The prevalence of coronavirus in fecal samples of neonatal calf diarrhea using electron microscopic examination

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Abstract:

BACKGROUND: Neonatal calf diarrhea (NCD), also known as calf scours, is caused by viruses, bacteria, and parasites. Coronavirus is one of the important causes of NCD syndrome. Electron microcopy has been used for demonstration of viruses in fecal samples of diarrheic calves since 1969. **OBJECTIVES:** The aim of this study was to determine the prevalence of coronavirus in fecal samples of neonatal calf diarrhea using electron microcopy examination of fecal samples. **METHODS:** For the purpose of this study, a total of 100 cases (under 2 months of age) of diarrheic calves from 25 commercial farms in four districts of East Azerbaijan province of Iran were used. Fresh Fecal samples using sterile swab were collected from every calf with clinical diarrhea. All samples were transferred into a sterile container and then were sent to the laboratory to be kept in -20^{o}C freezer until examination. Electron microscopic examination was applied to all fecal samples. Chi-square test was used to analyze the data. **RESULTS:** According to the results of this study, coronavirus was present in 15% of cases and a significant difference was found between the prevalence rate and the seasons ($p<0.05$). The highest prevalence was recorded in winter (28%) and the least prevalence was seen in summer (4%). There was not any significant difference in both age groups and different districts according to the prevalence rate of infection. **CONCLUSIONS:** Neonatal calf diarrhea is a multi-etiological syndrome. Among the viral diarrhea, bovine coronavirus is one of the major causes of this syndrome. For detection of coronavirus in feces, electron microscope examination is a gold standard diagnostic technique. It seems that coronavirus can be considered as one of the important etiologies of neonatal calf diarrhea in east Azerbaijan of Iran.

Introduction

Diarrhea in newborn calves under 30 days of age is one of the most common disease complexes. It is a significant cause of economic loss in both dairy and beef herds. The bovine coronavirus is an important cause of diarrhea in calves from birth to 3 months of age, but mostly between 1 and 2 weeks of age. Coronavirus was first isolated from calves with diarrhea in 1971 during an experimental field trial to evaluate a rotavirus vaccine. All known corona-viruses are single stranded RNA viruses of the

Coronaviridae family (Anderson 1992). Experimental studies showed that the incubation period is 20 to 30 hours. Generally, coronavirus diarrhea is more watery and greater in severity than rotavirus diarrhea, leading more rapidly to dehydration and acidosis (Andrews et al., 2004). The virus can be shed by up to 70% of adult cows despite the presence of specific antibodies in their serum and feces. The peaks of shedding are during the winter and at parturition in North America (Radostits et al., 2007). Calves born from carrier cows are at a higher risk of diarrhea. The pathogenesis of coronaviral enteritis in calves is similar to the rotavirus infection. The villous epithelial cells of the small and large intestines are commonly affected. In 1969, for the first time, electron microcopy was used to show the responsibility of a virus in calf diarrhea (Brugere-Picoux, Tessieer, 2010). Electron microscopy examination is a gold standard diagnostic technique for demonstration of the virus in feces. Subclinical persistence and recurrent infections are also common in both neonatal and older calves. Demonstration of the virus in feces using electron microscopy (EM) has been introduced as a standard diagnostic technique. The EM technique is currently used in Texas Veterinary Medical Diagnostic Laboratory in USA for calf diarrhea pathogen identification (Schroeder et al., 2012). It is easier to see the virus if it has been concentrated by ultracentrifugation or clumped by immune electron microscopy using specific antiserum. With electron microscopy, the virus can be detected for up to 6 to 10 days after the onset of the diarrhea. Immunofluorescent staining is used as another technique for detection of the virus in fecal samples and can be conducted in a few hours. The fluorescent antibody technique can only detect the virus within epithelial cells which are present in the feces for 4 to 6 h after the onset of the diarrhea. However, in some studies the fluorescent antibody technique detects the virus in only 20% of the samples, while electron microscopy detected the virus in about 60% of the samples (Radostits et al., 2007). ELISA technique has also been used for demonstration of coronavirus antibodies in serum samples (Rabbani et al., 2007). More recently, RT-PCR has been introduced as a modern test for detecting RNA of the virus in fecal samples (Zhu et al., 2011). The aim of this study was to determine the prevalence of coronavirus in neonatal calf diarrhea fecal samples in the East Azerbaijan province of Iran, during a 12-month period.

Materials and Methods

For this study, the following solution and materials were prepared, as described previously (Nourmohammadzadeh et al., 2012).

Antibiotic solution: To prevent the growth of the bacteria which can interfere with the existence of the virus in the samples, an antibiotic mixture was prepared and added into each sample. For this purpose, 10ml sterilized purified distilled water was added into a vial containing 1 million units of Penicillin G procaine and 1 gr of streptomycin. The mixture was shaken in order for the antibiotic to be completely dissolved. All sampled calves were located in the Province of Azerbaijan of Iran. The region was divided into four districts: northwest, northeast, southwest, and southeast. The number of the dairy farms in each district was determined, and the sampling of the diarrheic calves was done in a period of twelve months.

