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Effect of the Administration of Bacteriophages to Calves Infected with *Cryptosporidium* spp. on the Health Status, Nutrition and Survival Rate

L.N. Espinosa-García[1], F.G. Véliz-Deras[2], R.A. Delgado-González[2], L.R. Gaytán-Alemán[2], L. Jung-Jin[3], J. Moran-Martínez[4], [2]

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ABSTRACT

Background: *Cryptosporidium* spp., together with enteropathogenic bacteria, induces diarrhea, which could cause the calf's death. This work aimed to evaluate the use of bacteriophages in calves infected with *Cryptosporidium* spp. upon their health, nutritional status and survival rate.

Methods: A farm with calves infected with this protozoan was selected. For its control, decoquinate was used. Seventy-two pre-weaned calves, divided into two groups, were used. The treated group (n= 36) received 5 g of oral bacteriophages; the control group did not receive bacteriophages.

Result: The treated group had a higher survival rate (p=0.05) and fewer days with diarrhea (p=0.04). Also, diarrhea appeared earlier in the control group (p=0.03). A lower probability of receiving antibiotics treatment was observed in the treated group (OR = 0.05). Similarly, better results were obtained in the average daily gain (p=0.03), weight (p=0.01) and height (p=0.009). The supplementation with bacteriophages to calves infected with *Cryptosporidium* spp. improves the survival rate, delays the appearance of diarrhea, decreases days of diarrhea and reduces the use of antibiotics. In conclusion, using bacteriophages improves calves' health and nutritional state.

Key words: Bacteriophages, Bovine, *Cryptosporidium*, Diarrhea, Health, Survival rate.

INTRODUCTION

Enteric and respiratory diseases are calves' leading causes of morbidity and mortality (Tora *et al*., 2021; Diao *et al*., 2017). A frequent origin of these diseases is due to *Cryptosporidium* spp. infection (Thomson *et al*., 2017), a parasite that damages the epithelial cells of the gastrointestinal tract, causing diarrhea but with low mortality (Santin, 2020). However, when it is associated with bacteria, the disease becomes more severe and could cause death (Santin, 2013). To treat this enteric disease, antiparasitic and antimicrobial compounds are used. Nevertheless, antibiotics have ceased to be efficient in the last decade due to the increase in bacterial resistance (Grami *et al*., 2023; Hommerich *et al*., 2019). Therefore, new environmentally friendly alternatives are being searched for. An option is the use of bacteriophages, also known as phages. They are viruses that infect and lysate specific bacteria, even if those bacteria are resistant to antibiotics (Gaborieau and Debarbieux, 2023; Squires, 2018). In previous studies, the effectiveness of phages in controlling bacteria in the digestive system has been proven (Jeong *et al*., 2021; Schmoeller *et al*., 2021; Xie *et al*., 2021). In addition, it has been found that the same strain could sometimes lysate other varieties and even other bacterial species, which makes its range of action broader (Dueñas *et al*., 2017; Moreno-Switt *et al*., 2013). Hence, this study aims to evaluate the use of bacteriophages in calves infected with *Cryptosporidium* spp. upon their health, nutritional status and survival rate.

[1]Autonomous University of Chiapas, Faculty of Veterinary Medicine and Zootechnics, Tuxtla Gutierrez, Chiapas, México.
[2]Autonomous Agrarian University Antonio Narro, Laguna Unit, Torreón, Coahuila, Mexico.
[3]CTCBIO, Inc., Seoul 05724, Korea.
[4]Autonomous University of Coahuila, UAdeC. Department of Cellular Biology and Ultrastructure, Torreón, Coahuila, México.

Corresponding Author: D.I. Carrillo-Moreno, Autonomous Agrarian University Antonio Narro, Laguna Unit, Torreón, Coahuila, Mexico. Email: dalia.ivettecm@gmail.com

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MATERIALS AND METHODS

The present study was conducted on a dairy farm with the prevalence of *Cryptosporidium* spp. Animal management and experimental protocols were reviewed and approved

by the Committee of Animal Research Ethics of the Antonio Narro Autonomous Agrarian University (approval number 38111-425502002).

