

Occurrence and antimicrobial resistance profile of *Salmonella* spp. in calves from the Mesoregion Sertão of Alagoas, Brazil

Ocorrência de *Salmonella* spp. em bezerros e perfil de resistência antimicrobiana na Mesorregião do Sertão de Alagoas, Brasil

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ABSTRACT: The diarrhea in calves, caused by *Salmonella* spp., provokes serious economic damage to cattle farming. This study aimed to verify the occurrence of diarrhea and the isolation of strains of *Salmonella* spp. in diarrheal feces of calves from farms located in the Sertão from the state of Alagoas municipalities, and to characterize the *in vitro* resistance profile of *Salmonella* spp. isolated against conventional antimicrobials. The study was carried out with 431 calves from 10 to 90 days old. Of the total number of animals evaluated, 111 presented a diarrheal table, being analysed 111 samples of diarrhoeal faeces. The samples were sown in enrichment broths and selective culture media and the phenotypic and molecular characterization were performed. Among the animals evaluated, 25.75% (111/431) had diarrhea. The presence of *Salmonella* spp. was evidenced in 13.33% (2/15) of the studied establishments. Have been isolated 2.71% (3/111) strains of *Salmonella* spp. and have all been shown to be resistant to Cefotaxime and sensitive to Ciprofloxacin, Gentamicin, Amoxicillin, Ampicillin and Norfloxacin. *Salmonella* spp. and other infectious agents associated with diarrhea were confirmed in calves in the Sertão Alagoas mesoregion. The antimicrobial potential of the tested drugs reinforces the importance of their responsible and judicious use in the treatment of Salmonellosis, not being indicated the antibiotic therapy with Cefotaxima. The treatment based on Gentamicin, Amoxicillin, Ampicillin, Ciprofloxacin and Norfloxacin in the treatment of these animals, is recommended.

KEYWORDS: antibiogram, cattle, diarrhea, enteropathogens, Salmonellosis.

RESUMO: Objetivou-se verificar a ocorrência da diarreia e o isolamento de estirpes de *Salmonella* sp. em fezes diarreicas de bezerros provenientes de propriedades de municípios do Sertão Alagoano e caracterizar o perfil de resistência *in vitro* das estirpes de *Salmonella* spp. isoladas, frente a antimicrobianos convencionais. O estudo foi realizado com 431 bezerros com 10 a 90 dias de idade, sendo analisadas 111 amostras de fezes diarreicas, semeadas em caldos de enriquecimento e meios de cultura seletivos e feita caracterização fenotípica e molecular. 25,75% (111/431) dos animais apresentavam diarreia. A presença de *Salmonella* sp. foi evidenciada em 13,33% (2/15) dos estabelecimentos estudados. Foram isoladas 2,71% (3/111) estirpes de *Salmonella* sp. que revelaram-se em sua totalidade resistentes à Cefotaxima e sensíveis a Ciprofloxacina, Gentamicina, Amoxicilina, Ampicilina e à Norfloxacina. Confirmou-se a ocorrência de *Salmonella* sp. e outros agentes infecciosos associados a diarreia em bezerros no Sertão Alagoano. O potencial antimicrobiano das drogas testadas reforça a importância da utilização responsável das mesmas no combate a Salmonelose nesses animais, promovendo assim a minimização de casos de resistência bacteriana no Brasil e no mundo.

PALAVRAS-CHAVE: antibiograma, gado, diarreia, enteropatóticos, Salmonelose.

INTRODUCTION

In the state of Alagoas, Brazil, the bovine herd in 2015 reached 1,255,696 heads, of which 38.4% were in the East Mesoregion, 34.4% in the Agreste area and 27.2% in the

Sertão, highlighting the region of the Sertão for its dairy activity in the state, as an elementary source and relevant in the socioeconomic pattern (SEBRAE, 2013; BRASIL, 2016; ALAGOAS, 2017).

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Many disorders can interfere with the cattle production chain during the early age such as can compromise the development and productivity of these animals as adults (Borrielo *et al.*, 2012; Assis-Brasil *et al.*, 2013).

Diarrhea in calves often occurs due to infection with *Salmonella* spp., which is a bacterium of great zoonotic potential and considered one of the important gastrointestinal pathogens related to foodborne diseases, defined by Salmonellosis (Pereira *et al.*, 2004; Assis-Brasil *et al.*, 2013). The diarrhea caused by Salmonellosis determines serious economic losses to cattle farming, through high mortality rate, decrease in production and high cost of treating sick animals (Borrielo *et al.*, 2012; Strockbine *et al.*, 2015).

