Journal of Equine Veterinary Science 126 (2023) 104538

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Short Communication

Multi-Centered Field Evaluation of a *Salmonella* spp. Point-of-Care PCR Assay Using Equine Feces and Environmental Samples

Nicola Pusterla[a,*], Pramod Naranatt[b], Himani Swadia[b], Laramie Winfield[c], Ashley Hartwig[c], Samantha Barnum[a], Eric Mendonsa[b]

[a] *Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA*
[b] *Fluxergy, Irvine, CA*
[c] *Steinbeck and Peninsula Equine Clinics, Salinas, CA*

ARTICLE INFO

Article history:
Received 27 March 2023
Received in revised form 28 April 2023
Accepted 2 May 2023
Available online 5 May 2023

Keywords:
Point-of-care PCR
Salmonella spp
Environmental samples
Feces
Horses

ABSTRACT

The introduction of microfluidic card technology has opened the field for rapid point-of-care (POC) molecular assays, including fecal and environmental *Salmonella* spp. testing. The purpose of this study was to evaluate a novel POC PCR assay for the detection of *Salmonella* spp. in feces and environmental samples. A total of 143 fecal samples and 132 environmental samples were collected for POC PCR *Salmonella* spp. testing as well as qPCR testing. Each sample was inoculated into selenite broth and incubated for 18 to 24 hours. For the POC PCR assay, 14 μl of selenite broth were mixed with 126 μl of PCR reaction mix and pipetted into a microfluidic test card targeting the invA and ttrC gene of *Salmonella enterica.* For qPCR analysis, 200 µl of the selenite broth were processed for DNA purification and *Salmonella* spp. testing targeting the invA gene. The overall agreement between the POC PCR *Salmonella* spp. assay and qPCR assay was 88.1% for feces and 97.0% for environmental samples. Strong agreement and short turn-around-time make the POC device the first molecular diagnostic platform allowing detection of *Salmonella* spp. in a hospital setting without having to ship out samples to a veterinary diagnostic laboratory. The availability of an accurate POC PCR assay for the detection of *Salmonella* spp. will enhance the diagnostic capability of equine veterinarians to timely support a diagnosis of salmonellosis and also monitor the environment in order to reduce the risk of nosocomial infections.

1. Introduction

Salmonella spp. is an important enteric pathogen associated with colitis and subclinical infection in horses. It is well recognized that various factors often encountered among hospitalized horses, including stress of transportation, preexisting gastrointestinal diseases, changes in diet, use of antimicrobials, fasting, surgery and elevated ambient temperatures, can predispose horses to develop clinical salmonellosis [1–3]. Because of the contagious nature of *Salmonella* spp. and its zoonotic risk, the diagnostic challenge is to reach a timely shedding status in order to isolate infected horses and to institute appropriate biosecurity protocols, therefore, reducing the risk for environmental contamination and spread.

Salmonella spp. testing in large animal medicine is often done using the preferred method of the laboratory. Culture is considered the reference standard against which other tests or different culture protocols are compared. A range of culture techniques, including direct plating and various enrichment steps, are routinely used by veterinary microbiological laboratories. While the use of selective and enrichment steps prolongs the testing time, these steps are critical and have been repeatedly shown to increase the sensitivity of culture and molecular detection assays [4–8]. Modern molecular technologies for the detection of *Salmonella* spp. have become more commonplace in veterinary diagnostic laboratories, mainly because of shorter turn-around-time, greater diagnostic sensitivity and cost-effectiveness. Collectively, PCR assays for the detection of *Salmonella* spp. have shown a higher analytical sensitivity for the detection of *Salmonella* spp. in fecal and environmental samples compared to culture [9–15]. However, one of the drawbacks of molecular assays is the inability to distinguish viable *Salmonella* spp. organisms from nonviable cells or from free nucleic acids in biological samples.

Animal welfare/ethical statement: There were no animal welfare issues in the generation of the study data.

