Occurrence of *Salmonella* spp. in fecal samples from foals with and without diarrhea in the state of São Paulo: microbiological diagnosis, antimicrobial susceptibility profile, and molecular detection[1]

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ABSTRACT.- Braga P.R.C., Basso R.M., Martins L.S.A., Ribeiro M.G., Amarante A.F., Casas M.R.T., Nassar A.F.C, Pereira J.G., Pantoja J.C.F., Cerri F.M., Possebon F.S., Oliveira-Filho J.P. & Borges A.S. 2023. **Occurrence of *Salmonella* spp. in fecal samples from foals with and without diarrhea in the state of São Paulo: microbiological diagnosis, antimicrobial susceptibility profile, and molecular detection**. *Pesquisa Veterinária Brasileira 43:e07194, 2023*. Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Dr. Walter Maurício Correa s/n, Botucatu, SP 18618-681, Brazil. E-mail: alexandre.s.borges@unesp.br

The present study investigated *Salmonella* spp. in the feces of 200 foals up to one year of age (100 with clinical signs of diarrhea and 100 without clinical signs of diarrhea). Bacteriological culture, serotyping, antimicrobial susceptibility, and real-time PCR (qPCR SYBR® Green or a TaqMan®) for detecting the *invA* gene (with and without a selective pre-enrichment step in tetrathionate broth) were performed. Bacterial culture revealed 15% (n=30) of positive animals (21 animals with diarrhea and nine without diarrhea). Among the 30 isolates, 13 different serovars were identified: *S.* Infantis, *S.* Minnesota, *S.* I.4,5,12:i:-; *S.* Anatum, *S.* Cerro, *S.* Oranienburg, *S.* Braenderup, *S.* Give, *S.* Newport, *S.* IIIb 61:c:z35, *S.* 109:-:1.5, *S.* I.4.12:d:-, *S.* I.6.8:-:-. Multidrug resistance was found in 43.33% (n=13) of the isolates, with one isolate obtained from animals without diarrhea and 12 isolates from animals with diarrhea. All qPCR techniques used in the study classified more samples as positive for *Salmonella* spp. than the bacterial culture of feces. In addition, all qPCR techniques detected more positive animals in the diarrhea group than in the diarrhea-free group. The results confirm the utility of the qPCR method without the pre-enrichment step in tetrathionate as a rapid test for *Salmonella* spp. in carrier animals. In animals with clinical signs of diarrhea, it can be combined with bacterial culture (antimicrobial susceptibility testing and serotyping). The isolation of *Salmonella* spp. in nine animals without diarrhea confirms the importance of asymptomatic carrier animals in the epidemiology of the disease. The multidrug resistance observed highlights the importance of rational antimicrobial use in horses and adopting biosecurity protocols that are efficacious in controlling the spread of infections between animals and zoonotic transmission in farms.

INDEX TERMS: Horses, faeces, *invA* gene, multidrug resistance, serovars, *Salmonella* spp.

RESUMO.- [**Ocorrência de *Salmonella* spp. em amostras fecais de potros com e sem diarreia no estado de São Paulo: diagnóstico microbiológico, perfil de susceptibilidade aos antimicrobianos e detecção molecular.**] O presente estudo investigou a ocorrência de *Salmonella* spp. em fezes de 200 potros com até um ano de idade (100 com sinais clínicos de diarreia e 100 sem sinais clínicos de diarreia), utilizando as técnicas de cultivo bacteriológico e PCR em

tempo real (qPCR) pelos métodos de corante fluorescente (SYBR® Green) e sonda específica (Taqman®) para a detecção do gene *invA* com e sem etapa de pré-enriquecimento seletivo em caldo de tetrationato. O cultivo bacteriológico revelou 15% (n=30) de animais positivos (21 animais com diarreia e nove animais sem diarreia). Dentre esses 30 isolados, 13 sorovares diferentes foram identificados: *S.* Infantis, *S.* Minnesota, *S.* I.4,5,12:i:-; *S.* Anatum, *S.* Cerro, *S.* Oranienburg, *S.* Braenderup, *S.* Give, *S.* Newport, *S.* IIIb 61:c:z35, *S.* 109:-:1.5, *S.* I.4.12:d:-, *S.* I.6.8:-:-. Multirresistência foi constatada em 43,33% (n=13) dos isolados, sendo um isolado obtido de animal sem diarreia e 12 isolados de animais com diarreia. Todas as técnicas de qPCR empregadas no estudo apresentaram maior número de amostras classificadas como positivas para *Salmonella* spp. comparadas ao cultivo bacteriológico de fezes. Adicionalmente, em todas as técnicas de qPCR houve maior número de animais detectados como positivos no grupo de animais com diarreia em relação aos animais sem diarreia. Os resultados confirmaram a utilidade do método qPCR sem a etapa de pré-enriquecimento em tetrationato, como um teste rápido para detecção de *Salmonella* spp. em animais portadores ou em animais com sinais clínicos de diarreia. O cultivo bacteriológico deve ser associado para a realização do teste de sensibilidade aos antimicrobianos e sorotipificação. O isolamento de *Salmonella* spp. em nove animais sem diarreia, confirma a importância dos animais portadores assintomáticos na epidemiologia da doença. A multirresistência observada evidencia a importância do uso racional de antimicrobianos em equinos e a importância da adoção de protocolos de biossegurança que sejam eficazes para controlar a disseminação de infecções entre animais e a transmissão zoonótica nas fazendas.

TERMOS DE INDEXAÇÃO: Equinos, fezes, gene *invA*, multirresistência, sorovares, *Salmonella* spp.

INTRODUCTION

Diarrhea, defined as an increase in stool frequency, fluidity, or volume (Palmer 1985), is one of the most common problems in foals (Palmer 1985, Dunkel & Wilkins 2004, Frederick et al. 2009). Diarrhea in foals can be divided into infectious and noninfectious causes (Dunkel & Wilkins 2004, Slovis et al. 2014, Olivo et al. 2016).

In the first six months of life, up to 20% of foals have diarrhea of infectious origin (Slovis et al. 2014). The infectious agents most commonly listed as causing acute diarrhea in foals include *Clostridium perfringens*, *Clostridioides difficile*, rotavirus, *Salmonella* spp., *Cryptosporidium parvum,* and *Lawsonia intracellularis* (Browning et al. 1991, Netherwood et al. 1996, Dunkel & Wilkins 2004, Smith 2008, Wohlfender et al. 2009, Olivo et al. 2016). Determining the causal agent of diarrhea in foals is difficult, and the etiology is undetermined in 44% to 78% of cases (Netherwood et al. 1996, Mallicote et al. 2012). Moreover, understanding the mechanisms of coinfections is limited in cases of diarrhea in foals (Netherwood et al. 1996, Frederick et al. 2009, Oliver-Spinosa 2018), and coinfections may contribute to the worsening of gastrointestinal disorders (Slovis et al. 2014, Olivo et al. 2016).

In clinical situations, infectious agents are detected in the feces to establish the diagnosis and treatment, and this testing is always associated with the clinical history of the animal, epidemiology, physical and laboratory evaluation of the foal, and response to supportive and antimicrobial therapies (Palmer 1985, Spier 1993, Frederick et al. 2009). Salmonellosis is a frequent cause of diarrhea in foals and is associated with high mortality rates in these animals, in addition to being an important zoonosis. Therefore, the diagnosis and treatment of affected animals should be instituted early (Mallicote et al. 2012, Ferreira 2019).

