

## Research article

# ENTEROBACTERIA ISOLATED FROM DIARRHEIC CALVES AND THEIR PHENOTYPIC RESISTANCE PATTERN

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Diarrhea is a prevalent and economically impactful ailment in cattle herds, resulting in substantial losses attributed to mortality, treatment expenses, and impaired calf growth. The aim of this study was to establish data about the occurrence of the main bacterial agents involved in diarrhea in dairy calves in the state of Maranhão, as well as to determine the most effective and/or resistant antibiotics, establishing a foundation for treatment and prevention protocols. The samples were collected with rectal swabs and initially cultivated on sheep blood agar (5%) and MacConkey agar and later on *Salmonella-Shigella* agar and eosin-methylene blue agar. Gram staining, biochemical tests, and antibiograms were performed on the obtained colonies. Ten municipalities were studied, with 230 calves evaluated from ten dairy farms. A total of 21 animals exhibited clinical signs of diarrhea, representing a frequency of 9.13%. The frequency of positive farms was 35%, and in 50% of municipalities. Bacteria isolated belonged to the *Enterobacteriaceae* family, with 71.4% *Escherichia coli*, 14.3% *Enterobacter* sp., 9.5% *Proteus* sp. and 4.8% *Klebsiella* sp. On the antibiogram, the bacteria that showed the highest resistance levels were those of the *Proteus* genus, followed by *Enterobacter* sp. and *E. coli*. The bacteria found are relevant for both animal and human health due to their zoonotic potential and serve as a public health alert since the isolates in this study showed in vitro resistance to several antibiotics, which predisposes them to the emergence of multidrug-resistant bacteria.

**Keywords** Diarrhea; Dairy Farms; *Enterobacteriaceae*; Antibiogram; Multidrug resistance.

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## INTRODUCTION

Diarrhea is one of the most common diseases affecting cattle herds, causing significant economic losses, both due to mortality and treatment expenses, and delays in the growth of calves [1]. It is a common clinical sign in young calves, triggered by several pathogenic microorganisms and including noninfectious factors, such as immunity, nutritional status, hygiene conditions, environmental contamination and contact between healthy and infected animals.

Calves afflicted with diarrhea typically exhibit feces with a consistency that can vary from pasty to watery, which may or may not have a foul odor, with or without blood streaks and alterations in color. In the initial phase, fever, lack of appetite, depression, drooping ears, dehydration, rapid weight loss, and soiling of the perineal region and/or tail. Mortality is often not directly attributable to the pathogen, but rather to severe dehydration and loss of electrolytes can lead to death [2].

Bacteria of the family *Enterobacteriaceae*, including *Escherichia coli* and *Salmonella* spp., are frequently implicated in this calf syndrome. Their pathogenic effects stem from the local and systemic action of enterotoxins and endotoxins, as well as inflammation induced by the etiological agent and subsequent atrophy of intestinal villi [3,4].

Neonatal diarrhea treatment typically involves the use of antimicrobials, nonsteroidal anti-inflammatory drugs (NSAIDs), and supportive therapy comprising fluid administration to restore energy, fluids, and electrolytes [5]. Some broad-spectrum antimicrobials are commonly used indiscriminately in farms, leading to the emergence of bacterial resistance [6].

This syndrome also constitutes a public health alert, since failures in management or inadequate treatments may predispose human infection due to the zoonotic potential of certain bacterial agents, which can cause foodborne infections in humans, in addition to predisposing the emergence of resistance of those pathogens [7].

Thus, knowledge about the etiology of this syndrome is important, given its global prevalence, including within Brazil, since this study aims to establish data on the occurrence of bacterial agents associated with diarrhea in dairy calves in the state, as well as identify the most effective and/or resistant antibiotics, establishing a basis for effective treatment and prevention protocols, decreasing expenses and the impacts of diarrhea in calf production.

## MATERIAL AND METHODS

### Study area and sample collection

The present study was carried out in partnership with the State Health Defense Agency, focusing on small dairy farms across ten municipalities situated in Maranhão, northeastern Brazil. A total of 230 animals were initially submitted to clinical

examination, specifically for checking the mucous membrane color, degree of dehydration and alteration in feces, used to identify positive and negative animals for diarrhea.

