Bovine respiratory disease in naturally infected calves: clinical signs, blood gases and cytokine response

Y. OZKANLAR[1]*, M.S. AKTAS[1], O. KAYNAR[2], S. OZKANLAR[2], E. KIRECCI[3], L. YILDIZ[4]

[1]*Department of Internal Medicine, Faculty of Veterinary Medicine, Ataturk University, 25240 Erzurum, TURKEY.*
[2]*Department of Biochemistry, Faculty of Veterinary Medicine, Ataturk University, 25240 Erzurum, TURKEY.*
[3]*School of Health, Kahramanmaras Sutcuimam, University K. Maras, 46100 K. Maras, TURKEY.*
[4]*Department of Biochemistry, Faculty of Medicine, Ataturk University, 25240 Erzurum, TURKEY.*

**Corresponding author: ozkanlary@yahoo.com*

SUMMARY

Viral and bacterial aetiology, clinical symptoms, venous blood gases and cytokine production were aimed to investigate in naturally infected calves with bovine respiratory disease (BRD). Nasal swap and bronchoalveolar lavage samples and blood samples were obtained from 10 healthy control calves and from 48 calves with respiratory symptoms from 12 different herds and exposed to predisposing factors (insufficient aeration and hygiene) (BRD group) before tulathromycin and carprofen treatment. Virus isolations were achieved in 25% of the swap samples: BVDV (bovine viral diarrhoea virus), BRSV (bovine respiratory syncytial virus) and IBRV (infectious bovine rhinotracheitis virus) were identified in 12.5%, 8.3% and 6.2% of diseased calves respectively. Serum anti-BVDV antibodies were also detected in 68.75% of BRD calves using a sero-neutralization test. Lungs from all virally (directly and indirectly) infected calves were also invaded with bacteria (in majority with *Mannheimia hemolytica*, and *P. multocida*, α-haemolytic *Streptococcus* and coagulase negative *Staphylococcus*, at a lesser extend). The prevalence of the total bacterial infections was 100% and poly-infections were also frequent (47.92%). Respiration rate and rectal temperature were significantly increased ($P < 0.001$) in the affected calves. Nasal and ocular discharge, cough and dyspnoea were additional clinical findings. Venous gas analysis revealed the occurrence of a respiratory acidosis (decrease in blood pH coupled to increase in pCO_2 and decrease in pO_2) in diseased calves that was positively correlated with the respiratory frequency. Furthermore, serum concentrations of tumour necrosis factor-α and interleukin-6 were detected to be significantly high only in newly affected calves. Tulathromycin and carprofen treatment induced clinical recovery after 15 days and 30 days in 91.6% and 95.83% respectively of diseased calves. As a result, blood gases, respiratory rate, rectal temperature and respiratory signs have been deteriorated in these calves. A respiratory acidosis and an early inflammatory cytokine production occur during BRD, mainly related to the BVDV and *M. heamolytica* as most common agents involved in the disease.

Keywords: Bovine respiratory disease, calf, respiratory signs, cytokine, TNF-α, IL-6, respiratory acidosis, causative agents, BVDV, *Mannheimia hemolytica*.

RÉSUMÉ

La maladie respiratoire bovine chez les veaux naturellement infectés : signes cliniques, gaz sanguins et production de cytokines

L'étiologie virale et bactérienne, les symptômes cliniques, les gaz du sang veineux et la production de cytokines ont été analysés chez des veaux naturellement infectés par la maladie respiratoire bovine (MRB). Des écouvillonnages nasaux, des prélèvements de liquide de lavage broncho-alvéolaire et des prélèvements sanguins ont été réalisés sur 10 veaux témoins en bonne santé et sur 48 veaux présentant des signes respiratoires issus de 12 troupeaux différents et exposés à des facteurs prédisposants (défaut d'aération et d'hygiène) avant traitement par la tulathromycine et le carprofène. L'isolation virale a été positive sur 25 % des écouvillonnages nasaux : le BVDV (virus de la diarrhée bovine), le BRSV (virus respiratoire syncytial bovin) et l'IBRV (virus de la rhinotrachéite bovine) ont été identifiés dans respectivement 12,5 %, 8,3 % et 4,2 % des cas. Des anticorps circulants anti-BVDV ont également été détectés par un test de séro-neutralisation dans 68,75 % des cas. Les poumons de tous les veaux infectés par des virus (directement ou indirectement) étaient également envahis par des bactéries (en majorité *Mannheimia hemolytica*, puis *P. multocida, Streptococcus* α-hémolytique et *Staphylococcus* coagulase négatifs à un moindre degré). La prévalence totale des infections bactériennes a été de 100 % et les cas de poly-infections (47,92 %) sont apparus fréquents. La fréquence respiratoire et la température rectale ont été significativement augmentées ($P < 0.001$) chez les veaux malades. Les écoulements nasaux et oculaires, la toux et la dyspnée ont été considérés comme des signes additionnels. L'analyse des gaz sanguins a révélé l'existence d'une acidose respiratoire (diminution du pH sanguin associé à une augmentation de la pCO_2 et une diminution de la pO_2) chez les veaux malades, positivement corrélée à la fréquence respiratoire. En outre, des concentrations sériques en TNFα (tumour necrosis factor α) et en IL6 significativement élevées n'ont été observées que chez les veaux récemment atteints. Le traitement par la tulathromycine et le carprofène a induit une récupération clinique au bout de 15 jours dans 91,6 % et au bout de 30 jours dans 95,83 % des cas. Ainsi, les gaz sanguins, la fréquence respiratoire, la température rectale et les signes respiratoires ont été altérés chez les veaux malades. Une acidose respiratoire et une production précoce de cytokines inflammatoires surviennent durant la maladie respiratoire, impliquant le BVDV et M. hemolytica comme principaux agents infectieux.

