Acta Veterinaria Hungarica 53 (1), pp. 137–146 (2005)

DETECTION OF RESPIRATORY AND ENTERIC SHEDDING OF BOVINE CORONAVIRUSES IN CATTLE IN NORTHWESTERN TURKEY

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(Received January 15, 2004; accepted May 24, 2004)

Bovine coronavirus (BCoV) is an important cause of diarrhoea in calves, winter dysentery in adult cattle and respiratory tract disease in feedlot cattle. Serum, faecal and nasal swab samples were collected from a total of 96 cattle with clinical signs in 29 barns of 23 villages in Northwestern Turkey. The cattle were subdivided into 3 distinct age groups (0–30 days old, 4–12 months old and 2–7 years old). An indirect antigen-capture ELISA and an antibody-detection ELISA as well as geometric mean BCoV antibody titres were used to detect BoCV shed in the faeces and in the nasal secretions, respectively. Relationships between BCoV shedding and age group, seroconversion and clinical signs in cattle were also analysed. The rate of faecal shedding of BoCV was 37.1% (13/35) in 0–30 days old calves, 25.6% (10/39) in 4–12 months old feedlot cattle and 18.2% (4/22) in 2–7 years old cows. The overall rate of BCoV faecal shedding was 28.1% (27/96) in the cattle examined. Only one animal in the 4–12 months old age group was found to shed BoCV nasally. The analysis showed that there was a significant difference ($P < 0.0001$) with respect to faecal shedding between the clinical signs and the age groups. BCoV antibody titre in 50% of all cattle was ≤100 as detected by ELISA while 27.1% of the cattle had high titres ranging between 1,600 and 25,600. The seroconversion rate was 7.3% (7/96) in animals shedding BoCV in the faeces and 42.7% (41/96) in cattle negative for faecal shedding as detected by ELISA, and 20.8% of cattle with no seroconversion shed BCoV in the faeces. There was no statistically significant association between seroconversion and nasal or faecal BCoV shedding. These findings confirm the presence of BCoV infections in Turkey. Further studies are needed to isolate BCoV strains in Turkey and to investigate their antigenic and genetic properties.

Key words: Bovine coronavirus, ELISA, cattle, calf, Turkey

Bovine coronavirus (BCoV), a member of the family *Coronaviridae,* order *Nidovirales* (De Vries et al., 1997), is associated with severe diarrhoea in newborn calves (CD), winter dysentery (WD) in adult cattle and respiratory tract in-

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fections in calves and feedlot cattle (Saif et al., 1991; Clark, 1993). BCoV is enveloped and possesses a single-stranded, non-segmented RNA genome of positive polarity (De Vries et al., 1997). The virion contains five major structural proteins: the nucleocapsid (N) protein, the transmembrane (M) protein, the haemagglutinin/esterase (HE) protein, the spike (S) protein and the small membrane (E) protein (Lai and Cavanagh, 1997). The S glycoprotein is a type 1 membrane glycoprotein that carries distinct functional domains near the amino (S1) and carboxy (S2) termini (Gallagher and Buchmeier, 2001). The S1 subunit is peripheral and is associated with receptor binding functions whereas the S2 subunit is a transmembrane protein mediating fusion of viral and cellular membranes (Cavanagh, 1995).

BCoV was first recognised as a cause of potentially fatal diarrhoea of neonatal calves in 1972 (Saif et al., 1988). Economically important CD and WD BCoV outbreaks have been reported by many investigators (Saif et al., 1991; Traven et al., 1993; Ganaba et al., 1995; Fuente et al., 1999). In the early 1980s, Thomas et al. (1982) first isolated BCoV from lung washes and nasopharyngeal swabs from calves involved in two outbreaks of pneumonia. Subsequently, respiratory bovine coronavirus (RBCoV) strains were frequently detected by ELISA and isolated from nasal swab samples of feedlot cattle with respiratory tract disease after shipping (Saif et al., 1986; Tsunemitsu et al., 1991; Storz et al., 2000*a*; Cho et al., 2001*b*; Hasoksuz et al., 2002). Cattle shedding BCoV nasally after entering the feedlot were at an increased risk of developing respiratory disease (Lathrop et al., 2000*a*) and had high mortality due to BCoV infection (Storz et al., 2000*b*). Thus, BCoV infections may contribute to the bovine respiratory disease complex (BRDC), which is the single most important syndrome affecting 6- to 10-month-old beef cattle after entry into feedlots in North America (Martin et al., 1998). The BRDC is a multifactorial disease arising due to a combination of environmental, host, management, viral and bacterial factors. The viruses involved include: bovine viral diarrhoea virus (BVDV), bovine torovirus (BoTV), bovine respiratory syncytial virus (BRSV), infectious bovine rhinotracheitis virus (IBRV), parainfluenza-3 (PI-3) virus, and the main bacterial component, *Mannheimia (Pasteurella) haemolytica* serotype A1 (Thomas et al., 1982; Storz et al., 2000*a*). Although evidence of BCoV infections in feedlot cattle exists, the role of BCoV in the BRDC and its association with respiratory and/or enteric tract infections and the rate of seroconversion to BCoV in feedlot cattle are largely undefined.

