Lymph nodes and ground beef as public health importance reservoirs of *Salmonella* spp.

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Abstract:

This study aimed to determine the frequency of contamination, serovar diversity, and multilocus sequence typing (MLST) of *Salmonella enterica* (SE) in lymph nodes and ground beef. A total of 1,545 samples from 400 beef carcasses were analyzed. Samples included peripheral (PLN) and deep lymph nodes (DLN), lean and fatty ground beef obtained in warm (April-July) and cold (September-December) seasons during 2017 and 2018. The pure isolates were subjected to complete genome sequencing. With these data, the *in silico* prediction of serovars and the MLST profile was performed. In total, 78 SE isolates were obtained (5 % of the total analyzed samples). The frequency of contamination was associated with the type of sample ($\chi2=23.7$, *P*<0.0001) and the time of year ($\chi2=20.3$, *P*<0.0001), being higher in PLN (9.7%) and during the warm season (7.0%). The predominant serovars were Anatum and Reading (each one with n= 23), Typhimurium (n= 11), and London (n= 9). The MLST profile of strains of the Typhimurium (ST 19 and 34) and Kentucky (ST 198) serovars has been previously reported in isolates involved in clinical cases. It was concluded that lymph nodes and ground beef are reservoirs of SE of public health importance, especially during the warm months of the year. Therefore, it is necessary to establish measures to prevent dissemination throughout the production chain of strains associated with apparently healthy animals.

Key words: *Salmonella*, Cattle, Lymph nodes, Ground beef, Serovars, MLST.

Introduction

Foodborne salmonellosis is a public health concern worldwide[1]. The meat of different species, including beef, functions as a reservoir for its primary etiologic agent: *Salmonella enterica* subsp. *enterica*, from now on referred to as *Salmonella*[2]. In North America, ground beef has been linked to recent salmonellosis outbreaks[3], which is why it is considered one of the main vehicles of human exposure to *Salmonella*. In Mexico, percentages of positive samples range between 16 and 68 % in ground beef at points of sale[4,5], which is why research in this area is relevant from a public health perspective.

Recent experimental data report *Salmonella* isolates from apparently healthy cattle lymph nodes, in frequencies that range from <10 to >90%[6,7]. Furthermore, it has been proven that peripheral lymph nodes show a higher contamination rate as compared to deep lymph nodes, while the number of animals with contaminated lymph nodes is much higher in commercial feedlot cattle than in culled cattle[7,8]. However, results tend to vary significantly across geographical areas and season of the year, a phenomenon determined by unknown mechanisms.

In studies with *Salmonella* strains obtained from culled cattle, the typification of isolates by pulsed field gel electrophoresis showed clonality between lymph node and ground beef strains[9]. However, this type of study has not been performed in commercial feedlot animals.

Despite the high rates of positivity to *Salmonella* reported in bovine samples in Mexico[4-6], the contribution of lymph nodes to this phenomenon has not been addressed. Therefore, this study aimed to estimate the frequency of contamination and the diversity of *Salmonella* serovars in lymph nodes and the meat and fat associated with them at different seasons of the year.

Material and methods

Study design and sample size determination

The sample size was calculated with the statistical equation used to estimate a population proportion when the number of elements in that population is unknown[10]:

$$n = \frac{Z_\alpha^2 * p * q}{d^2} \quad ;$$

Where: n= sample size; Z_α^2= Z value in a normal distribution Z_α= 1.96 when α= 0.05; p= population proportion with the studied characteristic (if unknown, 0.5 is used, as in this case); q= population proportion without the studied characteristic (1-p); d= desired error or precision, fixed at 10% (0.1).

Thus, it was obtained a sample size of 96, which was rounded to 100. The sampling was performed twice a year for two consecutive years, and in two seasons of each year. The samples collected between April and July were labeled as "warm" season samples, and those collected between September and December were labeled as "cold" season samples.

Carcasses came from young bulls, crosses of *Bos Indicus*, with an average age of 24-36 months, processed in a Federal Inspection type slaughterhouse in Veracruz, and transported under refrigeration ($\leq$4 ºC) for approximately eight hours, until they arrived at a selling point in Mexico City. Upon arrival, carcasses were kept under refrigeration for two days until sample collection (72 to 96 h postmortem). The sale point was visited each week on Monday and Tuesday until completing between five and ten carcasses per week, depending on the number of carcasses available.

