum, S. meleagridis* and *S. infantis* were diphasic while in other serovars were monophasic. Parker (1983) reported that the flagellar characteristics of diphasic strains tend to be in equilibrium, but sometimes under certain conditions, separate colony may has one phase only. Therefore, it could not rely on such phenomenon to characterize the flagellar antigenicity.

Regarding the association of *Salmonella* serovars with calf species, Figure 3 showed that *S. typhimurium, S. anatum, S. entritidis* and *S. meleagridis* were isolated from both cow and buffalo-calves. No distinct variation between the two species in the selective susceptibility to salmonellosis was previously reported. On the other hand, Nakamura *et al.* (1989) and Aydn *et al.* (2001) reported that the serovars of *Salmonella* strains from cattle and buffaloes were found to be almost similar. Predomination of *S. typhimurium* among cow and buffalo-calves was obvious in the present study (Figure 3). It is documented that this serovar is endemic in Egypt (Farid *et al.,* 1987; Pasmas *et al.,* 2000). Veling *et al.* (2002) suggested that infection with such serovar may originate from milk as it could be isolated from bulk tank and milk filters at some dairies. *S. anatum* was recovered in percentage of 2.75% from cow-calves and 1.9% from buffalo-calves. This serovar constitutes special interest as it seems to be adapted to calves in Egypt and it was isolated previously by Hafez (1989) and Zaki (1994). *S. entritidis* could be isolated in the present study from cow and buffalo-calves in percentages of 1.4% and 0.7%, respectively while *S. Dublin* was isolated only from buffalo-calves in percentage of 0.93% (Table 5). No specific explanation for failure of isolation of *S. dublin* from cow-calves except the suggestion of geographical distribution because this serovar was previously isolated from cow-calves as well as buffalo-calves in Egypt by Farid *et al.* (1987) and Sobhi (1997). A similar observation was detected with *S. infantis* which was isolated from buffalo-calves in percentages of 0.93%, while could not be isolated from cow-calves.

It is important to pay attention to *S. meleagridis* which is pathogenic serovars specially for birds and isolated in the present study from cow and buffalo-calves (1.8% and 0.5%, respectively), the order that may reflect a certain role which could be played by birds in the transmission of such serovar. Further investigation regarding this point is recommended. In the present study

Salmonella organisms could be isolated from fecal samples of normal and diarrheic calves (5 %, 10% and 5.7%, 9.2%) in cow and buffalo-calves, respectively (Table 1). This obtained result gave no sharp discrimination between normal and diseased calves depending upon examination of fecal samples. A similar confusing explanation was reported by Corrier *et al.* (1990) who stated that because fecal excretion of Salmonellae is intermittent and may be induced by various stress factors in healthy calves, it was not possible to determine when *Salmonella* infection occurred in the fecal culture positive calves and on the other hand, excretion of *Salmonella* by infected calves may be intermittent and multiple fecal samples collected over several days were required to detect infection (Hume *et al.,* 2004). Subsequently, it was not possible to determine exactly the rate of *Salmonella* infection in a herd based on one day fecal sample collection. Most of *Salmonella* serovars were associated with diarrhea in buffalo-calves while *S. typhimuium, S. entritidis* and *S. meleagridis* were isolated from diarrheic cow-calves. Bad hygiene and possible contamination may lead to the dissemination of *Salmonella* serovars, resulting in diarrhea, which is one of the major symptoms associated with salmonellosis in calves (Lance *et al.,* 1992) recorded outbreaks of salmonellosis in calves and stressed on the importance of hygiene improvement and avoiding contamination in reducing transmission of infection among calves (Loefstroem *et al.,* 2010).

Regarding the influence of calf gender on the incidence of salmonellosis and distribution of *Salmonella* serovars, Table 3 revealed that there was no clear domination of certain *Salmonella* serovar than the other with regard to sex in both cow and buffalo - calves while the total recovery rates in female cow and buffalo-calves were higher than in males (10.0% and 10.2%, 9.4% and 7.7%, respectively). The reason for variation in susceptibility between male and female calves to *Salmonella* infection is not clear and unfortunate, no literature explaining this point is available but Lance *et al.* (1992) estimated that out of 10 culture positive calves to Salmonellae, only one was male and the other 9 were females. Hormones regulating sex differentiation during pregnancy may have interacted with the immunity of delivering calves (Smith *et al.,* 2004).

