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Original Article

Prevalence and Risk factors of *Salmonella* species in cattle presenting at Slaughter in Tripoli, Libya

Laila Alshaerik, Imad Buishi , Almabrouk Faris

Department of Preventive Medicine, Faculty of Veterinary Medicine,

University of Tripoli

ABSTRACT

Foodborne disease caused by *Salmonella* is an important public health concern worldwide. An observational study was conducted on apparently healthy cattle at four abattoirs, Tripoli, Libya from April 2017 to October 2017. The objective was to estimate the prevalence of *Salmonella* isolated from feces of cattle and to identify associated risk factors in abattoirs. From the total of 353 fecal samples examined for *Salmonella* using the conventional culture procedures outlined by International organization For Standardization, 15 (4.25%) were positive. A real-time PCR assay yielded 2 poitives out of 44 randomly selected negative-culture fecal samples. Origin of animals and shipping distance factors found to be significantly associated with the prevalence of *Salmonella* in cattle feces at the time of slaughtering (P<0.05). The results presented in this study confirm the presence of *Salmonella* in cattle feces at slaughter which may pose a considerable food safety hazard. In addition, the application of real-time PCR assay proved to be an important tool for rapid, sensitive detection of *Salmonella* in cattle feces.

Key words: *Salmonella*; Cattle; feces; conventional cultures; Real-Time PCR, Tripoli, Libya.

Introduction

Salmonella which are one of the most important foodborne pathogens in the world, is frequently implicated in foodborne disease outbreaks. It is estimated that *Salmonella* is responsible for approximately 1.3 billion cases of Salmonellosis worldwide each year (Desai *et al.*, 2013). Epidemiological studies have suggested that cattle carcass contamination can be resulted from exposure to feces and hides during both slaughtering and processing as a result of bacterial transfer from intestine evisceration and skinning stage (Narvaez-Bravo *et al.*, 2013) which can be a significant food-safety hazard. Microbiological methods used to detect *Salmonella* are vital tools in the food safety programs. Conventional culture methods remain the gold standard procedures for confirming the presence of *Salmonella* in different types of food and environment samples. However, the methods are laborious

Corresponding Author: Almabrouk Faris , Dep. Preventive Medicine- Fac. of Vet. Medicine.- Univ. of Tripoli. Libya.

Phone: + 218 926457288 **Email:** a.fares@uot.edu.ly

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and time-consuming, requiring 3-7 days to obtain a confirmed result. Thus, rapid and cost-effective detection of *Salmonella* is of major interest to food industry and the public. Polymerase chain reaction (PCR) technology offers several advatges compared with convetional culture methods in term of speed, detection limit and cost (Kasturi and Drgon 2017). Several studies have reported the prevalence of *Salmonella* in cattle at abattoirs (Claudia *et al.*, 2013; Gizachew and Mulugeta, 2015; Nouichi *et al.* 2018; Shaibu *et al.*, 2021;Wang *et al.*, 2020). To the best of our knowledge no study has been conducted in Libya on the prevalence and risk factors of contamination of *Salmonella* in cattle at the time of slaughtering. Therefore, the first objective of this work was to estimate the prevalence of *Salmonella* species in cattle presenting at abattoirs in Tripoli, Libya and the second objective was to identify risk factors associated with prevalence of *Salmonella* spp.

Materials and Methods

Study area

Four private abattoirs referred to as: (A. B, C, and D) (Table 1) were selected in Tripoli, Libya using a convenience sampling method since the abattoirs census (sampling frame) of the study area was unavailable. The abattoirs were with slaughtering capacity ranges from 10 to 40 animals per day. After Antemortem inspection, animals were slaughtered and dehided manually on the floor. Mixed species were found in the same pen at different days of sample collection.