In Brazil, comprehensive studies of salmonellosis in horses are scarce. Nevertheless, some case reports (Hayashi et al. 2017, Oliveira et al. 2019), studies conducted in slaughterhouses (Hofer et al. 2000), and postmortem studies of animals are available (Juffo et al. 2016, Ferreira 2019). Only two studies have examined substantial numbers of horses (Ribeiro et al. 2010, Olivo et al. 2016). Data on the prevalence and antimicrobial susceptibility profile of *Salmonella* spp. in horses are rare in Brazil (Ribeiro et al. 2010, Ferreira 2019, Oliveira et al. 2019), and few investigations have been examined in the field. Nevertheless, some studies in the United Kingdom (Browning et al. 1991, Netherwood et al. 1996), Trinidad and Tobago (Harris et al. 2012), the United States (Slovis et al. 2014), Brazil (Olivo et al. 2016), and Pakistan (Haq et al. 2018) have examined the occurrence of *Salmonella* spp. in foals. Most of the literature on salmonellosis in horses is focused on describing outbreaks in veterinary hospitals (Palmer 1985, Castor et al. 1989, Hartman et al. 1996, Ewart et al. 2001, Schott et al. 2001, Ward et al. 2005, Martelli et al. 2019). In the study by Ewart et al. (2001), the 14% PCR positivity rate for *Salmonella* spp. of environmental samples collected in a veterinary hospital demonstrated the importance of environmental contamination as a source of human infection.

Rapidly detecting *Salmonella* spp. in fecal and environmental samples is important to control this agent in equine populations effectively. In addition, the emergence of multidrug-resistant *Salmonella* spp. in horses is a global public health concern due to the international movement of horses. The objectives of this study were to evaluate the presence of *Salmonella* spp. in fecal samples of animals with and without diarrhea using the conventional bacteriological culture technique and real-time PCR (qPCR) with a fluorescent dye (SYBR® Green) and a specific probe (TaqMan®) for the *invA* gene, with and without pre-enrichment in tetrathionate broth. Other objectives of the study were to identify serovars, determine the susceptibility profile of isolates to antimicrobials used in treating enteric infections in humans and horses, and evaluate the presence of multidrug-resistant ones.

MATERIALS AND METHODS

Ethics Committee/ethics statement. This study was approved by the Committee on Ethics in the Use of Animals (CEUA), "Faculdade de Medicina Veterinária e Zootecnia", Unesp, Brazil. Protocol number 0095/2020.

Experimental groups and material collection/study design and sampling. A total of 200 fecal samples (100 samples from foals with diarrhea and 100 samples from foals without diarrhea) were collected from horses up to 12 months of age, regardless of sex or breed, from equine farms in the state of São Paulo, Brazil. During the visits to the farms, foals of equivalent ages and under the same management with the presence (sick group) or absence of diarrhea (control group) were selected simultaneously. The feces were obtained directly from the rectal ampulla of the animals, stored

refrigerated (2-8°C) in an 80-mL sterile universal collection bottle, and sent to the Laboratory of Microbiology, "Departamento de Produção Animal e Medicina Veterinária Preventiva" (Department of Animal Production and Preventive Veterinary Medicine), FMVZ-Unesp Campus Botucatu, to perform the specific microbiological culture for *Salmonella* spp. Fecal aliquots were frozen at -80°C for the molecular detection (qPCR) of *Salmonella* spp. at the Molecular Biology Laboratory of the "Departamento de Clínica Veterinária" (Department of Veterinary Clinics) of FMVZ-Unesp Campus Botucatu.

Microbiological culture for *Salmonella* spp. Swabs were prepared from the fecal samples, transferred to test tubes containing 10mL of tetrathionate broth, and incubated at 37°C for 12 hours to diagnose genus *Salmonella* spp. Next, the samples were grown on the surface of plates containing Salmonella-Shigella (SS) agar and kept under aerobic conditions at 37°C for 24-48 hours. Colonies 1-2mm in diameter with blackened centers on SS agar, suggestive of the genus *Salmonella* after 24 hours of incubation, were subjected to biochemical characterization: indole production, use of citrate, lysine, H_2S, urease production, glucose and lactose utilization, motility observation, phenylalanine deaminase production in MILI, EPM, and citrate media. Biochemically characterized isolates of the *Salmonella* genus in the biochemical tests were kept in Lignières inclined solid medium at room temperature and in a sterile freezer medium with 10% glycerol at -80°C.

Salmonella spp. isolates, conditioned in Lignières medium, were sent to the Centre for Enteric Diseases and Infections by Special Pathogens of the Bacteriology Centre of the "Instituto Adolfo Lutz" in São Paulo for serotyping and antimicrobial susceptibility testing.

Serotyping. Serotypes were identified with the agglutination test on slides, with antisera specific to *Salmonella* produced in the Enteropathogens Laboratory of the "Instituto Adolfo Lutz". Colony growth on nutrient agar was first tested with 2.0% saline solution and then with polyvalent and monovalent somatic antisera specific for *Salmonella*. Bacterial growth was allowed in a semisolid Svengard medium and tested with polyvalent and monovalent flagellar *Salmonella* antisera to detect flagellar antigens. The serotype was defined by the combination of the results of the agglutination tests with the somatic and flagellar antigens according to the White–Kauffmann–Le Minor scheme 2007 (Grimont & Weil 2007).

Antimicrobial susceptibility test. The antimicrobial susceptibility tests and the interpretation of results were based on the values established by the Clinical and Laboratory Standards Institute for Enterobacteriaceae (CLSI 2018, CLSI 2020).

Twenty-four antimicrobials belonging to seven classes were tested: (1) Beta lactams, divided into four subclasses: (a) Penicillins: ampicillin (AMP: 10μg) and amoxicillin with clavulanic acid (AMOX: 20/10μg); (b) Monobactams: aztreonam (AZT: 30μg); (c1) first-generation cephalosporins: cefazolin (CLINA: 30μg); (c2) second-generation cephalosporin: cefoxitin (CTIN: 30μg); (c3) third-generation cephalosporins: ceftiofur (CFUR: 30μg), ceftriaxone (CXONE: 30μg), cefotaxime (CXIMA: 30μg), ceftazidime (CDIMA: 30μg), and cefepime (CPIME: 30μg); and (d) carbapenems: imipenem (MPI: 10μg). (2) Aminoglycosides: gentamicin (GEN: 10μg), amikacin (AMI: 30μg) and streptomycin (EST: 10μg). (3) Quinolones/fluoroquinolones: nalidixic acid (ANL: 30μg), pefloxacin (PEF: 5μg) and ciprofloxacin (CIP: 5μg). (4) Sulfonamides: sulfamethoxazole + trimethoprim (SULT: 1.25/23.75μg) and sulfonamide (SUL: 30μg). (5) Tetracyclines: tetracycline (TET: 30μg), doxycycline (DOX: 30μg), and minocycline (MIN: 30μg). (6) Amphenicols: chloramphenicol (CLO: 30μg). (7) Macrolides: azithromycin (AZI: 15μg).

The standard strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used to control the susceptibility tests to antimicrobial agents.

