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Diagnostic performance and application of a real-time PCR assay for the detection of *Salmonella* in fecal samples collected from hospitalized horses with or without signs of gastrointestinal tract disease

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ABSTRACT

The main objective of this study was to assess the diagnostic performance of a real-time polymerase chain reaction (PCR) assay for the detection of *Salmonella* in fecal samples collected from hospitalized horses with or without signs of gastrointestinal (GI) tract disease. The PCR assay used primers and a probe that targeted the *invA* gene of *Salmonella*. Assuming a sensitivity of 100% and a specificity of 96.6%, and a disease prevalence of 2%, 5%, and 10–15% in study horses, the PCR assay had a high (100%) negative predictive value, and a positive predictive value that ranged from 37% in horses without signs of GI disease that tested *Salmonella* culture-negative, to 60% in horses with signs of GI disease that tested *Salmonella* culture-negative, to 76–83% in horses with signs of GI disease that tested *Salmonella* culture-positive. This study provides evidence that the real-time PCR that targets the *Salmonella invA* gene can be used as a screening test for the detection of *Salmonella* in feces of hospitalized horses with signs of GI disease. Horses that test PCR-positive can be tested in series using bacteriologic culture to reduce false positive results or to provide additional data (e.g., antibiogram and serotyping data) that can be used to identify potential nosocomial *Salmonella* infections.

Introduction

Early detection of *Salmonella* shedding in hospitalized horses is important for the rapid implementation of infection control measures in order to reduce the risk of an outbreak of nosocomial *Salmonella* infections. Several polymerase chain reaction (PCR) assays have been evaluated for their potential use to detect *Salmonella* in horse feces (Cohen et al., 1996; Gentry-Weeks et al., 2002; Kurowski et al., 2002; Ward et al., 2005; Bohaychuk et al., 2007; Pusterla et al., 2010). One advantage of PCR is that results can be obtained in less than 48 h if samples are enriched for 24 h or less, compared to bacteriologic culture (3–5 days).

Despite the availability of rapid PCR assays, the use of PCR as a surveillance tool for the early detection of *Salmonella* shedding in hospitalized horses has been limited. One concern has been the potential misclassification of non-infected horses because it can increase hospitalization costs. This concern was first revealed in two studies, where 26/152 (17%) horses without signs of gastrointestinal (GI) disease (Cohen et al., 1996) and 76/105 (72%) horses that tested culture-negative on ≥5 samples during hospitalization were classified as positive by PCR (when targeting the *Salmonella* histidine transport operon gene) (Ward et al., 2005). Four additional studies assessed the diagnostic performance of real-time PCR assays compared to bacteriologic culture (Gentry-Weeks et al., 2002; Kurowski et al., 2002; Bohaychuk et al., 2007; Pusterla et al., 2010). In these studies, the highest, combined diagnostic sensitivity and specificity were reported in assays that targeted the *SpaQ* gene with 100% sensitivity and 98.2% specificity (Kurowski et al., 2002), or the *invA* gene with 97.1–100% sensitivity and 91.3–100% specificity (Bohaychuk et al., 2007) or 100% sensitivity and 98% specificity (Pusterla et al., 2010). In all four studies, however, the clinical profile (i.e., horses with or without signs of GI disease), the number of samples collected from each study horse, and the predictive values of the PCR assay in horses with different clinical profiles were not reported. Thus, it is possible that diagnostic sensitivity and specificity were under- or overestimated.

The main objective of the current study was to assess the diagnostic performance of a real-time PCR assay targeting the *invA* gene for detection of *Salmonella* in multiple fecal samples collected from hospitalized horses with or without signs of GI disease. Among horses with signs of GI diseases, the specific objectives were (1) to compare the proportions of fecal samples classified as *Salmonella*

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positive by culture or PCR; (2) to estimate the agreement between culture and PCR for the diagnosis of *Salmonella* at the sample level; (3) to estimate the diagnostic sensitivity of PCR for the diagnosis of *Salmonella* using culture as a gold standard; and (4) to estimate among horses with or without signs of GI disease the predictive values of PCR for diagnosis of *Salmonella* using different disease prevalence estimates. A secondary objective was to investigate the specificity of the *Salmonella* genes targeted in previous real-time and conventional PCR studies on current bacterial bioinformatical databases.

