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Contribution to the study of diarrhea etiology in neonate dairy calves in São Paulo state, Brazil

Contribuição ao estudo da etiologia das diarréias em bezerros de aptidão leiteira no Estado de São Paulo, Brasil

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Resumo

Amostras fecais de 203 bezerros com diarréia, idade inferior a 30 dias, de ambos os sexos e de diferentes propriedades do Estado de São Paulo foram examinadas num período de dois anos. Cultivos para pesquisa bacteriana foram feitos em agar acrescido de 10% de sangue bovino e agar Levine. As placas foram incubadas por até 96 horas, em condições aeróbias, a 37°C, com observação dos aspectos de colônia e estudos morfológicos, bioquímicos e realização de outros testes, quando pertinentes. O teste de ELISA foi aplicado para pesquisa de *Rotavirus*. *Cryptosporidium spp*. também foi pesquisado e identificado. Resultados revelaram envolvimento de vários patógenos de forma isolada, assim como associados. *Rotavirus* foi encontrado em 51 (25,1%) das amostras, sendo em 58,8% só, em 41.7% associado a outros microrganismos. *Cryptosporydium spp* foi isolado em 43 (21.3%) das amostras, sendo só em 65,1% delas e associado a outros enteropatógenos em 34,9%. No exame parasitológico foram encontrados ovos de estrongilídeos em 5 (2,5%) das amostras, não excedendo mais de dois ovos por campo examinado. Ao exame microbiológico, um ou mais microrganismos foram isolados. *Escherichia coli* foi encontrada em 100% das amostras. As pesquisas de toxina termoestável e do antígeno de aderência K99 realizada nas 73 amostras de *E.coli* foram negativas, e o grupo sorológico das mesmas foi determinado, sendo 34,2%, 17,8% e 47,9% das amostras pertencentes aos sorogrupos O8, O11 e O101, respectivamente. *Salmonella Dublin* e *Salmonella typhimurium* foram isoladas em 5,4% e 6,1% das amostras examinadas, respectivamente.

Palavras-chave:
Diarréia.
Bezerros.
Etiologia.
Rotavirus.
Cryptospodium

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Introduction

Diarrheas cause serious economical damages by the delay in growth and death of animals, spending with treatments, in addition to professional expenses. Morbidity is usually high, affecting up to 90 to 100% of newborn calves, with a mortality of up to 50%.[1] In addiction to the influence of environment, management and physiological and nutritional factors, the infectious agents that cause diarrhea in neonate calves are numerous.[2] Among these agents, enterotoxigenic samples of *Escherichia coli* are marked.[3] The presence of adherence antigens on the surface of samples of *Escherichia coli* such as K99 is related to its enteropatogenicity.[4,5]

Ganaba et al.[6] did not find serotype K99 in 373 samples of feces of diarrheic

calves aging between 3 and 4 weeks. In Brazil, its occurrence is reported by Costa et al.[7] who found this microorganism in 39.3% of animals from the milky basin of Goiania. In the same way, Ávila et al.[8] in the region of Ribeirao Preto, marked 25.1% of positive presences of these agents in diarrheic feces of calves aging between 1 and 30 days.

Salmonella typhimurium and *Salmonella dublin* were isolated from calves with enteritis. Ploger et al.[9] found them in 16.8% of the necropsies of 726 calves up to 4 weeks old, as an only agent in 42.6% of cases and associated to other bacterial and viral enteropathogens in the remaining percentage. Taoudi, Meier and Amtsberg[10] also marked these agents in 27.6% of samples of intestinal content of calves. Other authors consider these agents important, and reported variable isolation frequencies from 1 to 10,6%.[11,12,13,14,15,16]

Among protozoan, *Cryptosporidium* spp is frequently found in calves with or without diarrhea. Heine[17] showed it in 30% of 620 samples of feces of neonate calves. In the region of Botucatu, Sao Paulo, Brazil, Modolo et al.[18] examined samples of feces of 23 calves between 11 days and 6 months old, in properties with diarrhea outbreak and found it in six (26.0%) of them with diarrhea and in five (23.0%) of animals without diarrhea. Still in Brazil, Garcia e Lima[13] and Souza Lopes[20] found it in 20.0% and 72.0% of samples of diarrheic feces examined, respectively.