Totally, one hundred fecal samples were collected from diarrheic calves of industrial dairy farms within the first week of the onset. Fecal samples were collected after cleaning the anus of the calf. Each fecal sample was taken directly from the rectum of the calf via a sterile swap, transferred into a sterilized glass vessel with lids, on which the number of each calf and the specimen were written. One ml of the antibiotic solution was added into each vessel. After fixing the lid, it was shaken to provide a mixture of the feces with antibiotic solution.

All samples were placed into an ice container at the farm and then within 12 hours transferred into a -20°C freezer until the time of examination. For conducting the electron microcopy examinations, all freezed samples were carried to the Virology section at Razi Vaccine and Serum Research Institute, in Karaj. In general, this process was set up so that each sample group was tested in less than one week.

To prepare the samples for the electron microscope examination, they were taken out of the freezer to be melted at room temperature. When the samples turned from the frozen state into the liquid, 5 mL of each sample was transferred into a centrifuge tube

and then spun at 10000 rpm for 15 minutes using a centrifuge. As the viruses are lighter than the other particles in the feces, they floated up to the upper part. One drop of the upper part was spread on the cooper grid. When it became dried, it was dyed by the negative PTA method, as described by Bozzola and Russell, and then observed by the electron microscope (Philips 400) (Bozzola and Russell, 1999). If a sample contained coronavirus virus, it would be photographed.

Results

Among the 100 collected fecal samples, 15 cases were found to have coronavirus using electron microscopic examination. This result indicated that the prevalence of coronavirus contamination in the whole area of East Azerbaijan province of Iran was equal to 15%. The rate of contamination in the dairy farms of northwest, northeast, southwest, and southeast was equal to 10.71%, 20%, 15.38%, and 12.5%, respectively (Table 1) which shows the highest prevalence rate in the northeast and the lowest rate in the northwest area.

Comparing the prevalence of coronavirus infections during the four seasons, as shown in table 2, indicates that the highest prevalence was recorded in winter (28%) and the lowest was found in the summer (4%). There is a significant difference between different seasons ($p<0.05$). Table 3 shows the rate of infection in five age groups, which indicates that the highest prevalence rate was seen at 2 to 4 weeks of age (19.23%) and the least during the first week of age (10%). Figure 1 shows a photograph of a complete and also an empty coronavirus particle from a positive fecal sample.

Table 1. Prevalence rate of coronavirus in fecal samples in four districts of Eastern Azerbaijan.

District	Number of Samples	Number of positive samples	Prevalence rate %
North - West	28	3	10.71
North - East	30	6	20
South - West	26	4	15.38
South - East	16	2	12.5
Total	100	15	15

Table 2. Prevalence rate of coronavirus in fecal samples in different seasons.

Season	Number of samples	Number of positive samples	Prevalence rate %
Spring	25	3	12
Summer	25	1	4
Autumn	25	4	16
Winter	25	7	28
Total	100	15	15

Table 3. Prevalence rate of coronavirus in fecal samples in different age groups.

Age groups (Week)	Number of samples	Number of positive samples	Prevalence rate %
1	20	2	10
1-2	26	5	19-23
2-4	21	3	14.23
4-6	18	3	16.66
6-8	15	2	13.33
Total	100	15	15

Discussion

Coronavirus is an important cause of diarrhea in calves with prevalence estimates ranging from 11 to 81%. (Radostits et al., 2007). Coronaviruses are particularly common in 5 to 30 days old calves, and they have been detected in feces of more than 70% of clinically normal cows. Calves born to carrier animals are at a higher risk for developing diarrhea. Calves may be infected with coronavirus by the oral or respiratory route (Smith, 2009). In Australia, it has been reported that 21.6% diarrheic calves were infected with coronavirus, using RT-PCR method (Izzo et al., 2011). In another study conducted by Zhu et al., 7.14% of diarrheic calves were found infected with coronavirus, using RT-PCR (Zhu et al., 2011). However, the study of the coronavirus infection using ELISA in India showed that 11.76% of the clinically diarrheic calves were positive (Rai et al., 2011).

In Iran, a study in 2007 showed that coronavirus antibodies are presented in 82% of the diarrheic calves and in 72% of the healthy calves (Rabbani et al., 2007). In an earlier serological study conducted in Markazi Province of Iran, it was shown that 34% of the diarrheic calves younger than 30 days old were positive against coronavirus, using ELISA test (Ghaemmaghami et al., 1999). The Study of coronavirus infection using cELISA in diarrhoic calves in Mashhad district showed that 3.7% of the calves were infected (Mayameei et al., 2010).

