Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Microbial Pathogenesis 145 (2020) 104165

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

Short communication

Immunofluorescence and molecular diagnosis of bovine respiratory syncytial virus and bovine parainfluenza virus in the naturally infected young cattle and buffaloes from India

Bhupesh Kamdi, Rajendra Singh*, Vidya Singh**, Shailendra Singh, Pawan Kumar, Karam Pal Singh, Neethu George, Kuldeep Dhama

Indian Veterinary Research Institute, Izzatnagar, Bareilly, Uttar Pradesh, 243122, India

ARTICLE INFO

Keywords:
BRSV
BPI-3
Immunofluorescence
Pneumonia
Syncytia
Bovine

ABSTRACT

Pneumonia in bovines is a multifactorial disease manifestation leading to heavy economic losses. Infections of bovine respiratory syncytial virus (BRSV) and bovine parainfluenza virus-3 (BPI-3) are among the important contributing factors for the development of pneumonia in young animals. These viral agents either primarily cause pneumonia or predispose animals to the development of pneumonia. Although, the role of BRSV and BPI-3 in the pathogenesis of pneumonia is well established, there are no reports of involvement of BRSV and BPI-3 from Indian cattle and buffaloes suffering from pneumonia. In the present investigation, we performed post-mortem examinations of 406 cattle and buffaloes, which were below twelve months of age. Out of 406 cases, twelve (2.95%) cases were positive for BRSV and fifteen (3.69%) cases were positive for BPI-3, screened by reverse transcriptase polymerase chain reaction (RT-PCR). Further, positive cases were confirmed by sequence analysis of RT-PCR amplicons and direct immunofluorescence antibody test (d-FAT) in paraffin-embedded lung tissue sections. BRSV positive cases revealed characteristic findings of bronchiolar epithelial necrosis, thickened alveolar septa by mononuclear cells infiltration and edema; alveolar lumens were filled with mononuclear cells and numerous syncytial cells were seen having intracytoplasmic inclusions. The BRSV antigen distribution was found to be in bronchiolar and alveolar epithelium and syncytial cells in the lung sections. In fifteen cases, where BPI-3 was detected, bronchointerstitial pneumonia in the majority of cases with thickened alveolar septa by mild macrophage infiltration, hyperplasia of type-II pneumocytes and bronchiolar necrosis along with syncytial cells having intracytoplasmic inclusions in the majority of cases were observed. The BPI-3 antigen distribution was found to be in bronchiolar and alveolar epithelium and syncytial cells in the lung sections. RT-PCR amplicons of BRSV and BPI-3 obtained were sequenced and their analysis showed homology with already available sequences in the NCBI database. It is the first report of detection of BRSV and BPI-3 from pneumonic cases by RT-PCR and d-FAT from cattle and buffaloes of India, indicating the need for more epidemiological studies.

1. Introduction

Livestock production plays a key role in India's agricultural economy as it offers employment opportunities and provides social and financial security. In the course of development of livestock industry, the health of replacement progeny calves is an important component of total operation profitability as the heifer calf is the foundation of the future herd [1]. However, calves are usually neglected as they do not bring any immediate financial return and the cost of their maintenance considerably adds to the production cost of milk. Pneumonia is associated with high morbidity and mortality of calves and incurs great economic losses to the farmers due to the losses by death, treatment costs, reduced growth and productivity [2]. It is estimated that calf mortality of 20% can reduce the net profit of an enterprise by 40% [3].

Some calf diseases result because of the complex interaction of the management practices, the environment factors, infectious agents and the calf's immune system [4]. Pneumonia is the second major cause of morbidity and mortality among calves after diarrhea and may account for 50% mortality in the affected animals [5]. It is the most common postmortem finding in calves at 1–5 months of age and calf morbidity may go up to 50% in a herd [6]. It is the most important contributor to the losses in the beef and dairy cattle industries on account of the cost

* Corresponding author. Division of Pathology, Indian Veterinary Research Institute, Izzatnagar, Bareilly, India.
** Corresponding author. Division of Pathology, Indian Veterinary Research Institute, Izzatnagar, Bareilly, India.
E-mail addresses: rajendra_singh5747@rediffmail.com (R. Singh), vidyasingh100@gmail.com (V. Singh).

https://doi.org/10.1016/j.micpath.2020.104165
Received 23 January 2020; Received in revised form 16 March 2020; Accepted 18 March 2020
Available online 20 March 2020

incurred on the metaphylactic and therapeutic uses of antibiotics and reduced growth performance of affected animals [7].

Pneumonia in young animals is associated with viruses, bacteria and mycoplasma, which, include bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus type 3 (BPI-3), bovine herpesvirus type 1, bovine viral diarrhea virus, bovine coronavirus, *Pasteurella multocida, Mannheimia. hemolytica, Haemophilus somnus, Salmonella dublin, Arcanobacterium pyogenes and Mycoplasma* spp. (e.g. *M. bovis* and *M. dispar*) [8]. Besides, bovine rhinovirus, bovine reovirus and Influenza A viruses have also been reported, occasionally to be associated with pneumonia in calves [9,10]. Moreover, sepsis and bacteriemia due to *E. coli*, *Salmonella dublin*, *Streptococcal* spp. or *Staphylococcal* spp. are also responsible for the development of pneumonia in the dairy calves [11]. Viruses are the primary pathogens and are generally detected during an early phase of pneumonia, and in the later stages, the bacterial infection becomes more predominant [12]. Respiratory viruses like BRSV and BPI-3 initiate primary damage to the upper respiratory tract and lungs, causing suppression of the local immune defense mechanism, which may facilitate rapid bacterial multiplication leading to acute to chronic pneumonia in a synergistic manner [13].

Looking at the site preference of viruses, the BRSV of the Pneumovirus genus under *Paramyxoviridae* family replicates in the upper and lower airways. It causes cellular damage and dysfunction of the cilliary defenses and predisposes for the secondary infection with respiratory pathogens [14]. In the endemic region, BRSV is associated with severe respiratory disease in young calves of 1–3 months of age [15]. The BPI-3, a negative sense RNA virus of Respirovirus genus under *Paramyxoviridae* family, contributes to tissue damage and immunosuppression in stressed calves resulting in severe bronchopneumonia from secondary bacterial infection. Clinically, BPI-3 infection may be manifested with asymptomatic to severe respiratory illness; however, in most cases, the clinical signs include coughing, fever and nasal discharge [16].

