Comparative detection of bovine herpesvirus-1 using antigen ELISA, immunohistochemistry and immunofluorescence methods in cattle with pneumonia

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Abstract: This study was conducted to detect the prevalence and presence of bovine herpesvirus-1 (BoHV-1) using immunohistochemistry (IHC), immunofluorescence (IF) and antigen enzyme-linked immunosorbent assay (ELISA) in the lung samples of cattle (n = 1023). In addition, three methods were compared using receiver operating characteristic (ROC) and chi-square test ($\chi^2$ test), and their usability were evaluated in laboratory conditions. Macroscopically, pneumonia was seen in 120 of the lung tissue samples (11.73%). Based on the microscopic examinations, the pneumonia types were classified as catarrhal-suppurative bronchopneumonia (6.7%), fibrinous bronchopneumonia (5%), interstitial pneumonia (84.1%), and granulomatous pneumonia (4.2%). The IHC, IF and antigen ELISA positivity were 48.33%, 50.83%, and 29.9%, respectively. Considering IF as a gold standard, IHC had more acceptable sensitivity and specificity than antigen ELISA. In conclusion, it has been observed that when IF method is not available, IHC is more reliable than antigen ELISA in the diagnosis of BoHV-1.

Key words: Bovine herpesvirus-1, diagnosis, pneumonia, sensitivity, specificity

1. Introduction
Bovine respiratory disease complex (BRDC) is a major health problem affecting cattle populations worldwide (1–3). Primarily, viral agents cause damage to the respiratory system in BRDC cases, while bacteria and mycoplasmas are secondary causes (1, 3, 4–6). The viral agents that cause respiratory system diseases in cattle include bovine herpesvirus-1 (BoHV-1), bovine coronavirus (BoCV), bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV), parainfluenza type 3 virus (PIV-3), bovine adenovirus, bovine enterovirus 1, 2 and 3, and bovine rhinovirus 1 and 2 (5,7).

BoHV-1 causes several clinical conditions, including infectious bovine rhinotracheitis (IBR) in respiratory system, infectious pustular vulvovaginitis (IPV) in genital system of female cattle, infectious pustular balanoposthitis (IPB) in genital system of male cattle, conjunctivitis, and generalized disease in newborn calves. Overall, it is an important agent causing livestock losses globally (8). BoHV-1 belongs to Alphaherpesvirinae subfamily, Varicellovirus genus of Herpesviridae family (9). After entering the body, it spreads locally or via neuro-invasion and viremia. The viral agent replicates at the location where it first enters the body and may settle into the axons of the local nerve cells. Then, the virus reaches the neurons via the intra-axonal pathway, and it can create latency in these regions (10). BoHV-1 can remain latent in some parts of the nervous system ganglia after the initial infection, resulting in replication in the upper respiratory system and ocular mucosa (11–13). In animals, a latent infection is characterized by the inability to isolate the infected virus and the detection of viral antigens via diagnostic methods (10). Many methods can be used for the laboratory diagnosis of BoHV-1 infections, including cell cultures, histopathology, serological tests, polymerase chain reaction (PCR), IHC and IF staining, Western blot, ELISA, and electron microscopy (11,13,14–17). Lung lesions are seen less frequently in BoHV-1 infections (3,18). Bronchial epithelial necrosis can be seen microscopically with the proliferation of type II pneumocytes in the lungs in the respiratory form of this disease. While this virus can cause the formation of giant cells in the lungs in many animal species, it can also form eosinophilic inclusion bodies in the alveolar epithelium (3,19). These intranuclear inclusion bodies can be found in the epithelial cells of the respiratory tract during the first stages of infection. Due
to bacterial complications, bronchopneumonic lesions can be seen in the lungs (20).

This study aimed to determine the presence and prevalence of BoHV-1 via IHC and IF staining and antigen ELISA testing in the lung samples of cattle in a slaughterhouse. In addition, these three methods were compared statistically, and their usability was evaluated in laboratory conditions.

2. Materials and methods

2.1. Sample collection
A total of 1023 slaughtered cattle were followed up in order to obtain the number of samples required for the study. The lung tissue samples were obtained from 120 (approximately between the ages of 2 and 10) Holstein and Brown Swiss crossbreds that were dairy and beef cattle.

