RESEARCH ARTICLE

Epidemiological investigation of six diarrhea pathogens in the main beef cattle breeding areas in Ningxia, China

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Beef cattle diarrhea seriously affects the healthy development of beef cattle farming and is caused by pathogenic microbial infections such as bacteria, viruses, and parasites or by various factors such as nutrition, environment, and management. Among them, *Mycobacterium avium subsp. Paratuberculosis* (MAP), *Escherichia coli* K99, *Cryptosporidium*, *Giardia duodenalis*, *Enterocytozoon bieneusi*, and *Eimeria coccidia* are the common pathogens causing diarrhea in beef cattle. In order to investigate the infections of six diarrhea pathogens in beef cattle, 144 fecal samples of beef cattle at different ages and in different breeding scales with diarrhea were randomly collected from different counties of Guyuan City, Ningxia, China. Different pathogen specific primers were used for polymerase chain reaction (PCR) amplification and chi square test was used for difference analysis. The results showed that the infection positive rate was the highest in Longde County, while the lowest one was in Jingyuan County. There was a significant difference between Longde County and the other four regions. Among the beef cattle at different age (months), the highest positive rate of 83.3%7 was 12 months of age. Under two different feeding modes, the positive rate of six diarrheal pathogens in large-scale beef farms was 73.3%, while the positive rate of six diarrheal pathogens in small-scale farms was 80.0%. Single pathogen infection was the most common one, accounting for 51.4%, and double pathogens infection accounted for 43.2%. The results indicated that the detection of 6 kinds of diarrhea pathogen should be further strengthened in the main beef cattle breeder areas in Ningxia, China, especially the mixed infection of multiple pathogens, which would improve the prevention and control of the disease.

Keywords: beef cattle; diarrhea; pathogen; *Mycobacterium avium subsp*. *Paratuberculosis*; *Escherichia coli* K99; *Cryptosporidium*; *Giardia duodenalis*; *Enterocytozoon bieneusi*; *Eimeria coccidia*.

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Introduction

Beef cattle diarrhea seriously affects the healthy development of beef cattle farming. Beef cattle diarrhea is caused by pathogenic microbial infections such as bacteria, viruses, and parasites or by various factors such as nutrition, environment, and management. Among them, *Mycobacterium avium subsp. paratuberculosis* (MAP), *Escherichia coli* K99, *Cryptosporidium*,

Giardia duodenalis, *Enterocytozoon bieneusi*, and *Eimeria coccidia* are the common pathogens causing diarrhea in beef cattle. Bovine paratuberculosis is an infectious disease caused by *Mycobacterium paratuberculosis* and characterized by persistent and intractable diarrhea [1]. *E. coli* K99 mainly causes watery diarrhea in calves, of which calves are highly susceptible to *Enterotoxigenic Escherichia coli* (ETEC) [2]. *Cryptosporidium*, *Giardia duodenalis*, and *Enterocytozoon bieneusi* are common protozoa causing diarrhea in humans and animals and can be transmitted through food and waterborne sources [3-5]. Beef cattle coccidiosis is a parasitic disease characterized by diarrhea and bloody stools caused by different species of the genus *Eimeria* parasitizing the intestinal tract of beef cattle [6]. With the development of the cattle industry toward an intensive scale, timely and accurate diagnosis of beef cattle diarrhea is particularly important for ranches, as well as having public health significance.

Guyuan City is the main production area for beef cattle breeding in Ningxia Hui Autonomous Region of China. Diarrheal diseases are common in beef cattle farming, but the diversity of diarrheal pathogens has not been investigated. In this study, the prevalence of 6 pathogens causing diarrhea in beef cattle in Guyuan city, Ningxia, China was investigated. The relationships between those 6 diarrheal pathogens and different areas, beef cattle monthly age, and farming scales were analyzed, respectively. The results of this study would enrich the epidemic information of beef cattle diarrhea pathogens and lay the foundation for the development of more effective disease prevention and control measures for breeding farms.