Pneumonia in bovines represents a great threat to animal health, farm welfare and profitability, despite the large availability of antimicrobials, vaccines and anti-inflammatory drugs for treatment and prevention in the country. Currently, among all respiratory pathogens, *Pasteurella* spp. has been most commonly reported to be associated with pneumonia, whereas BRSV and BPI-3 have usually been ignored which could be the primary pathogens and then various bacterial pathogens (*Pasteurella* Spp.) as a secondary pathogens. There is scanty information available about BRSV and BPI-3 associated calf pneumonia from India. Presently, there are no confirmed reports of the involvement of BRSV and BPI-3 in calf pneumonia cases from India, barring few seroprevalence studies [17–19]. The present investigation confirmed the involvement of BRSV and BPI-3 in calf pneumonia by RT-PCR and dFAT as well as described pathological alterations in the lungs from affected animals.

2. Materials and methods

2.1. Tissue samples

A total of 406 (166 cattle and 240 buffaloes) animals under 12 months of age were necropsied and representative tissues including lungs, mediastinal/bronchial lymph nodes and trachea were collected during March 2016 to June 2018. Study cases were selected either by calves having a history of respiratory signs or the identification of pulmonary lesions at post-mortem examination. The sampling was done from the postmortem facility of the division of pathology, Indian Veterinary Research Institute, Bareilly, India, abattoirs at Moradabad, Delhi and Chandigarh, and private dairy farms located in districts of Maharashtra, Andhra Pradesh and Madhya Pradesh (Table 1). Representative tissue samples (0.5 cm thick) from the pneumonic area of the lungs, upper respiratory tract (URT) and bronchial/mediastinal lymph nodes were collected in 10% neutral buffered formalin (NBF) for histopathology and dFAT in wide mouth container. The adjacent tissue samples were also collected in sterile, screw capped polypropylene vials containing RNA later using sterile scissors and forceps for molecular biology work. All the tissue samples were properly labeled and stored at room temperature (RT) and at -20 °C till further processing.

2.2. Processing of tissues for histopathology

After proper fixation in 10% NBF, tissues were cut into small sections with a thickness of 2–3 mm and embedded in the paraffin by standard procedures. The paraffin embedded tissues were cut into 5–6 μm thick sections and stained with hematoxylin and eosin [20].

2.3. Direct fluorescent antibody test (d-FAT)

For immunostaining, the primary antibody (FITC conjugated monoclonal antibody) against BRSV and BPI-3 (VMRD) was used. The paraffin embedded sections of 4–5 μm thickness were taken on the 3-aminopropyletriethoxysilane (APES) coated slides, deparaffinized in xylene twice (each for 10 min) and rehydrated through descending grades of alcohol to distilled water, rinsed in phosphate buffered saline (PBS) (pH 7.4) twice for 5 min each. Antigen retrieval was done using proteinase K (Merck-GeneiTM, India) diluted in PBS (20 μg/ml) at 37 °C in a humidified chamber for 15–20 min. Then the sections were rinsed in PBS (twice, 5 min each), covered with 0.3 M glycine in 1X PBS for 30 min. After incubation, the slides were rinsed in PBS (twice, 5 min each) and covered with 5% normal goat serum in PBS (pH 7.4) and incubated in a humidified chamber for 1 h at room temperature to avoid non-specific binding of antibodies. Slides were rinsed in PBS (twice, 5 min each). Two hour incubation with primary antibodies was done using specific monoclonal antibodies at 4°C in a humidified chamber. For negative control (antibody control), the sections were incubated with only PBS instead of primary antibodies. Slides were rinsed in PBS (5 min) and immediately mounted with fluorescent antibleaching mounting fluid (Vector Laboratories, USA) and viewed under a fluorescent microscope (Olympus BX-41, Japan) for positive signals, using 10X and 40X objectives.

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from collected tissue (lungs and lymph nodes) samples using commercial TRIzol® Reagent (Thermo Fisher Scientific, USA) as per manufacture's protocol. All extracted RNA samples were quantified by NanoVue plus (Thermo Fisher Scientific, USA) and the purity of RNA was checked by $A_{260}/_{280}$ and $A_{260}/_{230}$ ratio. The cDNA was synthesized from total RNA by using high-capacity cDNA reverse transcription kits (Applied Biosystems, USA). The synthesized cDNA was stored at -20 °C until further use.

Amplification of fragment of G (Glycoprotein) gene of BRSV and fragment of N (Nucleocapsid) gene of BPI-3 was carried out *via* RT-PCR. The self designed primer sequence (5′–3′) for bovine respiratory syncytial virus BRSV; F- GCACCACACTGTCCCAACCA and BRSV; R- GGC AGAGTGGTGAACAGGCA with a predicted amplicon length of 246 bp and the primer sequence (5′–3′) for bovine parainfluenza virus-3, BPI-3; F- CCTGCCCTTTGGAGTTATGCGA and BPI-3; R- GCATCACGTGCCAC TGCTTG have amplicon size 127 bp was used in the study. PCR reaction was carried out in 0.2 ml PCR tubes containing reaction mixture of 12.5 μL of Green Dream Taq Master mix (Genetix, Belgium), 0.5 μL of forward primer (10 pmol/μL) and reverse primer (10 pmol/μL), 2 μL of cDNA (1500 ng/μL) and 9.5 μl of nuclease free water. The tubes were then placed in a thermocycler and PCR cycling conditions were used with initial denaturation of 95 °C for 5 min, followed by 35 cycles [denaturation (94 °C, 10 s); annealing (56 °C, 15 s for BRSV; 57 °C 15 s for BPI-3), extension on (72 °C, 20 s)] and a single cycle of final extension at 72 °C for 8 min. Amplified products were resolved by agarose gel electrophoresis (1% w/v) at 100 V for 1 h in TAE buffer with 0.5 μg/

Table 1
Gross screening of the lungs and collection of representative lung samples and other tissues from cattle and buffaloes.

