DEMONSTRATION OF BOVINE RESPIRATORY SYNCYTIAL VIRUS RNA IN PERIPHERAL BLOOD LEUKOCYTES OF NATURALLY INFECTED CATTLE

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Summary. – RNA of Bovine respiratory syncytial virus (BRSV) was found in peripheral leukocytes and nasal mucosa of infected cows by nested reverse transcription–polymerase chain reaction (nRT-PCR). We suppose that this finding obtained in the convalescent phase of infection indicates possible persistence of the virus in cells of the immune system.

Key words: Bovine respiratory syncytial virus; cattle; leukocytes; nasal mucosa; persistent infection; serum antibodies; RT-PCR

BRSV (species *Bovine respiratory syncytial virus*, genus *Pneumovirus*, subfamily *Pneumovirinae*, family *Paramyxoviridae*) is one of significant pathogenic agents involved in the development of pneumonia in calves (Larsen, 2000). The virus is also occasionally isolated from adult cattle showing clinical signs of an acute respiratory disease (Ellis *et al*., 1996; Elvander, 1996). Major lesions occur in the airways and lungs of necropsied calves (Kimman *et al*., 1989), but viral RNA has been also demonstrated by RT-PCR outside the respiratory tract in lymphatic tissues and kidneys (van der Poel *et al*., 1997). In the respiratory tract BRSV is present mainly in bronchial and alveolar epithelial cells (Viuff *et al*., 1996) and virus antigen has been also demonstrated in alveolar macrophages (Meehan *et al*., 1994). *In vitro* BRSV can infect bovine and ovine alveolar macrophages, lymphocytes, and peripheral monocytes (Adair and McNulty, 1992; Keles *et al*., 1998; Sharma and Woldehiwet, 1996b). Host species of the virus include cattle and sheep (Masot *et al*., 1996). The virus has been demonstrated in blood mononuclear cells of experimentally infected lambs (Meehan *et al*., 1994; Sharma and Woldehiwet, 1996a). Presence of BRSV RNA in the blood of naturally infected animals has not yet been reported.

Studies on the related Human respiratory syncytial virus (HRSV, species *Human respiratory syncytial virus*, genus *Pneumovirus*) have demonstrated the presence of the viral antigen in circulating mononuclear leukocytes (Domurat *et al*., 1985) and its *in vitro* replication in human mononuclear leukocytes and alveolar macrophages (Midulla *et al*., 1989). HRSV-specific RNA (viral and mRNA) has been demonstrated by RT-PCR in circulating mononuclear cells of infected children (O'Donnell *et al*., 1998; Rohwedder *et al*., 1998).

This paper describes identification of BRSV in the acute phase of a respiratory disease in the blood of adult cows by RT-PCR.

We focused our attention to a herd of 210 cows with signs of an acute respiratory disease in 40% of animals. Specimens were collected from 15 lactating dairy cows suffering from the disease. The samples included 5 whole EDTA-treated bloods (cows Nos. 1, 2, 3, 4, and 7), 7 nasal swabs (cows Nos. 1, 2, 3, 4, 7, 8, and 11) and 15 sera. The cows Nos. 1, 2, 3, and 7 developed signs of an acute respiratory disease with body temperatures exceeding 39°C. The cows Nos. 4, 8, and 11 developed similar signs, but their body temperature was normal. The remaining animals showed only signs of a mild respiratory disease. Blood sampling was carried out again from the same animals 4 weeks later (the second sampling). A whole blood sample was collected after another 3 weeks from the

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Abbreviations: aa = amino acid; BRSV = Bovine respiratory syncytial virus; ELISA = enzyme-linked immunosorbent assay; HRSV = Human respiratory syncytial virus; nRT-PCR = nested reverse transcription–polymerase chain reaction; nt = nucleotide

cow No. 2 in which viral RNA was demonstrated in the blood from the second sampling.

Blood leukocytes were separated by mixing 6 ml of whole EDTA-treated blood with 15 ml of a hemolytic buffer (NH_4Cl 16.6 g/l, $NaHCO_3$ 2 g/l, and EDTA 0.184 g/l) and centrifuging at 2300 rpm for 15 mins at 4°C (Jouan BR4i centrifuge, rotor S40). The pelleted leukocytes were resuspended in 1 ml of the hemolytic buffer, centrifuged at 1300 rpm for 5 mins (Beckman MinifugeÔ 12), washed twice with 1 ml of DPBS (KCl 0.2 g/l, anhydrous KH_2PO_4 0.2 g/l, and Na_2HPO_4 x 12 H_2O 2.865 g/l, pH 7.0) and resuspended in 1 ml of DPBS.

The liquid soaked in the nasal swabs was squeezed out into a transport medium by syringe (Kovarcik, 1999). The leukocyte suspensions and nasal swab liquids were tested by nRT-PCR for the presence of BRSV RNA. Nasal swab liquids were further used for virus isolation (Kovarcik, 1999).