In the present study, using fecal electron microscopy examinations, 15% of the diarrheic

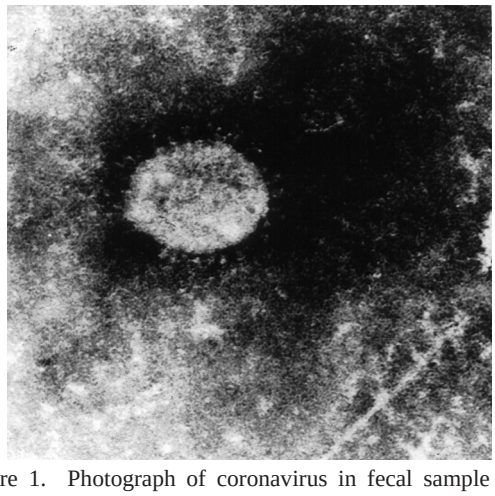

Figure 1. Photograph of coronavirus in fecal sample of a diarrheic calf using negative stain transmission electron microscopy.

calves showed coronavirus infection. Comparing the results of the present study with other studies revealed that the rate of coronavirus infection is different in different geographical and climate conditions. However, the high prevalence rate of coronavirus in serological studies in comparison to the virus detection methods showed that the serological methods could not demonstrate the accurate clinical pattern of the infection.

The results of our study show that the highest prevalence rate of infection was found in the northeast (20%) and the lowest rate was found in the northwest area (10.7%). However, statistical analysis using chi-square test shows that there is no significant difference between districts according to the prevalence rate of coronavirus infection. Nonetheless, results of chi-square test, shown in table 2, reveal that there is a significant difference between the prevalence rate and the season ($p<0.05$). The highest prevalence rate was recorded in winter (28%) and the lowest was found in the summer (4%). The high prevalence of coronavirus infections in winter in the present study is in agreement with other studies (Radostits et al., 2007). It seems that in cold conditions, as in winter, the absorption of the IgG1 by calves is decreased which can cause reduction of the serum IgG level. As a result, this can reduce the immunity of the calves and increase the rate of coronavirus infection in calves born during winter (Badiei et al., 2013).

The results of the present study also indicate that the highest prevalence rate was seen at 2 to 6 weeks of age and the least during the first week of age and 6 to 8 weeks of age. However, statistical analysis of the data shows that there is no significant difference between age groups.

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فراوانی کروناویروس در نمونه‌های مدفوع اسهال گوساله‌های نوزاد با استفاده از میکروسکپ الکترونی

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چکیده

زمینه مطالعه: اسهال گوساله‌های نوزاد یکی از بیماریهای است که عوامل متعدد ویروسی، باکتریایی و انگلی در بروز آن نقش دارند. کروناویروس یکی از عوامل مهم در بروز این سندرم می باشد. ازسال ۱۹۶۹ میلادی به بعد برای نشان دادن حضور ویروس در نمونه‌های مدفوع گوساله‌های مبتلا به اسهال استفاده از میکروسکپ الکترونی به‌عنوان یکی از روش‌های تشخیصی مورد توجه قرار گرفت. **هدف:** هدف از مطالعه حاضر تعیین فراوانی کروناویروس در نمونه‌های مدفوع اسهال گوساله‌های نوزاد با استفاده از مشاهده ویروس در نمونه های مدفوع به کمک میکروسکپ الکترونی بود. **روش کار:** برای انجام این مطالعه در مجموع ۱۰۰ راس گوساله مبتلا به اسهال با سن زیر دو ماه از ۲۵ گاوداری صنعتی از چهار منطقه در استان آذربایجان شرقی در طی ۱۲ ماه مورد مطالعه قرار گرفت. نمونه های مدفوع تازه با استفاده از سواب های استریل از هر یک از گوساله‌های مزبور اخذ گردید. هر نمونه به یک لوله آزمایش استریل منتقل وسپس به آزمایشگاه ارسال و تا زمان انجام آزمایش در فریزر $-20^{o}C$ نگهداری شد. در مطالعه حاضر از روش مشاهده ویروس با کمک میکروسکپ الکترونی استفاده شد. برای تجزیه و تحلیل داده‌ها از روش آماری مربع کای استفاده گردید. **نتایج:** نتایج مطالعه نشان داد که عامل کروناویروس در ۱۵٪ نمونه‌های مدفوع حضور داشته اختلاف معنی‌داری بین فراوانی عفونت و فصل وجود دارد ($p<0/05$). بیشترین فراوانی در زمستان (۲۸٪) و کمترین فراوانی در تابستان (۴٪) مشاهده شد. نتایج این بررسی همچنین نشان داد که اختلاف معنی داری بین گروه‌های سنی و نیز بین مناطق مختلف استان در رابطه با فراوانی حضور ویروس در گوساله‌های اسهالی وجود ندارد. **نتیجه گیری نهایی:** اسهال گوساله‌های نوزاد یک سندرم چند علتی می‌باشد. بین اسهال‌های ویروسی، کروناویروس گاوی یکی از سبب‌های مهم این سندرم است. برای نشان دادن کروناویروس در مدفوع، مشاهده با میکروسکپ الکترونی یک روش تشخیصی استاندارد می‌باشد. در این مطالعه مشخص شد که کروناویروس در استان آذربایجان شرقی می‌تواند به عنوان یکی از سبب‌های مهم سندرم اسهال در گوساله‌های نوزاد محسوب گردد.

واژه‌های کلیدی: اسهال گوساله‌ها، کروناویروس، میکروسکپ الکترونی

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