State	Place	Number	Particular of animals				Samples collected	
			Cattle calf		Buffalo calf			
			M	F	M	F	Lungs	Lymph nodes
Uttar Pradesh (152)	PMF	119	78	36	3	2	*	*
	Abattoir	33	–	–	33	–	*	*
Maharashtra	PMF	42	15	12	13	2	*	*
Delhi	Abattoir	70	–	–	70	–	*	*
Punjab	Abattoir	111	–	–	111	–	*	*
Andhra Pradesh	PMF	19	7	6	2	4	*	*
Madhya Pradesh	PMF	12	8	4	–	–	*	*

mL ethidium bromide and viewed under UV transilluminator (Geldoc, USA).

2.5. Sequencing and phylogenetic analysis

Gel fragments with specific PCR amplicons were cut using a sterile scalpel blade and transferred in a 1.5 ml microcentrifuge tube and stored at -20 °C till further use. The PCR products from the excised gel fragments were extracted by using the QIAEXII Gel extraction Kit (Qiagen, Netherland) as per the manufacturer's instructions and stored at -20 °C.

The purified PCR products were sequenced using pJET 1.2 forward and reverse sequencing primers by Eurofins Genomics Pvt Ltd. Bangalore, India to determine the nucleotide sequence of the amplicons. The sequence data so generated was received as colored electropherograms and the text files.

Nucleotide sequences of the gene fragments of the virus isolates were analyzed using 'EditSeq' programme of 'Lasergene' version.6 (DNASTAR Inc, USA). The nucleotide sequences were aligned separately by using the ClustalW method of 'MEGA6' programme. Multiple sequence alignment was carried out using ClustalW programme of MEGA v.6 software [21]. Phylogenetic tree was constructed based on the target genes using MEGA v.6 software with p-distance as nucleotide substitution model.

3. Results

In the present study, BRSV was detected in 12/406 (2.95%) cases and BPI-3 virus was detected in 15/406 (3.69%) cases by PCR and dFAT. PCR amplicons for BRSV and BPI-3 yielded 246 bp (Fig. 1a) and 127bp (Fig. 1b) products, respectively. In the positive cases of BRSV, grossly, lungs were consolidated cranio-ventrally with deep red atelectatic and collapsed areas with a rubbery consistency. Beside cranio-ventral consolidation, varying degrees of emphysema and edema in the diaphragmatic lobes with occasional deposition of fibrin on the pleura were observed (Fig. 2a). Mediastinal and tracheobronchial lymph nodes were swollen and edematous, and trachea had frothy exudate in the majority of the cases. Microscopically, BRSV infection was associated with typical lesions of bronchointerstitial pneumonia in ten cases, interstitial pneumonia in one case and fibrinous bronchopneumonia in one case. Characteristic findings of bronchiolar epithelial necrosis with thickened alveolar septa by mononuclear cells infiltration and edema; alveolar lumens were filled with mononuclear cells and numerous syncytial cells having intracytoplasmic inclusion were found (Fig. 2b and c) in numerous lung sections. Interlobular septae were widened by edema and dilation of lymphatic vessels in the majority of cases. The tracheal section showed mild to moderate congestion and infiltration of inflammatory cells. In one case, trachea had hemorrhages in the lamina propria and necrosis of the submucosal gland (Fig. 2e). There were depletion and necrosis of lymphoid follicles, congestion and edema of medullary sinuses with fibrin intermixed with lymphocytes, plasma cells (Fig. 2f) and occasional neutrophils and bacterial colonies in mediastinal and tracheobronchial lymph nodes. We observed immunopositivity in all twelve animals for BRSV by dFAT, which, were positive by RT-PCR. The BRSV antigen was distributed in the cytoplasm of bronchiolar and alveolar epithelium and syncytial cells. The apple green immunostaining in the epithelium was the mostly granular in type (Fig. 2d).

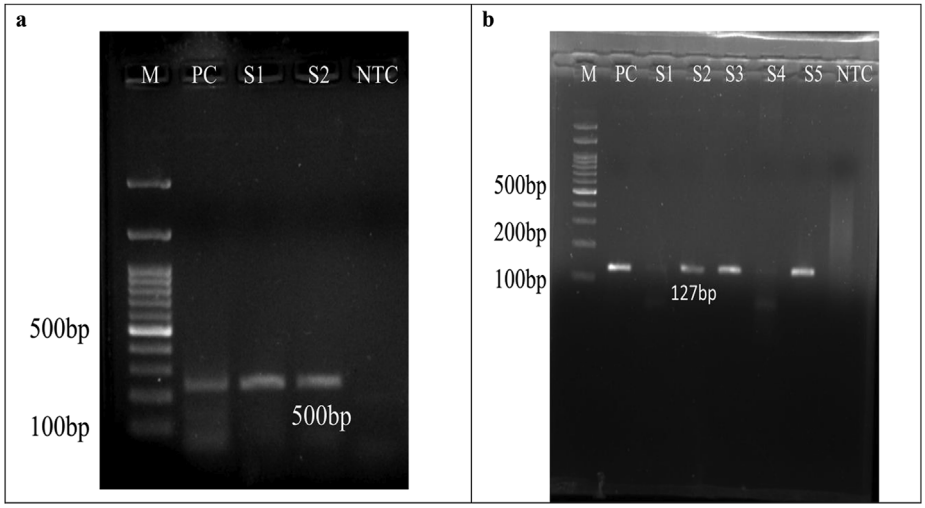

Fig. 1. Ethidium bromide stained agarose gel electrophoresis (2% agarose): positive samples (S1. Sn) for BRSV (a) and BPI-3 (b) with negative control (NTC), positive control (PC), 100bp DNA ladder (M).