The pathogenic activity of *Salmonella* serovars in the host cells seems to be monitored by one or more of virulence attributes that may facilitate their internalization survival and induced action. The present study aimed to throw light toward some virulence attributes of the isolated *Salmonella* serovars along the study. Motility was determined by stabbing semisolid agar with the isolated *Salmonella* strains. The obtained results shown that 47/56 strains isolated from diarrheic calves were motile. Motility in *Salmonella* is known to be associated with flagella; however flagellated Salmonellae may have non motile isogenic mutants. Lee *et al.* (1996) differentiated *S. typhimurium* strains into Fla+ Mot+, Fla- Mot- and Fla+ Mot-, the latter one named as paralyzed flagella and they added that the presence of paralyzed flagella did not offer any advantage over the loss of flagellation (Fla-) in adhesion or invasion. It was clear that not all *Salmonella* strains associated with diarrhea in calves are flagellated or have functional flagella.

The same observation was detected by Weinstein *et al.* (1984), who stated that flagella are not required for *Salmonella* to establish intestinal infection or disseminate

after oral inoculation. Detected motility with *Salmonella* strains could be explained by the suggestion of Weinstein (1984) who concluded that once flagellated organisms reached the reticuloendothelial system, net growth and secondary bacteraemia was readily detectable by a positive blood culture and added that flagellated *Salmonella* were more virulent than non flagellated ones.

Results of invasion of Hela cells by different *Salmonella* serovars either motile or non motile (Table 4), revealed that non motile isogenics of *S. meleagridis* were non invasive to Hela cells. It is important to note that the invasion of Hela cells is correlated with the invasion of the gastrointestinal mucosa by the organism which is an essential early step in the pathogenesis caused by *Salmonella* organisms (Giannella *et al.,* 1973) and so the Hela cells model is useful for investigating such parameter with the isolated *Salmonella* serovars. Portillo *et al.* (1997) declared the correlation between invasion to Hela cells by Salmonellae and the lethal toxicity produced by their lipopolysaccarides (LPS) and suggested that bacterial internalization which is expressed by the invasion capacity is required for formation of vesicles that is required for releasing LPS toxins while Kusters *et al.* (1993) proved that certain protein synthesis induced by contact of the bacterium to cells is needed to facilitate invasion which needs no metabolic activity of the bacteria or the cell. The similarity in invasiveness between motile and non motile *S. typhimurium* disagreed with that reported by Lee *et al.,* (1996) who proved that motility of the bacteria facilitated their contact to Hela cells. Invasion of host cells in the gastrointestinal tract by *Salmonella* was reported as the early step in pathogenesis (Giannella *et al.,* 1973).

The haemaglutination activity of the isolated *Salmonella* strains was studied against bovine, sheep, G. pig, chicken and human RBCs in mannose sensitive and mannose resistant forms (MSHA and MRHA). Table 5 showed that G. pig RBCs were the best indicator of such character. Results of mannose resistant haemagglutination (MRHA) are in agreement with the results of invasiveness obtained in the present study. This result confirms that obtained by Jones and Richardson. (1981) who concluded that MRHA activity was responsible for the attachment of *Salmonella* to Hela cells. MSHA also correlated with invasiveness as it reflects the action of type 1 fimbria which suggested promoting invasion. Earlier studies demonstrated also that *S. typhimurtum* expressing MSHA colonizing the intestine of mice more efficiently, but cleared from the blood more rapidly (Niemann *et al.,* 2011).

Concerning the use of PCR assay to develop a highly sensitive and specific diagnostic method for the detection of *Salmonella* species in fecal specimens, in this study 25 isolates of *S. typhimurium* were tested by using 2 primers for confirmation and the results showed that, PCR products of isolates include positive control, resulted in 243 base pairs amplified fragments and no amplified DNA fragments obtained from non *Salmonella* spp. The results agree with Eid (2010) who stated that PCR is more rapid and accurate method for detection of *Salmonella* spp. than conventional culture methods and can applied for routine diagnosis. Similar judging were reported by Koyuncu *et al.* (2010) who reported that PCR is a rapid, sensitive and accurate method for the detection of *Salmonella* species from fecal samples and confirmed that the isolated strains were developed and the method was highly valuable in a clinical setting to help the complication arising from an outbreak of salmonellosis in a herd or among patients of veterinary hospitals.