Mots clés : Maladie respiratoire bovine, veau, signes respiratoires, cytokine, TNF-α, IL-6, acidose respiratoire, agents causaux, BVDV, *Mannheimia hemolytica*.

Introduction

Bovine respiratory disease (BRD) is an infectious disease resulting from interactions of multiple predisposing stress factors and viral and bacterial agents [4]. The risk of BRD was significantly associated with involving agents. There are a number of viral agents that cause BRD such as bovine viral diarrhoea virus (BVDV), infectious bovine rhinotracheitis virus (IBR) and bovine respiratory syncytial virus (BRSV) [33]. The involving agents are being reported in numerous epidemiological studies. Often the virus infection causes lung tissue damage and then bacteria invade the tissue compromised. Bacterial

organisms grow inside the lung tissue resulting in pneumonia and deadly outcome [13, 14]. The antibacterial treatment is then the main part of the management of the disease. The most common bacterial agents are *Mannheimia (P.) haemolytica, P. multocida* and *Mycoplasma spp*. [4, 14, 33]. *M. haemolytica* is the most frequently bacterium isolated from the BRD [14]. Respiratory and clinical signs appear associated with the degree of damage in lung tissue. Acute onsets of pneumonia are characterized by fever, nasal discharge, coughing, tachypnea and dyspnoea along with the severe changes in blood gases.

Pneumonia is an inflammation of lung tissues that results from the response to the infectious agents, associating complex pathophysiological events and reactions of innate immune response [1]. There are a number of inflammatory and anti-inflammatory mediators in alveolar milieu. Among the major cytokines that mediate alveolar inflammation are tumour necrosis factor (TNF) and interleukins (IL) [17]. During the acute phase of alveolus injury, the inflammatory mediators set off both local and systemic inflammatory processes initiating the cellular and cytokine cascades involved in the process of the acute phase response (APR) [5, 29]. The cytokines, secreted by alveolar macrophages in the air space of lung, stimulate fever, local inflammation, chemotaxis and neutrophil activation. For example, the effects of BVDV on cytokine regulation have been studied in monocytes and macrophages *in vitro*. Interestingly, IL-1 inhibitor activity in bovine monocytes was increased by BVDV isolated from persistently infected cattle [19]. Furthermore, a decreased production of TNF-α was determined in bovine bone-marrow-derived macrophages by BVDVs [2]. Thus far, these results have been hypothesized for the immunosuppressive effects of BVDVs [30]. However, recently, interferon-γ, IL-1, IL-4, IL-6 and TNF- α productions have been found to be increased after the exposure of BVDV1b and/or *M. haemolytica* in an experimental model for BRD in calves [7]. Interferon regulated gene in white blood cells and APR have been determined to be increased in calves after experimental infection with BVDV [24].

Most of the previous studies about BRD etiopathogenesis have been carried out in experimental and/or *in vitro* models except for treatment and epidemiological studies. The innate response of the body against the disease, particularly in a predisposing environment, is further needed to be evaluated to understand the complex etiopathogenesis. Therefore, production of IL-6 and TNF-α were aimed to analyze in calves naturally infected with BRD in this study. Blood gas analysis and clinical symptoms were recorded to demonstrate the clinical severity of the disease. Viral and bacterial aetiology was also determined.

Material and Methods

ANIMALS AND SAMPLING

Forty eight calves, 1-6 months old, from 12 different herds in the north-east of Turkey and showing clinical symptoms of BRD constituted the BRD group. The north-east of Turkey is known as the coolest temperature around the country and has many conventional barns. The clinical signs used as criteria for inclusion in the BRD group were tachypnea, dyspnoea and nasal discharge. The animals in the BRD group have exhibited clinical symptoms within the last hours to 10 days. The calves were rearing in the same barns of adult cattle together with their mothers without having sufficient aeration. Additionally, 10 clinically healthy calves were recruited as control group from a modernized barn. The breeds in both groups were Holstein, brown Swiss and Anatolian black.

Blood samples for serum analysis were obtained by puncture of the jugular vein into sterile tubes without anticoagulant (BD Vacutainer, Plymouth, UK) on the day presentation in BRD group. The control animals were also sampled. After clotting at room temperature (21°C) for 1 hour, sera were obtained by centrifugation at 3 000 g for 10 minutes at room temperature (21°C) and stored at -86°C until analysis. Blood gas analysis was also performed from blood samples collected by jugular vein puncture into sterile syringes with lithium heparin (72 IU, Plasti-Med Ltd. Sti., Turkey) on the day presentation.

Nasal swap samples were collected following the antisepsis procedure to prevent possible bacterial contamination risk and obtained from the nasal cavity by rotation of a sterile cotton swab and placed in transport mediums (Eurotubo collection swap, Deltalab, Spain). Bronchoalveolar lavage (BAL) samples were taken via either endotracheal or transtracheal techniques performed in 1-3 months old calves and 3-6 months old ones respectively. In endotracheal intubation technique, trachea was intubated with the help of a laryngoscope and a catheter (50 cm length, 5 mm diameter, double ending holes) was passed through the intubation tube (size 7 and 8) towards diaphragmatic lobe of the lung. In transtracheal technique, after shaping and disinfected the caudal part of neck over trachea on the right side, a catheter (15G in size) was placed into trachea between two tracheal rings and a sterile catheter (30 cm length, 2 mm diameter, double ending holes) was passed towards the diaphragmatic lobe of the lung.

Because the endotracheal technique was too difficult to handle in 3-6 month old calves, the transtracheal technique was preferred. In both techniques, approximately 30-40 mL of sterile physiological saline solution (NaCl 0.15M) was slowly injected and 10-20 mL of lavage fluid was immediately aspirated. Samples were kept immediately in an ice box for further tests and processed to the microbiology and virology laboratory.