The *Rotavirus* was first isolated from feces of calves in the year of 1969 in the U.S.A..[21] It was isolated by different authors, being considered as an important diarrhea agent only or associated to other microorganisms. Working with feces of calves up to 20 days old in the beginning of diarrhea, as well as with clinically normal animals, Scherrer et al.[22] isolated the *Rotavirus* in 82.8% of the diarrhea cases and in 15.5% of the samples coming from normal animals.

Bachmann[1] examined 313 samples of feces of calves between 3 and 21 days through immunoenzimatic test and electronic microscopy and detected this agent in 36.0% of samples, coming from 64 (53.0%) of the 121 properties studied. In the same way, Reynolds et al.[23] studying 45 outbreaks of diarrhea in the southern part of England, observed that the *Rotavirus* was the main agent, being detected in 50.0% of the samples of diarrheic feces, in 23 (51.5%) of the outbreaks.

In Brazil, Jerez et al.[24] obtained 29.0% of positive samples for this agent in the immunoenzimatic test from 120 samples of feces of diarrheic calves aging up to 30 days old. Brito[25] found 7.77% of positive samples examining 223 samples of calves aging from 1 to 120 days old, coming from different rural properties in 14 towns of the state of Goias. Buzinaro et al.[26] detected *Rotavirus* in 11.2% and 17.2% of fecal samples from calves with and without diarrhea. Researches conducted by Bellinzoni et al.[27], Reinhardt et al.[28], Shah and Jhala[29] and Ishizaki et al.[30] about the occurrence of the *Rotavirus* in cases of diarrhea in calves showed prevalence of 53.0%, 28.12%, 20.0% and 22.0%, respectively.

The meaning of the associations of different enteropathogens aggravating enteritis, making therapeutics difficult and causing many deaths of animals, is well documented.[28,31,32]

Considering the complex etiology of diarrhea and its economical importance for Brazilian cattle raising, its etiology was studied in calves aging up to 30 days old by the examination of diarrheic feces of animals from different properties and regions of the state of Sao Paulo.

Materials and Methods

Samples of feces

Twohundred and three samples of diarrheic feces were studied, originating from Holstein calves, from both sexes, aged less than 30 days old, coming from 12 properties from different regions of the state of Sao Paulo. Samples were collected in sterile flasks

after cleaning of the anal area with a paper towel and beats by rectal stimulation with the index finger protected by disposable plastic glove. Immediately after collection, samples were kept at chilling temperature until they arrived at the laboratory where they were processed according to the type of examination they would be undergone to. The clinic symptoms of the calves were apathy and diarrhea with variable characteristics of the feces as presence of blood, mucus and fetid. The material were collected at the beginning of these symptoms

Bacteriological examination

Bacterial cultures of all samples collected were made in 10% bovine blood-agar and Levine mediums, incubated at 37°C, for 24, 48, 72 up to 96 hours, in aerobic conditions, followed by the observation of colonies and bacterial morphology, from smears stained by the Gram method. Samples of different microorganisms isolated were rang to a brain-heart broth medium, in order to obtain inocula to perform taxonomic tests according to Ewing.[33]

The thermo stable toxin research in 73 samples of *Escherichia coli* was carried out using the technique of re-isolation of the samples in brain-heart broth medium at 36°C, being agitated for 18 hours and subsequently centrifugated at 10.000g for 15 minutes, inoculating the floating solution in three to four-days-old lactant mice. After three to four hours, mice were sacrificed, calculating the ratio between intestine and body weight, considering results higher than 0.09 positive.

The detection of the adherence antigen K99 was carried out according to Guinée, Veldkamp and Jansen[34] through agglutination in laminas, using a specific antiserum prepared through immunization of rabbits with the K12:K99 sample. Samples were still studied using the agglutination method for serological grouping through the research of somatic antigens using specific antiserums.

