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Original article

Therapeutic potential of cristobalite in the treatment of calf diarrhea

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Abstract

Calf diarrhea continues to be the major problem of calves in the neonatal period. The effect of zeolites has been increasingly studied in ruminant health in recent years. In the present study, the efficacy of cristobalite, a zeolite, in neonatal calf diarrhea was studied first time. For this purpose, twenty-five neonatal calves with diarrheas were divided into two groups, and Group 1 (n=12) received conventional treatment and Group 2 (n=13) received cristobalite (Zoosorb 10 mg/kg) orally 3 times a day in addition to conventional treatment. *Escherichia coli* k99 and CS31a, bovine rotavirus and bovine coronavirus were isolated from fecal samples at the beginning of the treatment, on the third day and before discharge. It was determined that the recovery period in Group 2 was 0.95 (20.6%) days shorter than in Group 1 ($p<0.05$) while no viral agents were found on the fifth day in Group 2, viral shedding continued in 4 of 5 calves in Group 1. In conclusion, the study revealed that cristobalite speeds the recovery time and possibly decreases viral shedding in neonatal calf diarrhea, demonstrating a remarkable efficiency in the treatment.

Key words: calf diarrhea, cristobalite, CS31a, zeolite

Introduction

Calf diarrhea is an economic burden in bovine practice, with predominant infectious etiology. Major pathogens of the neonatal period are *bovine rotavirus*, *bovine coronavirus*, *Eschericia coli* (K99), and *Cryptosporidium parvum* (Izzo et al. 2015). Coinfections with more than one of these pathogens are frequently detected in diarrheic calves in clinical practice and are associated with increased morbidity and mortality rates (Carr et al. 2009). Major impact of diarrhea is economic losses in calf production due to treatment costs, growth

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retardation, or death (Cho et al. 2010). Neonatal calves (less than 14 days of age) are at risk of infection from enteric *rotavirus* and *coronavirus*, and most infections ocur in the first week of life. Morbidity is high as 50-100%, and mortality varies (70-100%) (Divers and Peek 2007). Treatment targets enterotoxigenic *Escherichia coli* (*E. coli*) and subtypes. Although antibiotic treatment is not essential in rotaviral enteritis alone, the possibility of mixed infections and pathological damage in enterocytes with bacterial invasion indicates antibiotics within the treatment protocol, especially for severely affected calves with systemic disease (Dives and Peek 2007).

Whatever the cause of diarrhea, bacteremia develops due to bacterial (*E. coli*) over- growth, especially in the small intestine, aggravating the severity of the disease. Therefore, it is constitutive to reduce the bacterial load and to bind endotoxins in the treatment (Valpotic et al. 2007, 2016). The conventional treatment of neonatal calf diarrhea includes parenteral administration of antibiotics, non-steroidal anti-inflamatory drugs (de Verdier et al. 2012), and intravenous fluid therapy that is primarily based on the degree of dehydration (Berchtold 2009). In antibiotic selection, resistant bacteria will inevitably emerge as a result of the non- isolation of the agent and uncontrolled use of antibiotics (de Verdier et al. 2012, Karamzadeh- Dehaghani et al. 2021). *E. coli* pathotypes, mainly K99, have been identified and classified based on their virulence factors, mechanisms of action, and promotion of disease. Some data obtained in recent years have shown that antimicrobial resistance is incresed in *E.coli* diarrhea. The gene sequence encoding the CS31A fimbrial protein was found in *E.coli* strains with multiple resistance (Gibbson et al. 2014). Hence, an antimicrobial susceptibility test is crucial for demonstrating bacterial resistance, drug selection, avoiding overuse of the same drugs resulting in further drug resistance of bacteria (da Silva Menezes Azola et al. 2021). Therefore, intensive research is performed on adjunct treatment options and non-antibiotic drugs to effectively treat diarrhea and avoid antimicrobial resistance (Renaud et al. 2019, Smulski et al. 2020). In accordance with this purpose, effect of zeolites in acute diarrhea was evaluated in humans (Tieroshyn et al. 2020) and veterinary medicine (Cerbu et al. 2020).