ISOLATION AND IDENTIFICATION OF BACTERIA AND VIRUSES

Swab and BAL samples were cultured in 5% sheep blood agar (Oxoid) and MacConkey Agar (Oxoid). Plates were inoculated and incubated aerobically and anaerobically at 37°C for 24-72 hours. Bacteria were identified by conventional methods. *Streptococcus spp*. and *Staphylococcus spp*. growing in the media were examined in terms of colony structure, Gram staining, microscopic appearance, tube coagulase, catalase and other biochemical specifications. For the identification of suspected *Mannheimia spp*. colonies growing in the media were again cultured and the isolates were purified. *Mannheimia haemolytica* were identified by biochemical tests such as oxidase, catalase, citrate utilisation, indol, urease and

sulfhydric acid production, carbohydrate fermentation and motility and additionally confirmed by API 20E identification system (bioMérieux, SA, France) [18, 21].

Examinations for viruses in swap and BAL samples were achieved in referenced laboratories (Erzurum and Pendik Veterinary Control and Research Institute, Turkey). Viral agents were isolated by routine methods. Isolations were completed in MDBK (Madin Darby Bovine Kidney) cells and identifications were made by neutralization test [26]. Antibody occurrence and corresponding serum titres against BVDV were determined by serum neutralization test [26].

ANALYSIS OF BLOOD GASES AND ELECTROLYTES

Blood gases and electrolytes were determined in whole blood samples by an automated blood gas and electrolyte analyzer using respiratory cassettes (Idexx VetStat, USA) according to the manufacturer instructions. Right after obtaining the fresh blood samples in a syringe with lithium heparin, the syringe sample was attached to calibrated cassettes into the analyzer. The overall time of whole process for the analyzing was finished within 60-70 seconds after blood collection.

ANALYSIS OF CYTOKINES

The commercially available ELISA kits for bovine TNF-α (Bovine TNF-α/TNFSF1A, DuoSet Elisa Development System, R&D System, USA) and bovine IL-6 (Bovine IL-6 Screening Set, Thermo Scientific, USA) were purchased. Sandwich ELISA technique was used to determine the concentrations in serum in duplicate. Capture/coating antibody for TNF- α and IL-6 were diluted by PBS (0.2 M, pH 9.6) in 1:100 dilutions to the working concentration. 100 µL per well of the diluted capture antibody was coated to a 96-well microplate. The plates were sealed and incubated overnight at room temperature. Each well was washed with wash buffer (PBS, pH 7.4) for a total three washes. Plates were blocked by adding 300 µL of block buffer (4% bovine serum albumin in PBS, pH 7.4) to each well and incubated at room temperature for 1 hour. The plates were washed again. 100 µL sample or standard diluent were added per well.

After incubation at room temperature for 2 hours for the TNF-α and 1 hour for IL-6, the plates were washed and 100 µL of the detection antibody were added to each well. The plates were incubated for 2 hours and washed again. 100 µL of the working dilution of Streptavidin-Horse Radish peroxidase (HRP) was added to each well and the plates were covered and incubated for 20 minutes at room temperature. Wash cycle was repeated. 100 µL of substrate solution was added to each well and incubated for 20 minutes at 25°C in a dark room. Finally, 50 µL of stop solution (0.2 M H_2SO_4) was added to each well and the optical density of each well was immediately determined using a microplate Elisa reader (Biotek µQuant, USA) at 450 nm and 550 nm. Concentrations of samples were calculated by subtracting the 450 nm absorbance to the 550 nm absorbance. Blank reading was then subtracted from the samples and logistic method standard curve was developed to calculate the concentrations.

TREATMENT PROCEDURE

Single doses of 2.5 mg/kg tulathromycin (Pfizer Animal Health, USA) and 1.4 mg/kg carprofen (Pfizer Animal Health, USA) were administrated subcutaneously in all 48 diseased animals on the day 0. Treatment was repeated in only 4 calves that were detected to be still having moderate BRD clinical symptoms 15 days after the first injection.

STATISTICAL ANALYSIS

Data were presented as mean ± standard deviations for each parameter. Statistically significant differences for the comparison of groups were determined by one-way ANOVA and the Tukey's *post hoc* test. The associations between variables were calculated by the Pearson product moment correlation test. A statistical significance was defined by $P < 0.05$. All analyses were performed using SigmaStat 2.03 statistical software for windows (SPSS Inc.).

Results

Tachypnea, dyspnoea, nasal discharge and fever were detected in all animals in BRD group. The additional clinical sings in some of the diseased animals (around 20%) were ocular discharge, cough and decreased appetite. Five of them also exhibited slight diarrhoea. Abnormal broncho-vesicular lung sounds were obtained in auscultation of chest region. Respiration rates and rectal temperatures were significantly higher in the BRD group before treatment than in the control group ($P < 0.001$) and these parameters significantly declined compared to initial values after treatment ($P < 0.001$) and became similar to control values (Table I). Administrations of tulathromycin and carprofen provided a clinical recovery in 44 out of 48 diseased calves (91.67 %) 15 days after the therapy initiation. Among the 4 resting calves, the same treatment was

Clinical parameters	Control (n = 10)		BRD group (n = 48)	
	Day 0	Day 15	Day 0	Day 15
Respiratory rates (cycle / min.)	45 ± 5^{a}	47 ± 6^{a}	89 ± 15^{b}	44 ± 11^{a}
Rectal temperature (°C)	39.0 ± 0.1^{a}	38.9 ± 0.2^{a}	40.1 ± 0.3^{b}	39.2 ± 0.2^{a}

Different superscripts ^{a,b} in the same row indicate significant differences ($P < 0.001$) between groups and between timepoints.