Farm selection

Feces samples were taken randomly from six different farms to diagnose *Cryptosporidium* spp. by the Ziehl Neelsen technique (Rekha *et al*., 2016); A farm with claves that presented some degree of infection (+, ++, +++, ++++) was chosen. During the experiment, all the calves received 2.5 mg/Kg of oral decoquinate as a prophylactic and the collection of samples continued at random to monitor the protozoan. On this farm, calves were kept in individual cages from birth to weaning and fed twice daily. The bedding was a mixture of dirt and sand that was not changed between each cycle; it was turned over. In addition, the cages were not periodically disinfected. Calves were vaccinated against respiratory (Bovine Infectious Rhinotracheitis, Bovine Viral Diarrhea type I and II, Bovine Respiratory Syncytial Virus and Parainfluenza 3) and gastrointestinal (rotavirus, coronavirus) diseases. The dams were not immunized to protect the calves against gastrointestinal diseases.

Experimental scheme and treatment

A random study was performed on a dairy farm in Torreón, Mexico, from February to June of 2021 (winter to spring). Seventy-two pre-weaned female Holstein-Friesian calves (37.5±4.3 kg body weight) were used from birth (d 1) to weaning (d 60). They were divided into two groups (control and phage) with 36 animals each, homogeneous in body weight. The groups were assigned randomly at birth.

To the phage group (treated), 5 g (5×10^6 PFU) of bacteriophages (eXolution ® CTCBIO, Inc., Seúl, Corea) were given per day, targeting *Salmonella typhimurium*, *Salmonella enteritidis*, *Salmonella dublin*, *Salmonella derby*, *Staphylococcus aureus*, *Escherichia coli* k99 and f41 and *Clostridium perfringens* of types A and C. The phages were added daily to the recipient, where milk was given to each calf. The control group didn't receive phages.

Accommodation and feeding

At birth, the calves were subjected to a physical exam; they were weighted, received 4 L of colostrum of the same quality and were kept in individual cages until weaning. The calves were fed with whole milk and balanced solid food (Iniciador supra®, Nuplen S.A. de C.V.). The balanced food contained protein (21.5%), fiber (10%), fat (2%) and ashes (14%). The calves had access to water *ad libitum* and their feeding protocol (milk and balanced solid food) was adjusted daily according to age (Table 1).

Evaluated variables

The presence of diarrhea and antibiotic treatment was evaluated according to the methodology used by Chamorro *et al*. (2017). Those calves that presented some nasal discharge (mucous, serous, or muco-serous) were considered positive for pneumonia. These evaluations were performed every day in the mornings and afternoons. On day 60, the survival rate was evaluated. When a calf presented diarrhea, a sample of the feces was taken from the rectum and then taken to the laboratory of the Universidad Autónoma Agraria Antonio Navarro to determine the growth of *Salmonella* spp. (Cho and Yoon, 2014) and *Cryptosporidium* spp. In the same laboratory, the diagnosis of the death of the calves was made.

The body weight was evaluated with a digital scale on the day of birth and after that, every 15 days until weaning. The average daily gain (ADG) was assessed by dividing the body weight of the period in evaluation by the 15 last days. At the same time, the height at withers was measured. These evaluations were carried out in the mornings before the morning feeding.

Use of antibiotics

The antimicrobial treatment of infectious diarrheas consisted of gentamicin sulfate and penicillin G procaine. For pneumonia, the calves were given a product containing florfenicol, paracetamol and bromhexine. The treatments used, including the decoquinate, were established by the farm.