The purpose of this study was to examine respiratory and enteric BCoV shedding patterns in three different age groups of cattle (0–30 days old, 4–12 months old and 2–7 years old) in Turkey by ELISA. Relationships between BCoV shedding, seroconversion and clinical signs (respiratory disease and diarrhoea) in cattle were also analysed.

Materials and methods

Reference BCoV strains and antisera

The BCoV strain DB2, three monoclonal antibodies directed against the S, HE and N components of a BCoV strain, and guinea pig as well as bovine hyperimmune serum to BCoV were obtained from the Food Animal Health Research Program run by the Ohio State University (USA).

Sample collection

From autumn 2001 through spring 2002, cattle of different age were surveyed for diarrhoea and respiratory signs in 35 villages of Northwestern Turkey (Fig. 1). Serum, faecal and nasal swab samples from 96 cattle showing clinical signs and belonging to three distinct age groups (1–30 days old, 4–12 months old and 2–7 years old) were collected from 29 barns in 23 villages. Sample collection procedures had previously been described by Hasoksuz et al. (1999*b*). None of the cattle had been vaccinated against BCoV prior to taking samples.

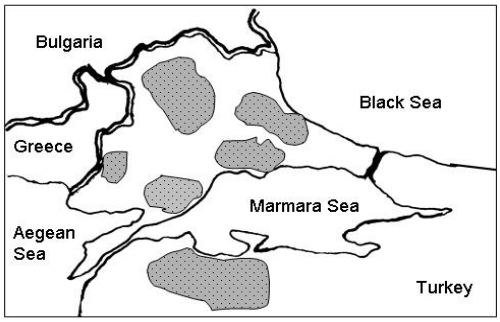

Fig. 1. Areas of sample collection in Northwestern Turkey. Faecal and nasal swabs and sera from 96 cattle with clinical signs were collected in 29 barns of 23 villages in the regions shown

Clinical signs

The consistency of the faeces at the time of sample collection was scored on a scale of 0–4, with 0, normal; 1, pasty; 2, semi-liquid; 3, liquid with some solid material; and 4, totally liquid. Cattle with scores ≥ 2 were considered as clinical cases of diarrhoea. Respiratory signs were scored on a scale of 0–4, with 0, normal; 1, mucopurulent or slight serous nasal discharge; 2, moderate serous or mucopurulent nasal discharge with mild to moderate coughing; 3, severe mucopurulent nasal discharge with moderate to severe coughing; and 4, clear signs of respiratory distress and dyspnoea. Scores ≥ 2 were considered as clinical respiratory disease. The scores of all samples taken from cattle with clinical respiratory disease and/or clinical cases of diarrhoea were ≥ 2.

ELISA for BCoV antigen

An indirect antigen-capture ELISA using a pool of three monoclonal antibodies was used to detect BCoV in faecal suspensions and nasal swab fluids as previously described (Smith et al., 1998). Briefly, 96-well plates were coated overnight at 4 °C with the pooled monoclonal antibodies, then blocked for 2 h at 23 °C with 5% non-fat dried milk. Specimens were applied at 23 °C for 1 h, and a secondary antibody (guinea pig hyperimmune serum against strain DB2 of BCoV) was then applied at 23 °C for 1 h. Goat anti-guinea pig IgG (H+L) conjugated to horseradish peroxidase was added and the plates were incubated for 1 h at 23 °C. Reactions were developed using the TMB substrate system (3,3,5,5-tetramethylbenzidine) at 23 °C for 5 min and then stopped with 1 M phosphoric acid. The absorbance value of each well was read with an ELISA reader, and the readings were saved as ASCII files. Specimens that had an absorbance > 0.1 after subtraction of background absorbance were considered positive for BCoV. Using the same ELISA protocol in our previous study, we reported that the sensitivity and specificity of RT-PCR as compared with ELISA were high, but the correlation between the ELISA and RT-PCR assays with nasal and faecal samples was considered good with 92.5 and 95.1% agreement and Kappa statistic (κ) of 0.66 and 0.81, respectively (Cho et al., 2001*a*).