Study design and sample size determination

A cross-sectional study was conducted over the period of time from April to October 2017. The sample size required for this study was determined by the formula given by Snedecor and Cochran (1967), based on previous published studies (Al-Saigh *et al.*, 2004; Beach *et al.*, 2002; Claudia *et al.*, 2013; Fedorka-Cray *et al.*, 1998; Payman *et al.*, 2014; Pengcheng *et al.*, 2014; Sarah *et al.*, 2014; Tadesse *et al.*, 2014; Van Donkersgoed *et al.*, 1999).

$n = 4PQ/L^2$ where; n = minimum sample size required

P = estimated prevalence of *Salmonella* based on previous studies.

Q = 100 – P

L = allowable error (absolute precision of 0.05).

Depending on the estimated mean prevalence of 10%, the calculated sample size was 144, but , to increase the precision of the study and to increase the number of *Salmonella* isolates, a total of 353 samples were collected from the four abattoirs (Table 1).

Table 1. Number of fecal samples collected from cattle pre-slaughter at four abattoirs in Tripoli, Libya.

Abattoirs	Number of samples collected
A	116
B	30
C	146
D	61
Total no.	353

Fecal sample collection

Fresh fecal samples were collected directly from cattle before slaughtering by rectal grab. Samples were collected using a new palpation sleeve for each sample. Sleeves were inverted upon collection, and were individually bagged in sealed plastic bags immediately after collection and kept on ice during transport (Callaway *et al.,* 2005) to the Microbiology Laboratory, National Centre for Animal Health, Tripoli city, Libya. Samples were analysed within 2-3 hr of collection.

Laboratory methods

Conventional culture method

The standard ISO 6579:2007 (ISO, 2007) designed for bacteria detection of *Salmonella* spp. in animal feces was used in this study. Briefly, 5 g of feces was preenriched in 45 ml of sterile buffered peptone water (BPW) (Oxoid, Basingstoc, UK) and incubated at 37 °C for 24 hr. A 100µl of each preenriched suspension was added to 9.9 ml of Rappaport-Vassiliades soya broth (RVS) (bioMerieux, Marcy l'Etoile, France) and incubated at 42 °C for 24 hr. Next, a loopful of RVS broth was plated onto Xylose Lysin Desoxycholate agar (XLD) (Oxoid) supplemented with 1.5% Novobiocin and incubated at 37 °C for 24hrs. According to ISO 6579:2002 (ISO, 2002), presumptive *Salmonella* colonies were examined by biochemical tests namely Triple Sugar Iron (TSI), Lysin Iron Agar (LIA), oxidase and urease. Isolates gave negative both oxidase and urease and gave gas on TSI were confirmed with Analytical profile index 20E (API20E) (bioMerieux, Marcy l'Etoile, France). An API 20E strip was inoculated for each isolate and incubated at 37 °C for 24 hr. positive results were evaluated for each of the 20 biochemical tests. Those colonies with typical *Salmonella* biochemical properties were further confirmed by using *Salmonella* Latex Agglutination test kit as described by the manufacturer (Difco Laboratories, Detroit, MI).

Real-time PCR

Randomly selected negative-culture samples (44 out of 338 negative-culture samples) were subjected to a real-time PCR assay using *Salmonella* real-time PCR kit (Liferiver™ , Shanghai ZJ Bio-Tech Co., Ltd, shanghai, China).

DNA extraction

DNA extraction kit was supplied with the *Salmonella* real-time PCR kit (Liferiver™). DNA extraction was performed according to the instructions of manufacturer. Approximately 50 mg of each fecal samples was mixed with1.0 ml of normal saline in 1.5 ml tube and vortexed vigorously. The sample was centrifuged at 13000 rpm for 2 min. then the supernatant was discarded carefully from the tube. A 100 µl of DNA extraction buffer was added to the tube and the pellet was vortexed. Lysis was completed by incubating the tube at 100 °C for 10 min. then centrifuged at 13000 rpm for 5 min. Finally, a volume of 4 µl of aliquots was used as the DNA template in the real-time PCR assay.

Salmonella real-time PCR assay

Rea-time PCR assay was performed according to the instructions of manufacturer, using the *Salmonella* Real-Time PCR kit ((Liferiver™).