Materials and Methods

Collection of samples

A total of 144 fecal samples of diarrheic beef cattle were collected from 5 counties of Guyuan City, Ningxia Hui Autonomous Region, China during September to December 2022, including 60 samples from Longde County, 12 samples from Jingyuan County, 18 samples from Xiji County, 36 samples from Pengyang County, and 18 samples from Yuanzhou County. All samples were collected based solely on the presence of diarrhea symptoms without distinguishing the gender of beef cattle. 36 samples were collected from beef cattle aged 0-6 months, 72 samples were collected from beef cattle aged 7-12 months, and 36 samples were collected from beef cattle aged over 12 months. 69 fecal samples were collected from large-scale farms with over 200 beef cattle, and 75 fecal samples were collected from small-scale farms with less than 200 beef cattle. The samples were collected by rectal sampling or fresh fecal samples, which were then immediately placed in an insulated box containing ice packs, and the details of the pen, cow number, month age, and fecal sample properties of the cattle from which the samples were collected were recorded. The samples were then taken back to the laboratory and stored in a 4℃ refrigerator for examination.

Polymerase chain reaction (PCR) amplification

MAP, *Escherichia coli* K99, *Cryptosporidium*, *Giardia duodenalis*, and *Enterocytozoon bieneusi* were identified by using PCR amplification technique. The sequences of different pathogen-specific primers for MAP, *Escherichia coli* K99 (K99), *Cryptosporidium* (SSU rRNA), *Giardia duodenalis* (bg), and *Enterocytozoon bieneusi* (ITS) were listed in Table 1 [2, 7-9]. The PCR reaction was set up as 25 µL of 2× taq PCR Master Mix (Tiangen, Beijing, China), 1 µL each of primers, 2 µL of sample DNA, and 21 µL of ddH_2O. The PCR amplification was performed by using the PCR machine (BIO-RAD, Hercules, California, USA) with the program of 95℃ for 2 min followed by 30 cycles of 95℃ for 30 s, annealing temperatures for different pathogens shown in Table 1 for 20 s, and 72℃ for 2 min, then 72℃ for 8 mins. *Cryptosporidium*, *Giardia duodenalis*, and *Enterocytozoon bieneusi* were amplified by using two sets of primers, with the second round of amplification using the first round PCR amplification product as the DNA template. The PCR amplification products were electrophoresis

Table 1. Primer sequence of 5 diarrhea pathogens.

Primers	Primer sequence (5'-3')	Annealing temperature (°C)	Fragment length (bp)
MAP-F	CAGCGGCTGCTTTATATTCC	58	446
MAP-R	ATGCTGTGTTGGGCGTTA		
K99-F	TATTATCTTAGGTGGTATGG	52.6	314
K99-R	GGTATCCTTTAGCAGCAGTATTTC		
SSU rRNA-F1	CCCATTTCCTTCGAAACAGGA	55	1325
SSU rRNA-R1	TTCTAGAGCTAATACATGCG		
SSU rRNA-F2	AAGGAGTAAGGAACAACCTCCA	55	830
SSU rRNA-R2	GGAAGGGTTGTATTATTAGATAAAG		
bg-F1	CCCATTTCCTTCGAAACAGGA	50	511
bg-R1	TTCTAGAGCTAATACATGCG		
bg-F2	AAGGAGTAAGGAACAACCTCCA	50	511
bg-R2	GGAAGGGTTGTATTATTAGATAAAG		
ITS-F1	GATGGTCATAGGGATGAAGAGCTT	55	400
ITS-R1	TATGCTTAAGTCCAGGGAG		
ITS-F2	AGGGATGAAGAGCTTCGGCTCTG	55	400
ITS-R2	AGTGATCCTGTATTAGGGATATT		

analysed by using 1.5% agarose gel with 10 µL of second round PCR products and 2 µL of 6× DNA Loading Buffer. The PCR amplification products were then separated by 1% agarose gel electrophoresis at 120 V for 30 min, and then, the PCR products were purified and recovered by using a gel recovery kit (OMEGA, Norcross, Georgia, USA). The obtained PCR-purified products were sent to Bioengineering (Shanghai) Co., Ltd. (Shanghai, China) for sequencing.

