Original Research Article

Differentiation of Acute and Latent Salmonella infection in Cattle Using the Glyoxylate Pathway Gene *aceA*, Flagellar Gene *fimA*, and an attempt at validation by bacterial culture

ABSTRACT

Aims: To test the hypothesis that it is possible to detect latently Salmonella infected cattle using a glyoxylate pathway gene as a qPCR target.
Study design: Convenience sample of tissue specimens.
Place and Duration of Study: Department of Biomedical Sciences and Pathobiology, Virginia Maryland College of Veterinary Medicine, between April 2015 and July 2017.
Methodology: Supra-mammary lymph nodes from 40 dairy cattle and mesenteric lymph nodes from 100 cattle from a slaughter house were collected, sampled, and bisected. One half of each sample was cultured on *Salmonella* chromogenic agar, while the other half was tested using qPCR for both an acute infection associated gene (*fimA*) and a chronic/latent infection associated gene (*aceA*). The *Salmonella* culture isolation results were compared with the qPCR results for the above two genes.
Results: All 40 dairy cattle lymph nodes were qPCR negative for both fimA and aceA, and none of the lymph nodes were culture positive. From the 100 cattle mesenteric lymph nodes, 11 were qPCR positive for aceA, none were qPCR positive for fimA, and 7 were culture positive for Salmonella isolation. Of those 11 aceA qPCR positive, only 5 of them were culture positive and six were culture negative.
Conclusion: The qPCR results for mesenteric lymph nodes showed high specificity and predictive value negative. The results between qPCR and bacterial culture in supra-mammary lymph nodes may suggest that the number of *Salmonella* in the sample cultured may be below detection limit for both the assays. But the results demonstrate the advantage of using gene(s) primers to identify latent *Salmonella* infections in clinically normal cattle from slaughter house samples. In addition, the assay may be able to differentiate between latent vs active *Salmonella* infection. The sample size might be the reason for the lack of results in the case of the dairy cattle samples.

Keywords: Salmonella, latent infection, cattle

1. INTRODUCTION

Salmonellosis has classically been a problem in the intensively cultivated/raised cattle, poultry, swine, and has been identified as an increasing problem in feedlot beef cattle as well. One of the factors that allow *Salmonella* to thrive on a farm is the widespread environmental contamination resulting from *Salmonella* shedding, contaminated feed and the organism's ability to survive for prolonged periods in suitable conditions outside the host

[1]. According to the National Animal Health Monitoring System, 52.6% of sampled swine farms had positive fecal cultures for *Salmonella* [2]. A study found that in cattle dairy farms, 24.7% of the bulk tank milk filters were positive for *Salmonella*, 10.8% of bulk tank milk were RT-PCR positive, and 39.7% of dairy cows were fecal culture positive for *Salmonella* [3]. In addition, being linked to human disease outbreaks, dairies affected with salmonellosis have increased mortality, treatment costs, reduced milk yield, weight loss. Therefore, regulatory and insurance costs may bankrupt such dairies [4]. Additionally, antimicrobial resistance is on the rise in such *Salmonella* strains, further clouding the diagnostic and treatment picture. In case of swine farms sampled, 57.7% of *Salmonella* isolates were resistant to three or more antimicrobials [2], and 17% of dairy cattle isolates were resistant to at least one antimicrobial drug [3]. This emerging resistance has heightened the need for accurate diagnostics, to develop effective control and eradication measures, for developing effective treatment protocols, to identify latent carriers to prevent transmission and outbreaks in humans.

There is increasing evidence that *Salmonella* species may reside/persist within lymph nodes of cattle [5-8]. Research on the ecology of *Salmonella* serovars within mandibular, mesenteric, mediastinal, and subiliac lymph nodes from Mexican cattle presented for harvest at an abattoir revealed varying prevalence within lymph node anatomic location, ranging from 91.2% in mesenteric to 7.4% in mediastinal lymph nodes [9]. Of these *Salmonella* isolates, 8 different serovars were identified [9]. This variety of *Salmonella* serovars and the infected lymph node distributions makes identifying latent carriers even more important for control and potentially eradicating one of important sources of zoonotic infection of human beings from animals.

