MOLECULAR CHARACTERISATION OF THE FIRST POLISH ISOLATES OF BOVINE RESPIRATORY SYNCYTIAL VIRUS

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Received for publication June 16, 2009

Abstract

This paper describes the first characterisation of bovine respiratory syncytial virus (BRSV) detected in the nasal swabs collected from young yearlings with clinical signs of lower respiratory tract illness from the northern regions of Poland. RT-PCR products of the BRSV gene encoding glycoprotein G were sequenced and subsequently compared to the reference nucleotide sequences of 19 BRSV strains from all over the world. This comparison suggested a close relationship of Polish isolates to the strains isolated in the Czech Republic in 2002-2003 and Danish strains from the 80’s and 90’s.

Key words: cattle, bovine respiratory syncytial virus, isolation, phylogenetic analysis.

Bovine respiratory syncytial virus (BRSV), together with human respiratory syncytial virus (HRSV), belongs to the subfamily Pneumovirinae of Paramyxoviridae family (5). BRSV is an enveloped virus with a negative single-stranded RNA genome, which encodes 10 different proteins. The most variable viral protein is the attachment protein (G), which is responsible for the virus entry to the cell, as well as the reaction with neutralising antibodies. The most immunogenic part of the G protein is externally accessible for the neutralising antibodies cystein-rich region between 174 and 205 aa position of the protein (6). The amino acids 180, 183, 184, and 205 are residues, which are described as most important for the group. The analysis of the genes-encoding nucleocapsid, glycoprotein, and fusion protein from 87 different BRSV strains revealed existence of six phylogenetically-distinct subgroups of BRSV genus (25). BRSV, together with BHV-1, BPIV-3, and BVD-MD virus is the main infectious factor involved in the bovine respiratory disease complex (BRDC). BRSV infections are of serious economic significance to the cattle industry around the world due to the mortality, treatment expenses, and slower growth of affected animals (2, 9, 12, 18, 22, 27). Infections with BRSV are usually asymptomatic in older cattle; however in young animals the infection can lead to severe respiratory disease. Especially high losses are recorded in young calves under the age of 9 months (15). The symptoms range from coughing, nasal and ocular discharge, hyperthermia, and accelerated respiration; in mild cases to pneumonia, lung emphysema and oedema, forced breathing, anorexia, and depression in severe outbreaks.

In some cases, BRSV infection in very young calves is fatal. Recovered animals often remain behind in development compared with the healthy animals of the same age in the herd (26).

Prevention from BRSV infection should be based on compliance with hygiene procedures, quarantine, and limitation of stress in the herd. There are BRSV vaccines available but the protection of the youngest, most susceptible animals is difficult because of the presence of maternal antibodies. Recently, new polyclonal vaccines against pathogens causing BRDC including BRSV are being developed and introduced into the Polish market. However, control of BRSV even in herds where the vaccination strategies were introduced should be carried over since it was shown that the virus can replicate in the presence of specific antibodies (after immunisation or infection) and reinfection is quite common (25). We should concentrate on the assessment of the infectiveness of Polish isolates and analysis of BRSV as a potential cause of BRDC in Polish cattle. In Poland, BRSV has not been the object of wider research yet. In 2008, the prevalence of the BRSV antibodies in bulls from Polish breeding herds was established (20). Serological tests showed that over 50% of animals had had earlier contact with BRSV virus, which could have had an important effect on the health status of Polish herds. This paper describes the first attempt at BRSV isolation using cell cultures and the use of RT-PCR for the diagnosis and identification of the virus infection in Polish herds. Both nucleotide and amino acid sequences of the isolates were compared with the sequences of the reference strains.
Material and Methods

Specimens. Eighty and nine nasal swabs from young animals (very young calves and yearlings, respectively) from 16 herds, were collected into a virus-transport medium (Copan) in winter 2008-2009 and spring 2009. The animals were showing clinical symptoms of respiratory disease such as nasal discharge, coughing, depression, and elevated body temperature (up to 41°C). Additionally, lung tissue samples were collected from six animals that had died of respiratory disorder.

Virus isolation. Bovine turbinate (BT) cells (ATCC CRL-1390), were grown on 24 well plates in Dulbecco Minimum Essential Medium (DMEM) supplemented with 10% of horse serum (ATCC 30-2040) at 37°C and in the atmosphere of 5% CO₂. When confluent, the growth medium was removed and replaced with 250 µl of diluted 1:10 nasal swab sample and incubated at 37°C for 1 h. Then, the inoculum was replaced with DMEM containing antibiotics. Plates were kept at 37°C in 5% CO₂ and monitored daily for the viral cytopathic effect (CPE, syncytium formation) until 7 d. Three subsequent passages were done for each tested sample.