* Corresponding author at: Nicola Pusterla, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, CA 95616.
E-mail address: npusterla@ucdavis.edu (N. Pusterla).

https://doi.org/10.1016/j.jevs.2023.104538
0737-0806/

A timely detection of *Salmonella* spp. in feces and environmental samples is important from a diagnostic and biosecurity standpoint. Clinical laboratories generally require at least 48 hours for presumptive detection of *Salmonella* spp., without taking into account the time of sample shipment. The development of point-of-care (POC) diagnostics for human and animal infectious pathogens has made tremendous leaps, as recently experienced with the COVID-19 pandemic [16,17]. The introduction of microfluidic card technology coupled with highly integrated sensor systems, allows for the molecular detection of selected equine respiratory and enteric pathogens. The recent introduction by Fluxergy (Irvine, CA) of *Salmonella* spp. testing in environmental samples represents a marked improvement and increased commodity, since the expected turn-around-time for an individual sample is less than 24 hours, including sample incubation, enrichment step and PCR analysis. Therefore, the objective of this study was to evaluate a POC PCR assay for the detection of *Salmonella* spp. in equine feces and environmental samples.

2. Materials and Methods

2.1. Study Samples

For the purpose of this study, 143 equine fecal and 132 environmental samples were collected from two equine veterinary hospitals (Steinbeck and Peninsula Equine Clinics, Salinas, CA and The William R. Pritchard Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California, Davis, CA). Fecal samples were collected from equine patients as part of the hospitals' biosecurity program. The fecal samples were collected either from the rectum of the patient or from the ground following defecation and placed in a fecal cup. Feces (1 g) were placed in 10 mL conical tubes containing phosphate-buffer saline (PBS). The environmental samples were collected using a sponge and placed into a bag containing 10 mL of neutralizing buffer (3M Sponge Stick, Maplewood, MN). For each environmental sample, a surface of approximately 12 x 12 inches was swabbed in two different directions.

2.2. Sample Analysis

Feces in PBS (1 mL) and environmental swabs in neutralizing buffer (1 mL) were inoculated into 20 mL of selenite cysteine broth (Becton, Dickinson and Company, Sparks, MD) and incubated at 35ºC for 16 to 24 hours.

For the POC PCR assay (Fluxergy, Irvine, CA), 14 µL of enriched cultured samples were mixed with 126 µL of thawed *Salmonella* spp. reaction mix. The solutions were mixed gently and spun down using a mini centrifuge. Once the step was completed, 130 µL of the mixture were pipetted and carefully dispensed into the loading port of the microfluidic card according to the manufacturer's instructions. The test card was inserted into the device and the *Salmonella* spp. testing was initiated. Fluxergy's proprietary microfluidic system handles sample preparation (including DNA extraction) by employing a sample type specific buffer system. Specifically, the inhibition-resistant buffer along with a mixture of surfactants and dispersants allows for rapid sample dispersion and lysis of raw sample matrix. The POC *Salmonella* spp. assay targets two pathogenicity genes, the invasion A (invA) and the tetrathionate reductase structural (ttrC) gene. Further, an internal control (exogenous, MS2 bacteriophage genomic fragment, primers and probe), included as part of the reaction mix, is used in the background of every test to validate a true negative sample. Amplification must occur in the internal control channel for a negative sample to be qualified as a negative. The cycling conditions of the PCR POC device were 94°C for 5 minutes followed by 45 cycles of 10 seconds at 94°C and 25 seconds at 56°C. The total time for each test was 50 minutes. Test output was one of three options: positive (*Salmonella* spp. DNA present), negative (*Salmonella* spp. DNA absent) or indeterminate (issue with reaction and/or analyzer). Indeterminate results were not re-run and classified as negative.

Concurrently to the POC PCR testing, 200 µL of the enriched cultured samples were processed for DNA purification using an automated nucleic acid extraction system (QIAcubeHT, Germantown, MD) according to the manufacturer's recommendations. Following DNA purification, the samples were analyzed using an already established and validated *Salmonella* spp. real-time PCR assay [14]. Briefly, the real-time PCR assay is based on the detection of a specific 132 base-pair product of the invA gene of *Salmonella* spp. (GenBank accession number U43271; oligonucleotides: forward primer invA-156f CATTTCTATGTTCGTCATTCCATTACC, reverse primer invA-283r CGTTGAAAAACTGAGGATTCTGTC, probe invA-236p FAM-TGTTTATGGGGTCGTTCTA-MGB). The samples were amplified in a combined thermocycler/fluorometer (QuantStudio 5, Applied Biosystems, Foster City, CA) with the standard thermal cycling protocol: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Furthermore, a real-time PCR assay targeting a universal sequence of the bacterial 16S rRNA gene was used as quality control (i.e., efficiency of DNA purification and amplification) and as indicator of fecal inhibition [18]. Total time for nucleic acid extraction and amplification was 180 minutes. All PCR analyses were performed in a masked fashion, where the outcome of the results was unknown to the laboratory personnel.