***Rotavirus* research**

The immunoenzimatic test according to Voller et al.[35] for the "double sandwich" technique was used, being components provided by the World Health Organization (ELISA-test kit), with hyperimmune serums prepared from AS-11 samples of *Rotavirus*. Each hollow of the micro-plate (Nunc, Denmark) received 100ml of rabbit antirotavirus hyperimmune serum, diluted in a 1:100 carbonate-bicarbonate buffer, with a pH of 9.8, incubated at 4°C for 12 hours. Next, the contents of the hollows were discarded and micro-plates had all their hollows washed six times with PBS/T (phosphate-saline bufer with 0.1 % v/v Tween 20 and 0.1M EDTA with a pH 7.2), except the ones reserved for controls, and, after, in pairs, hollows received 25ml of each suspension of feces to be analyzed (final dilution: 1:40). Suspensions were first prepared being diluted into 1:10 TRIS 0.1M buffer, with a pH 7.3, and shaken in a mixer for about five minutes. The fecal suspension was undergone to environment temperature for about thirty minutes, being shaken three times during this period. Next, the material was centrifugated at 10.000 rpm for thirty minutes. The floating solution was treated with equal volume of T.F. freon (trichlorofluorethane). After homogenization, all the material was centrifugated at 3.000 rpm for 15 minutes, using the floating suspension as the sample used for the research of the viral antigen through the immnuoenzimatic test.

Micro-plates were covered and incubated at 37°C for two hours, discarding contents, washing hollows again six times with PBS/T. After that, 100ml of anti-*Rotavirus* hyperimmune guinea-pig serum, diluted to 1:100 in PBS/T/BSA (phosphate-saline buffer with 0.1% Tween 20 and 1% albumin), was distributed in all hollows, except in the ones reserved to control, being the micro-plate covered and incubated for 30 minutes at 37°C.

Once more the content was discarded and hollows were washed six times with PBS/T. Next, each hollow received 100ml of alkaline phosphatase (p-nitrophenil-phosphate diluted in a 10% diethanolamine solution in distilled water added with 0.01% $MgCl_2x6H_2O$ and pH adjusted to 9.8 with concentrated hydrochloric acid; final concentration = 1 mg of p-nitrophenil-phosphate/ml of medium) and, next, plates were covered and incubated at 37°C for approximately 20 minutes. To interrupt the reaction, each depression was added with 50ml of 3M sodium hydroxide. Spectrophotometric reading was performed in a wave length of 405nm, using substrate control hollows as blank. Samples with absorption higher than 0.1 were considered positive.

Criptosporidium spp. research

Samples were examined according to Heine.[17] Smears were made on glass laminas for microscopy using 3ml of feces and 3ml of carbolic fuchsin and, immediately after drying, smears were added with a drop of immersion oil and covered with a glass laminula and examined with an optical microscope with a 400x magnitude.

Parasite examination

The helminthes research was performed according Willis[36] by adding approximately 3g of feces in a flask with large mouth, diluting it in a hyper-saturated NaCl solution, homogenizing with the help of a glass stick, stirring constantly until the mixture reaches the edge of the flask. At this time, the flask was covered with a microscopy lamina and stood still for 15 minutes. Then the lamina was removed, turned over and covered with a glass laminula for microscopic examination.

Results and Discussion

Table 1 shows the etiological agents responsible for the appearance of diarrheas in the calves, in the different properties. The *Rotavirus* was the most prevalent, with 25.1%, followed by *Cryptosporidium* spp., in 21.2% of the samples examined. Both were found associated in 36 (17.7%) of them.