The silicon dioxide (SiO_2) modifications are named as quartz in reference books. Zeolites (also called as Crystelline silicon dioxide, quartz) are chemical compounds of phosphorus, aluminum, oxygen, and silicon, intrinsically embedded in a stable structure with repeated topology (Mohammadkhani et al. 2016). Zeolites could be defined as porous aluminum silicates and classified into synthetic and natural types (Carr et al. 2009, Rhodes 2010). Zeolites are edible, biocompatible, and non-toxic substances and they have a few unique features like molecular sieve structure, ionic exchange ability, and water absorbance (Papaioannon et al. 2022). Zeolites have a great potential for a wide range of technical, industrial, agricultural, commercial and also biomedical applications, for this reason, zeolites have been called as “magic Stones” (Laurino and Palmieri 2015). More than 70% of the body is formed from water, hence zeolites can be used to control the body fluids volume (Auerbach et al. 2003). In this approach, zeolite is used as an anti-diarrheal drug, which is also beneficial for digestive disorders (Serati-Nouri et al. 2020).

Clinoptilolite, which is a form of crystal silica, provides essential minerals for individuals by way of food additives (Papainoannou et al. 2022). Clinoptilolite is used in agriculture as a potassium releasing agent and prolongs the irrigation time due to its suitable absorbent property (Polat et al. 2004, Rehakova et al. 2004). Although clinoptilolite is the main component of natural zeolite (25% SiO_2), it also contains different minerals such as sodium and magnesium. In addition, it has been reported to be beneficial in calf diarrhea (Sadeghi and Shawrang 2008, Cerbu et al. 2020). Cristobalite (100% SiO_2), another form of natural zeolite, forms at very high temperatures and low pressure (San et al. 2004). It has a similar chemical formula (SiO_2), but has a distinct crystal structure. Cristobalite is less common than quartz and can be found in volcanic rocks and glass (Mossman and Glenn 2013). The considerable efficiency of the pure SiO_2 tablet form has been demonstrated in acute diarrhea in human medicine (Tieroshyn et al. 2020).

To our knowledge, there is no study on the efficiency of pure SiO_2 in neonatal calf diarrhea as an adjunct treatment drug. This study aimed to demonstrate the effects of oral use of cristobalite in neonatal calf diarrhea.

Materials and Methods

The study was approved by The Ethics Committee of Ondokuz Mayıs University (ID: E-92 68489742--604.01.03-139889). The animal material was selected from forty-five diarrheic calves referred to our hospital between January and August 2020, and twenty-five calves without coccidiosis, cryptosporidium, or diet etiology were included in the study.

All calves included in the study were pre-weaned and had no vaccination or colostrum intake history. The calves were alternately assigned into one of two groups according to arrival date.

Table 1. Primers used in the study.

Primer and Probe	Sequence (5'---------- 3')	Agent (Target zone)	Source
BCov-fwd	CTAGTAACCAGGCTGATGTCAATACC	Bovine coronavirus (N)	Cho et al. (2010)
BCoV-rev	GGCGGAAACCTAGTCGGAATA		
BRV-fwdl	TCAACATGGATGTCCTGTACTCCT	Bovine rotavirus Group A (VP6)	Cho et al. (2010)
BRV-fwd2	TCAACATGGATGTCCTGTATTCCT		
BRV-fwd3	TCAACATGGATGTCCTTTATTCCT		
BRV-revl	TCCTCCAGTTTGGAACTCATT		
BRV-rev2	TCCCCCAGTTTGGAATTCATT		
BRV-rev3	CCCTCCAGTTTGGAATTCATT		

Conventional treatment was applied to the calves in Group 1 (n = 12). In addition to conventional treatment, cristobalite (Zoosorb, Biomedix, Prague, Czech Republic) (1 gr/10 kg) was given orally to calves in Group 2 (n = 13) three times a day along with the treatment.

Conventional treatment included isotonic NaCl solution (10 ml/kg/day), $NaHCO_3$ (1 ml $NaHCO_3$ = 1MEq, 100 ml in total) and 5% dextrose solution (5 ml/kg/day) given by auricular venous catheterization (18G) in 24 hr. Also, ceftiofur (Ceftisin, Teknovet, Istanbul, Turkey) was injected intramuscularly (5 mg/kg), once a day up to discharge. The amount of fluid therapy and bicarbonate solution varied according to the animal's dehydration and vital parameters (temperature, ability to stand, and suckling reflex). Cristobalite was administered by calf drencher (Kerbl, Buchbach, Germany) to two calves (one from each group) which did not prefer the oral intake.