Eimeria coccidia detection

The number of oocysts per gram of feces (OPG) was calculated according to the McMaster method. Briefly, 2 g of feces was mixed with 5 mL of water before adding saturated saline to bring up the total volume to 60 mL. The mixture was then filtered through a copper sieve. The fecal fluid was injected into the counting chamber of the McMaster counting plate, standing for 5 minutes before counting the number of oocysts in the two graduated chambers under a microscope. The average number of eggs in the two chambers was calculated as follows.

$$OPG = ((n1 + n2) / 2 / 0.15) \times (60 / 2)$$

where (n1 + n2) / 2 was the average number of oocysts in each chamber. 0.15 was the effective volume of each chamber (0.15 mL). 60 was the total volume of fecal fluid (60 mL). 2 was the number of grams of feces used (2 g) [10-12].

Statistical analysis

Microsoft Excel (Microsoft, Redmond, WA, USA) was used for preliminary analysis of six pathogens for different regions, months of age, and single and mixed infections. The differences of infection rates were compared by using chi-square test of SPSS 23.0 (IBM, Armonk, New York, USA) with $P < 0.05$ indicating significant differences and $P < 0.01$ indicating very significant differences.

Results

Infection of beef cattle with diarrhea in different areas

111 out of 144 beef cattle fecal samples were detected as positive samples with the average positivity rate of 77.1%. The risk of infection of these six diarrheal pathogens existed in five counties of Guyuan City, Ningxia Hui Autonomous Region, China, among which the positive rates of six diarrheal pathogens were 90.0% in Longde County, 50.0% in Jingyuan County, 66.7% in Xiji County, 75.0% in Pengyang

Table 2. Infection of beef cattle with diarrhea in different counties of Guyuan city, Ningxia, China.

County	Longde	Jingyuan	Xiji	Pengyang	Yuanzhou	Total
No. of samples	60	12	18	36	18	144
No. of positives	54	6	12	27	12	111
Infection rate (%)	90	50	66.7	75	66.7	77.08
Eimeria coccidia	6	3	0	0	3	12
***Escherichia coli*K99**	21	0	3	6	0	30
Mycobacterium avium subsp. paratuberculosis	9	0	0	0	3	12
Cryptosporidium	12	6	0	15	12	45
Giardia duodenalis	9	0	0	12	0	21
Enterocytozoon bieneusi	30	0	9	15	0	54

Table 3. Infection of diarrhea pathogens at different months of age in Guyuan city, Ningxia, China.

Months of age	0-6	7-12	Over 12	Total
No. of samples	36	72	36	144
No. of positives	27	60	24	111
Infection rate (%)	75	83.3	66.67	77.08
Eimeria coccidia	3	0	9	12
***Escherichia coli*K99**	15	15	0	30
Mycobacterium avium subsp. paratuberculosis	12	0	0	12
Cryptosporidium	12	24	9	45
Giardia duodenalis	9	12	0	21
Enterocytozoon bieneusi	3	39	12	54

County, and 66.7% in Yuanzhou County. Statistical analysis revealed that the positivity rates of six diarrheal pathogens in beef cattle in Longde County were very significant different from those in Jingyuan County ($P < 0.01$), and significant different from those in Xiji County, Pengyang County, and Yuanzhou County ($P < 0.05$). Among the different areas in Guyuan, Ningxia, *Enterocytozoon bieneusi* was common in Xiji County, Longde County, and Pengyang County, while *Cryptosporidium* was common in Jingyuan County, Pengyang County, and Yuanzhou County (Table 2).