Sampling

Peripheral (PLN, superficial cervical and subiliac) and deep lymph nodes (DLN, axillary and celiac) were collected from each carcass. Lymph nodes were selected based on the probability that they were included in the grinding process, due to their anatomical location. In addition to the lymph nodes, approximately 200 g of lean meat (LM, 50 % of the chuck roll and 50 % of the sirloin, as they are the most used cuts to produce ground beef) and fatty meat (FM) were collected from the surrounding areas of the PLN and DLN (approximately 50 % of each). Before analysis, the individual portions of each sample type were combined to form a single sample. On some occasions, certain parts of the carcass were compromised for sale and were not available for sampling. Therefore, it was not possible to obtain all sample types of 100% of the carcasses. Thus, the sampling unit was defined as the sample composites of PLN, DLN, LM, and FM. A total of 1,545 samples were collected from all sources in the two years of the study.

Table 1: Distribution of the 1,545 meat and lymph node samples analyzed by season and year between April 3, 2017 and December 14, 2018

Sample type	Warm season			Cold season		
	2017	**2018**	**Total**	**2017**	**2018**	**Total**
PLN	168	98	266	33	102	135
DLN	166	98	264	33	102	135
LM	130	98	228	33	102	135
FM	149	98	247	33	102	135
Total			1,005			540

Warm season: April-July, cold season: September-December.
PLN= peripheral lymph nodes, DLN= deep lymph nodes, LM= lean meat, FM= fatty meat.

The individual portions of each sample type were placed in previously identified sterile plastic bags and kept in coolers with cooling gels (at approximately 4°C) during their transportation to the laboratory (maximum two hours).

Microbiological analysis

Lymph node samples were prepared following the methods previously described[11], with some modifications. Lymph nodes were weighed and subsequently submerged in boiling water for 5 s to sterilize their surface. Then, half of the buffered peptone water (BPW) necessary to reach an approximate 1:10 dilution (8 g of DLN in 80 ml of BPW and 25 g of PLN in 225 ml of BPW) was added, and lymph nodes were ground for 3 s in a previously sterilized Oster blender. The ground samples were emptied in a previously identified Stomacher® bag, and, using the rest of the BPW, the remainder contained in the blender was recuperated, assuring the transfer of the whole sample to the Stomacher® bag, subsequently homogenizing the mixture for 1 min.

For the analysis of the lean meat (LM) samples, 25 g were ground in a sterile Oster blender for 30 s. Subsequently, the content was placed in a previously identified Stomacher® bag with 225 ml of BPW, and the mixture was homogenized for 1 min. Finally, fatty meat (FM) samples were ground in a sterile Oster blender for 30 s, approximately 1/3 of fat and 2/3 of meat from the surrounding areas of PLN and DLN (50 % from each type of lymph node). After grinding, 25 g were aseptically weighed and subjected to the same procedure described for lean meat.

Homogenates were left to rest for two hours at room temperature, before following the pre-enrichment, selective enrichment, isolation, and biochemical confirmation procedures for *Salmonella* spp., established in the current Official Mexican Standard[12]. According to previously described methods, presumptive positive *Salmonella* spp. isolates were also molecularly confirmed by PCR using the *invA* gene (284 bp)[13]. DNA was extracted with the Ez-10 Spin Column Bacterial Genomic DNA Miniprep Kit (BioBasic, Inc., Canada), following the instructions of the supplier, from pure strains, previously refreshed in tryptic soy broth (MCD Lab®, PRONADISA-CONDA®, Spain) for 18-24 h. Forward (CGCCATGGTATGGATTTGTC) and reverse (GTGGTAAAGCTCATCAAGCG) primers were used in PCR with a total volume of 10 μl, employing the MyTaq^{TM} Mix reagents (Bioline, U. K.) with the following final concentrations: 5 μl of MyTaq^{TM} Mix, 0.2 μl of each dNTP, and 2.1 μl of nuclease-free water. The thermocycling conditions were: 94 ºC/3 min of initial denaturation; 35 denaturation, annealing, and extension cycles (95 ºC/45 s, 62 ºC/30 s, 72ºC/45 s, respectively), and a final extension at 72 ºC/2 min. The PCR amplified products were subjected to a 2% agarose gel electrophoresis (SeaKem® LE