Statistical analysis

The data were analyzed with the statistical package SPSS ver. 25.0 IBM Corp. (2017). For the assumption of normality, the Kolmogorov-Smirnov test was used. The verification of homoscedasticity was made with the Levene test. The survival test was calculated by a chi-square test. For each group's total days of diarrhea, a univariate General Linear Model (GLM) was used, where the dependent variable was the duration of diarrhea in days and the independent variable was the presence of diarrhea. Latency until the day of the presence of diarrhea was analyzed through a Mann-Whitney U test. The probability of receiving antibiotic therapy or developing pneumonia among the groups was calculated with Odds Ratio; if any cell was lower than 5, Fisher's exact value was considered. For the analysis among the groups of weight, ADG and height at weaning, a t-student was used. It was considered statistically different with a value of $p\leq0.05$.

RESULTS AND DISCUSSION

The infection by *Cryptosporidium* spp. is frequent and produces chronic diarrhea, which brings high economic losses (Shaw *et al*., 2020). To our knowledge, this is the first study that evaluates bacteriophages in Holstein-Friesian pre-weaned calves infected with *Cryptosporidium* spp.

The diarrheas were not wholly eradicated during the study period, probably due to *Cryptosporidium* spp. (Santin, 2013), which was tried to be controlled by administrating decoquinate. Nevertheless, some moderate to severe (++, +++) infections were found in the random sampling. This drug reduces the number of oocysts, but sometimes it is not enough to avoid the appearance of diarrheas (Åberg *et al*., 2020). The origin of diarrheas is multifactorial and we cannot attribute them only to this protozoan.

Survival rate

A significant difference was observed between the two groups regarding the survival rate of the calves (p=0.05). In the group that received phages, 100% survived, while in the control group, only 86.11%. Most deaths were due to gastroenteric problems. This survival is similar to the 99% of Schmoeller *et al*. (2021), although there was no cryptosporidiosis in their study. The deaths in the control group could be caused by enteropathogens such as *E. coli*, *Salmonella* spp. and C. perfringens (Thomson *et al*., 2017). Not having mortality in the phage group complies with the most crucial indicator of animal well-being (Uetake, 2013). Also, the diarrheas in the control group were treated with antibiotics; therefore, not having deaths in the phage group would indicate that these are better at controlling bacterial populations than the antibiotic.

Diarrhea, antibiotic therapy and pneumonia

The total of days with diarrhea was lower in the phage group than in the control group (4.69 ± 2.29 and 13±4.88, respectively, p=0.04) (Fig 1). A significant difference was observed between the groups regarding the beginning of diarrheas (p=0.03). In the control group, they appeared faster (13 d) than in the bacteriophages group (18 d) where, in addition, the range of appearance was broader than in the control group (Fig 2); the phage group was the healthiest presenting fewer days with diarrheas and delaying their appearance even with the prevalence of *Cryptosporidium* spp. Similar results have been obtained in other studies, although they did not report infection by protozoans (Alomari *et al*., 2021; Schmoeller *et al*., 2021). This explains why bacteriophages are effective controllers for bacteria that cause diarrheas (Xie *et al*., 2021; Dueñas *et al*., 2017; Moreno-Switt *et al*., 2013) and oral administration is helpful despite the pH of the abomasum (Hong *et al*., 2016). This control was observed in the phage group, where there was no growth of *Salmonella* spp.; this did not happen in the control group. *Cryptosporidium* spp. opens the opportunity to other agents (Santin, 2020), but the diarrheas decrease if the enteropathogen bacteria are controlled.

Additionally, calves of the treated group tended to have a lower probability of receiving antibiotics treatment than the control group (OR = 0.05, p = 0.005). Furthermore, it was observed that the treated group had a lower risk of developing pneumonia (OR = 0.4; p = 0.32) (Table 2). Other bacteriophage therapies have been tried, with favorable results (Alomari *et al*., 2021; Schmoeller *et al*., 2021; Kwiatek *et al*., 2020). The phages are very effective for the lysate of bacteria, so technically, there is no need to use an antibiotic (Nobrega *et al*., 2018); this also avoids the destruction of intestinal microbiota (Low *et al*., 2021). Hence bacteriophages are an

Table 1: Feeding protocol (milk and balanced solid food) of calves from birth to weaning.