Materials and methods

The study protocol was approved on February 28, 2011 by the Institutional Animal Care and Use Committee at the University of Florida (Protocol number 201104908).

Study population

The study was conducted at the University of Florida's Large Animal Hospital (UF LAH). The hospital has an on-going surveillance program for the early detection of *Salmonella* fecal shedding in horses at the time of admission and during hospitalization. All horses with or without clinical signs of GI disease (colic, diarrhea, or fever and leukopenia) admitted to the UF LAH from January to December 2011 were eligible for inclusion in the study.

Study samples

Fecal samples from three groups of horses were included: (1) 67 samples from 20 horses with signs of GI disease that tested *Salmonella*-culture-positive at admission or during hospitalization and were sampled two to seven times; (2) 126 samples from 43 horses with signs of GI disease that tested *Salmonella*-culture negative on more than three consecutive fecal samples at admission and during hospitalization; and (3) 150 samples from 30 horses without signs of GI disease that tested *Salmonella*-culture negative on five consecutive fecal samples collected at 12 h intervals during hospitalization.

Collection of fecal samples

In horses with signs of GI disease, samples were collected by the hospital personnel as part of the UF LAH surveillance program. A fecal sample or rectal swab (if fecal sample was not available) was collected from each study horse within 12 h of admission and submitted for bacteriological culture. Thereafter, additional fecal samples were collected from the stall floor each morning prior to cleaning, every Monday and Thursday until the horse was discharged from the hospital. Samples collected outside regular business hours were refrigerated at 4 °C prior to laboratory submission (for a maximum of 24 h).

In horses without signs of GI disease, the first fecal sample was collected from the floor of each stall housing a study horse within 12 h of admission by one of the study investigators (ABE). Thereafter, two to five additional samples were collected at 12 h intervals during hospitalization. No rectal swab samples were collected in this group of horses.

As part of the bacteriological culture procedures at the UF Veterinary Clinical Microbiology Laboratory, all fecal and rectal swab samples collected from study horses were enriched in 10 mL of Hajna Tetrathionate broth (with Iodine-Iodide) (TTB) for 24 h at 37 °C. The following day, the broth was subcultured on Hektoen Enteric Agar (HEA) plates. The remainder of the broth (approximately 9.5 mL) was aliquoted and stored at –80 °C for *Salmonella* DNA extraction and real-time PCR testing.

Bacteriologic culture procedures

Bacteriologic culture for detection of *Salmonella* was performed at the UF Veterinary Clinical Microbiology Laboratory as previously described (Ekiri et al., 2009) with one modification (TTB was used for selective enrichment instead of selenite cystine broth).

Real-time PCR assay procedures

The goal for the bioinformatics analysis was to determine, in silico, the specificity of the primers and probes previously investigated for the detection of *Salmonella enterica* in equine fecal samples. Several gene targets from published PCR studies were analyzed, including the *invE*, *sip*, *spaQ*, and *invA* genes, and the *histidine transport operon* gene (Cohen et al., 1996; Kurowski et al., 2002; Bohaychuk et al., 2007; Pusterla et al., 2010). Primer and probe sequences from published studies were analyzed using the National Centre for Biotechnology Information (NCBI) database to determine the degree of similarity and homology of *Salmonella* against bacterial nucleotide sequences. Individual primers and probes were analyzed using NCBI blast and compared using the following information: number of hits, organisms hit, description (chromosome/plasmid), sequence similarity scores, query coverage, expected values, and percent maximal identity score. Based on the above criteria, one set of primers and probe was selected from published real-time PCR studies for use in this study. After selection, the target gene (*invA*; Genbank accession number U43271; 1950 bp) was analyzed as five overlapping fragments to verify the existence of sequence alignments with non-*Salmonella* organisms.