ELISA for BCoV antibody

An antibody-detection ELISA, previously described by Smith et al. (1998), was used to detect IgG antibodies to BCoV in the serum samples. Briefly, 96-well plates were coated with a mixture of the same three monoclonal antibodies as used in the antigen-capture ELISA described above. After blocking, clarified semi-purified human rectal tumor (HRT-18) cell culture supernatants containing BCoV were added to each well and incubated at 23 °C for 1 h. Serial twofold dilutions of serum samples in PBS (range 1:100 to > 1:25,600) were applied to two rows of wells and then the plates were incubated at 23 °C for 1 h. Goat anti-bovine IgG (H+L) conjugated to horseradish peroxidase was added and the plates were incubated at 23 °C for 1 h. The same chromogens as described for the antigen-capture ELISA were applied to each well at 23 °C for 5 min. The colour reaction was stopped with 1 M phosphoric acid and the plates were read with an ELISA reader; the readings were saved as ASCII files. The titre was defined as the inverse of the serum dilution at which the positive-coated wells had an absorbance value of ≥ 0.1 above the mean absorbance of the negative wells.

Statistical analysis

Chi-square analysis was used to determine the association between BCoV shedding and age group, clinical signs (diarrhoea and respiratory disease) and seroconversion.

Results

Respiratory disease, characterised by coughing and nasal discharge, along with diarrhoea was observed in the three distinct age groups of cattle (Table 1). The overall rates of cattle with diarrhoea or respiratory disease were 65.6% (63/96) and 28.1% (27/96), respectively, and altogether 6.1% (6/96) of the cattle had both diarrhoea and respiratory disease (Table 1).

The percentage and number of cattle of different age, shedding BCoV in the faeces, are shown in Table 1. The overall rate of cattle with faecal or nasal BCoV shedding was 28.1% (27/96) and 1% (1/96), respectively, as detected by ELISA. Only one animal in the 4–12 months age group of cattle with respiratory signs was found to shed BCoV nasally. No statistically significant association was found between the faecal and nasal shedding of BCoV as detected by ELISA and the clinical signs. However, 37.7% (26/69) of calves with diarrhoea were shedding BCoV in the faeces (Table 1). Among animals with diarrhoea, the rate of faecal shedding of BoCV was 39.3%, 38% and 44.4% in the 1–30 days old, 4–12 months old and 2–7 years old cattle groups, respectively (Table 1). There was a significant difference ($P < 0.0001$) between cattle of different clinical status and between the different age groups with respect to faecal shedding (Table 1).

Table 1

Results of testing for BCoV in faecal samples obtained from three distinct age groups of cattle using antigen-capture ELISA and number of cattle with clinical signs

Clinical status	1–30 days old	4–12 months old	2–7 years old	Total
Faecal shedding/ Diarrhoea and respiratory signs	0% (0/1)	25% (1/4)	0% (0/1)	16.6% (1/6)
Faecal shedding/ Diarrhoea, no respiratory signs	39.3% (13/33)	38% (8/21)	44.4% (4/9)	39.7% (25/63)
Faecal shedding/ No diarrhoea, respiratory signs	0% (0/1)	7.1% (1/14)	0% (0/12)	3.7% (1/27)
Total	37.1% (13/35)	25.6% (10/39)	18.2% (4/22)	28.1% (27/96)

The BCoV antibody titre of 50% of all cattle was ≤ 100 as detected by ELISA while 27.1% of the cattle had high titres ranging between 1,600 and 25,600 (Fig. 2). Animals with BCoV geometric mean antibody titres (GMT) over 1,600 did not shed BCV either in the faeces or nasally. Twenty-two cattle (22.9%) with faecal BCoV shedding had a low antibody titre ranging between 200 and 800 (Fig. 2). The seroconversion rate was 7.3% (7/96) in cattle positive for faecal shedding and 42.7% (41/96) in cattle found negative for faecal shed-

ding by ELISA (Table 2). In addition, 20.8% of the cattle with no seroconversion shed BCoV in the faeces as detected by ELISA (Table 2).