Fecal samples were directly collected from the rectal ampoule of the calves utilizing sterile swabs immersed in 0.9% saline solution. Subsequent evaluation included assessing fecal consistency, color, odor, and the presence of blood.

### **Isolation, phenotypic characterization, biochemical tests and antibiogram**

The samples were inoculated on plates containing sheep blood agar (5%) and MacConkey agar, incubated at 37°C under aerobic conditions and evaluated at 24 and 48 hours. Colony identification was based on colonial morphology and Gram stain reaction, along with biochemical testing using Triple Sugar Iron (TSI), Simmons Citrate, Lysine Iron Agar (LIA), Urease, Sulfide Indole Motility (SIM), Methyl red (MR), and Voges-Proskauer (VP).

Subsequently, the enteropathogens were submitted to a microbial sensitivity test with the antibiotics selected based on the available drugs indicated for diarrhea in calves. The antibiotics utilized were tetracycline (30 µcg), streptomycin (10 µcg), penicillin G (10 IU), gentamicin (10 µcg), amoxicillin + clavulanate (30 µcg), florfenicol (30 µcg), erythromycin (15 µcg), amoxicillin (10 µcg), ceftiofur (30 µcg), enrofloxacin (5 µcg), cephalexin (30 µcg), and cotrimoxazole (25 µcg, trimethoprim-sulfamethoxazole) from Sensifar-Vet/Cefar Diagnostic.

Following inoculation, the plates were incubated for 18 to 24 hours, and the diameters of the inhibition zones surrounding each antibiotic disc were measured. This measurement was carried out in accordance with the guidelines set forth by the Clinical and Laboratory Standards Institute [8], aiming to determine the sensitivity of the enteropathogens to the specific antibiotics.

### **Statistical analysis**

Epidemiological aspects concerning the occurrence of diarrhea were studied, including distribution by municipalities and properties, age, sex, probable etiology, and macroscopic fecal characteristics, defined through clinical information (history and physical examination) based on the data obtained through a checklist. The acquired data were subjected to analysis using the Epi Info statistical software.

### **Ethical Approval**

This study was approved by the Ethics and Animal Experimentation Committee - CEEA of the Veterinary Medicine Course at UEMA, according to protocol no. 30/2020.

## RESULTS

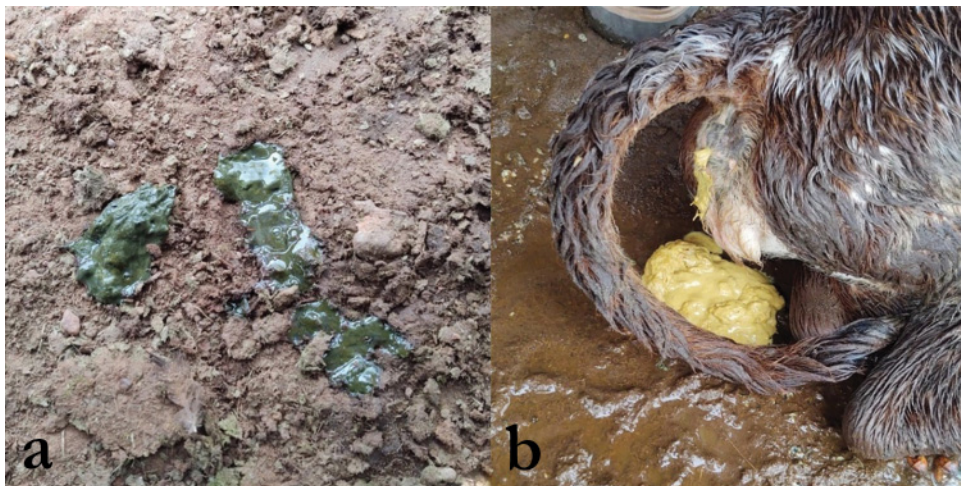
Among the 230 calves examined, 9.13% (n=21) showed clinical signs of diarrhea. The prevalence of positive cases in dairy farms was 35% (n=7), while the prevalence in municipalities was 50% (n=5). The age of positive animals ranged from 7 to 90 days, with calves younger than two months of age being more affected, mostly female (Table 1).