Agarose, Lonza, USA). Gels were run in a Tris/borate/EDTA buffer (TBE 1x) at 80 V for 50 min using SYBR Safe DNA Gel Stain (Invitrogen, USA) to reveal the DNA fragments. The visualization and digitization of images were performed in a Gel Logic 2200 imaging system (Kodak, USA) with the Care Stream® software (Carestream Health, Inc., USA). In each run, it was included a strain, from the laboratory, of *S. enterica* subsp. *enterica* ser. Typhimurium, previously confirmed by biochemical methods, PCR, and whole-genome sequencing. Confirmed isolates were preserved in two ways. In the first one, 1 ml inocula were prepared by taking fresh colonies and mixing them in brain-heart infusion broth (Merck, Germany) with 10% glycerol and kept at -70 °C in an ultra-low freezer. Moreover, a backup of the isolates was kept in tryptic soy agar (TSA, PRONADISA-CONDA®, Spain) at room temperature.

Serovar prediction and multilocus sequence typing (MLST)

The serovar of the obtained strains was predicted from the whole genome sequencing data (raw reads). Genomic DNA was extracted from fresh colonies in TSA broth with agitation at 37 °C for approximately 18 h. Then, it was centrifuged 1 ml of TSA broth at 5,000 xg for 10 min to obtain a cell pellet. Subsequently, following the instructions provided by the manufacturer, it was used the High Pure PCR Template Preparation Kit (Roche Molecular Systems, Inc., Switzerland) to obtain the genomic DNA.

Sequencing was performed in an Illumina NextSeq (Illumina, USA) equipment, using the Nextera XT version 3 kit (Illumina, USA) to prepare the DNA library, with an insert of 150 bp and a minimum estimated depth of 30X. The obtained raw reads were used to predict the serovar through *in silico* analysis, with the help of the SeqSero program[14]. Finally, a multilocus sequence typing (MLST) analysis was performed, based on seven house-keeping genes (*aroC, dnaN, hemD, hisD, purE, sucA*)[15], in the server of the Center for Genomic Epidemiology[16]. As MLST has been used for decades and there is a public access database[17], it is possible to estimate the epidemiological importance of the isolates through comparison with the ST previously reported in human and animal clinical samples. Furthermore, the allele profile was used to create a minimum spanning phylogenetic tree, using the GrapeTree[18] program, to analyze the ST diversity in the sample under study.

Statistical analysis

To determine if there was an association between the type of sample, the season of year, and the *Salmonella* serovar with the frequency of contamination, it was employed a chi-square test. If a significant association was observed, the odds ratio was used to estimate the factors with the greatest influence on the contamination rate of the different studied matrices. Data were analyzed using the Statgraphics Centurion XV program (StatPoint, Technologies, USA).

Results

Overall, it was observed a 5% *Salmonella* spp. contamination frequency, with 78 isolates obtained from the 1,545 samples analyzed in the two years (Figure 1). A strong association between the sample type and the pathogen positivity was observed ($\chi^2=23.7$, *P*<0.0001), with a higher probability of finding positive samples in PLN than in other sources (odds ratio 3.2, 95 % confidence interval 2.0-5.0, *P*<0.0001).