With respect to the distribution of BCoV antibody titres among the three distinct age groups of cattle with faecal positivity for BCoV, the antibody titres of 26% (7/27) of the cattle were found to be low (between 200 and 800) while those of the other animals were < 100. However, in the groups of cattle with faecal negativity for BCoV, antibody titres were high (between 1,600 and 25,600) in 30.4% (21/69) of the animals, and fell in the range of 200 to 800 in 13% (9/69) of the cattle (data not shown). There was no statistically significant association between seroconversion and the nasal or faecal shedding of BCoV.

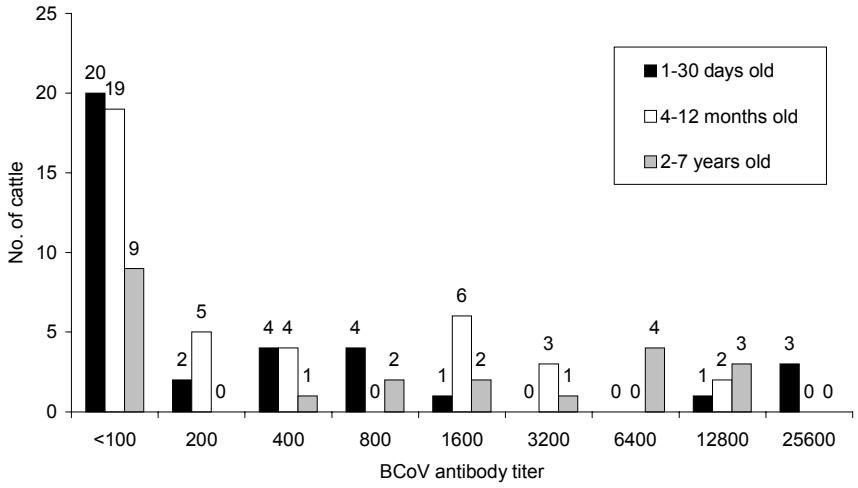

Fig. 2. Distribution of geometric mean BCoV antibody titres as determined by ELISA in 96 cattle belonging to different age groups

Table 2

Status of cattle shedding BCoV in the faeces and seroconversion to BCoV in three distinct age groups of cattle

BCoV status	1–30 days old	4–12 months old	2–7 years old	Total
No shedding, no seroconversion	31.4% (11/35)	28.2% (11/39)	27.3% (6/22)	29.2% (28/96)
No shedding, seroconverted	31.4% (11/35)	46.2% (18/39)	54.5% (12/22)	42.7% (41/96)
Shedding, no seroconversion	25.7% (9/35)	20.5% (8/39)	13.6% (3/22)	20.8% (20/96)
Shedding, seroconverted	11.4% (4/35)	5.1% (2/39)	4.5% (1/22)	7.3% (7/96)

Discussion

BoCV has been detected in faecal samples from diarrhoeic and winter dysenteric cattle of various ages in the USA (Saif et al., 1991; Storz et al., 2000*a*) and in other countries (Tsunemitsu et al., 1991; Traven et al., 1993; Ganaba et al., 1995; Fuente et al., 1999; Naciri et al., 1999). In these studies, investigators reported that BoCV and other pathogens, including BVDV, rotavirus, bovine torovirus (Breda virus), *Salmonella* spp., *E. coli,* coccidia and *Cryptosporidium parvum* were associated with diarrhoea in cattle. In addition, although previous investigators have described BoCV infection of the respiratory tract of feedlot cattle after shipping (Storz et al., 2000*a*; Storz et al., 2000*b*), the association between respiratory and enteric BoCV infections in cattle is unclear. Investigators have suggested that stress is a predisposing factor for BRDC (Filion et al., 1984). Animals are subjected to many stress factors including weaning, shipping, commingling, dietary changes, and long holding times in the sale barn (Stephens et al., 1980; Filion et al., 1984).