On dairy farms, outbreaks typically last for several months because of many factors, including carrier animals, environmental persistence, risk factor persistence, and reinfection. *Salmonella* has primarily been introduced into dairies via contaminated feed. Under appropriate moisture, temperature, and pH conditions, *Salmonella* species can replicate every 30 minutes [10]. In the environment, *Salmonella* can persist for up to 5 years in areas that are out of direct sunlight or within feed. *S. dublin* can survive in dry feces for more than a year. While rendering kills *Salmonella*, post processing contamination is common; it accounts for 50% of contaminated rendered feed products [11]. Aside from environmental contamination, carrier animals are also a significant source of cross contamination on a dairy. Chronically affected carriers can shed 10^8 to 10^9 bacteria per day in feces, and 10^2 to 10^5 organisms per mL of milk. These levels are alarming because the infectious dose in cattle is approximately 10^9 organisms [11]. With the increasing popularity of raw milk consumption this is even more important source of human infection.

The need to address shedding in cattle as a source of nontyphoidal *Salmonella* (NTS) infections in people has become increasingly more urgent [15]. Recently, researchers have described co-infections of human immunodeficiency virus (HIV) and NTS that disseminate beyond the gastrointestinal tract and cause septicemia [16]. Another trend is the emergence of multidrug-resistant (MDR) strains of both typhoid fever and NTS. The first line treatment for typhoid fever in humans consists of fluoroquinolones like nalidixic acid and ciprofloxacin [17], which are not permitted for extra-label use in food animals and are thus off limits for treating cattle. Even if those drugs were available to veterinarians for treating diarrheal disease, fluoroquinolone resistance has been reported [18]. In addition to affecting human health, *Salmonella* can spread rapidly within a herd. In an experimental evaluation of transmission within a herd, deliberately infected calves were released into a herd. These seeder calves were able to transmit S. *enterica* subsp. *enterica* serotype Typhimurium to up to 80% of naïve calves within one week. Of those newly infected calves, 23% became asymptomatic carriers [19]. Being able to identify carriers of *Salmonella* in livestock may

allow public health professionals to decrease the prevalence of infection, interrupt the transmission, and improve the health of both animals and humans.

Treating symptomatic cattle is an expensive and impractical prospect for dairies. Supportive care and prudent antimicrobial use are often necessary for up to 6 days, and even with such treatment prognosis is poor [12]. It is also challenging to implement preventive strategies into a dairy herd after an outbreak. The current standard is that at least 20% of the population must have their feces cultured, and these cultures must be performed for 3 to 6 months in order to distinguish recovering animals from latent carriers [10]. PCR based testing is available, but it requires the same timeframe as culturing and is more expensive. The primary strategy for salmonellosis in dairies is focused on prevention. Using "all in – all out" systems, maintaining a closed herd, minimizing new additions, separating calving cows and sick cows, separating heifers and calves from cows, restricting access to pond water, and disinfecting waterers with bleach twice daily are all effective methods to decrease *Salmonella* introduction. But with these measures, incidence of salmonellosis in animals and humans have not been dramatically reduced, and there are opportunities for further reduction.

This research aims to detect the presence of *Salmonella* within cattle lymph nodes and to determine the prevalence of *Salmonella* within populations of cattle using two tests: culture isolation and quantitative PCR. During chronic infections, however, the bacteria shifts priorities from growth to survival. The glyoxylate shunt is a mechanism that allows bacteria to do just that. The glyoxylate shunt is a bypass of the Krebs cycle that permits gluconeogenesis starting from acetyl-CoA following fatty acid catabolism. It avoids the CO_2 generating step of the TCA cycle and converts one molecule of acetyl-CoA and one molecule of isocitrate into two C_4 compounds that can be used for biosynthesis. This contrasts with acute infection, where activities such as fimbriae synthesis are prioritized. Isocitrate lyase is one of the glyoxylate cycle enzymes and is encoded by the *aceA* gene, while the *fimA* gene codes for a major subunit of *Salmonella* type 1 fimbriae. Therefore, the two targets for qPCR were used: *aceA* to identify latent infections due nonreplicating *Salmonella*, and *fimA* to identify any acute infections due to actively replicating *Salmonella*. Lymph nodes from two different population of cattle and two different anatomic locations were tested. The first population consisted of supra-mammary lymph nodes from dairy cattle collected in Tennessee. The second population tested was mesenteric lymph nodes that were collected from cattle that were presented to an abattoir in South Carolina. This research will also help assess the utility of using *aceA* as a tool to identify latent carriers of *Salmonella*.