Following completion of the study, the results from the POC *Salmonella* spp. PCR assay from fecal and environmental samples were compared to the results of the gold standard qPCR *Salmonella* spp. assay in order to determine the accuracy between the two detection assays and calculate sensitivity and specificity with 95% confidence interval of the POC PCR assay.

3. Results

A total of 275 fecal and environmental samples were available for *Salmonella* spp. testing. The POC PCR *Salmonella* spp. assay gave 35 positive, 225 negative and 15 indeterminate results (Table 1). The gold standard of qPCR yielded 33 positive and 242 negative results. Thirteen fecal and two environmental samples gave indeterminate results. All indeterminate results tested negative by the qPCR platform. The indeterminate samples were not retested and were included in the calculation to determine sensitivity and specificity (Table 1). For all samples combined, the POC *Salmonella* spp. PCR assay showed an overall agreement of 92.4% with qPCR. By sample type, the agreement of the POC *Salmonella* spp. PCR assay with qPCR was 88.1% and 97.0% for feces and environmental samples, respectively.

4. Discussion

The study results showed great overall agreement between the POC PCR assay and the gold standard of qPCR in the detection of *Salmonella* spp. in fecal and environmental samples. Because of its high analytical sensitivity and specificity, qPCR for the detection of *Salmonella* spp. has become a well-established testing platform in the veterinary diagnostic field. However, despite qPCR having a microbial limit of detection lower than conventional culture, biological samples such as feces, and environmental samples still require a selective enrichment step in order to reliably detect *Salmonella* spp [4–8]. The enrichment step is routinely performed after the arrival of the sample to the veterinary laboratory, further delaying the availability of test results. Even with rapid overnight courier and shortening the enrichment step to 16 to 20 hours, it takes at

Table 1
Detection of *Salmonella* spp. in fecal and environmental samples using a POC PCR and a reference qPCR platform.

POC PCR Platform	qPCR Reference Platform				
	Fecal Samples (143)		Environmental Samples (132)		Total Samples (275)
	Positive (27)	Negative (116)	Positive (6)	Negative (126)	
Positive	25	2	6	2	35
Negative	2	101	0	122	225
Indeterminate[a]	0	13	0	2	15

Feces: sensitivity 92.3% (25/27; 95% CI 76.8%–98.7%), specificity 87.1% (101/116; 95% CI 87.1%–88.5%)
Environmental samples: sensitivity 100% (6/6; 95% CI 55.9%–100.0%), specificity 96.8% (122/126; 95% CI 94.7%–96.8%)
[a] The PCR POC assay reports indeterminate results when the internal control gene (fragment of MS2 bacteriophage) is not detected in a biological sample and reflects a sample that did not pass quality control.

least 48 hours from sample collection to reliably finalize testing for *Salmonella* spp. by qPCR in a diagnostic laboratory. With recent advances in microfluidic technology and the development of POC instrumentation, testing of selected pathogens can be performed at hospital site in a timely fashion without having to ship out diagnostic material [19]. In 2022, Fluxergy launched the first POC PCR assay for the environmental detection of *Salmonella* spp. While the manufacturer of the POC PCR instrument and the *Salmonella* spp. test kit claims great analytical performance of the assay, the *Salmonella* spp. test has not been evaluated in a clinical setting. Further, while the test is labeled for the environmental testing of *Salmonella* spp., its use for the testing of feces has yet to be investigated.

Instrumentation needed to perform the testing requires the analyzer, a mini centrifuge, pipettes with tips and a conventional microbiological incubator. The *Salmonella* spp. assay comes with the sampling sponge, the enrichment media, the reaction mix, and the microfluidic card. Hands-on pipetting is limited to the initial inoculation of the enrichment medium and the loading of the microfluidic card after mixing the cultured medium with the reaction mix. Results were available often in less than 24 hours from sample collection, including sample incubation and enrichment (16–24 hours), and PCR analysis (50 minutes). The experience with the system used at two different veterinary hospitals was user friendly, easy to perform with little training needed.