Figure 1: Frequency of contamination with *Salmonella* spp. in bovine samples of lean meat (LM, n=363), fatty meat (FM, n=382), deep lymph nodes (DLN, n=399), and peripheral lymph nodes (PLN, n=401), collected between April 2017 and December 2018
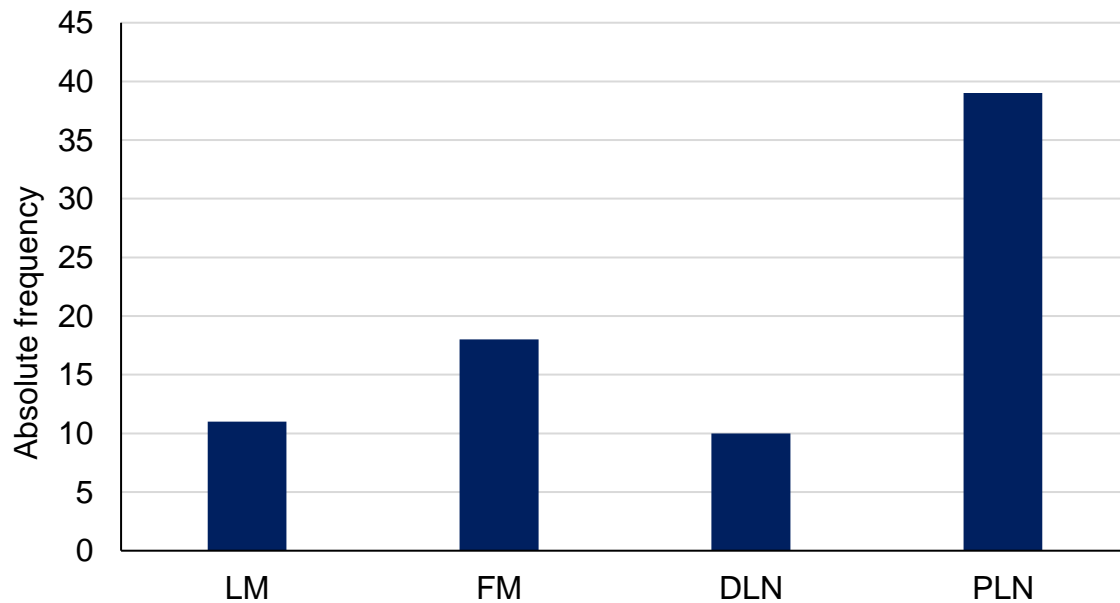
There was also a significant association between the frequency of contamination and the season of year (χ^2=20.3, *P*<0.0001). The probability of finding positive samples in the warm season was much higher than in the cold season (odds ratio 4.7, 95 % confidence interval 2.2-9.8, *P*<0.0001) (Figure 2).

Figure 2: Frequency of contamination with *Salmonella* spp. in bovine samples of lean meat (LM, n=363), fatty meat (FM, n=382), deep lymph nodes (DLN, n=399), and peripheral lymph nodes (PLN, n=401), collected between April 2017 and December 2018
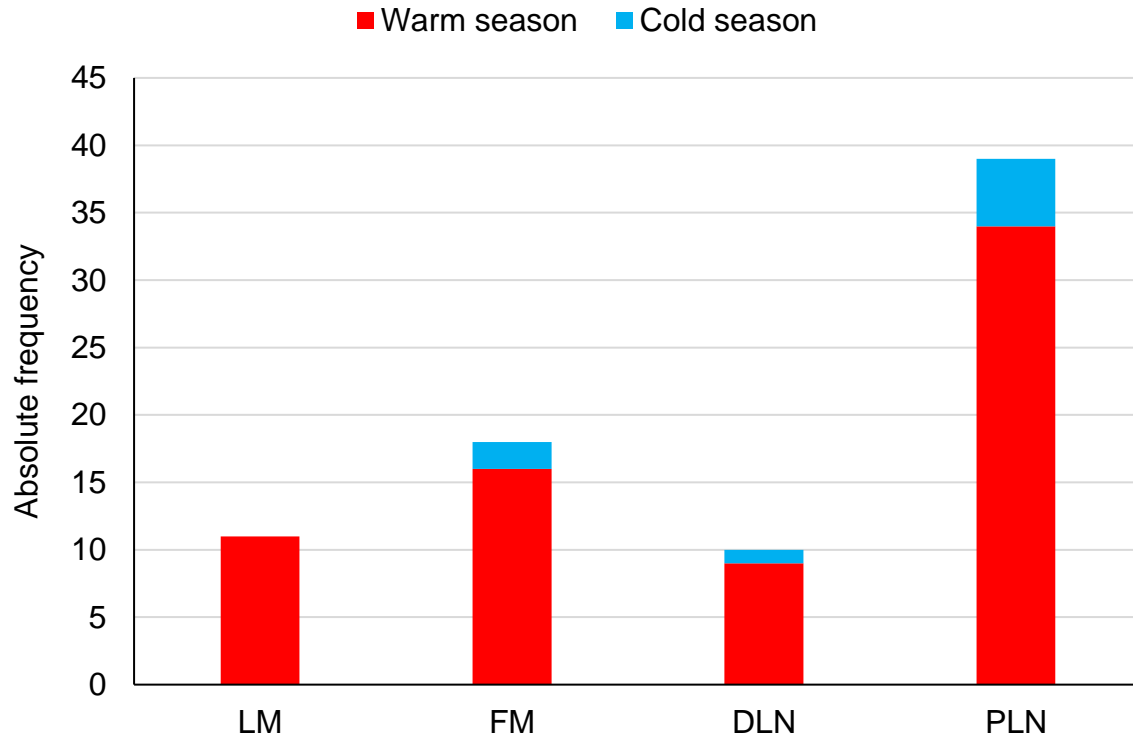
The serovar was also associated with the sample type (χ^2=43.8, *P*=0.0025). *Salmonella typhimurium* was only detected in meat samples from the warm season. However, the Muenster (n= 2) and Kentucky (n= 5) serovars were only found in lymph nodes, also from the warm season (Figure 3). Furthermore, the only strain of the Give serovar was isolated from PLN in the cold season. Although serovar diversity was higher in both types of lymph nodes than in the lean or fatty meat, in general, strains from the Reading and Anatum (n= 23 of each), Typhimurium (n= 11), and London (n= 9) serovars were predominant.