Table.1 Number of examined calves and the prevalence of Salmonellae

Animal species	*Salmonella* positive cases					
	Apparently healthy calves			Diseased calves		
	No. of examined cases	*Salmonella* positive cases	%	No. of examined cases	*Salmonella* positive cases	%
Buffalo-calves	35	2	5.7	393	36	9.2
Cow-calves	20	1	5	198	20	10.1
Total	55	3	5.4	591	56	9.5

% was calculated upon the No. of positive biochemical isolates.

Table.2 Serotyping and antigenic formula of isolated Salmonellae from newly born calves

Serovars	Group	Antigenic structure			No. of isolates	Incidence rate
		Somatic	Phase 1	Phase2		
S, typhimurium	B	1,4, 5, 12	i	1,2	25	3.8%
S. anatum	E	3, 10	eh	1,6	14	2.2%
S. dublin	Dl	1,9,12	g-P		4	0.62%
S. entritidis	Dl	1,9,12	gm		6	0.93%
S. meleagridis	El	3,10	eh	1,W	6	0.93%
S. infantis	Cl	6,7	r	1,5	4	0.62%
Total					59	9.1%

Table.3 Incidence of *Salmonella* serovars in calves regarding the calf species and sex

Serovars	Cow-calves				Buffalo-calves			
	Male (117)		Female (101)		Male (220)		Female (208)	
	No.	%	No.	%	No.	%	No.	%
S. typhimurium	3	2.5	5	4.9	7	3.1	10	4.8
S. anatum	5	4.3	1	1	3	1.4	5	2.4
S. dublin	-	-	-	-	2	0.9	2	1
S. entritidis	2	1.7	1	1	2	0.9	1	0.5
S. meleagridis	1	0.85	3	2.9	1	0.45	1	0.5
S. infantis	-	-	-	-	2	0.9	2	1
Total	11	9.4	10	10	17	7.7	21	10

( ) = No. of examined calves

Table.4 Invasion of Hela cells 3, 24 hrs after incubation

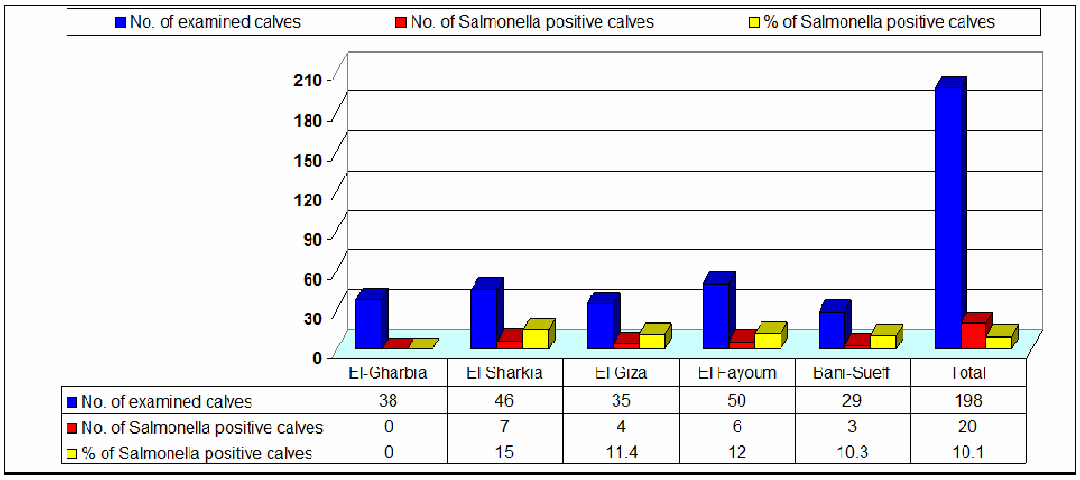
Salmonella serovars	3hrs		24hrs	
	% of infected cells	% of dead cells	% of infected cells	% of Dead Cells
Tm	40	60	2	98
Am	53.4	46.6	4	96
Dm	46.6	53.4	All cells died	
Em	33.3	66.6	All cells died	
Mm	33.3	66.6	All cells died	
Mn	40	60	2	98
Im	40	60	1.3	98.7