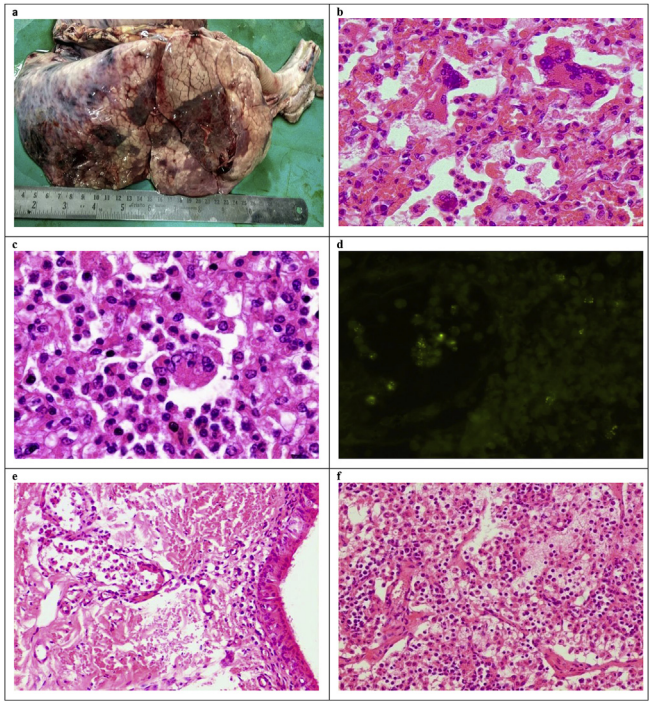

Fig. 2. Pathology of BRSV pneumonia: Dark brown areas of consolidation at right cranial, middle and anterior part of the diaphragmatic lobe with severe edema and multifocal hemorrhages (a); BRSV pneumonia: Photomicrographs of lung showing thickened alveolar septa, and alveolar lumen filled with macrophages and syncytia (b, 400x); alveolar lumen filled with macrophages and syncytial cells having intracytoplasmic inclusion (arrow) (c, 400x). H&E; granular apple green fluorescence in the epithelium cells and macrophages (d, 400x). dFAT; Photomicrographs of trachea showing hemorrhages in the lamina propria and necrosis of sub mucosal gland (e, 200x); medullary area of lymph node showing fibrinous exudate with moderate infiltration of plasma cells, lymphocytes and macrophages (f, 200x). H&E. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BRSV PCR amplicons from different animal flocks having good concentration of gene product were subjected for sequencing and sequence analysis for further confirmation. Phylogenetic analysis of the sequences of the PCR amplicons of BRSV positive samples for a partial fragment of BRSV (G gene) revealed that the sequence of all four amplicons was homologous to each other and were closely related to the sequences of the isolates reported from Belgium, UK and the USA in NCBI database. Further, the isolates were distantly related to the sequences of the isolates reported from Denmark, France and the Czech Republic (Fig. 3). Sequence distance of the BRSV isolates with respect to those reported in the NCBI database is presented in Fig. 3.

BPI-3 positive cases, grossly revealed multifocal areas of consolidation in the cranio-ventral lobes with varying degrees of consolidation, emphysema, edema, fibrin deposition in the lungs lobes (Fig. 4a) and edema of the lymph nodes. Histopathologically, BPI-3 positive cases were associated with bronchointerstitial pneumonia in six cases having thickened alveolar septa by mild macrophage infiltration, hyperplasia of type-II pneumocytes and bronchiolar necrosis; alveoli were filled with alveolar foamy macrophages and syncytial cell having intracytoplasmic inclusions (Fig. 4b and c). Interstitial pneumonia was noticed in four cases with characteristic thickening of the interalveolar wall due to mononuclear cell infiltration and engorgement of alveolar capillaries. Two cases revealed fibrinous bronchopneumonia having abandoned exudate in the alveoli lumen mixed with fibrin and inflammatory cells. Suppurative bronchopneumonia was observed in three cases. We observed immunopositivity in all fifteen animals for BRSV by dFAT which were positive by RT-PCR. Apple green immunosignals were detected in the cytoplasm of bronchiolar, alveolar epithelium and syncytial cells using the FITC anti BPI-3 antibody (Fig. 4d).

Phylogenetic analysis of the sequence of single PCR amplicon of BPI-3 positive samples for partial fragment of N gene of BPI-3 virus revealed that the sequence of all the PCR amplicons were homologous to each other and were closely related to the sequences of the isolates reported from the USA, Egypt and Japan and distantly related to those of other isolates reported from the USA. The phylogenetic tree and the sequence distance of the BPI-3V isolates with respect to those reported in the NCBI database are presented in Fig. 5.

4. Discussion

Pneumonia is associated with many abiotic and biotic factors and is the result of interactions between a wide range of pathogens, and other stresses [22]. There are multiple reports in the literature, indicating respiratory affections as one of the important causes of morbidity and mortality, specifically in young animals. Viruses like BRSV and BPI-3 play an important role in the development of pneumonia [23]. Although these viruses have been shown to be primary respiratory pathogens in experimental studies, they can compromise the pulmonary defenses thereby facilitating bacterial colonization and pathogenesis of pneumonia [24]. However, there are no reports of involvement of BRSV and BPI-3 in the cases of pneumonia from India in cattle and buffaloes.

Hence, the present study was planned to detect BRSV and BPI-3 and examine pathological processes involved in fatal cases of pneumonia. Young animals aged less than 12 months were selected, since pneumonia is primarily a problem in young calves less than 6 months with peak occurrence from 2 to 10 weeks, but it can be noticed more frequently in calves up to 1 year of age [25]. Calves *vis-a-vis* adult animals often suffer because of the waning influence of maternal immunity, and their own still relatively naïve immune system.

We reported BPI-3 and BRSV with an incidence of 3.69% and 2.95%, respectively, in pneumonic cases of bovines by RT-PCR. Although BRSV is well recognized as one of the major pathogens associated with bovine respiratory disease complex (BRDC) from many countries [26,27], no systematic studies have been carried out for the investigation of BRSV in the ruminant population of India, barring few reports *viz.* seroprevalence of 65.33% in cattle in Orissa [17], 47.06% in cattle and buffaloes in Punjab [18] and 50% in cattle in Himachal Pradesh [19] by using ELISA. In the present study, the frequency of BRSV detection was determined to be 2.95% (12/406) by RT-PCR which is lower as compared to the earlier seroprevalence reports of BRSV from India. This is because of repeated exposure of adult animals *vis-à-vis* calves to the virus infection throughout their life as well as possibility of re-infections [28]. Moreover, in the present study, the lung tissues were also screened for the detection of BRSV antigen unlike earlier reports of serosurveillance.