2.2. Histopathological examination
Cattle lung samples were washed and then, graded alcohol and xylene solutions were obtained for these samples. Following the routine histopathology procedure, all lung samples were embedded in paraffin and sections of 4 µm from paraffin blocks were taken and stained with hematoxylin-eosin (H&E) to evaluate standard histopathological findings. Slides were examined under the light microscope and pneumonia types were classified as interstitial pneumonia, embolic pneumonia, granulomatous pneumonia, and bronchopneumonia (3).

2.3. Immunohistochemical staining
After deparaffinization, 3% H2O2 solution was dropped on each slide to inactivate endogenous peroxidase activity for 10 min. Then, the slides were heated in antigen retrieval solution to unmask antigens for 15 min. Then, blocking solution was examined in sections to prevent nonspecific binding for 15 min. Sections were incubated with Bovine Herpesvirus type 1 primary antibody (BHV-1/IBR, MAb gC-gIII IgG2b Isotype monoclonal antibody, VMRD, Catalog no. F2, Dilution: 1/100) at 37 °C for 45 min. After incubation, EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC Kit (abcam, cat no: ab80436) was followed. 3,3'-diaminobenzidine (DAB) solution was treated to the sections and then these sections were counterstained with Mayer's hematoxylin (MH).

2.4. Immunofluorescence staining
After deparaffinization, endogenous peroxidase blocking, antigen retrieval, and protein blocking stages were performed as in immunohistochemical staining. The sections were incubated with bovine herpesvirus type 1 primary antibody (BHV-1/IBR, MAB gC - gIII IgG2b Isotype monoclonal antibody, VMRD, Catalog no. F2, Dilution: 1/100) at 37 °C for 45 min. Then, the secondary antibody (Abcam, Goat Anti-Mouse IgG H&L (FITC), Catalog No: ab6785) was dropped at the dilution of 1/100 on the sections and incubated for 45 min. in the dark. Finally, all sections were covered using diluted glycerin for evaluation by fluorescence microscopy.

2.5. Antigen ELISA test
Pulmotest BoHV-1 Kit (commercial kit Cat. No. BIO K 335) from the manufacturer was provided for the determination of BoHV-1 by ELISA from the lungs obtained in this study. The specimens were examined following the commercial kit procedure.

2.6. Statistical analysis
χ2 and ROC tests were used for intermethod comparisons (MedCalc Statistical Software version 13.1.2, Ostend, Belgium). The sensitivity and specificity of the test was evaluated taking immunofluorescence staining as relative gold standard (21,22).

3. Results

3.1. Macroscopic findings
In this study, 1023 cattle lungs were examined and pneumonia was observed macroscopically in the cranoventral and caudodorsal lobes of 120 cases. In the lungs with severe bronchopneumonia, the lesions were more cranial and had a dark red-gray hardness. Fibrinous bronchopneumonia cases were mostly observed in the lobar character and in cranial lobes. Due to the dark red and gray areas, the appearance of variegated marble was observed. The lungs, which were diagnosed with interstitial pneumonia, were not collapsed, and their colors were pale. Furthermore, in most cases, it was observed that mediastinal lymph nodes were enlarged.

3.2. Histopathologic results
Following microscopical examination, pneumonia was observed as catarrhal-purulent (n = 8, 6.7%), interstitial (n = 101, 84.2%), fibrinous (n = 6, 5%) and granulomatous (n = 5, 4.2%). Oedema, desquamated epithelial cells, and abundant neutrophil leukocytes were observed in the catarrhal-suppurative bronchopneumonia cases (Figure 1A, Figure 1B), and there were some necrotic areas in the bronchi, bronchioles, and alveolar epithelium. In the fibrinous bronchopneumonia cases, there were typical inflammatory hyperaemia, red-grey hepatization (Figure 1C, Figure 1D), and enlarged interlobular septal tissue. In the interstitial pneumonia cases, the lumen of the alveoli appeared to be empty, and there was interalveolar septal thickening due to dense mononuclear cell infiltration and increased connective tissue (Figure 1E). Bronchus-associated lymphoid tissue (BALT) hyperplasia (Figure 1F) and fibromuscular hypertrophy were observed around the bronchi and bronchioles. In cases with granulomatous pneumonia were detected Langhans giant cells, epithelioid cells, histiocytes, lymphocytes, and plasma cells around the necrotic center (Figure 1G), calcification (Figure 1H), and fibrous connective tissue forming the outermost capsule.
Figure 1. Histopathological results of pneumonia. A) Bronchiole filled with neutrophil leukocytes, suppurative bronchopneumonia, B) Cellular exudate of neutrophils in bronchiole (*), C-D) Fibrinous bronchopneumonia, grey hepatization areas (arrowhead), E) Thickness in the interalveolar septum, interstitial pneumonia (*), F) Hyperplasia in BALT (arrowhead), G) Caseous necrosis (*), langhans giant cells (arrow), granulamatous pneumonia, H) Calcification foci in necrosis area (arrowhead). H&E.
3.3. Immunohistochemical and immunofluorescence results
BoHV-1 immunopositivity was determined in 58/120 (48.33%) of the samples via IHC examination. The BoHV-1 immunopositivity based on the type of pneumonia is shown in Table. The immunopositivity was observed in BALT, the epithelium of the bronchi (Figure 2A), lumen of the bronchioles, the epithelium of bronchioles and peribronchiolar inflammatory cells (Figure 2B), desquamated epithelial cells, macrophages, and interstitial areas.