**Table 1.** Frequency of sex and age group of calves with diarrhea

| Variables     | n  | %  |
|---------------|----|----|
| Female        | 13 | 62 |
| Male          | 8  | 38 |
| < 1 month     | 5  | 24 |
| 1 to 2 months | 9  | 43 |
| 2 to 3 months | 5  | 24 |
| > 3 months    | 2  | 9  |

n= number %= percentage

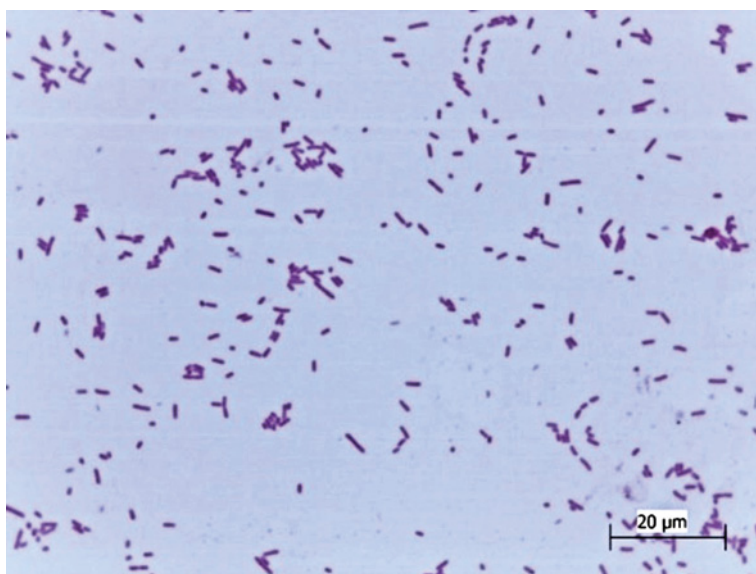
The majority (67%, n=14) of diarrheic feces exhibited a watery consistency, while 38% were pasty. The color distribution showed brown (48%, n=10) as the most common, followed by yellow (33%, n=7) and green (19%, n=4) hues (Figure 1). Additionally, a characteristic foul odor was detected in 81% (n=17), while no presence of blood was observed in any of the cases.



**Figure 1.** Liquid and greenish feces (A) and pasty and yellowish feces (B) obtained from the studied calves

Although the calves had diarrhea, their behavior remained normal during the examination with no severe signs of dehydration or other clinical signs of systemic infection. As they were dairy animals, they were reared in a semi-intensive system, but the place where they remained for most of the time did not have adequate hygiene conditions, containing a lot of mud and accumulated feces, and the calves were crowded together.

The growth of colonies on plates and the results of the biochemical tests revealed Gram-negative rod-shaped bacteria belonging to the *Enterobacteriaceae* family (Figure 2) with 71.4% (n=15) *Escherichia coli*, 14.3% (n=3) *Enterobacter* sp., (n=2) 9.5% *Proteus* sp. and 4.8% (n=1) *Klebsiella* sp.



**Figure 2.** Reaction to the Gram stain of bacteria of the *Enterobacteriaceae* family showing Gram-negative rods

It was not possible to establish a pattern that could correlate the macroscopic findings of the feces with their etiological agent, as animals affected by the same agent exhibited varying fecal characteristics (Table 2). Among *E. coli* cases, the majority displayed a watery consistency, followed by a pasty texture, along with the presence of all three colorations. Additionally, most of these cases emitted a noticeable odor.

In comparison, *Enterobacter* sp. cases also showed a predominance of watery consistency, followed by pasty texture, and presented all three colorations. These cases were predominantly brown in color and emitted an odor. For *Klebsiella* sp., the feces exhibited a pasty consistency, were yellowish in color, and emitted an odor.