Real-time PCR for *Salmonella* spp. Following the manufacturer's instructions, DNA was extracted from feces using the EZNA® Stool DNA Kit (Omega Bio-Tek). A set of primers described by Windsor et al. (2006) was used (PanB-283 F forward primer 5′-GGATGATCAGCCACACTGGA-3′ and PanB-352 R reverse primer 5′-CCAATATTCCTCACTGCTGCC-3′) to detect universal 16S rDNA as a control for DNA extraction from the stool. All purified DNA samples were evaluated for the presence of the invasion gene *invA* by qPCR using an intercalating agent (SYBR® Green) and the TaqMan® probe technique. The TaqMan® qPCR technique was performed using 10μL of GoTaq® Probe qPCR Master Mix, 0.4μM of each primer (forward primer *invA* -156f: 5′-CATTTCTATGTTCGTCATTCCATTACC-3′; reverse primer *invA* -288r: 5′-AGGAAACGTTGAAAAACTGAGGATTCT-3′), 0.3μM probe (*invA* -189p; FAM-TCTGGTTGATTTCCTGATCGCACTGAATATC-TAMRA), and 2μL of the sample, resulting in a 20-μL reaction (Pusterla et al. 2010). Similarly, SYBR® Green qPCR was performed in a final volume of 20μL using 10μL of GoTaq ® qPCR Master Mix, 0.3μM of each primer (forward primer *invA* -5- deg F: 5′-GATYTGAARGCCGGTATTATTG-3′; reverse primer *invA* -5 R: 5′-ATAAACTTCATCGCACCGTCA-3′), and 5μL of the sample (Barbau-Piednoir et al. 2013). qPCR was performed in a 7500 Real-Time PCR System (Applied Biosystems) using the standard thermal cycling protocol: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C. The dissociation curve for the reactions was drawn using intercalating agents.

The detection limit was measured using 10-fold dilutions of predetermined colony-forming units of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ATCC 14028) added to phosphate-buffered saline and feces of *Salmonella*-free horses. The detection limit was 0.4 per reaction using a probe (DNA isolated from predetermined colony-forming units by PBS and contaminated feces) and 1 and 0.1 organisms per reaction using an intercalating agent, with DNA isolated from colony-forming units of *Salmonella* spp. determined by PBS and contaminated feces, respectively.

RESULTS

Samples. The animals collected belonged to 26 farms in 25 municipalities in the state of São Paulo (Altinópolis, Americana, Amparo, Arandu, Assis, Atibaia, Avaré, Bauru, Botucatu, Bragança, Catanduva, Espírito Santo do Pinhal, Indaiatuba, Ipuã, Itapira, Jaguariúna, Mariapolis, Mogi-Mirim, Monte Mor, Porto Feliz, Quadra, Santo Antônio da Posse, São Carlos, Sorocaba, and Tatuí). The study population available in the 26 farms was 5,627 horses with a yearly birth rate of 1,168 foals. Stool samples were collected from December 2019 to December 2020 and were classified according to geographical location, sex, age, breed, and clinical presentation of the animal at the time of collection. Eighty-seven male animals (n=45 with diarrhea and n=42 without diarrhea) and 113 female animals (n=55 with diarrhea and n=58 without diarrhea) were sampled. According to breed, the animals were classified as follows: Arabian (n=11 with diarrhea and n=10 without diarrhea), Brasileiro de Hipismo (n=11 with diarrhea and n=10 without diarrhea), Mangalarga Marchador (n=42 with diarrhea and n=43 without diarrhea), Mangalarga (n=3 with diarrhea and n=3 without diarrhea), Paint Horse (n=1 with diarrhea and n=1 without diarrhea), Thoroughbred (n=6 with diarrhea and n=6 without diarrhea), and Quarter-horse (n=26 with diarrhea and n=27 without diarrhea).

Bacteriology. *Salmonella* spp. was isolated from 30 (15%) samples, 21 from foals with diarrhea and nine without diarrhea. The animals belonged to 15 different farms. On eight farms, one isolate of *Salmonella* spp. was identified; on four farms, two isolates were identified per farm; on two farms, three isolates were identified; and on a single farm, eight isolates of *Salmonella* spp. were identified. Regarding age, 80% of the animals that tested positive for *Salmonella* spp. were no older than two months old at the time of collection. The remaining animals with positive bacteriological cultures were between three (n=1), four (n=1), five (n=1), 10 (n=2), and 12 months of age (n=1), with four animals with diarrhea and two animals without diarrhea (Table 1).

Serotyping. Among the 30 isolates, 13 different serovars were identified: *S.* Infantis (26.7%), *S.* Minnesota (16.7%), *S.* I,4,5,12:i:- (10%); *S.* Anatum (6.7%), *S.* Cerro (6.7%), *S.* Oranienburg (6.7%), *S.* Braenderup (3.3%), *S.* Give (3.3%), *S.* Newport (3.3%), *S.* IIIb 61:c:z35 (3.3%), *S.* IO9:-:1.5 (3.3%), *S.* I.4,12:d:- (6.7%), and *S.* I.6.8:-:- (3.3%). The detection frequencies of the serovars identified in the study are described in Figure 1.

Antimicrobial susceptibility test. Of the 30 isolates, three (10%) were resistant to fewer than three classes of antimicrobials, 12 (40%) were susceptible to all tested antimicrobials, and 13 (43.33%) were multidrug-resistant (Fig.2).

The phenotypic patterns of antimicrobial resistance of *Salmonella* spp. drug-resistant drugs are shown in Table 2.

On the same farm, pansusceptible and multidrug-resistant strains were isolated. On Farm 4, *S.* Infantis isolated from an animal with diarrhea, which produces AmpC, was multiresistant to 12 antimicrobials (Table 2), and *S.* IIIb 61:c:z35 isolates from animals with diarrhea were pansusceptible (Table 1). On Farm 5, *S.* Infantis isolated from an animal with diarrhea, a producer of AmpC, was multidrug-resistant to 13 antimicrobials (Table 2), and serovar *S.* I.9.12:-:1.5 isolated from animals without diarrhea was pansusceptible (Table 1). On Farm 11, serovar *S.* I.4,5,12:i:- was identified from an animal without diarrhea that presented multidrug resistance to five antimicrobials (Table 2). Three serovars of *S.* Minnesota isolates were identified from animals with diarrhea, including a serovar multidrug-resistant to 19 antimicrobials, a serovar multidrug resistant to 16 antimicrobials, and a serovar resistant to 14 antimicrobials (Table 2). On this farm, serovars

Table 1. Classification of *Salmonella* spp. serovars identified in the study according to the susceptibility profile to antimicrobials, enzyme production characteristics, clinical signs, location, age, race, and sex of the animals