Infection of six diarrheal pathogens in beef cattle at different months of age

The positivity rate of six diarrheal pathogens in calves from 0 to 6 months of age was 75.0%. All six pathogens were detected with higher positivity rates for *E. coli* K99, *Cryptosporidium*, and MAP (Table 3). The positivity rates of six pathogens in cattle from 7 to 12 months and over 12 months of age were 83.3% and 77.08%, respectively, with *Enterocytozoon bieneusi* being the most common pathogen in both age groups. Among the different months of age, the rates of positivity for the six pathogens demonstrated very significance between 7 to 12 months of age and over 12 months of age groups ($P < 0.01$).

Detection of six diarrheal pathogens in two different feeding patterns

The statistical analysis of beef cattle farms with two feeding modes, large-scale and small-scale farms, showed that five of the six diarrheal pathogens were detected in large-scale beef cattle farms with a positive rate of 73.9 %, while all six diarrheal pathogens were detectable in small-scale farms with a positive rate of 80.0 %. However, there was no significant statistical difference between the two feeding modes (Table 4).

Statistical analysis of single and mixed infections

Table 4. Infection of six diarrheal pathogens under different feeding patterns in Guyuan city of Ningxia, China.

Feeding mode	Large scale farms	Small scale farms
No. of samples	69	75
No. of positives	51	60
Infection rate (%)	73.9	80
Eimeria coccidia	3	9
***Escherichia coli*K99**	18	12
Mycobacterium avium subsp. paratuberculosis	0	12
Cryptosporidium	21	24
Giardia duodenalis	9	12
Enterocytozoon bieneusi	24	30

Table 5. Statistical results of infection rate of six diarrheal pathogens.

Type of infection	Pathogen	No. of positives	Total number of positives	Infection rate (%)
Single pathogen infections	*Eimeria coccidia*	3	111	2.70
	Escherichia coli K99	9	111	8.11
	MAP	0	111	0.00
	Cryptosporidium	12	111	10.81
	Giardia duodenalis	6	111	5.41
	Enterocytozoon bieneusi	27	111	24.32
	Total	57	111	51.35
Mixed infection of two pathogens	*Escherichia coli* K99 and *Enterocytozoon bieneusi*	3	111	2.70
	Eimeria coccidia and *Cryptosporidium*	6	111	5.41
	Cryptosporidium and *Giardia duodenalis*	3	111	2.70
	Escherichia coli K99 and *Giardia duodenalis*	6	111	5.41
	Cryptosporidium and Enterocytozoon bieneusi	12	111	10.81
	Eimeria coccidia and Enterocytozoon bieneusi	3	111	2.70
	Escherichia coli K99 and MAP	6	111	5.41
	MAP and *Cryptosporidium*	3	111	2.70
	Giardia duodenalis and *Enterocytozoon bieneusi*	6	111	5.41
	Escherichia coli K99 and *Cryptosporidium*	3	111	2.70
	Total	48	111	43.24
Mixed infection of three pathogens	*Escherichia coli* K99, *Cryptosporidium*, and *Enterocytozoon bieneusi*	3	111	2.70
	Total	3	111	2.70
Mixed infection of four pathogens	MAP, *Cryptosporidium*, *Giardia duodenalis*, and *Enterocytozoon bieneusi*	3	111	2.70
	Total	3	111	2.70

Among the positive samples detected in this study, single infection positive samples accounted for 51.40% with 24.30% from *Enterocytozoon bieneusi*, 10.80% from *Cryptosporidium*, 8.10% from *E. coli* K99, and 2.70% from *Eimeria coccidia*. The proportion of positive samples with mixed infection of two pathogens was 43.20% with *Cryptosporidium* and *Enterocytozoon bieneusi* accounted for the largest proportion of 10.80%. Three pathogens mixed infection samples accounted for 2.70% of the positive samples, which were mixed infection of *E. coli* K99, *Cryptosporidium*, and *Enterocytozoon bieneusi*. Four pathogenic mixed

infection samples accounted for 2.70% of the positive samples, which were infected with *Paratuberculosis*, *Cryptosporidium*, *Giardia duodenalis*, and *Enterocytozoon bieneusi*. The variability between infection types was very significant ($P < 0.01$) (Table 5).