TABLE I: Clinical parameters (respiratory frequency and rectal temperature) recorded in control calves and in BRD (Bovine Respiratory Disease) affected calves before (day 0) and 15 day after treatment (2.5 mg/kg tulathromycin SC and 1.4 mg/kg carprofen SC). Results are expressed as mean ± standard deviation.

performed again on day 15: 2 died and 2 survived until the end of the course study (day 30). Therefore, overall success rate for the treatment can be calculated as 95.83%.

The bacterial and viral isolations and the results of serum BVDV neutralization test are shown in Table II. Virus isolations were completed in 25% of the nasal swap samples in diseased calves. Among the 12 positive swap samples, BVDV was identified in 6 cases (12.5% of BRD affected calves), BRSV in 4 cases (8.3%) and IBRV in 2 cases (4.2%) and only one virus type was identified per each positive nasal swap samples. Additionally, neutralizing antibodies against BVDV were detected in 68.75% of serum samples with titres ranging from 5 to 640, and particularly in the 6 calves positive for BVDV isolation in swap samples. The 2 other viruses, BRSV and IBRV, were not isolated from nasal swap samples from the 27 other seropositive calves. *Mannheimia hemolytica, Pasteurella multocida*, coagulase negative *Staphylococcus* and α haemolytic *Streptococcus* were the major bacterium types identified in BAL samples, detected in 62.50%, 25.00%, 18.75% and 16.67% cases of the BRD-affected calves, respectively, whereas the other bacteria types, *Bacillus spp., Neisseria spp., E. coli, Staphylococcus aureus, Heamophilus spp., Morexella spp., Pseudomonas aeroginosa* and *Mycoplasma spp*., were more rarely encountered. Nevertheless, at least one bacteria type was isolated and identified in all BAL collected from the diseased calves and bacterial co-infections were recorded in 23 cases (47.92%). Among the 30 *M. hemolytica* positive BAL samples, 17 (56.67%) were also positive for other bacteria types such as *P. multocida* (7 cases), coagulase negative *Staphylococcus* (6 cases), *Bacillus spp*. (4 cases), *Neisseria spp*., α haemolytic *Streptococcus* and *E. coli* (2 cases for each type) and occasionally with *Heamophilus spp*. and *Morexella spp*. Moreover, *P. multocida*, coagulase negative *Staphylococcus* and α haemolytic *Streptococcus* were also frequently associated together in the BAL samples and on the other hand, the coagulase negative *Staphylococcus* and the *Bacillus* spp. were never isolated alone from the BAL samples. Besides, all viral infections directly evidenced by virus isolation from nasal swap samples or indirectly by serum anti-BVDV neutralizing antibodies were associated to one or more bacterial infections: in the BDR group, *M. hemolytica* was isolated in BAL samples from the 6 calves infected with the BVDV and from one calf infected with the IBRV. Other bacteria (*P. multocida*, coagulase negative *Staphylococcus*, α haemolytic *Streptococcus, Heamophilus spp*. and *Bacillus* spp.) were also detected in virally infected calves. *M. hemolytica, P. multocida*, α haemolytic *Streptococcus* and coagulase negative *Staphylococcus* were also found in 59.26% (16 cases), 29.63% (8 cases), 18.52% (5 cases) and 14.81% (4 cases) respectively, BAL samples from the 27 strictly serologically positive calves.

The results of venous blood gas analyses are shown in Table III. The venous pH values and the plasma proton concentrations were significantly altered before treatment in diseased calves ($P < 0.05$). In addition to the observed weak acidosis, partial pressure of carbon dioxide (pCO_2) ($P < 0.01$) and total

Infectious agent	Total cases	Mono-infections	BRD group (n = 48)		
		(strictly)	With bacteria	With viruses	With a-BVDV Ab
Viruses	39 (81.25%)	0 (0%)	39 (100%)	6 (15.38%)	-
Direct diagnosis	12 (25.00%)	0 (0%)	12 (100%)	0 (0%)	0 (0%)
BRSV	4 (8.33%)	0 (0%)	4 (100%)	0 (0%)	0 (0%)
IBRV	2 (4.17%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)
BVDV	6 (12.50%)	0 (0%)	6 (100%)	0 (0%)	6 (100%)
SNT (BVDV)	33 (68.75%)	0 (0%)	33 (100%)	6 (18.18%)	-
Bacteria	48 (100%)	2 (4.17%)	23 (47.92%)	12 (25.00%)	33 (68.75%)
Mannheimia hemolytica	30 (62.50%)	1 (3.33%)	17 (56.67%)	7 (23.33%)	22 (73.33%)
Pasteurella multocida	12 (25.00%)	0 (0%)	9 (75.00%)	2 (16.67%)	8 (66.67%)
Coagulase - Staphylococcus	9 (18.75%)	0 (0%)	9 (100%)	2 (22.22%)	6 (66.67%)
α haemolytic *Streptococcus*	8 (16.67%)	0 (0%)	5 (62.50%)	2 (25.00%)	6 (75.00%)
Bacillus spp.	5 (10.42%)	0 (0%)	4 (80.00%)	1 (20.00%)	4 (80.00%)
Neisseria spp	4 (8.33%)	0 (0%)	4 (100%)	0 (0%)	4 (100%)
Escherichia coli	3 (6.25%)	0 (0%)	2 (66.67%)	0 (0%)	2 (66.67%)
Staphyloccus aureus	3 (6.25%)	0 (0%)	2 (66.67%)	0 (0%)	3 (100%)
Heamophilus spp	2 (4.17%)	0 (0%)	1 (50.00%)	1 (50.00%)	0 (0%)
Morexella spp	2 (4.17%)	0 (0%)	2 (100%)	0 (0%)	1 (50.00%)
Pseudomonas aeroginosa	1 (2.08%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
Mycoplasma spp	1 (2.08%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)

BRSV: Bovine Respiratory Syncytial Virus; IBRV: Infectious Bovine Rhinotracheitis Virus; BVDV: Bovine Viral Diarrhoea Virus; SNT: Serum Neutralization Test; a-BVDV Ab: serum anti-BVDV antibodies.