Serovar	Antimicrobial susceptibility profile	Enzyme production	Diarrhea	Farm	City	Age (months)	Breed	Sex
S. enterica subsp. *enterica* 6,8:-:-	Resistant		No	1	São Carlos	1	MM	M
S. Give	Resistant	ESBL	Yes	2	Bauru	2	QM	M
S. Infantis	Multiresistant	AmpC	Yes	4	Amparo	1	MM	M
S. IIIb 61:c:z35	Pansusceptible		Yes	4	Amparo	1	MM	M
S. Infantis	Multiresistant	AmpC	Yes	5	Atibaia	4	MM	M
S. I.:O9:-:1,5	Pansusceptible		No	5	Atibaia	5	MM	F
S. Minnesota	Multiresistant	AmpC	Yes	10	Jaguariúna	0	MM	F
S. Oranienburg	Multiresistant	AmpC	Yes	10	Jaguariúna	0	MM	M
S. Newport	Multiresistant	AmpC	Yes	10	Jaguariúna	2	MM	M
S. I.4,5,12:i:-(monophasic *S.* Typhimurium)	Multiresistant		No	11	Monte Mor	1	ARB	F
S. Minnesota	Multiresistant	AmpC	Yes	11	Monte Mor	0	ARB	F
S. Minnesota	Multiresistant	AmpC	Yes	11	Monte Mor	0	ARB	M
S. Minnesota	Multiresistant	AmpC	Yes	11	Monte Mor	0	ARB	F
S. Oranienburg	Pansusceptible		No	11	Monte Mor	1	ARB	F
S. Anatum	Pansusceptible		No	11	Monte Mor	1	ARB	M
S. Anatum	Pansusceptible		No	11	Monte Mor	1	ARB	F
S. Infantis	Pansusceptível		Yes	12	Santo Antônio da Posse	0	MP	M
S. Infantis	Pansusceptible		Yes	12	Santo Antônio da Posse	2	MP	M
S. Infantis	Multiresistant	AmpC	Yes	15	Espírito Santo do Pinhal	2	MM	F
S. Braenderup	Pansusceptible		Yes	16	Assis	1	QM	M
S. Cerro	Pansusceptible		Yes	18	Altinópolis	12	MM	F
S. Cerro	Pansusceptible		No	19	Ipuã	1	BH	M
S. Infantis	Intermediate		Yes	22	Avaré	2	QM	M
S. Infantis	Intermediate		Yes	22	Avaré	2	QM	F
S. Minnesota	Multiresistant		Yes	23	Porto Feliz	1	MM	F
S. I.4,5,12:i:- (monophasic *S.* Typhimurium)	Multiresistant		Yes	23	Porto Feliz	0	MM	F
S. I.4,5,12:i:- (monophasic *S.* Typhimurium)	Multiresistant		Yes	23	Porto Feliz	0	MM	M
S. enterica subsp. *enterica* 4,12:d:-	Resistant		No	24	Sorocaba	10	QM	M
S. enterica subsp. *enterica* 4,12:d:-	Pansusceptible		Yes	24	Sorocaba	10	QM	M
S. Infantis	Pansusceptible		Yes	25	Quadra	3	QM	M

MM = Mangalarga Marchador, ARB = Arabian, QM = Quarter-horse, MP = Mangalarga, BH = Brasileiro de Hipismo, F = female, M = male.
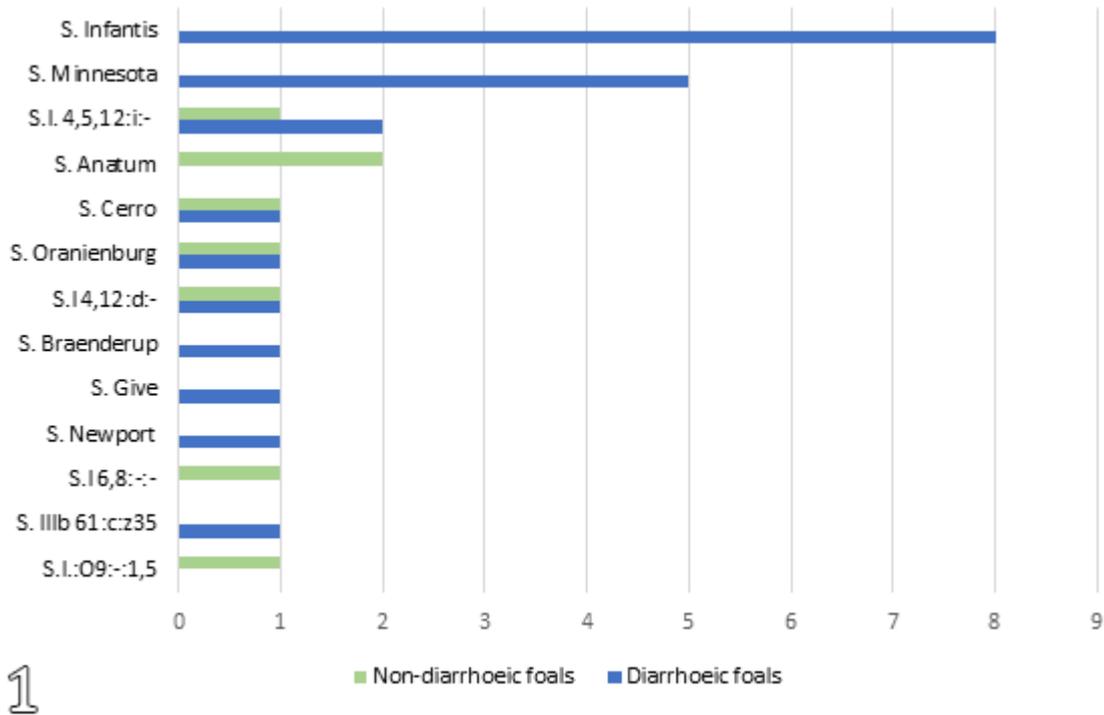
Fig.1. Number of foals affected by different serovars identified with the White-Kauffmann-Le Minor method. São Paulo, 2021.
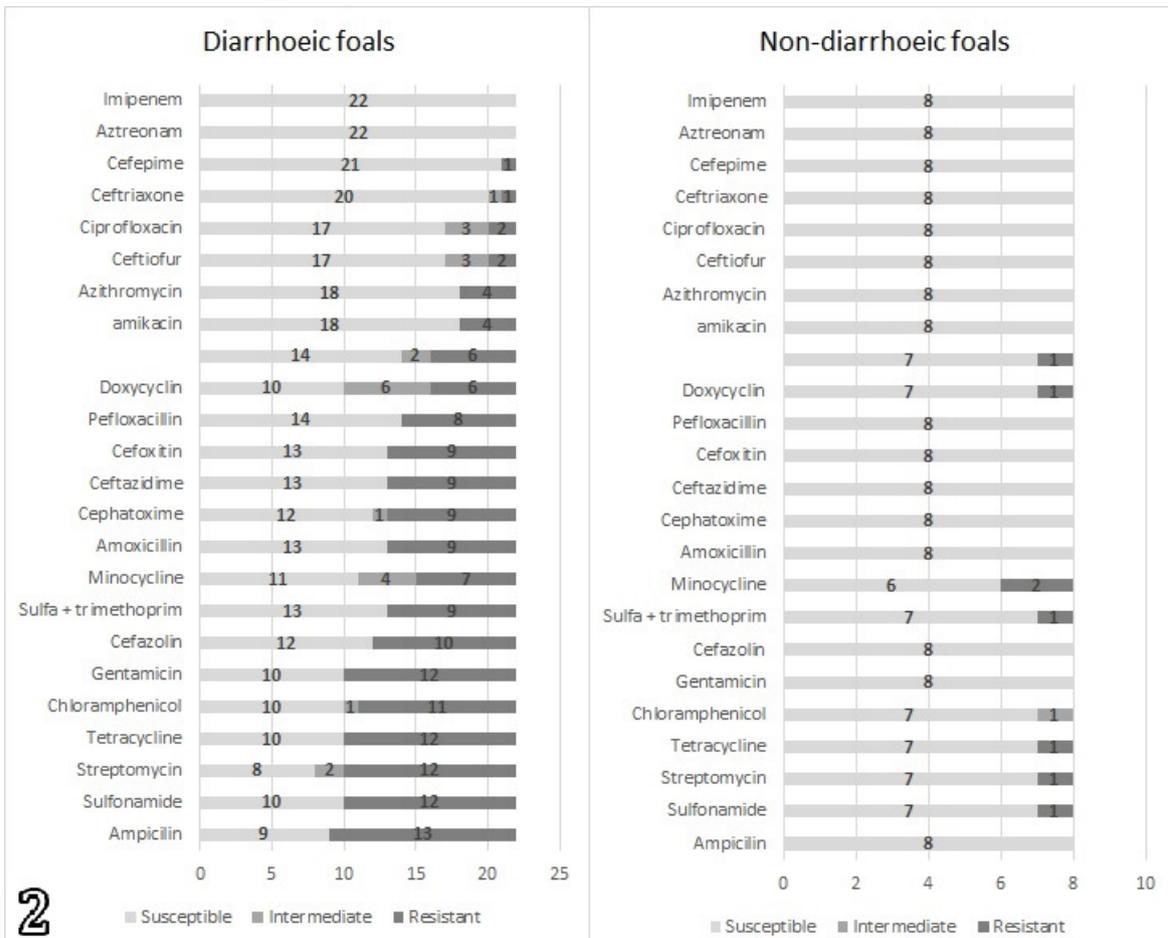

Fig.2. Phenotypic sensitivity profile of *Salmonella* spp. isolates from animals with and without diarrhea to the 24 antimicrobials tested in the study. São Paulo, 2021.