Age (d)	Balanced solid food (Kg)	Milk (L)
1-7	0.3	6
8-25	0.4	8
26-40	0.5	10
41-50	0.8	8
51-55	1.2	6
55-60	1.5	0

Balanced solid food was offered once and milk twice each day at 7:00 and 15:00 h in equal amounts.

Table 2: Probability of receiving antibiotics therapy or presenting pneumonia in calves infected with *Cryptosporidium* spp. supplemented with bacteriophages.

Variable	OR (PHA vs CTL)	95% CI OR	p-value
Antibiotic therapy	0.05	0.005-0. 533	0.005
Pneumonia	0.4	0.06-2.769	0.32

Shows results of the Odds Ratio (OR), the confidence interval (CI) and the value p (calculated through the Fisher exact) between the phage (PHA) and control (CTL) groups.

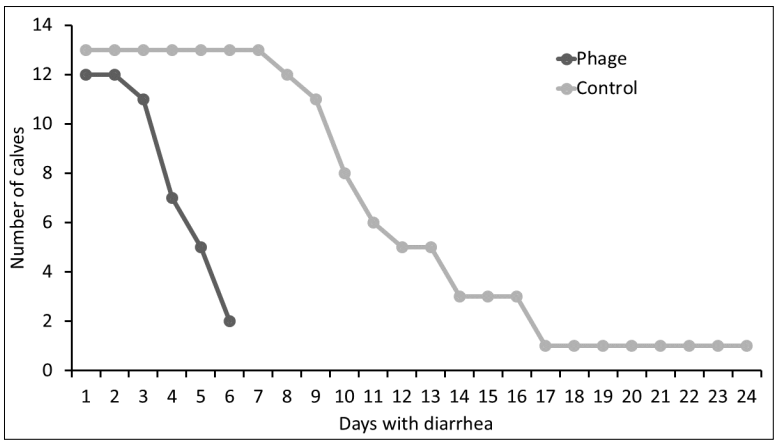

Fig 1: The total duration of diarrhea in calves infected with *Cryptosporidium* spp. supplemented with bacteriophages. The average of the treated group was 4.69±2.29 days and for the control group, it was 13±4.88 days (p = 0.04).

excellent alternative for addressing antimicrobial resistance despite phage resistance (Gaborieau and Debarbieux, 2023; Grami *et al*., 2019; Hommerich *et al*., 2019). If the diarrheas are eliminated, pneumonia could also be diminished (Pardon *et al*., 2015). This study obtained a preventive effect of the phages on the appearance of pneumonia but without an acceptable statistical significance. Some complementary studies are required to evaluate this effect in the absence of *Cryptosporidium* spp.

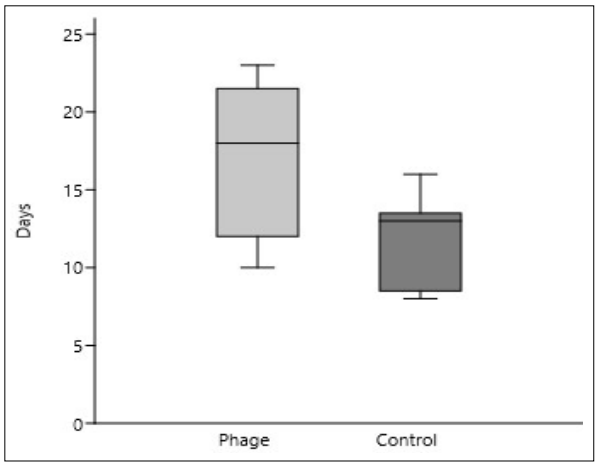

Fig 2: Latency days of diarrheas in calves infected with *Cryptosporidium* spp. and supplemented with bacteriophages. A Mann-Whitney U test was carried out (p = 0.03); it took 18 days for the phage group to show diarrhea, while the time for the control group was 13 days.