2. MATERIAL AND METHODS

Supra-mammary lymph nodes from 40 dairy cattle were obtained from a processing facility in Bean Station, Tennessee. One lymph node was dissected from each animal, giving a total of 40 lymph nodes from 40 individual dairy cattle. Similarly, the same methodology was used to collect mesenteric lymph nodes from 100 cattle obtained from a packing company in Gaffney, South Carolina.

The individual lymph nodes were placed in RNALater (Qiagen), a RNA storage and stabilizing solution, and placed on dry ice. Later that same day, the lymph nodes were bisected. The samples were appropriately catalogued so that it was known which two halves were from the same animal's lymph node.
To culture for *Salmonella*, one section of the bisected lymph nodes was placed in Selenite broth (Hardy Diagnostics), which reduces fecal coliform growth and selectively enriches for *Salmonella* species [13], and incubated at 37° C for 16 hours. After incubation, the enriched

samples were plated on HardyChrome agar (Hardy Diagnostics). Growth of non-*Salmonella* species result in gray or blue colonies. Hydrolysis of Magenta-caprylate by lactose negative *Salmonella* species results in magenta colonies [13].

The remaining sections of lymph nodes preserved in RNALater were used for qPCR. The lymph nodes were thawed, then disrupted using a roto-stator tissue homogenizer, and total RNA was isolated from the lysate using an RNeasy Mini Kit (Qiagen). The total RNA was then converted to single strand cDNA using a QuantiTect reverse Transcription Kit (Qiagen). Primers for *fimA* for acute infections and *aceA* for chronic infections were designed using Primer3Plus software (Table 1 showing forward and reverse primers for *fim*A and *ace*A). The qPCR was performed using SYBR Green and included housekeeping genes for both *Salmonella* (*rsmC*) and *Bos Taurus* (*GAPDH*). The primers for *aceA* and *fimA* were run through a Basic Local Alignment Search Tool (BLAST) to ensure there was no homology with other host or pathogen sequences. The qPCR mixture included 12.5 µL of SYBR Green PCR Master Mix reagent (Applied Biosystems), 0.5 µL of 10 µM of each primer, 10 µL of RNAse-free water, and 5 µL of DNA in a final volume of 25 µL. qPCR was performed in a Bio-Rad iCycler thermocycler (SABiosciences) with an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C.

3. RESULTS AND DISCUSSION

3.1 Supra-mammary lymph nodes in dairy cattle

On culturing on the chromogenic agar showed that none of the lymph nodes were culture positive for *Salmonella.* After standardization, none of the lymph nodes were positive for *Salmonella* via qPCR for *aceA* or *fimA* (0%) (Table 1).

None of the supra-mammary lymph nodes were positive by either culture or in qPCR with gene specific primers. This particular processing facility was located in central Tennessee, and there was no information on the prevalence of *Salmonella* in the cattle that fed into that facility. The absence of qPCR false positives indicates that the qPCR may have a high specificity for detecting *Salmonella*. This failure to detect could be due to a low relative number of Salmonella present in the samples that is below the detection limit of the tests, or a true absence of Salmonella in the lymph nodes selected. Repeating this experiment with a larger sample size would provide further information.

Table 1. Results of bacterial culture, qPCR *aceA*, and qPCR *fimA* tests for 40 individual dairy bovine supra-mammary lymph nodes.

Test result (n=40)	Culture	aceA	fimA
Positive	0	0	0
Negative	40	40	40

All 40 of the supra-mammary lymph nodes from dairy cattle were negative for *Salmonella* by culture, *aceA* qPCR, and *fimA* qPCR.

3.2 Mesenteric lymph nodes from beef cattle

On culturing the 100 mesenteric lymph node samples from slaughter house cattle on the chromogenic agar, 7 lymph nodes were culture positive for *Salmonella* and 93 were culture negative (7% positive) (Table 2). A positive culture result using chromogenic agar was

marked by a magenta colored colony, while a negative culture result was indicated by a gray-blue colored colony. After standardization, 11 of the lymph nodes were positive for *Salmonella* via qPCR for *aceA* (11%), and 0 lymph nodes were positive for *fimA* (0%) (Table 2). Of the 7 culture positive lymph nodes, 5 of them were also *aceA* PCR positive. There were 6 lymph nodes that were positive on *aceA* qPCR that were culture negative (Table 2).