Grossly, BRSV positive lungs were observed with cranioventral consolidation, emphysema, edema and deflation of diaphragmatic lobes and swollen edematous tracheobronchial lymph nodes. The observations are in concordance with the earlier findings of [27,29]. Microscopically, characteristic lesions of bronchointerstitial pneumonia were observed in ten cases and few were diagnosed as fibrinous bronchopneumonia and interstitial pneumonia. Similar findings have also been reported earlier [14,30]. The presence of bronchointerstitial pneumonia with multinucleated syncytia and inclusions in the present study are in consonance with the findings of [31] in the BRSV affected animals. It is because of the replication of the virus in the alveolar macrophages and F glycoprotein induces fusion of the infected cells, formation of syncytia and spreads to the adjacent cells [32]. BRSV is a known primary pathogen associated with initial damage to the lung tissues and subsequent colonization with bacterial pathogens as also reported earlier [33]. BRSV has a special predilection to the ciliated bronchiolar epithelium, it's attachment to the ciliated epithelial cells is

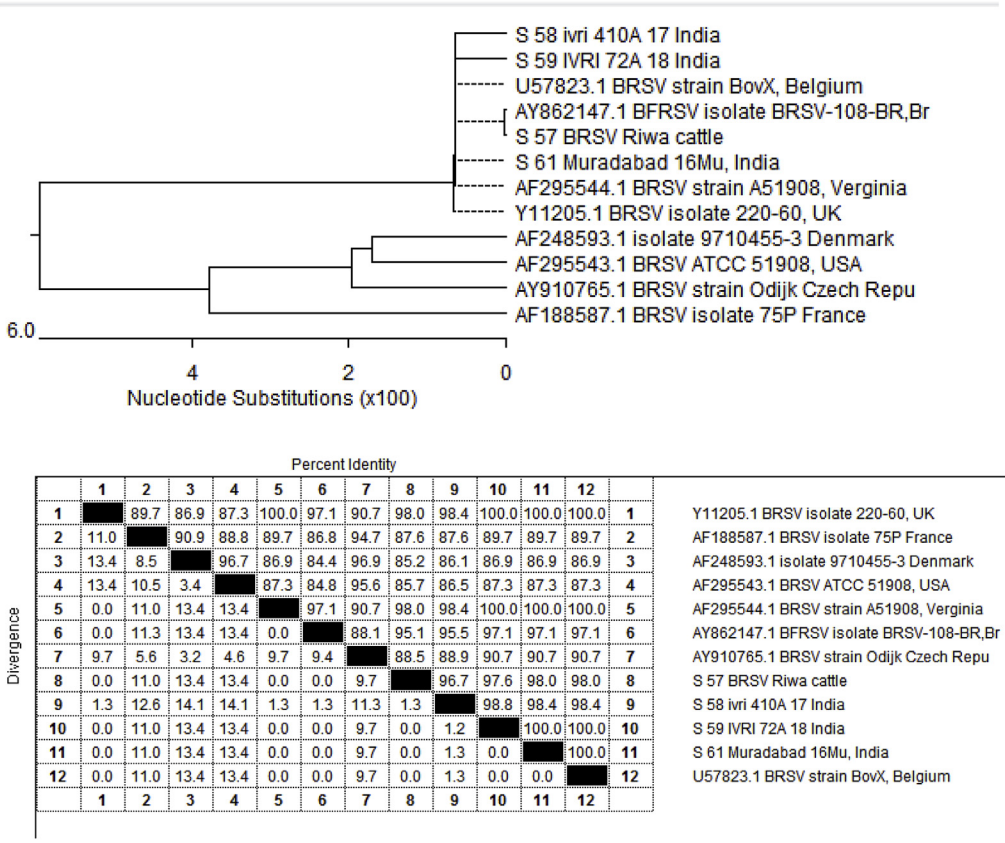

Fig. 3. Phylogenetic analysis, sequence divergence and percent identity of BRSV based on G Gene amplicons (246bp) using Megalign (DNA star).

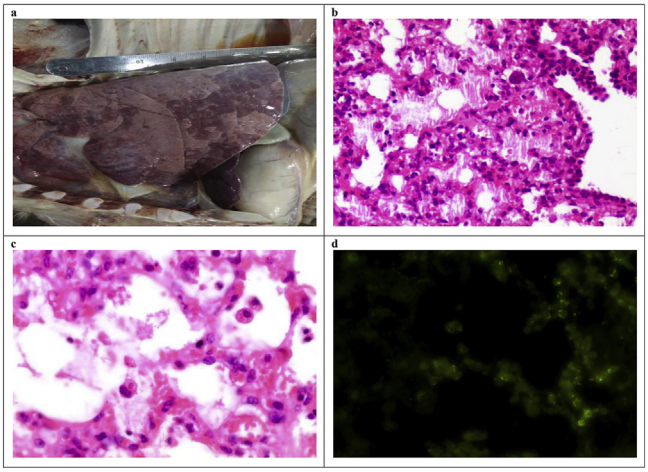

Fig. 4. Pathology of BPI-3 pneumonia: Light brown areas of consolidation in lobular pattern and pale inflated areas of emphysema (a); photomicrograph showing thickened alveolar septa and alveolar lumen filled with proteinacious fluid and few syncytia (b, 200x); thickened alveolar septa, few foamy macrophages and intracytoplasmic inclusion in alveolar macrophage (c, 400x). H&E; Lung section showing, granular apple green fluorescence in alveolar macrophages and pneumocytes (d, 100x). dFAT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mediated by the heparin binding domains on G glycoprotein [34]. Findings of fibrinous bronchopneumonia with the presence of fibrinous exudate with inflammatory cells and interstitial pneumonia were in accordance with earlier reports [23]. It may be due to the release of a cascade of pro-inflammatory cytokines in the infected cell, recruiting neutrophils, lymphocytes, and macrophages to the affected area (Caswell and Williams, 2016). The BRSV antigen was demonstrated in the cytoplasm of bronchiolar and alveolar epithelium and syncytial cells as observed previously [35,36]; Ceribasi et al., 2014) [35,36]. reported 19.5% (7/41) incidence by indirect immunofluorescence (IFA) in young cattle from Brazil. All the four sequences of partial fragment of BRSV were closely related to sequences from Belgium, Brazil, USA and UK [37–40] and distantly related to selected BRSV sequences from Denmark, Czech Republic and France [41–43]. Although amplicons size was very small and the small number of samples evaluated in the study, the obtained data suggest that BRSV is circulating in India among the bovine population.