The POC *Salmonella* spp. PCR assay performed well when compared to the molecular gold standard of qPCR. The overall agreement between the two molecular assays was 92.4%, with environmental samples showing a greater agreement compared to fecal samples. The discrepancy between the two sample types lays in the level of inhibition, with fecal samples known to contain various inhibitory compounds (bilirubin and bile salts) of PCR [20]. This was further supported by the 13 fecal samples yielding indeterminate results compared to the two environmental samples that failed quality control. Environmental samples are generally collected from stalls and high traffic areas after cleaning and disinfecting the area to sample, therefore, eliminating most if not all organic material known to harbor molecular inhibitors. One must keep in mind that in order to expedite the analysis, the POC *Salmonella* spp. POC assay combines sample lysis and amplification, while conventional qPCR separates the analysis into two steps, nucleic acid purification and nucleic acid amplification. From a practical standpoint, samples with indeterminate *Salmonella* spp. results should be retested using another aliquot of the cultured enrichment broth. If the sample still fails quality control, a fresh fecal sample should be collected and the entire process of pre-enrichment and analysis repeated with the goal to hopefully eliminate inhibition. Unfortunately, the study protocol did not further pursue indeterminate results and it would have been interesting to determine the outcome of retesting such samples. Another limitation of the study was the inability to culture each fecal and environmental sample. Culture has many advantages, including the assessment of viability, which is key to allow serotyping and to determine antimicrobial susceptibility [12]. The low limit of detection of the POC *Salmonella* spp. PCR assay (one colony forming unit/mL), coupled with the dilution effect of fecal and environmental samples during the pre-enrichment step and the small volume of cultured medium used for the analysis are in support of replicating microorganisms in the case of a PCR-positive result. Discrepant fecal and environmental test results between the POC PCR and the qPCR platform related to indeterminate results with small numbers of false positive and false negative results. While a false positive result may lead to unnecessary isolation and retesting of patients or extend the vacancy of a stall due to additional cleaning and disinfecting steps, a false negative result may have greater clinical impact with the risk for nosocomial transmission. It is therefore advised to collect and test 3 to 5 fecal samples from horses with enteric signs compatible with salmonellosis and also to collect and test multiple sponges from a stall, which previously housed a horse with clinical or subclinical salmonellosis [21,22].

In conclusion, the POC *Salmonella* spp. PCR assay used on feces and environmental samples yielded satisfactory results in terms of sensitivity, specificity, and overall agreement when compared to the gold standard of qPCR. When considering the use of a novel POC PCR assay, the end-user has to consider advantages and limitations of the technology. The PCR *Salmonella* spp. POC assay was easy to perform and suitable as an in-house assay. Further, the assay has low risk of contamination due to few sample handling steps and closed-tube amplification technology. The costs for a single *Salmonella* spp. test kit is similar to a qPCR assay offered by a commercial veterinary diagnostic laboratory, however, the lack of shipping costs, the rapid turn-around-time and the excellent performance of the test make this novel platform a relevant monitoring and diagnostic tool for equine veterinary hospitals.

Financial disclosure

This study was supported by Fluxergy, Irvine, CA.

Declaration of Competing Interest

Dr. Pusterla has served on the scientific advisory board of Fluxergy since 2023. Dr. Pramod Naranatt, Mrs. Himani Swadia and Mr. Eric Mendonsa work for Fluxergy. Dr. Laramie Winfield, Mrs. Ashley Hartwig and Mrs. Samantha Barnum have no conflict of interest to declare. There has been no financial compensation in exchange for the evaluation of the POC PCR test kit.

References

[1] Owen RR, Fullerton JN, Tizard IR, Lumsden JH, Barnum DA. Studies on experimental enteric salmonellosis in ponies. Can J Comp Med 1979;43:247–54.
[2] Hird DW, Pappaioanou M, Smith BP. Case-control study of risk factors associated with isolation of *Salmonella saintpaul* in hospitalized horses. Am J Epidemiol 1984;120:852–64.