Table 1
Detection of enteropathogens in diarrheic feces of 203 calves, younger than 30 days old. Botucatu, 2003

Enteropathogen	Number	%
Rotavirus	51	25.1
Cryptosporidium spp	43	21.2
Rotavirus + *Cryptosporidium* spp	36	17.7
Salmonella serovar *typhimurium*	14	6.1
Salmonella serovar *Dublin*	11	5.4
Strongylides spp.	5	2.5

The importance of *Rotavirus* spp. in the etiology of diarrheas is confirmed and agrees with Bellinzoni et al.[27] and Reynolds et al..[23] These authors detected it with higher prevalence, varying from 30% to 53%. The results obtained are similar to the present study, with isolation rates of 29%, 28.12%, 20% and 22%, respectively.[24,28,29,30]

As in the studies made by De Leeuw et al.[31], Perrin et al.[32] and Reinhardt et al.[28], the association of enteropathogens was observed, specifically the one between *Rotavirus* and *Cryptosporidium*, in 17.7% of samples, what reinforces the importance of laboratory diagnosis in diarrheas, for therapeutic orientation and control.

The participation of *Cryptosporidium* spp in the diarrhea of calves is also important as this protozoan is hard to treat and can be found in animals with or without diarrhea, what allow its permanence in the property for a longer time, making its control even more difficult. Contrasting with the results from national literature, it's similar to the ones obtained by Modolo et al.[18], with a prevalence of 26% against 21.1% in the present research; however, close to results obtained by Garcia and Lima[19], with 20% of positive samples. On the other hand, Souza and Lopez[20] reported its presence in 72% of cases examined.

Salmonella serovar *typhimurium* and *dublin* were detected in 6.1 % and 5.4% of samples.

Serovar *Dublin* is a specific bovine-adapted serotype which causes an epidemic disease in calves, usually infecting animals between 3 and 6 weeks old. Salmonellosis is characterized by septicemia, sometimes followed by diarrhea. In the present study, this serovar was found in only one property whose sick animals were 3 and 4 weeks old.

Ploger et al.[9] and Taoudi, Meier and Amtsberg[10] reported a higher isolation of *Salmonella* spp in cases of diarrhea when compared to the present study. The results obtained by several authors[11,12,13,15,16] are similar to this study and Saad[14] obtained *Salmonella dublin* isolation in 4% of his samples.

Although other authors emphasize the importance of samples of enterotoxigenic *Escherichia coli* in the diarrhea of neonate calves[4,5] the presence of a thermostable toxin and the adherence antigen K99 was not detected in the present work in the samples of *Escherichia coli* studied, as well by Ganaba et al.[6]. The serological grouping of these toxins configured as 34.2%, 17.8% and 47.9% of samples belonging to serological groups O8, O11 and O101, respectively.

The presence of helminthes mentioned in the present study is represented by a little amount of strongylids eggs in only 5 (2.5%) of animals, what may reflect the appropriate conditions of periodic treatment of animals in the properties, fact to be observed in modern raisings, especially milky bovines.

The results of this research allow us to conclude for the multiple etiology of diarrheas and the importance of microbiological diagnosis relating to the research of different enteropathogens of diarrheas in calves to better understand their etiology, epidemiology and help in the development of a specific treatment so that it can be controlled.

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Abstract

Two hundred and three samples of feces of calves aged less than 30 days old from both sexes and from different properties and regions of the state of São Paulo were examined in the period of two years. Bacterial cultures were carried out in bovine 10% blood-agar and Levine mediums, incubated for up to 96 hours in aerobic conditions at 37°C, with the observation of colonies and morphological and biochemical study to characterize isolated microorganisms, or other tests, when pertinent. ELISA was performed for the *Rotavirus* research. The *Cryptosporydium* spp was surveyed and its parasitological examination was made. Results revealed the involvement of several enteropathogens alone and associated. *Rotavirus* was found in 51 (25.1%) samples, being 58.8% alone and 41.7% associated. *Cryptosporidium* spp was obtained in 43 (21.3%) samples, being the only agent involved in 65.1% of them and associated to other enteropathogens in the remaining 34.9%. The parasitological examination showed strongylids eggs in only 5 (2.5%) of the animals and in little amount, not exceeding more than two eggs by examined field. In the microbiological examination, one or more microorganisms were isolated; *Escherichia coli* was found in 100% of samples. The thermostable toxin and the adherence antigen K99 researches, made in 73 samples of *E.coli*, were negative. The serological grouping of the same ones were configured as 34.2%, 17.8% and 47.9% of the samples belonging to serological

Key-words:
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groups O8, O11 and O101, respectively. *Salmonella dublin* and *Salmonella typhimurium* were isolated in 5.4% and 6.1 % of the examined samples, respectively.