Table 2. Results of bacterial culture, qPCR *aceA*, and qPCR *fimA* tests for 100 individual beef bovine mesenteric lymph nodes.

Test result (n=100)	Culture	aceA	fimA
Positive	7	11	0
Negative	93	89	100

In the beef cattle mesenteric lymph nodes, 7 samples were positive for *Salmonella* based on culture, 11 were positive based on *aceA* qPCR, and 0 were positive based on *fimA* qPCR.

While evaluating the qPCR results, it is important to first note that none of the lymph nodes tested positive on the qPCR for *fimA* alone. That indicates that none of the cattle were acutely infected with *Salmonella* (i.e., suffering from salmonellosis) when they were presented to the packing facility. These qPCR results are consistent with the environment from which the samples were collected; visibly sick or clinically affected animals might have been sent to be culled instead of slaughtered. It is entirely plausible that none of the animals presented to this slaughterhouse would have an active Salmonella infection.

When comparing the culture results to the *aceA* qPCR results, there are consistencies and discrepancies between the two tests. Of the 7 culture positive samples, only 5 of them were also *aceA* qPCR positive. Given that it appears likely that latently infected cattle harbor *Salmonella* bacteria within their lymph nodes [5-6], it is entirely reasonable that these culture positive and qPCR *aceA* positive samples represent cattle that are latently infected with *Salmonella*. Additionally, there were 2 samples that were culture positive and *aceA* qPCR negative, and 6 samples that were culture negative and *aceA* qPCR positive. Since the aceA primer was run through an algorithm designed to ensure that the sequence is unique to this particular gene, it is likely that the 6 culture negative *aceA* qPCR positive samples represent latent *Salmonella* infections that were not identified via culture. One possible explanation for this discrepancy is that *Salmonella* is not thought to be uniformly distributed throughout the lymph node [12]; these disconcordant samples may represent latently infected cattle whose lymph node sample did not contain viable bacteria for culture, but the bacterial DNA was present. It could also be due to presence of *Salmonella* at very low numbers (i.e., below detection limit for the culture assay). Previous studies found that *Salmonella* prevalence in subiliac lymph nodes of feedlot cattle was approximately 12% [11]. While the samples in this study are from a different anatomic location, the overall aceA qPCR prevalence (including culture+*aceA*+ and culture-*aceA*+ samples) is 11%, which is consistent with the aforementioned study.

By comparing the culture results with the *aceA* qPCR results (Table 3), it is possible to calculate a specificity, sensitivity, predictive value positive (PVP), and predictive value negative (PVN) for one test relative to the other. When comparing bacterial culture to the *fimA* and *aceA* qPCR, the culture has the following characteristics:

Sensitivity: 45.5% Specificity: 97.8% PVP: 71.4% PVN: 93.5%

The high specificity is consistent with what was noted in the supra-mammary lymph nodes as well, but these experiments need to be repeated with a larger sample size for further

validation. These results indicate that *aceA* qPCR may be able to detect latent *Salmonella* infections in mesenteric lymph nodes with more specificity than bacterial culture.

Table 3. Comparison of using enriched culture and qPCR for aceA for Salmonella detection in bovine mesenteric lymph nodes.

		qPCR *aceA*	
		Positive	Negative
Culture	Positive	7	11
	Negative	93	89

Comparing bacterial culture results to qPCR *aceA* results shows *aceA* qPCR to be highly specific (97.8%).

4. CONCLUSION

According to the Center for Food Safety and Public Health (CFSPH), biosecurity is the cornerstone of non-typhoidal Salmonella prevention on the farm. Part of that biosecurity is being able to identify carrier animals to be isolated and treated or culled [19]. Historically, identification of carrier animals has been limited to testing pooled fecal samples for the presence of bacteria or serological tests that detect antibodies against *Salmonella*. Culture is not always reliable because of the intermittent nature of shedding by latent carrier animals, and serology has limitations in individual animals because antibodies do not appear until two weeks after infection [19] and slowly but completely disappear after a certain period of time. There is a clear need for a better PCR-based diagnostic test to identify asymptomatic carriers within a herd, and these preliminary results indicate that *aceA* could serve as such a genetic target in qPCR based highly sensitive test.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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