Table.5 Haemagglutination patterns of Salmonellae in RBCs of different animal species

Serovars	Bovine RBCs			Sheep RBCs			Guinea Pig RBCs			Chicken RBCs			Human RBCs		
	MRHA	MSHA	-ve	MRHA	MSHA	-ve	MRHA	MSHA	-ve	MRHA	MSHA	-ve	MRHA	MSHA	-ve
Tm	9	5	11	7	7	11	13	2	10	3	7	15	1	7	17
Am	4	-	10	3	-	11	13	-	1	4	-	10	3	-	11
Dm	4	-	-	2	-	2	2	1	1	-	-	4	-	-	4
Em	-	-	6	5	-	1	5	-	1	-	2	4	-	1	5
Mm	2	-	2	2	-	2	4	-	-	2	-	2	-	-	4
Mn	-	-	2	1	-	1	-	-	2	1	-	1	-	-	2
Im	2	-	2	-	-	4	-	1	3	1	-	3	1	-	3
Total	21	5	33	20	7	32	37	4	18	11	9	39	5	8	46

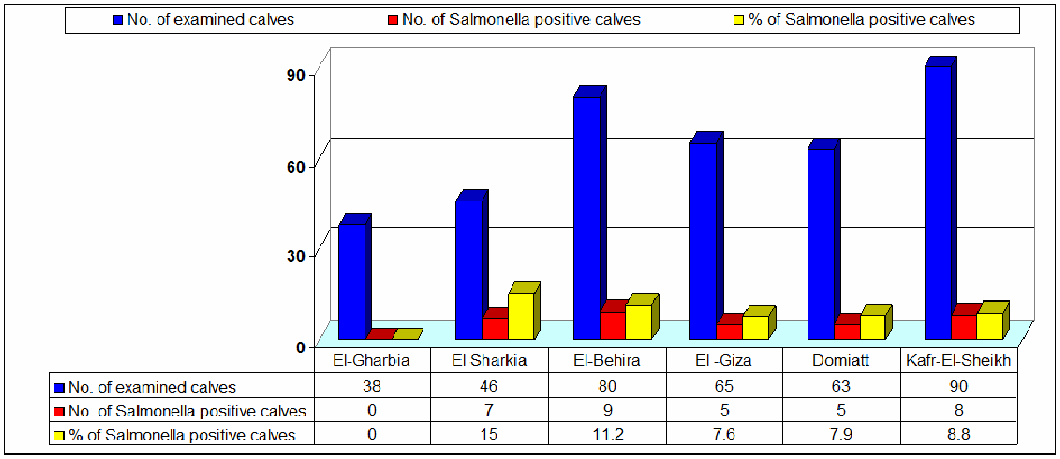
Tm = Typhimurium motile
Am = Anatum motile
Dm = Dublin motile
Em = Entritiidis motile
Mm = Meleagridis motile
Mn = Meleagridis non motile
Im = Infantis motile

Fig.1 Prevalence of *Salmonella* infected cow–calves in relation to its location



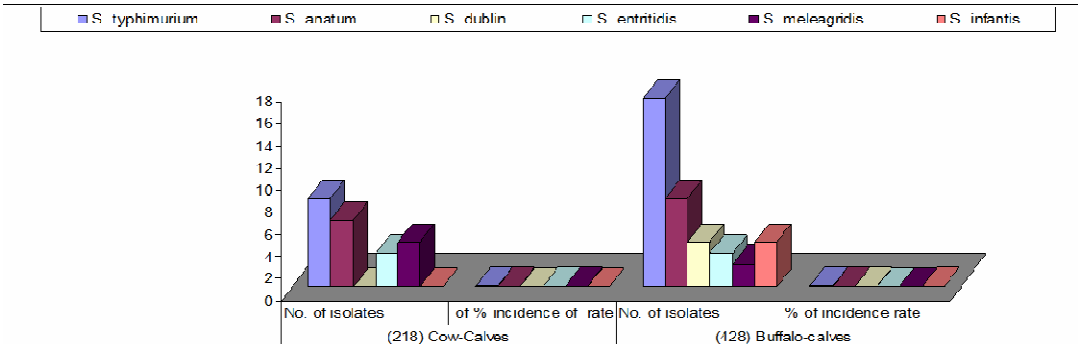
	El-Gharbia	El Sharkia	El Giza	El Fayoum	Bani-Suef	Total
No. of examined calves	38	46	35	50	29	198
No. of Salmonella positive calves	0	7	4	6	3	20
% of Salmonella positive calves	0	15	11.4	12	10.3	10.1