Table 2. Frequency of fecal characteristics related to bacteria found in calves with diarrhea

| Fecal characteristics | <i>Escherichia coli</i> | <i>Klebsiella sp.</i> | <i>Proteus sp.</i> | <i>Enterobacter sp.</i> |
|-----------------------|-------------------------|-----------------------|--------------------|-------------------------|
| Watery                | 67 % (n=10)             | -                     | 100 % (n=2)        | 67 % (n=2)              |
| Pasty                 | 33 % (n=5)              | 100% (n=1)            | -                  | 33% (n=1)               |
| Yellowish             | 33 % (n=5)              | 100% (n=1)            | -                  | 33.3% (n=1)             |
| Greenish              | 20% (n=3)               | -                     | -                  | 33.3% (n=1)             |
| Brown                 | 47% (n=7)               | -                     | 100 % (n=2)        | 33.3% (n=1)             |
| Odor Present          | 80% (n=12)              | 100% (n=1)            | 50 % (n=1)         | 100 % (n=2)             |
| Odor Absent           | 20% (n=3)               | -                     | 50 % (n=1)         | -                       |
| Blood Present         | -                       | -                     | -                  | -                       |
| Blood Absent          | 100%                    | 100%                  | 100%               | 100%                    |

For *Proteus* sp., all cases exhibited a watery consistency and appeared brown in color. Half of the cases emitted an odor, while the other half lacked any discernible odor. Furthermore, none of the cases indicated the presence of blood.

By establishing a pattern to correlate each bacterial species with their respective antibiogram results, the presence of multidrug resistance was evident. Among these, the genus *Proteus* showed the highest number of resistance to antibiotics, being resistant to seven of the 12 antibiotics tested, followed by *Enterobacter* sp. and *E. coli*. In addition, all genes showed resistance to penicillin (Table 3).

Table 3. Antibiogram results for each genus of bacteria isolated from calves with diarrhea.

| Antibiotics | <i>Escherichia coli</i> (%) |     |      | <i>Klebsiella sp.</i> (%) |     |     | <i>Proteus sp.</i> (%) |   |     | <i>Enterobacter sp.</i> (%) |      |      |
|-------------|-----------------------------|-----|------|---------------------------|-----|-----|------------------------|---|-----|-----------------------------|------|------|
|             | R                           | I   | S    | R                         | I   | S   | R                      | I | S   | R                           | I    | S    |
| AMC         | 6.7                         | -   | 93.3 | -                         | -   | 100 | 100                    | - | -   | 66.7                        | -    | 33   |
| AMO         | 33.3                        | -   | 66.7 | -                         | -   | 100 | 100                    | - | -   | 66.7                        | -    | 33   |
| CEF         | 6.7                         | 6,7 | 86.6 | -                         | -   | 100 | 100                    | - | -   | 33.3                        | 33.3 | 33.3 |
| CFT         | 6.7                         | -   | 93.3 | -                         | -   | 100 | -                      | - | 100 | -                           | -    | 100  |
| ENO         | -                           | -   | 100  | -                         | -   | 100 | -                      | - | 100 | -                           | -    | 100  |
| ERI         | 26.7                        | -   | 73.3 | -                         | 100 | -   | 100                    | - | -   | 33.3                        | 66.7 | -    |
| EST         | 6.7                         | -   | 93.3 | 100                       | -   | -   | -                      | - | 100 | -                           | -    | 100  |
| FLF         | 13.3                        | -   | 86.7 | -                         | -   | 100 | -                      | - | 100 | -                           | -    | 100  |
| GEN         | -                           | -   | 100  | -                         | -   | 100 | 100                    | - | -   | -                           | -    | 100  |
| PEN         | 100                         | -   | -    | 100                       | -   | -   | 100                    | - | -   | 100                         | -    | -    |
| SUT         | 6.7                         | -   | 93.3 | -                         | -   | 100 | -                      | - | 100 | -                           | -    | 100  |
| TET         | 60                          | -   | 40   | -                         | -   | 100 | 100                    | - | -   | -                           | -    | 100  |

AMC – Amoxicillin + Clavulanato; AMO – Amoxicillin; CEF – Cephalexin; CFT – Ceftiofur; ENO – Enrofloxacin; ERI – Erythromycin; EST – Streptomycin; FLF – Florfenicol; GEN – Gentamicin; PEN – Penicillin G; SUT – Cotrimoxazole; TET – Tetracyclin. S – Sensitivity; R – Resistance; I - Intermediary.