Antibiotic therapy is the main measure used by producers to combat diarrhea in calves. However, for the effective treatment of animals with diarrhea by Salmonellosis, it is recommended to previously perform the isolation and identification of these agents in the laboratory, as a definitive proof in the diagnosis of the disease, complemented with in vitro antimicrobial sensitivity analysis (antibiogram), and consider the judicious use of antibiotics (Mota *et al.*, 2005; Marques *et al.*, 2013).

The indiscriminate use of antibiotics in the treatment of animals leads to a gradual increase in antimicrobial resistance in microorganisms of their normal microbiota and pathogenic bacteria causing disease, which increases the number of cases of multi-resistant bacteria to antimicrobial drugs and highlights the seriousness of the shortage period and the non-commercialisation of products of animal origin during that time. The development of resistance by some strains of the genus *Salmonella* spp. and other pathogenic bacteria is faster than the industry's ability to produce new effective drugs for the treatment (Mota *et al.*, 2005; Cades *et al.*, 2017).

Even with the significant losses triggered by diarrhea in cattle breeding, there is no research in Alagoas, and specifically in the Sertão of state, which aims to identify the main enteropathogens related to the disease in calves. In Brazil, according to Salvatori *et al.*, (2003), research is considered to be scarce, and more studies on the subject are needed. The objective was to verify the occurrence of diarrhea and isolation of strains of *Salmonella* spp. in diarrheal calf feces from properties of municipalities in the Sertão Alagoano and to characterize the in vitro resistance profile of isolated strains of *Salmonella* spp. against conventional antimicrobials.

MATERIAL AND METHODS

This research was approved by the Ethics Committee on the Use of Animals of the Universidade Federal de Alagoas (CEUA-UFAL) with protocol number 65/2017. The study was performed in 15 properties in the backwoods from the state of Alagoas, Brazil, on the municipalities of Água Branca, Batalha, Delmiro Gouveia, Jacaré dos Homens, Jaramataia, Monteirópolis, Pariconha and São José da Tapera; with a total

of 431 calves with 10 to 90 days old, where 111 had diarrhea. The sample size was determined from the proportion calculation at Openepi (Dean *et al.*, 2019). For the calculation, a population of 5,274 calves from zero to 12 months was considered, according to 2019 data provided by the Defesa Agropecuária do Estado de Alagoas (ADEAL), with precision of 4.5%, confidence interval of 95% and an anticipated frequency of 50%, because the actual diarrhea situation is unknown in the region. The collections were made by means of rectal swab on symptomatic animals, under strict asepsis and antisepsis rules. After being collected, they were introduced into sterile test tubes containing Stuart Transport Medium (HiMedia Laboratories), and transported to the Microbiology laboratory of the Universidade Federal de Alagoas, under refrigeration in a hermetically sealed container at 8°C.

The samples were submitted to the *Salmonella* spp. isolation protocol, being sown in selective enrichment broths Rappaport-Vassiliadis and Tetrathionate and incubated for 24 hours at 37°C. Later, aliquots of the respective broths were sown in Petri dishes containing MacConkey Agar, Bright Green Agar, Enteric Hektoen Agar and an exclusively for differential isolation of *Pseudomonas aeruginosa*. Thus, the samples were sown in Cetrimide Agar, and placed in a bacteriological oven at 37°C for 24 hours, as recommended by Andrews *et al.*, (2016). The media and broths used for the isolation stages originated from HiMedia Laboratories.

The isolated colonies were morphologically and phenotypically characterized by biochemical tests: Triple iron sugar, Indol production, motility, H₂S production, methyl red, Voges-Proskauer, citrate, urease, lysine decarboxylase, lactose, glucose, dulcitol, mannitol, gas production and sucrose (Andrews *et al.*, 2016).

The molecular identification of *Salmonella* spp. samples started with the extraction of the total DNA from bacteria using the QIAamp Fast DNA Stool Kit (Qiagen), according to the protocol provided by the manufacturer. The extracted material was then submitted to a PCR reaction, following the protocol described by Rall *et al.*, (2009), using the following primer pair: invA1: TCATCGCACCGTCAAAGGAAC and invA2: GTGAAATTATCGCCACGTTCCGG. In all PCR reactions a positive and a negative control were added, being provided courtesy of the Pathology Laboratory of FMVZ-UNESP. For the amplification analyses, Agarose Gel Electrophoresis (1.5%) was performed. The in vitro susceptibility of strains of *Salmonella* spp. confirmed by biochemical and molecular evaluation against conventional antibiotics was evaluated by the diffusion disc technique on plates containing Mueller Hinton Agar according to CLSI (2005). The following antimicrobials were used: ampicillin 30 mcg, tetracycline 30 mcg, norfloxacin 10 mcg, gentamicin 10 mcg, polymyxin 300UI, cefalexin 30 mcg, amoxicillin 30 mcg, ciprofloxacin 05 mcg, cefoxitin 30 mcg and cefotaxime 30 mcg. The plates were incubated at 37°C and

analyzed after 24 hours. The results were interpreted according to CLSI (2005).