Fig.2 Prevalence of *Salmonella* infected buffalo–calves in relation to its location



	El-Gharbia	El Sharkia	El-Behira	El -Giza	Domiatt	Kafr-El-Sheikh
No. of examined calves	38	46	80	65	63	90
No. of Salmonella positive calves	0	7	9	5	5	8
% of Salmonella positive calves	0	15	11.2	7.6	7.9	8.8

Fig.3 Prevalence of *Salmonella* serovars regarding calve species



	No. of isolates	% of incidence rate	No. of isolates	% of incidence rate
	(218) Cow-Calves		(128) Buffalo-calves	
S. typhimurium	8	3.70%	17	3.97%
S. anatum	6	2.75%	8	1.90%
S. dublin	0	0%	4	0.93%
S. entritidis	3	1.40%	3	0.70%
S. meleagridis	4	1.80%	2	0.50%
S. infantis	0	0%	4	0.93%

( ) =No. of examined calves

Fig.4 Hela cells before invasion of *Salmonella* spp.

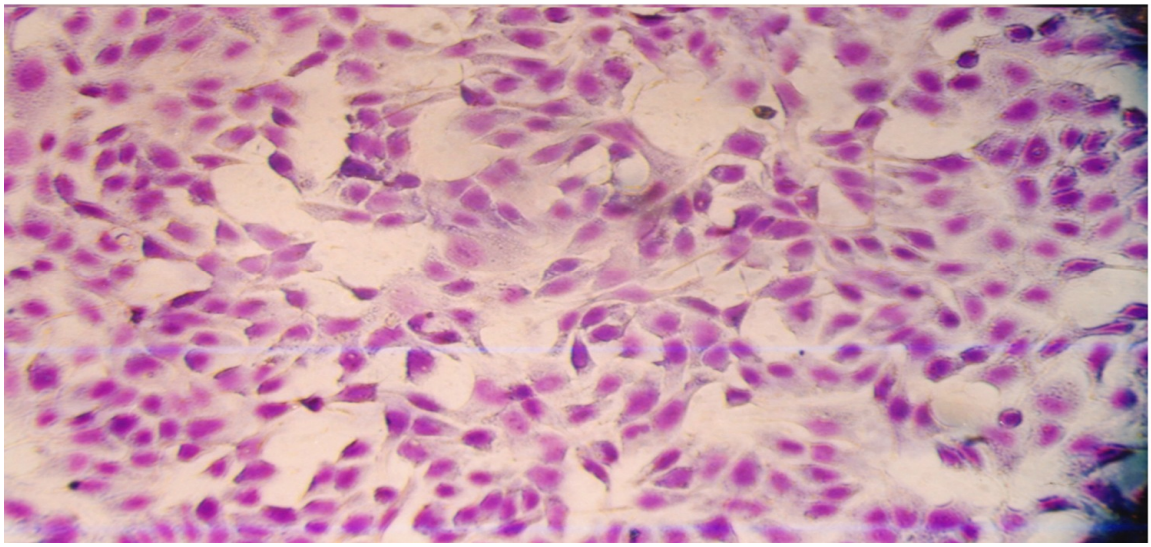


Fig.5 Invasion 3 hrs post incubation

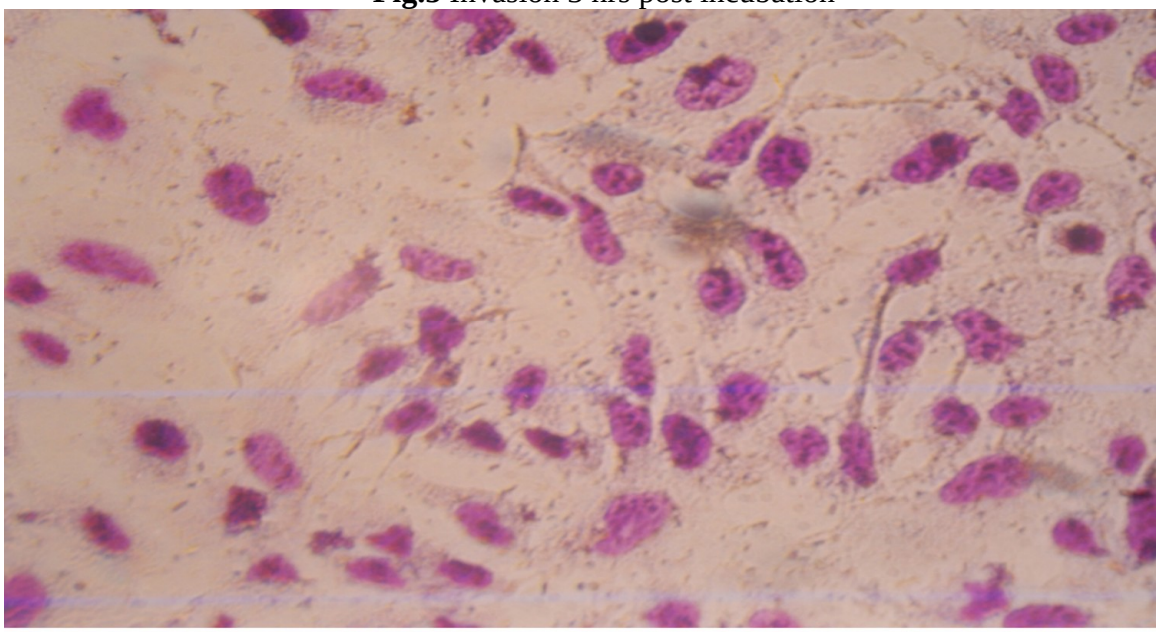