## DISCUSSION

The frequency of diarrhea found in this study is in line with Wilson et al. [9] when addressing that studies report occurrence between 5-23% of calves were affected in all production systems.

Furthermore, the higher frequency between females than males contrasts with the findings of Monney et al. [10], who reported a lower frequency of calf diarrhea in females. This discrepancy could be attributed to the demographic composition of the dairy herds under evaluation, where the number of female calves was significantly greater than that of males, as in the milk-cattle production system, males are commonly sold early, after birth, while females are used to replace herds. In addition, they found higher diarrhea frequencies among calves aged one to two months compared to those aged three months.

Hygienic management failures increase the possibility of the entry of infectious agents causing diseases, especially diarrhea. In situations when animal density is high, there is accumulation of organic matter, excessive humidity and animals of different age groups in the same facility, which determines a greater risk of contamination and a greater probability of occurrence of clinical cases or outbreaks [4].

From *E. coli* isolates, Singh et al. [11] and Mohammed et al. [12] found frequencies of 62.82% and 46.42%, respectively, in fecal samples from diarrheic calves. This work presented a lower frequency when compared to other similar studies; despite this, this bacterium was the main cause of diarrhea in dairy calves among the studied municipalities.

Additionally, colibacillosis is a very important disease in neonatal calves, and its occurrence may be associated with different facts, resulting in high mortality rates in the herd [12]. In addition, some serotypes of *E. coli* have been described as important food pathogens, in which cattle are considered the major reservoirs and main cause of human infection [13].

For *Enterobacter* sp., the results were close to those of Meshref et al. [14], whose frequencies were close to 6%. *Klebsiella* sp. species are not commonly identified as the main cause of diarrhea in calves, and other studies report its frequency in fecal samples, such as Meshref et al. [14], who found a frequency of 8.71%. This species is a commensal microorganism in the digestive system of animals; therefore, environments contaminated with feces are considered sources of infections for young ruminants [15]. Having found this genus in this study may be mainly associated with the hygienic-sanitary condition of the facilities where the calves were housed and the low immunity of these young animals.

The frequency of *Proteus* sp. observed in this study was higher than the findings of Singh et al. [11], who reported a frequency of 3.84%. It was also closer to the frequency reported by Hasson et al. [16] for enteric isolates from calves with diarrhea, at 5.4%.

The findings correlating bacteria and fecal scores suggest that different bacterial agents are associated with varied fecal characteristics, highlighting the complexity of the relationships between etiological agents and observable symptoms. Further investigation is warranted to elucidate the underlying mechanisms driving these associations.

The resistance of the bacteria found in this research may be justified based on the indiscriminate use of antibiotics in the dairy farms studied. This phenomenon mainly occurs due to the widespread use of broad-spectrum antibiotic drugs, which have ineffective mechanisms of action against certain bacteria, thereby promoting the emergence of resistant strains. Furthermore, resistance can also be explained by the production of enzymes that confer resistance to certain beta-lactam antimicrobials, including cephalosporin and penicillin [17].

In addition to becoming an obstacle in the treatment and control of calf diarrhea, bacterial resistance is also considered a public health threat. The inappropriate management and administration of antibiotics can lead to human infections. It is important to note that these bacteria have zoonotic potential, which is commonly associated with hospital infections caused by multidrug-resistant (MDR) bacteria [18].

The genus *Proteus*, as observed by Zappa et al. [17], exhibited resistance against the antimicrobials cefalexin and amoxicillin + clavulanate, which aligns with the findings of this study. However, they also reported resistance against ceftiofur and sulfamethoxazole + trimethoprim, in contrast to the bacteria studied in the present work. *E. coli* and *Klebsiella pneumoniae* quickly developed resistance mechanisms against new antibiotics, mainly by the formation of biofilms, providing them with the ability to resist the host's immune system and antimicrobials [19].