RNA extraction. RNA was extracted from 500 µl of nasal swabs and homogenised lung tissue using TRI reagent (Sigma) according to the producer’s instructions. Positive and negative controls were added to each extraction. As a positive control of extraction, BRSV A51908 reference strain grown in BT cells was used. Negative-for-BRSV nasal swab was used as negative control.

RT-PCR. RT-PCR was performed using Titan One Tube RT-PCR System (Roche). The pair of primers B7 (5’-CATCAATCCAAAGCACCATGTC-3’) and B8 (5’-GCTAGTTCTGTGAGTTTAGTTGTC-3’) published by Vlcek et al. (24), specific to the region of the BRSV genome encoding fragment of glycoprotein G of BRSV were used (29). The expected PCR product size was 381 base pair long. Reverse transcription was performed for 1 h at 48°C, followed by 2 min denaturation at 94°C. The amplification was done in 35 cycles using conditions as follows: 45 s at 94°C; 45 s at 50°C, and 1.5 min at 72°C. The reaction was completed by final elongation for 7 min at 72°C. Products of RT-PCR were analysed on 1.5% agarose gel stained with ethidium bromide.

Sequencing and phylogenetic analysis. RT-PCR products were gel purified using QIAquick Gel extraction kit (Qiagen) and suspended in qa total volume of 25 µl of DEPC water and sent for sequencing to the Genomed Company (Poland). The sequencing was done on both DNA strands separately using B7 and B8 primers. For sequence analysis, construct of consensus sequences and phylogenetic analysis, BioEdit Sequence Alignment Editor v.7.08, was used (26). Nucleotide sequences of the fragment of the glycoprotein gene of Polish BRSVs were compared with sequences of 19 reference BRSV strains, one HRSV strain and one ORSV strain (Table 1). All reference sequences were retrieved from the GenBank database. Phylogenetic trees were constructed using the neighbour–joining method with NJplot v.4.32. Additionally, predicted amino acid sequences of the isolates were compared with sequences of one of the strains from Czech Republic (NI02), one of the Danish strains (2022), two vaccine strains (375, RB94), and two reference strains (A51908, FS-1). Antigenic plots of all of these strains were constructed with Jameson-Wolf’s method using PROTEAN, Lasergene® v.7.0 (DNASTAR) and the data presented in a matrix using Microsoft Office Excel. The method is based on the data that each amino acid has a numerical value (hydrophilicity value) and averaging these values along the peptide chain we can locate antigenic determinants.

Results

None of nasal swabs or the lung homogenates was positive for virus isolation in BT cells; however in the wells with positive controls (reference BRSV strain) syncytium formation and CPE were observed. RT-PCR was successful only in three nasal swabs collected from young heifers from one herd in north-eastern Poland. The validity of the test was confirmed by successful amplification of the positive control and lack of amplification in the negative control sample (Fig. 1).

Fig. 1. Electrophoresis of RT-PCR products (M – pUC mix 8 marker; W1-W3 – nasal swabs, K1+ – extraction control K2+ – amplification control, K- – negative control).

A sequence of three RT-PCR products comprising glycoprotein G gene fragment of three different BRSV detected in the swabs were compared with relevant reference sequence fragments from GenBank to construct the phylogenetic tree shown in Fig. 2. None of the three Polish isolates showed any nucleotide differences, which indicated one source of the infection in that herd.
Table 1

Reference strains used in phylogenetic analysis (4, 22, 27)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Country</th>
<th>Subgroup</th>
<th>Strain</th>
<th>Country</th>
<th>Subgroup</th>
</tr>
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<tbody>
<tr>
<td>A51908</td>
<td>USA</td>
<td>I</td>
<td>FS-1</td>
<td>USA</td>
<td>III</td>
</tr>
<tr>
<td>BRSV-25-BR</td>
<td>Brazil</td>
<td>I</td>
<td>375</td>
<td>USA</td>
<td>III</td>
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<tr>
<td>RB-94</td>
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<td>II</td>
<td>Dorset</td>
<td>UK</td>
<td>IV</td>
</tr>
<tr>
<td>2020</td>
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<td>II</td>
<td>Snook</td>
<td>UK</td>
<td>IV</td>
</tr>
<tr>
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<td>II</td>
<td>WBH</td>
<td>Netherlands</td>
<td>IV</td>
</tr>
<tr>
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</tr>
<tr>
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<td>88P</td>
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<td>V</td>
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<tr>
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<td>Czech Republic</td>
<td>II</td>
<td>75P</td>
<td>France</td>
<td>VI</td>
</tr>
<tr>
<td>PO03</td>
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<td>II</td>
<td>K1</td>
<td>France</td>
<td>VI</td>
</tr>
<tr>
<td>HRSV B1</td>
<td>-</td>
<td>-</td>
<td>ORSV</td>
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Fig. 2. Dendrogram based on the analysis of the fragment of the glycoprotein G gene showing the phylogenetic relationship of Polish isolates (marked by shading), 19 reference strains of BRSV, HRSV, and ORSV.