The kit contained patented probes and enzymes for the specific amplification of the *Salmonella* DNA including *S.enterica. S.bongori, S.suberranae*, and suspecies of *S.enterica*. in addition, the kit also utilizes an internal positive control. All reactions were run using a QIAGEN's real-time PCR cycler, the Rotor-Gene Q (Qiagene, Hilden, Germany), using a 4 µl of DNA template in a total reaction volume of 36 µl. Thermal cycling parameters were set for an initial denaturation step of 37 ^{o}C for 2 min, followed by a holding stage of 94 ^{o}C for 2 min. This was followed by 40 cycles of amplification at denaturation of 93 ^{o}C, for 15 s with subsequent annealing and extension at 60 ^{o}C, for 60 s (Table 2). Finally, data were analysed and interpreted according to the manufacturer's instructions. Positive measurement were recorded as the threshold cycle values (C_T) that corresponded with PCR product whose measurement elicited fluorescence above the fixed threshold before the completion of 40 cycle.

Table 2. Thermal cycling program for each PCR reaction.

program	Target temp. (^{o}C)	Incubation time
First denaturation	93	2 min.
Amplification (40 cycles)		
• denaturation	93	15 s
• annealing	60	60 s
• extension	60	60 s

Statistical Analysis

All data were transferred to SAS release 9.3 for analysis (SAS Institute Inc. Cary. NC). The data were analysed comparing proportions by Chi-square (X^2) test on the number of observations per contingency table cells test association. For the strength of association odd ratios were computed using a logistic regression analysis. The explanatory variables considered (origin of animal, distance of shipping, presence of other animal species at the same pen, and the signs of diarrhoea). Values were considered to be statistically significant when P<0.05.

Results and discussion

Out of the total 353 cattle examined at four abattoirs for *Salmonella* shedding, 15 (4.25%) were positive. These findings are consistent with those previously reported for the prevalence of *Salmonella* in Cattle feces at abattoirs in different regions and countries (Genevieve *et al.*, 2003; Ghougal *et al.*, 2021; Hasan *et al.*, 2017; Keteme *et al.*, 2018). High results reported by Alfredo *et al.*, (2020); Nouichi *et al.*, (2018); Obaidat (2020) and Wang *et al.*, (2020), whereas lower results were reported by Al-Saigh *et al.*, (2004); Bonifait *et al.*, (2021); Shaibu *et al.*, (2021). The ability to

compare published prevalence studies with the prevalence rate obtained in the present work is difficult, due to variations in study population, study period, study site, number of samples examined, sampling procedures, microbiological techniques, differences in the hygiene conditions and design of abattoirs (McEvoy *et al.* 2003). All abattoirs included in the present study did not comply with good manufacturing practices and sanitation standard operating procedures, and none had implemented a food safety system. Therefore, there is a possibility of cross-contamination of carcasses by *Salmonella* from intestinal contents and hides of cattle during harvesting and dressing process. *Salmonella* contamination on beef carcasses has been reported at the preevisceration stage (Guteme *et al.*, 2021). To our knowledge no published data are available on the prevalence of *Salmonella* in fecal cattle at abattoirs in Libya. However, studies have been published in Libya on the incidence of *Salmonella* contamination in raw beef meat and meat products at retail stage of the food chain (Eshamah *et al.*, 2020; Hamad and Salah, 2019; Mansour *et al.*, 2019; Albie, 2019). These reports indicated that the possible sources of contaminates may result from unhygienic manner handling carcasses in abattoirs and/or inappropriate of sanitary conditions in meat retail shops.

Origin of animals and shipping distance factors were found to be associated with the prevalence of *Salmonella* (Table 4). Both factors have statistical significant effect ($P < 0.05$) on the prevalence of *Salmonella* (Table 5). In contrast, the presence of other animals at the same pen and the signs of diarrhoea had no significant effect on the prevalence of *Salmonella* in the collected fecal samples ($P > 0.05$) (Table 5).