Total RNA was isolated from 250 µl of leukocyte suspension or nasal swab liquid with the TRIZOL LS reagent (Gibco BRL, Life Technologies). RT-PCR was carried out using the Titan One Tube RT-PCR System (Roche). The primers were derived from the gene encoding the fusion (F) protein. The amplification using the outer sense primer 5'-TGGAGTTAGTGTCCTTACTAGC-3' (nt 502–523) and the outer antisense primer 5'-TCATCAGATCGACGTATG AAAGCC-3' (nt 1462–1485) yielded a 984 bp product. The reaction proceeded as follows: 5 µl of total RNA was added to 45 µl of the reaction mixture containing 0.2 mmol/l dNTPs each, 0.4 µmol/l of each primer, 0.5 mmol/l DTT, 40 U of RNAsin (Promega), 1x PCR buffer and 1 µl of the Titan enzyme mixture. The reaction mixture was subjected to the following thermal treatment: one cycle of 50°C/30 mins and 94°C/2 mins, and 35 cycles of 94°C/30 secs, 60°C/1 min, 68°C/1 min and 68°C/7 mins.

For the nested PCR the inner sense primer (Oberst *et al*., 1993) 5'-TTACCACACCCCTCAGTACA-3' (nt 681–700) and the inner antisense primer 5'-CATTGTGTCAC AGAACACTC-3' (nt 1044–1063) were used. They yielded a 383 bp product. For the nested PCR 2 µl of the PCR product was mixed with 98 µl of a reaction mixture containing 1 x PCR buffer (Promega), 1.5 mmol/l $MgCl_2$, 0.1 mmol/l dNTPs each, 0.5 µmol/l of each primer and 2.5 U of Taq DNA polymerase (Promega) The reaction conditions consisted of 25 cycles of 94°C/30 secs, 50°C/30 secs, 72°C/30 secs and 72°C/7 mins. An RNA isolated from uninfected MDBK cells was used as negative control. PCR products were identified in 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. The gel was subjected to electrophoresis at 80V for 30 mins. Positive PCR products were sequenced using the DNA ABI PRISM genetic analyser (Perkin Elmer). Blood serum samples were tested for the presence of antibodies to BRSV using an indirect ELISA (Kovarcik, 2001).

On the first blood sampling, a specific 383 bp product was detected by nRT-PCR in leukocyte samples from the cows Nos. 1, 2, 3, 4, and 7 (Fig. 1). On the second blood sampling, viral RNA was demonstrated only in the leukocyte sample from the cow No. 2. The nRT-PCR of the sample from this cow repeated 3 weeks later yielded a negative result. The identity of the PCR product was confirmed by nucleotide sequencing (237 nt, the GenBank Acc. No. AF503544) and deduction of amino acid (aa) sequence (79 aa). The obtained nt and aa sequences were matched against the sequences of glycoprotein F of BRSV strains provided by the GenBank using the ClustalX 1.8 program (Thompson *et al*., 1997) and the data were processed using the BioEdit software (Hall, 1999).

The nt and aa sequences were homologous within 96–100% and 98–100%, respectively. Fully identical were the nt sequences with those of the BRSV strains 220-69 (Acc. No. AF188576) and RB-94 (Acc. No. D00953), and up to 4% variability of the nt sequence was found in comparison with the strain 4642 (Acc. No. AF188575). The range of homology was consistent with the known variability of F protein sequences of BRSV isolates (Pastey and Samal, 1993).

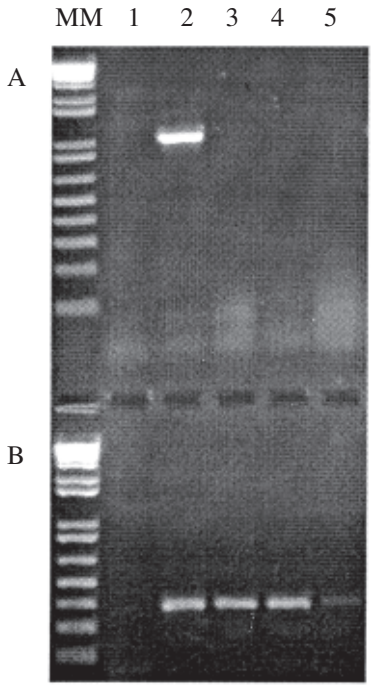

Fig. 1
Ethidium bromide-stained 1.5% agarose gel of RT-PCR (A) and nRT-PCR (B) products

RNA of MDBK cells (lane 1), RNA of MDBK cells infected with BRSV (lane 2), RNAs from leukocytes of cows Nos. 1, 2 and 4 (lanes 3, 4, and 5), 1 kb DNA ladder (lane MM).