TABLE II: Isolation and identification of viral and bacterial infections in the BRD (Bovine Respiratory Disease) affected calves (n = 48) on the presentation day (before treatment) by direct diagnosis from nasal swab samples and indirect sero-neutralization diagnosis for the BVDV infection.

Parameter	Control (n = 10)	BRD group (n = 48)	*P*
pH	7.467 ± 0.010	7.412 ± 0.020	< 0.05
H^+ (mmol/L)	34.04 ± 0.49	39.37 ± 2.05	< 0.05
pCO_2 (mmHg)	40.71 ± 1.46	51.23 ± 1.89	< 0.01
tCO_2 (mmol/L)	28.63 ± 0.91	32.05 ± 0.79	< 0.05
pO_2 (mmHg)	32.71 ± 1.12	27.92 ± 1.46	< 0.05
SO_2 (%)	65.0 ± 1.40	62.0 ± 0.84	NS
HCO_3^- (mmol/L)	27.37 ± 0.87	30.49 ± 0.79	< 0.05
BE (mmol/L)	3.90 ± 0.64	4.75 ± 0.99	NS
AnGap (mmol/L)	15.77 ± 0.64	14.21 ± 0.71	NS
tHb (g/L)	112.57 ± 2.80	129.92 ± 6.70	< 0.05
Na (mmol/L)	141.86 ± 0.49	140.54 ± 0.98	NS
K (mmol/L)	4.24 ± 0.12	4.11 ± 0.13	NS
Cl (mmol/L)	103.29 ± 0.53	100.00 ± 0.89	< 0.05

pCO_2: partial pressure of carbon dioxide; tCO_2: total carbon dioxide; pO_2: partial pressure of oxygen; SO_2: oxygen saturation; BE: base excess; AnGap: Anion Gap; tHb: total haemoglobin; NS: not significant.

TABLE III: Venous blood gas analysis and electrolyte concentrations in the BRD (Bovine Respiratory Disease) affected calves (n = 48) and in healthy control calves (n = 10) on the presentation day (before treatment). Results are expressed as mean ± standard deviation.

carbon dioxide concentration (tCO_2) ($P < 0.05$) were also markedly increased compared to the controls whereas the partial pressure of oxygen (pO_2) ($P < 0.05$) was significantly decreased and that the oxygen saturation (SO_2) was lowered but not significantly. In parallel, the plasma bicarbonate concentrations (HCO_3^-) and the haemoglobinemia (tHb) were significantly higher in BRD-affected calves than in controls ($P < 0.05$) and in the same way, base excess (BE) also tended to enhance but not significantly. A significant positive interaction between the increase in pCO_2 and the high respiration rate was also evidenced (r = 0.763, $P < 0.001$). Whereas the plasma Na and K concentrations were unchanged in diseased animals, chloride concentrations were significantly depressed compared to the healthy controls ($P < 0.05$). No anion significantly accumulated in plasma from BRD-affected cattle.

No increase in the cytokine production was detected in the calves exhibiting clinical symptoms since at least 2 days (n = 34) compared to the healthy controls but surprisingly, the serum TNF-α and IL-6 concentrations were dramatically increased ($P < 0.001$ and $P < 0.05$, respectively) in newly affected calves (n = 14) presenting clinical and respiratory symptoms within the last 24 hours (hyperthermia and accelerated respiratory rate) (Table IV). In addition, significant positive associations between cytokine concentrations and rectal temperatures (r = 0.627 and r = 0.559 for TNF-α and IL-6, respectively, $P < 0.05$) or respiratory rate (r = 0.695 and r = 0.601 for TNF-α and IL-6, respectively, $P < 0.05$) were found.

Discussion

Pneumonia is an inflammation of lung tissues that results from the response to some infectious agents. A wide range of pathogens, basically viruses and bacteria, can cause respiratory diseases, separately or simultaneously [4]. Certainty diagnosis can be achieved by isolation of organisms from nasal swaps and/or directly from lungs via BAL technique in calves presenting clinical symptoms such as cough and nasal discharge. In the present study, the causative viral agents directly isolated from nasal swap samples were BVDV, BRSV and IBRV. In addition, serum antibodies against BVDV were detected in 68.75% of diseased calves. It can be thought that neutralizing antibodies against BVDVs are produced after the beginning and the onset of the viral infection [16] and low antibody titres have been detected in some cases during the initiation stage of the disease [7].

The present results indicate that there was a significant relationship between the BVDV infection and the occurrence of pneumonia in the calves used herein. Similar findings have been demonstrated between the viral agent and the respiratory

Cytokine	Control (n = 10)	Newly BRD affected (n = 14)	*P*
TNF-α (ng/L)	107 ± 15	402 ± 96	< 0.001
IL-6 (ng/L)	196 ± 8	275 ± 34	< 0.05

BRD: Bovine respiratory disease.