## CONCLUSIONS

It was possible to perceive that diarrhea was present in the studied dairy calves, emphasizing the importance of studying this syndrome. Half of the municipalities had positive animals for diarrhea, which suggests the immunological vulnerability of calves due to inadequate hygiene conditions and facilities in these locations, confirming the need to implement veterinary technical assistance to establish effective control and prophylaxis measures to avoid the spread of diseases.

The bacteria found are relevant for both animal and human health due to their zoonotic potential and serve as a public health alert since the isolates in this study showed *in vitro* resistance to several antibiotics, which predisposes them to the emergence of MDR bacteria.

Therefore, for the treatment of diarrhea in calves, it is recommended to carry out a precise clinical investigation and antibiograms, when necessary the use of antibiotics, to establish the correct dosage. Based on the antimicrobial sensitivity pattern identified



in this study, the recommended antibiotic treatment protocol for dairy calves with diarrhea may include enrofloxacin, cotrimoxazole, ceftiofur, and gentamicin.

As future perspectives, it is planned to incorporate molecular techniques to identify resistance genes in the bacteria isolated in this study, aiming to provide deeper insights into antibiotic resistance mechanisms. Additionally, constant monitoring is essential, increasing the sample size and obtaining a statistically significant number of isolates, as this ensures more representative and accurate data, enabling more detailed decisions on treatment protocols and public health measures.

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### Authors' contributions

All authors equally contributed to the experimental execution and preparation of the scientific article.

### Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## ENTEROBAKTERIJE IZOLOVANE IZ TELADI SA DIJAREJOM I NJIHOVA FENOTIPSKA REZISTENCIJA

Lucilene Martins TRINDADE GONÇALVES, Caroline Lima SANTOS, Wendel Adelino POLICARPO, Lisa Hauane de MELO SANTANA, Kely Janine MEDEIROS de OLIVEIRA, Ana Catarina Pinheiro Angelim BEZERRA, Jaize Viana RIBEIRO SOUSA, Luísa ARAÚJO PIANCÓ, Diego Marques Costa SILVA, Leandro Henrique VEIGA de SOUSA, Giselle Cutrim de OLIVEIRA SANTOS, Robert Ferreira BARROSO de CARVALHO, Isabel AZEVEDO CARVALHO, Hamilton PEREIRA SANTOS, Helder de MORAES PEREIRA

Dijareja je kod goveda veoma rasprostranjena, ekonomski štetna i rezultira značajnim gubicima koji se pripisuju mortalitetu, troškovima lečenja i smanjenom rastu teladi. Cilj ovog istraživanja bio je da se utvrde podaci o pojavi glavnih bakterijskih uzročnika dijareje kod mlečne teladi u državi Maranhao, kao i da se ustanovi rezistencija i utvrde najefikasniji antibiotici i postave protokoli lečenja i prevencije. Uzorci su uzeti sakupljanjem rektalnih briseva i prvobitno kultivisani na ovčijem krvnom agaru (5%) i *MacConkey* agaru, a kasnije na *Salmonella-Shigella* agaru i eozin-metilen plavom agaru. Na dobijenim kolonijama izvršeno je bojenje po Gramu, biohemijski testovi i antibiogrami. Proučavano je deset okruga sa ukupno 230 teladi poreklom sa deset farmi mlečnih krava. Ukupno 21 životinja je ispoljavala kliničke znake dijareje, što predstavlja učestalost od 9,13%. Učestalost pozitivnih farmi bila je 35%, a u 50% oblasti. Izolovane bakterije pripadale su porodici *Enterobacteriaceae*, sa 71,4% *Escherichia coli*, 14,3% *Enterobacter* sp., 9,5% *Proteus* sp. i 4,8% *Klebsiella* sp. Na antibiogramu, bakterije koje su pokazale najveći nivo otpornosti bile su iz roda *Proteus*, a zatim *Enterobacter* sp. i *E. coli*. Pronađene bakterije su relevantne i za zdravlje životinja i ljudi zbog svog zoonotskog potencijala i služe kao upozorenje u oblasti javnog zdravlja, imajući u vidu isolate koji su prema ovoj studiji pokazali *in vitro* rezistenciju na nekoliko antibiotika, što ih potencijalno uključuje u multirezistentne bakterije.