In this study, only one animal from the 4–12 months old group shed BcoV as detected by ELISA. In earlier investigations by our group and in other studies it was reported that cattle of this age group were shedding BCoV especially after entering the feedlot (Hasoksuz et al., 1999*a*; Lathrop et al., 2000*b*; Cho et al., 2001*b*). The aim of this investigation was to detect BCoV in three different age groups of cattle (0–30 days old, 4–12 months old and 2–7 years old) with respiratory and/or diarrhoeic signs detected in their barn, and in animals that had been living for more than three months on the same farm. This explains why the number of cattle with respiratory shedding was much lower in this study than in other investigations. The isolation of BCoV in nasal fluids from clinically affected calves and from experimentally inoculated calves (Saif et al., 1986; Hasoksuz et al., 1999*a*; Hasoksuz et al., 1999*b*; Cho et al., 2001*a*; Cho et al., 2001*b*) has substantiated speculation that aerosol transmission and respiratory replication occur in the pathogenesis of BCoV associated with the Bovine Respiratory Disease Complex or Shipping Fever (Martin et al., 1998; Storz et al., 2000*a*; Storz et al., 2000*b*). In addition, some authors reported that in some cases under field conditions BCoV respiratory infections occurred prior to enteric infections (Heckert et al., 1990), which indicates the possible importance of this route of transmission in the spread and pathogenesis of BCoV infections and in the production of both enteric and respiratory disease.

No statistically significant association was found between faecal and nasal shedding of BCoV as detected by ELISA and the clinical signs. This may be due to the relatively small number of animals (n = 96), which influences the statistical power to detect such differences. However, we found that the overall rate of cattle with BCoV faecal shedding was 28.1% (27/96) as detected by ELISA while 37.7% (26/69) of calves with diarrhoea shed BCoV in the faeces. One to

30 days old calves were found to be a potential risk group for shedding BCoV in other age groups. On the other hand, the analysis showed that there was a significant difference ($P < 0.0001$) between the clinical signs and the age groups with respect to faecal shedding (Table 1). In previous investigations, 15% of cattle with diarrhoeic signs in Quebec, Canada, and 9% in Ohio, USA, were found to be BCoV positive (Athanassious et al., 1994). Alkan (1998) reported that 15.4% of diarrhoeic calves in central Turkey were shedding BCoV and that 13.4% of those calves were concurrently shedding bovine rotavirus. This was the first and so far the only study on BCoV shedding in calves in Turkey.

Another aim of this investigation was to detect the prevalence of seroconversion to BCoV in cattle in this region. Serological analysis showed that half of the cattle (48/96) seroconverted to BCoV. This high antibody prevalence to BCoV observed in cattle agrees with the high seroprevalence ranging from 45–75% reported in the cattle population (Saif et al., 1991; Lathrop et al., 2000*a*; Lathrop et al., 2000*b*; Hasoksuz et al., 2002).

In this study, the BCoV antibody titres in the three distinct age groups of cattle which had tested positive for faecal BCoV were low (between 200 and 800) for 26% (7/27) of the animals and < 100 for the remaining animals. However, in the group negative for faecal BCoV shedding the antibody titres were high, between 1,600 and 25,600 in 30.4% (21/69), and between 200 and 800 in 13% (9/69) of the cattle. The seroconversion rate was 7.3% (7/96) in cattle positive for faecal shedding and 42.7% (41/96) in cattle found negative for faecal shedding by ELISA (Table 2). These results are also in agreement with similar findings of previous studies which showed that calves aged between 4 and 12 months seroconverted to BCoV when they were under stressful conditions such as mixing with young calves and adult cattle (Lathrop et al., 2000*b*; Cho et al., 2001*b*; Hasoksuz et al., 2002).

In conclusion, BCoV infections may have a potentially negative impact on the performance of cattle, reflected by an increase in production costs. Cho et al. (2001*b*) and Hasoksuz et al. (2002) reported a 8.1 kg and 5.9 kg decrease, respectively, from starting weights over a 21-day period. Therefore, BCoV infections may directly contribute to economic losses in cattle by impacting weight gains or milk production by predisposing cattle to secondary bacterial infections. In the future, BCoV vaccines may be needed to decrease such economic losses due to BCoV infection in cattle. Further studies are needed to isolate BCoV strains in Turkey and to investigate their antigenic and genetic properties.

Acknowledgements

We thank Linda J. Saif from The Ohio State University for monoclonal antiserum and reference BCoV strains. We also thank Mustafa Özcan and Bulent Ekiz for technical assistance. This work was supported by the Research Fund of Istanbul University, project number UDP-248 / 29032004.

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