TABLE IV: Serum cytokine (TNF-α and IL-6) concentrations on the presentation day in healthy control calves (n = 10) and in newly BRD affected calves (n = 14) presenting clinical signs (hyperthermia and high respiratory frequency) within the last 24 hours. Results are expressed as mean ± standard deviation.

disease [10]. Furthermore, one or more bacteria types were found in the BAL samples from all diseased calves in which viruses were directly isolated or having circulating anti-BVDV antibodies in the present study, suggesting that bacterial, often multiple (in 47.92% cases here), infections have occurred secondary to the viral infections. After viral infection, the bacteria become attached to the lining of the respiratory tract and spread throughout the lungs. *M. haemolytica* and *P. multocida* are often associated with BRD or shipping fever in cattle [14]. The major bacterial infections in the present study involved *M. haemolytica, P. multocida*, α-haemolytic *Streptococcus* and coagulase negative *Staphylococcus*, whereas other bacterium types (*Bacillus spp., Neisseria spp., E. coli, Staphylococcus aureus, Heamophilus spp., Morexella spp., Pseudomonas aeroginosa* and *Mycoplasma spp*.) have occurred scarcely. *M. hemolytica* was identified in most of the cases (30/48) in this study and was associated with directly evidenced viral infections in 7 calves, with serum anti-BVDV antibodies in 23 cases and with other bacteria, mainly with *P. multocida*, coagulase negative *Staphylococcus* and *Bacillus spp*. Other bacteria were also frequently associated in the BAL samples and particularly, 2 types (coagulase negative Staphylococcus and the Bacillus spp.) were systematically associated with other bacteria. Finally, strictly mono-infections (with only one causative agent identified) were found only in 2 cases (4.17%). Viral infections, involving mainly the BVDV and complicated with bacterial infections cause considerable economic losses in the dairy industry, mainly attributable to reduced milk production, reduced reproductive performance, delayed growth, increased susceptibility to other diseases, early culling and increased mortality among young stock [15].

The lung tissue and organism respond to cope with the infection when pneumonia occurred and the severity of the disease depends upon the pathogenecity of the bacterial organism(s) and the associated viral infection(s). The lung inflammation leads to occurrence of clinical signs. Increased respiratory frequency and hyperthermia were the most important clinical findings in the BRD group. Dyspnoea and coughing were additional clinical findings. This clinical profile is in agreement with previous reports [8, 31].

Marked decrease in pO_2 and increase in pCO_2 in diseased animals revealed a severe respiratory failure. Similarly, NAGY *et al.* [25] reported marked hypoxemia and hypercapnia in the calves with severe respiratory disease. In addition, the changes in blood gases were significantly associated with a high respiratory frequency suggesting that the breath rhythm and intensity were insufficient to eliminate CO_2 and supply O_2. Consequently, a respiratory acidosis has occurred, evidenced throughout a significant decrease in the blood pH and a significant increase in the circulating proton concentrations. The fact that in one hand the anion gap (corresponding to the eventual accumulation of organic acids) was not altered and in the other hand the alkaline reserve (bicarbonate and base excess) was not severely depressed allows excluding the metabolic origin for acidosis. On the contrary, the plasma bicarbonate concentrations were markedly enhanced in BRD affected calves, confirming the insufficient CO_2 elimination. Additionally, the concomitant moderate hypochloremia suggests that the renal reabsorption of bicarbonates exchanged against chloride in nephrocytes was amplified [9]. In the same way, the significant increase in haemoglobinemia in diseased calves may be related to increase in haemoglobin biosynthesis induced by hypoxia [27]. It can be thought that clinical response is almost always consistent with the high respiratory rate resulting in significant changes of blood gases and acid-base balance in blood stream associated with lung failure which were demonstrated in the present data.

The immune system activates inflammatory reactions to limit tissue damage against invading agents [1]. Internalization of lipopolysaccharide (LPS) causes the transcription of many cytokine genes and release of pro-inflammatory cytokines, in which TNF-α, IL-l and IL-6 are the most important in large animals [28]. TNF-α is an inflammatory cytokine produced by macrophages during acute inflammation and is responsible for a diverse range of signalling events within cells [16]. The cytokine inhibition by BVDV reported in previous *in vitro* studies [2, 18] may, partly, explain the persistent infection of adult cattle [30]. However, in the present study, TNF-α and IL-6 were detected to be significantly increased in the newly BRD affected calves. Furthermore, significant positive associations between cytokine (TNF-α and IL-6) concentrations and respiratory frequency or rectal temperature during the initiation of the disease were found. Some cytokines, called pyrogene cytokines, such as the TNF-α, IL-1 and IL-6 may cause fever [1, 7]. Bacterial and viral products might induce the synthesis of pyrogene cytokines [1, 2]. The results of this study are in agreement with BURCIAGA-ROBLES *et al.* [7] in which elevated cytokine (IL-1β, IL-6 and TNF-α) concentrations were determined in steers 72 hours after an experimental infection with BVDV and/or M. hemolytica. Similarly, the interferon-α activity was increased by BVDV inoculation in calves [13] and the $CD4^+$, $CD8^+$ and total lymphocyte counts were decreased significantly in experimentally infected calves [12]. However, increases in cytokine concentrations recorded in the present study in recently infected calves were not so important than those observed in the study of BURCIAGA-ROBLES *et al*. [7]. It is probable that the cytokine production increases early after the infection and abruptly declines when clinical signs appear. As cytokine production happens in the early stage of the infection [7, 28], the cytokine concentrations were not significantly altered in calves in the more advanced stage of the disease.