The research data were statistically analyzed through the study of frequencies, absolute and relative.

RESULTS AND DISCUSSION

We evaluated 431 calves with ages ranging from 10 to 90 days. From these, 25.75% (111/431) had diarrhea and the presence of *Salmonella* spp. was evidenced in 2.71% (3/111). Of the properties visited, 13.33% (2/15) were positive for *Salmonella* spp., more precisely in the neighbours municipalities of Monteirópolis and Jacaré dos Homens, of which one property presented more than one positive calf. The existence of diarrhea from *Salmonella* spp. was also reported in 36% (18/50) of the properties in the study by Bilbao *et al.*, (2018) carried out in the Mar y Sierras dairy basin in Argentina, where eight establishments presented more than one positive calf.

The results obtained could be exponential, if the performance of periodic sampling were increased. Based on the research by Fossler *et al.*, (2004), who followed 110 establishments in the United States, taking different faecal samples from healthy cows, calves and samples of milk, water and feed supplied to the animals, in their five visits during the one-year period, the prevalence reached 90% for *Salmonella* spp.

The reference method for making a definitive diagnosis of Salmonellosis is microbiological isolation from stool samples. The recommended tests for the isolation of *Salmonella* spp. and other enteropathogens are based on pre-enrichment steps, enrichment in selective broths, plating in specific semi-solid culture media, followed by phenotypic characterization of suggestive colonies in biochemical tests (Marques *et al.*, 2013; Machado *et al.*, 2017). The investigation and bacterial isolation of diarrheal stool samples collected, through rectal swab, from symptomatic calves are evidenced in the results of analysis and phenotypic characterizations by means of biochemical tests, in Table 1.

From the processed stool samples, 2.71% (3/111) of *Salmonella* spp. were isolated, and it should be noted that this result was obtained on properties with no known history of Salmonellosis in the animals and no history of occurrence of the disease in calves in the region of the outbreak. Bilbao *et al.*, (2018) detected in 726 symptomatic and asymptomatic calves a prevalence of 5.5% (40/726) of *Salmonella* spp. in establishments, which, like the properties of the Sertão Alagoas, were located in an important milk producing region. It is worth noting that the author did not attribute the positivity and susceptibility of the animals studied to factors, such as: race, breeding system, temperature or geographical conditions. There was conformity between the studies in the age group, where the isolates of *Salmonella* spp. also came from feces of calves under 90 days of age. Marques *et al.*, (2013), in a Salmonellosis outbreak in calves reported on a farm in the municipality of Timon, Maranhão, revealed that of a total of 62 calves, 35.5% (22/62) fell ill and of these 40.9% (9/22)

died. The analysis of mortality in Alagoas calves was not performed, because the sick animals included in the study were kept alive.

In the investigation of possible triggers factors of diarrhea in calves, the identification of the causative agent is of notable relevance. *Salmonella* spp. and *E. coli* are the most commonly identified pathogens in calves under 3 months of age (Achá *et al.*, 2004; Izzo *et al.*, 2011).

The occurrence of *Samonella* spp. in the properties add scientific innovation to the detriment of the absence of data associated with the presence of the etiological agent in the Sertão from the state of Alagoas' mesoregion. Awosile *et al.*, (2018) found a frequency of 3.3% (16/488) enteric *Salmonella* in calves in New Brunswick, Canada, as well as the important finding made by El-Seedy *et al.*, (2016), finding an 18.1% (23/127) prevalence of *Salmonella* spp. in calf faeces with diarrhea corroborating for relevance among the prevalence and cosmopolitan potential of the bacterium. In contrast, Izzo *et al.*, (2011), that in quantify the occurrence of the main enteric pathogens in dairy calves with diarrhea on farms in Australia, obtained 23.8% (142/597) of positivity for *Salmonella* spp.

The differences of prevalence rates of diarrheal calves due to Salmonellosis compared with previous studies can be explained for reasons such as: inadequate hygiene measures, especially with regard to the management of faeces, the non-separation of the animals by age and keeping the sick in contact with the healthy. The correction of these factors will significantly influence the prevalence of the disease in calves (Younis *et al.*, 2009; Al Mawly *et al.*, 2015).

The diarrhea in calves, mainly when caused by *Salmonella* spp. remains as one of the most frequent and important problems faced by these animals, and only the adoption of prophylactic practices are able to avoid the animals from being affected and thus minimize the economic losses. Taking into consideration the adequate nutritional management is determinant for the decrease of sick animals in the herd. The highest rates of mortality and morbidity due to Salmonellosis occur in

Table 1. Number and percentage of enteropathogens isolated from diarrheal calf faeces on properties in the Sertão from the state of Alagoas, Brazil, 2019.