Calves were fed with 2 l of calf milk replacer (Vitamilk, Rougham, England) twice a day with a nipple bucket. If calves refused oral intake, feeding was performed with a calf drencher. Besides, oral rehydration fluid (Effydral, Zoetis, Leiden, Holland) was diluted with 2 l of warm water and supplied to the animals once a day up to discharge. Calves were hospitalized individually, and there was no contact between calf boxes. Daily cleaning and the infrared heater were provided for each box. After a calf was discharged, the box was cleaned and disinfected with bleach.

All the calves underwent the same management conditions. All the feeding and treatment procedures were conducted by the same operators. Another operator who was blinded for group information noted the clinical and fecal scores.

Blood samples were collected from the jugular vein when calves were included in the study, on the third day of treatment and right before discharge, using a holder and a vacutainer. Hematological samples were taken into 2 ml tubes containing EDTA and analyzed immediately (Table 3) (Mindray BC-5000vet, Mindray Bio-medical Electronics Company, Shenzhen, China). In addition, fecal samples were collected on the same days in order to determine the etiological factors. The fecal samples were obtained directly from the rectum using sterile latex gloves, were separated into two aliquots in sterile containers, and were stored at -80°C for microbiological and virological analysis.

Determination of Escherichia coli (CS31a and K99)

DNA extractions from feces were performed by DNA isolation kit (Pure Link® Genomic DNA mini kit, Invitrogen, Thermo Fisher, CA, USA), according to the manufacturer's instructions. Extracted DNAs were used as a template for identifying as being Escherichia coli CS31a or K99. The specific oligonucleotide primers used for PCR analysis are presented in Table 1. PCRs for determining clpG gene for Escherichia coli CS31a were performed as described by Bertin et al. (1998). The DNAs giving a band of 403 bp were evaluated as positive for the clpG gene and identified as Escherichia coli CS31a (Fig. 1). K99 gene was investigated in DNAs for being Escherichia coli K99. The DNAs giving a band of 314 bp were evaluated as positive for the K99 gene and identified as Escherichia coli K99 (Fig. 2).

Determination of viral pathogens

RNA extractions were performed by RNA extraction kit (Macherey-Nagel, 1605/010, Düren, Germany) according to the manufacturer's instructions. Primers targeting N gene segment for Bovine coronavirus and VP6 gene segment for Bovine rotavirus suggested by Cho et al. (2010) were used in Real-Time PCR test (Table 1). The Real-time PCR with SYBR green was performed using iTaqTM universal SYBR® Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA, USA).

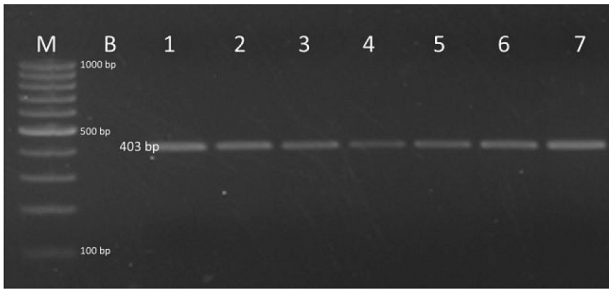

Fig. 1. Demonstration of PCR results for Escherichia coli CS31a. M: Marker (100-1000 bp), Lane 1, 3, 5, 7: positive results of CS31a gene in Group 1 of calves, Lane 2, 4, 6: positive results of CS31a gene in Group 2 of calves, B: negative control.

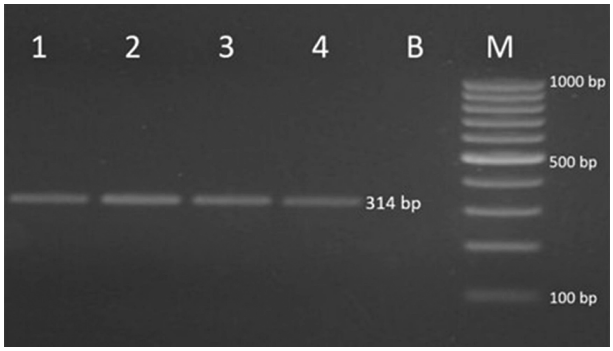

Fig 2. Demonstration of PCR results for Escherichia coli K99. B: negative control, M: Marker (100-1000 bp), Lane 1-3: positive results of K99 gene in Group 1 of calves, Lane 2-4: positive results of K99 gene in Group 2 of calves.