Discussions

Diarrhea is a common and frequent gastrointestinal disease in beef cattle, which can occur throughout the year and has a complex etiology, especially for unweaned calves. Therefore, breeders should make accurate diagnosis of diarrhea samples in a timely manner and use accurate medication to treat the disease. In this study, 144 fecal samples of beef cattle were collected from 5 counties in Guyuan City, Hui Autonomous Region, China, and the infections of MAP, *E. coli* K99, *Cryptosporidium*, *Giardia duodenalis*, *Enterocytozoon bieneusi*, and *Eimeria coccidia* were detected. Among them, the highest infection rate of 31.0% was found from *Enterocytozoon bieneusi* infection. *Enterocytozoon bieneusi* is an important opportunistic intestinal protozoan in cattle, which can cause diarrhea in beef cattle and seriously hinder the development of animal husbandry, and as a human-animal commensal intestinal pathogen, it also poses a serious threat to human health [13-15]. In this study, the serious infection of *Enterocytozoon bieneusi* in beef cattle farms in Guyuan City area was investigated. The results confirmed that *Enterocytozoon bieneusi* was one of the main causes of diarrhea in beef cattle in the region. The positive rates of *Cryptosporidium*, *E. coli* K99, and *Giardia duodenal* in the feces of beef cattle were 25.9%, 17.2%, and 12.1%, respectively, which were consistent with that in other regions of China [16-18]. The lowest infection rate of 6.9% was from *Eimeria* and MAP, which indicated that MAP in the beef cattle population in the Guyuan region was consistent with the findings in the other part of China [19], while the infection rate of *Eimeria* was lower than that from the previously reported results, suggesting that the infection might not be as severe as in herds in the other regions [20, 21].

Among the five counties in Guyuan City, the positive rate of six diarrheal pathogens ranged from 50.00% to 90.00%. The highest positive rate of six diarrheal pathogens was 90.0% in Longde County and the lowest was 50% in Jingyuan County with significant differences between Longde County and the other four counties. The results indicated a more serious infection of the six pathogens in Longde County and the need to strengthen diarrheal pathogen surveillance and improve prevention and control techniques. In the analysis of mixed infections of different pathogens in this study, it was found that single infections and mixed infections of two pathogens accounted for the largest proportion, especially the mixed infections of parasites among two pathogens were more serious, indicating that the deworming program of farmers was unreasonable or not dewormed. Therefore, the studied area should develop a feeding management system in accordance with the local actual situation, establish a perfect disinfection and deworming program to reduce the infection rate of diarrheal pathogens and reduce economic losses.

The positive rate of infection with the six diarrheal pathogens was higher in small-scale farms than that in large-scale farms under different feeding patterns, which was related to feeding management as well as disease prevention and control in large-scale farms. The scientific beef cattle breeding can greatly reduce breeding risks while greatly improving breeding efficiency. In this study, it was found that calves from 7 to 12 months of age had the highest positive rate of infection with six diarrheal pathogens, especially *Cryptosporidium* and *Enterocytozoon bieneusi* infection. In contrast, the diarrheal pathogens in beef cattle above 1 year of age were mainly three parasitic infections, while the positive rate of MAP was 0, which was consistent with the results of Hu, *et al*. [22]. Therefore, *Cryptosporidium*, *E. coli*, *Giardia duodenalis*, and MAP diarrheal pathogens should

be of concern in calves up to 6 months of age during beef cattle breeding in Guyuan area. Cattle above 6 months of age should be carried out in a timely manner with regular deworming and enhanced feeding management.

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