Figure 3: Number of *S. enterica* subsp. *enterica* isolates by serovar and source in the warm (a) and cold (b) season. LM: lean meat (n=363), FM: fatty meat (n=382), DLN: deep lymph nodes (n=399), PLN: peripheral lymph nodes (n=401)
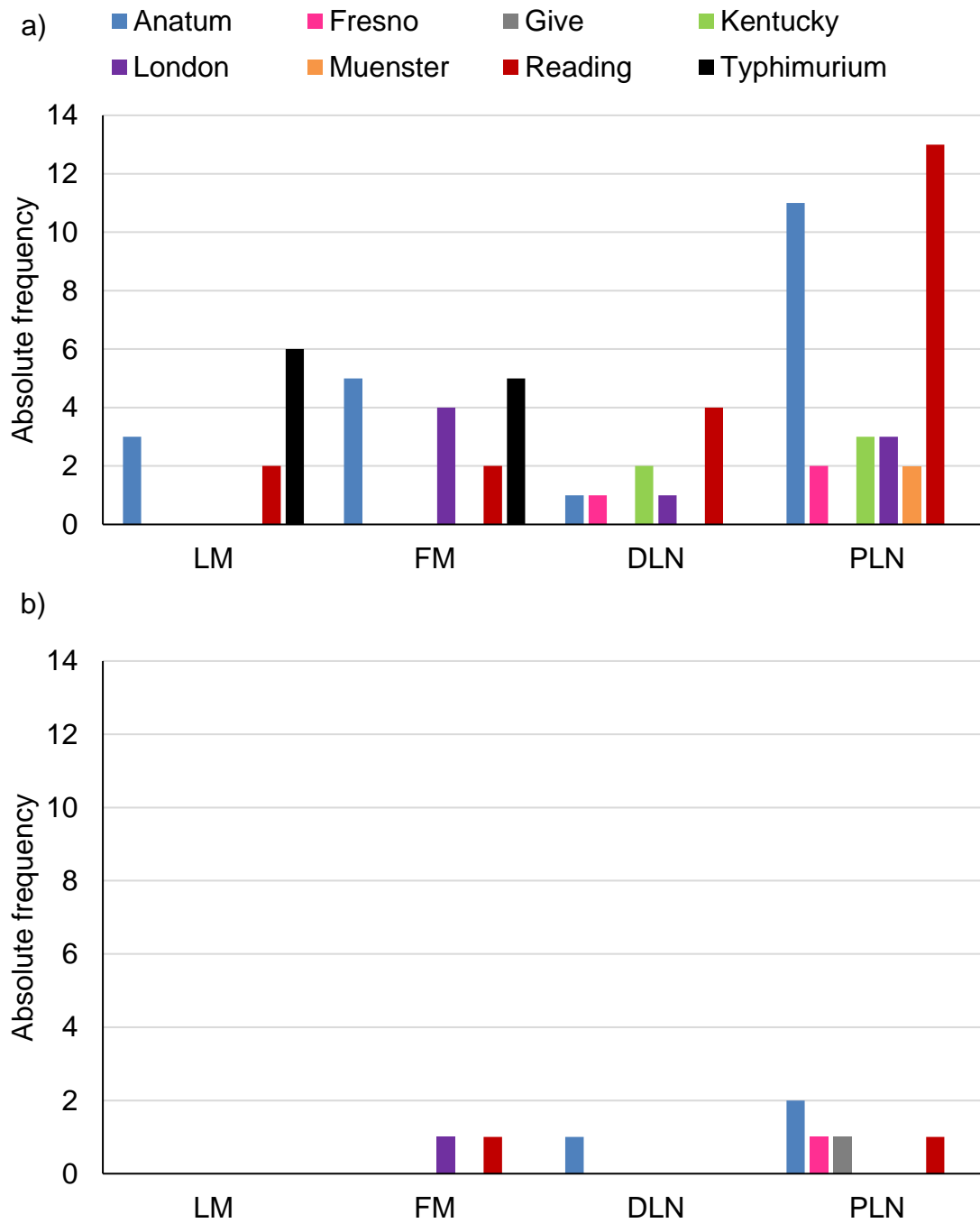
The MLST showed that the isolates of each serovar corresponded to the same ST (Figure 4). The exception was *Salmonella typhimurium*, which had two ST (19 and 34). However, both ST only differed in the *dnaN* allele. Therefore, they satisfy the criteria to be

