Comparative Immunology, Microbiology and Infectious Diseases 58 (2018) 52–57

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

# Genetic analysis of bovine respiratory syncytial virus in Croatia

Nina Krešić[a,*], Tomislav Bedeković[a], Dragan Brnić[a], Ivana Šimić[a], Ivana Lojkić[a], Nenad Turk[b]

[a] Virology Department, Croatian Veterinary Institute, Savska cesta 143, 10 000 Zagreb, Croatia[1]
[b] Faculty of Veterinary Medicine, Heinzelova 55, 10 000 Zagreb, Croatia[2]

ARTICLE INFO

*Keywords:*
BRSV
HRSV
Genetic analysis

ABSTRACT

Bovine respiratory syncytial virus (BRSV) represents an important causative agent of respiratory tract disease in cattle. This study describes the genetic diversity of BRSV strains detected in beef cattle herds in Croatia during four consecutive years, from the end of 2011 to April 2016. Genetic diversity of circulating Croatian strains is reflected in their clustering within three different genetic subgroups. Analysis of representative BRSV G gene sequences revealed that infections in Croatia were caused by BRSV strains belonging to two new subgroups (VII and VIII identified herein for the first time). In 2014–2016, the subgroup VII strains were replaced with BRSV strains clustered in the previously unidentified subgroup VIII. Furthermore, co-circulation of subgroup II and new subgroup VIII strains in Croatia was recorded in the same time period. Sequences of Croatian BRSV strains within subgroups II and VII revealed unique mutations within an essential immunodominant region, demonstrating continuous evolution of viral mechanisms for immune escape.

## 1. Introduction

Bovine respiratory syncytial virus (BRSV) and human respiratory syncytial virus (HRSV) are members of the *Orthopneumovirus* genus within the *Pneumoviridae* family. BRSV, a major cause of respiratory tract disease in cattle [1], is genetically closely related to HRSV, an important cause of lower respiratory tract disease (LRTD) in children. These related viruses share common epidemiological, clinical, and pathological characteristics [2].

The well-understood sensitivity of viruses has resulted in inefficient virus isolation from clinical samples [3]. Consequently, molecular methods are becoming the golden standard for accurate identification and genetic analysis of BRSV strains [4]. The gene encoding the attachment glycoprotein G has the highest reported mutation rate among BRSV strains and is commonly used as a target for molecular epidemiological investigations and phylogenetic analysis [2].

Based on sequence variability of the G protein, BRSV has been classified into six different genetic subgroups [1,2,5], while similar analysis of the N and F genes resulted in only five subgroups, illustrating the higher rate of evolution of the G gene [1,2,6]. Thus, it would not be surprising if the bovine immune system previously stimulated by infection or vaccination could select new variants [2]. A recent study provided evidence for the circulation of new, genetically unique BRSV strains in Italy; the rapid evolution of the virus highlights the importance of updating vaccination strategies [7].

The characteristic genomic heterogeneity of the BRSV genome and its low fidelity in replication [4] are some of the features used for survival and resistance to vaccination. The analysis of BRSV strain diversity is essential for development of efficient vaccines. Because there is a lack of data on the diversity of circulating BRSV strains in Croatia and Southeast Europe, the aim of this study was to analyse the genetic diversity of circulating BRSV strains in Croatia.

## 2. Materials and methods

### 2.1. Study design

The presence of BRSV was investigated by collecting nasal swabs, blood, lungs, and lymph nodes (Table 1) of cattle aged 1–6 months belonging to 10 herds in 10 counties (Table 2) in which the cattle industry is important. The study also included samples collected during regular diagnostic activity. Samples were collected during December

*Abbreviations:* aa, amino acid; BRSV, bovine respiratory syncytial virus; DMEM, Dulbecco's modified Eagle's medium; HRSV, human respiratory syncytial virus; JC, Jukes Cantor; mAb, monoclonal antibody; NJ, neighbour joining; nt, nucleotide; RT-PCR, reverse transcription polymerase chain reaction

* Corresponding author.
*E-mail addresses:* lemo@veinst.hr (N. Krešić), bedekovic@veinst.hr (T. Bedeković), brnic@veinst.hr (D. Brnić), simic@veinst.hr (I. Šimić), ilojkic@veinst.hr (I. Lojkić), turk@vef.hr (N. Turk).

[1] http://www.veinst.hr/.
[2] http://www.vef.hr/.

https://doi.org/10.1016/j.cimid.2018.09.004
Received 9 March 2017; Received in revised form 1 August 2018; Accepted 3 September 2018

**Table 1**
Number and type of collected samples.

| Number and type of collected samples | | | | | | |
|---|---|---|---|---|---|---|
| | Number of animals included | Number of samples collected | Nasal swabs | Lungs | Lymph nodes | Blood |
| Total (%) | 729 (100) | 864 | 604 (69.9) | 102 (11.8) | 35 (4.05) | 123 (14.23) |