References

1.BACHMANN, P. A. Neue virale Durchfallerkrankungen beim kalb. **Tierärztliche Umschau**, v. 10, p. 524-526, 1977.

2.SNODGRASS, D. R. et al. 1980. Cryptosporidia associated with *Rotavirus* and *Escherichia coli* in a outbreak of calf scour. **Veterynary Record**. v. 31, p. 458-459, 1980.

3.BALDER, G. Erfahrungen mit der oralen Schutzimpfung von Kälben gegen *Escherichia coli*. **Tierärztliche Umschau**, v. 32, p. 527-538, 1977.

4.ACRES, S. D. Enterotoxigenic *Escherichia coli* infections in newborn calves: A review. **Journal of Dairy Science**, v. 68, p. 229-256, 1985.

5.BARRANDEGUY, M. E. et al. *Rotavirus*, enterotoxigenic *Escherichia coli* and others agents in the feces of dairy calves with and without diarrhoea. **Revista Latino Americana de Microbiologia**, v. 30, p. 239-245, 1988.

6.GANABA, R. et al. Importance of *Escherichia coli* in young beef calves from Northwestern Quebec. **Canadian Jorunal of Veterinary Research**, v. 59, p. 20-25, 1995.

7.COSTA, R. C. et al. Freqüência e causas de doenças do aparelho digestivo em bezerros na bacia leiteira de Goiânia. **Anais da Escola Superior de Medicina Veterinária**, v. 9, p. 108-127, 1979.

8.AVILA, F. A. et al. Caracterização bacteriológica e sorológica de amostras de *Escherichia coli* de origem bovina. **ARS Veterinária**, v. 10, p. 59-64, 1994.

9.PLÖGER, W. et al. Untersuchung über die Ursachen der Kälbersterblickeit im Nord-westdeuschen Küstengebeit. **Tierärztliche Umschau**, v. 35, p. 659-671, 1980.

10.TAOUDI, A., MEIER, C., AMTSBERG, G. Untersuchung zum derzeitigen Vorkommen von bakteriellen Infektionserregern im Sektionsmaterial von Kälbern. **Der Praktich Tierärzt**, v. 3, p. 221-226, 1983.

11.LANCE, S. E. et al. *Salmonella* infections in neonatal dairy calves. **Journal of the American Veterinary Medicine Association**, v. 201, p. 864-868, 1992.

12.OLIVEIRA, A. A.; PEDREIRA, P. A. S.; ALMEIDA, M. F. R. S. Doenças de bezerros. I. Diarréias bacterianas no Estado de Sergipe, Brasil. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 41, p. 213-222, 1989.

13.OPUDA-ASIBO, J.; ROBINSON, R.A.; PULLEN, M. M. Prevalence of *Salmonella* in healthy calves following transportation to the stockyards and at slaughter. **Bulletin of Animal Health and Production in Africa.** v. 38, p. 101-102, 1990.

14.SAAD, M. K. Salmonellosis in newborn calves in a closed dairy farm. **Veterinary Medical Journal Giza**, v. 41, p. 43-45, 1993.

15.WRAY, C. et al. The epidemiology of *Salmonella* infection of calves: The role of dealrs. **Epidemiology & Infection,** v. 105, p. 295-305, 1990.

16.ZRELLI, M. et al. Infective agents associated with neonatal diarrhoea of calves in Tunisia. **Revista de Medicina Veterinaria**, v. 141, p. 861-872, 1990.27.BELLINZONI, R. C. et al. Microbiology of diarrhoea in young beef and dairy calves in Argentina. **Revista Argentina de Microbiolgía**, v. 22, p. 130-137, 1990.