BPI-3 is an important pathogen contributing to the development of BRDC in young and adult cattle [44] and a high level of seroprevalence has been reported in cattle across the world, however, it has rarely been isolated from the outbreaks of pneumonia. In India, there are few reports of BPI-3 associated sero-prevalence of 27.81% [18] and 57.58% [19] in Punjab and Himachal Pradesh, respectively. Variable incidence of BPI-3 has been reported as 13% and 14.81% [23,45]; respectively in young cattle from North America and Ireland. In the present study, an incidence of 3.69% of BPI-3 was observed by RT-PCR from pneumonic young cattle and buffaloes, which, could be due to theselection of younger animals and their less exposure to the viral agents.

Grossly, BPI-3 positive lungs were associated with focal to multifocal consolidation of cranio-ventral lobes with firm to a rubbery consistency and emphysema/edema in the diaphragmatic lobes leading to failure of the lungs to collapse. The observations are in agreement with those of earlier descriptions [46]. Histologically, they were diagnosed mostly as bronchointerstitial pneumonia in six cases, interstitial pneumonia in four cases, fibrinous bronchopneumonia in two cases and suppurative bronchopneumonia in three cases. Similar findings were also described by Refs. [47]. Presence of thickened alveolar septa, atelectasis, infiltration of lymphocytes and formation of syncytial cells and intracytoplasmic inclusions in the alveolar lumen observed in the

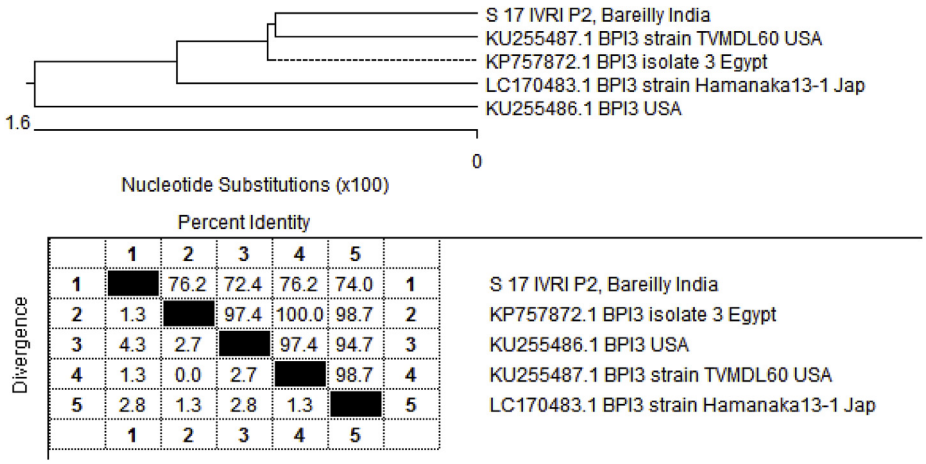

Fig. 5. Phylogenetic analysis and sequence divergence and percent identity of BPI-3 based on N Gene amplicons (127bp) using Megalign (DNA star).

present study were similar to the findings of Cerabasi et al. (2014) in spontaneous cases. The presence of inclusion bodies is attributed to the replication of the virus in the cytoplasm of respiratory epithelium and alveolar macrophages. BPI-3 has an immunosuppressive effect on the leucocytes as it inhibits the formation of phagolysosomes and increases the production of arachidonic acid metabolites, thereby inhibiting phagocytosis by the alveolar macrophages [10]. It also destroys the mucociliary system [48]. These mechanisms lead to the suboptimal mucociliary escalator and depressed local cellular immune response which is may be the reason for the development of severe lesions of fibrinous and suppurative bronchopneumonia observed in the present study. BPI-3 antigen was found in the cytoplasm of bronchiolar, alveolar epithelium and syncytial cells using d-FAT. This result ties well with Cerabasi et al. (2014) who located the BPI-3 antigen by using dFAT and found the incidence of 10.53% (26/247). Amplified single BPI-3 fragment was sequenced and analyzed with already available BPI-3 sequences at the NCBI database to confirm the disease. It was closely related to the BPI-3 sequences from the USA, Egypt and Japan [49–51]. This confirms the circulation BPI-3 in the Indian bovine population. Moreover, further studies with considering larger fragments and the complete genome are needed to identify various strain and genotype circulating in the country. This will help to design preventive strategies and develop appropriate diagnostics.

The present study confirms for the first time the presence of BRSV and BPI-3 in pneumonic cases of cattle and buffaloes from India. These findings indicate region wise molecular epidemiological studies to explore the exact prevalence of viruses circulating in the country. Hence suitable preventive and vaccination strategies can be implemented to prevent the disease occurrences. The present study adds information regarding the incidence of BRSV and BPI-3 in the bovine population from India to the literature.

CRediT author statement

1. Bhupesh Kamdi: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original Draft.
2. Rajendra Singh: Conceptualization, Writing - Original Draft, Project administration, Writing - Review & Editing.
3. Vidya Singh: Conceptualization, Visualization, Writing - Original Draft, Project administration, Writing - Review & Editing.
4. Shailendra Singh: Resources, Investigation, Formal analysis.
5. Pawan Kumar: Supervision, Formal analysis.
6. Karam Pal Singh: Supervision, Methodology.
7. Neethu George: Resources, Investigation, Formal analysis.
8. Kuldeep Dhama: Visualization, Writing - Review & Editing.

Declaration of competing interest

None.

Acknowledgements

The authors are thankful to the director, ICAR-Indian Veterinary Research Institute for providing providing financial support and the facilities for carrying out the above research work.