In the present study, all positive animals were local originated from abattoir (C), while other abattoirs showed no positive *Salmonella* samples (Table 3). The majority of cattle slaughtered at abattoir (C) is either adult local or cull cattle as most local cattle tested in the abattoir were above 48 months of age. In Libya, older and/or cull cattle are permitted for human consumption. Various studies showed a high prevalence of *Salmonella* in cull or older cattle (Beach *et al.*, 2002; Troutt *et al.*, 2001; Davies *et al.*, 2004). Moreover, culture positive samples in local animals rather imported ones can be attributed to the differences in the management program and feeding composition between the local and imported animals. Davies *et al.* (2004) indicated that feeding composition was considered as an important factor for the prevalence of *Salmonella* in cattle feces. In addition, positive samples in abattoirs may also be related to stress due to transportation as most cattle brought from distanced markets or farms especially for abattoir (C). It is well-known that there is an increase in shedding of *Salmonella* if live animals are subjected to stress during transportation (Beach *et al.*, 2002; Troutt *et al.*, 2001). Barham *et al.*, (2002) observed a significant increase in *Salmonella* contamination in abattoir during transport by approximately 2 to 14 fold increase in *Salmonella* levels in feces and hide, respectively.

Table 3. Prevalence of *Salmonella* spp. isolated from cattle fecal samples as tested by conventional culture method at selected abattoirs Tripoli, Libya

Abattoir	No. of fecal samples	No. of samples tested positive	Percentage (%)
A	116	0	0
B	30	0	0
C	146	15	10.27
D	61	0	0
Total No.	353	15	4.25

Table 4. Association between the rate of *Salmonella* and risk factors Cattle presented for slaughter, Tripoli-Libya

Risk factor	Number of samples	Number positive	Number negative	X^2	*P* value*
Origin of animal				29.243	0.008
local	I54	15	139		
imported	199	0	199		
Shipping distance				11.049	0.01
>20 Km	207	14	193		
<20 Km	146	1	145		
Presence of other animals				3.27	0.70
Yes	61	4	57		
No	292	11	281		
Signs of Diarrheal				1.56	0.511
Yes	32	2	30		
No	321	13	308		

* Statistically significant at $P<0.05$

Table 5. Regression analysis on the rate of *Salmonella* and risk factors in cattle presented for slaughter, Tripoli-Libya

Risk factor	Odds ratio	Confidence interval	*P*value *
Origin of animal	32.7	4.6-147.3	0.008
Shipping distance	9.3	2.1-22.2	0.01
Presence of other animals	2.83	1.1-6.5	0.70
Signs of Diarrheal	1.2	0.7-3.2	0.50

* **Statistically significant at *P*<0.05**

In the current study, positive *Salmonella* samples were observed over the period of July to October. Even though seasonal variation was not taken into consideration in this study, it was previously proved to be associated with *Salmonella* shedding (McEvoy *et al.*, 2003). Therefore, seasonal variation should be considered in future studies, when a risk analysis of *Salmonella* on beef is concerned.

The real-time PCR assay in this study showed two positive PCR products out of 44 negative culture sample (Figure 1), one positive is a sample of imported animal in abattoir A, and the other positive is a sample of local animal in Abattoir C. The remaining selected negative culture samples gave negative PCR results. Testing all negative culture samples was not possible because of the lack of resources in our laboratory. Conventional culture methods have been reported to show poor sensitivity for low-level contamination of *Salmonella* in feces which result in an increased false-negative rate explained by several factors such as the presence of stressed bacteria which is difficult to culture, inadequate handling of fecal sample before culture, presence of substances that inhibit the growth of *Salmonella,* and shedding very low numbers of *Salmonella* in feces (Eriksson and Aspan, 2007). Postollec *et al.* (2011) have indicated that t the PCR assays as real-time PCR are considered faster and usually more sensitive than the conventional culture methods which is of great benefit to the food industry and to public health authorities engaged in food safety and the managements of salmonellosis.