PCR mixtures, both bovine rotavirus and bovine coronavirus were tested by real-time multiplex PCR assay. Assay was performed using the Real Time PCR systems (Bio-Rad CFX96, Bio-Rad Laboratories, Hercules, CA, USA). The samples whose melting curves shared the same Tm with the positive control standard and Ct values below 40 were interpreted as positive (Fig 3-a). Tm value was 75.5°C for bovine rotavirus and 80.5°C for bovine coronavirus (±1.0°C) (Fig. 3-b). The animals with vital physiological parameters, appetite, and normal feces were considered to have completed the study.

Statistical analysis

Data were analyzed using the statistical program SPSS v21. The normality of the hematological parameters was assessed using the Shapiro-Wilk normality test. Independent group t-test was used to compare age and hospitalization time. The fecal score was transformed to scale by use in RT1 transformation for the needs of analysis (Conover and Iman 1981). Then, the fecal score was analyzed using the Mann-Whitney U test. Descriptive statistics and differences are presented in Table 4.3. In addition, Fisher's exact test and Chi- square Test was used to compare the efficacy of Group 2 vs. Group 1 regarding the ill and healthy calves on the same day. For hematological parameters, the significance between groups in the same day was determined using Student T-test or non-parametric two-tailed Mann-Whitney U test (Monocytes, Eosinophils). The significance within the group before and after the treatment was assessed using Paired Sample T-test. A value of $p<0.05$ was considered significant in all comparisons.

Results

Forty-five calves were presented to our hospital with a complaint of diarrhea, and 25 of these calves were included in the study. Twenty calves were excluded due to etiologies other than *E. coli*, bovine coronavirus, or bovine rotavirus. Group 1 was composed of 12 calves with a mean age of 6.91 days, and Group 2 was consisted of 13 calves with a mean age of 6 days old. Baseline data of calves included in the study are listed in Table 2. There was no statistically significant difference between age in both groups ($p=0.53$).

Fecal score

Changes in the daily fecal score are given in Table 2. Fecal scoring of calves was as previously described (McGuirk 2008), it was scored as 0 "no diarrhea, normal feces", 1 "soft feces", 2 "watery particulate feces", 3 "watery feces", and the mean values of the fecal scores of the groups were taken.

Efficiency of the treatment was observed with

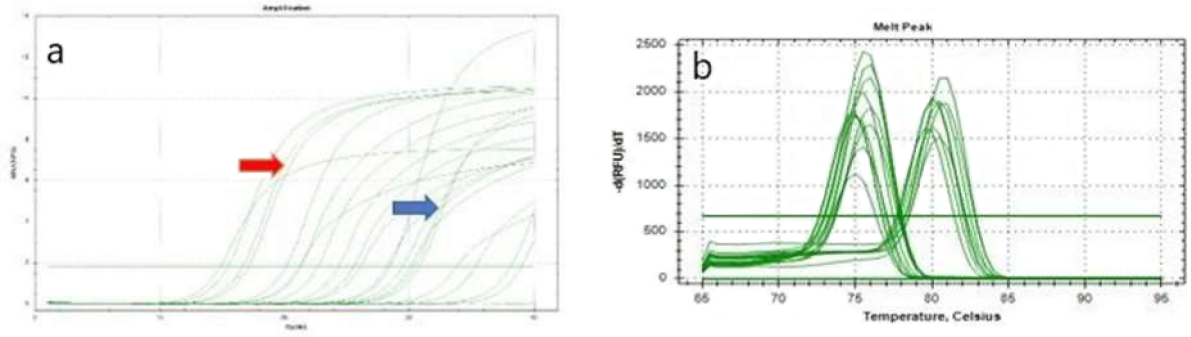

Fig. 3. a – Real-Time RT-PCR amplification curve for the bovine rotavirus (red) and bovine coronavirus (blue) positive control, b – Dissociation curve for RT-PCR positive controls for bovine rotavirus and bovine coronavirus showing an average temperature (Tm value was 75.5°C for bovine rotavirus and 80.5°C for bovine coronavirus (±1.0°C)

Table 2. Daily fecal score changes in groups of calves by mean scores. (mean±standart deviation).