ADG, weight and height

In the results at weaning, it was observed a significant difference in the ADG, weight (77.35±1.47 vs 71.38±1.62 kg) and height (92.21±0.8 vs 89.2±0.59 cm). The phage group had the highest mean (p=0.03, 0.01 and 0.009, respectively) (Table 3 and Fig 3). Although the difference in ADG was not significant when being evaluated every 15 days, it was during the entire trial period (60 d); the phage group gained more weight. These results agree with Jeong *et al*. (2021) but disagree with Schmoeller *et al*. (2021), who obtained statistical differences in all the evaluated periods. Regarding body weight, there was a difference only in the second half of the study, including the weaning. Some studies reported differences (Schmoeller *et al*., 2021), but others showed no difference (Jeong *et al*., 2021). The statistical equality in the first month could be because it is the period where the highest infection of *Cryptosporidium* spp. (Åberg *et al*., 2020). Otherwise, the difference could be observed since day 11 (Alomari *et al*., 2021). The most evident effect was in height, where in the whole period of

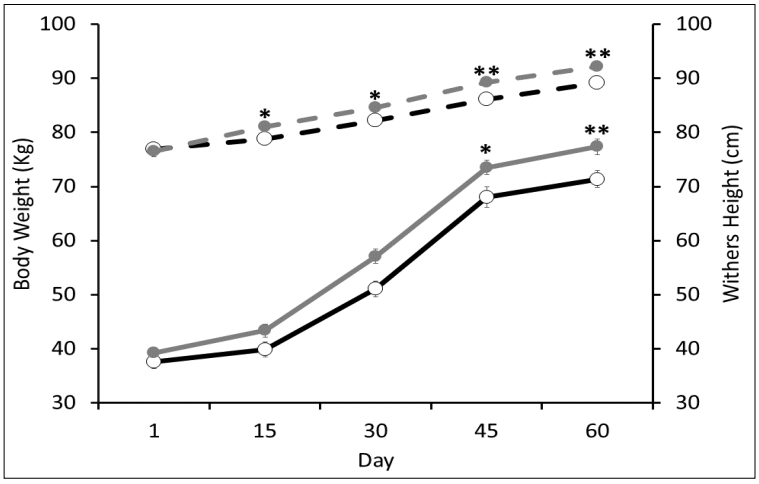

Fig 3: Effect of supplementation with bacteriophages on calves infected with *Cryptosporidium* spp. on the body weight and height at the withers from birth to weaning. Continuous lines = body weight; discontinuous lines = height at withers. Bold circles (•) = phage group; circles (o) = control group. Asterisk (*) = p = ≤0.05; two asterisks (**) = p = ≤0.01.

Table 3: Effect of the supplementation with bacteriophages to calves infected with *Cryptosporidium* spp. on the DWG from birth to weaning.

Item \ Day	ADG				
	1-15	15-30	30-45	45-60	1-60
Control	0.148±0.11	0.748±0.06	1.1±0.05	0.244±0.06	0.562±0.02
Phage	0.273±0.05	0.91±0.05	1.07±0.03	0.277±0.06	0.634±0.01
p-value	0.32	0.06	0.6	0.72	0.03

ADG: Average daily gain. Data are shown as mean±Mean standard error and a value of p≤0.05 was considered statistically different.

study, there was a statistical difference except in birth, similar to that obtained by Schmoeller *et al*. (2021). This indicates that although weights vary, height growth is relatively constant. The positive effects on the parameters of nutrition could be explained by the low morbidity in the phage group, which allowed better nutrition (Tora *et al*., 2021). Hence, bacteriophages improve health and, consequently, enhance nutritional efficiency.

CONCLUSION

Cryptosporidium spp. causes diarrheas in pre-weaned calves which could complicate by opportunistic bacteria, increasing the mortality rate. However, if bacteriophages are administered, the survival rate is improved, days of diarrhea are decreased and the onset of diarrhea is delayed. Additionally, the use of antibiotics is reduced, contributing to the strategy of mitigating bacterial resistance to these compounds. While improving the health of calves, the nutritional state is also enhanced.

Conflict of interest

Author Jung-Jin Lee is listed as the inventor on patent applications filed by CTCBIO Inc. All other authors declare no conflict of interest.

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