References

[1] M.A. Razzaque, M. Bedair, S. Abbas, Performance of pre-weaned female calves confined in housing and open environment hutches in Kuwait, Pak. Vet. J. 29 (2009) 1–4.
[2] M. Mellado, E. Lopez, F.G. Veliz, M.A. De Santiago, U. Macias-Cruz, L. Avendaño-Reyes, J.E. Garcia, Factors associated with neonatal dairy calf mortality in a hot-arid environment, Livest. Sci. 159 (2014) 149–155.
[3] D.C. Blood, O.M. Radostits, C.C. Gay, K.W. Hinchcliff, P.D. Constable, Veterinary Medicine: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats, Saunders Ltd, 2007.
[4] T. Wudu, Calf Morbidity and Mortality in Dairy Farms in Debre Zeit and its Envirions, Ethiopia, Doctoral dissertation, Msc thesis Faculty of Veterinary Medicine, Addis Ababa University, Ethiopia, 2004.
[5] M.P. Veena, B.R. Sumathi, Therapeutic management of neonatal calf pneumonia in HF calf-a case report, Vet. World 4 (2) (2011) 84.
[6] P. Manjari, Singh Purnima, Kumar Suresh, Kumari Anjali, Kumari Susavi, P. Thulasiraman, Management of calf pneumonia for economical dairy farming, Ind. Farm. 2 (4) (2015) 302–306.
[7] M.I. Gagea, K.G. Bateman, T. Van Dreumel, B.J. McEwen, S. Carman, M. Archambault, R.A. Shanahan, J.L. Caswell, Diseases and pathogens associated with mortality in Ontario beef feedlots, J. Vet. Diagn. Invest. 18 (1) (2006) 18–28.
[8] D. Bednarek, M. Szymańska-Czerwińska, K. Dudek, Bovine respiratory syndrome (BRD) etiopathogenesis, diagnosis and control, A Bird's-Eye View of Veterinary Medicine, InTech, Croatia, 2012, pp. 363–379.
[9] C.C. Sreenivasan, M. Thomas, R.S. Kaushik, D. Wang, F. Li, Influenza A in bovine species: a narrative literature Review, Viruses 11 (2019) 561, https://doi.org/10.3390/v11060561.
[10] D.C. Hodgins, J.A. Conlon, P.E. Shewen, Respiratory viruses and bacteria in cattle, Polymicrobial Diseases, ASM Press, 2002.
[11] K.P. Poulsen, S.M. McGuirk, Respiratory disease of the bovine neonate, Vet. Clin. Food Anim. Pract. 25 (1) (2009) 121–137.
[12] S. Kapil, R.J. Basaraba, Infectious bovine rhinotracheitis, parainfluenza-3, and respiratory coronavirus, Veterinary Clinics: Food Animal Practice 13 (3) (1997) 455–469.
[13] J.L. Caswell, Failure of respiratory defenses in the pathogenesis of bacterial pneumonia of cattle, Veterinary pathology 51 (2) (2014) 393–409.
[14] K. Blodörn, S. Hägglund, D. Gavier-Widen, J.F. Eléouët, S. Riffault, J. Pringle, G. Taylor, J.F. Valarcher, A bovine respiratory syncytial virus model with high clinical expression in calves with specific passive immunity, BMC Vet. Res. 11 (1) (2015) 76.
[15] L.E. Larsen, C. Tegtmeier, E. Pedersen, Bovine respiratory syncytial virus (BRSV) pneumonia in beef calf herds despite vaccination, Acta Vet. Scand. 42 (2001) 113–121.
[16] S.S. Maidana, P.M. Lomonaco, G. Combessies, M.I. Craig, J. Diodati, D. Rodriguez, V. Parreño, O. Zabal, J.L. Konrad, G. Crudelli, A. Mauroy, Isolation and characterization of bovine parainfluenza virus type 3 from water buffaloes (*Bubalus*