Group	Age (Days)	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Treatment Period (Days)
		Fecal Score Ill/Total	Fecal Score Ill/Total	Fecal Score Ill/Total	Fecal Score Ill/Total	Fecal Score Ill/Total	Fecal Score Ill/Total	
Group 1	6.91	2.66±0.49 12/12	1.75±0.75 12/12	0.75±0.75 12/12	0.50±0.67 11/12	0.16±0.38 9/12	0 2*/12	4.61
Group 2	6	2.76±0.59 13/13	1.92±0.86 12/13	1.61±0.96 12/13	1.00±0.81 6/13	0.46±0.66 5/13	0.23±0.43 0/13	3.66
P	0.53			0.027				0.012

significant clinical improvement. For this purpose, fecal scoring was followed. There were no significant differences in health status of calves at the second day of the treatment. On the third day, six calves were completely recovered and discharged from the hospital in Group 2. On the same day, only one calf was recovered and discharged from the hospital in Group 1 (Fig. 4).

In Group 1, two calves whose coronavirus were isolated on the first day were excluded from the study due to the lack of improvement of the clinical and fecal scores on the 5th day of the treatment and were considered unsuccessful. There was no non-response to the treatment in Group 2. None of the calves were dead during the study.

Duration of diarrhea in calves was to be considered. Moreover, this can be evaluated as the duration of hospitalization. The mean hospitalization period in Group 2 was 3.66 days, while the average in Group 1 was 4.61 days. It was determined that the hospitalization period of the animals included in Group 2 was significantly shorter than in Group 1 ($p = 0.012$).

Etiological findings

Pathogens isolated from fecal samples collected before the treatment were, K99 *E. coli* in 4 samples (2 in Group 1, 2 in Group 2), CS31a in 17 samples (10 in Group 1, 7 in Group 2), *bovine rotavirus* in 7 samples (3 in Group 1, 4 in Group 2), *bovine coronavirus* in 7 samples (5 in Group 1, 2 in Group 2) (Fig. 4). It was observed that five calves in Group 1 were infected with at least two agents, while three calves in Group 2.

On the third day of the treatment, viral agents were not isolated in Group 2, but rotavirus/coronavirus was isolated in 4 calves in Group 1. Two calves of coronavirus isolated on the 1st and third days with ongoing diarrhea were also isolated on the 5th day. On the same day of the treatment, CS31a *E. coli* was isolated in 4 animals in both groups.

As a result of the isolations made from the fecal samples taken on the day the animals were discharged from the hospital, CS31a was isolated in two calves in the Group 2, and K99 was isolated in 3 calves that were not isolated with K99 earlier. In Group 1, CS31a was continued to be isolated in 2 samples. In this group, two calves of coronavirus isolated on the first and third days with ongoing diarrhea were also isolated on the fifth day.

Hematological results

Hematological results were summarized in Table 3. As a result of the statistical analyzes performed, no significant difference was found between the groups in terms of hematological parameters before or after the treatment.

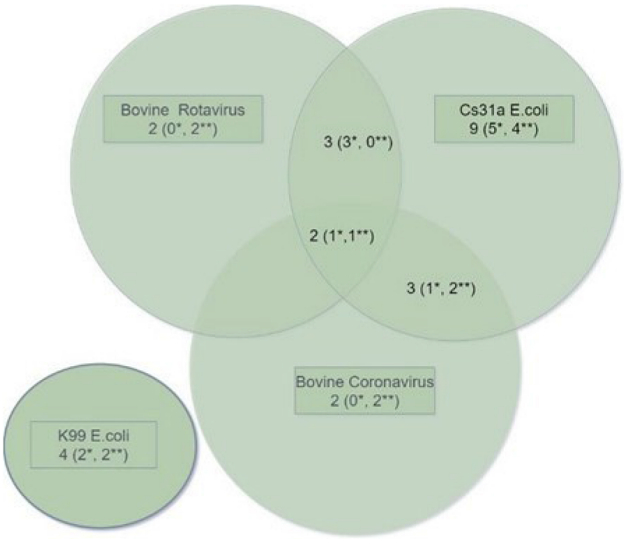

Fig. 4. Etiological findings on the first day of the treatment presenting comordities,
* Group 1 of calves, ** Group 2 of calves.

Discussion

Neonatal calf diarrhea is known to occur frequently in the first week of life, and the disease is more severe in younger calves (Divers and Peek 2007). The average age of the calves included in the study was 6.44, and it was determined that there was no difference between the groups.