Because most of the current knowledge has been accumulated thanks to experimental studies, the mechanisms for occurrence and response to pneumonia are not apparent in respect to innate immunity in naturally infected calves under natural predisposing factors for BRD. The complexity of BRD inevitably happens by complex interactions between predisposing factors and multiple causative agents. All involving factors should also be taken into account and cannot be separately explored in naturally infected animals. Firstly, in the natural occurrence of the disease, in which the viral infection is followed by bacterial invasion mainly with *M. hemolytica*, the secondary involvement of bacteria may again induce inflammatory reaction. In a model of bovine pneumonic pasteurellosis, TNF-α, IL-1β, and IL-8 was significantly increased in the airways and lung lesions of infected calves after endobronchial *M. hemolytica* inoculation [23]. Secondly, treatment may also interfere with cytokine production. Fluoroquinolones reduced the serum IL-6 and TNF-α concentrations

in LPS treated mice [20], and tetracyclines inhibited the secretion of TNF-α by porcine cells exposed to LPS *in vitro* [3]. After treatment, cytokine production decreases and this may be critical for integrating systemic responses, balancing antibacterial/antiviral host defences and inflammatory injury caused by pneumonia.

The treatment of BRD has included antibiotics and anti-inflammatory drugs. The clinical recovery obtained by administrations of tulathromycin and carprofen provided a clinical healing. Administrations of tulathromycin and carprofen provided a clinical recovery in 91.67 % of the calves 15 days after the therapy initiation in this study. The efficacy of tulathromycin has been confirmed for treatment of naturally occurring BRD in calves [31, 32]. Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be effective as an adjunct to antimicrobial therapy in cattle improving the pulmonary status because of analgesic, antipyretic and anti-inflammatory effects [6, 11, 22].

In conclusion, BVDV and *M. hemolytica* are the most common viral and bacterial agents, respectively, in the calves with BRD reared in barns having predisposing factors (insufficient aeration and hygiene). Blood gases, respiratory frequency, rectal temperature and respiratory signs are deteriorated in these calves. The cytokine production has been detected only in the calves at initiation stage of the disease associated with proinflammatory and inflammatory innate immune response mostly related to BVDV and *M. hemolytica* infections.

Acknowledgement

The authors would like to thank to Ataturk University for supporting this research financially (BAP-2008/191), to Erzurum and Pendik Veterinary Control and Research Institutes for virus isolations and to the Pfizer Company for kindly supplying tulathromycin and carprofen.

References

1. - ACKERMANN M.R., DERSCHEID R., ROTH J.A.: Innate immunology of bovine respiratory disease. *Vet. Clin. North Am. Food Anim. Pract*., 2010, **26**, 215-228.

2. - ADLER H., JUNGI T.W., PFISTER H., STRASSER M., SILEGHEM M., PETERHANS E.: Cytokine regulation by virus infection: bovine viral diarrhoea virus, a flavivirus, downregulates production of tumour necrosis factor alpha in macrophages *in vitro. J. Virol*., 1996, **70**, 2650-2653.

3. - AKUNDA J., JOHNSON K.E., AHRENS F.A., KRAMER T.T.: Chlortetracycline modulates acute phase response of *ex vivo* perfused pig livers, and inhibits TNF-alpha secretion by isolated Kupffer cells. *Comp. Immunol. Microbiol. Infect. Dis*., 2001, **24**, 81-89.

4. - AUTIO T., POHJANVIRTA T., HOLOPAINEN R., RIKULA U., PENTIKAINEN J., HUOVILAINEN A., RUSANEN H., SOVERI T., SIHVONEN L., PELKONEN S.: Etiology of respiratory disease in non-vaccinated, non-medicated calves in rearing herds. *Vet. Microbiol*., 2007, **31**, 256-265.

5. - BAUMANN H., GAULDIE J.: The acute phase response. *Immunol. Today*, 1994, **15**, 74-80.

6. - BEDNAREK D., KONDRACKI M., FRITON G.M., TRELA T., NIEMCZUK K.: Effect of steroidal and non-steroidal anti-inflammatory drugs on inflammatory markers in calves with experimentally-induced bronchopneumonia. *Berl. Munch. Tierarztl. Wochenschr*., 2005, **118**, 305-308.

7. - BURCIAGA-ROBLES L.O., STEP D.L., KREHBIEL C.R., HOLLAND B.P., RICHARDS C.J., MONTELONGO M.A., CONFER A.V., FULTON R.W.: Effects of exposure to calves persistently infected with bovine viral diarrhoea virus type 1b and subsequent infection with *Mannheima haemolytica* on clinical signs and immune variables: Model for bovine respiratory diseases via viral and bacterial interaction. *J. Anim. Sci*., 2010, **88**, 2166-2178.

8. - CATRY B., DUCHATEAU L., VAN DE VEN J., LAEVENS H., OPSOMER G., HAESEBROUCK F., DE KRUIF A.: Efficacy of metaphylactic florfenicol therapy during natural outbreaks of bovine respiratory disease. *J. Vet. Pharm. Therap*., 2008, **31**, 479-487.

9. - CARRARO-LACROIX L.R., MALNIC G.: Acid-base transport by the renal distal nephron. *J. Nephrol.*, 2010, **23** (Suppl 16), S19-S27.

10. - DIEGUEZ F.J., YUS E., VILAR M.J., SANJUAN M.L., ARNAIZ I.: Effect of the bovine viral diarrhoea virus (BVDV) infection on dairy calf rearing. *Res. Vet. Sci*., 2009, **87**, 39-40.

11. - ELITOK B., ELITOK O.M.: Clinical efficacy of carprofen as an adjunct to the antibacterial treatment of BRD. *J. Vet. Pharm. Therap*., 2004, **27**, 317-320.

12. - GANHEIM C., JOHANNISSON A., OHAGEN P., WALLER K.P.: Changes in peripheral blood leukocyte counts and subpopulations after experimental infection with BVDV and/or *Mannheimia haemolytica*. *J. Vet. Med. B*., 2005, **52**, 380-385.