Table 2. Phenotypic profile of antimicrobial resistance of the 13 *Salmonella* spp. that presented resistance to multiple tested antimicrobials. São Paulo, 2021

Serovars	Farms	Diarrhea	Number of antimicrobial resistance	Penicillins		Beta lactams — Cephalosporin						Aminoglycosides			Quinolones			Sulfonamides		Tetracycline			Amphenicol	Macrolide
						1st	2nd		3rd															
				AMP	AMOX	CLINA	CTINA	CFUR	CXONA	CXIMA	CDIMA	GEN	AMI	EST	ANL	PEF	CIP	SUL	SULT	TET	DOX	MIN	CLO	AZI
S. Minnesota	23	Yes	10	R	S	S	S	S	S	S	S	R	S	R	R	R	I	R	S	R	R	R	R	S
S. Minnesota	11	Yes	14	R	R	R	R	S	S	I	R	R	R	R	R	R	I	R	R	R	I	S	R	S
S. Minnesota	11	Yes	19	R	R	R	R	I	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S. Minnesota	11	Yes	16	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	I	R	R	S
S. Minnesota	10	Yes	15	R	R	R	R	S	S	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R
S. Infantis	15	Yes	13	R	R	R	R	R	S	R	R	R	S	R	S	S	S	R	R	R	I	S	R	R
S. Infantis	5	Yes	13	R	R	R	R	S	S	R	R	R	S	R	I	R	I	R	R	R	I	I	R	S
S. Infantis	4	Yes	12	R	R	R	R	I	S	R	R	R	S	R	S	S	S	R	R	R	I	I	R	R
S. I.4,5,12:i:-	23	Yes	10	R	S	S	S	S	S	S	S	R	R	R	S	S	S	R	S	R	R	R	R	S
S. I.4,5,12:i:-	23	Yes	9	R	S	S	S	S	S	S	S	R	R	R	R	R	R	R	S	R	R	R	I	S
S. I.4,5,12:i:-	11	No	5	S	S	R	R	S	S	S	S	S	S	R	R	R	S	S	S	R	R	R	I	S
S. Newport	10	Yes	16	R	R	R	R	I	S	R	R	R	S	R	I	S	S	R	R	R	R	R	R	S
S. Oranienburg	10	Yes	15	R	R	R	R	R	S	R	R	R	S	R	S	S	S	R	R	R	I	I	R	R

R = resistant, I = intermediate, S = susceptible, AMP = ampicillin, AMOX = amoxicillin, CLINA = cefalexin, CTINA = cefazolin, CFUR = ceftiofur, CXONA = ceftriaxone, CXIMA = cefotaxime, CDIMA = ceftazidime, GEN = gentamicin, AMI = amikacin, EST = streptomycin, ANL = nalidixic acid, PEF = pefloxacin, CIP = ciprofloxacin, SUL = sulfonamides, SULT = sulfamethoxazole + trimethoprim, TET = tetracycline, DOX = doxycycline, MIN = minocycline, CLO = chloramphenicol, AZI = azithromycin.

S. Oranienburg and *S.* Anatum, isolated from animals without diarrhea, were pansusceptible (Table 1). The same serovar was identified in different animals on the same farm with distinct antibiotic susceptibility profiles. As in the case of Farm 24, where *S. enterica* subsp. *enterica* 4.12:d:- isolated from an animal without diarrhea was resistant to the antimicrobial minocycline. The same serovar isolated from an animal with diarrhea was pansusceptible (Table 1 and 2).

The tests also revealed that the same animal could harbor different *Salmonella* spp. with different susceptibility profiles to antimicrobials. One animal with diarrhea had two serovars that exhibited other resistance profiles to the tested antimicrobials: *S.* IIIb 61:c:z35 was pansusceptible, but serovar *S.* Infantis was multidrug-resistant to 12 antimicrobials (Table 1 and 2).

Of the 13 multidrug-resistant isolates, one was obtained from animals without diarrhea, and 12 were obtained from animals with diarrhea. Thirteen animals with multidrug-resistant strains were identified on six farms. The multidrug-resistant strains were found in seven animals (53.8%) up to 30 days of age, three animals (23.1%) aged one month, two animals (15.4%) aged two months, and one animal (7.7%) aged four months. The serovars that presented a multidrug-resistance profile to the tested antimicrobials were *S*. Minnesota, *S.* Newport, *S.* Oranienburg, and *S*. Infantis.

Detection of *Salmonella* spp. by qPCR for the *invA* gene. All qPCR techniques used in the study identified more samples positive for *Salmonella* spp. than the bacterial culture of feces. In addition, all qPCR techniques detected more positive animals in the diarrhea group than in the non-diarrhea group (Table 3).

Between 95.2% and 100% of the animals detected as positive by the four qPCR techniques were younger than six months. Of the 26 farms sampled in our study, 18 harbored positive animals as identified by qPCR, and bacterial culture identified positive animals in 14 of these farms. Only qPCR detected animals positive for *Salmonella* spp. on four farms; on one farm, only bacterial culture detected a positive animal. The SYBR® Green qPCR run directly on feces identified 21 out of 30 animals that were positive by bacterial culture. SYBR® Green/tetrathionate qPCR identified 18 animals that were positive by bacterial culture. The TaqMan® qPCR technique run directly on feces identified 19 animals that were positive by bacterial culture, while the TaqMan®/tetrathionate qPCR technique identified 18 animals that were positive by bacterial culture (Table 4).

DISCUSSION

Diagnosing salmonellosis in horses is highly important to select and administering treatment, and treatments are more likely to be effective if salmonellosis is diagnosed early. Rapid and accurate methods facilitate the identification of the disease and permit the selection of the most specific antimicrobial when combined with the evaluation of the resistance of the isolate. Different methods are available to detect *Salmonella* spp. in clinical samples, including bacterial culture, PCR, and lateral flow immunoassays, but these tests require varying levels of experience, cost, and time (Burgess & Morley 2014). Confirming the importance of *Salmonella* spp. in foals, we found positive cultures in 15% of the samples. Studies conducted only in foals with diarrhea identified *Salmonella* spp. in 12% of the animals evaluated in the United States (Frederick et al. 2009) and 17.9% of animals in Pakistan (Haq et al. 2018). Lower isolation rates were found in studies of foals with and

without diarrhea: in the United Kingdom, 0.1% (Browning et al. 1991) and 3% (Netherwood et al. 1996) of samples were positive; in Trinidad and Tobago, 4.4% of samples were positive (Harris et al. 2012); and in the United States, 8% of samples were positive (Slovis et al. 2014).