The effect of clinoptilolite in bovine diarrhea has been studied before, but these studies did not reveal the etiological causes of diarrhea (Sadeghi and Shawrang 2008, Cerbu et al. 2020). Furthermore, two studies have revealed that the oral use of clinopotilolite was limited the excretion of giardia cysts in feces and were reduced in goat kids (Alıc et al. 2017) and dogs (Ayan and Erdogan 2019). As in these studies using oral zeolite, no drug related side effects were observed in our study. Before the treatment, there were no statistically significant differences between the two groups in term of hematological parameters. While there was a decreasing trend in Group 2 in the amount of neutrophils and monocytes in the post-treatment hematological parameters compared to the pre-treatment, it was observed that there was a tendency to increase in Group 1.

The effect of clinoptilolite on hematologic parameters in mice had also investigated and no diffrerences and side effect was observed (Auerbach et al. 2003). The hematological data we obtained, zeolites at appropriate doses did not form a negative effect on hematological parameters.

On the first day of hospitalization, bovine rotavirus was isolated in 6 samples, and bovine coronavirus was isolated in 7 samples. Etiological data and also animals with mixed infections were presented in Fig. 4. It was determined that 16% of the calves were infected with K99 *E.coli* alone, while 32% of the animals were infected with at least two pathogens.

While CS31a *E. coli* was isolated as a single pathogen in 9 calves, it was not co-isolated with K99 *E. coli* in any animal. The isolation of at least one viral agent in 52% of the calves necessitates consideration of antiviral treatment options in addition to antibacterial treatment. In the fecal samples taken on the third day of the treatment, no viral agents were found in Group 2 (6 samples positive on the first day), it was determined that viral shedding continued in 4 out of 5 animals diagnosed with viral enteritis in Group 1. In the conventional treatment group, two calves isolated with coronavirus were considered unsuccessful in the study due to the lack of clinical improvement, and the coronavirus was isolated again in the feces of these animals on the sampling. Although the number of animals was limited, the fact that this situation was not observed in Group 2 made the opinion that cristobalite exhibited antiviral activity. The antiviral efficacy of cristobalite has been previously studied in humans and shown to reduce viral load (Grce and Palvelić 2005). There is no publication in antiviral efficiency of cristobalite up to time. Bovine rotavirus is limited to enteritis, but diarrhea caused by bovine coronavirus may continue for 1 week due to severe enterocolitis. Systemic antibiotics are used in the fight against secondary bacterial infections of mostly lung, intestine and other systems of calves. The use of non-antibiotic drugs to reduce intestinal bacterial load increases importance in recent years, and it aims to prevent the development of antibiotic resistance.

On the first day of hospitalization, *E.coli* was isolated

Table 3. Pre- and post-treatment hematological results of calves.

Parameter (Unit)		Pre-treatment	Post-treatment
White Blood Cells (10^9/l)	Group 1	10.05±3.03	14.15±5.49
	Group 2	13.16±4.22	12.25±5.16
Neutrophils (10^9/l)	Group 1	5.51±1.73	7.50±5.30
	Group 2	8.18±3.99	6.71±4.24
Lymphocytes (10^9/l)	Group 1	3.22±1.68	4.43±1.16
	Group 2	3.83±0.79	4.12±0.94
Monocytes (10^9/l)	Group 1	1.13±0.78	1.93±3.20
	Group 2	1.01±0.92	0.70±0.33
Eosinophiles (10^9/l)	Group 1	0.16±0.14	0.26±0.46
	Group 2	0.12±0.09	0.69±1.98
Basophils (10^9/l)	Group 1	0.004±0.009	0.004±0.005
	Group 2	0.003±0.006	0.004±0.006
Red Blood Cell (10^{12}/l)	Group 1	6.82±1.37	7.49±1.57
	Group 2	7.05±0.94	7.21±1.17
Hemoglobin (g/dl)	Group 1	9.20±1.85	9.85±2.20
	Group 2	9.58±1.35	10.47±3.61
Hematocrit (%)	Group 1	0.27±0.05	0.29±0.06
	Group 2	0.28±0.03	0.30±0.08
Mean Cell Volume (fl)	Group 1	40.44±2.07	39.81±2.14
	Group 2	39.97±1.89	42.12±9.8
MCH (pg)	Group 1	13.50±0.78	13.12±0.72
	Group 2	13.59±0.58	14.48±4.37
MCHC (g/l)	Group 1	333.54±7.96	329.57±2.63
	Group 2	340.00±10.77	341±20.73
RDV-CV	Group 1	0.22±0.01	0.22±0.01
	Group 2	0.23±0.03	0.23±0.04
RDV-SD	Group 1	31.40±3.32	31.22±3.65
	Group 2	32.20±4.84	32.41±5.19
Platelets (10^9/l)	Group 1	633.81±303.87	437.80±165.47
	Group 2	613.72±425.59	414.30±131.01
Mean Platelet Volume (fl)	Group 1	4.85±0.52	4.54±0.24
	Group 2	4.84±0.30	5.31±1.68
PDW	Group 1	15.20±0.22	15.20±0.27
	Group 2	15.06±0.26	15.12±0.37
PCT (mL/l)	Group 1	3.51±1.06	4.21±1.44
	Group 2	3.89±1.52	4.38±1.05