Agents	Samples	
	Number	Percentage (%)
<i>Salmonella</i> spp.	3	2,71
<i>Enterobacter aerogenes</i>	3	2,71
<i>Klebsiella</i> spp.	4	3,60
<i>Serratia odorifera</i>	6	5,40
<i>Escherichia coli</i>	47	42,34
<i>Pseudomonas aeruginosa</i>	17	15,31
Other enteropathogens	31	27,93
Total	111	100,0

Table 2. Antibiogram of the three samples of *Salmonella* sp. isolated from diarrheal faeces of calves from properties of the backwoods from the state of Alagoas, against ten conventional antimicrobials.

Samples	Antimicrobials agents									
	CTX	CFE	GEN	CFO	POL	AMO	TET	AMP	CIP	NOR
1	R	S	S	S	S	S	S	S	S	S
2	R	R	S	R	R	S	R	S	S	S
3	R	R	S	S	S	S	S	S	S	S

CTX = cefotaxime ; CFE = cephalixin ; GEN = gentamicin ; CFO = cefoxitin ; POL = polymyxin ; AMO = amoxicillin ; TET = tetracycline ; AMP = ampicillin ; CIP = ciprofloxacin ; NOR = norfloxacin ; R = resistant ; S = sensible.

the first months of life and the incidence of diarrhea decreases with age (Assis-Brasil *et al.*, 2013; Da Silva *et al.*, 2019).

The three isolated strains of *Salmonella* spp. were confirmed by molecular diagnosis type PCR, producing approximately 284bp each, when Machado *et al.*, (2017), using the same primers that also confirmed the presence in seven isolated samples of chicken carcasses, demonstrating the hazards when zoonoses and consumption of contaminated food are involved.

The admission of the antibiogram as a primary factor for the establishment of the therapeutic protocol favors the reduction of the inefficiency rates of the antimicrobial. According to Marques *et al.*, (2013), who reported the importance of previous accomplishment and priority of antibiogram, verifying the levels of antimicrobial resistance, based on an outbreak of Salmonellosis in calves on a farm in the state of Maranhão, Brazil.

The antibiogram of the three samples of *Salmonella* spp. from the diarrheal calves mentioned above, against ten conventional antimicrobials, revealed sensitivity of the three bacterial samples to 50% (5/10) of the drugs tested and resistance to 10% (1/10), as shown in Table 2.

Given that all strains of *Salmonella* spp. have been shown to be resistant to cefotaxime and sensitive to ciprofloxacin, the report of Arias and Carrilho (2012), which showed sensitivity to ciprofloxacin, pointing out the difference only in the action of cefotaxime where its isolates of *Salmonella* spp. showed sensitivity.

Similarly, the absolute sensitivity to Gentamicin, Amoxicillin, Ampicillin and Norfloxacin is noted. In contrast to the results of this research, in Chile, Claude *et al.*, (2017) found in newborn calves the infection with multi-resistant *Salmonella*, and warns that in the suspicion of *Salmonella* spp., the use of amoxicillin should be avoided due to the resistance presented.

The second and third samples of *Salmonella* spp. showed resistance to cefalexin, where only sample two were resistant to cefoxitin, polymyxin and tetracycline. Mulvey *et al.*, (2009) detected that 2.1% of the animals in the study served cefoxitin-resistant enterobacteria. The second and third samples of *Salmonella* spp. showed resistance to cefalexin, only sample 2 being resistant to cefoxitin, polymyxin and tetracycline. Mulvey *et al.*, (2009) found that 2.1% of the animals in the study had enterobacteria resistant to cefoxitin and could be attributed to the indiscriminate use of antimicrobial drugs in the treatment of disease.

The evidence of the large number of enterobacteria in the analyzed samples, such as: *Enterobacter aerogenes*, *Klebsiella* sp., *Serratia odorifera*, *Escherichia coli*, *Pseudomonas aeruginosa* and others enteropathogens, alerts for the presence of different infectious agents involved in diarrhea. Awakening to the high mortality rates in calves, Assis-Brasil *et al.*, (2013) asserted that among the main causes of death of these animals are associated with respiratory and enteric conditions, corroborating with the potential risk expressed in the results of 15.31% to *Pseudomonas aeruginosa* and 84.69% to other enteropathogens isolated in the study.

CONCLUSION

Salmonella spp. and other infectious agents associated with diarrhea were confirmed in calves in the Sertão Alagoas mesoregion. The antimicrobial potential of the tested drugs reinforces the importance of their responsible and judicious use in the treatment of Salmonellosis, not being indicated the antibiotic therapy with Cefotaxima. The treatment based on Gentamicin, Amoxicillin, Ampicillin, Ciprofloxacin and Norfloxacin in the treatment of these animals, is recommended.

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