Fig. 3. Comparison of deduced amino acid sequences of Polish isolates, selected Czech and Danish isolates, and vaccines strains (375, RB94). The box indicates the immunodominant region of glycoprotein G * and four conserved cystein residues in the cystein-rich region. Residues 180, 183, 184, and 205 are the most important for group specificity (6).
The similarity of glycoprotein G coding nucleotide sequences between Polish strains and foreign strains ranged from 83% (strain 75P) to 98% (strain NI02). Polish strains showed the highest similarity to strains isolated in the Czech Republic in 2002-2003 (98%-99%) and to Danish strains isolated in the 80’s and 90’s (up to 98%), which belong to phylogenetic subgroup II of BRSV. The level of similarity to strains representing other phylogenetic subgroups was lower and was 91% for subgroup I, 89%-90% for subgroup III, 92% for subgroup IV, 87% for subgroup V, and only 83% for subgroup VI. The analysis of predicted amino acid sequences showed 100% identity between Polish isolates and Danish isolate 2,022. The homology level of Polish isolates to Czech NI03 strain was only slightly lower and was influenced by a single G→S substitution detected in amino acid 173 of the glycoprotein G. In contrast, in the analysed fragment of 91 amino acids, 17 substitutions were found between Polish isolates and vaccine strain BRSV 375 (Fig 3.), including three in the immunodominant region of glycoprotein G (171 V→E, 177 K→E, 183 S→L). Only six amino acid substitutions were detected between Polish isolates and the other vaccine strain RB94. It included one residue in the immunodominant region (177 K→E).

To assess the possible influence of amino acid differences between Polish strains and BRSV vaccine strains, the antigenic plots were generated using Jameson-Wolf’s algorithm (Fig. 4). The comparison of antigenic profile of the fragment of 91 amino acids of glycoprotein G showed a higher similarity between Polish BRS viruses (represented by PL 01-09 in Fig. 4) and RB94 vaccine strain than to BRSV 375 strain. In the fragment of viral immunodomain between positions 174-188, all the strains had very similar antigenic profiles. Minor differences in the fragment between 168 and 175 aa and single peaks of higher antigenicity index between 147-152 and around 193 aa in BRSV 375 strain were specific to Polish BRSV isolates and other European strains included in the analysis (data not shown).

**Discussion**

Despite the negative isolation of BRSV in BT cells, RT-PCR turned positive in three nasal swabs. There can be several reasons for the failure of BRSV isolation in BT cells. One of them is the fact that BRSV, as an RNA-virus, can be quite sensitive to transport conditions. It was previously shown that repeated changes at temperature (especially freeze/thaw cycles), and prolonged storage (more than 24 h) at 4°C after acquisition of the sample, could lead to a serious reduction in the positive results in the viral isolation test (31). This effect could be limited by avoiding freeze/thawing of the sample or careful choice of the proper medium for the swab transportation (31). In case of this study, a transportation medium designed for the transfer of nasal swabs for virology tests was used. However, prolonged transportation time could still have had some effect on the infectivity of viral particles. The presence of BRSV in the blood of an infected animal and the period of virus shedding through respiratory routes is very short and therefore hard to spot. In various studies this period was estimated to last between 2 and 5 d after infection (4, 7, 30, 31). Therefore, if the animals are not sampled every day starting from the first clinical signs, the virus in the swabs can be missed.

Another problem is the susceptibility of BT cells used for virus isolation. The established BT and
MDBK cell lines are susceptible to infection with BRSV and the first one is recommended and most often used. However, it was shown that primary cell cultures of bovine origin are the most suitable for virus multiplication. Partial fragmentation of the viral RNA or cytotoxicity of the samples do not usually influence RT-PCR, so this method was more successful in identifying BRSV RNA in three out of 89 samples tested. As in previous studies (16, 29), RT-PCR proved to be useful for the diagnosis of the BRSV infection; however more studies are needed to determine the resistance of BRSV to different physico-chemical conditions.