We isolated *Salmonella* spp. more often from animals with diarrhea (21%) than those without diarrhea (9%). This finding was similar to other studies conducted in Brazil, which reported a 25% isolation rate from sick animals and a 7% isolation rate from healthy animals (Olivo et al. 2016). Previous studies in the United States described an 8% isolation rate from foals with diarrhea and a 0% isolation rate from horses without diarrhea (Slovis et al. 2014); in the United Kingdom, 2% isolation rates from animals with diarrhea and 1% isolation rates from healthy foals that did not have contact with diarrhoeal foals have been reported (Netherwood et al. 1996). The isolation of *Salmonella* spp. in nine animals without diarrhea confirms the importance of asymptomatic carrier animals in the epidemiology of the disease, promoting the maintenance and spread of resistant bacteria in the environment.

From an epidemiological perspective, the most important aspect was the incidence of serovars common in human enteric processes, such as *S.* Infantis, *S.* Newport, and *S.* I 1,4,5,12:i:-. The study identified serovars already described in horses as *S.* Infantis, monophasic *S.* Typhimurium, *S.* I1,4,5,12:i:-, *S.* Anatum, *S.* Oranienburg, *S.* Give, *S.* Newport, and *S.* Cerro. However, the serovar *S. enterica* subsp. *Diarizonae*, *S.* IIIb 61:c:z35, *S.* 109:-:1.5, *S.* I.4.12:d:-, *S.* I.6.8:-:-, which, according to the compiled literature, has not yet been described in equine species, was mentioned for the first time in the context of the equine population in Brazil. Moreover, its pathogenicity in this species is unknown (Hofer et al. 2000, Dunowska et al. 2006, Singh et al. 2007, 2009, Dallap Schaer et al. 2010, Ribeiro et al. 2010, Harris et al. 2012, Cummings et al. 2014, Juffo et al. 2016, Olivo et al. 2016, Haq et al. 2018, Leon et al. 2018, Martelli et al. 2019, Soza-Ossandón et al. 2020, Rivera et al. 2021).

Phenotypic characteristics of antimicrobial resistance were observed in 53.3% of the isolates in the present study. High resistance to β-lactams, sulfonamides, amphenicols, tetracyclines, and aminoglycosides was observed, corroborating

Table 3. Distribution of the number of animals detected as positive for *Salmonella* spp. according to the qPCR technique used and classified by health status

	qPCR SYBR® feces[a]	qPCR SYBR® tetrathionate[b]	qPCR Taqman® feces[c]	qPCR Taqman® tetrathionate[d]
With diarrhea	36	32	31	28
Without diarrhea	12	13	9	16
Total	48	45	40	44

[a] qPCR SYBR® feces = qPCR by fluorescent dye method for *invA* gene without pre-enrichment step in tetrathionate broth, [b] qPCR SYBR® tetrathionate = qPCR by fluorescent dye method for *invA* gene with pre-enrichment step in tetrathionate broth, [c] qPCR Taqman® feces = qPCR by the *invA* gene-specific probe method without pre-enrichment step in tetrathionate broth, [d] qPCR Taqman® tetrathionate = qPCR by *invA* gene-specific probe method with pre-enrichment step in tetrathionate broth.

Table 4. Distribution of the number of animals by qPCR and bacterial culture results

qPCR technique	Bacterial culture (+)	Bacterial culture (-)	Total
qPCR total (+)	25	41	66
qPCR total (-)	5	129	134
qPCR SYBR® feces (+)	21	27	48
qPCR SYBR® feces (-)	9	143	152
qPCR SYBR® tetrathionate (+)	18	27	45
qPCR SYBR® tetrathionate (-)	12	143	155
qPCR Taqman® feces (+)	19	21	40
qPCR Taqman® feces (-)	11	149	160
qPCR Taqman® tetrathionate (+)	18	26	44
qPCR Taqman® tetrathionate (-)	12	144	156

qPCR total (+) = sum of the positive results of the 4 qPCR techniques by the fluorescent dye (SYBR® Green) and specific probe (Taqman®) methods for the *invA* gene with and without a pre-enrichment step in tetrathionate broth; qPCR total (-) = sum of the negative results of the 4 qPCR techniques by the fluorescent dye (SYBR® Green) and specific probe (Taqman®) methods for the *invA* gene with and without a pre-enrichment step in tetrathionate broth; qPCR SYBR® faeces (+) = positive results in the qPCR technique by the fluorescent dye method for the *invA* gene without the pre-enrichment step in tetrathionate broth; qPCR SYBR® faeces (-) = negative results in the qPCR technique by the fluorescent dye method for the *invA* gene without the pre-enrichment step in tetrathionate broth; qPCR SYBR® tetrathionate (+) = positive results in the qPCR technique by the fluorescent dye method for the *invA* gene with the pre-enrichment step in tetrathionate broth; qPCR SYBR® tetrathionate (-) = negative results in the qPCR technique by the fluorescent dye method for the *InvA* gene with the pre-enrichment step in tetrathionate broth; qPCR Taqman® faeces (+) = positive results in the qPCR technique using the specific probe method for the *invA* gene without a pre-enrichment step in tetrathionate broth; qPCR Taqman® faeces (-) = negative results in the qPCR technique by the specific probe method for the *invA* gene without a pre-enrichment step in tetrathionate broth; qPCR Taqman® tetrathionate (+) = positive results in the qPCR technique by the specific probe method for the *invA* gene with the pre-enrichment step in tetrathionate broth; qPCR Taqman® tetrathionate (-) = negative results in the qPCR technique by the specific probe method for the *invA* gene with the pre-enrichment step in tetrathionate broth; Bacterial culture (+) = positive results for *Salmonella* spp. in the bacterial culture of faeces; Bacterial culture (-) = negative results for *Salmonella* spp. in the bacterial culture of faeces; Total = sum of positive and negative results for *Salmonella* spp. in the bacterial culture of faeces.

the latest panels on the worldwide use of antibiotics that identify tetracyclines, penicillins, macrolides, sulfonamides, aminoglycosides, and amphenicols as the main antibiotics used in production animals. Furthermore, these panels also report high resistance rates to antibiotics commonly used to treat clinical infections in these animals. Other authors have also demonstrated a tendency towards increasing resistance to drugs commonly used to treat *Salmonella* spp. infections in horses (Leon et al. 2018). Thus, high resistance indices in this species have been observed and confirmed in our study. Similar results were also found in horses hospitalized in the United States (Castor et al. 1989, Hartmann & West 1995, Schott et al. 2001, Ward et al. 2005, Dunowska et al. 2006, Dallap Schaer et al. 2010, Cummings et al. 2014, Leon et al. 2018), the Netherlands (van Duijkeren et al. 2002), Australia (Amavisit et al. 2001), and Chile (Soza-Ossandón et al. 2020, Rivera et al. 2021), which showed high resistance to β-lactams, aminoglycosides, sulfonamides, tetracyclines, amphenicols, and quinolones. Diseased horses in Brazil (Ferreira 2019, Oliveira et al. 2019), the United States (Dargatz & Traub-Dargatz 2004), and Pakistan (Haq et al. 2018) showed high resistance to antibiotics of the β-lactam, aminoglycoside, sulfonamide, quinolone, tetracycline, lincosamide, and fluoroquinolone classes.

According to the World Health Organization and the European Medicines Agency, third- and fourth-generation cephalosporins, polymyxins, quinolones, and fluoroquinolones are critically important antimicrobials in human medicine, and their use in animals should be restricted to mitigate risks to public health. As in the present study, resistance to third-generation cephalosporins has also been described in isolates from horses with salmonellosis (Hartmann & West 1995, Dargatz & Traub-Dargatz 2004, Ward et al. 2005, Dunowska et al. 2006, Singh et al. 2007, Singh et al. 2009, Dallap Schaer et al. 2010, Cummings et al. 2014, Haq et al. 2018). In the current study, nine of the 13 multidrug-resistant strains harbored the gene related to the production of AmpC-type β-lactamase, which has been correlated with increased antimicrobial resistance to extended-spectrum cephalosporins (Rankin et al. 2005).