considered a clonal complex, as they coincide in six of the seven alleles included in the MLST scheme[15].

Figure 4: Minimum spanning phylogenetic tree obtained from the MLST profile of 78 isolates of *S. enterica* subsp. *enterica*.
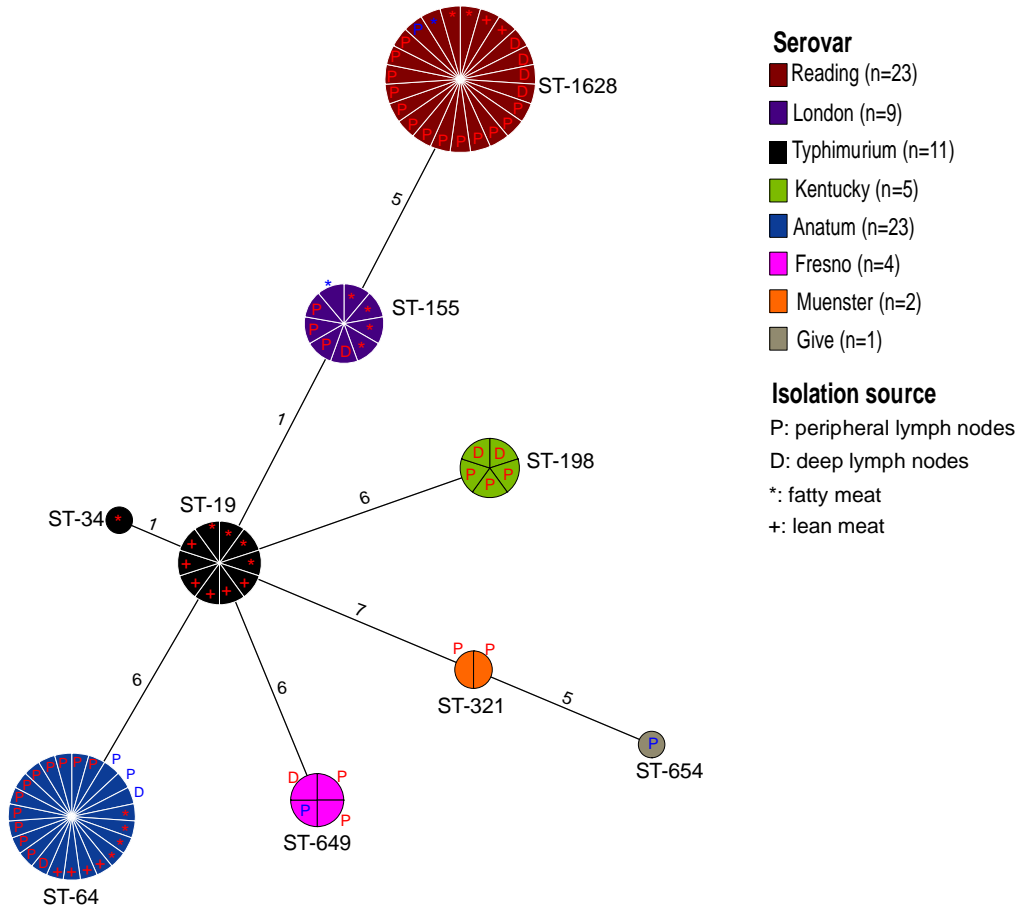
Each circle corresponds to a ST, and the divisions inside correspond to an isolate. The numbers in the tree branches indicate the number of alleles with different sequences between ST. Serovars are color-coded, and the source of isolation is indicated inside or adjacent to each circle (in red text, if they come from the warm season; or in blue, if they come from the cold season).