BoHV-1 immunopositivity was observed in 61/120 (50.83%) of the samples in the IF examination. The immunopositivity based on the pneumonia types is shown in Table. The immunopositivity was observed in the alveolar macrophages, bronchi, bronchioles, alveoli, macrophages around the blood vessels, and in the inflammatory cellular exudate (Figure 3A, 3B).

3.4. Antigen ELISA results
A commercial antigen ELISA Kit (Pulmotest BoHV - 1, Cat. No. BIO K 335) was used to test 117 (120 sample test kit consists of 117 suspicious specimens and 3 positive controls) of the 120 lungs tissue samples in which pneumonia was detected macroscopically. Thirty-five positive samples (29.9%) were detected.

3.5. Statistical analysis results
The results of the statistical analyses showed that the disease detection rate of IHC was 85%, while the disease detection rate of ELISA was 55% (P < 0.0001). Considering IF as a gold standard, IHC had more acceptable sensitivity and specificity (sp., 89.3%; se., 85.2%, P < 0.0001) than antigen ELISA (sp., 98.21%; se., 55.74%, P < 0.0001).

4. Discussion
Previous studies conducted by different researchers to determine the epidemiological prevalence of cattle pneumonia reported that the pneumonia rates vary between 3.6% and 65.83% (23–25). Pneumonia was determined in 120 of 1023 (11.73%) cattle lungs in the present study. Various epidemiological factors have been considered as reasons for the different pneumonia rates, such as the cattle variety, uncontrolled animal movements, adverse climatic conditions, and inadequate management conditions in the region in which the research was carried out. The cranioventral lung lobes are mostly affected due to direct exposure of the infectious agent via inhalation (26). However, viral pneumonia is usually found in the dorsocaudal lobes (3). Purulent bronchopneumonia and fibrinous bronchopneumonia are found most often in the cranial lobes, while interstitial pneumonia is found in the caudal lobes (27). In the present study too, it was determined that suppurative and fibrinous bronchopneumonia were mostly observed in the cranial lobes, whereas interstitial pneumonias were located in the caudal lobes. In particular, the observation of BoHV-1 immunopositivity in interstitial pneumonia in the study has been associated with the tendency of viral agents to settle in the caudal lobes. Narita et al. reported a mild and limited lesion in the right caudal lobe of lung as a result of endobronchial inoculation of the BoHV-1 agent. These lesions were characterized by mild necrotic bronchitis-bronchiolitis and fibrin bronchopneumonia (27). Furthermore, Loneregan et al. found BoHV-1 immunopositivity in 2 out of 108 acute interstitial pneumonia lungs and 3 out of 50 bronchopneumonia lungs (28). In the present study, more than 50 % BoHV-1 immunopositivity in fibrin bronchopneumonia and interstitial pneumonia cases was associated with predisposition to viral agents and secondary bacterial agents such as pneumonic pasteurellosis (29,30).