The difference between the high percentage of the BRSV antibodies detected in the earlier study (20) and the small number of positive reactions confirming BRSV presence in the specimens collected from sick animals could be explained based on the pathogenesis of BRSV infection. Clinical signs can be noticed in the late phase of BRSV infection when the level of active virus starts to decrease as an effect of the immunological reaction of the host (15, 4).

Additionally, previous research showed that in some cases, in the late phase of infection, virus could not be detected in the upper respiratory tract, even though it still persisted in the lungs (16). This could limit the reliability of the nasal swabs as source of material for diagnostics. As an alternative source of the sample for the diagnosis of RSV nasopharyngeal aspirate could be used. It was shown that this could allow the higher sensitivity of the diagnostic tests (11). If this is not possible, to maximise the chance of isolating virus from nasal swabs gathering them from most caudal region of nasal cavity (4) is recommended.

In comparison, BRSV IgG antibodies could be detected in blood after more than two months from the occurrence of infection (24, 10) and long after the disappearance of the active-virus from the lung tissue and respiratory tract discharges.

We have detected and analysed the first Polish BRSV isolates and further linked them to other known reference strains from the GenBank. The BRSV genus is divided into six known genetic subgroups. In each subgroup, geographical and isolation time clustering of virus strains has been observed (27). Although in our study we used the NJ (neighbour-joining) method for creating a phylogenetic tree, both branching and subgroup clustering of reference strains was identical with the trees constructed previously using the ML (maximum likelihood) method (26) and the ME (minimal evolution) method (27). Based on the glycoprotein G coding sequence analysis of Polish BRSV isolates that showed the highest similarity to the strains clustered in the II^th phylogenetic subgroup containing mainly European isolates (25), it can be suggested that our isolates should also be classified in this subgroup comprising strains from the same geographical area. It is possible either that animals from the same area affected by BRSV were transferred both to Poland and Czech Republic, or the source of the Polish isolates is the Czech Republic. Due to the fact that animals transferred to Poland from other countries are rarely tested for BRSV, both explanations are possible, and further investigation is needed (with more isolates) for more precise assessment of the diversity of Polish strains of BRSV and determination the possible source and routes of transmission.

In the study, we also tried to analyse the deduced AA sequence of viral glycoprotein G to predict the practical importance of the differences between field BRSV strains and vaccine strains. Glycoprotein G, together with viral fusion protein F, is the main target for the immunological response of the host (13, 23). The analysis was concentrated on the region of glycoprotein G including the immunodominant domain, which spreads between residues 171 and 186 (15).

Due to the fact that this domain is a preferred region for the binding of neutralising antibodies, its sequence and structure should be the object of special interest when designing vaccination strategies and choosing viral strains for the construction of the vaccine. Genetic differences existing in immunodominant regions of the vaccine strains and the field isolates could have had an effect on the efficiency of the vaccines in the prophylaxis of respiratory tract infections. Two kinds of vaccines are registered in Poland: Bovilis Bovipast RSP (Intervet), based on a modified live RB94 strain (isolated in Belgium in 1975), and Rispensal RS+PI3 (Pfizer), based on a 375 strain (isolated in the USA in 1979). Seventeen non-synonymous substitutions out of a sequence of 91 amino acids of partial glycoprotein G between Polish BRS viruses and American vaccine strain 375 were detected, while the European strain RB94 was much more similar to Polish strains, with only six amino acid differences in the analysed region. Additionally, a difference in one of the most immunogenic region of glycoprotein G – codon 183 was found between Polish BRSV isolates and the BRSV 375 strain. We also compared the antigenic plots of the glycoprotein G part covering the immunodominant domain, which were drawn using Jamson-Wolf’s algorithm, which showed higher variability between Polish strains and vaccine strain 375 than the closely-related RB94 strain (Fig. 4). Only a minor difference was seen between Polish isolates and the second vaccine strain RB94, which was both seen in the deduced aa sequence alignment and the antigenicity plot. Although the general efficiency against a BRSV challenge of both of these vaccines has been already proved (28, 17), none of these tests compared vaccine-induced protection against viral strains originating from different regions of the world. Therefore, it cannot be excluded that vaccines based on the strain 375 isolated in United States could be less effective in preventing infections with BRSV strains circulating in Poland. For a more accurate assessment of this risk, more data on the genetic diversity of BRSV should be collected. If similar variability would be found in other immunodominant regions, it would suggest that more attention should be given when selecting the appropriate vaccine strain to be used in vaccination programmes in different regions.
References