According to our results, the antimicrobials with the lowest resistance levels were amikacin, ceftiofur, ciprofloxacin, ceftriaxone, aztreonam, and imipenem. All 30 isolates of *Salmonella* spp. were 100% susceptible to the antibiotics aztreonam and imipenem. This result is important because carbapenems are the drugs of choice for severe infections caused by multidrug-resistant strains. Still, they currently represent the last options for treatment, and their use should be restricted to the hospital environment. The absence of new antibiotics against gram-negative bacteria and the emergence of resistance mechanisms that eliminate the therapeutic options available for treating infectious diseases caused by Enterobacteriaceae are the causes of serious public health problems worldwide. We must emphasize that the use of any antimicrobial should be rational, giving preference to drugs with a more restricted spectrum of action that is effective against the pathogens responsible for the infection whenever possible (Cummings et al. 2014, Leon et al. 2018). Other studies in horses have demonstrated the high sensitivity of *Salmonella* spp. to imipenem (Dargatz & Traub-Dargatz 2004, Dallap Schaer et al. 2010, Cummings et al. 2014).

The study identified 13 multidrug-resistant isolates (43.3%). A similar result in horses was found in Chile, with 45.45% of isolates being multidrug-resistant (Soza-Ossandón et al. 2020). Higher rates of multidrug-resistant isolates in horses were found in the United States, at 57% (Cummings et al. 2014), and in India, at 97% (Singh et al. 2007) or 75.7% (Singh et al. 2007, 2009). Lower rates in horses have been found in the Netherlands, at 13% (van Duijkeren et al. 2002), and in the United States, at 10.2% (Leon et al. 2018). Virtually all (12/13) of the multidrug-resistant isolates in the present study were found in foals with diarrhea. Although multidrug-resistant isolates are commonly reported in sick and hospitalized animals, they may also occur in healthy animals (Schott et al. 2001, Ward et al. 2005, Dallap Schaer et al. 2010, Cummings et al. 2014). The present study identified one multidrug-resistant isolate (11.11%) from a healthy animal belonging to serovar *S*. I.4.5.12:i:-, and a large study conducted in the United States showed that less than 5% of *Salmonella* spp. in the general population of healthy horses was resistant to the tested antimicrobials (Traub-Dargatz et al. 2000).

More reports of multidrug resistance have emerged among the serovars that cause clinical diseases (Cummings et al. 2014). Antibiotic-resistant serovars cause more severe infections, increase the risk of treatment failure, and limit treatment options. These facts increase the risk of failure in the treatment of animals and humans, representing a great concern for public health since most of the drug-resistant isolates of *Salmonella* spp. in humans are of animal origin (Cummings et al. 2014, Pan et al. 2018, Soza-Ossandón et al. 2020, Rivera et al. 2021). Infection with a resistant isolate is more likely to result in hospitalization and death than infection with a pansusceptible isolate (Cummings et al. 2014). Since animals are the main source of human contamination by *Salmonella* (Ferrari et al. 2019), the detection and early culling of animals with diarrhea or bacterial contamination in the environment allows the implementation of sanitary measures that effectively control the spread of infections between animals and zoonotic transmission.

Serotyping and antimicrobial susceptibility tests revealed the occurrence of 13 different serovars: *S*. Infantis, *S*. Minnesota, *S*. I.4,5,12:i:-, *S*. Anatum, *S*. Cerro, *S*. Oranienburg, *S*. Braenderup, *S*. Give, *S*. Newport, *S*. IIIb 61:c:z35, *S*. 109:-:1.5, *S*. I.4.12:d:-, and *S*. I.6.8:-:-, which presented 15 different phenotypic profiles of antibiotic resistance. The same farm presented isolates of *Salmonella* spp. with characteristics of pansusceptibility to multidrug resistance. In addition, the same serovar isolated from different animals on the same farm exhibited distinct antibiotic susceptibility profiles (Table 1). The tests also revealed that the same animal could harbor various *Salmonella* spp. with different antibiotic susceptibility profiles (Amavisit et al. 2001).

According to the number of classes of antibiotics, the serovars that presented the profile of resistance to most antimicrobials in our study were *S*. Minnesota, *S*. Newport, *S.* Oranienburg, and *S*. Infantis. With the exception of *S.* Minnesota, all serovars have already been described as resistant in studies in horses (Castor et al. 1989, Hartmann & West 1995, Dunowska et al. 2006, Dallap Schaer et al. 2010, Cummings et al. 2014, Leon et al. 2018). Bacterial culture and *in vitro* antimicrobial susceptibility testing of clinical cases are recommended to monitor the epidemiological characteristics of the farm.

The frequent implementation of these diagnostic tools will provide important information on the prevalence of the main pathogens and the characteristics of local antimicrobial resistance, guiding the clinician to choose the best treatment for an animal that shows clinical signs.

Developing rapid and reliable test methods for *S. enterica* in fecal samples is essential to facilitate infection control in horse populations. Enriched aerobic culture and PCR are currently the most widely used detection methods to identify *Salmonella* spp. DNA amplification using the PCR technique is a rapid detection tool with high sensitivity capable of detecting microorganisms at levels below those detected by culture, and this feature is valuable in the case of elimination of bacteria at low concentrations that are nonviable or when isolation is difficult (Cohen et al. 1993). Since its discovery, the invasion gene A (*invA*) has become the most frequently used gene for identification (Rahn et al. 1992, Gentry-Weeks et al. 2002, Mainar-Jaime et al. 2008, Pusterla et al. 2010, Wilkins et al. 2010, Ekiri et al. 2016) and is a specific marker for *Salmonella* spp. (Ekiri et al. 2016). PCR for the *invA* gene is considered a highly sensitive and specific technique for diagnosing salmonellosis in samples after selective enrichment (Rahn et al. 1992, Schrank et al. 2001, Malorny et al. 2003, Pusterla et al. 2010).

Notably, the present study was the first in Brazil to use qPCR to detect *Salmonella* spp. in fecal samples of foals. A pre-enrichment period in tetrathionate did not increase the detection of positive animals with the SYBR® qPCR and TaqMan® qPCR techniques compared to the SYBR® qPCR and TaqMan® qPCR tests with direct stool extraction. This study demonstrates the efficacy and applicability of qPCR using a fluorescent dye (SYBR® Green) and a specific probe (TaqMan®) for the *invA* gene without pre-enrichment in the tetrathionate step as a diagnostic tool for *Salmonella* spp. in clinical samples. Specifically, this method provides rapid results with high sensitivity and specificity to detect the agent in genetic material extracted directly from the feces of clinically diseased animals.

The need for pre-enrichment has limited the use of PCR as a rapid test for detecting *Salmonella* spp. in feces (Bohaychuk et al. 2007, Mainar-Jaime et al. 2008). Early detection of carrier animals, especially in hospitals, training centers, and breeding centers, is important for rapidly implementing control measures to reduce the risk of outbreaks (Palmer 1985, Spier 1993). In foals, in particular, the speed of obtaining results is important because implementing a therapy that prevents bacteremia early is imperative to promote higher survival rates. However, a concomitant bacterial culture is recommended to identify the sensitivity profiles.