bubalis) in Argentina, BMC Vet. Res. 8 (1) (2012) 83.
[17] S. Hazari, H.K. Panda, B.C. Kar, B.R. Das, Comparative evaluation of indirect and sandwich ELISA for the detection of antibodies to bovine respiratory syncytial virus (BRSV) in dairy cattle, Comp. Immunol. Microbiol. Infect. Dis. 25 (1) (2002) 59–68.
[18] P. Goswami, H.S. Banga, V. Mahajan, N.D. Singh, S. Deshmukh, R.S. Brar, Detection of multiple antibodies and risk factor Association of common respiratory viruses in the state of Punjab, India, Int. J. Curr. Microbiol. App. Sci 6 (3) (2017) 567–577.
[19] S. Katoch, S. Dohru, M. Sharma, V. Vashist, R. Chahota, P. Dhar, A. Thakur, S. Verma, Seroprevalence of viral and bacterial diseases among the bovines in Himachal Pradesh, India, Vet. World 10 (12) (2017) 142121426, https://doi.org/10.14202/vetworld.2017.1421-1426.
[20] L.G. Luna, Histological Staining Methods of the Armed Forces Institute of Pathology, 3edn., Mcgraw Hill Book Co., New York, 1972, pp. 12–159.
[21] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (12) (2013) 2725–2729.
[22] I. Lorenz, B. Earley, J. Gilmore, I. Hogan, E. Kennedy, S.J. More, Calf health from birth to weaning. III. housing and management of calf pneumonia, Ir. Vet. J. 64 (1) (2011) 14, https://doi.org/10.1186/2046-0481-64-14.
[23] G.M. Murray, S.J. More, D. Sammin, M.J. Casey, M.C. McElroy, R.G. O'Neill, W.J. Byrne, B. Earley, T.A. Clegg, H. Ball, C.J. Bell, Pathogens, patterns of pneumonia, and epidemiologic risk factors associated with respiratory disease in recently weaned cattle in Ireland, J. Vet. Diagn. Invest. 29 (1) (2017) 20–34.
[24] C.W. Booker, S.M. Abutarbush, P.S. Morley, G.K. Jim, T.J. Pittman, O.C. Schunicht, T. Perrett, B.K. Wildman, R.K. Fenton, P.T. Guichon, E.D. Janzen, Microbiological and histopathological findings in cases of fatal bovine respiratory disease of feedlot cattle in western Canada, Can. Vet. J. 49 (5) (2008) 473.
[25] N.J. Sivula, T.R. Ames, W.E. Marsh, R.E. Werdin, Descriptive epidemiology of morbidity and mortality in Minnesota dairy heifer calves, Prev. Vet. Med. 27 (3–4) (1996) 155–171.
[26] J.F. Valarcher, G. Taylor, Bovine respiratory syncytial virus infection, Vet. Res. 38 (2) (2007) 153–180.
[27] R.E. Sacco, J.L. McGill, A.E. Pillatzki, M.V. Palmer, M.R. Ackermann, Respiratory syncytial virus infection in cattle, Veterinary pathology 51 (2) (2014) 427–436.
[28] I.B.A.L. Hoppe, A.S.R. de Medeiros, C.W. Arns, S.I. Samara, Bovine respiratory syncytial virus seroprevalence and risk factors in non-vaccinated dairy cattle herds in Brazil, BMC Vet. Res. 14 (1) (2018) 208.
[29] G.A. Andrews, G.A. Kennedy, Respiratory diagnostic pathology, Vet. Clin. Food Anim. Pract. 13 (3) (1997) 515–547.
[30] L.J. Gershwin, A.L. Van Eenennaam, M.L. Anderson, H.A. McEligot, M.X. Shao, R. Toaff-Rosenstein, J.F. Taylor, H.L. Neibergs, J. Womack, Single pathogen challenge with agents of the bovine respiratory disease complex, PloS One 10 (11) (2015) e0142479.
[31] B.W. Brodersen, Bovine respiratory syncytial virus, Veterinary Clinics: Food Animal Practice 26 (2) (2010) 323–333.
[32] R.S. Schrijver, J.P. Langedijk, G.M. Keil, W.G. Middel, M. Maris-Veldhuis, J.T. Van Oirschot, F.A. Rijsewijk, Comparison of DNA application methods to reduce BRSV shedding in cattle, Vaccine 16 (2–3) (1998) 130–134.
[33] R.J. Panciera, A.W. Confer, Pathogenesis and pathology of bovine pneumonia, Veterinary Clinics: Food Animal Practice 26 (2) (2010) 191–214.
[34] R. Sacco, J. McGill, M. Palmer, J. Lippolis, T. Reinhardt, B. Nonnecke, Neonatal calf infection with respiratory syncytial virus: drawing parallels to the disease in human infants, Viruses 4 (12) (2012) 3731–3753.
[35] E.F. Flores, R. Weiblen, M. Medeiros, S.A. Botton, L.F. Irigoyen, D. Driemeier, L.F. Schuch, M. Moraes, A retrospective search for bovine respiratory syncytial virus (BRSV) antigens in histological specimens by immunofluorescence and immunohistochemistry, Pesqui. Vet. Bras. 20 (4) (2000) 139–143.
[36] E.F. Flores, R. Weiblen, M. Medeiros, S.A. Botton, L.F. Irigoyen, D. Driemeier, L.F. Schuch, E.M. Moraes, A retrospective search for bovine respirator A retrospective search for bovine respiratory syncytial virus y syncytial virus (BRSV) antigens in histological specimens by (BRSV) antigens in histological specimens by immunofluorescence and immunohistochemistry, Pesqui. Vet. Bras. 20 (4) (2000) 139–143 out./dez. 2000.
[37] D. Prozzi, K. Walravens, J.P. Langedijk, F. Daus, J.A. Kramps, J.J. Letesson, Antigenic and molecular analyses of the variability of bovine respiratory syncytial virus G glycoprotein, J. Gen. Virol. 78 (2) (1997) 359–366.
[38] R.S. Almeida, H.G. Domingues, F.R. Spilki, L.E. Larsen, S. Hagglund, S. Belak, C.W. Arns, Circulation of bovine respiratory syncytial virus in Brazil, Vet. Rec. 158 (18) (2006) 632–634.
[39] A.S. Yunus, S.K. Khattar, P.L. Collins, S.K. Samal, Rescue of bovine respiratory syncytial virus from cloned cDNA: entire genome sequence of BRSV strain A51908, Virus Gene. 23 (2) (2001) 157–164.
[40] J.M. Furze, S.R. Roberts, G.W. Wertz, G. Taylor, Antigenically distinct G glycoproteins of BRSV strains share a high degree of genetic homogeneity, Virology 231 (1) (1997) 48–58.
[41] L.E. Larsen, K. Tjørnehøj, B. Viuff, Extensive sequence divergence among bovine respiratory syncytial viruses isolated during recurrent outbreaks in closed herds, J. Clin. Microbiol. 38 (11) (2000) 4222–4227.
[42] V. Valentova, A.F.G. Antonis, K. Kovarcik, Restriction enzyme analysis of RT-PCR amplicons as a rapid method for detection of genetic diversity among bovine respiratory syncytial virus isolates, Vet. Microbiol. 108 (1–2) (2005) 1–12.
[43] J.F. Valarcher, F. Schelcher, H. Bourhy, Evolution of bovine respiratory syncytial virus, J. Virol. 74 (22) (2000) 10714–10728.
[44] T. Autio, T. Pohjanvirta, R. Holopainen, U. Rikula, J. Pentikäinen, A. Huovilainen, H. Rusanen, T. Soveri, L. Sihvonen, S. Pelkonen, Etiology of respiratory disease in non-vaccinated, non-medicated calves in rearing herds, Vet. Microbiol. 119 (2–4) (2007) 256–265.
[45] C.L. Klima, R. Zaheer, S.R. Cook, C.W. Booker, S. Hendrick, T.W. Alexander, T.A. McAllister, Pathogens of bovine respiratory disease in North American feedlots conferring multidrug resistance via integrative conjugative elements, J. Clin. Microbiol. 52 (2) (2014) 438–448.
[46] A. Lopez, Respiratory system, mediastinum, and pleurae, in: J.F. Zachary, M.D. McGavin (Eds.), Pathologic Basis of Veterinary Disease, Mosby Elsevier, St. Louis, 2012, pp. 458–538.
[47] J.A. Ellis, Bovine parainfluenza-3 virus, Veterinary Clinics: Food Animal Practice 26 (3) (2010) 575–593.
[48] B.M. Adair, H.E.L. Bradford, D.G. Bryson, J.C. Foster, M.S. McNulty, Effect of parainfluenza-3 virus challenge on cell-mediated immune function in parainfluenza-3 vaccinated and non-vaccinated calves, Res. Vet. Sci. 68 (2) (2000) 197–199.
[49] J.D. Neill, J.F. Ridpath, B.T. Valayudhan, Identification and genome characterization of genotype B and genotype C bovine parainfluenza type 3 viruses isolated in the United States, BMC Vet. Res. 11 (1) (2015) 112.
[50] N.M. Sobhy, S.K. Mor, I.M. Bastawecy, H.M. Fakhry, C.R. Youssef, S.M. Goyal, Surveillance, isolation and complete genome sequence of bovine parainfluenza virus type 3 in Egyptian cattle, International journal of veterinary science and medicine 5 (1) (2017) 8–13.
[51] Y. Oue, M. Sakakibara, M. Narita, Y. Sato, T. Kanno, Genomic characterization of bovine parainfluenza virus type 3 in Hokkaido and development of rapid diagnostic method, J. Jpn. Vet. Med. Assoc. 70 (2017) 363–369.