Currently, virus isolation has been used to detect BoHV-1, as well as FAT examinations, ELISA antigen detection and immunoperoxidase testing (31). Seroprevalence of BoHV-1 has been reported to be 33%–36%, 35.8%, 37.7%, 39.2%, and 61% in Belgium (32), China (33), Poland (34), India (35), and Italy (36), respectively. A 19.5% to 74% seroprevalence of BoHV-1 has been reported in previous studies in Turkey (37–39). BoHV-1 seropositivity was reported as 59.48% in a study in the cattle in Northeastern Anatolia. In the same study, 31 (37.8%) of 82 blood samples were found positive in Erzurum province (40). Antigen ELISA tissue studies to determine BoHV-1 are rarely performed in Turkey, as well as in the world. Collins

<table>
<thead>
<tr>
<th>Pneumonia types</th>
<th>Histopathological diagnose</th>
<th>IHC positive</th>
<th>IF positive</th>
<th>ELISA positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catarrhal- Suppurative Bronchopneumonia</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Fibrinous Bronchopneumonia</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Intersitial Pneumonia</td>
<td>101</td>
<td>50</td>
<td>52</td>
<td>27</td>
</tr>
<tr>
<td>Granulomatous Pneumonia</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>120</strong></td>
<td><strong>58 (%48.33)</strong></td>
<td><strong>61 (%50,83)</strong></td>
<td><strong>35 (%29.9)</strong></td>
</tr>
</tbody>
</table>

Table. The positive results of three methods in types of pneumonia.
et al. detected the *BoHV-1* positivity in 21 out of 457 nasal swap samples by antigen ELISA method (41). Elhassan et al. found the *BoHV-1* positivity as 1.7% in nasal swap, 33.3% in placenta, 20% in whole blood (42). *BoHV-1* positivity was found as 2% in 250 camel lung samples in a study via antigen ELISA (43). It has been reported that no positivity was detected in lung samples of 24 cattle in antigen ELISA study performed to determine *BoHV-1* in Turkey (44). So, in the light of the examined literature, the present study is the first study to detect *BoHV-1* positivity from cattle lung tissues by antigen ELISA method. In the present study, 35 (29.9%) positive cases were found in 117

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**Figure 2.** *BoHV-1* immunopositive stainings: A) Epithelium of bronchiole (arrowhead), and in inflammatory cellular exudate (arrow), B) Epithelium (arrow) of the bronchiole, and peribronchiolar inflammatory cells (arrowhead). IHC.

**Figure 3.** *BoHV-1* IF positivity: A) Inflammatory cellular exudate (arrow) and peribronchiolar inflammatory cells (arrowhead), B) Interstitial areas (arrowhead). IF.
pneumonic cattle lung samples via antigen ELISA. The reasons why the positivity rate of the antigen ELISA was lower than that of the serological studies may be linked to the viral agent being located in the ganglions in latent infection cases and not having a sufficient antigen amount in the tissue.

The immunopositivity was found to range between 2.5% and 55.6% in previous studies conducted to determine BoHV-1 antigens from lung tissue via the IHC method (45,46). BoHV-1 immunopositivity was found as 2.43% in a study that was conducted to determine BoHV-1 antigens from 247 pneumonic cattle lung samples. It was reported that immunopositivity was observed intracytoplasmically in the epithelium of the bronchi, bronchioles, alveoli, alveolar macrophages, and in the exudate in the lumen of the bronchioles (47). In the present study, 48.3% BoHV-1 immunopositivity was determined in 120 pneumonic cattle lung samples. The immunopositivity was observed in alveolar macrophages, in the cytoplasm of epithelial cells of bronchi, bronchiole and alveoli, in macrophages around bronchi-bronchioles. Therefore, it was observed that our findings were consistent with the literature.

Intisar et al. observed 1.6% BoHV-1 positivity in 186 cattle lungs with pneumonia using ELISA test and confirmed the correctness of ELISA positivity by IF (48). In another study, 11 (4.45%) positives were found with the direct fluorescent antibody method in 247 pneumonic cattle lung samples. Immunopositivity was found intracytoplasmically in the epithelium of the bronchi, bronchioles, and alveoli (47). In the present study, 61 (50.8%) immunopositive cases were detected via the IF staining method in 120 pneumonic cattle lung samples. Immunopositivity was found intracytoplasmically in the epithelium of the bronchi, bronchioles, and alveoli (47). In the present study, 48.3% BoHV-1 immunopositivity was determined in 120 pneumonic cattle lung samples. The immunopositivity was observed in alveolar macrophages, in the cytoplasm of epithelial cells of bronchi, bronchiole and alveoli, in macrophages around bronchi-bronchioles. Therefore, it was observed that our findings were consistent with the literature.

In conclusion, it has been detected that BoHV-1 plays an important role in the occurring and pathogenesis of pneumonia in cattle. Furthermore, although the ELISA test used for diagnostic purposes is quick and easy to perform in laboratory conditions, IHC and IF methods are more sensitive and reliable methods for determining the presence of infection.

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References