17.HEINE, J. Eine einfache Nachweismethods für *Kryptosporidien* im Kot. **Zentralblatt für die Veteriärmedizin B**, v. 29, p. 324-327, 1982.

18.MODOLO, J. R. et al. Ocorrência de criptosporidiose em bezerros na Região de Botucatu, SP. **Revista Brasileira de Medicina Veterinária**, v. 10, p. 9-10, 1988.

19.GARCIA, A. M.; LIMA, J.D. Frequência do *Cryptosporidium* em bezerros lactentes de rebanhos leiteiros de Minas Gerais. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 45, p. 195-198, 1993.

20.SOUZA, J. C. P. D., LOPEZ, C. W. G. Cryptosporidiosis in calves from milk-producing in the southern fluvial basin of Rio de Janeiro State. **Revista Brasileira de Parasitologia Veterinária**, v. 62, p. 1632-1638, 1991.

21.MEBUS, C. A. et al. Calf diarrhea (Scours): Reproduced with a virus from a field outbreak. **University of Nebraska Research Bulletin,** v. 23, p. 30-35, 1969.

22.SCHERRER, R. et al. *Reovirus* like agent (*Rotavirus*) associated with neonatal calf gastroenteritis in France. **Annales de Recherche Vétérinaire,** v. 2, p. 25-31, 1976.

23.REYNOLDS, J. et al. **Veterinary Record**, v. 12, p. 34-39, 1986.

24.JEREZ, J. A. et al. Evidenciação de *Rotavírus* através de ensaio imunoenzimático em fezes diarréicas de bezerros. **Revista de Microbiologia**, v. 18, p. 201-204, 1987.

25.BRITO, W. M. E. D. Bovine *Rotavirus* in the State of Goias. **Revista de Microbiologia**, v. 25, p. 229-234, 1994.

26.BUZINARO, M. G. et al. Detection of rotavirus in darrheia faeces sample from beef herd calves in São Paulo State, Brazil In: ENCONTRO NACIONAL DE VIROLOGIA, 11., 2000, **Anais..**. São Lourenço.

28.REINHARDT, G. et al. Aetiological diagnosis of neonatal diarrhoea in calves by means of ELISA. **Archivos de Medicina Veterinaria**, v. 23, p. 189-192, 1991.

29- SHAH, N. M., JHALA, V. M.. Epizootiological investigations on *Rotavirus* infection in cases of neonatal calf diarrhoea. **Indian Veterinary Journal**, v. 69, p. 5-7, 1992.

30.ISHIZAKI, H. Persistence of a single electro pherotype and serotype (G6P5) of bovine *Rotavirus* in calves on a closed dairy farm from 1990 to 1993. **American Journal of Veterinary Research**, v. 56, p. 1019-1024, 1995.
31.DE LEEUW, P. W. et al. *Rotavirus* infections in calves in dairy herds. **Research Veterinary Science**, v. 29, p. 135-141, 1980.
32.PERRIN, B. et al. Fréquence du *Rotavirus* et l´association *Rotavirus* et *Escherichia coli* k99 chez aux de quelques departments Français. **Annales de Recherche Vétérinaire**, v. 12, p. 259-263, 1981.
33. EWING, W. H. **Edwards and Ewing's identification of Enterobacteriaceae**. New York: Elsevier, 1986. 536 p.
34.GUINÉE, P. A. M.; VELDKAMP, J.; JANSEN, W. I. T. Improved medium for the detection of K99 antigen in calf enterotoxigenic strains of *Escherichia coli*. **Infection and Immunity**, v. 15, p. 676-678, 1977.
35.VOLLER, A. et al. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). **Journal of General Virology**, v. 33, p. 165-176, 1976.
36.WILLIS, H. H. A simple levitation method for the detection of hookworm ova. **Medicine Journal of Australia**, v. 2, p. 375-376, 1921.