The selected primers and probe were optimized using the Applied Biosystems Fast 7500 real-time PCR system (Applied Biosystems), and SYBR green and TaqMan probe PCR chemistries. A second set of primers and probe that universally targets the 16S *rRNA* gene of bacteria was used as a control of the efficiency of DNA purification and amplification and as an indicator of fecal inhibition (Windsor et al., 2006). The efficiency of the PCR assay was determined for the primers and probe by using seven 10-fold dilutions of *Salmonella* DNA (10 ng/μL) to generate standard curves. The slope of the log-linear phase and r^2 values were examined to verify if the amplification of the PCR assay was efficient.

Analytical specificity and sensitivity of the PCR assay

Analytical specificity of the assay (using the selected set of primers/probe) and cross-reactivity with non-*Salmonella* enteric and non-enteric organisms was determined as described by Pusterla et al. (2010) with modifications. Enteric and non-enteric organisms tested included *E. coli*, *Klebsiella pneumoniae*, *Bordetella bronchiseptica*, *Streptococcus equi* subsp. *zooepidemicus*, *Staphylococcus aureus*, *Clostridium difficile*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Enterococcus casseliflavus*, *Paenibacillus*, *Bacteroides fragilis*, *Proteus mirabilis*, and *Streptococcus equi* subsp. *equi*. All the non-*Salmonella* strains were obtained from the University of Florida Shands Diagnostic Microbiology laboratory and collections at the UF College of Veterinary Medicine.

The analytical sensitivity of the assay (using the selected primers/probe) was determined using 13 *Salmonella* isolates of equine origin from five serogroups: serogroup B (S. Saintpaul and S. Typhimurium); serogroup C1 (S. Mbandaka, S. Braenderup); serogroup C2 (S. Newport, *S. Litchfield*, and S. Muenchen); serogroup D (S. Enteriditis, S. Miami, S. Javiana) and serogroup E (S. Anatum, S. Meleagridis, S. Muenster). All *Salmonella* strains were obtained from the UF Veterinary Hospitals Microbiology laboratory.

PCR assay detection limit

The detection limit of the PCR assay was evaluated using an isolate of S. Typhimurium suspended in liquid media (PBS) and fecal samples as described previously (Cohen et al., 1993, 1994, 1995; Pusterla et al., 2010) with modifications. After enumeration of *Salmonella* colony forming units (10^9 CFU/mL), PBS and fecal samples were spiked, DNA was extracted using the UltraClean fecal kit (MOBIO, California), and real-time PCR was performed later.

Detection of Salmonella *DNA in study samples*

To reduce bias, the investigator responsible for PCR testing (ABE) was blinded from bacteriological culture results or source of study samples (i.e., horses with or without signs of GI disease). Each sample was identified with a unique number by a laboratory technician before DNA extraction and real-time PCR testing. Each study sample was tested in duplicate and results reported as positive or negative. If a sample were to test positive and negative in a given duplicate, then such results would have been considered inconclusive and the sample would be retested. A threshold cycle value (Ct) ≤37 was considered a positive result based on the standard curve where the lowest concentration of template was detected at a Ct of 37 ($1 \times 10e^{-5}$ ng/uL; *Salmonella* DNA). After PCR testing, results were unmasked for data analysis.

Data collection

For each horse, the following data were collected: medical record number, admission date, discharge date, age, gender, breed, presenting complaint, clinical findings and procedures at admission and during hospitalization (colic, diarrhea, fever, leucopenia), type of sample collected (fecal or rectal swab), sample identification number, date sample was collected, number of samples collected, culture and PCR results.

Data analyses

In each group of horses, the null hypotheses that the proportions of fecal samples classified as positive by culture or PCR were not different were tested using the McNemar's chi-square test. In addition, observed agreement between PCR and culture results was tested using kappa statistic (Dohoo et al., 2010). Values of $P \leq 0.05$ were considered significant. Using culture as gold standard, the diagnostic sensitivity and specificity of the PCR were calculated at the sample and horse levels; samples and horses from all three study groups were included. At the sample level, the sensitivity of PCR was calculated by dividing the number of samples that tested PCR-positive by the total number of samples that tested culture-positive. The specificity of PCR was calculated by dividing the number of samples that tested PCR-negative by the total number of samples that tested culture-negative.

Furthermore, at the horse level, the sensitivity of PCR was calculated by dividing the number of horses that tested PCR-positive by the total number of horses that