13. - GANHEIM C., HULTEN C., CARLSSON U., KINDAHL H., NISKANEN R., WALLER K.P.: The acute phase response in calves experimentally infected with bovine viral diarrhoea virus and/or *Mannheimia haemolytica. J. Vet. Med. B*., 2003, **50**, 183-190.

14. - GRIFFIN D., CHENGAPPA M.M., KUSZAK J., MCVEY D.S.: Bacterial pathogens of the bovine respiratory disease complex. *Vet. Clin. North Am. Food Anim. Pract.*, 2010, **26**, 381-394.

15. - HOUE H.: Economic impact of BVDV infection in dairies. *Biologicals*, 2003, **31**, 137-143.

16. - HOWARD C.J.: Immunological responses to bovine virus diarrhoea virus infections. *Rev. Sci. Tech*., 1990, **9**, 95-103.

17. - IDRISS H.T., NAISMITH J.H.: TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc. Res. Tech*., 2000, **50**, 184-195.

18. - JARAMILLO-ARANGO C.J., HERNANDEZ-CASTRO R., SUAREZ-GÜEMES F., MARTINEZ-MAYA J.J., AGUILAR-ROMERO F., JARAMILLO-MEZA L., TRIGO F.J.: Characterization of *Mannheimia* spp. strains isolated from bovine nasal exudate and factors associated to isolates, in dairy farms in the Central Valley of Mexico. *Res. Vet. Sci*., 2008, **84**, 7-13.

19. - JENSEN J., SCHULTZ R.D.: Effect of infection by bovine viral diarrhoea virus (BVDV) in vitro on interleukin-1 activity of bovine monocytes. *Vet. Immunol. Immunopathol.*, 1991, **29**, 251-265.

20. - KHAN A.A., SLIFER T.R., ARAUJO F.G., SUZUKI Y., REMINGTON J.S.: Protection against lipopolysaccharide-induced death by fluoroquinolones. *Antimicrob. Agents Chemother.*, 2000, **44**, 3169-3173.

21. - KIRECCI E., ERGUN Y., DOGRUER G., SARIBAY M.K.: Usefulness of the e-test for the determination of the susceptibility of staphylococcus sp. isolated from milk of sheep and goats with subclinical mastitis to amikacin and amoxicillin-clavulanic acid. *Bull. Vet. Inst. Pulawy*, 2009, **53**, 401-405.

22. - LOCKWOOD P.W., JOHNSON J.C., KATZ T.L.: Clinical efficacy of flunixin, carprofen and ketoprofen as adjuncts to the antibacterial treatment of bovine respiratory disease. *Vet. Rec*., 2003, **152**, 392-394.

23. - MALAZDREWICH C., AMES T.R., ABRAHAMSEN M.S., MAHESWARAN S.K.: Pulmonary expression of tumour necrosis factor alpha, interleukin-1 beta, and interleukin-8 in the acute phase of bovine pneumonic Pasteurellosis. *Vet. Pathol*., 2001, **38**, 297-310.

24. - MULLER-DOBLIES D., ARQUINT A., SCHALLER P., HEEGAARD P.M., HILBE M., ALBINI S., ABRIL C., TOBLER K., EHRENSPERGER F., PETERHANS E., ACKERMANN M., METZLER A.: Innate immune responses of calves during transient infection with a non cytopathic strain of bovine viral diarrhoea virus. *Clin. Diagn. Lab. Immunol*., 2004, **11**, 302-312.

25. - NAGY O., SEIDEL H., PAULIKOVA I., MUDRON P., KOVAC G.: Use of blood gases and lactic acid analyses in diagnosis and prognosis of respiratory diseases in calves. *Bull. Vet. Inst. Pulawy*, 2006, **50**, 149-152.

26. - OIE: Bovine Viral Diarrhoea. *In*: Manual of Standards Diagnostic Tests and Vaccines, 2008, Chapter 2.4.8. (http://www.oie.int)

27. - OLVER C.S., ANDREWS G.A., SMITH J.E., KANEKO J.J.: Erythrocyte structure and function, *In*: Schalm's Veterinary Haematology, 6th edition, WEISS D.J. and WARDROP K.J. (eds), Blackwell, USA, 2010, Chapter 20, pp.:128.

28. - RADOSTITS O.M., GAY C.C., HINCHCLIFF K.W., CONSTABLE P.D.: General systematic states, *In*: Veterinary Medicine, Textbook of the disease of cattle, horses, sheep, pigs and goats, 10th edition. Saunders, USA, 2007, Chapter 2, pp.: 54.

29. - RAYNES J.G.: The acute phase response. *Biochem. Soc. Trans*., 1994, **22**, 69-74.

30. - RIDPATH J.: The contribution of infections with bovine viral diarrhoea viruses to bovine respiratory disease. *Vet. Clin. North Am. Food Anim. Pract.*, 2010, **26**, 335-348.

31. - ROBB E.J., TUCKER C.M., CORLEY L., BRYSON W.L., ROGERS K.C., STURGESS K., BADE D.J., BRODERSEN B.: Efficacy of tulathromycin or enrofloxacin for initial treatment of naturally occurring bovine respiratory disease in feeder calves. *Vet. Therap.*, 2007, **8**, 127-135.

32. - SKOGERBOE T.L., ROONEY K.A., NUTSCH R.G., WEIGEL D.J., GAJEWSKI K., KILGORE W.R.: Comparative efficacy of tulathromycin versus florfenicol and tilmicosin against undifferentiated bovine respiratory disease in feedlot cattle. *Vet. Therap.*, 2005, **6**, 180-196.

33. - WOOLUMS A.R., AMES T.R., BAKER J.C.: Lower respiratory tract diseases, In: Large animal internal medicine, SMITH B.P. (ed.), 4th edition, Mosby, USA, 2009, pp.: 601-643.