The samples of nasal swabs were used for BRSV isolation in secondary cultures of calf kidney cells (Kovarcik, 1999), while individual passages were tested for the presence of virus RNA by nRT-PCR. Although the specific amplification product was usually detected in the first two passages, the subsequent passages were negative. We failed to isolate the virus from any of the nasal swab samples.

Seroconversion could be demonstrated only in antibodies to BRSV. Differences of A_{450} in ELISA between convalescent

Table 1. Examination of serum BRSV antibodies by ELISA and BRSV RNA in leukocytes by nRT-PCR

Cow No.	Signs of acute respiratory disease [a]	Samples taken for examination		Net A_{450} in ELISA[b]		Serocon version[e]	nRT-PCR of leukocytes[f]		
		Blood serum	EDTA-treated blood	A[c]	B[d]		Date of blood sampling		
							29.2	29.3	19.4
1	+	+	+	0.050	0.742	+	+	–	ND
2	+	+	+	0.350	0.598	+	+	+	–
3	+	+	+	0.035	0.394	+	+	–	ND
4	+	+	+	0.614	0.712	–	+	–	ND
5	–	+	–	0.135	0.468	+	ND	ND	ND
6	–	+	–	0.652	0.575	–	ND	ND	ND
7	+	+	+	0.019	0.452	+	+	–	ND
8	+	+	–	0.550	0.617	–	ND	ND	ND
9	–	+	–	0.068	0.500	+	ND	ND	ND
10	–	+	–	0.034	0.412	+	ND	ND	ND
11	+	+	–	0.757	0.656	–	ND	ND	ND
12	–	+	–	0.558	0.586	–	ND	ND	ND
13	–	+	–	0.108	0.369	+	ND	ND	ND
14	–	+	–	0.090	0.423	+	ND	ND	ND
15	–	+	–	0.499	0.443	–	ND	ND	ND

[a]Acute respiratory signs included serous nasal discharge and significantly increased respiratory rate.
[b]A_{450} values >0.2 were scored as positive.
[c]Serum samples collected in acute phase of infection.
[d]Serum samples collected in convalescent phase of infection.
[e]Values (B–A) >0.2 were scored as positive seroconversion.
[f]Leukocytes isolated from EDTA-treated blood. ND = not done.

and acute sera exceeding 0.2 were scored as positive seroconversion (Kovarcik 2001). A significant increase in specific antibody titers between the 1st and 2nd blood sampling was observed in 9 (Nos. 1, 2, 3, 5, 7, 9, 10, 13, and 14) of 15 cows tested (Table 1).

The outbreak of BRSV infection in the herd was diagnosed by a direct demonstration of specific viral RNA by nRT-PCR in blood samples and nasal swabs collected from the affected animals, and indirectly by serological examination. No seroconversion was observed in the cow No. 4, which was positive for BSRV RNA and showed a high antibody titer already on the first sampling (Table 1). This finding may be explained as a result of sampling at the convalescent phase of the infection when the antibodies were already formed. The same explanation may also be applied to the cows Nos. 6, 8, 11, 12, and 15.

A positive finding of viral RNA in circulating leukocytes was obtained in one cow in the acute phase of the infection and again 4 weeks later. This result is in agreement with those of Valarcher and coworkers (Valarcher *et al*., 2001), who have demonstrated viral RNA in peripheral blood mononuclear cells in some experimentally infected calves during acute phase of the disease and 36 days later. The available data do not allow us to speculate on the length of the viremic phase or on the long-term persistence of BRSV in the blood. A long-term search for viral RNA in the blood and monitoring of its nucleotide sequence variability will be necessary for demonstration of persistent infection.

Our results are consistent with those reported on the closely related HRSV (Domurat *et al*., 1985; O'donnell *et al*., 1998; Rohwedder *et al*., 1998). To our knowledge it is the first report on BRSV RNA in leukocytes of naturally infected cows. It is evident that both HRSV and BRSV can spread in their hosts also outside the respiratory tract (Sharma and Woldehiwet, 1996b; van der Poel *et al*., 1997), however, the viremic phase of infection is not routinely detected. Unknown is the fate of BRSV in herds affected by yearly outbreaks of BRSV infections. A low rate of reinfections throughout the year and presence of permanently infected BRSV carriers in herds are possible. Van der Poel and coworkers (1997) have observed a four-fold increase in specific BRSV antibody titers in some corticosteroid-treated-animals after BRSV infection and one immunotolerant carrier of bovine diarrhea virus experimentally infected with BRSV. Although they have failed to detect the virus, it is apparent that the immunosuppressive treatment induced its replication. The affinity of BRSV to cells of the immune system, demonstrated in this as well as other studies (Keles *et al*., 1998; Sharma and Woldehiwet, 1996b; Sharma and Woldehiwet, 1996a), and the recently published data on persistent infection of B lymphocytes of lymph nodes with BRSV (Valarcher *et al*., 2001) indicate that survival of BRSV in tissues of the host immune system is possible.

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