The evaluation of the effectiveness of the diagnosis of *Salmonella* spp. in serial fecal samples from hospitalized animals with and without clinical signs of diarrhea by qPCR for the *invA* gene showed that the technique could be used in animals with or without clinical signs of gastrointestinal disease and can be used as a screening tool in animals with clinical signs of diarrhea (Ekiri et al. 2016). However, animals with positive qPCR results should have serial bacterial cultures of feces performed to obtain additional data, such as the antibiogram and serotype (Ekiri et al. 2016). Wilkins et al. (2010) compared qPCR and culture results. They found that qPCR had similar results to culture and can be considered a useful tool for screening many samples, particularly when the prevalence of *Salmonella* spp. is low. Again, the bacteriological culture of positive qPCR results should always be performed when serotyping or other analysis is needed (Wilkins et al. 2010).

SYBR® Green qPCR and TaqMan® qPCR with or without enrichment in tetrathionate identified more positive samples than bacterial culture (Schrank et al. 2001, Malorny & Hoofar 2005, Ward et al. 2005, Bohaychuk et al. 2007, Mainar-Jaime et al. 2008, Gal-Mor 2019). Bacterial culture identified 15% (n=30) of animals as positive, SYBR® Green qPCR with tetrathionate identified 22.5% (n=45) of animals as positive, TaqMan® qPCR with tetrathionate identified 22% of animals as positive (n=44), SYBR® Green qPCR without combination with the selective enrichment medium identified 24% (n=48) of animals as positive, and TaqMan® qPCR without selective enrichment identified 20% (n=40) of animals. The higher detection rate by qPCR than by bacterial culture may be due to the presence of nonviable microorganisms in the samples analyzed (Malorny & Hoofar 2005, Ward et al. 2005), the testing of one sample per animal (Kurowski et al. 2002), the treatment of animals with antimicrobials, the presence of asymptomatic carrier animals that may have shed lower amounts of bacteria in the feces (Palmer 1985, Cohen et al. 1993, Spier 1993, Ward et al. 2005), and the uneven distribution of bacteria in the samples (Ekiri et al. 2016).

For some samples, qPCR produced negative results, and bacterial culture produced positive results, as described in other studies conducted on horses (Ward et al. 2005, Chapman 2006, Mainar-Jaime et al. 2008, Wilkins et al. 2010). In a study to identify the elimination of *Salmonella* spp. in racehorses, six animals positive by culture were negative by PCR, which the author attributed to a longer freezing time that may have damaged the DNA of the samples or the presence of residual inhibitors in the stool samples (Chapman 2006). Another study, which identified a positive sample for serovar Mbandaka by bacterial culture but negative by PCR, attributed the result to the presence of PCR inhibitors (Mainar-Jaime et al. 2008). Unlike the serovars *S*. Anatum, *S*. Infantis, and *S*. Oranienburg isolates in this study, the serovars *S*. Cerro, *S*. Minnesota, *S*. I.4,5,12:i:- and *S. enterica* subsp. *enterica* 4.12:d:- were not included in the PCR standardization technique by Pusterla et al. (2010). Bacterial mutations may result in the deletion of genomic segments, and the loss of some virulence factors, such as the *invA* gene, may not be a terminal event for bacterial invasion. However, using this gene in PCR may not detect *invA* mutants of *Salmonella* spp. (Ginocchio et al. 1997, Chapman 2006). PCR based on the *invA* gene has failed to detect at least three serovars, *S*. Saintpaul, *S*. Litchfield, and *S*. Senftenberg (Rahn et al. 1992, Malorny et al. 2003). Amplicon loss has explained the nine negative PCR results for the *invE* and *invA* genes and positive results in the bacterial culture of feces (Gentry-Weeks et al. 2002). Thus, the results may be due to the presence of serovars in our study that were not included in the PCR and qPCR standardization techniques for *Salmonella* spp. in horses. These serovars could also have genetic characteristics that prevent detection by *invA*-targeted qPCR. These results confirm that the association of isolation and qPCR techniques could be useful for identifying *Salmonella* spp. in horse feces.

Study limitations. Among the limitations of this study is the use of antimicrobials in the animals before collecting samples for the bacteriological culture test. Of the animals that presented isolates of *Salmonella* spp., 16 (53.33%) have received antibiotic treatment. Of the 200 animals included in the study, 141 animals (70.5%) were not under treatment with antibiotics at the time of sample collection, whereas 59 animals (29.5%) had currently been receiving this treatment, and these animals were all classified as sick. Thus, 59% of the sick animals had already received antibiotic therapy before sample collection. The main antibiotics used were azithromycin + rifampicin (n=15), sulfamethoxazole + trimethoprim (n=13), metronidazole (n=12), amikacin (n=11), ceftiofur (n=10), gentamicin (n=4), penicillin (n=2), florfenicol (n=2), clarithromycin (n=1), and oxytetracycline (n=1). Another study limitation was the 11 months of freezing the samples at -80°C until the molecular biology techniques were performed. The long freeze may have influenced the ability of the selective enrichment medium tetrathionate to promote the multiplication and selection of bacteria, thus preventing the differential expression in the molecular biology tests in which it was used.

CONCLUSIONS

Salmonella spp., an important causative agent of diarrhea in young foals, should be included in the differential diagnosis of animals up to one year old with gastrointestinal disorders. Regarding age, 80% of the animals that were positive for *Salmonella* spp. were less than two months old, and 95.2-100% of the animals detected as positive by qPCR were less than six months old.

Bacterial culture revealed 15% (n=30) of animals as positive. *Salmonella* spp. was isolated more often from animals with diarrhea (21) than animals without diarrhea (nine). Serotyping revealed 13 different *Salmonella* spp. We identified serovars as important in enteric processes in humans and horses. Conversely, we also identified serovars that have not been described in horses. This study seems to be the first to mention these serovars in the context of the equine population in Brazil; their pathogenicity in this species is unknown.

The analysis of the antimicrobial susceptibility profile found multidrug resistance in 43.33% (n=13) of the isolates. The existence of multidrug-resistant serovars in the studied equine population, with a high potential for zoonotic transmission, emphasizes the importance of implementing biosecurity measures on the properties, especially by people who deal directly with the animals. This finding also supports the rational use of antimicrobials in animals to decrease selection pressure. Clinical and laboratory diagnosis of diseases should be promoted. Due to the uniqueness of some of the isolates, the antimicrobial resistance profile should also be monitored so that recommendations and effective treatments can be instituted in the animals. Most of the farms sampled do not isolate sick animals, and many of the animals considered healthy consequently had contact with the sick animals. This contact increases the risk of spread in the population, which may have contributed to the presence of isolates with resistance in the healthy animals of the study and hindered the implementation of adequate sanitary practices to control the infection.

All qPCR techniques used in the study identified more samples as positive for *Salmonella* spp. than the bacterial culture of feces, all qPCR techniques detected more positive animals in the diarrhea group than in the non-diarrhea group. SYBR® Green direct feces qPCR showed the highest agreement with the results obtained by bacterial culture, and it also yielded the fewest negative qPCR results in samples positive by bacterial culture.

The earlier detection of infection in a clinical sample from a given animal by qPCR, rather than by bacterial culture, together with the lack of need to pre-enrich the sample, enables qPCR tests to screen animals that present clinical symptoms of gastrointestinal illness. This strategy will be very important in foals with diarrhea, in which early diagnosis and initiation of therapy improve the prognosis. It will also allow the implementation of preventive measures more quickly and efficiently. Furthermore, culture and sensitivity evaluation remain important steps to guide the treatment of sick animals.

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