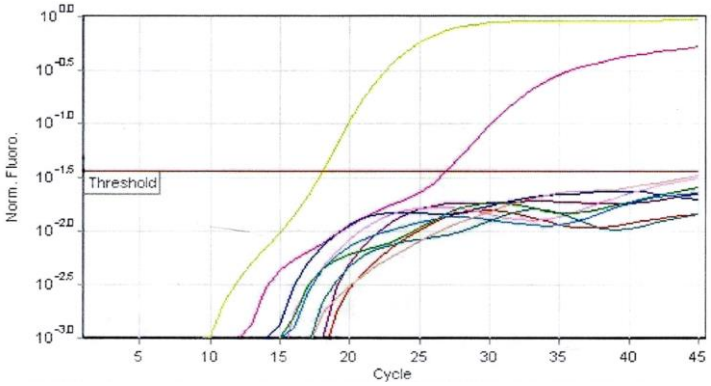

Figure 1. Amplification curves of *Salmonella* in fecal samples. The two positives samples show amplification curve above the fixed threshold before the completion of 40 Cycles.

The general goal of this study is to provide an accurate prevalence of *Salmonella* in cattle at the slaughtering step of the meat chain as a part of a large-scale epidemiologic study aimed to develop quantitative risk assessment model of salmonellosis linked to the consumption of beef. The identification of false-negative culture samples by the real time PCR in this study may indicate that the estimated prevalence rate is underestimated.

Conclusion

The detection of *Salmonella* in cattle feces presenting at abattoirs in Tripoli city in this study can increase the chance of carcass contamination and poses significant threat to the consumer health. To reduce the risk represented by zoonotic agents such as *Salmonella* to consumer health, it is essential to reduce the contamination of carcasses during the slaughtering process. Therefore, the maintenance of slaughter hygiene is of central importance in meat production which can be measured by microbiological monitoring of carcasses according to Hazard Analysis Critical Control Point principles (HACCP). In addition, the results of this study re-confirm the use of PCR as a sensitive and rapid technique for *Salmonella* detection in cattle fecal sample.

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معدل انتشار وعوامل اختطار السالمونيلا في الأبقار عند الذبح بمسالخ مدينة طرابلس، ليبيا

ليلى محمد الشريك، عماد الهادي البوعيشي، المبروك رمضان فارس

قسم الطب الوقائي- كلية الطب البيطري– جامعة طرابلس

المستخلص

الامراض المنقولة عن طريق الغذاء والتي تسببها السالمونيلا هي من أهم مشاكل الصحة العامة في جميع أنحاء العالم. الهدف من هذا البحث هو تحديد معدل انتشار وعوامل الاختطار المرتبطة بمعدل انتشار السالمونيلا في براز الأبقار أثناء عملية الذبح بمسالخ مدينة طرابلس. من مجموع 353 عينة براز تم تجميعها، 15 (4.25٪) كانت إيجابية للسالمونيلا. النتائج الاحصائية أظهرت ان مصدر الحيوانات ومسافة الشحن لهما تأثير معنوي على معدل انتشار السالمونيلا في براز الماشية قبل الذبح (P >0.05). تؤكد النتائج بالدراسة الحالية وجود السالمونيلا في براز الأبقار عند الذبح في مسالخ مدينة طرابلس، ليبيا والتي قد تشكل خطرا كبيرا على سلامة الأغذية. اضافة إلى ذلك، أثبت اختبار تفاعل البلمرة المتسلسل (PCR) في الدراسة أنه اختبار سريع وحساس للكشف عن السالمونيلا في براز الأبقار.

الكلمات الدالة: السالمونيلا، براز الأبقار، اختبارات الزراعة المخبرية ، تفاعل البلمرة المتسلسل ، طرابلس، ليبيا.

للاتصال: المبروك فارس. قسم الطب الوقائي، كلية الطب البيطري، جامعة طرابلس.

هاتف: 926457288 218 + **البريد الالكتروني:** a.fares@uot.edu.ly

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