Fig.6 Invasion 24 hrs post incubation

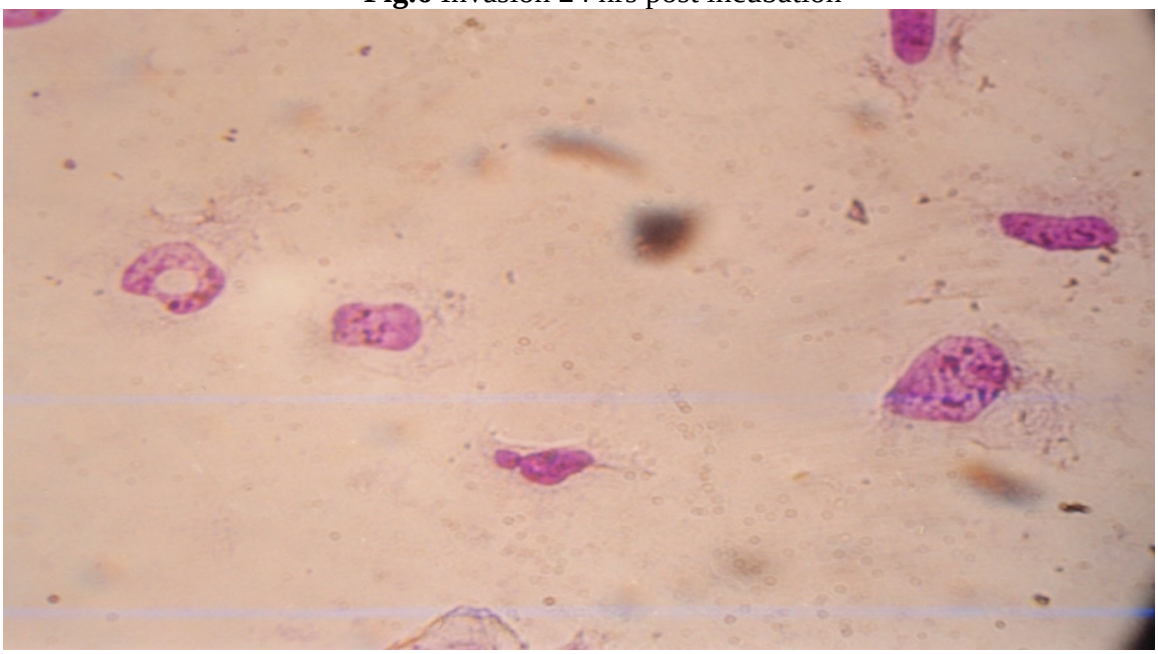
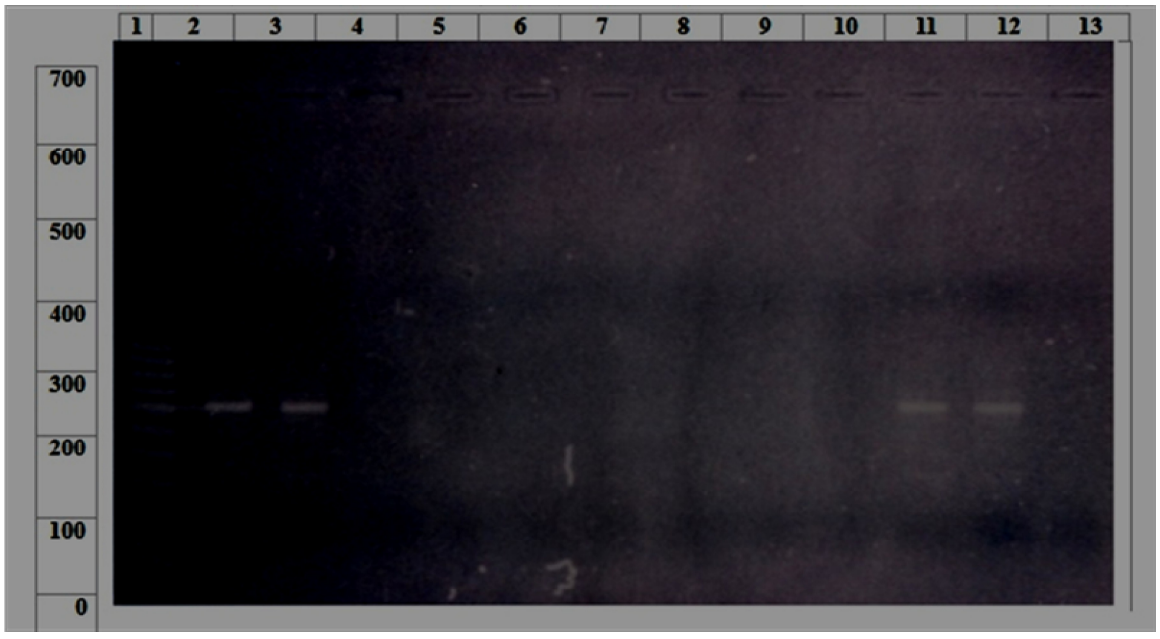


Fig.7 Agarose gel electrophoresis analysis of DNA products with specific primers for the invA gene of *S. typhimurium* produced and PCR product of 243 bp



Lane 1: DNA size marker. Lane 2, 3: Positive strains. Lane 4, 5, 6, 7, 8, 9, 10: Negative strains (Gram positive). Lane 11: Positive strains. Lane 12: Positive control (*S. typhimurium*). Lane 13: Negative control (*E. coli*).

Also, Salehi *et al.* (2007) concluded that, the PCR results showed that detection of Salmonellae at the genus level and specific primers can potentially permit to more readily evaluate fecal and other types of samples for the presence of this organism. As well as PCR system proved to be suitable for characterization of *Salmonella* flagellin gene and confirmed serological identification (Gentry-Weeks *et al.,* 2002). PCR assays need 24 hours for results obtaining whereas it needs from 5-7 days to identify *Salmonella* spp. by culture methods.

Conclusion

The overall prevalence of Salmonellae among young diarrheic cow and buffalo-calves was appeared to be quite high. However, this would not represent the incidence of Salmonella infection in Egyptian farms due to the small proportion of calves, all governorates were not covered and the investigation was restricted to farms that had previous history of salmonellosis. In addition, the application of PCR assay is important to develop a highly sensitive and specific diagnostic method for rapid detection of Salmonella species in calves.

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