**Table 2**
BRSV G gene presence in clinical samples.

| County | Total / positive animals | Collected/positive samples | | | | |
|---|---|---|---|---|---|---|
| | | Collected / positive samples | Nasal swab | Lungs | Med. lymph node | Blood |
| Bjelovarsko-bilogorska | 3/0 | 3/0 | 0 | 3/0 | 0 | 0 |
| Istarska | 7/0 | 7/0 | 7/0 | 0 | 0 | 0 |
| Koprivničko-križevačka | 323/12 | 323/12 | 321/11 | 2/1 | 0 | 0 |
| Krapinsko-zagorska | 13/0 | 13/0 | 13/0 | 0 | 0 | 0 |
| Osječko-baranjska | 195/38 | 205/38 | 122/33 | 55/5 | 0 | 18/0 |
| Požeško-slavonska | 1/0 | 1/0 | 0 | 1/0 | 0 | 0 |
| Sisačko-moslovačka | 10/0 | 10/0 | 10/0 | 0 | 0 | 0 |
| Vukovarsko-srijemska | 7/0 | 7/0 | 0 | 2/0 | 0 | 5/0 |
| Zagrebačka | 163/5 | 298/6 | 125/4 | 38/1 | 35/1 | 100/0 |
| Zadarska | 7/0 | 7/0 | 6/0 | 1/0 | 0 | 0 |
| **Total %** | **729/55 7.54** | **874/56 6.40** | **604/48 5.49** | **102/7 0.8** | **35/1 0.11** | **123/0 0** |

2011–January 2016. Sampling at all farms was performed by the same veterinarian in the early phase of acute respiratory infection.

### 2.2. Clinical samples

#### 2.2.1. Nasal swabs

Nasal swabs (n = 604) were collected from all animals within each herd (animals presenting clinical signs of respiratory infection and asymptomatic animals). The sampling was conducted by inserting a sterile swab into each animal's nostrils, briefly rotating the swabs. The swabs were placed into a plastic holder containing special transport medium composed of Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) and 1% v/v antibiotics (penicillin/streptomycin) (Sigma-Aldrich). Samples were delivered the same day to the laboratory and immediately placed in the procedure.

#### 2.2.2. Lungs

Lung tissue samples (n = 102) (Table 1) were obtained from deceased animals that had shown clinical signs of respiratory infection. The samples were homogenised in a mortar with the addition of DMEM and centrifuged at 3000 rpm (2100 $\times g$) for 10 min. The supernatant was filtered through a 0.45-μm filter and used for RNA isolation.

#### 2.2.3. Lymph nodes

Mediastinal lymph nodes were collected and processed using the procedure described in Section 2.2.2.

#### 2.2.4. Blood

Blood samples were collected to estimate the presence of BRSV (viremia). A total of 123 whole blood samples were collected from the jugular vein using a vacuum system.

### 2.3. Methods

#### 2.3.1. Nucleic acid extraction

RNA isolation was performed using the transport medium of the nasal swab samples, lung and lymph node tissue homogenate supernatants, and blood. RNA was extracted using the QIAamp® Viral RNA Kit (Qiagen, Hilden, Germany) and Rneasy Mini Kit (Qiagen) according to manufacturer's instructions.

#### 2.3.2. Reverse transcription polymerase chain reaction (RT-PCR) and semi-nested PCR

RT-PCR was performed for viral RNA detection, and semi-nested PCR was conducted to investigate the gene encoding viral glycoprotein G. RT-PCR and semi-nested PCR were performed according to the protocol described by Valentova et al. [1]. The primers used were as follows (5′ to 3′): G1F, CCATCAACCAATCAAGCAACT; G2F, GATAAC CTTGACATCACTCGTC; G6R, CTCTACTAACTGCACTGCATGT. RT-PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and primers G1F and G6R. The reaction mixture containing 7.5 μl sterile PCR water, 12.5 μl reaction buffer, 1 μl of each primer, 1 μl enzyme mixture, and 2 μl of isolated RNA was added to a 0.2-ml tube. Cycling conditions were as follows: 50 °C for 30 min; 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 1 min, and 68 °C for 1 min; and a final elongation at 68 °C for 7 min.

Semi-nested PCR was performed by adding 3 μl of the first-round product into 12.5 μl of GOTaq Hot Start Green Master Mix (Promega, Madison, WI, USA) with 1 μl of each primer (G2F and G6R) and 7.5 μl PCR water. Cycling conditions were as follows: 94 °C for 3 min; 25 cycles of 94 °C for 1 min and 53 °C for 30 s; and a final elongation at 72 °C for 30 s.

PCR products were visualised by gel electrophoresis, purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, and sequenced by Macrogen Inc. (Amsterdam, The Netherlands).

#### 2.3.3. Phylogenetic analysis

Phylogenetic analysis was performed using representative sequences of Croatian BRSV isolates (Table 3) and the reference sequences available in GenBank. Multiple alignments were performed in Mega 7 using Clustal W. Reliability of the multiple alignments was assessed by estimation of the overall mean distance of protein sequences with the *p-distance* model, also in Mega 7.

The neighbour-joining (NJ) method with the *p-distance* model in Mega 7 was also used for analysis. Suitability of the data for the NJ method was assessed by estimation of the overall mean distance of protein sequences with the Jukes Cantor (JC) model. The bootstrap method with 2000 repetitions was used.

### 2.4. Statistical analysis

Data were analysed using descriptive statistical methods for organisation, summarizing, and visualisation. Statistical significance was estimated using Fisher's exact test ($p < 0.05$ was considered significant).

## 3. Results

### 3.1. Viral RNA detection

Viral RNA was detected in 7.54% of tested animals (55/729)