Discussion

The frequencies of positivity to the pathogen observed here (2.5 to 9.7 %) are lower than those reported in other studies with ground beef (16-68%)[4,5] and lymph nodes (50-

94 %)[6,19,20]. However, the variability of this phenomenon between geographical areas and season of the year is well documented[20,21]. Overall, the study confirms the importance of apparently healthy cattle as a reservoir of various *Salmonella* serovars of epidemiological importance. This is demonstrated by the detection of ST 19 and 34 of the Typhimurium serovar, which are associated with human clinical cases and with the globally distributed DT104 strain[22]. Similarly, isolates of the Kentucky serovar (ST 198) have been associated with human and animal infections in the United States[23]. These findings highlight the need to continue investigating *Salmonella* populations of non-clinical origin, associated with animal production, due to their role as a reservoir of human infections.

The results also support previous observations on the higher positivity rates to the pathogen in peripheral lymph nodes, especially in warm climate conditions[7,8]. Although the environmental factors responsible for this variability have not been deciphered, the higher incidence of flies and other insect bites during the summer has been suggested as a conditioning factor of this seasonal variation[19,24]. However, the scant experimental evidence related to this factor does not come from natural contexts but from challenge studies with flies artificially infected with *Salmonella*.

The efforts made so far to prevent asymptomatic *Salmonella* infection in cattle have been unsuccessful. The use of vaccines based on genes involved in the uptake of iron, a mineral with a central role in the infectious process, had no effect on the frequency of contamination in the lymph nodes of fattening cattle[25]. This is not an unexpected finding, considering the functional redundancy of *Salmonella*, which has multiple genes for the uptake and transport of iron (*iroBCDE, fepBCDEG, fhuBCD, exbBD, sitD,* and *tonB*)[26].

Moreover, the intracellular survival of the bacterium, internalized in eukaryotic cells vacuoles[27], such as macrophages, suggests that antibiotics are an unlikely strategy. Thus, the administration of increasing concentrations of tylosin in the diet of Holstein cattle, previously inoculated with the pathogen, did not show any effect, as *Salmonella* was still recovered from the lymph nodes of treated animals[28].

Apparently, the functional redundancy of *Salmonella* and its intracellular survival mechanisms indicate that the eventual pathogen elimination will ultimately depend on the immune system of the host. In animals experimentally inoculated with strains of the Montevideo serovar, the total elimination of the bacteria took about a month[29]. In this context, the screening of *Salmonella* subclinical infections in feedlots, a poorly applied measure, could function as a method to segregate carrier animals and limit the spread of the pathogen. Additionally, the presence of strains of the same serovar and ST in ground beef and lymph nodes, observed in this study, suggests removal of lymph nodes could be a good strategy to drastically reduce the frequency of contamination with *Salmonella* in ground beef. This measure is relatively easy to perform at slaughterhouses, although only for

peripheral lymph nodes, not for the deep lymph nodes. However, it is precisely the peripheral lymph nodes that are of the greatest epidemiological relevance. Therefore, establishing this measure as mandatory in national regulations could function as a strategy to mitigate the risks associated with the presence of *Salmonella* in ground beef.

Moreover, it is interesting to analyze why some serovars were only present in meat samples (e.g., Typhimurium), while others were detected in all matrices (e.g., Anatum and Reading). Notably, the Anatum serovar was previously reported as a predominant strain in non-clinical samples, especially in lymph nodes[19,20]. These evidences suggest the possibility that some *Salmonella* strains are better adapted to colonize and survive in particular ecological niches. However, in the context of the present study, it is difficult to determine whether the relative representation of serovars in lymph nodes depends on specific genetic factors. It is also necessary to use analyses with greater discriminatory power than MLST to explore more precisely the intra- and interserovar phylogenetic relationships, and the evolutionary dynamics of these populations. This will be the focus of future contributions in the comparative genomics field.

Conclusions and implications

The study shows that the lymph nodes and ground beef from animals approved for slaughter are reservoirs of *Salmonella enterica* strains of clinical importance in humans. Therefore, it is necessary to establish control measures to prevent the spread of this pathogen throughout the production chain.

Acknowledgments and conflicts of interest

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