in 21 (84%) samples, however, in previous studies, Mohammed et al. (2019) found 46%, Paul et al. (2010) 76% and Dereje (2012) 43.1%. These results may be due to regional differences, the number of animals included in the study, and hygiene conditions. However, besides these factors, unlike other studies, our isolation of CS31a *E. coli* caused this rate to be quite high. Yeshiwas and Fentahun (2017) concluded that *E. coli* is one of the most common diseases of newborn calves (9-10 days of age) characterized by watery diarrhea and the affected calves die within 2–3 days.

Diarrhea is the primary cause of morbidity and mortality in the neonatal period of calves, resulting in significant economic losses worldwide. Uncontrolled use of antimicrobials in veterinary practice accelerates the continuously increasing number of resistant bacteria strains. An antimicrobial resistance study conducted on CS31a *E. coli* revealed 88.1% multi-resistant antibiotic resistance (MAR), and only 1% of these strains were susceptible to antibiotics (Gibbons et al. 2014). The clpG gene encoding the CS31A antigen and it is plasmid borne and has previously been found in association with clusters of resistance genes on conjugative plasmids (Girardeau et al. 1988, Jallat 1994). In addition, the etiological data we obtained have been published in terms of caution against CS31a *E. coli* in terms of preventing calf deaths (Ozcan et al. 2021). A rise in multi-drug resistant isolates is of concern for researchers. A high resistance rate was noticed on the assessment of the MAR indices of isolates (Assumpção et al. 2015, da Silva Menezes Azola et al. 2021). On the third day, CS31a *E. coli* was determined in 4 animals in Group 2, while determined in five animals in Group 1. K99 *E. coli* was also present in three calves without previously isolated in the last fecal samplings. This has been interpreted as plasmid transfer (Nagy and Fekete 2005).

The addition of the SiO2 allowed a significant ($p<0.01$) reduction in the treatment duration averagely for 0.95 (20.6%) days in comparison with Group 1. Longer periods of diarrhea will lead to an increased loss of fluids, leading to the development of dehydration, strong ion acidosis, electrolyte abnormalities, and the development of a negative energy balance. In this extremely important period, especially in the absence of active immunity, it is crucial to minimize the duration of the disease in order to avoid the negative effects of each sick day in the neonatal period on factors such as productivity, average daily gain, immunity, where metabolic disorders due to diarrhea may create different predisposition in the future (Smith 2009). Thus, shortening the duration of diarrhea is critical; in our study, the shortened hospitalization period of the cristobalite group contributed to both treatment cost and prognosis.

Naturally the study has few limitations. The major limitation is that a larger subject number possibly would more clearly demonstrate the antiviral efficiency. Other limitation is the lack of a negative control group, which actually was an elective situation, since the study is a clinical trial rather than being an experimental design due to ethical reasons. Nevertheless, the advantage of maximally stabilized external factors (such as all the calves were transported to the animal hospital and all were treated and cared by the same practitioner throughout the study), created a good basis for reliable results. Certainly, further investigation with larger subject numbers is needed, still the present study is the first investigation on the effects of cristobalite in neonatal calf diarrhea.

The present results demonstrate that the high rate of CS31a *E.coli* isolated in calves and the glpG gene encoding CS31a is associated with antimicrobial resistance, which should be considered in the protection, control and treatment of calf diarrhea. In conclusion, crystobalite "Zoosorb®" is well tolerated and accelerates the achievement of clinical recovery at the acute episode of the diarrhea. And thus must be considered in the conventional treatment strategy of calf diarrhea.

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