[3] House JK, Mainar-Jaime RC, Smith BP, House AM, Kamiya DY. Risk factors for nosocomial *Salmonella* infection among hospitalized horses. J Am Vet Med Assoc 1999;214:1511–16.
[4] Van Duijkeren E, Sloet van Oldruitenborgh-Oosterbaan MM, Houwers DJ, van Leeuwen WJ, Kalsbeek HC. Equine salmonellosis in a Dutch veterinary teaching hospital. Vet Rec 1994;135:248–50.
[5] Ruple-Czerniak A, Bolte DS, Burgess BA, Morley PS. Comparison of two sampling and culture systems for detection of *Salmonella enterica* in the environment of a large animal hospital. Equine Vet J 214;46:499-502.
[6] Burgess BA, Noyes NR, Bolte DS, Hyatt DR, van Metre DC, Morley PS. Rapid *Salmonella* detection in experimentally inoculated equine faecal and veterinary hospital environmental samples using commercially available lateral flow immunoassays. Equine Vet J 2015;47:119–22.
[7] Agga GE, Arthur TM, Schmidt JW, Wang R, Brichta-Harhay DM. Diagnostic accuracy of rectoanal mucosal swab of feedlot cattle for detection and enumeration of *Salmonella enterica*. J Food Prot 2016;79:531–7.
[8] Goeman VR, Tinkler SH, Hammac GK, Ruple A. Evaluation of environmental sampling methods for detection of *Salmonella enterica* in a large animal veterinary hospital. Can Vet J 2018;59:408–12.
[9] Cohen ND, Martin LJ, Simpson RB, Wallis DE, Neibergs HL. Comparison of polymerase chain reaction and microbiological culture for detection of salmonellae in equine feces and environmental samples. Am J Vet Res 1996;57:780–6.
[10] Amavisit P, Browning GF, Lightfoot D, Church S, Anderson GA, Whithear KG, Markham PF. Rapid PCR detection of *Salmonella* in horse faecal samples. Vet Microbiol 2001;79:63–74.
[11] Kurowski PB, Traub-Dargatz JL, Morley PS, Gentry-Weeks CR. Detection of *Salmonella* spp in fecal specimens by use of real-time polymerase chain reaction assay. Am J Vet Res 2002;63:1265–8.
[12] Sibley J, Yue B, Huang F, Harding J, Kingdon J, Chirino-Trejo M, Appleyard GD. Comparison of bacterial enriched-broth culture, enzyme linked immunosorbent assay, and broth culture-polymerase chain reaction techniques for identifying asymptomatic infections with *Salmonella* in swine. Can J Vet Res 2003;67:219–24.
[13] Ward MP, Alinovi CA, Couëtil LL, Wu CC. Evaluation of a PCR to detect *Salmonella* in fecal samples of horses admitted to a veterinary teaching hospital. J Vet Diagn Invest 2005;17:118–23.
[14] Pusterla N, Byrne BA, Hodzic E, Mapes S, Jang SS, Magdesian KG. Use of quantitative real-time PCR for the detection of *Salmonella* spp. in fecal samples from horses at a veterinary teaching hospital. Vet J 2010;186:252–5.
[15] Goodman LB, McDonough PL, Anderson RR, Franklin-Guild RJ, Ryan JR, Perkins GA, Thachil AJ, Glaser AL, Thompson BS. Detection of *Salmonella* spp. in veterinary samples by combining selective enrichment and real-time PCR. J Vet Diagn Invest 2017;29:844–51.
[16] Gupta N, Augustine S, Narayan T, O'Riordan A, Das A, Kumar D, Luong JHT, Malhotra BD. Point-of-care PCR assays for COVID-19 detection. Biosensors 2021;11:141.
[17] Zhang JY, Bender AT, Boyle DS, Drain PK, Posner JD. Current state of commercial point-of-care nucleic acid tests for infectious diseases. Analyst 2021;146:2449–62.
[18] Mapes S, Rhodes DM, Wilson WD, Leutenegger CM, Pusterla N. Comparison of five real-time PCR assays for detecting virulence genes in isolates of *Escherichia coli* from septicaemic neonatal foals. Vet Rec 2007;161:716–18.
[19] Yang SM, Lv S, Zhang W, Cui Y. Microfluidic point-of-care (POC) devices in early diagnosis: a review of opportunities and challenges. Sensors 2022;22:1620.
[20] Widjojoatmodjo MN, Fluit AC, Torensma R, Verdonk GP, Verhoef J. The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples. J Clin Microbiol 1992;30:3195–9.
[21] Van Duijkeren E, Sloet van Oldruitenborgh-Oosterbaan MM, Houwers DJ, van Leeuwen WJ, Kalsbeek HC. Equine salmonellosis in a Dutch veterinary teaching hospital. Vet Record 1994;135:248–50.
[22] Palmer JE, Benson CE. *Salmonella* shedding in the equine. In: Snoyenbos GH, editor. Proceedings of the International Symposium on *Salmonella*. American Association of Avian Pathologists; 1994. p. 161–4.